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Microencapsulation de bactéries probiotiques dans des matrices laitières : Etude des mécanismes de formation par une approche multi-échelle

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Liste des abréviations

°C	Degré Celsius
2D, 3D	Two dimensional, Three dimensional
AFM	Atomic Force Microscopy
ANP	Azote Non Protéique
ATP	Adenosine triphosphate
Bdi	Breadth of the distribution
CLSM	Confocal Laser Scaning Microscopy
CMP	Caseino-macro-peptide
DLVO	Derjaguin-Landau-Verwey-Overbeek
(XDLVO)	(Extended Derjaguin-Landau-Verwey-Overbeek)
DO (OD)	Densité Optique (Optical Density)
E/H, H/E	Eau dans Huile, Huile dans Eau
EFSA	European Food Safety Authority
EPS	Exopolysaccharide
EQPC	Circle of equal projection area
ER	Encapsulation Rate
ESEM	Environmental Scanning Electron Microscopy
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FJC	Freely Jointed Chain
g	gramme
GI	Gastro-Intestinal
GRAS	Generally Recognised as Safe
h	Heure
IDF	International Dairy Federation
IEP	Isoelectric Point
IMCU	International Milk Clotting Units
LAB	Lactic Acid Bacteria
LGG (wt)	*Lactobacillus rhamnosus* GG (wild type)

LGR-1	*Lactobacillus rhamnosus* GR-1
LTA	Lipoteichoic Acid
MATH	Microbial Adhesion To Hydrocarbons
MATS	Microbial Adhesion To Solvents
MC	Micellar Casein
ME	Micro-Encapsulation
MFGM	Milk Fat Globule Membrane
min	Minute
MRS	De Man, Rogosa and Sharp
PBS	Phosphate Buffer Saline
Pa	Pascal
PEI	Polyethylenimine
PG	Peptidoglycane
PSD	Position Sensitive Detector
RPM	Rotation par minute
s	Seconde
SEM	Scanning Electron Microscopy
SLp	S-Layer protein
SMFS	Single Molecule Force Spectroscopy
SR	Survival Rate
TA	Teichoic Acid – Acide téichoique
TEM	Transmission Electron Microscopy
UFC (CFU)	Unité Formant Colonie (Colony forming Unit)
XPS	X-ray Photoelectron Spectroscopy
WLC	Worm-Like Chain
(v/v)	Volume/Volume
w/v	Weight/Volume
w/w	Weight/Weight

Chapitre 1 :

Introduction

1. Contexte

Depuis quelques années, les habitudes des consommateurs ont évolué vers des aliments pouvant influencer positivement leur santé. Les aliments fonctionnels sont en fait des aliments conventionnels dans lesquels des micronutriments ont été ajoutés comme par exemple des vitamines et minéraux, des acides gras oméga-3, des peptides bioactifs, des polyphénols, des prébiotiques ou encore des enzymes. Parmi eux, les aliments contenant des bactéries probiotiques détiennent une place importante puisqu'ils représentent 65% du marché mondial en termes d'aliments fonctionnels (Agrawal, 2005). Le marché global des probiotiques était de 14 milliards d'euros en 2008 (Scott-Thomas, 2009) et une forte croissance est attendue dans ce secteur avec une prévision de plus de 20 milliards d'euros pour 2015 (Starling, 2010). En règle générale, la plupart des aliments sur le marché comportent des bactéries libres et très peu contiennent des bactéries encapsulées (Kailasapathy and Champagne, 2011).

Les bactéries probiotiques sont des microorganismes vivants qui, lorsqu'ils sont ingérés en quantité suffisante, ont des effets bénéfiques sur l'équilibre et les fonctions physiologiques du microbiote intestinal (FAO/WHO, 2002). Le maintien de la viabilité et de la fonctionnalité des bactéries probiotiques jusqu'à ce qu'elles atteignent leur destination dans le tube digestif est primordial (Mattila-Sandholm et al., 2002). Cependant, de nombreuses études rapportent une perte importante de ces bactéries lorsqu'elles sont incorporées dans un aliment (de Vos et al., 2010). Ainsi, véhiculer des bactéries probiotiques protégées par une barrière physique, leur permettant de résister aux conditions environnementales défavorables rencontrées au niveau de l'aliment puis, au niveau de l'estomac, est une approche recevant actuellement un intérêt considérable (Kailasapathy, 2009). Dans cette optique, la microencapsulation est une technique développée pour une utilisation industrielle et qui permet d'augmenter la survie des bactéries probiotiques pendant leur conservation dans l'aliment puis durant le transit gastrique (Borgogna et al., 2010).

Au regard des nombreuses études qui ont été réalisées sur la microencapsulation de bactéries probiotiques, il est surprenant que si peu de produits comportant des bactéries encapsulées soient retrouvés sur le marché. D'un point de vue économique, pour que le système soit rentable, une perte maximale de 1 log doit être observée. En effet, il faut garder à l'esprit que les produits contenant des bactéries probiotiques

microencapsulées sont plus chers, par conséquent les bénéfices sur la survie des bactéries doit au moins dépasser l'augmentation du coût.

Les stratégies de communication des entreprises proposant des produits dans lesquels des bactéries probiotiques encapsulées sont introduites diffèrent. En effet, certains choisissent de ne pas révéler cette information au consommateur. D'autres au contraire, mettent en avant la présence des microparticules et dans ce cas, c'est un tout nouveau produit qui est décrit. L'acceptation par le consommateur de ce type de produit peut prendre du temps mais, s'il est averti des propriétés sensorielles de l'aliment, il ne sera pas surpris et n'assimilera pas cette différence à un défaut.

Ainsi la maitrise du procédé d'encapsulation est primordiale et tous les aspects doivent être pris en considération au moment de sa mise au point : taille, forme et consistance des microparticules, comportement dans des milieux hostiles mais aussi et surtout maintien de la viabilité et de la fonctionnalité des bactéries probiotiques.

2. Objectifs de la thèse

Les objectifs de la thèse sont les suivants :

- Mettre au point un système d'encapsulation de bactéries probiotiques dont la matrice encapsulante n'est faite que de protéines laitières.

  Le procédé d'encapsulation ne doit recourir qu'à des constituants utilisés dans l'industrie alimentaire et le procédé doit être extrapolable pour une utilisation à l'échelle industrielle.

  Ce même procédé doit également être adaptable à l'encapsulation d'autres molécules bioactives.

- Comprendre les mécanismes mis en jeu lors de l'encapsulation de bactéries probiotiques par l'utilisation d'outils qui recouvrent plusieurs échelles d'étude : macroscopique (ex. : taux d'encapsulation), microscopique (ex. : microscopies), nanoscopique (ex. : interactions molécule-molécule).

Pour répondre à ces objectifs, la stratégie suivante a été adoptée. Dans un premier temps, une étude de la littérature a permis de déterminer les principaux enjeux et les difficultés rencontrées lors de l'encapsulation de bactéries probiotiques. La sélection du

procédé par émulsification et son dimensionnement ont permis la production de microparticules dont la teneur en bactéries était excellente et la structure de la microparticule idéale. Finalement la mise au point d'une méthode d'étude des interactions et sa combinaison avec des techniques de microscopie a permis d'élucider le mécanisme de formation de microparticules.

3. Originalité de la thèse

De nombreuses études se sont intéressées à l'encapsulation de bactéries probiotiques avec pour variable l'espèce de la souche encapsulée, le type de méthode d'encapsulation, la nature de la matrice ou encore la catégorie de l'aliment dans lequel les microparticules ont été introduites. Quelques études se sont également focalisées sur le devenir des microparticules lors de la digestion. Toutefois, aucune étude ne s'est intéressée aux mécanismes mis en jeu dès le début du procédé et qui pourtant ont une grande influence sur le résultat obtenu.

Le procédé développé dans ce travail, propose une matrice d'encapsulation uniquement composée d'ingrédients laitiers ce qui permet leur ajout dans une grande diversité de vecteurs alimentaires. De plus, leur stabilité dans des milieux à forte teneur en eau permet de véhiculer des molécules bioactives qui pourraient être affectées par de tels milieux.

La compréhension du mécanisme de formation n'a pas uniquement reposé sur l'observation du résultat c'est-à-dire le taux d'encapsulation ou la localisation des bactéries dans les microparticules comme c'est le cas dans la majorité des travaux. La mise au point d'une technique d'exploration des interactions entre les bactéries et les protéines composant la matrice par microscopie de force atomique est une avancée majeure dans l'optimisation du procédé et donc de l'amélioration des propriétés des microparticules.

4. Structure du manuscrit

Ce travail de thèse est à l'interface de trois disciplines qui sont : le génie des procédés, la physico-chimie et la biophysique. C'est donc naturellement que ce manuscrit a été structuré en trois parties (Figure 1).

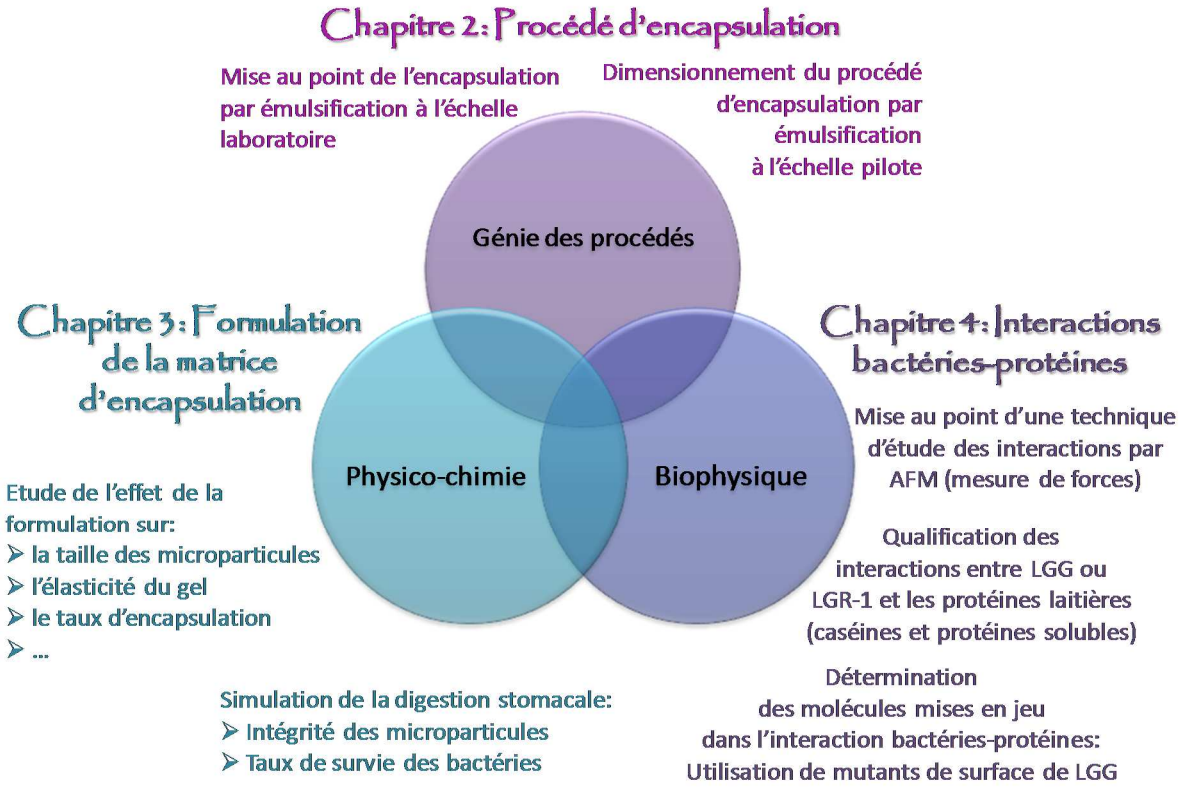

Figure 1 : Pluridisciplinarité de la thèse.

La première partie (Chapitre 2 : Procédé d'encapsulation) débute par une étude bibliographique des systèmes d'encapsulation disponibles pour les bactéries probiotiques ainsi que les utilisations dans des matrices alimentaires que ce soit à l'échelle laboratoire ou à l'échelle industrielle. La méthode d'encapsulation par émulsification choisie pour cette étude est ensuite décrite à l'échelle laboratoire. La mise au point des différentes étapes est détaillée. Finalement, le dimensionnement de l'encapsulation à l'échelle pilote a été réalisé et son mode de fonctionnement est présenté de même qu'une proposition d'extrapolation à l'échelle industrielle.

La seconde partie (Chapitre 3 : Formulation de la matrice d'encapsulation) traite du choix de la nature et des proportions en protéines laitières requises. La taille des microparticules, l'élasticité du gel ou encore le taux d'encapsulation de la souche probiotique sont autant de paramètres affectés par un changement de formulation. L'exposition des microparticules à un milieu simulant les conditions gastriques a

également été réalisée. La préservation de l'intégrité des microparticules mais également le suivi du taux de survie des bactéries microencapsulées ont été réalisés. L'objectif de cette partie a donc été la sélection d'une matrice encapsulante idéale pour *Lactobacillus rhamnosus* GG.

La troisième partie (Chapitre 4 : Interactions bactéries-protéines) débute par une étude bibliographique sur les interactions bactéries – ingrédients laitiers. Dans cette dernière partie, l'étude biophysique des interactions entre les bactéries et les protéines laitières a été réalisée. Dans un premier temps, la mise au point d'une méthode utilisant la microscopie de force atomique a permis de qualifier la nature des interactions entrant en jeu entre les protéines et les bactéries. Dans un second temps, l'utilisation de mutants de surface de *Lactobacillus rhamnosus* GG a permis de déterminer la nature des molécules, présentes à la surface de la bactérie, impliquées dans les interactions.

5. Valorisation scientifique

5.1. Publications

C. Gaiani, P. Boyanova, R. Hussain, I. Murrieta Pazos, M.C. Karam, **J. Burgain** & J. Scher (2011). Morphological descriptors and colour as a tool to better understand rehydration properties of dairy powders. *International dairy journal*, 21, 462-469.

J. Burgain, C. Gaiani, M. Linder & J. Scher (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of food engineering*, 104, 467-483.

J. Burgain, C. Gaiani, C. Jeandel, C. Cailliez-Grimal, A-M. Revol, & J. Scher (2012). Maldigestion du lactose: formes cliniques et solutions thérapeutiques. *Cahiers de Nutrition et de Diététique*, 47, 201-209.

J. Burgain, C. Gaiani, G. Francius, , A.M. Revol, C. Cailliez-Grimal, S. Lebeer, H.L.P. Tytgat, J. Vanderleyden & J. Scher (2013). In vitro interactions between probiotic bacteria and milk proteins probed by atomic force microscopy. *Colloids and Surface B: Biointerfaces*, 104, 153-152.

J. Burgain, C. Gaiani, C. Cailliez-Grimal, C. Jeandel, & J. Scher (2013). Encapsulation of *Lactobacillus rhamnosus* GG in microparticles: Influence of casein to whey proteins ratio on bacterial survival during digestion. *Innovative Food Science and Emerging Technologies*, 19, 233-242.

M. I. Afzal, C. C. Gonzalez Ariceaga, E. Lhomme, N. Kamel Ali, S. Payot, **J. Burgain**, C. Gaiani, F. Borges, A-M. Revol-Junelles, S. Delaunay & C. Cailliez-Grimal (2013). Characterization of *Carnobacterium maltaromaticum* LMA 28 for its positive technological role in soft cheese making. *Food Microbiology*, 36, 223-230.

J. Burgain, J. Scher, S. Lebeer, J. Vanderleyden, C. Cailliez-Grimal, M. Corgneau, G. Francius & C. Gaiani. Importance of *Lactobacillus rhamnosus* GG surface composition on interaction with milk proteins during microencapsulation. Soumise.

J. Burgain, J. Scher, G. Francius, F. Borges, M. Corgneau, A.M. Revol, C. Cailliez-Grimal & C. Gaiani. Bacterial interaction with milk components: Physico-chemical aspects and interest for food industry. Soumise.

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5.2. Brevets et offres de compétences

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J. Burgain, C. Gaiani & J. Scher. Système de simulation des conditions gastro-intestinales avec suivi en temps réel des phénomènes observables. Offre de compétence publiée.

5.3. Chapitres de livre

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5.4. Présentations orales

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- Bourse du comité scientifique
- Prix de la meilleure communication

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- Communication invitée

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J. Burgain, C. Gaiani, G. Francius & J. Scher. Probing in vitro interactions between probiotic bacteria and milk proteins using atomic force microscopy. *5th International symposium on spray-dried dairy products*, St-Malo, France, Juin 2012.

J. Burgain, C. Gaiani, G. Francius & J. Scher. Interactions between bacteria and milk proteins: Influence on encapsulation efficiency. 6th International Granulation Workshop, Sheffield, United-Kingdom, Juin 2013.

C. Gaiani, I. Murrieta Pazos, R. Hussain, P. Boyanova, **J. Burgain** & J. Scher. Relationship between milk powders morphology and its rehydratation properties. 5th International Granulation Workshop, 20-22 June, 2011, Lausanne, Switzerland.

5.5. Présentations par affiche

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La nécessaire encapsulation des probiotiques (2011). Actif's magazine, interview publiée par Mme Cyrille Costa, 23, 52-53.

Troubles digestifs : et si c'était une intolérance alimentaire ? (2012). Le généraliste, interview publiée par Mme Cyrille Costa, 2608, 30.

Chapitre 2 :

Procédé d'encapsulation

1. Introduction

Les bactéries probiotiques doivent être véhiculées jusqu'à leur site d'action (c'est-à-dire l'intestin) dans un état viable et fonctionnel. Toutefois, la tolérance de la majorité de ces bactéries aux conditions défavorables rencontrées d'une part lorsqu'elles sont introduites dans un aliment et d'autre part lorsqu'elles sont confrontées à l'acidité gastriques sont autant de paramètres défavorables à leur survie.

La nécessité de protéger ces cellules devient donc une évidence et, la microencapsulation est une solution prometteuse. L'encapsulation de bactéries probiotiques doit se faire dans des conditions qui préservent l'intégrité des cellules tout en restant une méthode utilisable à l'échelle industrielle dans le but de produire suffisamment de microparticules pour pouvoir les introduire dans des aliments financièrement acceptable pour la majorité des consommateurs.

Cette partie décrira les protocoles d'encapsulation utilisés pour les bactéries probiotiques dans une revue de la littérature. L'utilisation de bactéries microencapsulées que ce soit à l'échelle laboratoire ou industrielle sera également abordée.

Par la suite, la méthode d'encapsulation par émulsification qui a été retenue dans ce travail sera décrite en détail. Cette technique a l'avantage d'être extrapolable à l'échelle industrielle. Si elle est mise en œuvre avec précaution, elle permet de préserver la viabilité des bactéries probiotiques.

Dans un premier temps, le travail à l'échelle laboratoire sera développé. Ensuite, le procédé à l'échelle pilote sera décrit et une extrapolation du procédé vers une échelle industrielle sera proposée.

2. Synthèse bibliographique sur la microencapsulation de bactéries probiotiques

Encapsulation of probiotic living cells: From laboratory scale to industrial applications

J. Burgain, C. Gaiani, M. Linder & J. Scher (2011). *Journal of Food Engineering*, 104, 467-483.

Abstract

In the recent past, there has been a rising interest in producing functional foods containing encapsulated probiotic bacteria. According to their perceived health benefits, probiotics have been incorporated into a range of dairy products but the major current challenge is to market new probiotic foods. In the research sector, many studies have been reported using dairy products like cheese, yogurt and ice cream as food carrier, and non-dairy products like meat, fruits, cereals, chocolate, etc. However, in the commercial sector only few products containing encapsulated probiotic cells can be found. Nutraceuticals are another important vector for probiotics already developed by several companies in a capsule or a tablet form.

The review compiles the technologies used to encapsulate the cells in order to keep them alive and the food matrices used in the research and commercial sector for delivery to the consumer.

Résumé

Depuis quelques années, l'intérêt pour la production d'aliments fonctionnels contenant des bactéries probiotiques encapsulées est grandissant. Grâce à leurs effets bénéfiques sur la santé, les bactéries probiotiques ont été incorporées dans une large gamme de produits laitiers. Le défi majeur actuel est de fournir de nouveaux aliments contenant ces bactéries. Dans le secteur de la recherche, de nombreux travaux étudient l'incorporation de probiotiques dans des produits laitiers comme les fromages, les yaourts et les crèmes glacées mais également d'autres produits tels que les charcuteries, les fruits, les céréales, le chocolat, etc. En revanche, dans le secteur commercial peu de produits contenant des bactéries probiotiques encapsulées sont retrouvés. Les produits nutraceutiques sont un autre vecteur important pour les probiotiques, plusieurs industries les proposent sous la forme de comprimés ou de capsules.

Cette revue compile les technologies utilisées pour encapsuler les bactéries probiotiques dans le but de préserver leur survie. Les matrices alimentaires utilisées dans les secteurs de la recherche et de l'industrie permettant de les véhiculer jusqu'au consommateur sont également détaillées.

2.1. Introduction

Modern consumers expect their food to be healthy and to prevent illness as they are increasingly interested in their personal health (Kailasapathy, 2009). This explains the reason for a rising interest in probiotic health-based products. Probiotic is a term that means ‘‘for life’’ and defined as ‘‘live microorganisms that beneficially affect the host’s health by improving its microbial balance’’ (Fuller, 1989). More recently, probiotics have been defined as ‘‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’’ (FAO/WHO, 2002). In fact, probiotic products are important functional foods as they represent about 65% of the world functional food market (Agrawal, 2005) and the market for probiotic products continues to expand (Jankovic et al., 2010). Probiotic bacteria have been incorporated into a wide range of foods, including dairy products (such as yogurt, cheese, ice cream, dairy desserts) but also in non-dairy dairy products (such as chocolate, cereals, juices) (Anal and Singh, 2007). The viability of probiotic cells is of paramount importance because to have their beneficial effects on the host’s health they must stay alive as far as their site of action. Many reports indicated that there is poor survival of probiotic bacteria in products containing free probiotic cells (de Vos et al., 2010). Providing probiotic living cells with a physical barrier to resist adverse environmental conditions is therefore an approach currently receiving considerable interest. Microencapsulation (ME) is a powerful technology which has been developed for use in the food industry and allows the protection of bacterial cells (Borgogna et al., 2010).

However, ME of probiotic cells requires some specific processing steps which complicate the manufacture of the food product and increase its cost. Many challenges exist when considering ME of probiotic living cells like, probiotic strain selection for its health benefits and quantity required to have positives effects, but also stability of the cells during the processing steps and storage and finally, effects on sensory properties of the food (Champagne and Fustier, 2007a).

Currently, the probiotic market is affected by global regulatory requirements which have become stricter in recent years. In fact, manufacturers have to take into account cell viability and probiotic function in order to make a health claim (Jankovic et al., 2010). In Europe, the European Food Safety Authority (EFSA) is responsible for judging the health claims suggested by industrialists. In December 2006, EU makers adopted a regulation on the use of health claims and since then, many of them have been

rejected. The main reason unveiled by EFSA is a lack of probiotic strain characterization and that strains referenced are different from those present in the food products for which the claims were made. EFSA has confirmed that it does not plan to issue probiotic health claim guidance as has been done by the Canadian regulatory authority. In fact, Health Canada recently published a guidance document for the use of probiotic bacteria in food and the use of health claims associated with these products (Canada, 2009). In the United States there are no such government standards for probiotics and it is essential to have scientific substantiation to make a health claim. The Food and Drug Administration (FDA) expects manufacturers to provide scientific justification for use of any health claim. In 2009, guidance for Industry on ''Evidence-based review system for the scientific evaluation of health claims'' was published and describes some recommendations to manufacturer to substantiate a claim (FDA, 2009).

In this report, the probiotic term will be firstly defined and its beneficial effects for the human health will be mentioned. Then, a definition of ME and the technologies used to encapsulate probiotic cells will be developed. The last part will focus on the use of encapsulated probiotic cells in food on a laboratory scale. Finally, probiotic foods already available on the market will be listed and the tendency of development at an industrial scale will be approached.

2.2. Probiotics

2.2.1. Definition

Probiotics are feed and food supplements that beneficially affect the host's health. Strain identity is important in order to link a strain to a specific health effect and to enable accurate surveillance and epidemiological studies (Pineiro and Stanton, 2007).

The term ''probiotic'' includes a large range of microorganisms, mainly bacteria but also yeasts. Because they can stay alive until the intestine and provide beneficial effects on the host health, lactic acid bacteria (LAB), non-lactic acid bacteria and yeasts can be considered as probiotics. LAB are the most important probiotic known to have beneficial effects on the human gastro-intestinal (GI) tract. These bacteria are Gram-positive and usually live in a non-aerobic environment but they can also support aerobic conditions (Anal and Singh, 2007, Holzapfel et al., 2001). Bifidobacteria are also Gram-positive and can grow at a pH range of 4.5–8.5 but the most important characteristic is the fact that they are strictly anaerobic (Anal and Singh, 2007, Holzapfel et al., 2001). Other LAB (*e.g.*

Lactococcus lactis, *Enterococcus faecium*, etc.) and non-lactic acid bacteria (*e.g*. *Escherichia coli* strain nissle) but also some yeasts (*e.g. Saccharomyces cerevisiae*, *Saccharomyces boulardii*, etc.) are also considered as probiotics. It has been mentioned that dead bacteria, products derived from bacteria or end products of bacterial growth could provide some health benefits. However, because they are not alive when administrated they cannot be considered as probiotics (Sanders et al., 2007).

The effects of probiotics are strain-specific (Luyer et al., 2005, Canani et al., 2007, Kekkonen et al., 2007) and that is the reason why it is important to specify the genus and the species of probiotic bacteria when proclaiming health benefits. Each species covers various strains with varied benefits for health. The probiotic health benefits may be due to the production of acid and/or bacteriocins, competition with pathogens and an enhancement of the immune system (Chen and Chen, 2007). Dose levels of probiotics depend on the considered strain (Sanders, 2008), but 10^6–10^7 CFU/g of product per day is generally accepted (Krasaekoopt et al., 2003).

Overall, probiotics are orally administrated and are available in various forms such as food products, capsules, sachets or tablets. The advantage of food products such as dairy products is that they may additionally provide essential nutrients (e.g. calcium, proteins) and the addition of probiotics to these products is a natural way to enhance their functionality (Weichselbaum, 2009). Orally ingested probiotics have to survive adverse conditions during their passage through the GI tract to be able to influence the human gut microflora. The intestinal flora is made up of harmless microorganisms, present in appropriate proportions that are essential for its normal functioning. Ingested probiotic strains do not become established members of the normal intestinal flora but generally persist only for the period of consumption and for a relatively short period thereafter (Corthésy et al., 2007).

2.2.2. Health benefits

2.2.2.1. The mechanism of action of probiotic bacteria

There is evidence that probiotics have the potential to be beneficial for our health (Weichselbaum, 2009). Probiotics have been reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers (Kailasapathy and Chin, 2000, Sanders et al., 2007). The effects of probiotics can be classified in three modes of action Figure 2.

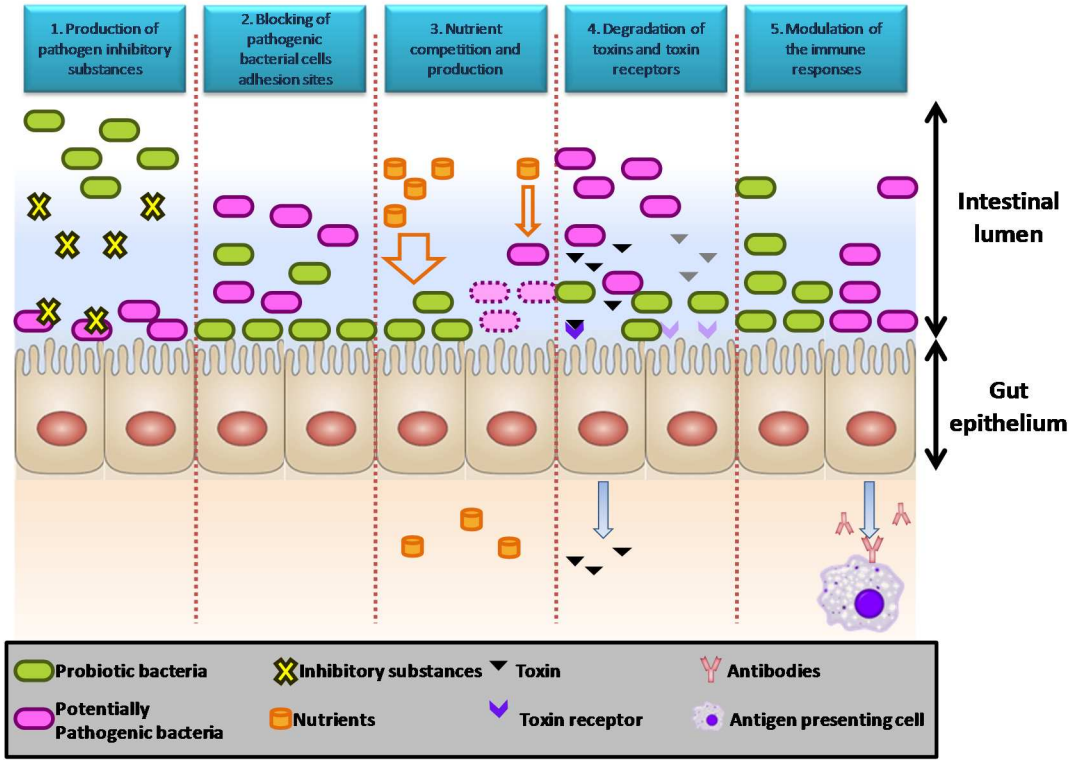


Figure 2: Modes of action of probiotic bacteria.

The first is related with the modulation of the host's defences which is most likely important for the prevention and treatment of infectious disease and also for treatment of intestinal inflammation (Collado et al., 2009). Probiotics may influence the immune system by means of products such as metabolites, cell wall components or DNA. In fact, these products can be recognised by the host cells sensitive for these because of the presence of a specific receptor (Cummings et al., 2004). In this context, the main target cells are generally the gut epithelial and the gut-associated immune cells. Finally, the interaction between probiotics and the host's immune cells by adhesion might be the triggering signaling cascade leading to immune modulation (Corthésy et al., 2007).

The second mechanism of action can be described by a direct effect on other microorganisms which can be commensal and/or pathogenic. In this case, the therapy and the treatment of infections are concerned but restoration of the microbial balance in the gut is an important factor too (Kaur et al., 2002). Probiotics have the ability to be competitive with pathogens and therefore allow for preventing their adhesion to the intestine (Tuomola et al., 1999).

Eventually, probiotics have the ability to affect some microbial products such as toxins and host products like bile salts and food ingredients (Patel et al., 2010). However, it is important to know that these three mechanisms of action are strain-dependent, and to date the modes of action of probiotic bacteria are not yet fully known (Oelschlaeger, 2010).

2.2.2.2. Examples of published health benefits: the gut and the immune system

In Europe, gut health has been shown to be the key sector for marketing functional foods (Mattila-Sandholm et al., 2002) and probiotics have a considerable potential for preventive or therapeutic effects on GI disorders (Wohlgemuth et al., 2010). The details of probiotics' health benefits related to the gut and the immune system are developed in the following paragraphs.

Inflammatory bowel disease is a chronic recurrent pathology, which mainly consists in ulcerative colitis and Crohn's disease. Recent studies have shown that some probiotic strains (*E. coli* Nissle 1917 and *Lactobacillus rhamnosus* GG) can prevent relapses of inflammatory bowel diseases and are able to decrease the recurrence of ulcerative colitis (Rembacken et al., 1999, Kruis et al., 2004, Zocco et al., 2006, Henker et al., 2008). Nevertheless, in the case of Crohn's disease current evidences suggest that probiotic are ineffective at treating patient with this pathology (Schultz et al., 2004, Bousvaros et al., 2005, Lomax and Calder, 2009). Evidence on the efficacy of probiotics on constipation is limited but it seems that some strains could bring relief to patients suffering from this pathology (Chmielewska and Szajewska, 2010). A number of probiotic strains are effective in preventing antibiotic associated diarrhea (Fitton and Thomas, 2009) and there is also promising evidence of a preventive effect of probiotics in *Clostridium difficile* associated diarrhea (Parkes et al., 2009). Acute diarrhea is a health problem which is well studied, particularly in children and studies have shown

that selected probiotic strains seem to be effective in reducing the duration of acute diarrhea (Lomax and Calder, 2009). Studies investigating the preventive effect of probiotics in the context of the common cold and flu infections show that the studied strains failed to lower the incidence of episodes but that they have the potential to decrease the duration of episodes, which suggests that the immune system may be more efficient in fighting off common cold or flu infections after consuming these strains (Weichselbaum, 2009).

Finally, there is no evidence so far proving that probiotics are effective in preventing or treating eczema and allergies. Some probiotic strains seem to lower the risk of developing eczema if taken by pregnant women and their infants in early life (Weichselbaum, 2009).

2.2.3. Synbiotics: a combination of the probiotics and prebiotics positives effects

Overall, products available on the market that positively influence the intestinal microflora are probiotics and prebiotics. The properties of probiotics have been shown in the previous section. Prebiotics can be defined as 'non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits' (Ouwehand et al., 2007). When considering probiotics, viability and dose level are important parameters for their efficacy and prebiotics have the potential to improve probiotic's viability and vitality, its survival in the GI tract and its further attachment and growth in the intestine. Inulin and fructo-oligosaccharides are the most common prebiotics used, because of their resistance against gastric acid and pancreatic enzymes (Ramchandran and Shah, 2010). Thereby, the symbiotic concept can be defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GI tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health promoting bacteria, and thus improving host welfare'. Synbiotics are not only a mixture of probiotics and prebiotics but a synergy between the two components (Ouwehand et al., 2007).

2.3. Encapsulation

2.3.1. Definition and goals of encapsulation

Encapsulation is a physicochemical or mechanical process to entrap a substance in a material in order to produce particles with diameters of a few nanometres to a few millimeters (Chen and Chen, 2007). Encapsulation of bioactive components can be used in many applications in the food industry: controlling oxidative reaction, masking flavours, colours and odours, providing sustained and controlled release, extending shelf life, etc. Probiotic encapsulation is used to protect the cells against an adverse environment more than controlled release (Champagne and Kailasapathy, 2008, Zuidam and Shimoni, 2009). The encapsulated substance called the core material is dispersed in a matrix also named coating or shell. This carrier material must be food grade if used in food industry, and able to form a barrier to protect the encapsulated substance.

As can be seen in Figure 3, different types of encapsulates can be found, the reservoir type and the matrix type.

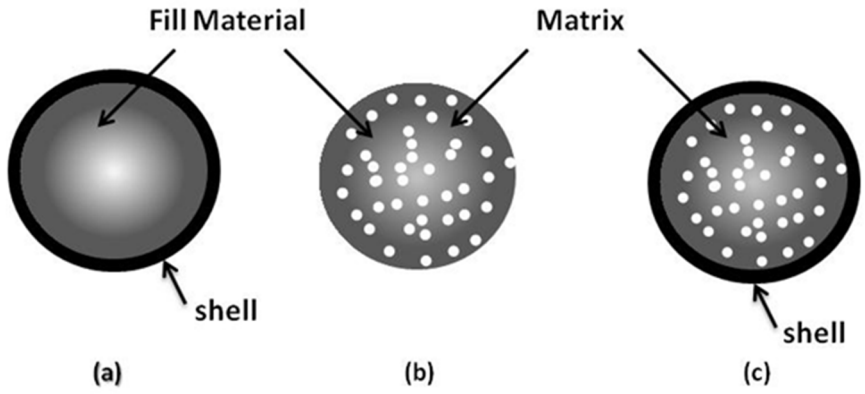


Figure 3 : Schematic representation of encapsulation systems: (a) reservoir type, (b) matrix type, and (c) coated matrix type.

The reservoir type has a shell around the core material and this is why it can also be called a capsule. In the case of matrix type, the active agent is dispersed over the carrier material and can also be found on the surface. A combination of these two types gives a third type of capsule: the matrix where the active agent is recovered by a coating (Zuidam and Shimoni, 2009).

Finally, encapsulation gives a structure and allows creating new function or innovative systems (Poncelet et al., 2007) for probiotic products. The technology of encapsulation of probiotic living cells evolved from the immobilised cell culture technology used in the biotechnological industry. Probiotics present two sets of

problems when considering encapsulation: their size (typically between 1 and 5 µm diameter), which immediately excludes nanotechnologies, and the fact that they must be kept alive. This latter aspect has been crucial in selecting the appropriate ME technology (Champagne and Fustier, 2007b, Zuidam and Shimoni, 2009).

Several technologies can be applied to probiotic encapsulation and each of them provides microcapsules with different characteristics in terms of range size of particles and of type of capsule (Figure 4).

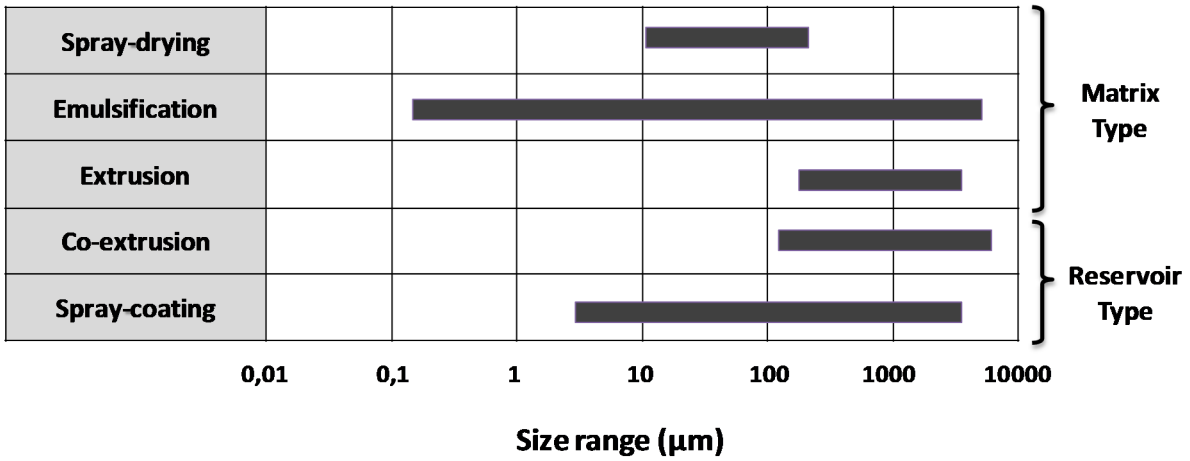


Figure 4 : Probiotic encapsulation technologies: size range provided by each technique.

For example, emulsification allows the production of a wide particle size range from 0.2 to 5000 µm whereas, extrusion gives a smaller range size but it does not provide particles under 300 µm. In Figure 4 it can be seen the different types of particles obtained (matrix or reservoir type) by each method.

The ability of microorganisms to survive and multiply in the host strongly influences their probiotic benefits. Studies have reported low viability of probiotics in dairy products such as yogurt and frozen dairy desserts due to the concentration of lactic acid and acetic acid, low pH, the presence of hydrogen peroxide, and the high oxygen content (de Vos et al., 2010). Encapsulation has been investigated for improving the viability of microorganisms in both dairy products and the GI tract (Krasaekoopt et al., 2003, Picot and Lacroix, 2004). The viability of encapsulated probiotic cells depend on the physico-chemical properties of the capsules. In fact, the type and the concentration of the coating material, particle size, initial cell numbers and bacterial strains are some parameters which are important to master (Chen and Chen, 2007). In the case of probiotic encapsulation, the objective is not only to protect the cells against

adverse environment, but also to allow their release in a viable and metabolically active state in the intestine (Picot and Lacroix, 2004). The obtained microparticles have to be water-insoluble to maintain their integrity in the food matrix and in the upper part of the GI tract and finally, particle properties should allow progressive liberation of the cells during the intestinal phase (Picot and Lacroix, 2004, Ding and Shah, 2007).

As shown in Figure 5, encapsulation technology is usually held in three stages.

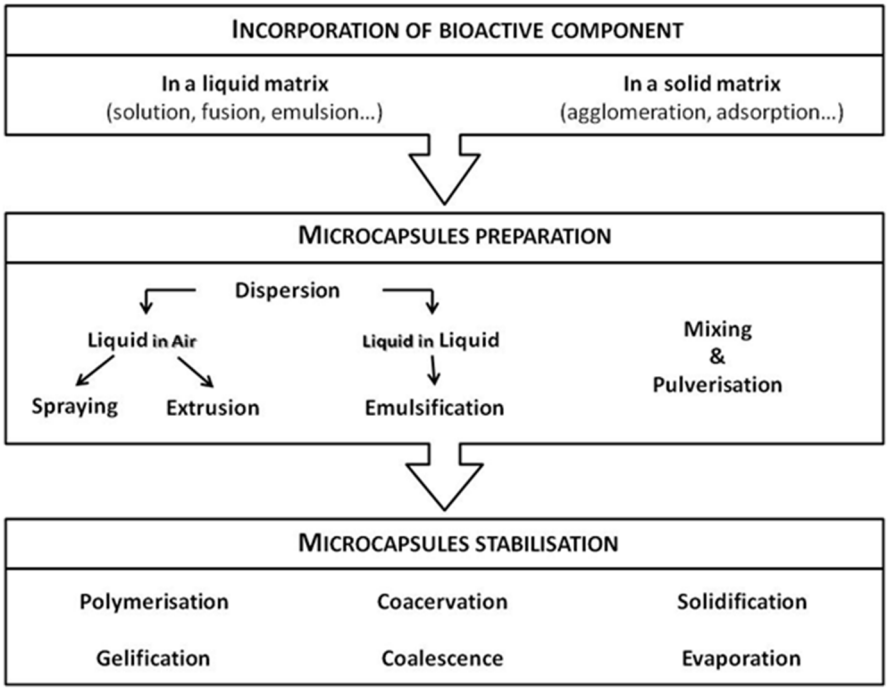


Figure 5 : General plan describing steps to produce microcapsules.

The first step consists in incorporating the bioactive component in a matrix which can be liquid or solid. In case of the core is liquid, incorporation will be a dissolution or a dispersion in the matrix whereas if the core is solid the incorporation will be an agglomeration or an adsorption. For the second step, the liquid matrix is dispersed while a solution is pulverised on the solid matrix. The last step consists in stabilisation by a chemical (polymerisation), a physicochemical (gelification) or a physical (evaporation, solidification, coalescence) process (Poncelet and Dreffier, 2007).

In the next part, techniques used to encapsulate probiotic living cells will be described. However, other techniques can provide microparticles and we can quote liposome, coacervation, co-crystallisation, molecular inclusion, but their use is limited because of their cost, or the large size of bacteria for example (Champagne and Kailasapathy, 2008).

2.3.2. Materials used to encapsulate probiotic cells

2.3.2.1. Alginate

Alginate is a naturally derived polysaccharide extracted from various species of algae and composed of β-D-mannuronic and α-L-guluronic acids. The composition of the polymer chain varies in amount and in sequential distribution according to the source of the alginate and this influences functional properties of alginate as supporting material. Alginate hydrogels are extensively used in cell encapsulation (Rowley et al., 1999) and calcium alginate is preferred for encapsulating probiotics because of its simplicity, non-toxicity, biocompatibility and low cost (Krasaekoopt et al., 2003). However, some disadvantages are attributed to the use of alginate. For example, alginate beads are sensitive to the acidic environment (Mortazavian et al., 2008) which is not compatible for the resistance of the microparticles in the stomach conditions. Others disadvantages concern the scaling-up of the process that is very difficult. In addition, the microparticles obtained are very porous which is a drawback when the aim is to protect the cells from its environment (Gouin, 2004).

Nevertheless, the defects can be compensated by mixing alginates with other polymer compounds, coating the capsules by another compound or applying structural modification of the alginate by using different additives (Krasaekoopt et al., 2003). For example, mixing alginate with starch is commonly used and it has been shown that this method results in an improvement of probiotic encapsulation effectiveness (Sultana et al., 2000, Sun and Griffiths, 2000, Hansen et al., 2002, Krasaekoopt et al., 2003).

2.3.2.2. Gellan gum and xanthan gum

Gellan gum is a microbial polysaccharide derived from *Pseudomonas elodea* which is constituted of a repeating unit of four monomers that are glucose, glucuronic acid, glucose and rhamnose (Chen and Chen, 2007). A mixture of xanthan–gelan gum has been used to encapsulate probiotic cells (Sultana et al., 2000, Sun and Griffiths, 2000) and contrary to alginate, the mixture presents high resistance towards acid conditions.

2.3.2.3. κ- Carrageenan

κ-Carrageenan is a natural polymer which is commonly used in the food industry. The technology using the compound requires a temperature comprised between 40 and 50 °C at which the cells are added to the polymer solution. By cooling the mixture to room temperature, the gelation occurs and then, the microparticles are stabilised by adding potassium ions (Krasaekoopt et al., 2003). The encapsulation of probiotic cells in κ-carrageenan beads keeps the bacteria in a viable state (Dinakar and Mistry, 1994) but the produced gels are brittle and are not able to withstand stresses (Chen and Chen, 2007).

2.3.2.4. Cellulose acetate phthalate

Because of having a safe nature, cellulose acetate phthalate is used for controlling drug release in the intestine (Mortazavian et al., 2008). The advantage of this component is that it is not soluble at acidic pH (less than 5) but it is soluble at pH higher than 6. The encapsulation of probiotic bacteria using cellulose acetate phthalate provides good protection for microorganisms in simulated GI conditions (Favaro-Trindade and Grosso, 2002).

2.3.2.5. Chitosan

Chitosan is a linear polysaccharide composed of glucosamine units which can polymerise by means of a cross-link formation in the presence of anions and polyanions. This component has not shown a good efficiency for increasing cell viability by encapsulation and it is preferably use as a coat but not as a capsule (Mortazavian et al., 2008). In fact, encapsulation of probiotic bacteria with alginate and a chitosan coating provides protection in simulated GI conditions and therefore, it is a good way of delivery of viable bacterial cells to the colon (Chavarri et al., 2010). However, chitosan has some disadvantages and it seems to have inhibitory effects on LAB for example (Groboillot et al., 1993).

2.3.2.6. Starch

Starch is a polysaccharide consisting of a large number of glucose units joined together by glucosidic bonds. Starch consists mainly of amylose, a linear polymer of D-glucopyranose joined by α-1-4 glucosidic bond and amylopectin, a branched polymer of

glucose joined by α-1-4 glucosidic bond and α-1-6 glycosidic bond for ramification (Sajilata et al., 2006). Resistant starch is the starch which is not digested by pancreatic enzymes (amylases) in the small intestine. Resistant starch can reach the colon where it will be fermented (Sajilata et al., 2006, Anal and Singh, 2007). This specificity provides good enteric delivery characteristic that is a better release of the bacterial cells in the large intestine. Moreover, by its prebiotic functionality, resistant starch can be used by probiotic bacteria in the large intestine (Mortazavian et al., 2008). Finally, resistant starch is an ideal surface for the adherence of the probiotic cells to the starch granules (Anal and Singh, 2007) and this can enhance probiotic delivery in a viable and a metabolically active state to the intestine (Crittenden et al., 2001).

2.3.2.7. Gelatin

Gelatin is a protein gum, which makes a thermoreversible gel and was used for probiotic encapsulation, alone or in combination with other compounds. Due to its amphoteric nature, it is an excellent candidate for cooperation with anionic polysaccharides such as gellan gum. These hydrocolloids are miscible at a pH higher than 6, because they both carry net negatives charges and repel each other. However, the net charge of gelatin becomes positive when the pH is adjusted below the isoelectric point and this causes the formation of a strong interaction with the negatively charged gellan gum (Krasaekoopt et al., 2003, Anal and Singh, 2007).

2.3.2.8. Milk proteins

Milk proteins are natural vehicles for probiotics cells and owing to their structural and physico-chemical properties, they can be used as a delivery system (Livney, 2010). For example, the proteins have excellent gelation properties and this specificity has been recently exploited by Heidebach et al. (Heidebach et al., 2009a, Heidebach et al., 2009b) to encapsulate probiotic cells. The results of these studies are promising and using milk proteins is an interesting way because of their biocompatibility (Livney, 2010).

2.3.3. Dispersion methods

2.3.3.1. Atomization

Spray-drying (Figure 6).

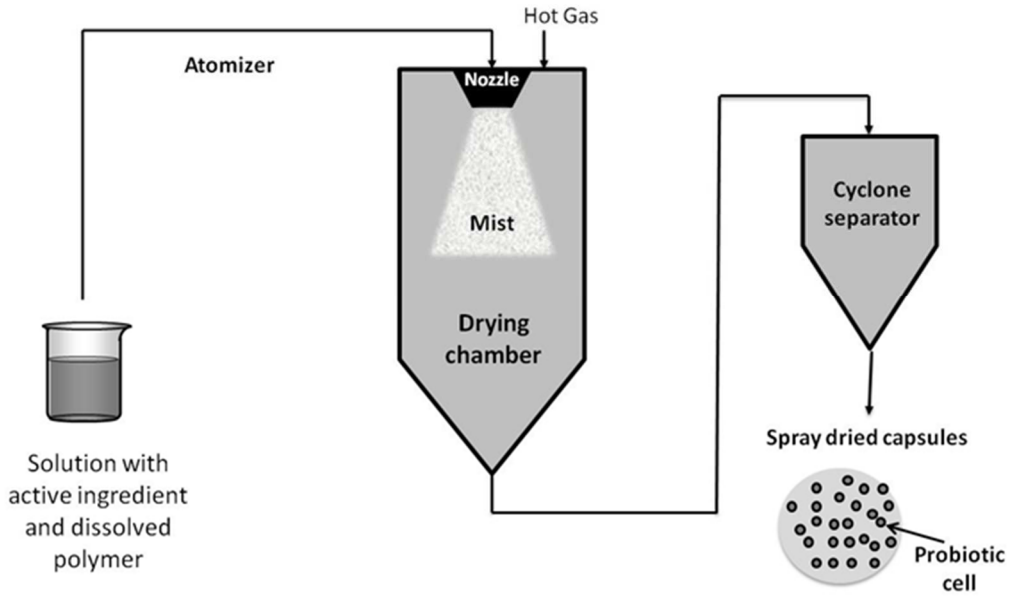


Figure 6 : Schematic presentation of the spray-drying procedure.

A solution containing the probiotic living cells and the dissolved polymer matrix is prepared. The polymer matrices are generally gum arabic and starches because they tend to form spherical microparticles during the drying process (Chen and Chen, 2007, Kailasapathy, 2009, de Vos et al., 2010). The advantages of spray drying are the rapidity and the relatively low cost of the procedure. The technique is highly reproducible and the most important is that it is suitable for industrial applications. One disadvantage of spray-drying is the fact that the technique has a small field of application but the main problem is the use of high temperature which is not compatible with the survival of bacteria. In order to improve probiotic survival, protectants can be added to the media prior to drying. For example, granular starch improves culture viability during drying and storage, soluble fibre increase probiotic viability during storage and trehalose is a thermoprotectant. Moreover, spray-dried capsules can be coated by an additional layer in order to give a protection against acidic environment of the stomach or to reduce the deleterious effect of bile salts (Semyonov et al., 2010).

Spray Freeze drying. Spray freeze drying method combines processing steps that are common to freeze-drying and to spray-drying. Probiotic cells are in a solution which

is atomized into a cold vapour phase of a cryogenic liquid such as liquid nitrogen. This step generates a dispersion of frozen droplets. Frozen droplets are then dried in a freeze-dryer (Wang et al., 2006, Kailasapathy, 2009, de Vos et al., 2010, Semyonov et al., 2010). Spray freeze drying presents various advantages, like providing controlled size, larger specific surface area than spray-dried capsules. The technique also has some disadvantages including the use of high energy, the long processing time and the cost which is 30–50 times more expensive than spray-drying (Zuidam and Shimoni, 2009). Capsules can be coated by an additional shell to give protection against adverse environmental conditions (Semyonov et al., 2010).

2.3.3.2. Emulsification

Emulsification and ionic gelification. Emulsification is a chemical technique to encapsulate probiotic living cells and use hydrocolloids (alginate, carrageenan and pectin) as encapsulating materials (Figure 7).

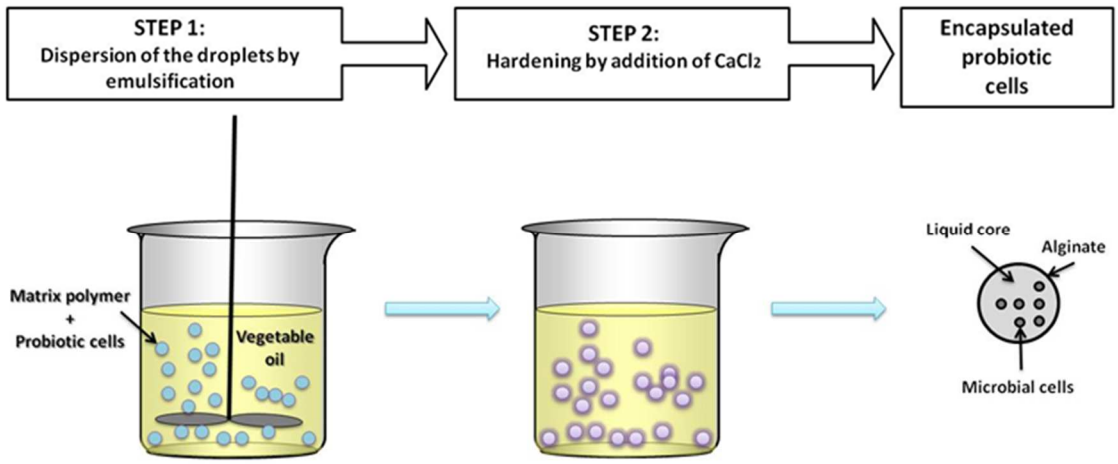


Figure 7 : Schematic presentation of the emulsification procedure.

The principle of this technique is based on the relationship between the discontinuous and the continuous phases. For encapsulation in an emulsion, an emulsifier and a surfactant are needed. A solidifying agent (calcium chloride) is then added to the emulsion (Chen and Chen, 2007, Kailasapathy, 2009, de Vos et al., 2010).

The emulsion technique is easy to scale-up and gives a high survival rate of the bacteria (Chen and Chen, 2007). The obtained capsules have a small diameter but the main disadvantage of this method is that it provides large size range and shape. The emulsion procedure enables the production of the targeted microcapsules size by

variation of agitation speed and the water/oil ratio (Kailasapathy, 2009). The gel beads can be introduced into a second polymer solution to create a coating layer that provides added protection to the cell or maybe give improved organoleptic properties (Kailasapathy, 2009).

Emulsification and enzymatic gelification. One problem with classical encapsulation technologies is the use of coatings such alginate, κ-carrageenan, gellan-gum or xanthan which are not allowed in dairy products in some countries (Picot and Lacroix, 2004). The solution can be the use of milk proteins in which probiotics will be encapsulated by means of an enzymatic induced gelation (Heidebach et al., 2009a, Heidebach et al., 2009b). Milk proteins have excellent gelation properties and they are natural vehicles for probiotics (Livney, 2010). This method gives water insoluble and spherical particles. Heidebach et al. (Heidebach et al., 2009a, Heidebach et al., 2009b) detailed an example of encapsulation by means of rennet gelation (Figure 8).

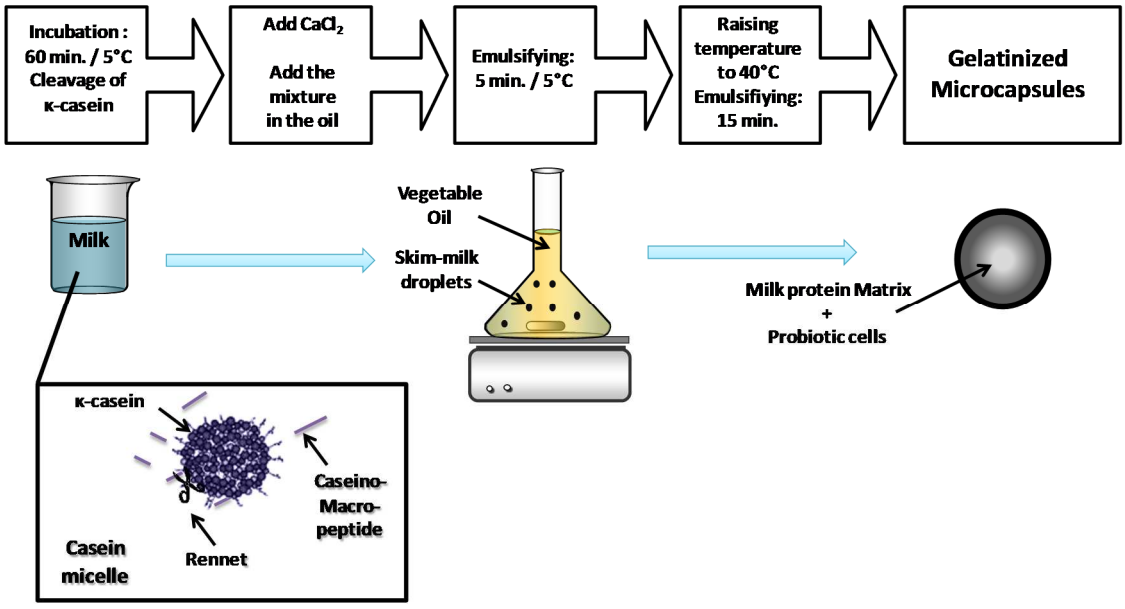


Figure 8 : Schematic presentation of the microencapsulation of probiotic cells by means of rennet-gelation of milk proteins.

Emulsification and interfacial polymerisation. Interfacial polymerization is an alternative technique which is performed in a single step. The technique requires the formation of an emulsion: the discontinuous phase contains an aqueous suspension with the probiotic cells and the continuous phase is an organic solvent. To initiate the

polymerisation reaction, a biocompatible agent which is soluble in the continuous phase, is added. The droplets obtained containing probiotic cells are enveloped in a thin and strong membrane (Kailasapathy, 2002). Interfacial polymerisation is used to encapsulate microorganisms in order to improve their productivity in fermentation (Yáñez-Fernández et al., 2008).

2.3.3.3. Extrusion method

Extrusion is a physical technique to encapsulate probiotic living cells and uses hydrocolloids (alginate and carrageenan) as encapsulating materials. The ME of probiotic cells by extrusion consists in projecting the solution containing the cells through a nozzle at high pressure. If the formation of droplets occurs in a controlled environment way (as opposed to spray-drying), the technique is known as prilling. This is preferably done by the pulsation or vibration of the jet nozzle. The use of coaxial flow or an electrostatic field is the other common technique to form droplets (Kailasapathy, 2002). The principle of the technique is explained in Figure 9 (Krasaekoopt et al., 2003, Chen and Chen, 2007, Kailasapathy, 2009, de Vos et al., 2010).

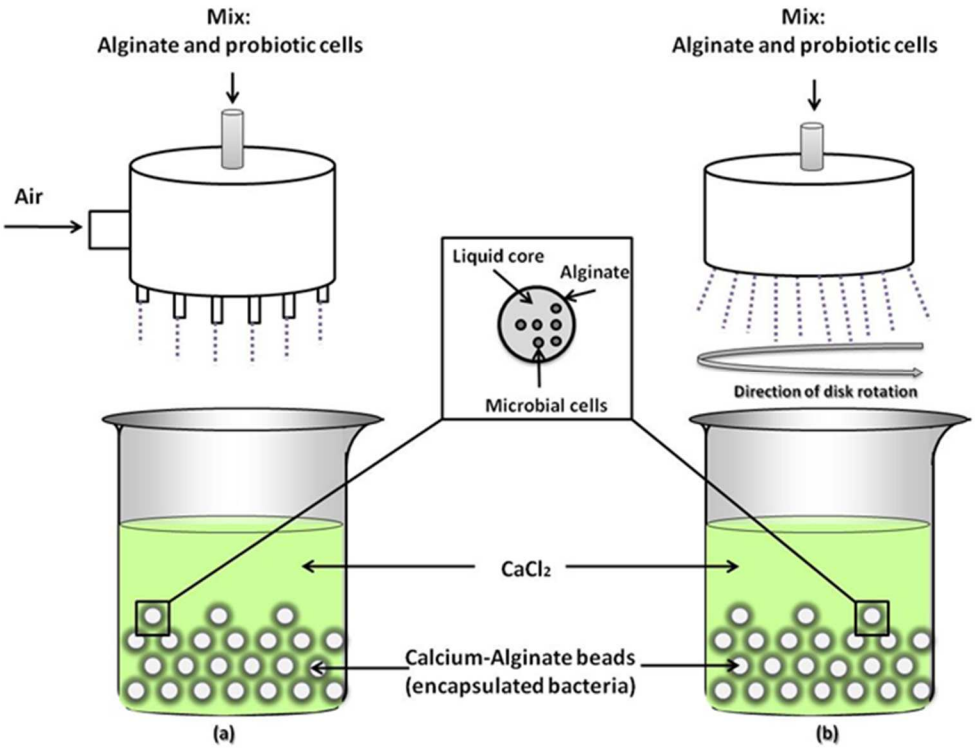


Figure 9 : Extrusion technologies: simple needle droplet-generator that usually is air driven (a) and spinning disk device (b).

Extrusion is a simple and cheap method that uses a gentle operation which causes no damage to probiotic cells and gives a high probiotic viability (Krasaekoopt et al., 2003). The technology does not involve deleterious solvents and can be done under aerobic and anaerobic conditions. The most important disadvantage of this method is that it is difficult to use in large scale productions due to the slow formation of the microbeads.

2.3.4. Encapsulation by coating and agglomeration

In spray-coating, the core material needs to be in a solid form and is kept in motion in a specially designed vessel as can be seen in Figure 10 (Champagne and Fustier, 2007b, de Vos et al., 2010).

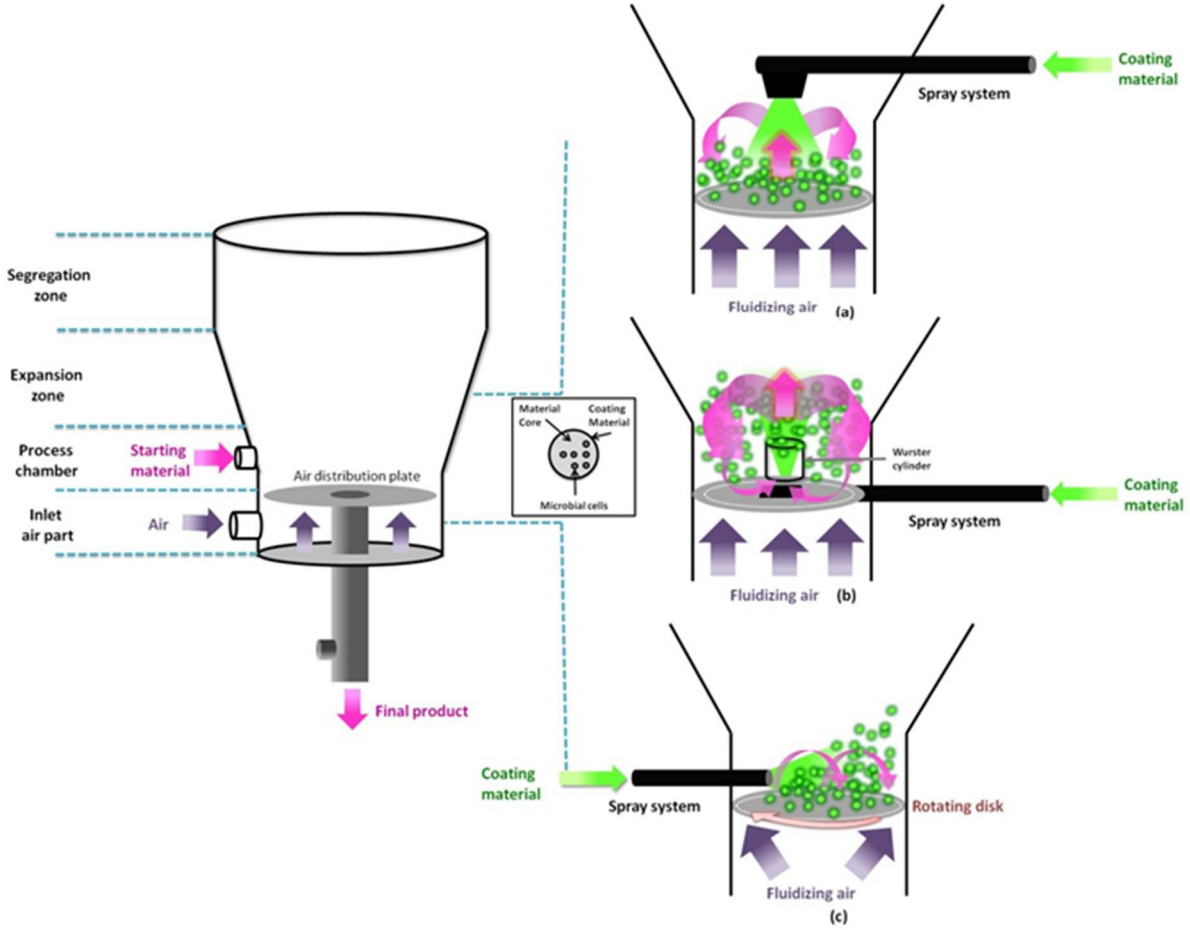


Figure 10 : Schematic presentation of the spray-coating technology. A liquid coating material is sprayed over the core material and solidifies to form a layer at the surface. The liquid coating material can be injected from many angles over the core material: fluid-bed top spray-coating (a), fluid-bed bottom spray-coating with the Wurster device (b), and fluid bed tangential spray-coating (c).

The advantage of spray-coating is that it is easy to scale up, which is why it is mostly use to encapsulate probiotics for nutraceuticals for example. Spray-coating is particularly adapted to give multilayer coatings. However, it is important to highlight that spray coating is a technology which is difficult to master.

Institut Rosell and Lal'food are part of the Lallemand group, a private Canadian company which develops products containing bacteria and particularly probiotic products. The company has developed and has patented a ME technology known as Probiocap (Durand and Panes, 2001). The process is based on coating freeze-dried LAB with fatty acids. The technology allows strains to resist harsh effects of temperature, gastric acidity and compression. Probiocap offers a number of opportunities to develop new food products and supplements.

Cell Biotech is a successful Danish-Korean company dedicated to probiotics which has developed and patented a dual coating technology for LAB, that are marketed under the brand name Duaolac. The first layer of coating is made of soy peptides and the second layer is made of cellulose and gum. The technology allows an increase in probiotic viability during processing shelf life and during their passage through the GI tract. The coating system is based on a pH-dependent release mechanism which protects the cells against acidic environment in the stomach and release the coating in the pH-neutral environment of the intestines.

2.4. Encapsulated probiotics in food products

ME is important for the survival of probiotics during storage and its passage through the digestive tract. Addition of microcapsules should not affect the sensory properties of food products (Champagne and Fustier, 2007b). To avoid negative sensorial impacts of microcapsules in food; it is desirable to obtain a size below 100 µm (Hansen et al., 2002).

2.4.1. Recent developments in the research sector

2.4.1.1. Cheese

Many studies have reported the use of encapsulated probiotic cells (Tableau 1) and particularly in Cheddar cheese.

Tableau 1 : Examples of encapsulated probiotics and their applications in cheese

	Probiotic strain	Technology	Materials	References
Fresh	*L. bulgaricus* *S. thermophilus*	Extrusion	Ca-alginate	**(Prevost and Divies, 1987)**
Cheddar	*B. bifidum*	Emulsification	κ-carrageenan	**(Dinakar and Mistry, 1994)**
Fresh	*L. lactis spp. lactis*	Emulsification	κ-carrageenan	**(Sodini et al., 1997)**
Crescenza	*B. bifidum* *B. infantis* *B. longum*	Freeze-drying	Ca-alginate	**(Gobbetti et al., 1998)**
Cheddar	*L. paracasei*	Spray-drying	Skim milk	**(Gardiner et al., 2002)**
Cheddar	*L. acidophilus* *B. infantis*	Emulsification	Alginate/ Starch	**(Godward and Kailasapathy, 2003a)**
Feta	*L. acidophilus* *B. lactis*		Alginate	**(Kailasapathy and Masondole, 2005)**
Kasar	*L. acidophilus* *B. bifidum*	Extrusion & Emulsification	Alginate	**(Özer et al., 2008)**
White Brined	*L. acidophilus* *B. bifidum*	Extrusion & Emulsification	Alginate	**(Özer et al., 2009)**

Due to a relative high pH (pH 5.5), Cheddar cheese presents the advantage of being a good carrier of probiotic microorganisms. In addition, its good buffering capacity and its relatively high fat content may offer a protection to probiotic bacteria against enzymatic degradation and acidic environment of the GI tract (Gardiner et al., 1998, Stanton et al., 1998). Dinakar and Mistry (Dinakar and Mistry, 1994) immobilized *Bifidobacterium bifidum* with an emulsification technique and the obtained gel beads were frozen and lyophilised. The addition of the immobilised cells in the cheese was not uniform, but the survival of bifidobacteria in the cheese was not affected. In fact, the cells remained viable until 24 weeks and did not affect the flavor and the flavor intensity, texture and appearance of the cheese. These observations can be explained by a lack of bifidobacteria metabolism. To produce acetic and lactic acid, bifidobacteria

need substrates such as lactose but in this case it was not available. The low temperature ripening (between 6 and 7 °C) avoids bifidobacterial growth, whose optimal growth temperature is 37 °C, but they remain viable. The composition of the cheese was not affected by probiotic incorporation and the addition of bifidobacteria in Cheddar is valuable, since the bacteria can stay alive for at least 6 months. Others authors have also made the observation that incorporation of bifidobacteria into Cheddar cheese does not negatively impact cheese quality in aroma, flavour and texture (Stanton et al., 1998). Moreover addition of *E. faecium* strain may have a positive influence on cheese properties (Gardiner et al., 1999b).

Spray-drying is another technology used to encapsulate probiotics before their incorporation into Cheddar cheese. The spray-dried culture was stable for 7 weeks while it was kept at room temperature and during refrigeration as well (Gardiner et al., 2002). Advantages are the cost-effectiveness and the applicability to large scale production compared to traditional methods like freezing or freeze-drying. However, 7 weeks does not seem to be sufficient when considering cheese ripening. Indeed, 5 weeks was defined as the minimum ripening time by the Codex Alimentarius. Overall, ripening time is longer than 5 weeks in order to develop cheese aroma for example.

Otherwise, it seems that the ME is not necessary for increasing the probiotic viability in Cheddar cheese but in the case of fresh cheese. For example, the ME is a good way of enhancing probiotic viability because of the low pH value of the product (Kailasapathy, 2002). This assertion was resumed in a study in which a comparison of viability was carried out between free and encapsulated probiotic cells in Cheddar cheese. Cheddar cheese was found as an ideal carrier for probiotics but the ME does not increase survival of probiotics. Moreover, the physical barrier inhibits the release of acids (produced by bacteria) and their accumulation in the bacterial surrounding leads to cell death (Godward and Kailasapathy, 2003a). Contradictory results were observed by Dinakar and Mistry (Dinakar and Mistry, 1994). Nevertheless, different probiotic strains were used and may not react in a similar way during the ripening period.

Encapsulated probiotic bacteria have also been incorporated in Crescenza cheese. This soft Italian cheese requires a brief ripening period. In this case, probiotic incorporation in the cheese did not require any change in flavour, appearance, and microbial and physico-chemical properties (Gobbetti et al., 1998). Two probiotic strains (*L. acidophilus* and *B. Bifidum*) have been incorporated into Kasar cheese using extrusion

or emulsification technology to encapsulate the cells. No difference was noticed between the two techniques when considering bacterial counts, proteolysis and organoleptical properties of the final product. Özer et al. (Özer et al., 2008) concluded that ME can be a good way to enhance probiotic viability in Kasar cheese. In another study, Özer et al. (Özer et al., 2009) introduced the same strains by the same techniques but in white-brined cheese. The use of probiotic cells in an encapsulated form enhances their viability and does not affect sensory properties of the cheese even though the ME has induced the formation of acetaldehyde and diacetyl.

Prevost and Divies (Prevost and Divies, 1987) described a process to continuously produce pre-fermented milk by means of entrapped cells in Ca-alginate particles. The final product had constant characteristics which were not the case with batch wise processing applied in the industry. In addition, the incubation time was reduced by 50 % compared with standard starter culture fermentation. The feasibility of a continuous milk pre-fermentation process on an industrial scale was confirmed by Sodini et al. (Sodini et al., 1997) and also added that it could be easily automated.

2.4.1.2. Yogurt

The incorporation of probiotic living cells in yogurt enhances its therapeutic value (Chen and Chen, 2007, Weichselbaum, 2009). However, there is poor level of probiotic viability in yogurt because of the low pH (from 4.2 to 4.6). Studies have shown that the use of encapsulated probiotic bacteria was better for their survival. Furthermore, the incorporation of probiotic cells into yogurts could be carried out without making many modifications from the traditional process (Kailasapathy, 2009). Many authors used encapsulated probiotic cells to incorporate into yogurts (Tableau 2).

Firstly, the encapsulation of probiotic cells in beads comprised of gellan–xanthan gum mixtures is a way to increase their tolerance to acidic environments (Sun and Griffiths, 2000). The introduction of encapsulated probiotic cells in set yogurts appears to increase probiotic survival in the product. However, bacteria have low metabolic activity and there is poor acetic acid produced (Adhikari et al., 2000). This compound gives a sour sensory property to yogurt and the lack of it is considered as a defect. In another study, Adhikari et al. (Adhikari et al., 2003) encapsulated bifidobacteria and incorporated them into stirred yogurt. However, consumers detected a grainy texture in these yogurts (size range particles about 22 – 50 μm). Even though the ME gives a

protection to bifidobacteria in yogurt, the sensory quality affected which is a major problem for consumer acceptance.

Tableau 2 : Examples of encapsulated probiotics and their applications in yogurt.

Probiotic strain	ME technology	Materials	References
L. casei	Extrusion		**(Lacroix et al., 1990)**
B. infantis	Extrusion	Gellan/Xanthan gum	**(Sun and Griffiths, 2000)**
B. longum	Emulsification	κ-carrageenan	**(Adhikari et al., 2000)**
L. acidophilus	Emulsification	Alginate-Starch	**(Sultana et al., 2000)**
B. adolescentis	Emulsification	Alginate	**(Hansen et al., 2002)**
B. longum	Emulsification	κ-carrageenan	**(Adhikari et al., 2003)**
L. acidophilus *B. infantis*	Emulsification	Alginate/Starch	**(Godward and Kailasapathy, 2003c)**
B. breve *B. longum*	Emulsification Spray-drying	Milk fat & whey protein	**(Picot and Lacroix, 2004)**
L. acidophilus	Extrusion	Ca-alginate	**(Krasaekoopt et al., 2004)**
L. acidophilus *B. infantis*	Extrusion	Raftilose, Raftiline & starch	**(Anjani et al., 2004)**
L. acidophilus	Extrusion	Ca-alginate Chitosan	**(Iyer and Kailasapathy, 2005)**
L. acidophilus *L. casei,* *L. rhamnosus* *B. infantis*	Emulsification	Alginate	**(Capela et al., 2006)**
L. acidophilus *B. lactis*	Emulsification	Alginate/starch	**(Kailasapathy, 2006)**
L. acidophilus *B. bifidum* *L. casei*	Extrusion	Alginate-chitosan	**(Krasaekoopt et al., 2006)**
L. acidophilus *B. longum*	Spray-drying	Maltodextrin/ Gum arabic	**(Su et al., 2007)**
L. acidophilus	Extrusion	Alginate-chitosan	**(Urbanska et al., 2007)**
L. acidophilus *B. lactis*	Extrusion		**(Kailasapathy et al., 2008)**
L. acidophilus *B. lactis*	Extrusion		**(Mortazavian et al., 2008)**
L. casei	Extrusion	Alginate/ Pectin	**(Sandoval-Castilla et al., 2010)**

Probiotic cells can be encapsulated with prebiotic ingredients (e.g. resistant starch) or cryoprotectants (e.g. glycerol) to improve their viability (Sultana et al., 2000,

Capela et al., 2006). It has been shown that this technique enhances probiotic survival in the product but not under simulated GI conditions (Sultana et al., 2000).

The co-encapsulation is another way to enhance probiotic viability and consists in encapsulating two different probiotic bacterial strains together (Godward and Kailasapathy, 2003c). A comparison was made between survival of free and encapsulated cells. Encapsulation and co-encapsulation were found to increase survival and in particular, freeze-dried cells after encapsulation survived better in the yogurt. Thus, yogurt can be a good probiotic carrier if the cells are encapsulated. The protected bacteria which are in a viable state at the moment of consumption, will survive through the GI tract and arrive in the intestine in a viable state (Godward and Kailasapathy, 2003c).

Other studies have demonstrated the use of co-encapsulating two probiotic strains with prebiotics such as raftilose in order to stimulate bacterial growth and to protect the cells against adverse environmental conditions. The co-encapsulation concept allows an increase of functional food efficiency thanks to the synergy between probiotic and prebiotic. The use of probiotic ingredients is also a factor to enhance probiotic viability. It was also shown that coating the capsule with chitosan gives better protection to probiotic cells than alginate when considering survival in yogurt and in simulated GI conditions (Anjani et al., 2004, Iyer and Kailasapathy, 2005).

Whey proteins have been used to encapsulate bifidobacteria and it was shown that this technique could be useful for the delivery of viable probiotic cells to the GI tract, when incorporated into dairy fermented products. However, spray-drying technology used in the study requires high temperature so, it is important to consider strain properties for heat resistance (Picot and Lacroix, 2004).

Kailasapathy (Kailasapathy, 2006) explained that the incorporation of capsules containing probiotic cells did not significantly alter yogurt's properties such as appearance, colour, flavour, taste and acidity. In contrast, by incorporating probiotic cells, textural properties were affected, in particular smoothness and consumers have detected grittiness in yogurts. Concerning acidity, it was shown that the addition of probiotic cultures slows down the post-acidification during the storage of yogurt. In another study, Kailasapathy et al. (Kailasapathy et al., 2008) demonstrated a correlation between post-storage pH and the survival probiotic bacteria which is negatively affected

by the presence of fruit pulp. However, all the obtained yogurts contained the recommended levels of probiotic bacteria even after 35-day shelf life.

The ME allows the delivery of a high number of bacteria to desired targets in the GI tract and the capsules containing probiotic cells seems to be good for oral administration by cell therapy (Urbanska et al., 2007). However, the technology used to encapsulate the cells and the probiotic strain, are two important parameters which significantly influence encapsulation yield (Picot and Lacroix, 2004). Talwalkar and Kailasapathy (Talwalkar and Kailasapathy, 2004b) observed that a beneficial effect of ME on probiotic cell survival occurs when there is oxygen in the medium, and the microenvironment in this case has a lower oxygen level. Champagne and Fustier (Champagne and Fustier, 2007a) hypothesised that discrepancies in cell viability between some studies could be due to various oxygen sensitivities between the strains or to different oxygen levels in yogurts. Another reason could be the type of coating material (e.g. chitosan) or the addition of starch in alginate core which improves the viability of the cells. Finally, ME of probiotics for addition into yogurts appears to prevent losses of oxygen-sensitive strains more than protecting the cells against acidic environment (Talwalkar and Kailasapathy, 2004a).

To conclude this part, the addition of encapsulated probiotic cells into yogurts is clearly detected in the mouth by the consumer. However, according to Champagne and Fustier (Champagne and Fustier, 2007a), the effects on sensory properties can become desirable if the consumer is forewarned and expects the presence of the particles.

2.4.1.3. Frozen dairy dessert

It is not easy to incorporate probiotic microorganisms into frozen desserts because of high acidity in the product, high osmotic pressure, freeze injury and exposure to the incorporated air during freezing (Chen and Chen, 2007). The introduction of probiotic bacteria in an encapsulated form into frozen desserts (Tableau 3) may overcome these difficulties and could produce useful markets and health benefits (Chen and Chen, 2007).

Entrapment of lactobacilli in Ca-alginate provides a higher survival rate (40%) compared to free cells, when freezing ice cream (Sheu and Marshall, 1993, Sheu et al., 1993). Godward and Kailasapathy (Godward and Kailasapathy, 2003b) studied the incorporation of probiotic cells in ice cream in different states. In fact, the cells can be free, freshly encapsulated, encapsulated and freeze-dried and finally co-encapsulated

and freeze-dried. The results have shown that free cells survive better than encapsulated cells. Freshly encapsulated probiotic cells had greater survival than those which were freeze-dried after encapsulation and co-encapsulation of *L. acidophilus* and *B. bifidum* enhances the survival of both strains. Finally, addition of probiotics does not affect air incorporation into ice cream. Another study demonstrated that there was no significant difference between the survival of encapsulated probiotic bacteria and the free cells. The protection of free cells in ice cream may be due to the high total solids making ice cream a suitable food for delivering probiotic living cells to the consumer (Kailasapathy and Sultana, 2003).

The encapsulation of probiotic cells enhances their viability when incorporated into ice cream and this addition had no effect on the sensory properties of the product. The high rate of total solid encountered in ice cream, and resistant starch added as prebiotic, provide further protection for probiotics in this case (Homayouni et al., 2008). The number of viable probiotic cells was between 10^8 and 10^9 CFU/g after three months of storage while the International Dairy Federation (IDF) recommended a viable number of 10^7 CFU/g in food product at the time of consumption (Homayouni et al., 2008).

Tableau 3 : Examples of encapsulated probiotics and their applications in frozen dairy dessert.

Probiotic strain	ME technology	Materials	References
L. bulgaricus	Emulsification	Alginate	**(Sheu and Marshall, 1993)**
L. casei *B. lactis*	Emulsification	Alginate	**(Sheu et al., 1993)**
L. acidophilus *B. infantis*	Emulsification	Alginate/ Starch	**(Godward and Kailasapathy, 2003b)**
L. acidophilus *B. lactis*	Emulsification		**(Kailasapathy and Sultana, 2003)**
L. casei *B. lactis*	Emulsification	Ca-Alginate	**(Homayouni et al., 2008)**

2.4.1.4. Other food products

Most of the products containing probiotic cells are dairy products and it is necessary to develop other food carrier for probiotics owing to lactose intolerance in certain populations (Ranadheera et al., 2010). Efforts have been made to identify new food carriers (Tableau 4).

Tableau 4 : Examples of encapsulated probiotics and their applications in various food systems.

	Probiotic strain	ME technology	Materials	References
Cream	*L. lactis*	Extrusion	Ca-alginate	**(Prevost and Divies, 1992)**
Mayonnaise	*B. bifidum* *B. infantis*	Emulsification	Alginate	**(Khalil and Mansour, 1998)**
Dry beverage	*Bifidobacterium* PL1	Spray-drying	Starch	**(O'Riordan et al., 2001)**
Banana	*L. acidophilus*	Extrusion	κ-carrageenan	**(Tsen et al., 2004)**
Soft foods	*B. lactis*	Extrusion	Gellan/Xanthan gum	**(McMaster and Kokott, 2005)**
Tomato Juice	*L. acidophilus*		Ca-Alginate	**(King et al., 2007)**
Sausages	*L. reuteri*	Extrusion Emulsion	Alginate	**(Muthukumarasamy and Holley, 2006)**
Sausages	*L. reuteri* *B. Longum*	Extrusion	Alginate	**(Muthukumarasamy and Holley, 2007)**
Biscuits **Cranberry & Vegetable juices**	*L. rhamnosus*	Extrusion	Whey protein	**(Reid et al., 2007)**
Orange and Apple Juices	*L. rhamnosus* *L. salivarius* *B. longum* *L. plantarum* *L. acidophilus* *L. paracasei* *B. lactis*	Emulsification		**(Ding and Shah, 2008)**
Chocolate	*L. helveticus* *B. longum*	Spray-coating	Fatty acids	**(Maillard and Landuyt, 2008)**
Swine feeding	LAB	Extrusion	Ca-Alginate	**(Ross et al., 2008)**
Tomato Juice	*L. acidophilus*	Extrusion		**(Tsen et al., 2008)**
Chocolate	*L. helveticus* *B. longum*	Spray-coating		**(Possemiers et al., 2010)**

For example, good quality mayonnaise was obtained when incorporating encapsulated bifidobacteria. Calcium alginate provides protection for bifidobacteria

against the bactericidal effects of vinegar. Other advantages can be quoted when considering the use of encapsulated probiotic cells such as growth inhibition of yeasts over 10 weeks (probably due to the antibacterial effect of the probiotics) and the improvement of mayonnaise's sensory properties (Khalil and Mansour, 1998). McMaster et al. (McMaster and Kokott, 2005) produced beverages and soft foods containing viable encapsulated probiotic cells with a range size of 20–2200 μm by an extrusion method.

The fermentation of banana media by using encapsulated probiotic cells was carried and the obtained product is proposed to be a synbiotic (Tsen et al., 2004). The possibility to use encapsulated probiotic cells in the fermentation of tomato juice was demonstrated. ME allows probiotic cells to survive against the unfavourable pH encountered in tomato juice. Furthermore, the sensory quality of the product has been improved upon incorporation of encapsulated cells compared to free cells (King et al., 2007, Tsen et al., 2008).

The potential use of ME to protect the cells in meat products was investigated (Muthukumarasamy and Holley, 2006, Muthukumarasamy and Holley, 2007). The introduction of encapsulated probiotic cells into dry fermented sausages did not affect sensory properties of the product. Moreover, the viability of encapsulated probiotic cells was improved compared to free cells. In addition, it was shown that probiotics could reduce *E. coli* O157:H7 in number but ME decreased this potential.

The incorporation of probiotic cells encapsulated by spray-coating technology, has been carried out in chocolate (Maillard and Landuyt, 2008). According to these authors, probiotic viability in the small intestine was three times higher when incorporated in chocolate than in dairy product. In this case, probiotic chocolate process was transposed to a larger scale but the challenge here was to obtain a process which is compatible with probiotic survival because high temperatures are required in the usual process. Possemiers et al. (Possemiers et al., 2010) also incorporated encapsulated probiotic cells in chocolate. Results have shown that the introduction of encapsulated probiotic strains into chocolate can be an excellent solution to protect them from environmental stress conditions. In chocolate, the lipid fraction of cocoa butter was shown to be protective for bifidobacteria (Lahtinen et al., 2007).

The encapsulation of probiotic cells in whey protein gel particles could offer protection during processing and storage, as well as extending the food applications to

biscuits, vegetable and frozen cranberry juice. Proteins have a protective effect on probiotics and ME is beneficial because it creates a microenvironment appropriate to survival of the cells against adverse conditions (*e.g.* low pH in cranberry juice) (Reid et al., 2007). Using protein-based technology to encapsulate probiotic cells is an alternative to ME with alginate-type gels or spray-coating which are the two most common encapsulation methods.

2.4.1.5. Importance of food carrier

Various product factors can affect probiotic growth and survival, such as concentration of proteins, sugar and fat, and pH levels (Ranadheera et al., 2010).

Godward and Kailasapathy (Godward and Kailasapathy, 2003a, Godward and Kailasapathy, 2003b, Godward and Kailasapathy, 2003c) applied the same condition to encapsulate probiotic cells but they introduced them into various food matrices: Cheddar cheese, yogurt and ice cream. The conclusion of each study was different. In Cheddar cheese and ice cream encapsulation is not necessary for the survival of the cells whereas it is important when considering yogurt. This showed that the food carrier is important for probiotic survival.

Cheddar cheese and yogurt are both able to protect probiotic cells by giving a favourable environment during manufacturing and storage (Sharp et al., 2008). However, Cheddar cheese seems to be better than yogurt as a delivery food for probiotic because cells were better able to resist the low pH in the stomach.

A potential breakthrough in probiotic encapsulation was reported by investigating the capabilities of various prebiotic fibres to protect the stability and viability of probiotic *L. rhamnosus* strains. In fact, this protection has been revealed during freeze-drying, storage in freeze-dried form and after formulation into apple juice and chocolate-coated breakfast cereals (Saarela et al., 2006). In this case, fibres are considered as prebiotics because they are non-digestible components and they stimulate probiotic viability (Gibson and Roberfroid, 1995). This study highlighted the fact that the optimal carrier for probiotic might depend, in part on how it is eventually used.

Rößle et al. (Rößle et al., 2010) dipped apple wedges into a solution containing probiotic cells. The resulted product was acceptable in terms of quality and the number

of probiotics was sufficient to have a beneficial effect. However, the authors concluded that the use of encapsulation would be useful to enhance probiotic resistance.

The importance of choosing the matrix has been discussed in the study of Klayraung et al. (Klayraung et al., 2009). These authors described how the production of a tablet with lyophilised bacterial cells is carried out. In fact, the matrix influences the survival of the probiotic cells in the stomach. For example, sodium alginate allows higher cell survival in simulated GI conditions and Ross et al. (Ross et al., 2008) highlighted advantages of this technique as its ease of treatment, nontoxic nature and low cost. Efforts have been made to innovate in term of food matrices to incorporate probiotics.

2.4.2. Recent developments in the industrial field

During the past few years, food products containing encapsulated probiotic cells have been introduced on the market (Tableau 5).

Institut Rosell and Lal'food with the Probiocap technology have developed new food products like probiotic chocolate and Probio'Stick. Probio'Stick is an orodispersible powder which contains *Bifidobacterium* and *Lactobacillus* strains. The product allows a reduction of physical symptoms related to stress (Diop et al., 2008), particularly abdominal pain, nausea and vomiting.

In 2007, Barry Callebaut developed a process to produce chocolate containing encapsulated probiotic cells with the Probiocap technology in partnership with Lal'food. According to Barry Callebaut, the addition of encapsulated probiotic cells has no influence on chocolate taste, texture and mouth feel. A consumption of 13.5 g per day of probiotic chocolate seems to be sufficient to ensure the balance of the intestinal microflora.

Chocolate has also been used by DSM Food Specialities which produces a bar called Attune launched in the United States in January 2007. The product also contains the prebiotic inulin which supports a healthy digestive function. Attune's innovative product line is found in the refrigerated yogurt section and advertising of this product highlights more input in calcium, fibre and less sugar than in most yogurts (www.attunefoods.com).

Tableau 5 : Examples of the use of encapsulated probiotics for industrial applications.

Food Product	Company	Observations
Probiotic Chocolate	Institut Rosell & Lal'food	Using the Probiocap® ME technology
Probio'stick		
Innovance Probiotique	Ysonut Laboratories	
Yogurt **Nutrient Bars** **Tablets**	Balchem Encapsulates & Institut Rosell	
Chewing gum	Cell Biotech	
Cernivet® LBC ME10	Cerbios-Pharma SA	Using for animal food
Bio-*tract*® tablets	Nutraceutix	
Probio-Tec® capsules	Chr Hansen	
Probiotic whey drink		German market
Orange juice "Dawn"	Chr Hansen & Kerry Group	Irish market
Probiotic ice cream	Dos Pinos	Central American Industry
Doctor-Capsule (Yogurt)	Bingrae Co.Kyunggi-do	Korean market
Attune (Chocolate)	DMS Food Specialities	American market
Yogurt	Jinta Capsule Technology	
Bifina-constipation		Pharmaceutical product
Geneflora™	BioPlus Corporation	Symbiotic product
Granio+reducys®	EA Pharma®	Combination of probiotic strain and Cranberry
ThreeLac™	GHT™ Global Health Trax	Powder sprinkled into the mouth

After two years of collaboration, Balchem Encapsulates and Institut Rosell, have developed a stabilised form of encapsulated probiotics (Balchem newsletter, August 16, 2001). Institut Rosell has incorporated encapsulated probiotic cells into yogurt-covered raisins, nutrient bars, chocolate bars and tablets (Siuta-Cruce and Goulet, 2001). According to information available on the website of Balchem, the clinical testing has revealed ''an unprecedented 100 % delivery rate''. The tests carried out on chocolate bars also revealed a high recovery rate (www.balchem.com).

Chr Hansen distributes Probio-Tec capsules for dietary supplement, infant formula and pharmaceutical industry (www.chr-hansen.com). With the Kerry Group in Ireland,

they have developed the first probiotic orange juice ''Dawn'' for the Irish market. According to these companies, the probiotic cells remain viable throughout the product's shelf life. The use of encapsulated probiotic cells can be better suited to survive harsh conditions in juices (www.chr-hansen.com).

In Latin America, Chr Hansen and Dos Pinos, one of the top players in Central American ice cream industry, have developed a probiotic ice cream. The product is described as an innovative yogurt ice cream with a number of health benefits (www.chr-hansen.com). However, a probiotic ice cream has already been launched by Unilever in 1999 but at that time, consumers were unwilling to accept this innovation. Thus, taking into consideration the expectations of consumers is an important factor in creating new probiotic food products.

In Korea, yogurts containing encapsulated LAB are available on the market under the brand name Doctor-Capsule (Bingrae Co.Kyunggi-do) as described by Lee and Heo (Lee and Heo, 2000). Jinta Capsule Technology markets a range of products containing encapsulated probiotic cells. For example, yogurt containing encapsulated bifidobacteria and bifina-constipation a pharmaceutical product which contains encapsulated bifidobacteria available in a capsule form.

Most of the products containing encapsulated probiotic cells are available in a tablet/capsule form (Forever Active Probiotic, Probiotic 7, Multi-probiotic) or in a powder form (PureBaby Probiotic, ThreeLac™). ThreeLac™ proposed by GHT™ Global Health Trax, is a powder containing encapsulated probiotic cells, and which has to be sprinkle into the mouth. Encapsulation technology used here ensure the probiotic cells to get through the hostile stomach acids to reach the intestine (www.ghthealth.com).

Ysonut Laboratories markets Innovance Probiotiques, a nutraceutical with probiotic cells encapsulated by the Probiocap technology. This product contains a mix of three probiotic strains produced by Institut Rosell (www.ysonut.com).

Cernivet LBC ME10 distributed by Cerbios-Pharma SA is a pelletable microbial feed for the stabilisation of intestinal microflora and containing the encapsulated probiotic strain *E. faecium* SF68. The product is registered for use in animal food, for example for chicken and pig fattening. The shelf life of Cernivet LBC ME10 covers a period of 24 months if stored at refrigerated temperature (www.cerbios.ch).

Nutraceutix is an American company which provides probiotic cells in a variety of forms, from bulk powder to capsules and advanced tablets. Patented Bio-tract tablets

gives protection for probiotic living cells against adverse conditions in the stomach (low pH) (www.nutraceutix.net).

America's BioPlus Corporation proposed Geneflora™, a symbiotic containing encapsulated *Lactobacillus sporogenes* as a probiotic and a fructo-oligosaccharide as a prebiotic (www.yeastbuster.com).

Capsules containing cranberry (Granio + reducys) were marketed by EA Pharma and present recognised effects on urinary disorders as cystitis. In this product, encapsulated probiotic cells have been incorporated and the chosen strain has positive effects on urinary flora. The combination of these two ingredients makes this product particularly useful in preventing cystitis (www.ea-pharma.com).

Nowadays, encapsulated probiotic cells are essentially introduced in nutraceutical products, but efforts have been made to develop novel food as an ideal carrier for the bacteria.

2.5. Future trends

The ME technology has been explored by many companies as a way of enhancing the resistance of probiotic cells in the GI tract and for prolonging the shelf-life of bacterial strains in food products.

In most cases, to encapsulate probiotic living cells, natural biopolymer such as alginate, κ-carrageenan or gellan gum have been used. However, although the results are promising on a laboratory scale, the technologies used present difficulties for scaling-up. For example, in the case of extrusion methods, low production capacities and large particle sizes have been reported. Regarding the emulsion method, large-size dispersions have been obtained. Another major problem is that the addition of some polysaccharides is not allowed in dairy products in some European countries (Picot and Lacroix, 2004).

Most of the foods containing probiotic microorganisms are found in the refrigerated section of supermarkets this being due to the fact that the bacteria are sensitive and can be destroyed by heat. Thus, the dairy sector has a major advantage in probiotic foods. Nevertheless, the researches focus actually on expanding the food categories currently available.

ME has to face many challenges for its application on an industrial scale. On one hand, technological challenges to obtain microcapsules with the best properties must be

enhanced. On the other hand, consumer behaviour towards novel foods should be taken into account. ME can achieve a wide variety of functionalities according to the development of the technology and nowadays, encapsulated probiotic cells can be incorporated in many types of food products. In fact, probiotics can be found not only in dairy products, but also in chocolate or cereals too.

An important challenge for probiotic encapsulation is to reduce the particle size because it can negatively affect the textural and the sensorial properties of the product. In laboratory applications, the chosen method is generally emulsification but this technique presents disadvantages for food applications for many reasons. The presence of residual oil on capsule surface is detrimental to the texture and the organoleptic properties of the product. The presence of residual oil hampers capsules incorporation in diet products and the residual oil, surfactant, or emulsifier can be toxic for probiotic cells.

The incorporation of probiotic cells into a wider range food product is limited by an unsuitable food matrix (e.g. low pH or competing microbes) and the non-optimal storage conditions. During the past few years, the use of prebiotics and fibre as protectant for probiotic cells has gained increasing interest.

The development of novel functional foods is a major challenge to address the expectation of consumers who expect healthy and beneficial food products for their health. Industries and laboratories have now to provide possible technologies to an industrial scale with adequate cost. The last decade, efforts were done to improve relations between these two entities. Nevertheless, further researches have to be carried out to optimize the use of encapsulated probiotic cells while considering numerous factors as safety and ecological production. In conclusion, it is evident that the probiotic market has a strong future as the consumers demand is increasing. As benefits provided by probiotics are now well documented, consumer requirements for food, beverage and supplement products enriched with these ingredients will increase.

3. Matériel et méthodes

3.1. Protéines laitières et enzymes

Les caséines micellaires ont été fournies par International Dairy Ingredient (IDI SAS, Arras, France). Elles sont obtenues par microfiltration et diafiltration de lait écrémé, suivies d'une atomisation. La poudre contient 0,3 % de matière grasse et environ 88 % de protéines (Karam et al., 2012). Les isolats de protéines du lactosérum natives proviennent de Lactalis (Lactalis Ingrédients, Bourgbarré, France). L'obtention des protéines du lactosérum dénaturées est réalisée de la façon suivante : 200 ml d'une solution de protéines solubles natives sont portés à une température de 78 °C durant 10 min. Le passage d'une solution translucide à une solution blanche opaque est le témoin visuel de la dénaturation thermique des protéines.

Pour la coagulation enzymatique des protéines laitières à l'échelle laboratoire, la présure Naturen™ (Christian Hansen, Hambourg, Allemagne), avec une activité déclarée de 140 IMCU/mL, a été utilisée. Une solution stock de présure est préparée quotidiennement en diluant 1 g de la solution dans 4 g d'eau distillée.

Concernant la coagulation enzymatique des protéines laitières à l'échelle pilote, la chymosine CHY-MAX™ Plus (Christian Hansen, Hørsholm, Denmark), avec une activité déclarée de 199 IMCU/mL, a été utilisée. Une solution stock est préparée quotidiennement en diluant 100 fois la solution initiale dans de l'eau distillée.

Le Tween® 80 a été acheté chez Sigma-Aldrich et l'huile de tournesol dans un supermarché local.

3.2. Croissance des bactéries probiotiques

La souche probiotique utilisée au cours de cette étude, *Lactobacillus rhamnosus* GG (ATCC 53103) (LGG), nous a été fournie par Dr Sara Lebeer et Pr Jos Vanderleyen de K.U. Leuven. Une collaboration a été mise en place entre les deux équipes.

Dès la réception de la souche, son intérêt à être encapsulée a été vérifié (perte d'un grand nombre de bactéries durant la simulation des conditions gastriques).

La souche bactérienne a été stockée à -80 °C dans du milieu MRS (De Man et al., 1960) contenant 20 % de glycérol (v/v) afin de conserver un stock suffisant que ce soit en termes de quantité que de qualité.

3.2.1. Culture statique

Une pré-culture est tout d'abord réalisée en inoculant 9 ml de bouillon MRS avec 100 µL de culture stock. Cette pré-culture est ensuite utilisée pour inoculer du milieu MRS frais. La culture des bactéries est ensuite conduite jusqu'à la fin de la phase exponentielle de croissance quand la DO_{660} atteint 1.2 (Figure 11).

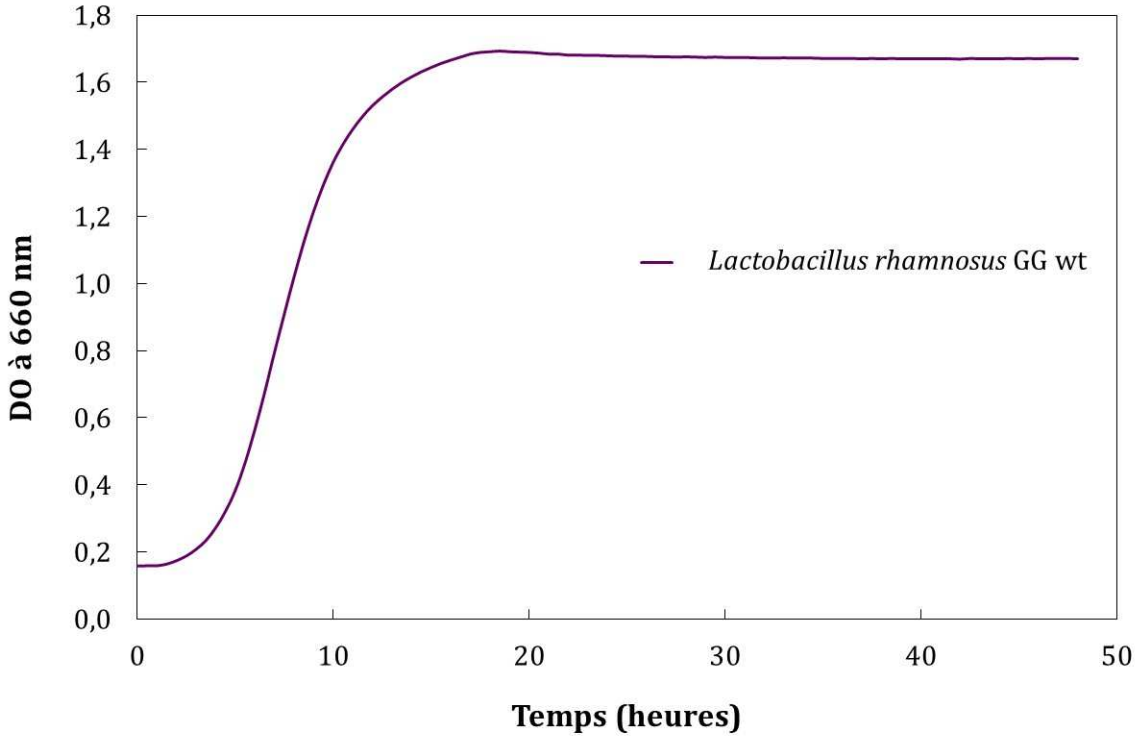


Figure 11: Suivi de la DO_{660} au cours de la croissance de LGG wt et de LGR-1.

La récupération des cellules se fait par centrifugation (3000 g, 10 min, 20 °C) puis, le culot est lavé grâce à de l'eau physiologique (pH 7,4) et une nouvelle centrifugation est réalisée dans les mêmes conditions que la précédente. Le culot est ensuite congelé dans de l'azote liquide puis lyophilisé (Christ alpha 1-2, freeze-dryer, Osterode, Allemagne). Le lyophilisat obtenu présente une concentration en bactéries de 10^{11} UFC/g.

3.2.2. Culture en fermenteur

Lors du passage de l'encapsulation à l'échelle laboratoire vers l'encapsulation à l'échelle pilote, la quantité de bactéries produite en culture statique n'était plus suffisante. C'est la raison pour laquelle une culture en fermenteur a été mise au point.

3.2.2.1. Préparation de la fermentation

La fermentation est réalisée dans un réacteur de deux litres selon un mode d'alimentation par batch et nécessite la préparation de la culture bactérienne (pré-culture puis culture) et la stérilisation préalable du réacteur.

La pré-culture est réalisée en inoculant 50 mL de bouillon MRS avec 1 mL de solution stock de cellules. Les pré-cultures sont incubées à 37 °C durant une nuit sous agitation (150 RPM).

La veille du lancement de la fermentation, le réacteur (Figure 12) contenant le milieu de culture (1,3 L de MRS liquide) et l'anti mousse (500 µL) ainsi qu'une bouteille contenant de la soude à 6 $mol.L^{-1}$ sont stérilisés en autoclave (Lequeux, Paris, France) durant 15 min à 120 °C.

Figure 12: Fermenteur utilisé pour la production de bactéries probiotiques.

3.2.2.2. Suivi de la fermentation

Le bioréacteur (Setric Genie Industriel, Toulouse, France) est relié à une console de contrôle permettant de suivre la température, le pH, la vitesse d'agitation et la quantité d'oxygène dissous dans le milieu de culture. Avant de commencer la fermentation, les sondes de température, de pH et d'oxygène sont connectées et calibrées. Pour la sonde à oxygène, le zéro est fait en faisant buller de l'azote dans le milieu et le 100 % en faisant buller de l'air via la pompe. L'alimentation en oxygène se fait par l'intermédiaire d'un spargeur. Un cryostat permet de refroidir le milieu tandis qu'une résistance permet de le chauffer : la combinaison des deux permet de maintenir la température à 37 °C.

Les bactéries lactiques étant acidifiantes, l'ajout régulier, à l'aide d'une pompe péristaltique, d'une solution de NaOH à 6 mol.L^{-1} permet de maintenir le pH à une valeur de 6,5, tandis l'agitation du milieu est maintenue à 300 RPM.

Après stérilisation, la pré-culture est transférée stérilement dans une fiole Schott connectée au réacteur. L'ensemencement a lieu en pompant la pré-culture grâce à la pompe péristaltique. L'ensemencement correspond au temps zéro de la fermentation et la DO_{660} est vérifiée à ce moment.

Des prélèvements stériles du milieu de culture sont régulièrement effectués de façon à mesurer sa turbidité (DO_{660}) et donc de connaitre la phase de croissance atteinte par les bactéries (Figure 13). Pour cela, les mesures sont réalisées à l'aide d'un spectrophotomètre (Genesys 20, Thermoscientific, United States) à une longueur d'onde de 660 nm. Ainsi, lorsque la phase stationnaire est atteinte, la fermentation est stoppée.

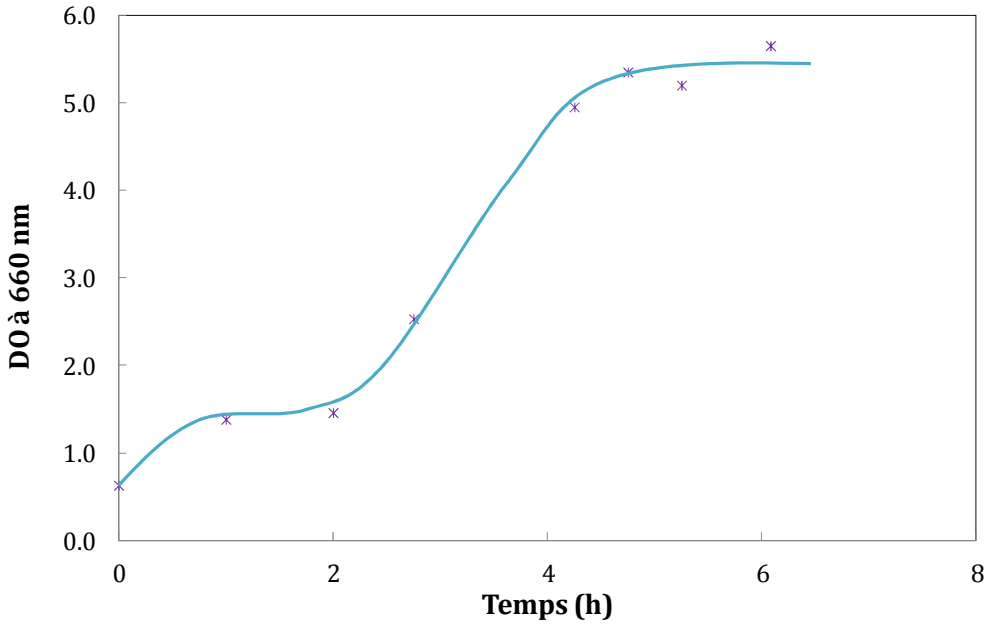


Figure 13: Evolution de la DO_{660} en fonction du temps lors de la fermentation de LGG wt en bioréacteur.

A la fin de la fermentation, le bioréacteur est vidé stérilement de son contenu par la canule de prélèvement, dans un contenant lui aussi stérile. Le milieu est centrifugé (3000 g, 10 min, 20 °C) puis, le culot est lavé grâce à de l'eau physiologique (pH 7,4) et une nouvelle centrifugation est réalisée dans les mêmes conditions que la précédente. Le culot est ensuite congelé dans de l'azote liquide puis lyophilisé (Christ alpha 1-2, freeze-dryer, Osterode, Allemagne).

3.2.2.3. Relation entre la DO_{660} et la concentration massique

Généralement, une densité bactérienne est exprimée en gramme de biomasse par litre de milieu, et non en valeur de densité optique. Pour obtenir la correspondance, il convient de réaliser une courbe d'étalonnage de la DO en fonction de la concentration massique en biomasse (Figure 14).

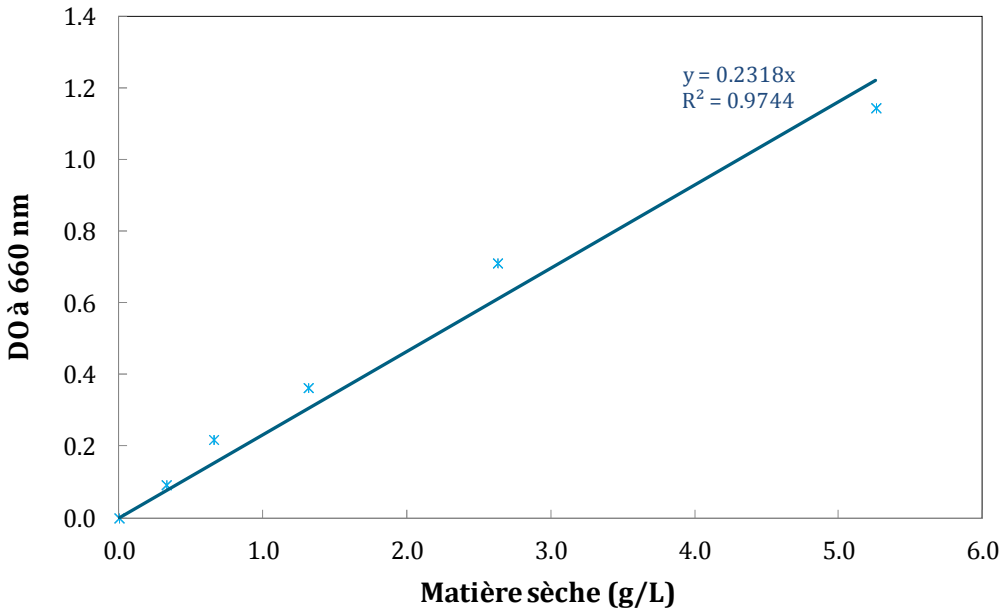


Figure 14: Corrélation entre la quantité de matière sèche et la DO_{660} au cours de la fermentation de LGG wt.

Pour cela, trois creusets sont mis à sécher à 103 °C durant une nuit. La culture bactérienne (40 mL) ayant une DO_{660} comprise entre 1,5 et 2 est filtrée à travers un filtre d'acétate de cellulose ayant une porosité de 0,2 µm. Les bactéries sont récupérées dans 40 mL d'eau physiologique et remises en suspension. Cette solution correspond à la solution mère. Cette solution (5 mL) est répartie dans chacun des trois creusets et est placée à l'étuve à 103 °C durant 24 h. Pendant ce temps, des dilutions de la solution mère sont réalisées et la valeur de la DO_{660} est relevée. Au bout des 24 h, la masse des creusets est relevée et la masse de matière sèche présente dans chacun d'eux peut être déduite. Il convient cependant de retrancher à cette masse la masse de matière sèche apportée par les 5 ml d'eau physiologique. Connaissant la DO_{660} des différentes dilutions, il est possible d'en déduire leur masse de matière sèche et donc de tracer une courbe d'étalonnage de la DO_{660} en fonction de la concentration massique (Figure 14).

L'équation de la droite nous permet alors d'en déduire l'évolution de la concentration massique en biomasse tout au long de la fermentation.

3.3. Matériel d'encapsulation

3.3.1. Echelle laboratoire

Le réacteur thermostaté utilisé pour l'encapsulation à l'échelle laboratoire possède une capacité d'environ 0,7 L (Figure 15).

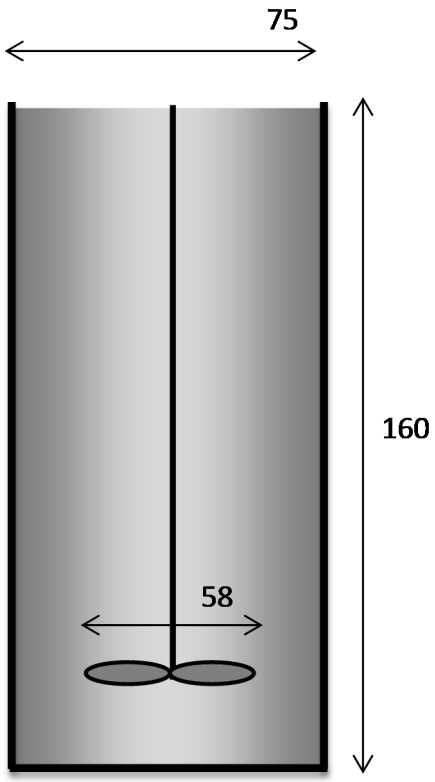


Figure 15: Dimensions caractéristiques (en mm) du réacteur utilisé à l'échelle laboratoire.

Pour la réalisation de l'émulsification, une turbine de Rushton à 6 pales est entrainée par un moteur (Lightin® LabMaster). La régulation de la température du réacteur se fait grâce au passage d'eau dans sa double paroi (Thermo HAAKE K20, Thermo Electron Corporation). Pour la séparation des microparticules de l'huile, une centrifugation a été requise (SW12, Firlabo).

3.3.2. Echelle pilote

Deux réacteurs principaux sont utilisés pour l'encapsulation à l'échelle pilote : un réacteur pour la réaction enzymatique et un réacteur d'émulsification (Figure 16).

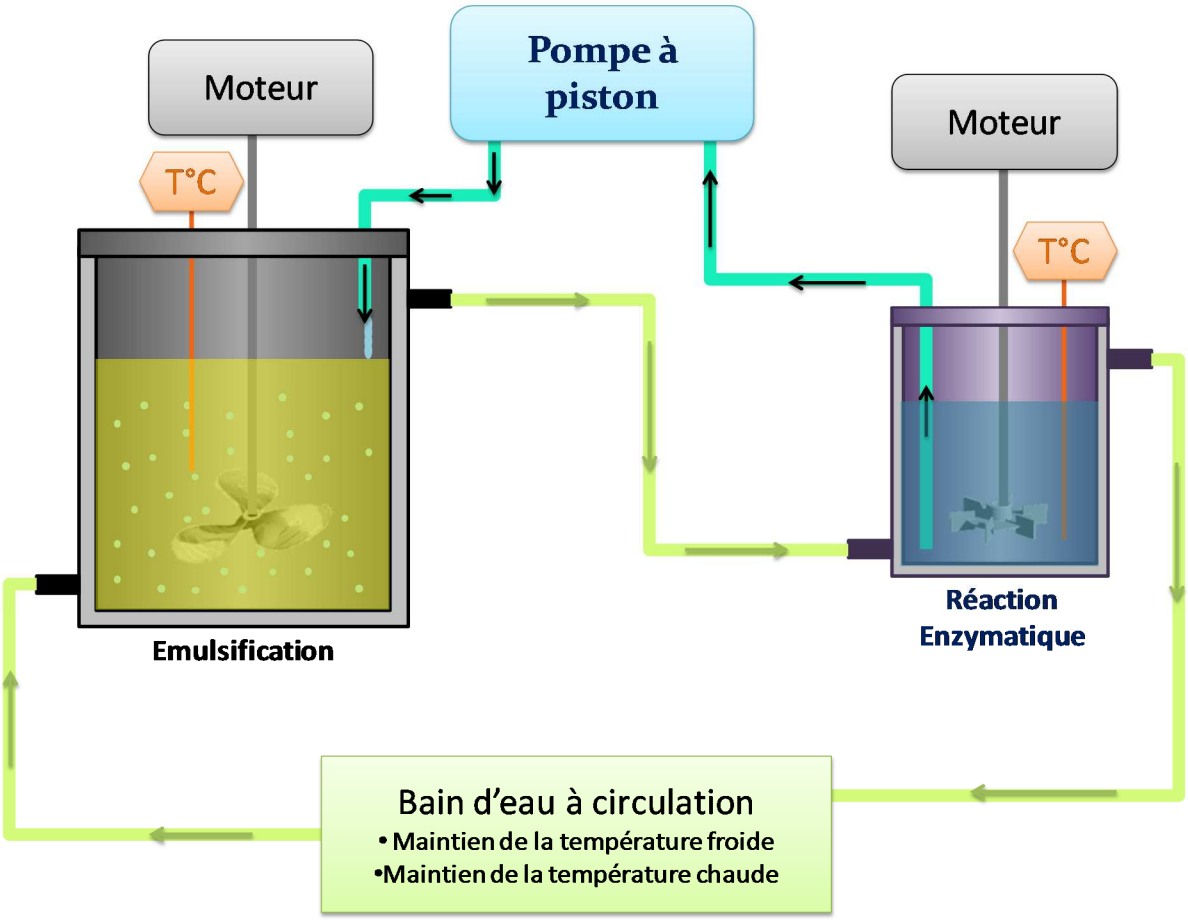


Figure 16 : Schéma général de l'organisation du matériel pour l'encapsulation à l'échelle pilote.

Un premier réacteur thermostaté, fermé et agité, d'une capacité de 0,7 L, est utilisé pour la réalisation de la phase enzymatique. L'agitation est réalisée au moyen d'une turbine de Rushton à 6 pales couplée à un moteur (Lightin® LabMaster) dont la rotation est fixée à 150 RPM.

Pour le transfert de la solution contenue dans le réacteur où se déroule la réaction enzymatique vers celui où se déroule l'émulsification, une pompe de type piston (FMI lab pump, Modèle QG-400) a été utilisée.

Un second réacteur thermostaté, fermé et agité, d'une capacité de 1,5 L, est utilisé pour l'émulsification. Ses caractéristiques sont résumées sur la Figure 17.

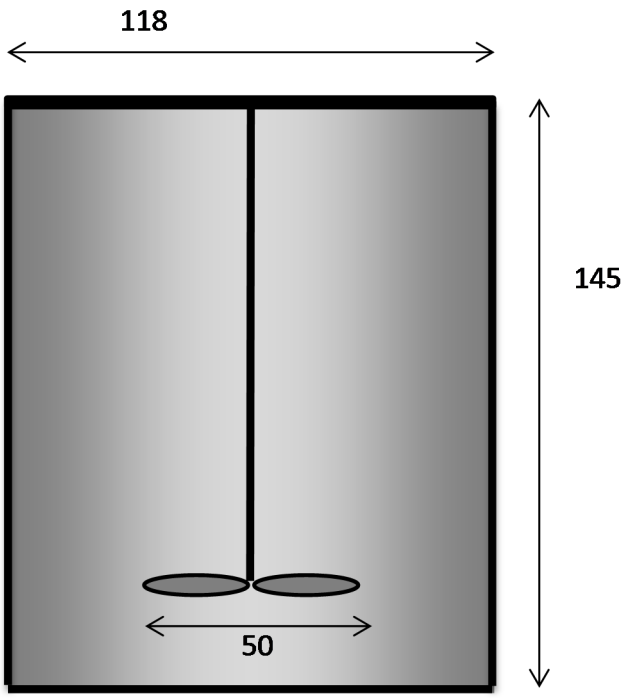


Figure 17: Dimensions caractéristiques (en mm) du réacteur d'émulsification à l'échelle pilote.

La régulation de la température au sein des deux réacteurs se fait grâce au passage d'eau dans leur double paroi (Lauda Eco RE 415, Modèle LBT0277-11-02).

3.4. Suivi et optimisation de la phase primaire enzymatique

La réaction de coagulation du lait peut être divisée en trois processus cinétiques principaux. Le premier est la réaction enzymatique qui consiste en une hydrolyse de la caséine-κ, le second est un processus non-enzymatique de floculation des micelles de caséine et le troisième consiste en la formation de liaisons au sein du gel (Carlson et al., 1987).

La présure est un complexe enzymatique protéolytique essentiellement constitué de chymosine (EC.3.4.23.4). Cette enzyme est une aspartyl protéinase, c'est-à-dire une enzyme protéolytique caractérisée par deux résidus aspartiques au niveau du site actif. La molécule est constituée de deux lobes et d'une vaste fissure où se situe le site actif.
Au cours de la phase primaire, la chymosine clive la liaison Phe_{105} – Met_{106} de la caséine-κ. La vitesse d'hydrolyse de cette liaison étant 1000 fois plus rapide que l'action de l'enzyme sur tout autre substrat, on peut considérer qu'il s'agit d'une réaction enzymatique à un seul substrat et ainsi retenir la théorie de Michaëlis-Menten retenue

pour cette cinétique (Fox, 1984, Hyslop, 2003). La réaction peut donc être écrite de la façon suivante :

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_{cat}} P + M + E$$

E : Enzyme (la chymosine)

S : Substrat de l'enzyme (la caséine- κ)

ES : Complexe formé entre l'enzyme et le substrat

P : Produit de la réaction : la para-caséine- κ

M : Produit de la réaction : le CMP (Caseino-macro-peptide)

$\mathbf{k_1}$ et $\mathbf{k_2}$: Constantes associées à l'état pré-stationnaire

La présure est utilisée en fromagerie, elle permet de libérer le CMP par clivage de la liaison Phe_{105}-Met_{106} de la caséine- κ, située à la surface des micelles de caséine dans le lait. Son activité optimale se situe vers 40 °C et à un pH voisin de 5,5. La libération de ce peptide entraîne une diminution de la charge nette négative et une augmentation de l'hydrophobicité de la para- κ -caséine, ce qui va conduire à l'agrégation des micelles de caséine.

Toutefois, si la présure est mise au contact du lait à faible température, la caséine-κ est clivée mais les micelles ne coagulent pas ou très peu, et ce n'est qu'en augmentant la température que la formation du gel sera rendue possible. Ce découplage de la phase primaire enzymatique et de la phase secondaire non-enzymatique peut être utilisé pour la mise au point d'un système d'encapsulation où la coagulation serait déclenchée au moment adéquat par une augmentation de la température (Carlson et al., 1987, Bansal et al., 2007).

Dans le but de quantifier le CMP libéré au cours de la réaction primaire, la méthode de Kjeldahl a été utilisée (AOAC, 1984). En effet, le CMP est un peptide retrouvé dans l'azote non protéique (ANP) qui correspond au filtrat obtenu après précipitation et filtration des protéines laitières par de l'acide trichloroacétique à 6 %. L'ANP étant contenu dans d'autres molécules que le CMP, la quantité d'ANP avant incubation avec la présure sera systématiquement soustrait à l'ANP mesuré après incubation. On considère donc que l'augmentation de l'ANP au cours de l'incubation est due à la libération du CMP par la présure.

Dans le but d'optimiser la phase enzymatique primaire, le CMP libéré a été quantifié en fonction de plusieurs paramètres. Un plan d'expériences a été utilisé afin

d'organiser les essais de manière à obtenir un maximum de renseignements avec un minimum d'expériences.

La construction et l'analyse du plan d'expériences a été possible grâce à l'utilisation du logiciel NemrodW (LPRAI, Marseille).

Pour la réalisation du plan d'expériences, le choix s'est porté sur une matrice de Doehlert où les points expérimentaux sont situés sur une sphère de rayon 1. Les trois paramètres étudiés sont : la quantité de protéines, la quantité d'enzyme et la température d'incubation. Le domaine expérimental et les niveaux des facteurs sont présentés dans le Tableau 6.

Tableau 6: Domaine expérimental et niveaux des facteurs pour la matrice de Doelhert.

Facteurs	**Niveaux des facteurs**						
Caséines Micellaires (%)		7,7	9,3	10,8	12,4	13,9	
Enzyme (µL/g)	18	21	24	27	30	33	36
Température (°C)			1	5	9		

La réponse mesurée est la quantité de CMP libérée après clivage de la caséine-κ par la présure. L'objectif est de trouver la combinaison des trois paramètres qui permet la libération la plus importante de CMP.

Le premier facteur concerne les protéines, c'est-à-dire le substrat de la présure. Dans le plan d'expériences les valeurs indiquées correspondent à la quantité de poudre (en g) ajoutée à 100 g d'eau. Le second facteur concerne la quantité d'enzyme utilisée lors de l'incubation. Il s'agit ici de la quantité en microlitres d'enzyme prélevée dans la solution stock par gramme de protéines laitières. Enfin, le troisième paramètre concerne la température à laquelle l'incubation est réalisée et est exprimée en degrés Celsius.

Une fois les caséines micellaires réhydratées, la solution est maintenue à la température souhaitée et la présure est ajoutée. L'incubation dure 60 min et la réaction est stoppée par l'ajout d'acide trichloroacétique à 6 %. La détermination de l'ANP retrouvé dans le filtrat est réalisée selon la méthode Kjeldahl.

3.5. Rhéologie : analyse viscoélastique

3.5.1. Principe

Les liquides présentent des propriétés visqueuses mais également des propriétés élastiques si la structure est complexe. L'élasticité est observée en appliquant ou en supprimant brutalement un cisaillement, il apparait alors un régime transitoire pendant lequel la structure de l'échantillon évolue avant de se stabiliser.

L'analyse viscoélastique est un test qui consiste à imposer à l'échantillon un cisaillement oscillatoire. Au cours de ce mouvement périodique, la contrainte et la vitesse de cisaillement évoluent sinusoïdalement au cours du temps avec la même pulsation mais en présentant un déphasage φ l'une par rapport à l'autre. L'analyse de ces signaux sinusoïdaux permet de définir des grandeurs viscoélastiques dynamiques caractéristiques de l'échantillon. Tout d'abord, le déphasage δ entre la contrainte et la déformation de cisaillement peut être relié à φ par la relation suivante : $\delta = (\pi/2) - \varphi$. En pratique, deux grandeurs fondamentales de la rhéologie dynamique sont utilisées, il s'agit du module de conservation G' et du module de perte G''. Ces deux grandeurs peuvent être reliées au déphasage δ qui vient d'être défini : $G' = G^* * \cos\delta$ et $G'' = G^* * \sin\delta$, où G* représente le module de cisaillement. Les modules G' et G'' ont la dimension d'une contrainte et s'expriment donc en Pascal (Pa). L'énergie élastique emmagasinée et restituée au cours d'une période est proportionnelle à G', ce module décrit donc les propriétés élastiques de l'échantillon. L'énergie dissipée par frottement visqueux au cours d'un même cycle est proportionnelle à G'', ce module décrit donc les propriétés visqueuses de l'échantillon. Ainsi, le déphasage δ est relié à ces deux modules par la relation : $\tan\delta = G''/G'$, déphasage qui peut varier de 0° (solide élastique, G''= 0) à 90° (liquide visqueux, G'= 0). La valeur de δ (aussi appelé angle de perte) indique le partage entre les propriétés élastiques et visqueuses de l'échantillon : pour $0° < \delta < 45°$ l'échantillon a un comportement plus élastique que visqueux alors que c'est l'inverse pour $45° < \delta < 90°$ (Grossiord and Quemada, 2012) .

3.5.2. Appareillage

Les mesures ont été réalisées grâce au rhéomètre rotationnel Kinexus Pro (Malvern Instruments, KNX 2100, UK). L'appareil est équipé d'un cylindre coaxial avec une base conique et est utilisé en mode oscillatoire à une fréquence de 1 Hertz et une contrainte de 0,06 dans la gamme linéaire de viscoélasticité. L'avantage de l'appareil est

que la montée et la descente en température sont rapides et précises (0,01 °C) grâce à l'effet Peltier.

Dans le but de caractériser le processus de coagulation, des analyses viscoélastiques ont été réalisées sur des échantillons de protéines laitières. Pour cela, les modules d'élasticité (G') et de viscosité (G'') ont été enregistrés en fonction du temps et de la température.

3.6. Suivi conductimétrique

La conductivité de l'émulsion dans le réacteur d'émulsification a été mesurée grâce à une électrode (Schott-Geräte GmbH, Germany) reliée à un conductimètre (Tacussel, CDRV 62 ENR).

3.7. Analyse granulo-morphométrique

Les microparticules ont été analysées grâce au granulo-morphomètre (Qicpic, Sympatec Inc.Clausthal-Zellerfeld, Allemagne) équipé du module de dispersion Lixell et du logiciel Windox5.0 (Sympatec Inc.Clausthal-Zellerfeld, Allemagne). L'appareil utilise l'analyse d'images dynamiques pour déterminer la taille et la forme des microparticules (Köhler et al., 2008). Pour réaliser les mesures, les microparticules ont été dispersées dans de l'eau.

Le diamètre EQPC des particules représente le diamètre d'un cercle qui a la même aire que celle projetée par la particule. A partir de ce diamètre, la sphéricité peut être déterminée ; il s'agit du rapport du périmètre équivalent du cercle sur le périmètre réel. La valeur de la sphéricité varie entre 0 et 1 et, plus la valeur est faible plus la particule présente une forme irrégulière. La convexité est un paramètre important puisqu'elle représente la compacité de la particule. Théoriquement, la convexité peut avoir une valeur maximale de 1 s'il n'existe aucune région concave. Toutefois, en raison de la conception du détecteur (pixels carrés), toutes les particules semblent présenter une petite région concave. Par conséquent, le maximum de convexité mesuré sera limité à 0,99.

4. Procédé d'encapsulation à l'échelle laboratoire

Le choix de la méthode d'encapsulation des bactéries probiotiques s'est porté sur l'émulsification et en particulier la production des microparticules a été réalisée au moyen d'une coagulation enzymatique de protéines laitières.

Selon ce procédé, les proportions finales des phases aqueuse et huileuse sont directement ajoutées dans le réacteur puis, l'agitation est mise en place afin d'obtenir l'émulsion. Cette méthode est communément désignée comme la méthode standard et semble être la plus simple à mettre en œuvre pour produire une émulsion à petite échelle. Cependant le temps d'agitation ainsi que la position de l'agitateur sont deux facteurs qui influencent le résultat. En effet, il a été vérifié que la position de l'agitateur détermine le type d'émulsion et ce, surtout dans les systèmes en absence de tensioactif comme c'est le cas ici. La phase dans laquelle est placé l'agitateur devient la phase continue. Ainsi si l'agitateur se trouve dans la phase huileuse, l'émulsion formée sera de type eau dans huile (E/H).

4.1. Optimisation de la phase primaire enzymatique

Les résultats pour le dosage du CMP libéré en fonction des paramètres donnés par le plan d'expériences sont présentés dans le Tableau 7.

Les résultats peuvent être divisés en trois groupes:

- les points de 1 à 12 permettent la construction du modèle,
- les points de 13 à 15 sont les répétitions au centre,
- et finalement des points tests (de 16 à 19) ont été ajoutés.

Tout d'abord, les trois répétitions au centre ont des résultats proches ce qui nous permet de dire que les expériences sont répétables.

Pour valider le modèle, il faut comparer les valeurs expérimentales des points tests aux valeurs calculées à partir du modèle. Dans notre cas, les valeurs calculées et les valeurs mesurées sont proches, le modèle proposé est donc validé. On peut conclure que le modèle proposé représente bien la réponse expérimentale dans le domaine expérimental étudié. Ainsi, il est possible d'estimer en n'importe quel point du domaine expérimental d'intérêt, la valeur de la réponse avec une qualité acceptable.

Les statistiques des coefficients dépendent du modèle, des résultats obtenus aux points expérimentaux et du nombre de points expérimentaux. L'analyse de la variance

nous permet de valider le modèle aux points expérimentaux mais pas dans tout le domaine d'intérêt (Tableau 8).

Tableau 7: Matrice d'expériences et résultats obtenus pour chaque essai : Réponse expérimentale et réponse calculée d'après le modèle.

N°Essai	Caséines micellaires (%)	Enzyme (µL/g)	Température (°C)	CMP_{exp} (mg/L)	CMP_{calc} (mg/L)
1	13,9	27	5	331	327
2	7,7	27	5	200	204
3	12,4	36	5	353	359
4	9,3	18	5	305	299
5	12,4	18	5	330	337
6	9,3	36	5	281	274
7	12,4	30	9	417	415
8	9,3	24	1	292	295
9	12,4	24	1	302	298
10	10,8	33	1	287	288
11	9,3	30	9	291	295
12	10,8	21	9	349	348
13	10,8	27	5	308	308
14	10,8	27	5	308	308
15	10,8	27	5	309	308
16	9,5	25	4	288	281
17	12,1	25	4	313	318
18	10,8	32	4	301	306
19	10,8	27	8	322	333

Tableau 8: Analyse de la variance pour l'étude de la libération du CMP.

Source de variation	Somme des carrés	Degrés de liberté	Carré moyen	Rapport	Significativité
Régression	$3,0 * 10^4$	9	$3,4 * 10^3$	10067	***
Résidus	$4,3 * 10^2$	9	$4,8 * 10^1$		
Validité	$4,3 * 10^2$	7	$6,1 * 10^1$	183	**
Erreur	$6,7 * 10^{-1}$	2	$3,3 * 10^{-1}$		
Total	$3,1 * 10^4$	18			

* P < 0,05 ; ** P < 0,01 ; *** P < 0,001

Les coefficients (Tableau 9) révèlent que la quantité de protéines a une forte influence sur la quantité de CMP libéré, c'est-à-dire que plus on met de protéines plus on libère de CMP, ce qui est logique. En revanche, la quantité d'enzyme utilisée a peu d'influence sur la réponse. Enfin, la température a une forte influence sur la réponse et tout comme pour les protéines, plus on élève la température meilleure est la réponse, ce qui parait logique puisque la réaction primaire de libération du CMP possède un Q_{10} compris entre 2 et 4 (Bansal et al., 2007).

Tableau 9 : Estimations et statistiques des coefficients pour l'étude de la libération du CMP.

Nom	Coefficient	Significativité
b0 (Moyenne)	306,6	***
b1 (Proteines)	60,8	***
b2 (Enzyme)	-1,6	*
b3 (Température)	34,8	***
b1-1	-40,5	***
b2-2	27,9	***
b3-3	27,0	***
b1-2	28,2	***
b1-3	62,2	***
b2-3	35,4	***
Ecart Type de la réponse	0,58	
R^2	0,986	

Les interactions entre les facteurs sont toutes positives ce qui traduit une synergie des facteurs pour maximiser la réponse.

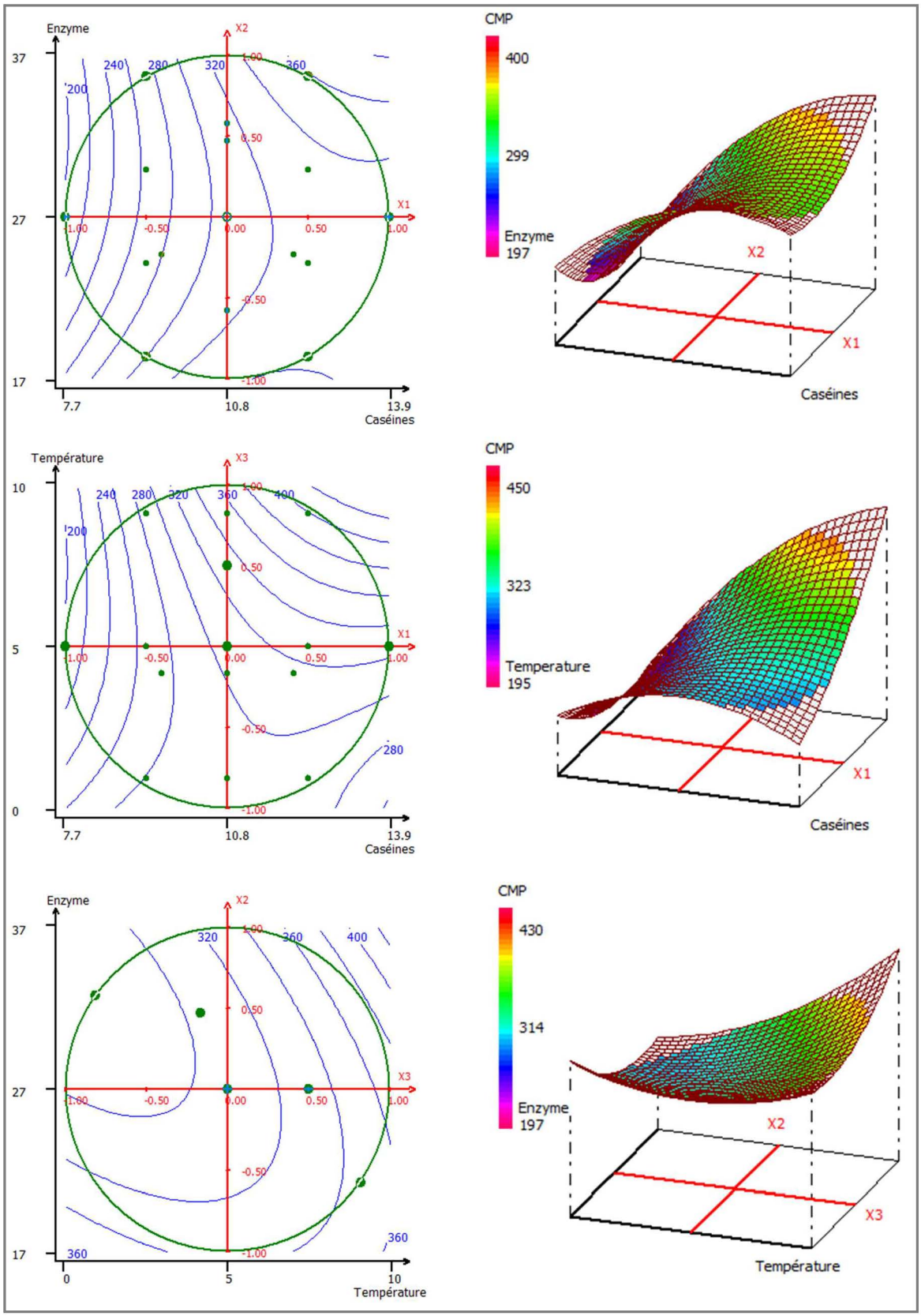


Figure 18: Courbes d'iso-réponses et surface de réponse pour la libération du CMP.

L'étude du chemin optimal (Figure 19) permet de connaître les valeurs des paramètres pour lesquelles une réponse maximale sera obtenue.

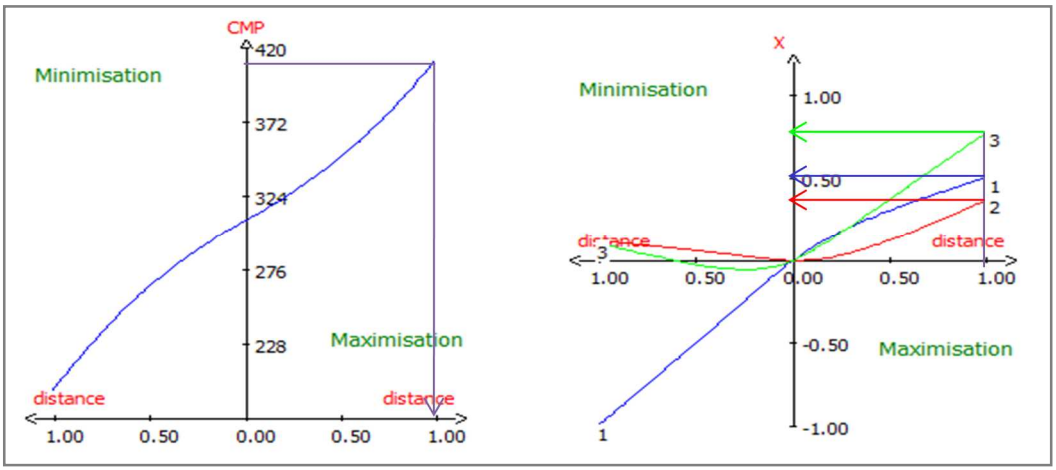


Figure 19: Etude du chemin optimal pour la libération du CMP.

Ainsi on détermine facilement que la quantité de protéines doit être de 12,5 %, que la quantité d'enzyme doit être de 31 µL.g^{-1} et que la température doit être de 9 °C pour avoir une libération maximale de CMP (Figure 18, Figure 19).

L'optimisation nous a permis d'accéder aux valeurs des trois paramètres permettant une réponse maximale et ce sont ces valeurs qui seront retenues dans la suite de l'étude.

La quantité de CMP libérée au cours du temps a été suivie en incubant des caséines micellaires (12,5 %) avec de la présure (31 µL.g^{-1}) à 9°C. Les échantillons sont prélevés à des intervalles de 5 min au début puis toutes les 10 min. Les résultats sont présentés sur la Figure 20.

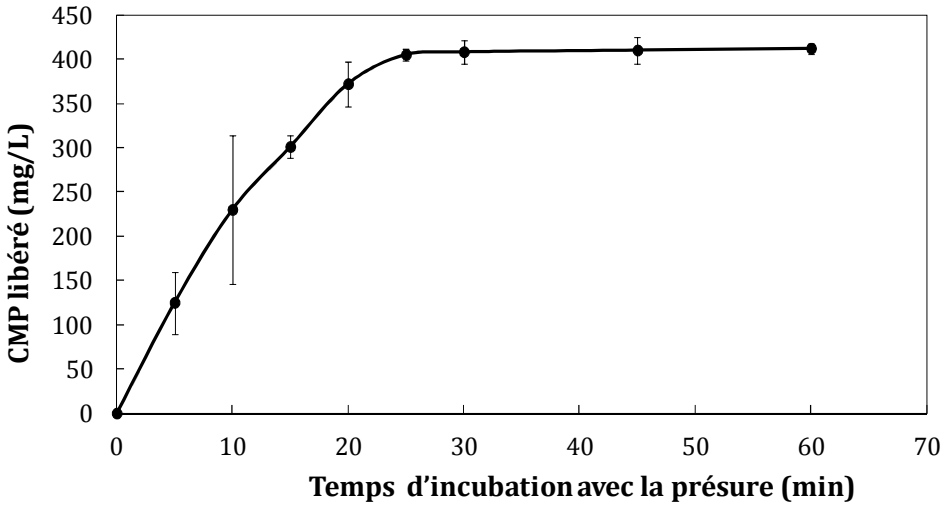


Figure 20 : Cinétique de libération du CMP en fonction du temps d'incubation avec la présure (n = 2).

Globalement, la libération du CMP se fait durant les 30 premières min, au-delà un plateau est atteint et cette quantité ne varie plus. Pour les essais d'encapsulation, il ne sera donc pas nécessaire d'attendre 60 min comme c'est le cas dans l'étude de Heidebach et al. (Heidebach et al., 2009a). L'important dans cette phase enzymatique primaire est que 85 % à 90 % du CMP soit clivé au moment où la température est augmentée. En effet, il est nécessaire que la phase primaire ait atteint cet avancement pour que la phase secondaire puisse s'initier (Gunasekaran and Ak, 2003).

4.2. Caractérisation de la phase secondaire : rhéologie

Dans le but d'initier la phase secondaire, la température de la solution doit être augmentée. Pour cela, une rampe de température allant de 9 °C (température d'incubation avec la présure) à 40 °C (température d'action optimale de l'enzyme) permet d'induire la coagulation des caséines. Afin de caractériser cette étape, il est possible de reproduire la rampe de température et de mesurer les modules d'élasticité (G') et de viscosité (G'') tout au long de la montée en température. Les résultats sont présentés sur la Figure 21.

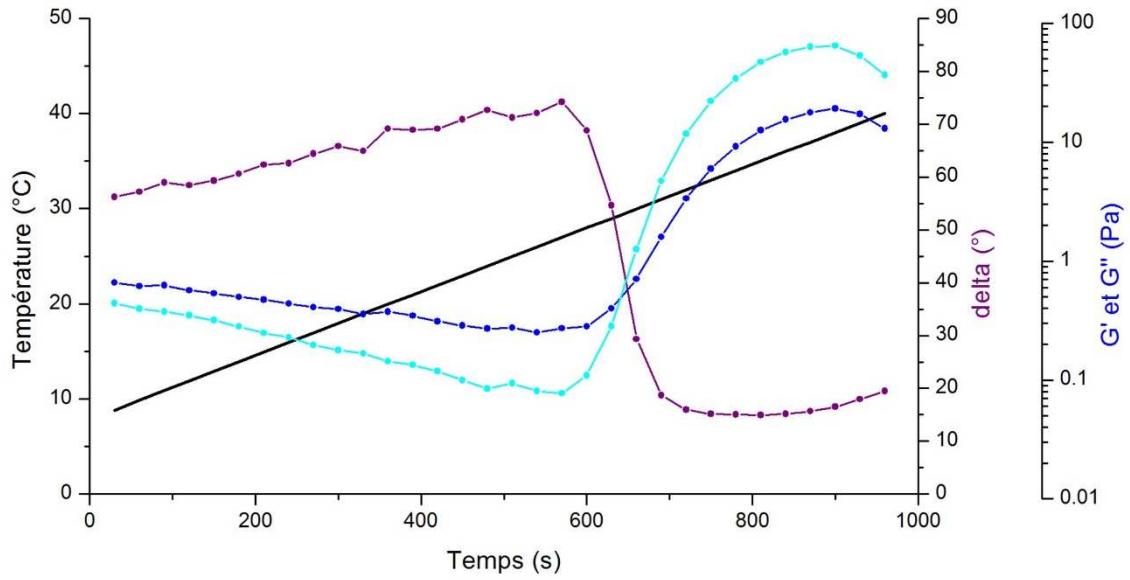


Figure 21: Suivi des modules d'élasticité et de viscosité lors de la coagulation enzymatique de protéines laitières.

Lorsque le lait est maintenu à 9 °C, il n'y a pas ou peu de variation des modules G' et G'' et ce n'est que lorsqu'on applique la rampe de température que l'on observe des modifications.

En effet, lorsqu'on augmente la température, les courbes G' et G'' se rapprochent pour finalement se croiser et c'est cette intersection qui détermine le point de coagulation des caséines (Gunasekaran and Ak, 2003).

De même, durant l'augmentation de la température la valeur de δ diminue fortement, passant de 85 ° à 15 °. On remarque que la valeur de 45 ° est atteinte vers 29 °C, ce qui correspond à la température pour laquelle le croisement des courbes G' et G'' est observé. Avant 29 °C, donc avant la coagulation, les caséines micellaires ont un comportement plus visqueux qu'élastique alors que cette tendance s'inverse au-delà du point de coagulation, d'où un comportement plus élastique que visqueux pour le gel formé. Ceci est confirmé par l'augmentation rapide de la viscosité suite à la coagulation.

Les mesures réalisées confirment la possibilité de séparation des deux phases (enzymatique et coagulation) en fonction de la température et l'on peut donc utiliser ce phénomène pour encapsuler des bactéries probiotiques en maitrisant le moment auquel la coagulation se produira.

4.3. Procédé d'obtention des microparticules

Grâce aux précédentes expériences, plusieurs paramètres ont pu être déterminés pour la réalisation de l'encapsulation de bactéries probiotiques. Le schéma général d'obtention des microparticules à l'échelle laboratoire est présenté sur la Figure 22.

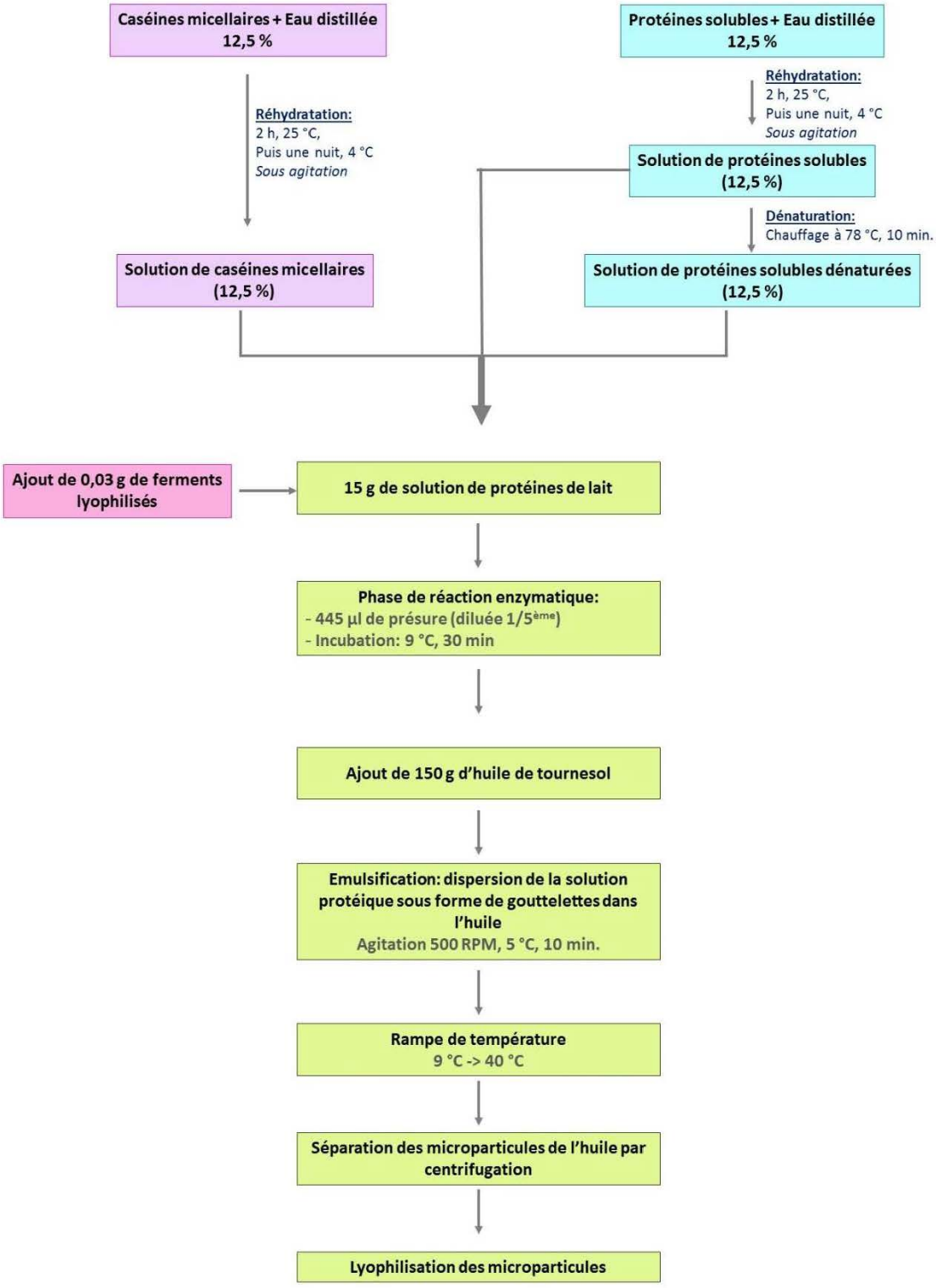


Figure 22 : Diagramme de fabrication des microparticules à l'échelle laboratoire.

Tout d'abord, 12,5 g de caséines micellaires ou de protéines solubles sont pesées et ajoutées à 100 g d'eau. Le tout est mis sous agitation pendant 2 h à 25 °C puis placé à 4 °C durant toute la nuit tout en maintenant l'agitation. Le lendemain, 0,03 g de bactéries probiotiques sont ajoutés à 15 g de solution de protéines laitières réhydratées et maintenus à 9 °C. L'ajout de la présure (445 µL) déclenche le début de la phase primaire qui durera 30 min. Au bout de ce temps, 150 g d'huile de tournesol sont ajoutés et l'émulsification est mise en place, d'abord durant 5 min à 9 °C puis la température est augmentée et va progressivement atteindre 40 °C. Ainsi on obtient des microparticules de protéines de lait coagulées dispersées dans l'huile. Afin de se débarrasser de cette huile, deux lavages à l'eau distillée suivis d'une centrifugation (500 g, 1 min) sont réalisés.

4.4. Analyse des microparticules obtenues

La distribution de taille des microparticules a tout d'abord été mesurée à l'aide du granulo-morphomètre (Figure 23).

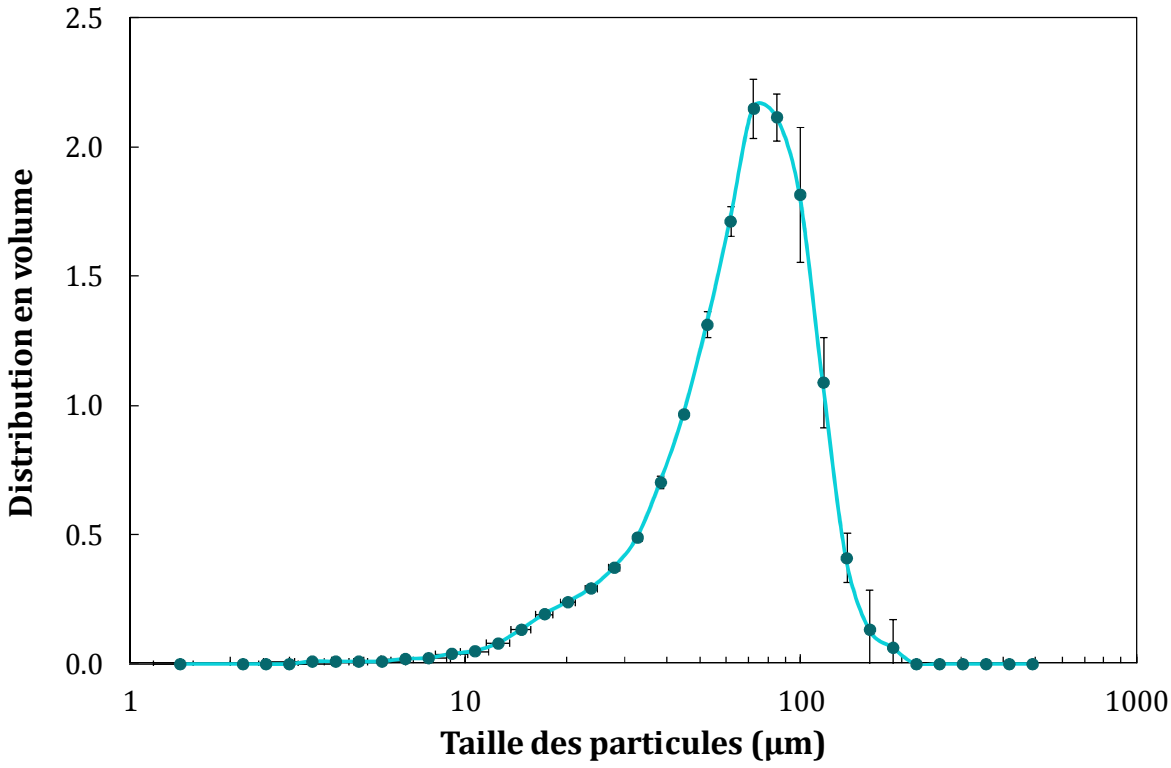


Figure 23: Distribution de taille pour des microparticules obtenues par un procédé d'encapsulation à l'échelle laboratoire (matrice composée uniquement de caséines micellaires).

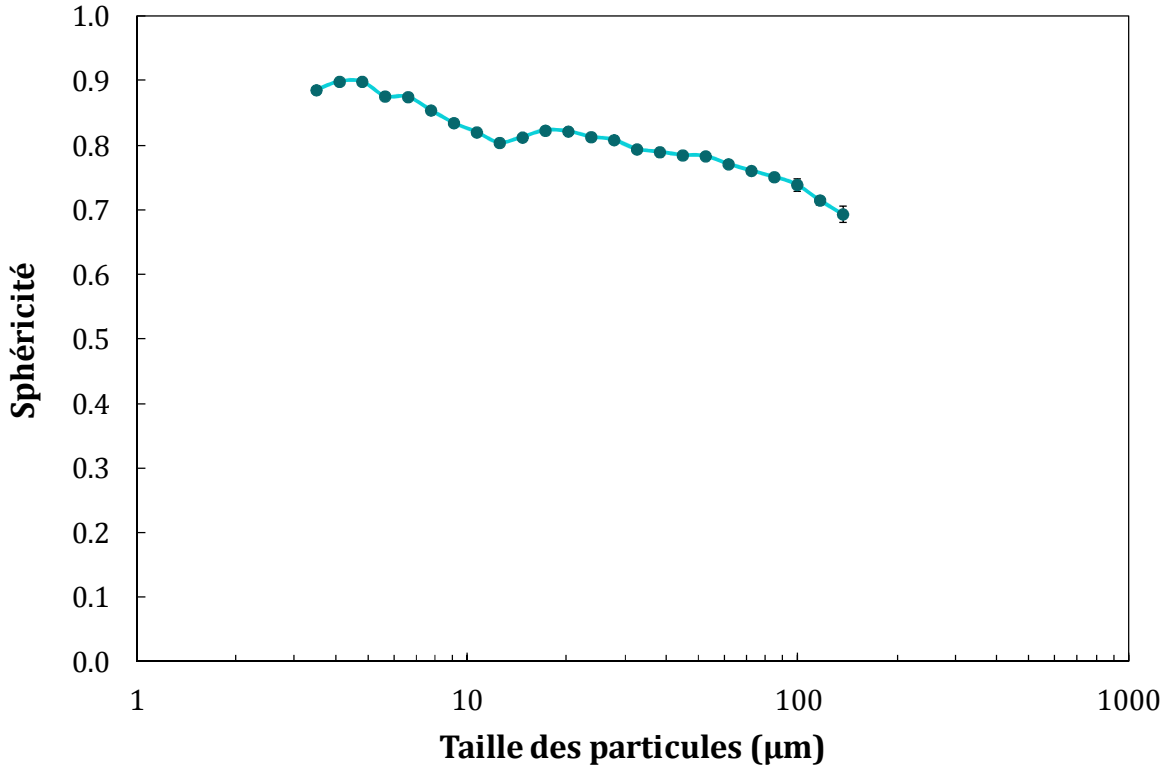
La gamme de taille obtenue est large comme le prévoyait la méthode utilisée, avec une valeur moyenne de 70 µm. L'objectif final de la production des microparticules étant de pouvoir les incorporer dans des matrices alimentaires, il est important que cette addition n'affecte pas les propriétés sensorielles du produit (Champagne and Fustier, 2007b).

Lors de l'analyse des images, la présence de gouttelettes d'huile résiduelle a pu être observée ce qui indique qu'elle n'a pas totalement été éliminée par la technique de lavage par centrifugation.

En ce qui concerne la sphéricité des particules, sur la Figure 24A on remarque que plus la taille des particules augmente plus la sphéricité diminue. Ceci est en accord avec les résultats de la Figure 24B qui présente une diminution de la convexité lorsque la taille des particules augmente.

Ces observations peuvent en partie s'expliquer par le fait que le lavage des microparticules est réalisé par plusieurs centrifugations douces. Ainsi, les particules ont tendance à s'agréger et, malgré la resuspension dans l'eau, leur séparation n'est pas complète. L'étude de la convexité et de la sphéricité des particules est un point clé puisque les propriétés de dégradation des particules semblent être liées à leur microstructure (Matalanis et al., 2011).

(A)



(B)

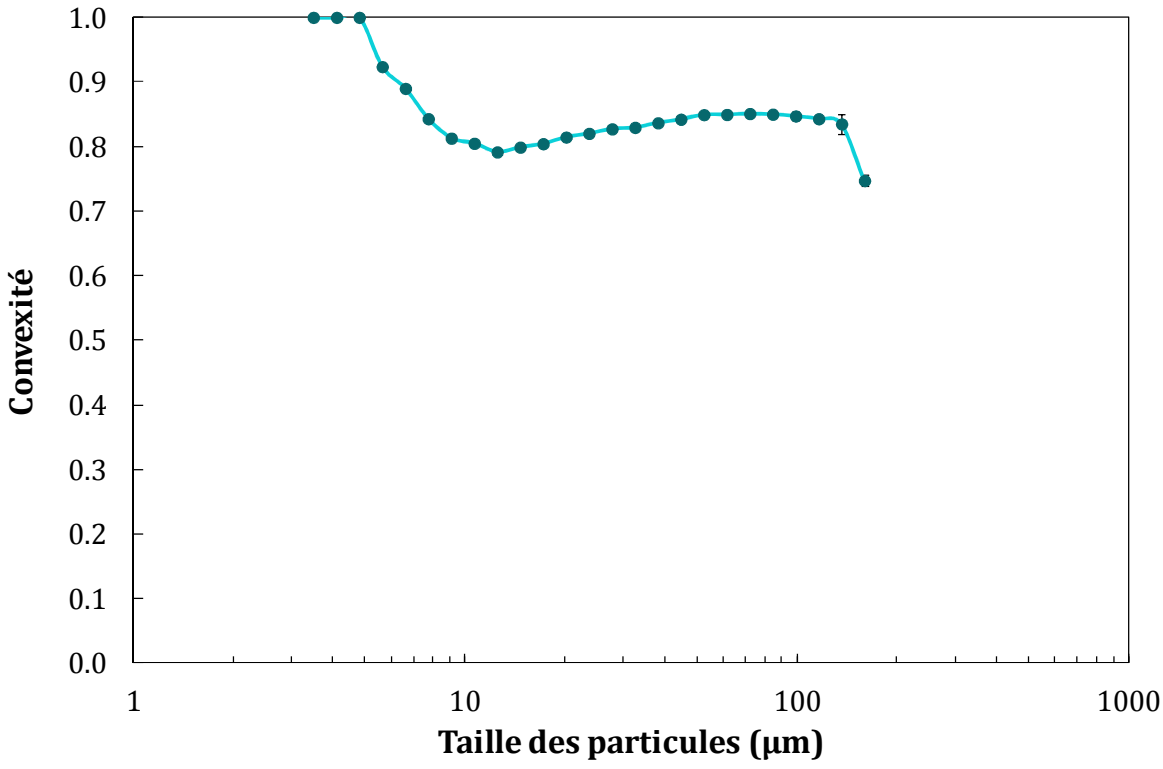
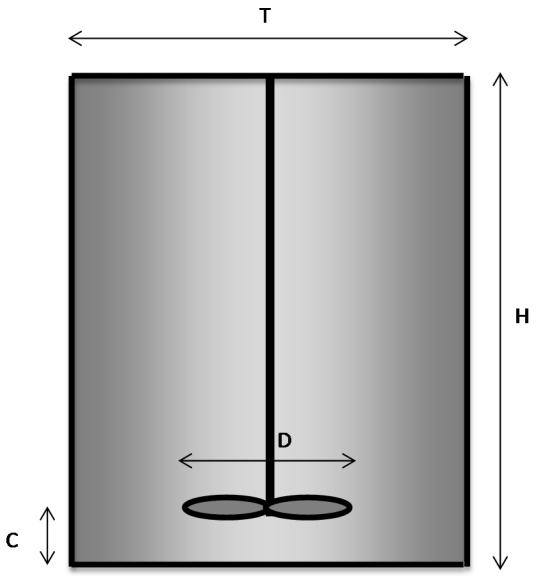


Figure 24 : Evolution de la sphéricité (A) et de la convexité (B) en fonction de la taille des microparticules.

5. Mise au point et optimisation du procédé à l'échelle pilote

5.1. Symboles et unités



C Distance fond de la cuve – mobile (m)

D Diamètre du mobile d'agitation (m)

H Hauteur de liquide (m)

T Diamètre de la cuve (m)

Symbole	Signification	Unité	Valeur (échelle pilote)
γ	Tension superficielle	$Kg.s^{-2}$	
μ_c	Viscosité phase continue (huile de tournesol)	Pa.s	$1.2*10^{-1}$
μ_d	Viscosité phase dispersée (solution protéique)	Pa.s	$3.1*10^{-2}$
ρ_c	Masse volumique phase continue	$Kg.m^{-3}$	920
ρ	Masse volumique de l'émulsion	$Kg.m^{-3}$	
Φ	Fraction volumique	-	0.2
d	Diamètre de la goutte	m	
D	Diamètre de l'hélice	m	0.05
F	Facteur d'échelle (Facteur d'extrapolation)	-	
Fr	Nombre de Froude	-	
N	Vitesse de rotation	s^{-1}	
Np	Nombre de puissance	-	
P	Puissance d'agitation	W	
Re	Nombre de Reynolds	-	
V	Volume de liquide	m^3	
V_c	Volume phase continue (huile)	m^3	$0.8*10^{-3}$
V_d	Volumes phase dispersée (solution protéique)	m^3	$0.2*10^{-3}$
Vp	Vitesse en bout de pale	$m.s^{-1}$	
We	Nombre de Weber	-	

5.2. Choix du matériel et définition des conditions d'utilisation

5.2.1. Définition des conditions pour la phase enzymatique

A l'échelle laboratoire, la présure Naturen™ était l'enzyme utilisée pour la coagulation des caséines. Pour l'encapsulation à l'échelle pilote, la chymosine CHY-MAX™ Plus a été utilisée. L'avantage de cette enzyme est qu'elle ne contient pas de pepsine comme c'est le cas pour le mélange enzymatique qui constitue la présure Naturen™. La chymosine CHY-MAX™ Plus sera de ce fait beaucoup plus spécifique pour la caséine-κ et les hydrolyses résiduelles observées avec la présure seront par conséquent évitées.

La concentration en enzyme a été choisie selon les spécifications du fabricant. Ainsi, la réaction enzymatique de clivage de la caséine-κ a été réalisée avec 1,4 IMCU/g de solution de protéines de lait. La température de cette étape a été abaissée à 5 °C puisque lors de l'étape de pompage une légère remontée en température est observée du fait de l'installation. Ainsi, pour ne pas dépasser la limite critique de 10 °C lors de cette étape de pompage et gagner en souplesse sur le choix de la vitesse de pompage, la température a été réduite à 5 °C.

La durée de la phase enzymatique reste de 30 min sauf que dans ce cas le réacteur est fermé à son extrémité supérieure et une légère agitation permet d'assurer une bonne interaction entre l'enzyme et son substrat.

5.2.1. Transfert de la phase enzymatique vers le réacteur d'émulsification

La nature de la dispersion créée, c'est-à-dire E/H, est dépendante de la formulation mais également du procédé et en particulier du protocole opératoire. Ce dernier fixe l'ordre et le mode d'introduction des constituants, les températures à respecter et enfin, le type, la vitesse et la durée d'agitation. De plus, le protocole d'incorporation, et notamment la dynamique d'introduction de la phase dispersée, joue un rôle primordial sur la taille des gouttes et sur sa distribution.

Pour cette raison, il a été choisi d'apporter progressivement la solution protéique dans le réacteur d'émulsification grâce à une pompe à piston. Toutefois, un autre facteur limitant est la remontée en température observée lors de cette étape de pompage. En effet, lors du passage de la solution dans les tuyaux et dans la pompe, aucune réfrigération n'est utilisée, il faut donc être suffisamment rapide pour ne pas que les

protéines coagulent hors de la cuve d'émulsification tout en restant à une vitesse adaptée à l'obtention d'une émulsion de type E/H.

Plusieurs vitesses ont donc été testées et le meilleur compromis a été de 8,2 $L.h^{-1}$.

5.2.2. Réacteur d'émulsification

5.2.2.1. Cuve et mobile d'agitation

Pour la réalisation de l'encapsulation à l'échelle pilote, une cuve cylindrique à fond plat été utilisée.

L'objectif est l'obtention de microparticules dont la matrice est composée de protéines laitières dans lesquelles des bactéries probiotiques sont dispersées. La taille des microparticules doit être suffisamment petite pour que leur introduction dans des aliments ne soit pas assimilée à un défaut sensoriel par le consommateur. La largeur de la distribution de taille doit également être resserrée afin de maitriser la libération des bactéries.

L'encapsulation de bactéries probiotiques est délicate dans le sens où des biomolécules existent à leur surface et qu'un cisaillement trop important dans la cuve pourrait entrainer leur détachement voire une atteinte de la bactérie. De plus, l'intérêt de la microencapsulation de ces cellules étant la préservation de leur viabilité et de leur fonctionnalité, ces contraintes doivent être prises en compte pour la mise au point du système. Néanmoins, afin de générer des gouttelettes de petite taille, un taux de cisaillement suffisant doit être observé. Un compromis a été trouvé grâce à l'utilisation d'une hélice marine comme mobile d'agitation. Celle-ci permet d'obtenir un cisaillement suffisant pour la formation de l'émulsion mais à la fois pas trop important pour ne pas endommager les cellules. Ce type de mobile est dit axial du fait de l'écoulement principal qu'il développe par rapport à son axe de rotation (Poux, 2006a).

Le rapport du diamètre du mobile d'agitation sur le diamètre de la cuve (D/T) doit être compris entre 0,2 et 0,7. Dans notre cas, ce rapport vaut 0,42 et respecte donc cette contrainte.

5.2.2.2. Détermination de la vitesse d'agitation

La vitesse de rotation du mobile dans la cuve d'émulsification (notée N) est primordiale puisqu'elle influence la distribution de taille des gouttelettes ainsi formées. Le terme vitesse périphérique (ou vitesse en bout de pale - Vp) est le plus souvent

utilisé. Cette vitesse a une influence directe sur la consommation énergétique lors de l'émulsification, mais également sur le cisaillement maximum obtenu en bout de pale. Deux vitesses ont été testées lors de la mise au point du procédé d'encapsulation qui sont de 0,7 $m.s^{-1}$ et 3,3 $m.s^{-1}$. Des vitesses supérieures ne sont pas acceptables au niveau industriel.

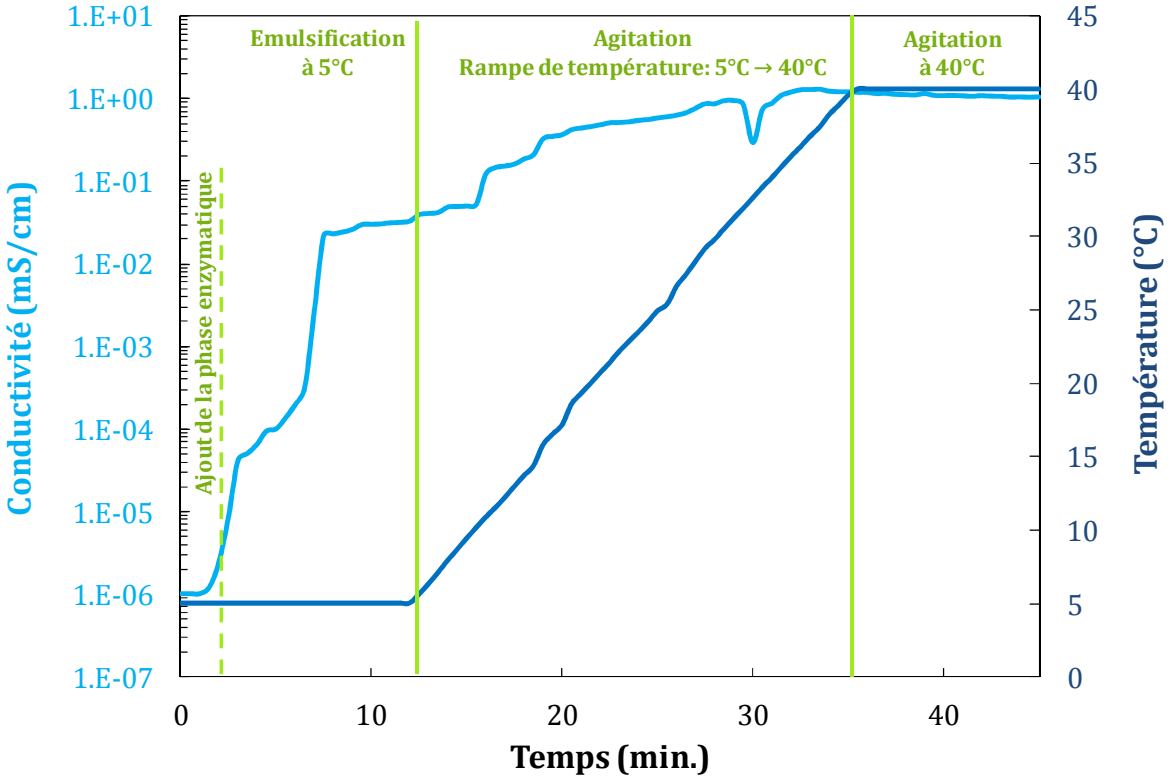
5.2.2.2.1. Suivi de la conductivité

En raison de l'évidente différence de résistivité électrique entre les phases huileuse et aqueuse d'une émulsion, le suivi de la conductivité nous informe sur les phénomènes qui se déroulent dans le réacteur d'émulsification (Figure 25).

La conductivité initiale correspond à celle de l'huile en mélange avec le Tween® 80. Au fur et à mesure de l'ajout de la solution de protéines laitières la conductivité augmente sous l'influence de l'apport d'électrolytes. La valeur maximale qu'atteint cette courbe correspond à la valeur de la conductivité de la solution protéique.

Pour une vitesse d'agitation de 8,3 $tours.s^{-1}$, la coagulation des protéines a lieu vers 25 °C et c'est à ce moment que l'on remarque une augmentation rapide de la conductivité. A l'inverse, lorsque la vitesse d'agitation n'est que de 4,2 $tours.s^{-1}$ ce changement brutal n'est pas observé mais la conductivité augmente progressivement durant toute la durée de la rampe de température.

(A) Vitesse d'agitation : 4.2 tours.s^{-1}



(B) Vitesse d'agitation : 8.3 tours.s^{-1}

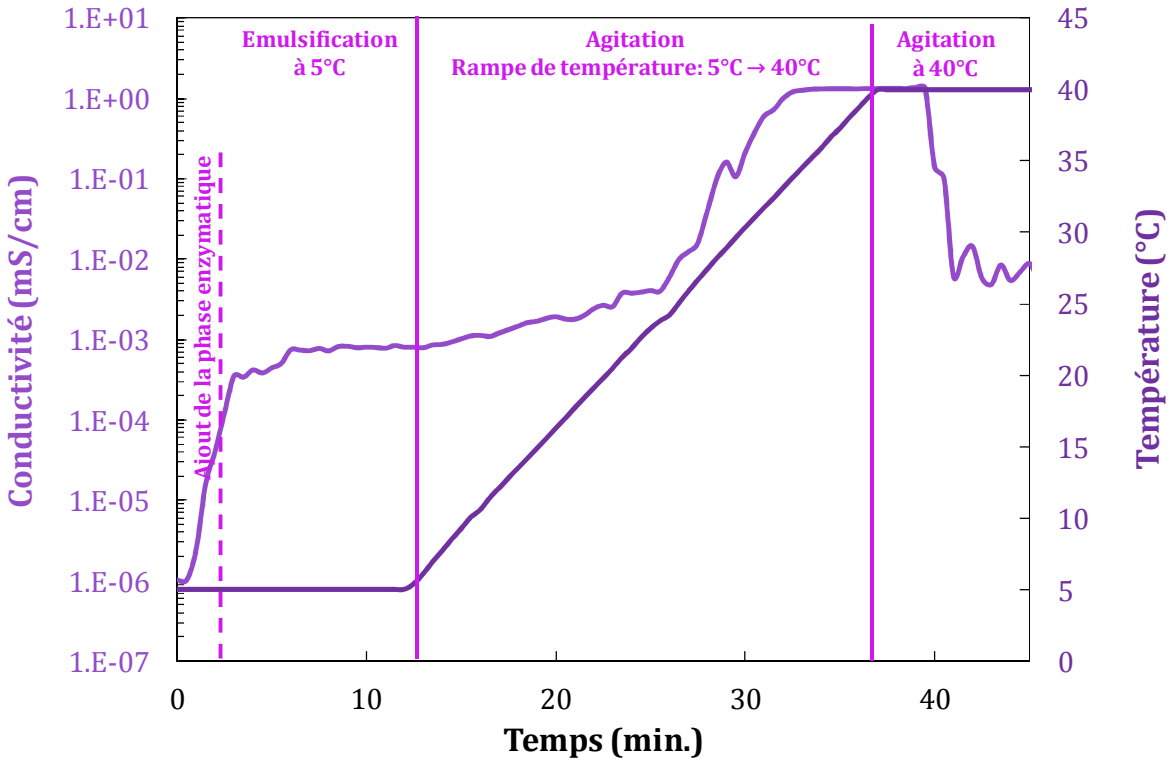
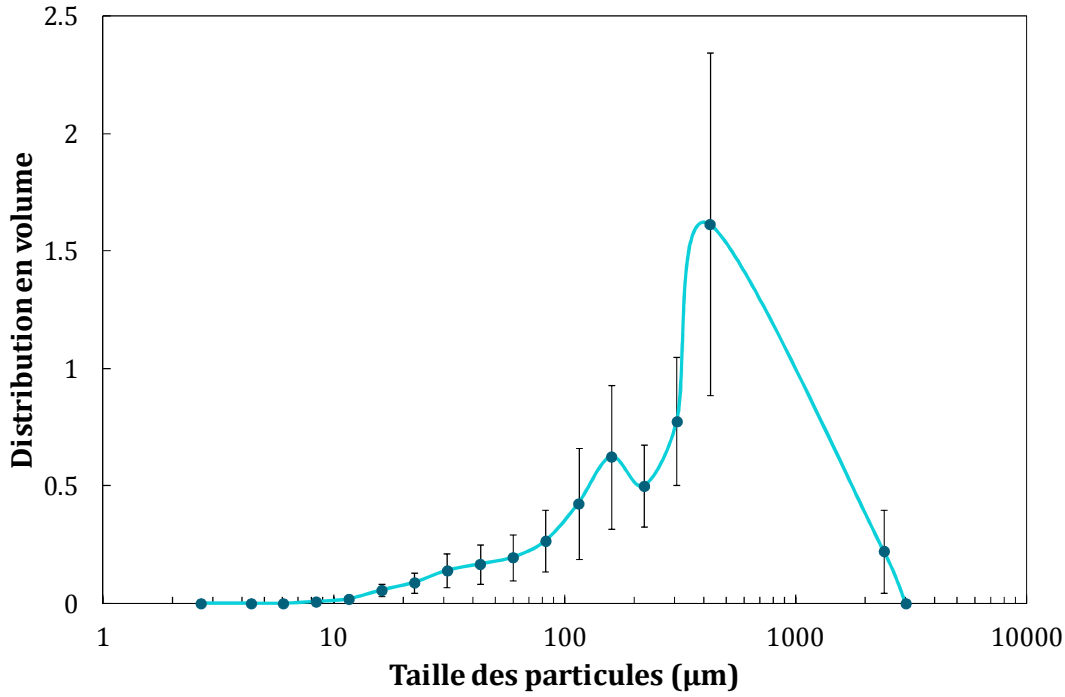


Figure 25 : Evolution de la conductivité dans la cuve d'émulsification au cours du procédé d'encapsulation pour une vitesse d'agitation de 4,2 tours.s^{-1} (A) et 8,3 tours.s^{-1} (B).

5.2.2.2.2. Taille et forme des microparticules

La vitesse d'agitation a une influence directe sur la taille des microparticules obtenues (Figure 26) mais également sur leur forme (Figure 27).

(A) Vitesse d'agitation : 4,2 tours.s^{-1}



(B) Vitesse d'agitation : 8,3 tours.s^{-1}

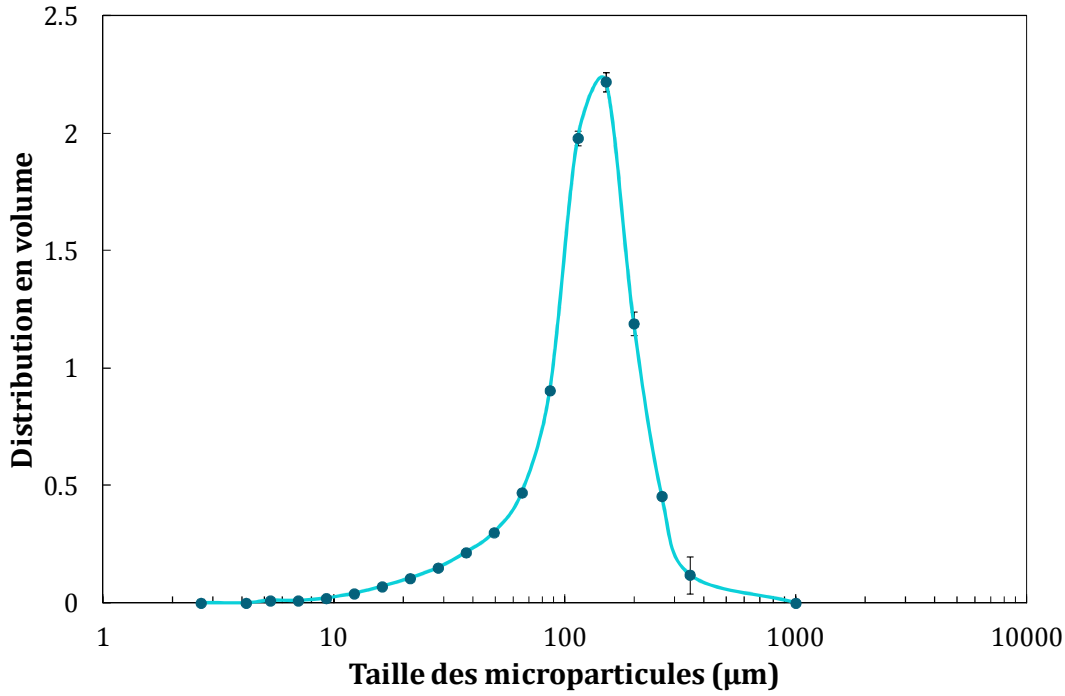


Figure 26: Effet de la vitesse d'agitation sur la taille des microparticules obtenues (A : 4,2 tours.s^{-1}; B : 8,3 tours.s^{-1}).

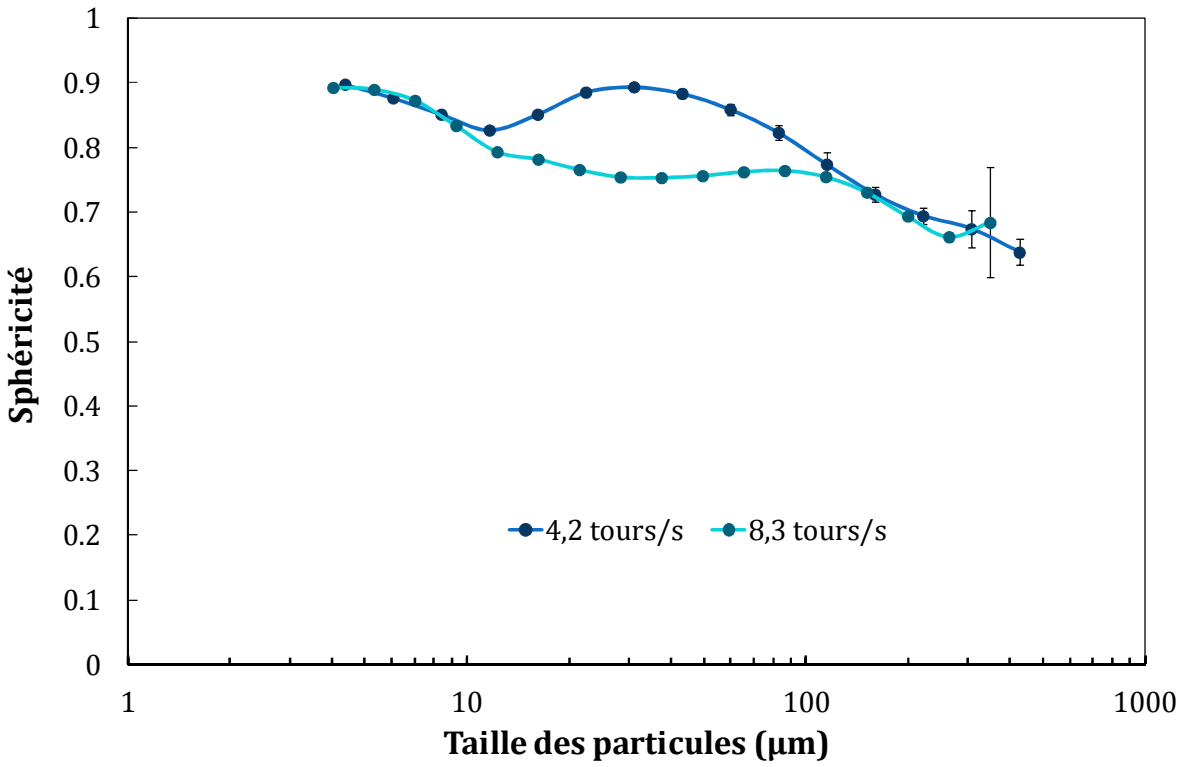


Figure 27 : Effet de la vitesse d'agitation sur la forme des microparticules obtenues.

On voit facilement que les microparticules produites à une petite vitesse d'agitation auront un diamètre plus élevé que celle produites avec une agitation plus rapide. Pour une vitesse d'émulsification de 8,3 tours.s^{-1}, un seul pic est observé vers 130 µm alors que pour l'émulsification à plus faible vitesse, une gamme de taille plus large est observée. Concernant la forme, des différences sont observées uniquement pour les microparticules ayant une taille inférieure à 100 µm : une vitesse d'agitation plus faible produira des microparticules plus sphériques alors que pour des tailles supérieures à 100 µm les mêmes valeurs sont à peu près retrouvées dans les deux cas.

5.2.2.2.3. Taux d'encapsulation

Le taux d'encapsulation de LGG est influencé par la vitesse d'agitation. Pour les deux vitesses d'agitation le taux d'encapsulation est supérieur à 100 % en particulier, on observe un taux de 243 % ± 37 % pour la plus faible vitesse et 175 % ± 48% pour la seconde. Ces valeurs s'expliquent par le fait que les bactéries (LGG) s'associent en chainettes et lors de l'encapsulation, le cisaillement entraine une rupture des chaines et donc le nombre d'UFC en sera augmenté.

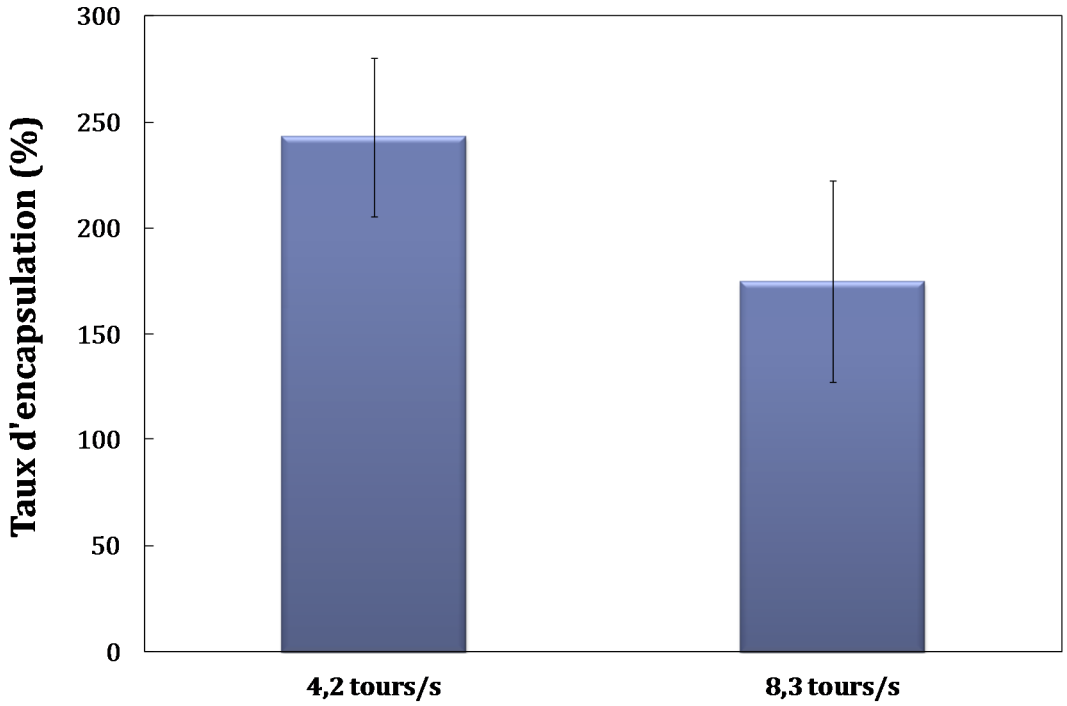


Figure 28 : Variation du taux d'encapsulation en fonction de la vitesse d'agitation

5.2.2.3. Détermination de la rampe de température

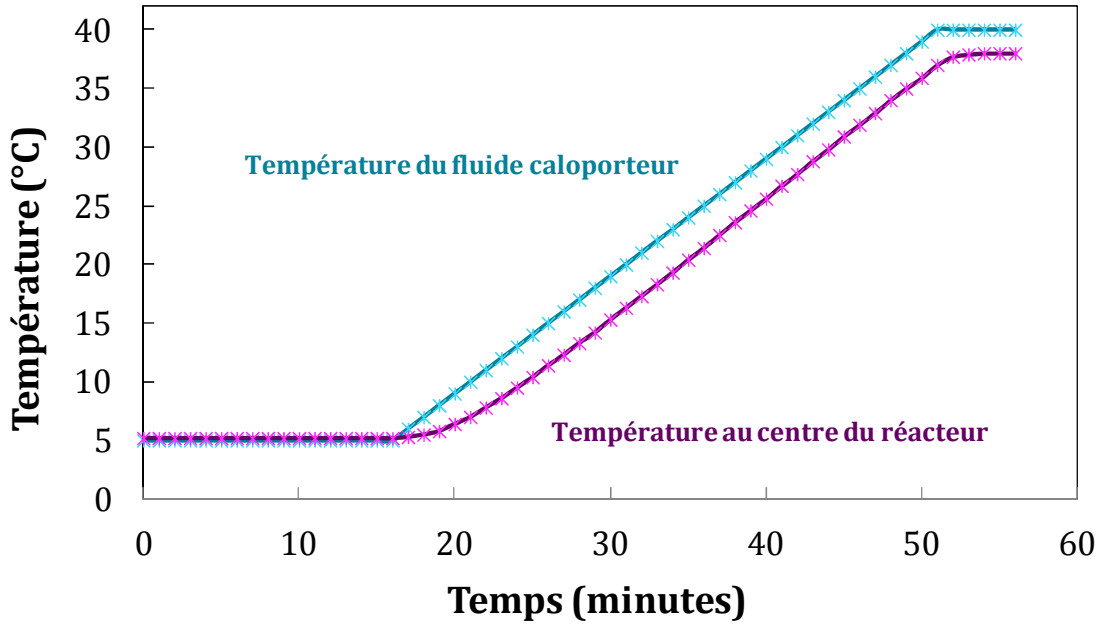
Pour induire la coagulation des caséines au cours du procédé, la température doit être augmentée jusque 40 °C. Le pas de cette rampe de température doit être suffisant pour que le transfert de chaleur puisse se faire de la double enveloppe dans laquelle circule le fluide caloporteur vers le centre du réacteur.

Une sonde de température a donc été placée au centre du réacteur et plusieurs rampes ont été testées. Deux exemples sont donnés sur la Figure 29 pour une rampe de 1,0 °C.min^{-1} et une rampe de 1,4 °C.min^{-1}.

Dans les deux cas présentés ci-dessous, un certain laps de temps est nécessaire avant que la température augmente à l'intérieur du réacteur. En effet, le transfert thermique doit dans un premier temps se faire au travers de la paroi en acier du réacteur puis, dans le fluide contenu dans l'enceinte, l'huile.

Par la suite, on remarque que la pente de la rampe de température est conservée entre l'augmentation de température du fluide caloporteur et celle au centre du réacteur tout en conservant le décalage initial.

(A) Rampe de température à 1,0 °C.min^{-1}



(B) Rampe de température à 1,4 °C.min^{-1}

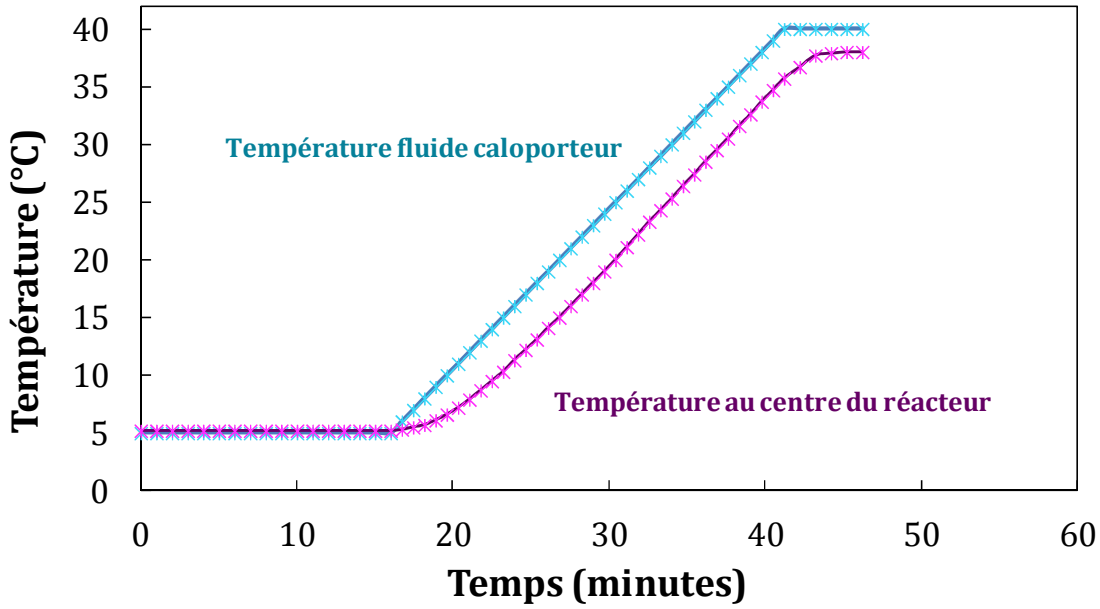


Figure 29 : Températures du fluide caloporteur et de l'huile au centre du réacteur pour une rampe de température à 1,0 °C.min^{-1} (A) et 1,4 °C.min^{-1} (B).

Le transfert thermique est important dans le procédé d'encapsulation dans le sens où c'est l'augmentation de la température de la solution protéique, dispersée sous forme de gouttelettes à ce moment-là, qui va permettre la coagulation et donc de figer le système sous la forme de microparticules. Lorsque la rampe de température est trop rapide, le gel obtenu est friable, le réseau ne s'est pas formé de façon efficace. Au

contraire, lorsque l'augmentation de la température est progressive, le réseau a le temps de se former et les microparticules sont compactes et bien individualisées.

Les microparticules obtenues en appliquant les deux rampes présentées ci-dessus étant de qualité similaire, c'est celle à 1,4 °C.min^{-1} qui a été choisie puisqu'elle permet de réduire la durée nécessaire à l'encapsulation.

5.2.2.4. Séparation et lavage des microparticules

L'utilisation de la centrifugation comme procédé de séparation des microparticules de l'huile s'est avérée être une technique peu adaptée puisqu'elle entrainait une agrégation des microparticules et une grande perte en matière était alors observée.

A l'échelle pilote un nouveau procédé de séparation a été utilisé, les microparticules sont séparées de l'huile grâce à l'utilisation de tamis (100 µm et 50-75 µm). Ensuite, de l'eau distillée est pulvérisée sur les microparticules toujours déposées sur le tamis.

Toutefois, l'encapsulation réalisée sans émulsifiant ne permet pas une bonne séparation des microparticules de l'huile dans laquelle elles sont dispersées.

Dans un premier temps, afin de s'affranchir de l'utilisation d'un émulsifiant, plusieurs essais ont été réalisés. Tout d'abord, une modification du support de lyophilisation des bactéries a été testée. En effet, il peut être intéressant d'utiliser un cryoprotectant lors de la lyophilisation des bactéries ; ce dernier serait choisi de façon à ce qu'il ait en plus une influence sur l'efficacité de séparation des microparticules à la fin du procédé d'encapsulation. Dans cette optique, le lait écrémé et le glycérol ont été utilisés mais les résultats ont été décevants puisque les microparticules se séparaient mal de l'huile. Cette proposition n'a donc pas été retenue. Les bactéries ont néanmoins été lyophilisées dans du lait pour assurer leur protection durant leur conservation avant encapsulation.

Deux émulsifiant (Span® 20 et Tween® 80) ont ensuite été testés : ces derniers sont ajoutés dans l'huile contenue dans le réacteur d'émulsification.

L'utilisation du Tween® 80 (Monooléate de sorbitane polyoxyéthylène) a conduit aux meilleurs résultats, il s'agit d'un tensioactif non-ionique.

L'addition de ce tensioactif n'a pas permis de réduire la taille des gouttelettes (comparé à une encapsulation sans Tween® 80) et donc des microparticules obtenues

par la suite, mais son ajout a été en faveur d'une meilleure séparation des microparticules de l'huile (Figure 30).

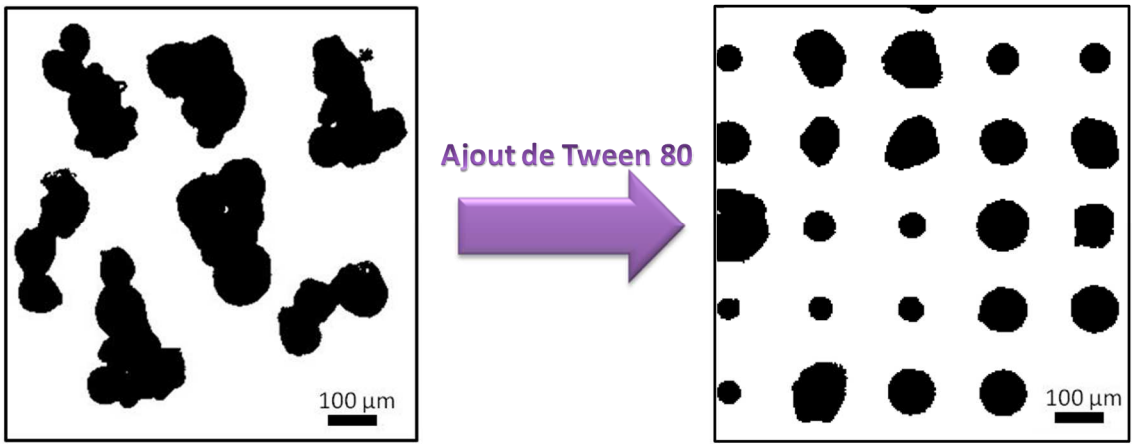


Figure 30 : Images enregistrées par le granulo-morphomètre lors d'une encapsulation sans Tween® 80 et une encapsulation avec Tween® 80.

La quantité de Tween® 80 à ajouter lors de l'émulsification a fait l'objet de nombreux essais. En effet, à faible teneur (0,1 %) les microparticules se séparaient facilement de l'huile et n'étaient pas agrégées mais la taille des microparticules était très grande (de l'ordre de 1 – 2 mm). L'augmentation du taux de Tween® 80 a permis de réduire ce paramètre jusqu'à obtenir une taille de microparticules autour de 100 µm avec 1 % de Tween® 80.

5.3. Dimensionnement du procédé d'émulsification

Le procédé d'encapsulation à l'échelle pilote fait l'objet de différentes étapes bien distinctes (Figure 31). La première est la phase enzymatique (1), la seconde est une étape de pompage de la solution protéique (2) contenue dans le réacteur enzymatique vers le réacteur d'émulsification. Ensuite, vient l'étape importante d'émulsification (3) qui permet de former les gouttelettes qui deviendront par la suite les microparticules. L'induction d'une rampe de température (4) permet la coagulation des protéines laitières. Finalement, la récupération et le lavage (5) des microparticules clôturent cette succession d'opérations unitaires.

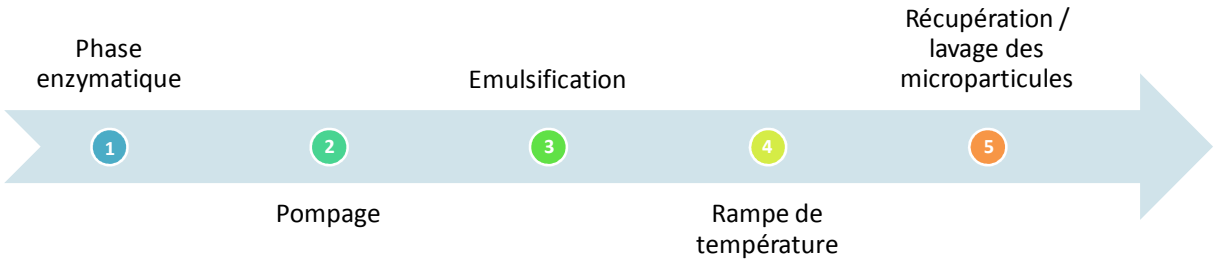


Figure 31 : Etapes du procédé d'encapsulation par émulsification à l'échelle pilote.

L'émulsification étant l'étape clé dans la formation des gouttelettes qui deviendront les microparticules, elle a donc fait l'objet d'une étude plus approfondie.

5.3.1. Emulsions : généralités

Le terme émulsion désigne un système hétérogène comprenant au moins un liquide immiscible dispersé dans un autre sous la forme de gouttelettes. Un tel système se caractérise par une stabilité minimale qui peut être accrue par l'ajout de tensioactifs. Les deux phases formant l'émulsion sont appelées symboliquement eau (E) et huile (H).

Pour des systèmes contenant des gouttelettes de tailles supérieures à quelques micromètres, on parle d'émulsion ou de macroémulsion ; ces tailles correspondent à la valeur minimale à laquelle on peut accéder par agitation mécanique. Dans le cas d'une macroémulsion, les gouttelettes formées sont de tailles supérieures au micromètre et la quantité de tensioactif est faible, ce qui justifie le fait que le système soit qualifié d'instable ou hors équilibre (Poux, 2006b).

La fabrication classique d'une émulsion nécessite un apport d'énergie afin d'accroître l'aire interfaciale entre les deux phases. Une agitation mécanique, par exemple, permet la déformation de l'interface et ainsi la formation des gouttelettes. Ces

gouttelettes peuvent coalescer aussitôt après leur formation. Ces deux phénomènes (rupture et coalescence) constituent les étapes critiques du processus d'émulsification.

La fragmentation individuelle des gouttelettes est accomplie par le liquide constituant la phase continue, ce dernier étant mis en mouvement par le système mécanique de dispersion. La fragmentation sera rendue possible si un cisaillement local suffisant existe (Brochette, 1999).

5.3.2. Fraction volumique

La fraction volumique de phase dispersée (notée Φ) caractérise la concentration en phase dispersée dans l'émulsion. Ce paramètre est défini à partir des volumes des deux phases selon la relation :

$$\Phi = \frac{V_D}{V_D + V_C}$$

Où V_D représente le volume de la phase dispersée et V_C le volume de la phase continue.

La fraction volumique détermine la nature de la dispersion obtenue. Le système est considéré comme dilué lorsque $\Phi < 0{,}01$, dans ce cas le phénomène de coalescence est négligeable et la dispersion est essentiellement due à l'hydrodynamique.

Lorsque $0{,}01 < \Phi < 0{,}2$ le système est qualifié de moyennement concentré alors que pour $\Phi > 0{,}2$ le système est dit concentré.

Dans notre cas la valeur de la fraction volumique vaut 0,2, on se trouve donc à la limite supérieure pour un système moyennement concentré.

5.3.3. Emulsification

La formation des gouttelettes résulte de la rupture d'une goutte de plus grosse taille sous l'action de forces extérieures. Ces forces agissant sur la goutte sont en majorité transmises par la phase continue. Elles peuvent être dues aux gradients de vitesse ou de pression, aux forces de cisaillement ou d'inertie. Ces forces motrices s'opposent aux forces de résistance qui sont les forces de cohésion de la goutte liées à la tension interfaciale existant entre les deux fluides et la viscosité de la phase à disperser.
Le fractionnement de la goutte est donc le résultat d'un équilibre entre les forces motrices et les forces cohésives. Les paramètres qui déterminent la rupture sont : les propriétés rhéologiques de la phase continue et celles de la phase dispersée, les propriétés interfaciales des deux fluides et l'hydrodynamique du système.

Le nombre de Weber macroscopique est souvent utilisé pour discriminer les phénomènes de rupture. Ce nombre adimensionnel compare les contraintes de déformation inertielles aux contraintes de cohésion (pression de Laplace). Si ce rapport excède une certaine valeur, il y a rupture de la goutte (Poux, 2006b).

$$We = \frac{\rho_c * N^2 * D^3}{\sigma}$$

D'après l'équation de Young Laplace appliquée au cas d'une sphère, la déformation d'une gouttelette est directement proportionnelle à la tension interfaciale, σ, et inversement proportionnelle au diamètre de la goutte d.

$$\Delta P = 4 * \frac{\sigma}{d}$$

Cette équation met bien en évidence l'influence du tensioactif qui, généralement, en abaissant la tension interfaciale, diminue la quantité d'énergie nécessaire et favorise ainsi l'émulsification, jouant un rôle considérable sur le phénomène de rupture et donc sur la distribution de taille de l'émulsion.

Dans le procédé de dispersion en cuve agitée, l'agitation joue un rôle prépondérant dans le processus de formation des gouttes. Deux zones hydrodynamiques principales peuvent être définies : l'une de coalescence (macromélange), qui correspond au mouvement global induit des gros agrégats, et une zone de rupture (micromélange), localisée vers l'agitateur où le cisaillement très intense permet la rupture des gouttes.

Dans la zone de rupture, le cisaillement est réalisé par des tourbillons de taille inférieure à celle des gouttes de la phase dispersée et dont l'énergie cinétique est suffisante pour compenser les énergies de cohésion des gouttes. Dans la zone de coalescence, loin de l'agitateur, la coalescence est réalisée par des tourbillons de taille supérieure à celle des gouttes qui causent des collisions efficaces. Ces deux phénomènes, de rupture et de coalescence, conduisent à définir les diamètres minima et maxima stables d'une émulsion.

Le fractionnement des gouttes dépend de la nature de l'écoulement. En effet, dans le cas d'un écoulement laminaire les forces visqueuses prédominent, alors que dans la cas d'un écoulement turbulent les forces d'inertie et les forces visqueuses sont toutes les deux présentes. La nature de l'écoulement est déterminée par le nombre de Reynolds calculé selon l'équation suivante :

$$Re = \frac{\rho_c * N * D^2}{\mu_c}$$

Ainsi, un nombre de Reynolds élevé sera le résultat d'un régime turbulent.

5.3.4. Consommation énergétique

La puissance consommée par le mobile peut être calculée à partir du nombre de puissance Np. Ce nombre traduit la capacité d'un mobile à mettre en mouvement un fluide et sa capacité à le cisailler. Ainsi, plus le mobile assurera un cisaillement intense, plus Np sera élevé. En régime turbulent, Np est constant et est défini par la relation suivante :

$$Np = \frac{P}{\rho * N^3 * D^5}$$

En régime turbulent, la puissance d'agitation (P) ne dépend que de la masse volumique de la dispersion et non de sa viscosité. Pour un mobile donné, il existe des courbes caractéristiques reliant Np et Re (Figure 32).

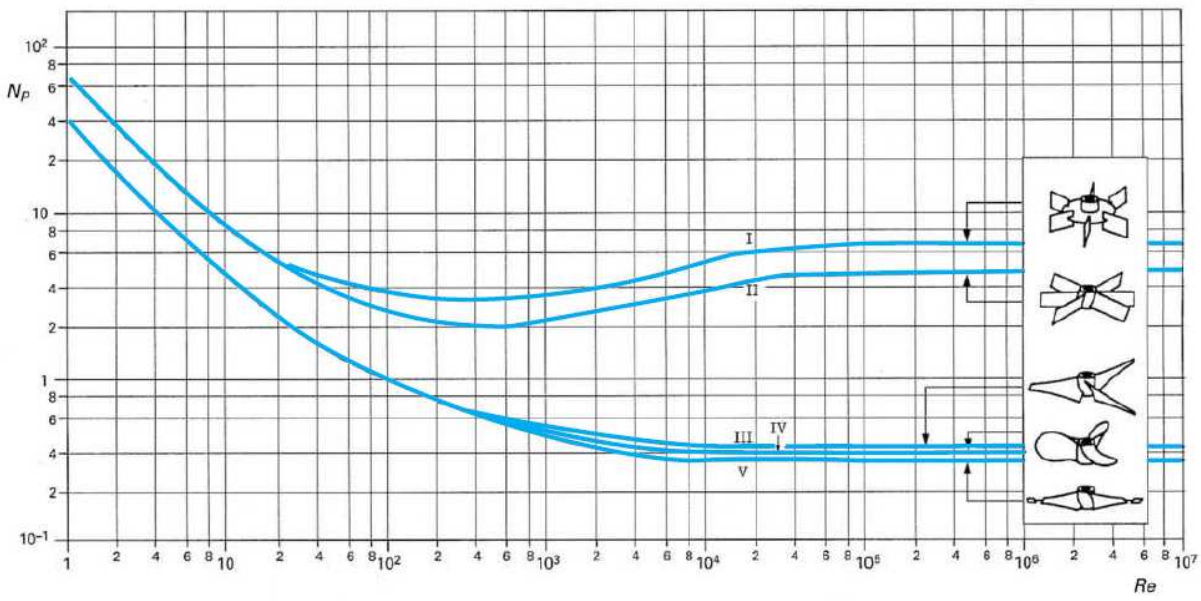


Figure 32 : Nombre de puissance de 5 mobiles d'agitation en fonction du nombre de Reynolds (Roustan, 1997).

Pour terminer, le nombre de Froude caractérise l'action des forces d'inertie sur les forces de gravité.

$$Fr = \frac{N^2 * D}{g}$$

Ce nombre permet par exemple de prédire la formation d'un vortex s'il est élevé.

5.4. Extrapolation à l'échelle industrielle

L'objectif de l'extrapolation est de pouvoir reproduire à l'échelle industrielle les résultats obtenus à l'échelle pilote. L'extrapolation est fondée sur le principe de similitude, ce qui signifie que les rapports d'un ou plusieurs paramètres sont les mêmes aux deux échelles (Poux, 2006c).

On peut distinguer 5 similitudes :

- Similitude géométrique : implique que les rapports des dimensions géométriques soient les mêmes aux deux échelles (exemple : D_1/D_2),
- Similitude dynamique : implique que les rapports des forces soient identiques (exemple : Nombre de Reynolds),
- Similitude cinématique : implique que les rapports des vitesses en des points homologues soient identiques,
- Similitude chimique : implique que les concentrations soient identiques en des points homologues,
- Similitude thermique : implique que les températures soient les mêmes en des points homologues.

5.4.1. Similitude géométrique

Afin de comparer les grandeurs aux deux échelles, l'indice 1 est utilisé pour l'échelle pilote alors que l'indice 2 est utilisé pour l'échelle industrielle. Les rapports géométriques entre les deux échelles sont reliés par le facteur d'échelle F.

Un exemple de similitude géométrique est proposé sur la Figure 33. Dans ce cas le facteur d'échelle est de 2, ce qui engendre un rapport de volume de 8 entre les deux installations. Grâce à ce type de similitude, les grandeurs caractéristiques du mobile d'agitation seront conservées aux deux échelles (exemple : Np). En revanche, l'augmentation du volume entraine une modification du rapport volume/surface. Le volume par unité de surface sera bien plus élevé à l'échelle industrielle qu'à l'échelle

pilote. L'extrapolation ira dans le sens d'une augmentation des effets de volume. Il est important de tenir compte de ce paramètre dans le cas d'un transfert thermique (Poux, 2006c).

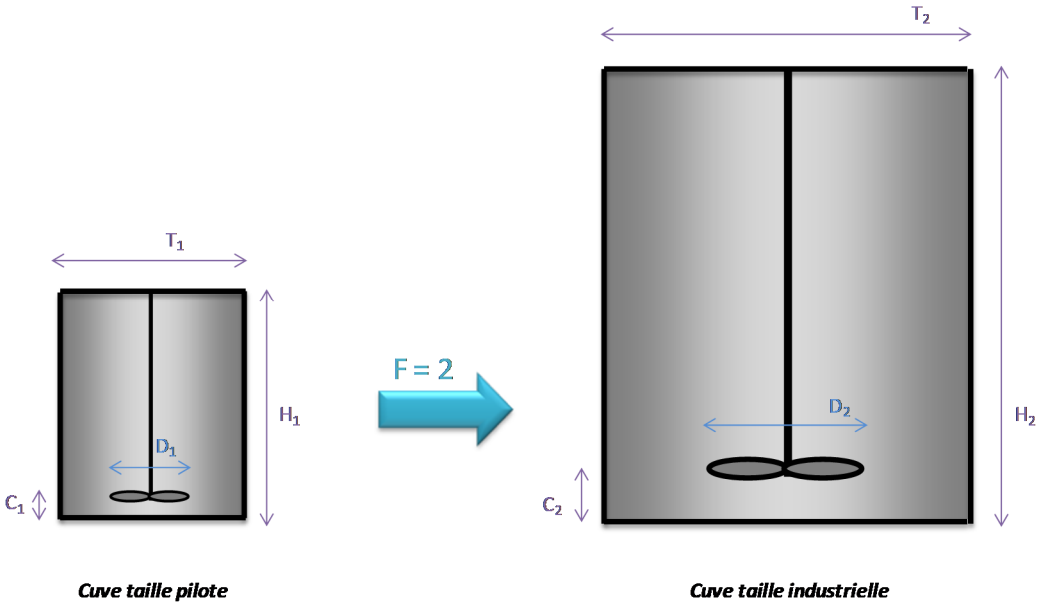


Figure 33 : Dimensions caractéristiques d'un pilote et d'une cuve industrielle extrapolée avec un facteur F=2.

5.4.2. Identification des invariants

L'extrapolation du procédé d'émulsification repose sur l'identification des invariants. Pour cela, il est nécessaire de définir au préalable les grandeurs caractéristiques qui vont être utilisées (Tableau 10).

Tableau 10 : Grandeurs caractéristiques utilisées pour l'extrapolation du procédé d'émulsification.

Grandeur	Equation	Proportionnalité
Puissance par unité de volume	$P/V = \frac{Np * \rho_c * N^3 * D^5}{\pi * r^2 * H}$	$P/V \propto N^3 * D^2$
Nombre de Reynolds	$Re = \frac{\rho_c * N * D^2}{\mu_c}$	$Re \propto N * D^2$
Nombre de Froude	$Fr = \frac{N^2 * D}{g}$	$Fr \propto N^2 * D$
Nombre de Weber	$We = \frac{\rho_c * N^2 * D^3}{\sigma}$	$We \propto N^2 * D^3$
Vitesse en bout de pale	$Vp = \pi * N * D$	$Vp \propto N * D$

Le maintien de l'un de ces paramètres constant va entraîner une variation des autres d'un facteur multiplicatif faisant intervenir le facteur d'échelle F.

L'influence d'un invariant sur les autres grandeurs est décrite dans la démonstration qui suit.

Invariant : Puissance par unité de volume

$$\frac{N_1^3 * D_1^2}{N_2^3 * D_2^2} \propto 1 \qquad \text{D'où} \qquad N_2 \propto N_1 * F^{-2/3}$$

- ✓ Effet sur le nombre de Reynolds :

$$Re \propto N_2 * D_2^2 \propto N_1 * D_1^2 * F^{4/3}$$

- ✓ Effet sur le nombre de Froude :

$$Fr \propto N_2^2 * D_2 \propto N_1^2 * D_1 * F^{-1/3}$$

- ✓ Effet sur le nombre de Weber :

$$We \propto N_2^2 * D_2^3 \propto N_1^2 * D_1^3 * F^{5/3}$$

- ✓ Effet sur la vitesse en bout de pale :

$$Vp \propto N_2 * D_2 \propto N_1 * D_1 * F^{1/3}$$

Invariant : Nombre de Reynolds

$$\frac{N_1 * D_1^2}{N_2 * D_2^2} \propto 1 \qquad \text{D'où} \qquad N_2 \propto N_1 * F^{-2}$$

- ✓ Effet sur la puissance par unité de volume :

$$P/V \propto N_2^3 * D_2^2 \propto N_1^3 * D_1^2 * F^{-4}$$

- ✓ Effet sur le nombre de Froude :

$$Fr \propto N_2^2 * D_2 \propto N_1^2 * D_1 * F^{-3}$$

- ✓ Effet sur le nombre de Weber :

$$We \propto N_2^2 * D_2^3 \propto N_1^2 * D_1^3 * F^{-1}$$

- ✓ Effet sur la vitesse en bout de pale :

$$Vp \propto N_2 * D_2 \propto N_1 * D_1 * F^{-1}$$

Invariant : Nombre de Froude

$$\frac{N_1^2 * D_1}{N_2^2 * D_2} \propto 1 \qquad \text{D'où} \qquad N_2 \propto N_1 * F^{-1/2}$$

- ✓ Effet sur la puissance par unité de volume :

$$P/V \propto N_2^3 * D_2^2 \propto N_1^3 * D_1^2 * F^{1/2}$$

- ✓ Effet sur le nombre de Reynolds :

$$Re \propto N_2 * D_2^2 \propto N_1 * D_1^2 * F^{3/2}$$

- ✓ Effet sur le nombre de Weber :

$$We \propto N_2^2 * D_2^3 \propto N_1^2 * D_1^3 * F^2$$

- ✓ Effet sur la vitesse en bout de pale :

$$Vp \propto N_2 * D_2 \propto N_1 * D_1 * F^{1/2}$$

Invariant : Nombre de Weber

$$\frac{N_1^2 * D_1^3}{N_2^2 * D_2^3} \propto 1 \qquad \text{D'où} \qquad N_2 \propto N_1 * F^{-3/2}$$

- ✓ Effet sur la puissance par unité de volume :

$$P/V \propto N_2^3 * D_2^2 \propto N_1^3 * D_1^2 * F^{-1}$$

- ✓ Effet sur le nombre de Reynolds :

$$Re \propto N_2 * D_2^2 \propto N_1 * D_1^2 * F$$

- ✓ Effet sur le nombre de Froude :

$$Fr \propto N_2^2 * D_2 \propto N_1^2 * D_1 * F^{-1}$$

- ✓ Effet sur la vitesse en bout de pale :

$$Vp \propto N_2 * D_2 \propto N_1 * D_1 * F^{-1/2}$$

Invariant : Vitesse en bout de pale

$$\frac{N_1 * D_1}{N_2 * D_2} \propto 1 \qquad \text{D'où} \qquad N_2 \propto N_1 * F^{-1}$$

- ✓ Effet sur la puissance par unité de volume :

$$P/V \propto N_2^3 * D_2^2 \propto N_1^3 * D_1^2 * F^{-5/2}$$

- ✓ Effet sur le nombre de Reynolds :

$$Re \propto N_2 * D_2^2 \propto N_1 * D_1^2 * F^{1/2}$$

- ✓ Effet sur le nombre de Froude :

$$Fr \propto N_2^2 * D_2 \propto N_1^2 * D_1 * F^{-2}$$

- ✓ Effet sur le nombre de Weber :

$$We \propto N_2^2 * D_2^3 \propto N_1^2 * D_1^3 * F$$

A partir de ces équations, le Tableau 11 décrit l'influence d'un changement d'échelle d'un facteur F sur les grandeurs caractéristiques du système d'émulsification considéré, en conservant une similitude géométrique.

Tableau 11 : Effet d'un changement d'échelle sur les grandeurs caractéristiques du système d'émulsification (régime turbulent).

	Grandeurs invariantes				
	P/V	**Re**	**Fr**	**We**	**Vp**
P/V	1	F^{-4}	$F^{0,5}$	$F^{-2,5}$	F^{-1}
Re	$F^{1,33}$	1	$F^{1,5}$	$F^{0,5}$	F
Fr	$F^{-0,33}$	F^{-3}	1	F^{-2}	F^{-1}
We	$F^{1,67}$	F^{-1}	F^{2}	1	F
Vp	$F^{0,33}$	F^{-1}	$F^{0,5}$	$F^{-0,5}$	1

Ce tableau décrit parfaitement l'incompatibilité qu'il existe entre les critères, il est donc nécessaire de déterminer les grandeurs dont l'influence est prépondérante sur le phénomène d'émulsification et celles que l'on garde constantes.

Pour obtenir un produit de même qualité à l'échelle pilote et à l'échelle industrielle, il est intéressant de maintenir P/V et Vp constants. Toutefois ces deux critères sont incompatibles en similitude géométrique, il est donc nécessaire de trouver un compromis (Poux and Canselier, 2004).

Pour le passage d'un volume de 1 L à 1 m^3, le facteur F peut être calculé :

$$F = \left(\frac{1}{1 * 10^{-3}}\right)^{-1/3} = 10$$

Les valeurs des grandeurs caractéristiques calculées à l'échelle pilote sont résumées dans le Tableau 12.

Tableau 12 : Valeurs des grandeurs caractéristiques à l'échelle pilote.

Grandeur	Valeur à l'échelle pilote
P/V_1 ($W.m^{-3}$)	8.20E+01
Re_1	1.59E+02
Fr_1	3.50E-01
We_1	2.47E+02
Vp_1 (m/s)	1.30E+00

A partir des équations obtenues dans l'analyse des invariants, du facteur F et des valeurs à l'échelle pilote (Tableau 12), il est possible de prédire les valeurs obtenues à l'échelle industrielle (Tableau 13).

Tableau 13 : Détermination des valeurs caractéristiques à l'échelle industrielle en fonction de l'invariant choisi.

	Grandeurs invariantes				
	P/V	**Re**	**Fr**	**We**	**Vp**
P/V	8.20E+01	8.20E-03	2.59E+02	2.59E-01	8.20E+00
Re	3.40E+03	1.59E+02	5.03E+03	5.03E+02	1.59E+03
Fr	1.64E-01	3.50E-04	3.50E-01	3.50E-03	3.50E-02
We	1.15E+04	2.47E+01	2.47E+04	2.47E+02	8.61E-02
Vp	2.79E+00	1.30E-01	4.12E+00	4.12E-01	1.30E+00

L'analyse de ce tableau permet de choisir quel paramètre sera maintenu constant lors du passage de l'échelle pilote à l'échelle industrielle.

Lorsque la puissance par unité de volume est maintenue constante, la vitesse en bout de pale se trouve augmentée entrainant dans le même temps une augmentation du cisaillement. Etant donné que l'objectif ici est de minimiser le cisaillement, ce choix ne sera donc pas retenu. Si le nombre de Reynolds est conservé entre les deux échelles, la puissance par unité de volume se trouve fortement diminuée ce qui n'est pas très favorable pour effectuer une émulsification avec efficacité. Cet invariant ne sera donc pas choisi et, quel que soit l'invariant choisi ici, le régime d'écoulement est conservé (il est situé à la limite du régime turbulent). La vitesse en bout de pale se trouve également affectée lorsque le nombre de Froude et le nombre de Weber sont conservés aux deux

échelles : dans le premier cas elle sera augmentée alors que l'inverse est observé dans le second cas. Finalement, lorsque la vitesse en bout de pale est conservée entre les deux échelles, les mêmes vitesses locales du liquide sont retrouvées et il en est de même pour les contraintes locales en cisaillement. Le choix de cet invariant conduit à une puissance dissipée par unité de volume plus faible à l'échelle industrielle qu'à l'échelle pilote.

Le maintien de la vitesse en bout de pale lors du passage de l'échelle pilote à l'échelle industrielle semble donc être le meilleur compromis. En effet, le cisaillement ne sera pas augmenté ce qui représente un point crucial dans notre système afin de ne pas endommager les bactéries.

6. Conclusion du chapitre

L'encapsulation de bactéries probiotiques dans des matrices composées de protéines laitières a été réalisée avec succès.

La mise au point d'un système d'encapsulation à l'échelle laboratoire a dans un premier temps permis de se confronter aux problèmes rencontrés lors de l'encapsulation de bactéries probiotiques.

Des points sensibles ont pu être identifiés tels que :

- L'absence d'agitation de la phase enzymatique affecte les capacités d'interaction entre l'enzyme et son substrat.
- L'ouverture du système au moment de la phase enzymatique et de l'émulsification le rend sensible aux contaminations extérieures.
- La non maitrise du pas de la rampe de température entraine des variations dans la structure même du gel obtenu.
- La dynamique d'ajout des deux phases est réalisée selon la méthode standard.
- Le lavage des microparticules par des successions de centrifugation/suspension dans de l'eau distillée entraine l'agrégation des microparticules.

Les différences majeures observées entre le système d'encapsulation à l'échelle laboratoire et celui à l'échelle pilote sont présentées dans le Tableau 14.

Lors du passage à l'échelle pilote, en plus de la volonté de produire une plus grande quantité de microparticules, tous ces points ont été pris en considération dans le but d'améliorer le système.

L'objectif majeur du passage par une encapsulation à l'échelle pilote est le dimensionnement qui permet ensuite l'extrapolation. Un exemple d'extrapolation selon une similitude géométrique a été décrit mais d'autres critères pourraient être choisis dans le cas où un industriel souhaiterait réaliser l'encapsulation dans une cuve qu'il possède déjà.

La mise au point et le dimensionnement du procédé d'encapsulation ont fait l'objet d'un dépôt de brevet présenté en annexe.

Tableau 14 : Différences majeures entre le procédé d'encapsulation à l'échelle laboratoire et celui à l'échelle pilote.

	Echelle laboratoire	**Echelle pilote**
Volume d'huile utilisé (ml)	150	800
Volume de lait utilisé (ml)	15	200
Quantité de microparticules produites (g)	3	41
Extrapolation à l'échelle industrielle	Impossible	Possible
Maitrise de la température	Approximative	Parfaite
Système	Cuve ouverte donc risques de contaminations externes	Tout le dispositif est fermé donc aucune contamination possible
Phase enzymatique	Non agitée = action non optimale de l'enzyme	Agitée = meilleure répartition de l'enzyme sur son substrat
Nature de l'enzyme	Naturen™ (CHR Hansen) = activité résiduelle de l'enzyme au-delà du procédé d'encapsulation	CHY-MAX Plus (CHR Hansen) = ABSENCE d'activité résiduelle de l'enzyme au-delà du procédé d'encapsulation
Dynamique d'ajout des phases	Standard	Batch
Mobile d'agitation pour l'émulsification	Turbine de rushton	Hélice marine pour limiter de cisaillement
Nature de l'émulsifiant	Pas d'émulsifiant (particules agrégées)	Tween® 80 (particules bien individualisées)
Procédé de séparation	Centrifugation (tendance à l'agrégation)	Filtration (facilement industrialisable et individualisation des particules)

Chapitre 3 :

Formulation de la matrice d'encapsulation

1. Introduction

Parmi les différents matériaux d'encapsulation qui existent, les protéines sont des candidates de choix pour la protection des bactéries probiotiques. Les protéines du lactosérum ont été régulièrement utilisées comme matrice encapsulante (Picot and Lacroix, 2004, Picot and Lacroix, 2003b, Picot and Lacroix, 2003a) dans le but de protéger les bactéries probiotiques durant les étapes de fabrication et le stockage dans l'aliment dans lequel les microparticules sont introduites. L'utilisation de protéines du lactosérum permet d'élargir la gamme de produits dans lesquels les bactéries encapsulées sont incorporées (Reid et al., 2007) mais également d'assurer une libération des cellules au niveau de l'intestin.

La formation d'un gel à partir de la coagulation de protéines laitières a démontré être une bonne solution pour produire des microparticules insolubles dans des milieux aqueux. Pour cela, l'utilisation de caséines est requise de même qu'une enzyme protéolytique telle que la présure ou la transglutaminase. La création d'un microenvironnement favorable à la survie de la bactérie est probablement responsable de la réussite des procédés utilisant les protéines laitières.

Néanmoins, selon les études, les taux d'encapsulation et de survie dans l'estomac sont très variables. Plusieurs paramètres peuvent être à l'origine des différences observées dont les proportions des protéines présentes qui influencent directement la qualité du réseau formé.

Dans l'objectif de définir une matrice d'encapsulation optimale pour LGG, un plan de mélange a tout d'abord été utilisé afin de comprendre l'effet des proportions en caséines et protéines solubles, dénaturées ou non, sur la taille des particules obtenues, leur matière sèche mais également sur l'élasticité du gel formé.
Cette première approche a permis de différencier des formulations très opposées. Ainsi 4 de ces formulations ont été utilisées pour produire des microparticules contenant LGG puis soumises à une étude de leur devenir dans des conditions simulant l'estomac.

Pour terminer, la structure des microparticules issues de ces 4 formulations a été observée par différentes techniques telles que la microscopie électronique à balayage, la microscopie électronique à transmission, la microscopie confocale ou encore la microscopie de force atomique.

2. Material and methods

2.1. Formulation selection according to microparticle physico-chemical properties

2.1.1. Mixture design description

In order to select the best formulation for the production of microparticles, a mixture experimental design with lower and upper bound restrictions on the component proportions was applied (Tableau 15).

Tableau 15: Description of upper and lower constraints required for the construction of domain.

Component (w%)	Limits (%)	
	Lower	Upper
Micellar casein (X1)	80	100
Native whey protein (X2)	0	20
Denatured whey protein (X3)	0	20

A three-component mixture was set up, corresponding to the amount of micellar casein (X1), whey proteins in a native (X2) or denatured (X3) state. The casein percentage was allowed to vary between 80 and 100 % and native or denatured whey proteins were both between 0 and 20 %. These proportions were selected as a reference to milk composition which contains around 80 % of caseins and 20 % of whey proteins. The constrained region represented a centred simplex where seven experimental points and three test points were distributed. A centred cubic design was used to construct a third order Scheffé polynomial model for fitting the experimental data.

The equation was of the form:

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3$$

where Y is the dependent variable, β_1, β_2, etc., are the coefficient estimates and X_1, X_2 and X_3 are the concentrations of micellar casein, native and denatured whey proteins respectively.

In the study, 10 experiments were performed according to the plan given by experimental mixture design. As shown in Tableau 16, seven mixtures were realised (grey lines) and three additional test points were done. Each line corresponds to a

specific mixture presenting variable percentages of milk proteins in a native or denatured state.

Tableau 16 : Amount of micellar casein (X1), native whey (X2) and denatured whey (3) proteins used in each of the 10 formulations of the experimental design.

Experiment	Experimental plan		
	Micellar casein	Native Whey	Denatured whey
1	1.00	0.00	0.00
2	0.80	0.20	0.00
3	0.80	0.00	0.20
4	0.90	0.10	0.00
5	0.90	0.00	0.10
6	0.80	0.10	0.10
7	0.8667	0.0667	0.0667
8	0.9333	0.0333	0.0333
9	0.8333	0.1333	0.0333
10	0.8333	0.0333	0.1333

Grey lines: experimental points; white lines: tests points.

For each of the ten experiments three responses were recorded: the particle size (Y1), the dry matter (Y2) and the elastic modulus (Y3).

2.1.2. Dry matter

The percentage of water and dry matter were determined for microparticles produced by each formulation. For this, 1 g of microparticles was dried in a forced convection oven (WTB binder, Amilabo, Germany) for 5 h at 105 °C until they reached a constant weight. The initial and the weight loss after drying provides information on water content (ADPI, 2002).

2.2. Formulation selection according to microparticle resistance in a gastric environment

2.2.1. Material used

Micellar casein powder (Promilk 872B) was obtained from Ingredia IDI (Arras, France). Whey protein isolate powders (Prolacta 90) were purchased from Lactalis

Ingredients (Bourgbarré, France). The chemical characterization of the powders has been already studied (Karam et al., 2012). Micellar casein and whey proteins present respectively a protein content around 87% and 90% (w/w).

The rennet preparation (Naturen™) was provided by CHR Hansen (Arpajon, France) and presented an activity of 140 IMCU.mL^{-1}. The rennet solution was prepared just before use by diluting 3 g of rennet preparation in 12 g of distilled water (28 IMCU.mL^{-1}). The sunflower oil was purchased from a local store.

LGG (ATCC 53103) was used throughout this study. Bacteria were first subcultured at 37 °C in MRS medium. This preculture was then used to inoculate 500 mL of MRS broth which was incubated overnight at 37 °C until an early stationary phase. Cells were centrifugated (15 min, 3500 g, room temperature), washed with physiologic water (pH 7.4) and finally harvested by centrifugation (15 min, 3500 g, room temperature). The pellet was then frozen and placed on the shelves of a freeze-dryer (Christ alpha 1–2, freeze-dryer, Osterode, Germany). The microbial powder obtained after freeze-drying has a content of 10^{11} CFU/g.

2.2.2. Microparticulation procedure

2.2.2.1. Preparation of the carrier material

All the solutions were prepared by adding 12.5 g of protein powder into 100 g of distilled water (Figure 34). The rehydration was done by stirring for 2 h at room temperature and then overnight at 4 °C. The denatured whey proteins were obtained by heating the native whey solution at 78 °C for 10 min then cooling it to room temperature. Four formulations were used to produce variable microparticles with different milk protein composition (micellar casein and whey proteins in a native or denatured state). The amount of each solution used to prepare the initial mix of protein solution is detailed in Figure 34.

The four formulations were tested for the microencapsulation of LGG. The strain was added after mixing the protein solution and before enzymatic incubation (Figure 34). 0.03 g of freeze-dried LGG was added to 15 g of protein mixture.

2.2.2.2. Microparticle production

Microparticles were produced by using an emulsification method (Heidebach et al., 2009a) and the procedure is detailed in Figure 34.

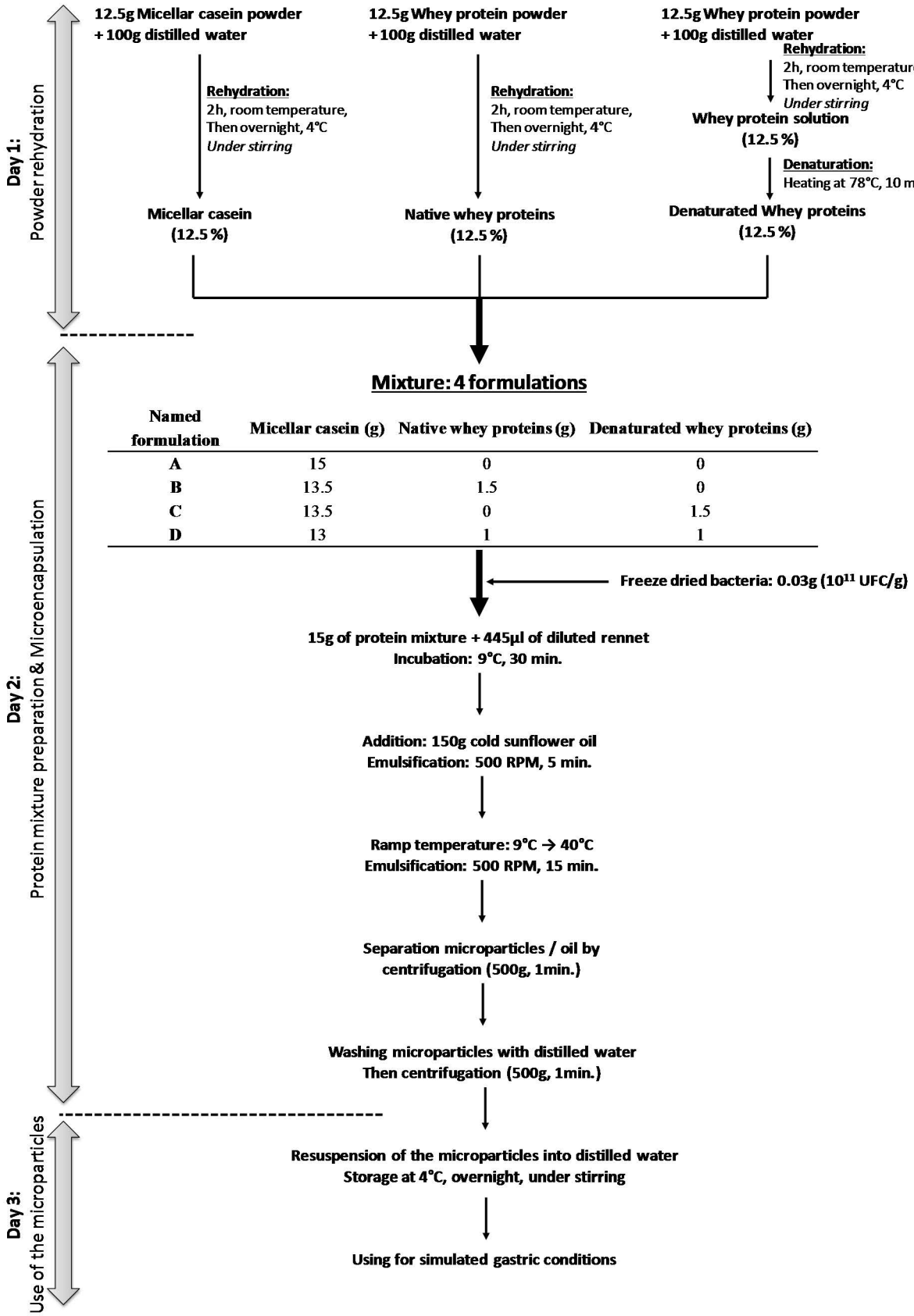


Figure 34: Flowchart for the production of microparticles.

The encapsulation procedure was completed in a double-walled, temperature controlled reactor, made of stainless steel. An amount of 15 g of protein mixture was added into the reactor held at 9 °C. Then, 445 µL of diluted rennet preparation was added and mixed with the solution. The mixture was left for 30 min at 9 °C allowing the rennet enzyme to cut the κ-casein. This step was followed by an emulsification. For this purpose, 150 g of cooled sunflower oil was added and stirred with the mixture at 500 RPM for 5 min. Afterward, the temperature was raised to 40 °C for 15 min and under mechanical agitation.

Microparticles were removed from the reactor and separated by centrifugation (500 g, 1 min). The harvested particles were washed with distilled water. Afterwards, the microparticles were stripped from the container and shaken for 12 s. Another centrifugation following the same conditions was then performed. Finally, the microparticles were removed, re-suspended in distilled water and stored at 4 °C under stirring until their use the next day.

2.2.2.3. Encapsulation rate (ER)

The enumeration of LGG living cells was done by serial dilutions. The samples (0.1 mL) were plated in duplicate on MRS agar. CFU were determined after incubation (48 h at 37 °C). To enumerate the entrapped probiotic cells, the microparticles were mechanically broken with a spatula.

The ratio between the number of bacteria added in the protein mixture and the number of bacteria in the final particles was done. The initial number of bacteria introduced in the protein mixture was obtained by determining the CFU in 1 g of solution. The result was multiplicated by 15 which is the total mixture quantity used for microencapsulation. At the end of encapsulation the microparticle total weight was measured. The final CFU in 0.2 g of microparticle was also determined and multiplicated by the total quantity of obtained microparticles. The encapsulation rate (ER) was calculated by applying the following equation:

$$ER\ [\%] = \frac{[\mathrm{CFU/g}]^{\text{Final microparticles}} * [\text{quantity of microparticles (g)}]}{[\mathrm{CFU/g}]^{\text{Initial solution}} * 15} * 100$$

2.2.3. Simulated gastric digestion

Simulated gastric fluid was formulated with sodium chloride (34 mM) and hydrochloric acid with a final pH of 2.5 (Pharmacopeia, 2008). This pH value was chosen according to (Gbassi et al., 2011) and corresponds to the encountered pH in stomach after consuming a classical meal. Finally, 0.64 g of pepsin was added to 200 mL of gastric juice. The simulation of the stomach digestion was done in a temperature-controlled reactor, made of glass, with a magnetic stirrer (150 RPM). The particles (2 g) were introduced in 200 mL of gastric solution and left for 2 h at 37 °C to imitate human body temperature. During particle digestion, the reactor was directly connected to a particle size and shape analyzer (Figure 35).

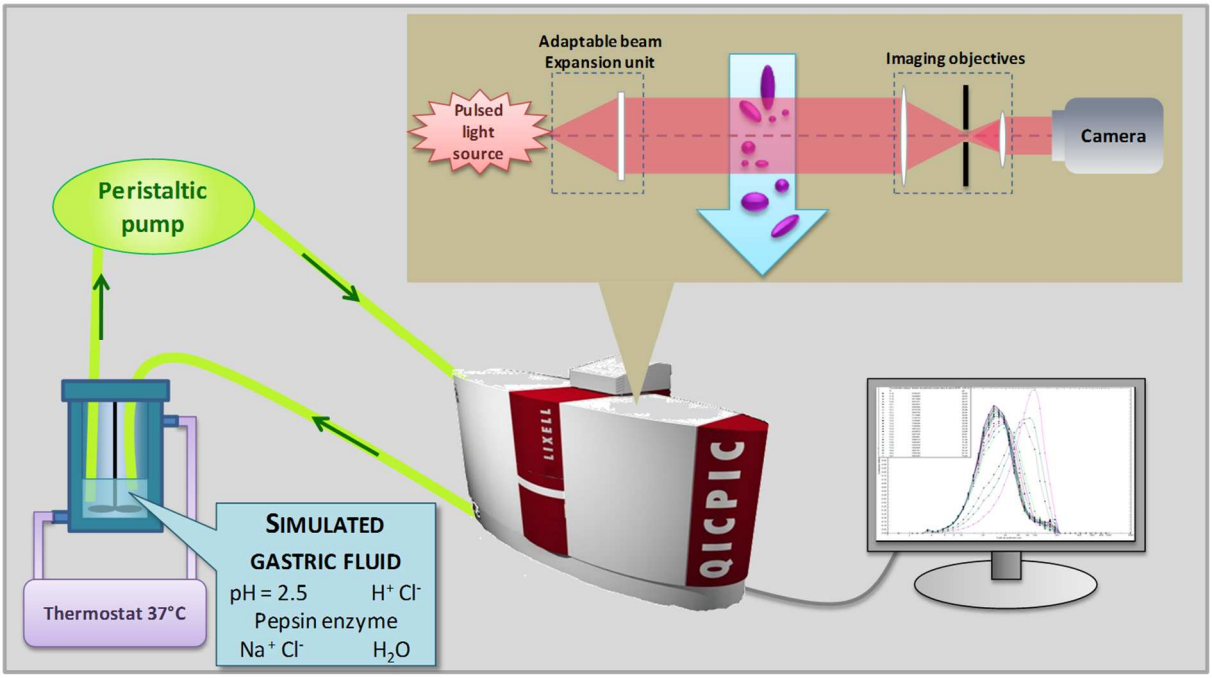


Figure 35: Installation allowing the monitoring of "*in situ*" microparticles digestion.

In order to determine the survival rate (SR) of free or encapsulated LGG during simulated gastric digestion, aliquots of 1.5 mL were collected at different times: 5, 30, 60 and 120 min. The SR was calculated by the following equation:

$$SR\ [\%] = \frac{CFU/g^{(t=\ t_i)}}{CFU/g^{(t=\ 0)}} * 100$$

where $CFU/g^{(t\ =\ 0)}$ is the cell count before incubation in the simulated gastric juice and $CFU/g^{(t\ =\ ti)}$ is the cell count at various incubation times t_i.

2.2.4. Size and morphology analysis

Particle size and shape were determined by using a QICPIC™ analyzer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) equipped with a module specific for dispersions. This equipment allows instantly evaluating and characterizing particle sizes and shapes even in acid and corrosive media like gastric mimic media. Here, the analyzer was directly connected to the reactor of digestion (Figure 35) and planned to take measurements every 5 min during the 120 min of digestion. The gastric liquid was pumped into the reactor and passed through the measuring cell. Concurrently, images captured were recorded. The analysis of these images enabled size and shape data collection.

Image analysis provides 2D image of a complex 3D particle then determines several size and shape parameters from the 2D image. The diameter of a circle of equal projection area (EQPC) was calculated. It corresponds to the diameter of a circle with the same area as the 2D image of the particle (Figure 36A). Because different shaped particles may have the same EQPC, other parameters were used to describe the particles. The first shape parameter measured was the sphericity (Figure 36B) defined by the ratio between the EQPC perimeter with the real particle perimeter. The sphericity values comprised between 0 and 1. For example, a particle with a sphericity of 1 is a perfect circle whereas a particle with a sphericity close to 0 is irregular. Thus, sphericity is a good way to describe the particle shape deviation from a perfect circle. The second shape parameter determined was the convexity (Figure 36B). This measure provides information about the roughness of the particle. Convexity values also comprised between 0 and 1. A particle with smooth edges has a convexity value of 1 whereas a particle with irregular ones has a lower convexity (Gaiani et al., 2011).

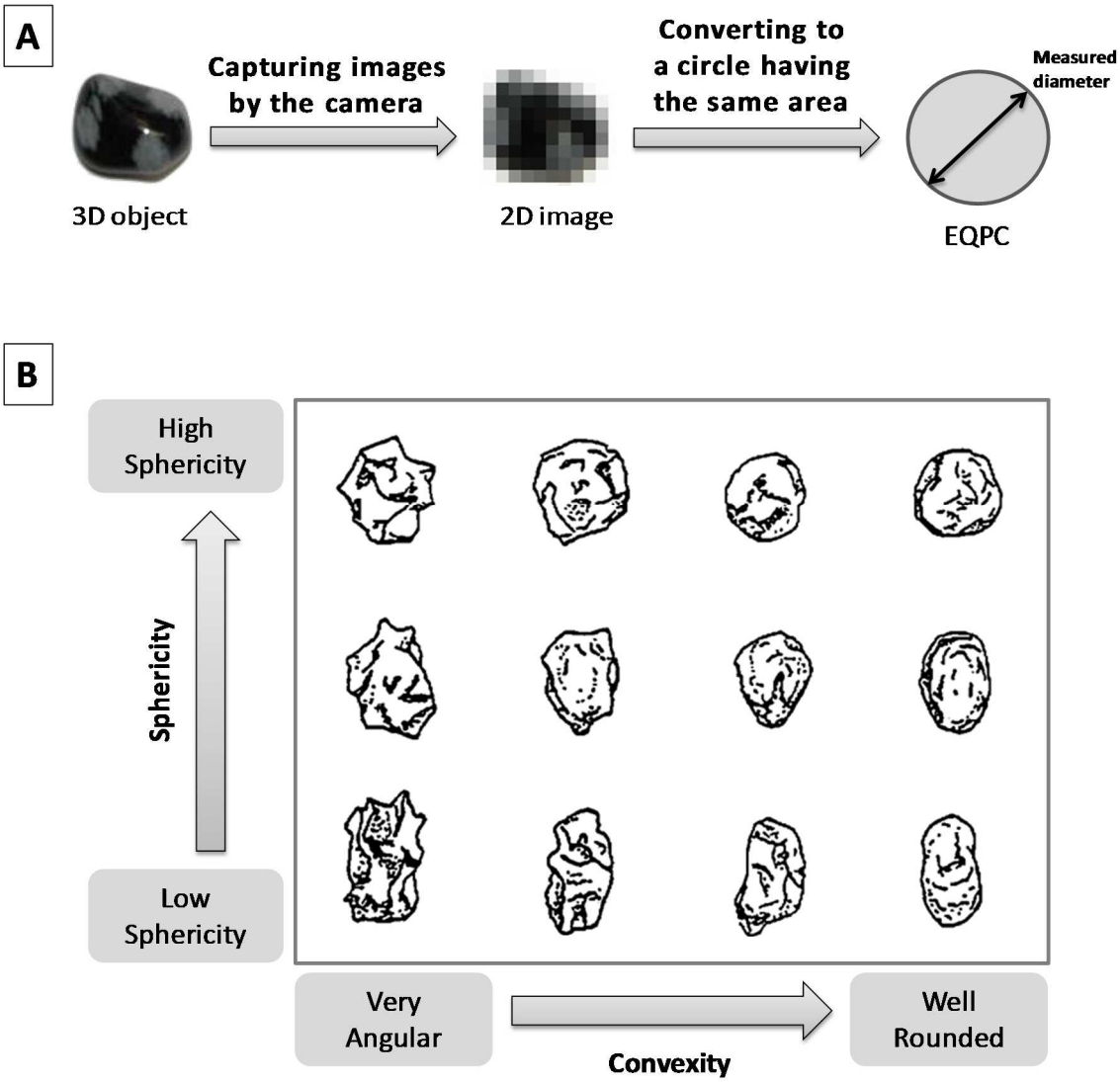


Figure 36: Particle size and shape descriptors provided by the QICPIC™ analyser. A- Representation of how the EQPC is calculated from a 3D object. B- Representation of the sphericity and the convexity variations as a function of real particle morphology.

The size distribution was characterized by the breadth of the distribution (Bdi) calculated by the following equation:

$$\mathrm{Bdi} = (d_{90} - d_{10})/d_{50}$$

The diameter values for d_{10}, d_{50} and d_{90} reflect that 10 %, 50 % or 90 % of the population has a diameter below the value. The calculation of the Bdi is a criterion to describe the population polydispersity.

During digestion, the particle sizes were recorded continuously. The d_{50} evolution was normalized and represented as a function of digestion time according to the following equation.

$$y_t = \frac{d_{50}\ (t = t_i)}{d_{50}\ (t = 0)}$$

where d_{50} (t = 0) is the first particle size measurement during simulated gastric digestion and d50 (t = ti) is the measurement between 5 and 120 min of digestion. A linear-exponential equation was used to fit the model (Kong and Singh, 2008):

$$y_t = (1 + k\beta t)e^{-\beta t}$$

k, is dimensionless and represents the lag phase. Thus, a large k means that a delayed-sigmoïdal disintegration occurred whereas a small k represents an exponential decay. β (L min−1) allows the measurement of the gastric emptying of the particles as a function of digestion time (β > 0).

The k and β values can be obtained with a regression analysis. When the parameters k and β are determined, the half time ($t_{1/2}$) can be calculated using y_t = 0.5. The half time corresponds to the time when size was reduced by 50 %.

2.2.5. Dairy gel rheological properties

Rheological properties of the gel (viscous and elastic components as well as the final value of the storage modulus and the phase angle) were measured with a Kinexus rotational rheometer (Malvern Instruments, KNX 2100, UK). The rheometer was equipped with a cup and bob geometry consisting of coaxial cylinders. The oscillatory mode was used with a shear strain of 6 % and a frequency of 1 Hz. The kinetics of rennet coagulation were followed *in situ* for each formulation. Incubation of the protein mixture with the rennet enzyme was realized at 9 °C for 30 min and then, a temperature ramp (2 °C/min) from 9 °C to 40 °C was applied.

2.2.6. Statistical analysis

The evolution of d_{50} during digestion (y_t) was analyzed and fitted into an empirical model. Modeling of data was performed with XLStat™ 2010.5.06 software (AddinSoft, France) as an add-in to Microsoft Excel™ 2007. Estimates for parameters were obtained by minimizing the mean square error (MSE) according to:

$$\mathrm{MSE} = \frac{\sum_{i=1}^{n}(y_{exp} - y_{fit})^2}{n}$$

where y_{exp} represents the experimental value, y_{fit} is the fitted value, and n is the number of experimental points. The value of MSE provides information of the goodness of fit. Additionally, the correlation coefficient (r^2) is also a criterion for assessing the performance of the model. A value closer to 1 indicates that the prediction model is effective.

All measurements presented in this paper were performed on three independent samples (excepted SR). The KyPlot software version 2.0 was used and a parametric multiple comparison test (Tukey test) was performed. The significance levels were: ***P < 0.001, **P b 0.01, *P < 0.05 and ^{NS}P > 0.05.

2.3. Formulation selection according to microparticle microstructure

2.3.1. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe freeze-dried microparticles set on a double-sided adhesive tape and fixed to SEM stubs. Excess particles were removed with a dry air projection at the surface of the stubs. Samples were then covered with gold by sputtering. Finally, the samples were studied with a Hitachi SEM instrument (Hitachi S-4800, Tokyo, Japan) operating at 10 kV.

2.3.2. Transmission electron microscopy

Microparticles were fixed during 2 hours in glutaraldehyde (2.5 %), then flushed with cacodylate buffer during 3 hours. A post fixation with osmium tetroxide during 1 hour was performed at ambient temperature. Successive dehydration in increasing ethanol baths was then done (30, 50, 70, 80, 90, and two times at 100 %). The sample is finally embedded in epoxy resin followed by a resin polymerisation at 56 °C during at least 48 h.

The day after, ultrathin slides (70 nm) were obtained with an Ultramicrotome (Reicher-Yung). Acetate Uranyle was used as a slide contrast agent. Microstructure of particle was examined with a CM12 Philips Scanning Electron Microscope.

2.3.3. Confocal laser scanning microscopy

The staining of protein network was performed by using fast green FCF, lipids around microparticles were stained with Nile Red dye (10 µg/mL) for 30 min, then washed in PBS, and mounted onto glass slide. Preparations were visualized and digitized images were taken by using an FV10i-W confocal microscope (Olympus, Rungis, France).

2.3.4. Atomic force microscopy: Topography

For topographic images, Olympus micro cantilevers OMCL-TR400 were purchased from Atomic Force (Mannheim, Germany) with a spring constant of 20 pN/nm.

Topography images were made at room temperature using an Asylium MFP-3D atomic force microscope (Santa Barbara, CA, USA) with IGOR Pro 6.04 (Wavemetrics, Lake Osewego, OR, USA) as operation software. It was used in contact mode with an applied force lower than 250 pN and images were acquired at a scan rate of 1 Hz and scan size of 30 µm × 30 µm.

2.4. Experimental mixture design

Three measures were selected to be the responses of the experimental design: the size (Y1), the dry matter (Y2) and the elastic modulus of the gel (Y3). Tableau 17 summaries the experimental responses. In all three cases, the calculated values are close to those obtained by experiment, especially for the particle size response where calculated and experimental values are similar.

Tableau 17 : Experimental values for the three responses: Particle size (Y1), Dry matter (Y2) and Elastic modulus of the gel (G') (Y3).

Experiment	Experimental responses		
	Y1 (µm)	Y2 (%)	Y3 (Pa)
1	70	29.0	61
2	68	29.5	20
3	58	28.4	23
4	56	32.2	56
5	59	29.4	96
6	46	31.6	66
7	47	25.3	79
8	56	28.5	107
9	51	29.0	99
10	48	27.6	118

- Response model and validation

The analysis of variance (ANOVA) indicated in Tableau 18 shows how the total calculated sums of squares are distributed among the different sources of variations. As it can be seen, the regressions sums of square are statistically significant. The evaluations of the residual sum of squares with 3 degrees of freedom allow the validation of adequacy of the fitted model for Y1, Y2 and Y3. We can then conclude that the model is adequate and can be used as prediction equation for both three responses (Y1, Y2 & Y3).

Tableau 18 : Analysis of variance of the responses.

Source of variation	Sum of squares	Mean square	F ratio	Significance
Y1				
Regression	$6.22 *10^{2}$	$1.04 *10^{2}$	370.4	0.02^{***}
Residuals	$8.00 *10^{-1}$	$3.00 *10^{-1}$		
Total	$6.22 *10^{2}$			
Y2				
Regression	$3.31 *10^{1}$	$5.51 *10^{0}$	29.2	0.94^{**}
Residuals	$6.00 *10^{-1}$	$1.89 *10^{-1}$		
Total	$3.36 *10^{1}$			
Y3				
Regression	$1.5 *10^{4}$	$2.5 *10^{3}$	71.9	$< 0.001^{***}$
Residuals	$4.7 *10^{3}$	$3.6 *10^{2}$		
Total	$2.0 *10^{4}$			

Model exploitation

The relationship between each response and the variables can be illustrated by iso-response curves which represent lines of constant response in a two variables plane. Such plots are helpful in studying the effects on each response of the three factors in the studied domain.

Figure 37 represents the iso-response curves of Y1, Y2 and Y3. It shows that the particle size increase with a higher content of micellar casein. On the contrary, the addition of whey proteins allows a diminution.

The objective is to formulate an encapsulation matrix with a particle as small as possible. However, the size cannot be too low, because the microparticles must contain enough bacteria ranging in size from 1 to 3 µm and bigger microparticles better resist in stomach (Lee and Heo, 2000). Matrix density is indirectly described by the elastic modulus and in order to have a solid network, high elasticity must be obtained ensuring a better retention of probiotic bacteria within the microparticles.

Tableau 19 shows the coefficient estimates and the determination coefficients of the Scheffé models that were fitted to the experimental data. The interaction between the three components leads to a positive effect on the elastic modulus. It can be the

result of network organization that is different when caseins are the only constituent than when whey proteins are added.

- Model exploitation

The relationship between each response and the variables can be illustrated by iso-response curves which represent lines of constant response in a two variables plane. Such plots are helpful in studying the effects on each response of the three factors in the studied domain.

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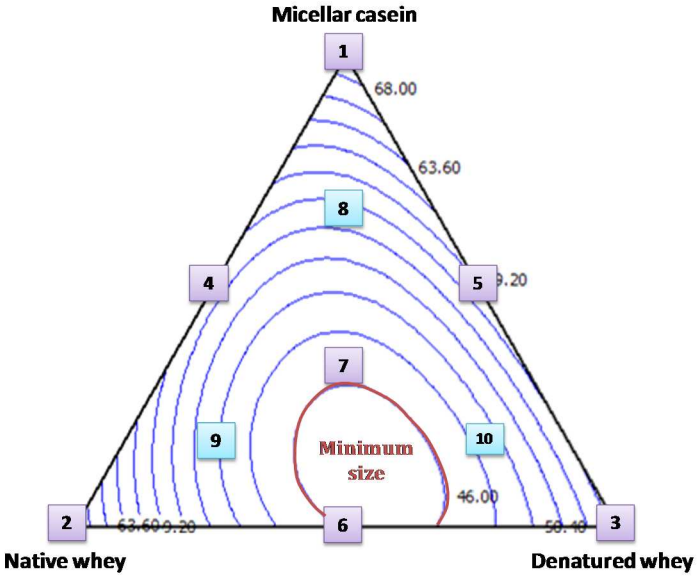
Tableau 19 : Derived Scheffé's coefficients for the particle size, the dry matter and the elastic modulus for microparticles formulated with micellar casein (80-100 %), native whey (0-20 %) and denatured whey proteins (0-20 %). The determination of the coefficient r^2 for the calculated model is included below each column (grey line).

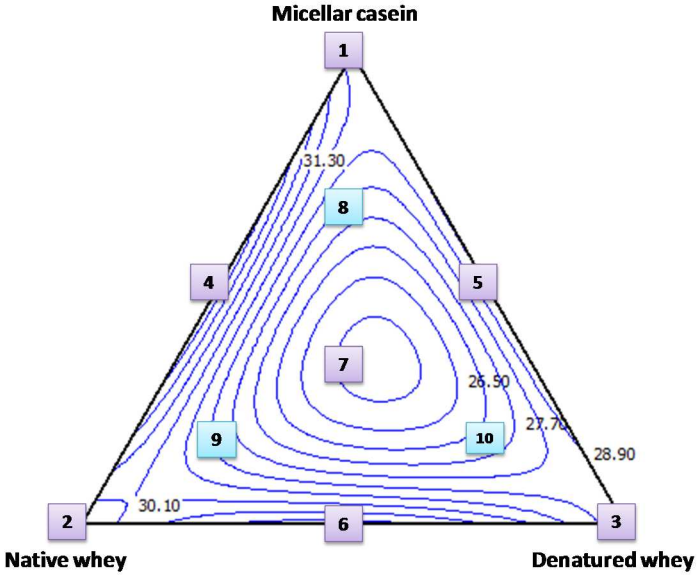
	Coefficient	Particle Size	Dry matter	G'
Micellar casein	β_1	70.0 ***	29.13 ***	62 ***
Native Whey	β_2	1099.5 ***	-210.86 *	-1522 **
Denatured Whey	β_3	415.8 **	-35.40	-4699 ***
Interactions of first	β_{1-2}	-1299.3 ***	302.43 *	1740 **
order between	β_{1-3}	-508.4 **	76.06	5747 ***
components	β_{2-3}	6761.1 *	16878.37 **	-34228 *
Interaction between the three components	β_{1-2-3}	-10586.9 *	-20765.95 **	48917 *
Statistic of coefficients: r^2		0,999	0.98	0.751

* P < 0.05, ** P < 0.01, *** P < 0.001

(A) Particle size (µm)



(B) Dry matter (%)



(C) Elastic modulus (Pa)

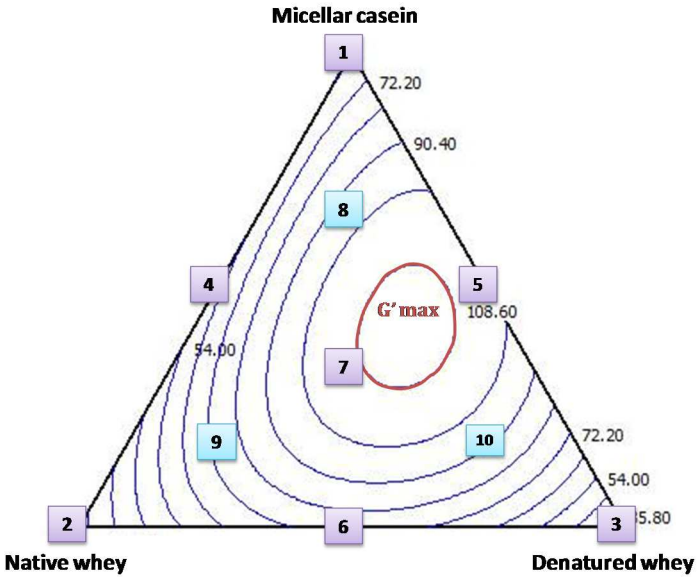


Figure 37 : Iso-response curves for the mixture design: particle size (A), Dry matter (B) and elastic modulus (C).

This experimental design doesn't allow selecting one formulation as the maximum particle size obtained is acceptable. The dry matter provides important information but is not determinant for selecting the formulation. Finally, elastic modulus is an interesting parameter to explore as a high value reflects a dense matrix which can further provide matrix resistance when microparticles are submitted to gastric medium. In fact, proteolytic enzymes cannot penetrate into the structure. The observed results for this parameter are significant meaning that the prediction made by the software can be considered.

As mentioned just before, the elastic modulus is an important parameter to consider. For that reason we decided to select the two formulations that are the closest to the iso-coutour line providing the highest value. The formulation 5 and formulation 7 were thus the first selected. In order to compare their behavior in a simulated gastric digestion, formulation 1 only composed of micellar casein which is the base of this study was also selected. Finally, in order to see if the denaturation of whey proteins plays a role in microparticle resistance in stomach digestion, the formulation 4 was also selected.

The summary of selected formulations is listed in Tableau 20.

Tableau 20 : Protein composition of selected formulations.

	Micellar casein	**Native whey**	**Denatured whey**	**New name**
Formulation 1	1	0		Formulation A
Formulation 4	0.9	0.1	0	Formulation B
Formulation 5	0.9	0	0.1	Formulation C
Formulation 7	0.8667	0.0667	0.0667	Formulation D

3. Microencapsulation of LGG : influence of matrix formulation on bacterial survival during digestion

Encapsulation of Lactobacillus rhamnosus GG in microparticles: Influence of casein to whey protein ratio on bacterial survival during digestion

J. Burgain, C. Gaiani, C. Cailliez-Grimal, C. Jeandel & J. Scher (2013). *Innovative Food Science and Emerging Technologies*, 19; 233-242.

Abstract

Encapsulation of *Lactobacillus rhamnosus* GG in various microparticles made of only milk proteins (casein, native whey and/or denatured whey proteins) was done. The microparticles obtained were rather similar in shape (mostly round) and size (around 60 µm) whatever the formulation but the obtained gel presented different elasticity (varying between 61 and 96 Pa). An original equipment involving a granulo-morphometer coupled to a thermostated reactor was developed and validated to visualize *in situ* the microparticles during digestion. Although the initial particles were similar, their disintegration in simulated gastric media was totally different and characterized by two stages. An initial decrease in particle size more or less quick depending on the protein composition was followed by a stable phase characterized by the particle size and shape retention. At the end of gastric digestion, a significant amount of intact particles was still noticeable for each formulation. Nevertheless, the formulation containing a mix of casein and denatured whey presented the best bacterial survival (99%) and encapsulation rate (97%) in comparison with formulations containing either only casein or casein and native whey or casein in mixture with native and denatured whey proteins.

Résumé

L'encapsulation de LGG dans des microparticules constituées uniquement de protéines laitières (caséines, protéines solubles natives et/ou dénaturées) a été réalisée. Les microparticles obtenues sont rondes et leur taille est d'environ 60 µm quel que soit la formulation alors que le gel obtenu présente des différences significatives d'élasticité. Un nouvel équipement comprenant un granulo-morphomètre couplé à un réacteur thermostaté a été développé et validé pour visualiser la digestion *in situ* des microparticules. La dégradation des microparticules dans le milieu simulant les conditions gastriques a été différente en fonction de la formulation mais dans tous les cas caractérisée par deux étapes : d'abord une diminution de la taille plus ou moins rapide puis, une phase de stabilité durant laquelle la taille et la forme ne varient plus. A la fin de la digestion de nombreuses particules intactes sont conservées pour chacune des formulations.

Finalement, une formule optimale a été identifiée. Elle contient un mélange de caséines et de protéines solubles dénaturées et présente le meilleur taux de survie (99%) et d'encapsulation (97%) des bactéries comparé aux trois autres formulations.

Industrial relevance:

This paper is part of a global project entitled “Structured dairy matrices to enhance probiotic efficiency”. The entire project will provide milk structured matrices allowing the stabilization and the vectorization of LGG. This project will consist of four main axes: milk constituent's interactions with LGG, stabilization process implementation, and structural and functional characterization of the matrices obtained. The scientific objective is to propose models connecting process parameters, matrix structure (from an atomic, molecular to a macro scale) and their functionality. This implies the in-depth study of interactions between milk components and probiotic strain. For this purpose, the use of genetically modified strain of LGG will allow the identification of biomolecules interacting with milk matrices. The industrial aims are to optimize and control the processes to suit the needs of industrial criterions: encapsulation rate, gastric resistance, intestinal release, storage in the final food…

3.1. Introduction

Modern consumers are increasingly buying into the concept that probiotic yogurt and other fermented milk products improve digestion, boost immunity and provide other health benefits long claimed by food companies (Burgain et al., 2011). However, regulatory authorities, particularly in Europe, have not supported probiotic health claims on the grounds that the provided data were not sufficient. This year, the EFSA has rejected almost all health claims put forward by the probiotic industry. Several reasons are invoked to justify the refusal: microbes have not been sufficiently characterized, the claimed effect was not considered beneficial or because human studies to support the claims were not provided (Schmidt, 2013). The design of biopolymer based microparticles to encapsulate, protect and release a specific bioactive component believed to benefit human health is now gaining interest (Matalanis et al., 2011). In most cases, the encapsulated substance needs to be released at a specific site in the body. Thus, developing a model identifying the physico-chemical mechanism leading to the release could be a useful tool to predict microparticles' future (Matalanis et al., 2011).

In the past few years, the use of bioactive ingredients derived from the dairy sector has gained interest. Dairy ingredients are widely used in the food industry because of their technological properties. In fact, they are recognized for their surface-active and

colloid stabilizing characteristics (Horne, 2009). Moreover, their health benefits are increasingly promoted (Mackie and Macierzanka, 2010). Milk proteins can be used as a carrier for health-promoting delivery system. For example, nanoparticles are used to solubilize and protect hydrophobic nutraceuticals. In this case, the natural digestibility of caseins is exploited (Livney, 2010). Milk contains two major protein groups: casein and soluble proteins. These proteins differ in their physico-chemical properties and more particularly in their amino acid composition. Caseins and whey proteins make up respectively around 80% and 20% of the proteins in milk. Due to their emulsifying properties and amino-acid composition, caseins play an important role in human nutrition. These characteristics explain their wide use as additives in food. Casein micelles are composed of α_{s1}-, α_{s2}-, β- and κ-casein proteins. Rennet coagulation of milk results from κ-casein proteolysis by chymosin enzyme. In fact, by releasing hydrophilic fragments, repulsive forces disappear allowing thereby micelle aggregation via calcium bond formation (Horne, 2009). Gelation properties of milk proteins can be exploited for probiotic encapsulation. Many health promoting claims attributed to probiotic bacteria are dependent on the cells being both viable and sufficiently numerous in the intestinal tract (Cook et al., 2012). Nevertheless, the passage through the stomach of most bacteria results in an important loss of viability which lowers the efficacy of the administered supplement. Formulation of probiotics into microcapsules is an interesting method to reduce cell death during the gastrointestinal passage (Burgain et al., 2011). The use of milk protein based microparticles was poorly exploited whereas it was demonstrated that dairy matrices such as cheeses can protect bacterial cells (Stanton et al., 1998) thanks to the good buffering capacity of milk proteins for example (Livney, 2010).

Rennet gelation of milk proteins was used to develop a microencapsulation technology for the protection of two strains: *Lactobacillus paracasei* ssp. paracasei F19 and *Bifidobacterium lactis* Bb12 (Heidebach et al., 2009a). The obtained microparticles were spherical and water insoluble due to the use of an emulsification process. This technique allows a high encapsulation rate and a good microparticle resistance in simulated gastric conditions leading hence to an important survival rate of bacteria. By using this technique, the authors claimed that probiotic cells can resist to adverse conditions encountered in the stomach. Another enzyme (transglutaminase) was also tested to produce microparticles where milk protein gelation was exploited (Heidebach et al., 2009b). The heat induced gelation of whey proteins can be used to produce

microparticles containing probiotic bacteria. In this way, *Bifidobacterium* Bb-12 was microencapsulated by spray drying with whey proteins and the entrapped cells presented better survival during simulated gastric digestion (De Castro-Cislaghi et al., 2012). Another example is the microencapsulation of LGG with gelled whey protein isolates. The produced matrix was able to protect the cells during *in vitro* stomach incubation (Doherty et al., 2011) and also during *ex-vivo* digestion (Doherty et al., 2012). Finally, all of these trials for microencapsulation with dairy proteins led to an increase in bacterial survival during digestion.

In this context, the first objective of this study was to compare the resistance of four matrices (without bacteria) constituted by only milk proteins in simulated gastric environment. For this purpose, an original equipment allowing the *in situ* determination of particle size and shape during digestion was developed. Then, the same matrices were used to encapsulate LGG. Survival rate during gastric digestion was followed during the 2 h. The selection of the best formulation should be done by the confrontation of results: particle size, shape and Bdi, encapsulation and gastric resistance rates.

3.2. Microparticle characterization

3.2.1. Size, shape and breadth of the distribution

Different initial sizes depending on the formulation were observed (Figure 38, black column). The biggest average particles were obtained from formulation A (69 µm) and the smallest from B (56 µm). Formulations C and D provided intermediate microparticles of 59 and 65 µm respectively. Significant differences between A and B ($P < 0.01$) and between A and C formulations ($P < 0.05$) were measured. The convexity and sphericity values of all microparticles whatever the formulation were comprised between 0.80–0.85 and 0.68–0.81 respectively (Tableau 21). These values reflected that the microparticles were initially relatively well rounded.

The breadth of the distribution (Bdi) provided information on the polydispersity of the produced microparticles (Figure 38). Formulation C presented the lowest polydispersity (1.08) whereas formulation B had the highest one (1.50). These two formulations had the same amount of casein however whey proteins were in a native form in formulation B and denatured in formulation C. The polydispersity of the two other formulations A and D comprised between these two extremes with values of 1.22 and 1.28 respectively.

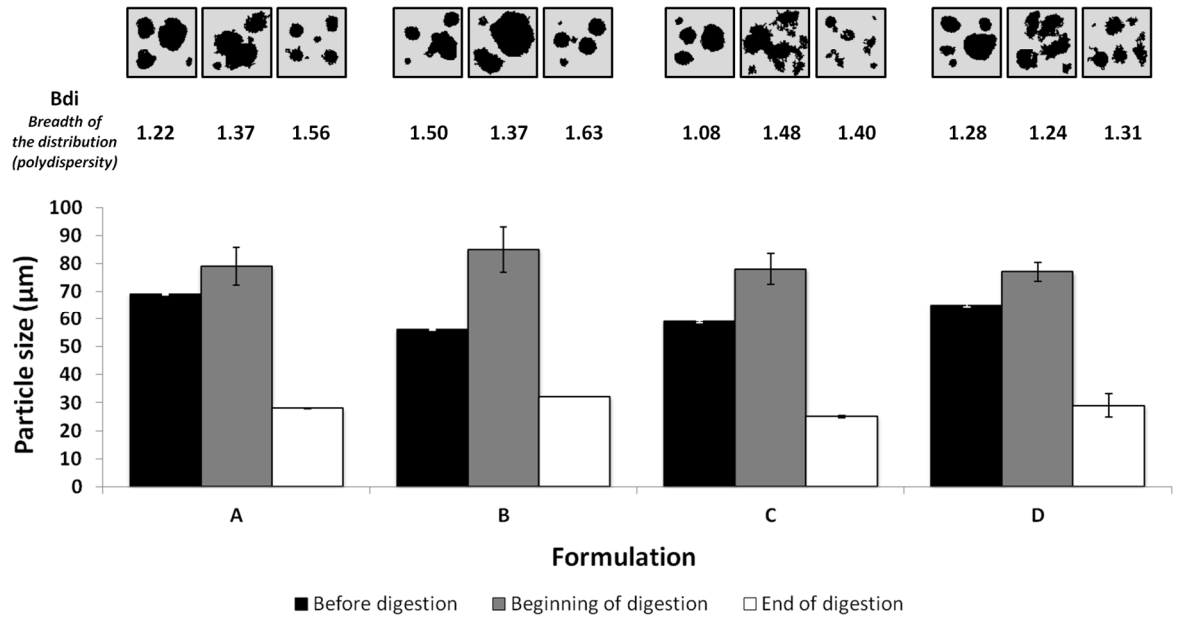


Figure 38: Particle size measurements (d_{50}) realised at different times (mean ± SD, n=3) for the four formulations (A, B, C & D): before gastric digestion (in black) means that the size was recorded for a particle dispersion in water, beginning of digestion (in grey) means that the measurement was realised just after the particles introduction in the gastric medium and end of digestion (in white) for a recording after the 120 min. of digestion. For each measurement, the breadth of the distribution (Bdi) is indicated and reflects the polydispersity of the produced particles. For each record, associated particle images are provided. The digestion was realised in a gastric medium containing the pepsin enzyme.

Tableau 21: Particle shape measurements recorded at different time of digestion. Before digestion means that the size measurement was done in water before adding the particles in the acidic fluid. The beginning of digestion represents the first measurement done during digestion and the end corresponds to the one realized after 120 min of contact with the gastric fluid.

Micro-particle composition	Time of digestion	Convexity			Sphericity		
		X_{10}	X_{50}	X_{90}	X_{10}	X_{50}	X_{90}
A	Before	0.82	0.85	0.84	0.81	0.76	0.72
	Beginning	0.72	0.72	0.68	0.63	0.54	0.46
	End	0.77	0.74	0.74	0.78	0.70	0.64
B	Before	0.82	0.84	0.82	0.81	0.76	0.70
	Beginning	0.75	0.73	0.75	0.76	0.66	0.63
	End	0.77	0.77	0.79	0.78	0.72	0.70
C	Before	0.81	0.83	0.81	0.78	0.74	0.68
	Beginning	0.67	0.61	0.62	0.58	0.42	0.37
	End	0.77	0.71	0.72	0.80	0.67	0.61
D	Before	0.80	0.83	0.82	0.80	0.75	0.71
	Beginning	0.67	0.64	0.56	0.58	0.46	0.34
	End	0.77	0.69	0.64	0.78	0.63	0.51

3.2.2. Influence of microparticle composition

One goal of this study was to produce microparticles with a dense protein matrix in order to assess their resistance to gastric conditions. To achieve a high network density, milk protein solutions with a concentration of 12.5 % were used (Figure 34). Indeed, this concentration of milk proteins was already found as optimal (data not shown). As presented in Figure 39, the matrix density was measured indirectly by the gel elasticity (G'). Significant differences were observed between the formulations. Formulation C presents the highest elasticity. Formulation D was slightly less elastic whereas formulations A and B were similar with lower G' values.

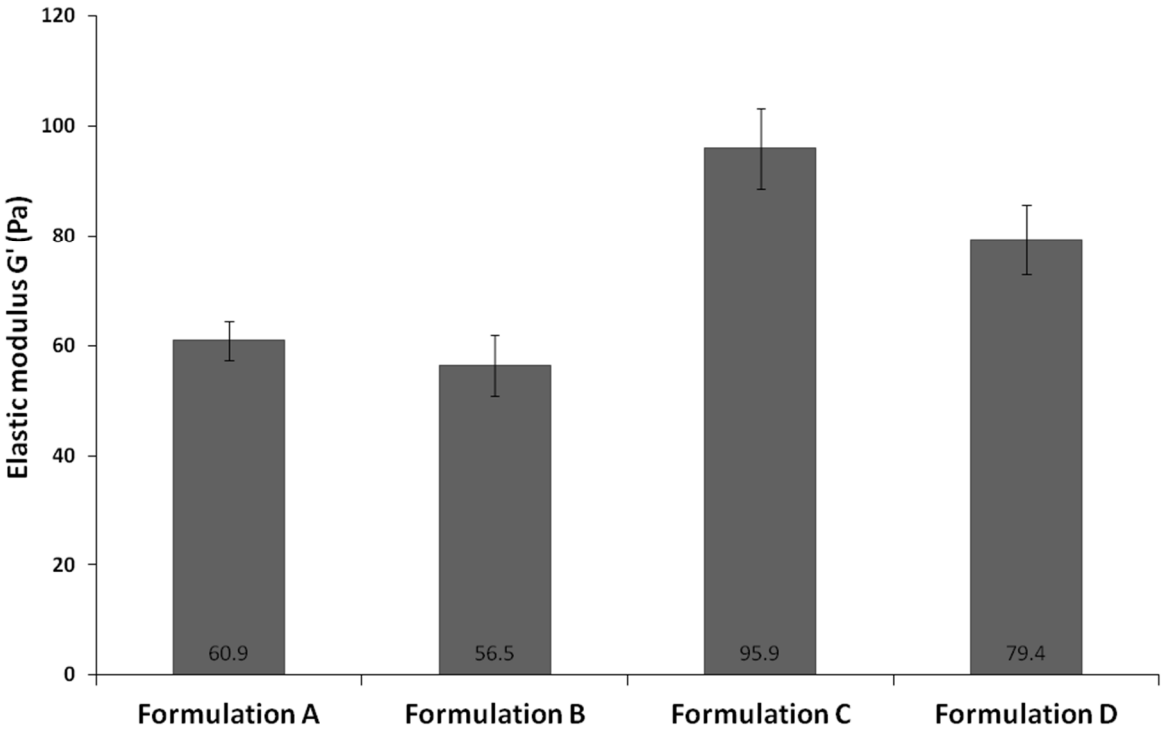


Figure 39: The elastic modulus (G') was determined by rheology for the four formulations (A, B, C & D) (mean ± SD, n=3).

It was already demonstrated that the development of microparticles with a dense matrix offers resistance against proteolytic degradation since it is difficult for the enzymes to penetrate into the particles (Gunasekaran et al., 2007). Creation of new links between proteins (Zoon, 1988) was found to increase the gel network and was reflected in the storage modulus (G') (Walstra and Vliet, 1986). These may be related to a lower tendency to breakdown (Mellema et al., 2002) and a better resistance.

The protein nature seems to influence the particle size, the Bdi and the gel elasticity. It was observed that a higher amount of micellar casein led to significant bigger particles and lower gel elasticity. The presence of denatured whey proteins was beneficial for producing microparticles with a small polydispersity and a high elasticity. The shape of the microparticles was not significantly modified by the proteins used in the different formulations. However, the protein composition can also influence other factors like porosity, surface and internal microstructure (Matalanis et al., 2011). Indeed, the nature of the proteins presenting variable physical properties (density, refractive index), size, charge, and stability may be of influence (LaClair and Etzel, 2010). By modifying these properties a more or less porous structure may be obtained. And

consequently a porous structure should allow an easier access and release of an encapsulated bioactive while a dense structure would limit access and release.

3.3. Microparticle during digestion

Microparticle size, shape and Bdi were recorded continuously during the entire process of digestion: before their addition into the simulated gastric medium (previously discuss), at the very beginning of digestion and at the end of digestion (Figure 38).

3.3.1. Beginning of digestion

When the microparticles were added to the gastric environment, a particle diameter increase was systematically observed (Figure 38, grey column). For experiment A, the particles had an initial d_{50} of 69 µm and after their addition into acid the d_{50} was increased up to 79 µm corresponding to a size increase of 14 %. A similar behavior was observed for experiments B, C and D with size increases of 52 %, 32 %, and 18 % respectively. The size increase was only significant for formulations B, C ($P < 0.001$) and D ($P < 0.05$). For formulations A and C, a Bdi increase was observed once the particles approach the acidic environment. Relying on the above Bdi value provided by the images, it can be seen that bigger particles were produced just after their addition into the gastric liquid. These new particles were the result of physical interactions between existing particles explaining thus the increase in size observed. In the case of formulation A the interactions were very important whereas for formulations C and D, initial particles retained their identity and the interactions were less important.

The particle shape parameters (convexity and sphericity) tended to decrease and microparticles became more irregular (Tableau 21). The most important decrease in convexity value was observed for formulation C with a reduction from 0.83 to 0.61. A comparable decline in sphericity value from 0.74 to 0.42 was also observed for this formulation. It has already been noticed with recorded images (Figure 38, grey column) that the microparticles from formulation C presented a high deformation (the physical interactions between initial particles being less important than in other formulations). These are particles that most deviate from a spherical shape. On the contrary, sphericity and convexity in formulation B were higher after microparticle addition in the gastric medium. As previously stated, these particles already stood out from the others with

their higher compacity in the visual analysis of images in Figure 38. The creation of aggregates for formulations A, C and D caused a reduction in sphericity as the microparticles were placed into the gastric solution. Nevertheless, the lowest sphericity for the big particles in each experiment was obtained for formulation D. In fact, at the beginning of digestion, the large particles reflected the generated aggregates. With particle images (Figure 38), it can be seen that aggregates for this formulation seem to be more flocked than fused particles. In contrast, it seems that for the microparticles from formulation B, the swelling phenomenon was responsible for the increase in size as no aggregates can be noted.

3.3.2. During digestion and end of digestion

The size distributions were recorded every 10 min during the digestion (Figure 40).

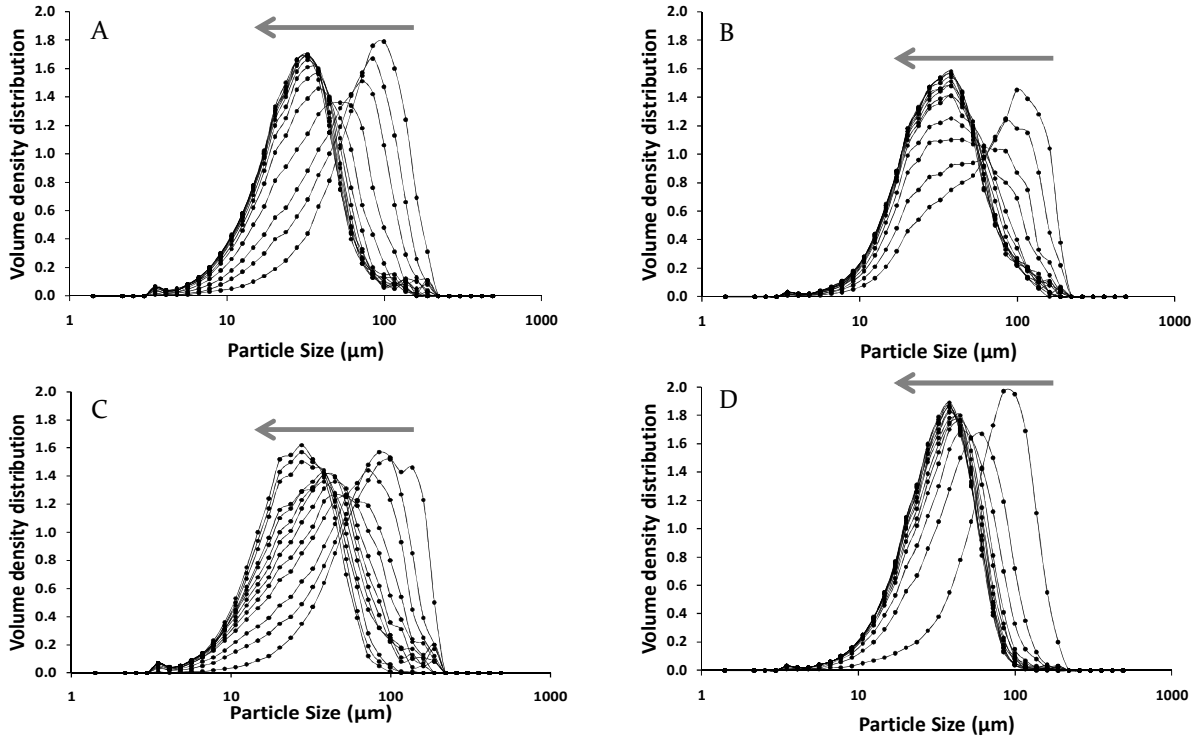


Figure 40: Particle size distribution recorded as a function of digestion time. The curves are plotted each 10 min. The arrow indicates the direction of evolution from the beginning to the end of digestion for the four formulations (A, B, C and D). The digestion was realized in a gastric medium containing the pepsin enzyme.

Whatever the formulation, an instantaneous particle size decrease is observed during the first minutes. This was noticed by a curve shift from the right to the left

corresponding to smaller sizes. After a while, the particle size reached a plateau. This was observed by a superposition of the curves principally for experiments A, B and D. Formulation C presents a slower size decrease but continue without reaching a plateau. At the end of digestion, particles recovered higher convexity and sphericity in all cases because of the individualization of microparticles (Tableau 21). However, the initial sphericity and convexity values were not reclaimed. The highest value in these both shape parameters was obtained by the particles produced with formulation B. Conversely, microparticles that have suffered greater deterioration are the ones produced with formulation D. This information is reinforced by the particle images in Figure 38, at the end of digestion the microparticles were spherical and compact for formulation B and the opposite for formulation D.

3.3.3. Data modelling of the entire process of digestion

In order to obtain a general view of the entire process of digestion, the particle size retention (y_t) was plotted (Figure 41) with the calculated values and experimental data were fitted.

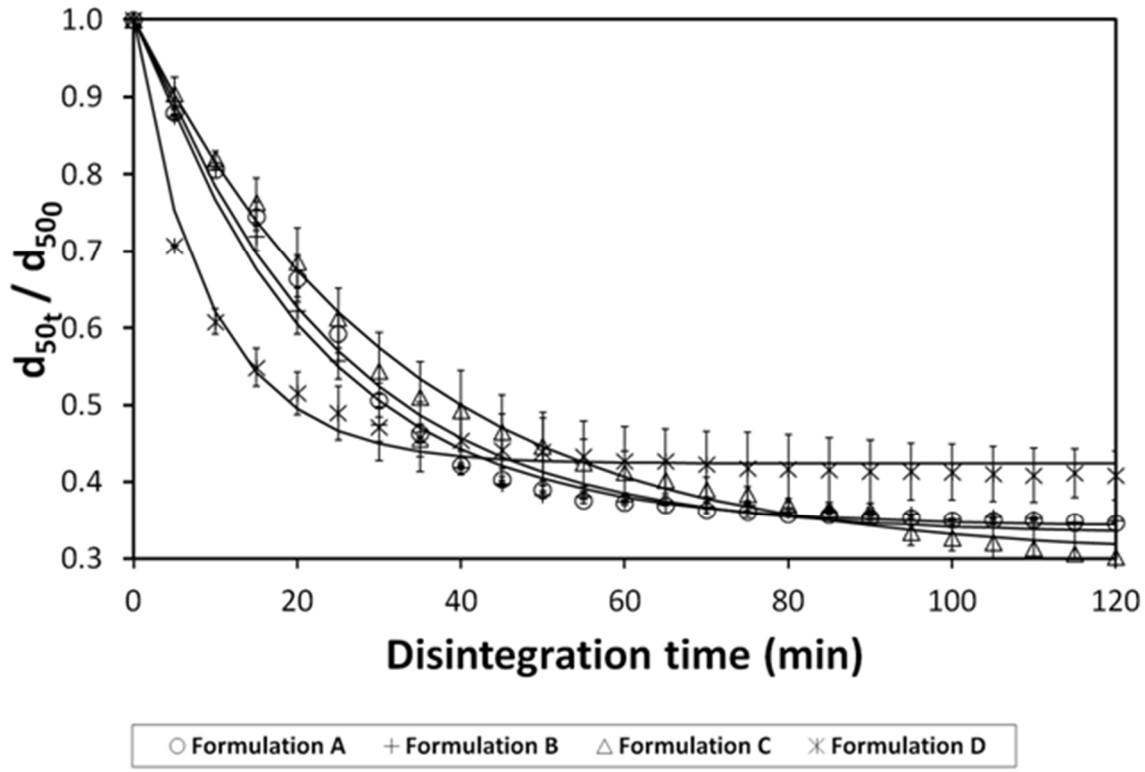


Figure 41: Normalised diameter (mean ± SD, n=3) decrease as a function of digestion time. The experimental points are fitted with an empirical model.

Correlation coefficient (r^2) and MSE were also reported (Tableau 22).

Tableau 22: Determination of the kinetic parameters of gastric disintegration of microparticles for each formulation (A, B, C and D) using the linear exponential equation proposed by (Kong and Singh, 2008).

Formulation	k	β	MSE x 10^3	r^2	$t_{1/2}$ (min)
A	0.630	0.043	0.477	0.988	25.6
B	0.640	0.048	0.377	0.990	22.4
C	0.590	0.032	0.199	0.995	37.3
D	0.642	0.103	0.314	0.983	8.3

All the curves presented an exponential disintegration profile with digestion parameters k and β that are nearly the same for the four formulations as shown in Figure 41 and Tableau 22. These profiles are typical of soft foods due to their fast advancing erosion front that dominates the reactions of food during gastric digestion (Ferrua et al., 2011). Nevertheless, profiles of disintegration (sigmoidal, exponential, and delayed exponential…) during food digestion are strongly linked to the force applied. From the four formulations studied here, differences were evident and related to the particle size distribution evolution during digestion. For experiment C, the decline was slow with a $t_{1/2}$ of around 37 min in contrary to experiment D which presented a rapid decrease and a steady particle size with a smaller $t_{1/2}$ of around 8 min. This half time can be used as an indicator of the food disintegration rate. The two other formulations (A and B) presented intermediate behaviours. Nevertheless, the size of the particles produced in this study was small (around 60 µm); thus they cannot be assimilated to the digestion of a solid meal and could be related to a liquid meal. Liquid meals are emptied faster than solid meals, which generally present a lag phase prior to gastric emptying (Fox et al., 2004). Gastric emptying is governed by physical and chemical characteristics of the ingested meal. For example, whey proteins are digested faster than micellar casein (Calbet and Holst, 2004).

(1) The initial increase in size observed (formulations A, C and D) at the beginning of digestion could be explained by particle aggregation. Aggregation was confirmed on recorded images during digestion. On the contrary, a swelling phenomenon was observed for microparticles produced by formulation B. The high

presence of hydrogen ions in simulated gastric solution was already found to modify steric protein structure, in this case native whey proteins. Consequently, the particle porosity could increase and the microparticles could uptake the solution (Matalanis et al., 2011).

(2) Then a decrease in size occurred along the digestion. Indeed, cohesive forces of food matrices are known to be different as a function of the protein composition (Lundin et al., 2008). During digestion the particles could be influenced as follows: hydrolyzation by acid, proteolysis by pepsin, shearing forces by peristaltic stomach movements and finally body temperature (Lundin et al., 2008). Gastric pepsin enzyme may cause the protein hydrolysis into polypeptides, oligopeptides and some free amino acids. Nevertheless, the determining factor enabling hydrolysis is cleavage site accessibility to the enzyme. One of the reasons explaining the good resistance of milk microparticles could be that the cleavage sites were partially hidden in the structure. Indeed, casein resistance under simulated gastric digestion has already been shown (Dupont et al., 2010). The high resistance of κ- and α_{s2}-casein was primarily highlighted. It has been demonstrated that hydrophobic and post-translational modified (phosphorylated and glycosylated) fragments could maintain the protein integrity.

3.4. Selection of the better matrices for the encapsulation of LGG

3.4.1. Selection of the better matrices from microbiological measures

The formulations previously studied without probiotic bacteria were finally tested as an encapsulation material for LGG. The best formulation for bacterial survival during gastric digestion was formulation C followed by D. Formulations A and B presented the worse results (Figure 42).

The curve of non encapsulated bacteria is also interesting as it is a proof of the efficiency of encapsulation (the population decline from 100 to 0.0005 %). When looking at the encapsulation rates, formulation C was again the best one with an excellent rate around 97 % (Tableau 23).

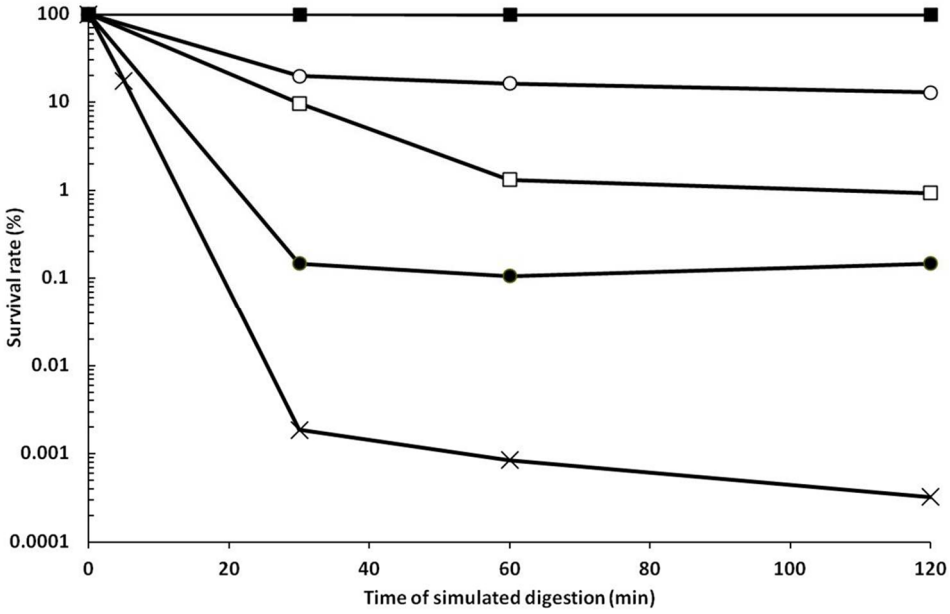


Figure 42: Bacterial survival during simulated gastric digestion: non encapsulated bacteria ×; formulation A ● ; formulation B □; formulation C ■; formulation D ○).

Tableau 23: Encapsulation rate for the probiotic strain LGG encapsulated into different milk protein matrices.

Formulation	Encapsulation rate (%)	Standard deviation (%)
A	64	4
B	88	6
C	97	2
D	84	2

It is worth noting that even the worse encapsulation rate obtained for formulation A around 64 % was not so bad compared to the other authors that microencapsulated bifidobacteria with whey protein and obtained an encapsulation rate comprised between 0.03 % and 25 % (Picot and Lacroix, 2004). The improvement of encapsulation efficiency when LGG is entrapped in a matrix not only composed of micellar casein but also of whey proteins can be explained by the specific interaction settling between the strain and whey proteins (Burgain et al., 2013b). Thanks to these adhesion forces, probiotic bacteria can be better retained in the gel explaining why the encapsulation rate was higher with the addition of whey proteins.

3.4.2. Selection of the better matrices from morphology and physical concerns

Microparticle sizes are an important consideration since the microparticles must i) have a high volume-to-surface ratio for increasing the protective effect and ii) be sufficiently small to avoid a negative sensory impact (Anal and Singh, 2007). The particle detection by the consumer will be assimilated as a defect and the sensory perception is affected by the food matrix in which the microparticles are introduced (van Vliet et al., 2009). In the study, the sizes were about the same order and comprised between 56 µm and 70 µm so, formulations cannot be discriminated by the size criterion. The shape is also important for consumer acceptance because a round particle is less detectable than an irregular particle (Imai et al., 1999). However, the produced microparticles have almost the same sphericity and convexity values relying that they were well rounded and spherical.

According to Tableau 22, it was measured that microparticles from formulation D were the most quickly affected by the digestion (small $t_{1/2}$), and for this reason it cannot be selected for future probiotic microencapsulation. It was explained in this study that at the beginning of digestion the microparticles tend to form aggregates but in the case of formulation B it seems that swelling was responsible for microparticle size increase. The tendency of swelling depends on the type of bonds holding the matrix (Matalanis et al., 2011) and the risk with this phenomena is the diffusion of the encapsulated substance out of the particle (Matalanis et al., 2011). Even if for some delivery system this effect can be exploited for controlled release, in the case of probiotic encapsulation it is not desired since the gastric liquid will have access to the interior of the particle and will be in contact with the cells. For this reason, formulation B will not be kept for probiotic microencapsulation.

Microparticles prepared by this emulsification method are shown to be polydisperse in size, which is not desirable for controlled release (Cheng et al., 2010). However, microparticles produced with formulation C have the lowest polydispersity so they can be selected from others. Moreover, according to the half-time obtained during digestion, formulation C had the longest one meaning that it can better resist gastric environment and also has the strongest gel elasticity (G'). For all of these reasons, formulation C, composed of micellar casein and denatured whey proteins seems to be the more effective to protect probiotic bacteria.

3.5. Conclusion

The different parameters observed in this study enable discriminating the best formulation to produce microparticles able to protect LGG into simulated gastric conditions. Clearly, formulation C consisting of micellar casein and denatured whey proteins was the best one: particle size, Bdi, G', half time of gastric digestion, rates of encapsulation and survival.

In addition and for the first time, an original equipment was used to follow *in situ* the microparticle size and shape evolution during digestion in the stomach. This equipment could be used in the future to improve the target delivery of bioactives by a simple, direct and easy method.

The next step of this work is to use the microparticles for the protection of other sensitive bioactives against adverse environmental conditions encountered in the stomach. Studies are under progress for polyphenolic compounds and other probiotic bacteria.

4. Microstructure of produced microparticles

The production of microparticles with a dense protein matrix is crucial for protecting probiotic cells. Freeze-dried microparticles were observed by SEM revealing differences in the matrix structure and bacterial location (Figure 43).

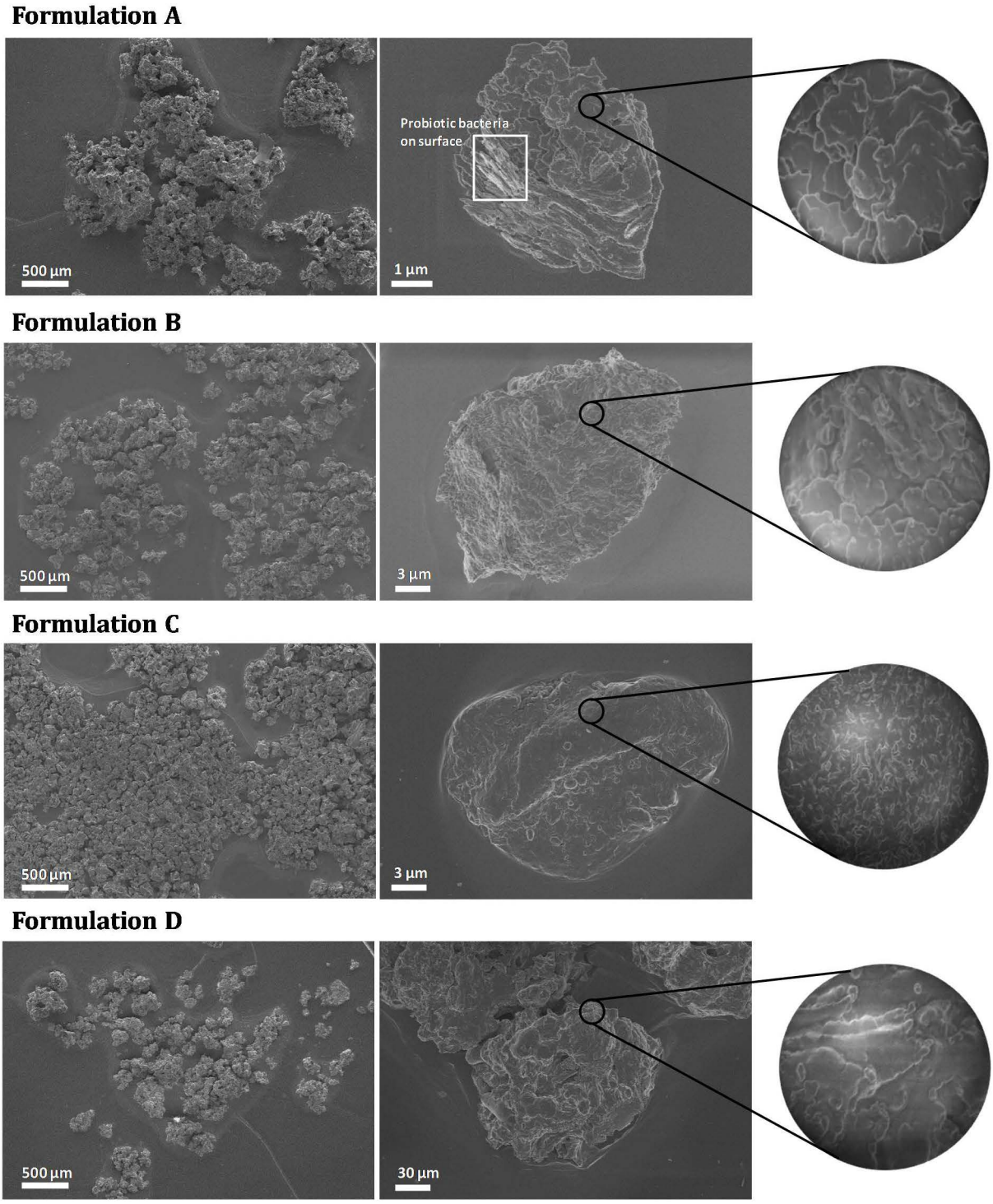


Figure 43: SEM images of microparticles made of milk proteins.

Regarding microparticles produced with only micellar casein, the structure is not very compact and groups of bacteria can be found on the surface. On the contrary, for microparticles formulated with micellar casein and whey proteins no bacteria were observed outside the microparticle. These observations can be linked to the measured encapsulation rate: it was of 64 % for microparticles only composed of micellar casein whereas the addition of denatured whey proteins causes an increase in this rate to up 97 % (for the formulation C for example).

As seen in the previous section, probiotic bacteria encapsulated in a matrix composed of casein and whey proteins better resist in a simulated gastric medium. It can be assumed that the preferential location of the bacteria inside the matrix can be responsible for their protection against detrimental conditions. On the contrary, bacteria entrapped only in micellar gel can be found on the surface and are not protected by the matrix so, they are in direct contact with the gastric medium and their survival is affected by the low pH and the presence of proteolytic enzymes.

The microparticle compactness is also improved when using whey proteins in addition to micellar casein. Moreover, the highest compactness seems to be assigned to the formulation C. Against, by comparing with results obtained during simulated digestion, the high compactness of the network can influence the bacterial survival. In fact, this kind of system is almost impervious to acids and enzymes encountered in the gastric medium. Bacteria remain in a protected system where the local pH is maintained acceptable for their survival. Buffering capacity of milk proteins is also important as it enables the maintenance of a local pH higher inside the microparticle than the one encountered in the outside environment. Buffering capacity is substance-specific and the buffering effect occurs on a defined range. Micellar casein, which are the major proteins in the four formulations are those that most affect the buffering capacity (Salaün et al., 2005). Micellar caseins are able to buffer between pH 6 and 3.25 with a maximum buffering capacity around pH 4.5 to 5.5. The maximum buffering capacity of whey proteins ranges between pH 3 and 4.

The observation of the inner part of microparticles is possible by producing slices that will be further observed by TEM (Figure 44).

Presented images are obtained from the microparticles produced with the formulation C. The high density of the network can be once again highlighted (Figure 44 A & C). The bacterial location within the network can also be observed (Figure 44 B &

D). The bacteria are found as chaplets or in an individual form. The visualization of EPS around the bacteria is evident and their interaction with the surrounding matrix can be noted.

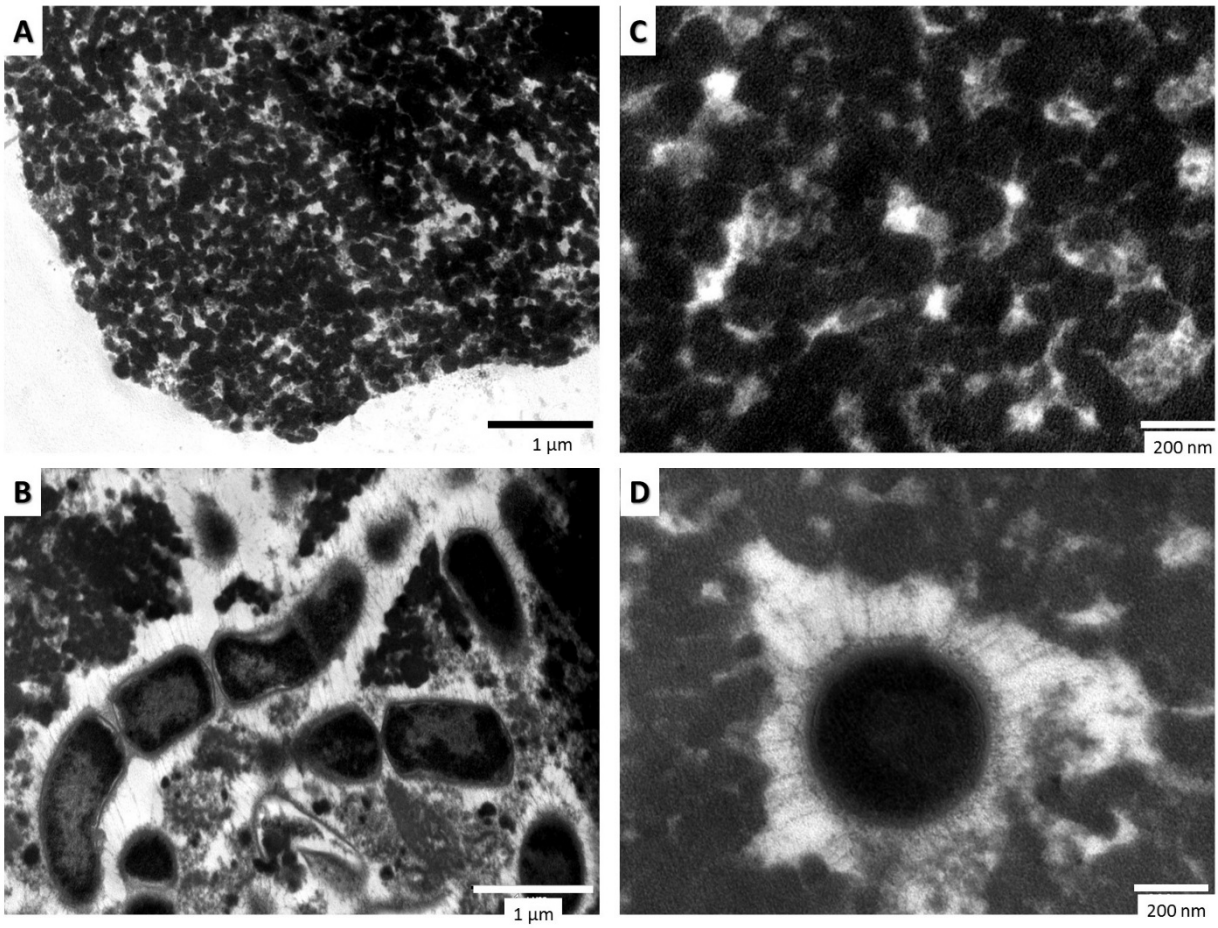


Figure 44 : TEM images of microparticles (from formulation C) containing LGG wt.

The observation of microparticles just after their production, in a wet form, by CLSM was realized (Figure 45). Microparticles observation before freeze-drying reveals a porous structure and a lipid layer remaining after encapsulation.

This adsorbed lipid layer was also outlined when recording AFM images on freeze-dried microparticles with the tapping mode (Figure 46A). On the contrary, when used in a contact mode, AFM provides good quality images of microparticle surface (Figure 46B).

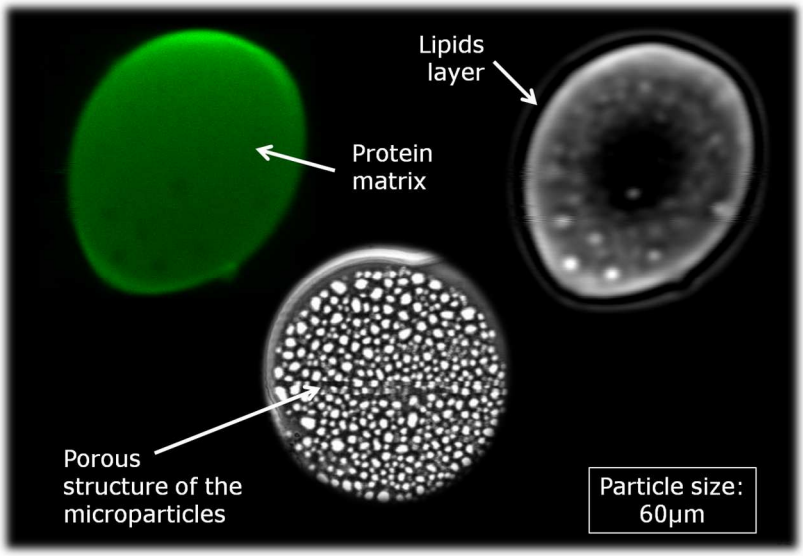


Figure 45 : CLSM images of wet microparticles composed of micellar casein and whey proteins.

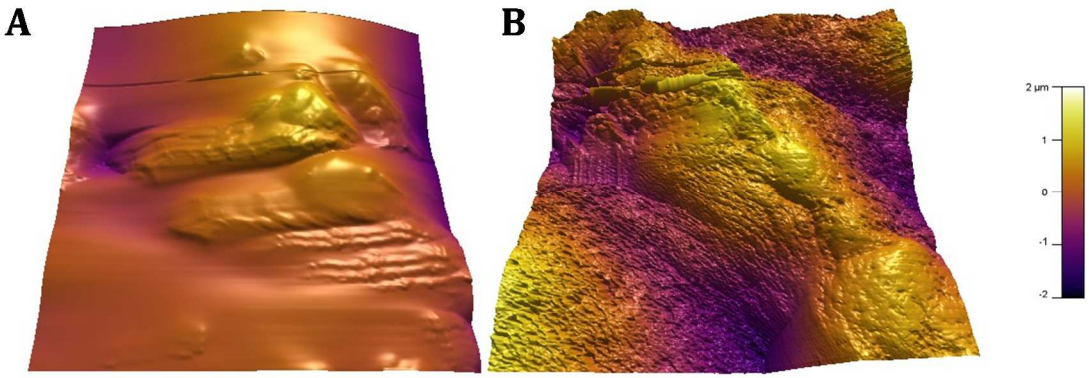


Figure 46 : AFM images of microparticle surface recorded in a tapping mode (A) and in a contact mode (B).

Because microparticles have small size, the total surface is high and the cumulative lipid layers cause a significant amount of residual oil. This aspect is negative as high level in lipids is not compatible for the nutritional profile of health food. However, this residual oil layer can be beneficial for protecting the inner part of the microparticle from its environment. In fact, fatty acids were already used as coating material in order to protect freeze-dried bacteria (Durand and Panes, 2001). The presence of fatty acids in cheese (Dinakar and Mistry, 1994, Godward and Kailasapathy, 2003a) and in chocolate (Maillard and Landuyt, 2008) are also believed to be responsible for bacterial survival improvement when they are introduced in these products.

5. Conclusion du Chapitre

L'optimisation de la formulation de la matrice d'encapsulation a nécessité le recours à plusieurs critères :

- Morphologiques : la taille et la forme des microparticules produites,
- Rhéologiques : l'élasticité du gel produit à la suite de la coagulation,
- Microbiologiques : le taux d'encapsulation,
- Structuraux : l'aspect visuel de la surface et de l'intérieur des microparticules,
- Environnementaux : la résistance des microparticules dans un milieu simulant l'estomac et le taux de survie des bactéries.

La matrice sélectionnée à la suite de ce chapitre est donc composée de 90 % de caséines micellaires et 10 % de protéines solubles dénaturées. Cette formulation permet la production de microparticules sphériques dont la taille est d'environ 60 µm. D'après ses propriétés rhéologiques et structurales, le réseau formé est compact ce qui lui permet de bien retenir les bactéries à l'intérieur de la microparticule et de les préserver d'un milieu environnant éventuellement agressif. L'intégrité des microparticules est préservée lorsqu'elles sont soumises à des conditions telles que celles rencontrées dans l'estomac, où acidité et enzymes protéolytiques sont défavorables au maintien de la survie et de la fonctionnalité des bactéries probiotiques.

Le suivi de la digestion par le couplage d'un granulo-morphomètre et d'un réacteur de digestion a fait l'objet d'une offre de compétences, actuellement publiée et qui est présentée en annexe.

Chapitre 4 :

Interactions bactéries-matrice

1. Introduction

L'encapsulation de bactéries probiotiques dans des matrices laitières a montré qu'en fonction de la formulation de la matrice choisie, le taux d'encapsulation mais également le taux de survie dans des conditions gastriques ne sont pas identiques. L'imagerie a également démontré que la localisation des bactéries dans la matrice était affectée par la nature des protéines utilisées.

Il parait ainsi intéressant d'étudier les mécanismes d'interaction qui opèrent entre les bactéries et la matrice au moment de l'encapsulation. Au lieu de s'intéresser au résultat final (les bactéries microencapsulées) pour essayer d'expliquer ce qu'il s'est produit au moment de l'encapsulation, nous avons choisi de sonder directement les interactions qu'il existe entre les bactéries et les protéines au moment où elles sont mises en contact.

Dans un premier temps, la caractérisation de la surface des entités présentes semble être une évidence. Des méthodes comme l'XPS ou la mobilité électrophorétique permettent d'accéder à ces informations. Pour l'étude des interactions à l'échelle moléculaire, la mesure des forces par AFM s'est révélée être un outil adapté et performant. Finalement, la combinaison de toutes ces informations permet de formuler des hypothèses quant aux mécanismes qui régissent les interactions entre les bactéries et les protéines expliquant ainsi les phénomènes observés à l'échelle microscopique.

2. Bibliographie sur les interactions bactéries-ingrédients laitiers

Bacterial interaction with milk components: Physico-chemical aspects and interest for food industry.

J. Burgain, J. Scher, G. Francius, F. Borges, A.M. Revol-Junelles, C. Cailliez-Grimal & C. Gaiani. Submitted.

Abstract

The understanding of bacterial interaction with milk components is of huge importance as LAB are introduced in many dairy products and have technological role. However, only few researches try to describe and understand how the bacteria interact with their environment which is constituted by milk proteins, lipids, minerals when considering dairy matrices. It can be conceived that, based on the solid knowledge of bacterial adhesion to surfaces (for biofilm formation for example), hypothesis concerning their interaction with biomolecules from dairy matrices could be formulated.

In this review, biomolecules present on bacterial surface are firstly described then, the composition of dairy components is provided. In order to understand how bacteria can interact with dairy molecules, a description of the adhesion mechanism is provided and the environmental conditions affecting the bacterial adhesion. The methods that can be used to investigate the bacterial surface and the ones that can probe bacterial interactions with other components are also detailed. Finally, the interest in studying bacterial interactions with milk components is illustrated by relevant examples, as the influence of bacterial surface biomolecules on yogurt structure or the bacterial location in a dairy matrix.

Résumé

La compréhension des interactions entre les bactéries et les composants laitiers est d'une grande importance puisque les LAB sont introduites dans de nombreux produits laitiers et ont un rôle technologique majeur. Cependant, seulement peu de recherches ont été réalisées concernant la description et la compréhension des mécanismes d'interaction entre les bactéries et leur environnement. Néanmoins, sur la base des solides connaissances concernant l'adhésion bactérienne aux surfaces (pour la formation de biofilms par exemple), des hypothèses peuvent être formulées.

Dans cette revue, les biomolécules présentes à la surface des bactéries ainsi que la composition des ingrédients laitiers sont tout d'abord décrits. Le mécanisme d'adhésion bactérienne est ensuite détaillé ainsi que l'influence des conditions environnementales sur ce dernier. Les méthodes actuellement disponibles pour examiner la surface des cellules ou pour sonder les interactions sont également décrites. Pour terminer, l'intérêt d'étudier les interactions entre les bactéries et les composants laitiers est illustré par des exemples industriels pertinents comme l'influence des biomolécules de surface sur la structure du yaourt ou encore sur la localisation des cellules dans les matrices laitières.

2.1. Introduction

LAB form a phylogenetically highly heterogeneous bacterial group generally having the GRAS (Generally Recognized as Safe) status (Limsowtin et al., 2002). Bacteria belonging to this group are also the subject of numerous applications in food industry, especially in the dairy sector (Leroy and De Vuyst, 2004). The value of consumption of LAB appeared to the early 20th century when Metchnikoff suggested that the absorption of these living microorganisms, present in yogurt, increased the longevity of the consumer. The author attributes the positive effects observed on the host health to a reduction in the population of spoilage bacteria and/or producing toxins in the digestive tract (Metchnikoff, 1907). The concept of probiotic bacteria was then just getting started (Fuller, 1992). Since the development and consumption of food or food supplements containing probiotic microorganisms has grown considerably (Foligné et al., 2013). In over the past decades, major leaders in food groups have widely exploited the concept and put on the market a wealth of new products based on probiotic bacteria, which come within the range of functionalized food or health food or nutraceuticals (Foligné et al., 2013). However, this growth was affected as a result of the regulations published by the EFSA requiring manufacturers to take into account cell viability and probiotic function in order to make a health claim (Jankovic et al., 2010). Recently, many of health claims were rejected due to a lack of probiotic strain characterization. In addition, the strains referenced were different from those present in the food products for which the claims were made. These bacteria are usually conveyed through dairy products such as cheese, yogurt, and ice cream (Burgain et al., 2011). Their survival during storage in the food product is of great importance and this is dependent of the nature of nutrients that are present in the food matrix but also to the environmental conditions such as pH or ionic strength (Corcoran et al., 2005, Gomes da Cruz et al., 2009). In fact, these factors are critical for bacterial interaction with other components, their location in the matrix and the preservation of bacterial survival and functionality until they reach their site of action.

Bacterial interaction with milk components potentially plays a fundamental role in defining their function and in the observed protective effect of dairy matrices on bacterial survival during exposure to adverse conditions (Brisson et al., 2010). However, the way the bacteria interact with dairy components has not been elucidated. In this review, we first describe the surface structure of LAB and milk components then, the

techniques available to characterize their surface and the ones that allow the visualization of the bacteria in the dairy matrix. Finally, examples of bacterial interaction with dairy components and their impact on the product are discussed.

2.2. Lactic acid bacteria

2.2.1. Description, classification and physiologic characteristics

LAB are defined as living cells, prokaryote, heterotrophic and chemo-organotrophic, that is to say they require complex organic molecules as an energy source (Axelsson, 2009). Bacteria are distinguished by differences in the bacterial cell-wall architecture and are referred to as either Gram-positive or Gram-negative. LAB constitute a group of Gram-positive bacteria that exhibit a thick layer varying between 30 and 100 nm (Lebeer et al., 2010, Vollmer and Seligman, 2010) of peptidoglycan (PG) (Delcour et al., 1999). According to Stiles and Holzapfel (Stiles and Holzapfel, 1997) LAB include the following genera: *Aerococcus, Alliococcus, Bifidobacterium, Carnobacterium, Dolosigranulum, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella*. However, it is mostly the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus* and *Weissella* which are subject to applications in food industries for the development of functionalized foods (Vandamme et al., 1996).

2.2.2. Bacterial adhesion: general concern

As a starting point, the knowledge on cell adhesion can be used to describe the binding capacity of bacterial cells. Adhesion is an action that is characterized by all the physicochemical and biological phenomena allowing bacteria to unite with a surface (Quirynen and Bollen, 1995). It depends on the environment (temperature, pH, ...), the appearance and surface roughness, the surface free energy of couple bacteria/substrate, the hydrophilic or hydrophobic character of bacteria or substrate, the surface charge, the ionic strength of the medium, the presence of specific structures on bacterial surface or substrate (An and Friedman, 1998).

In a general way, the bacterial adhesion occurs in two steps. Firstly, non-specific and reversible interactions (including hydrophobicity/hydrophilicity, electric charge and the Lewis acid-base) are established, followed by specific and non-reversible

interactions (Busscher and Weerkamp, 1987, An and Friedman, 1998) involving adhesins and complementary receptors. There are also non-protein adhesins such as teichoic acids (TA) or lipoteichoic acids (LTA) and polysaccharides (Granato et al., 1999).

2.2.3. Description and role of the bacterial cell wall

The bacterial cell is surrounded by a lipid layer which is itself enclosed by the cell wall (Figure 47).

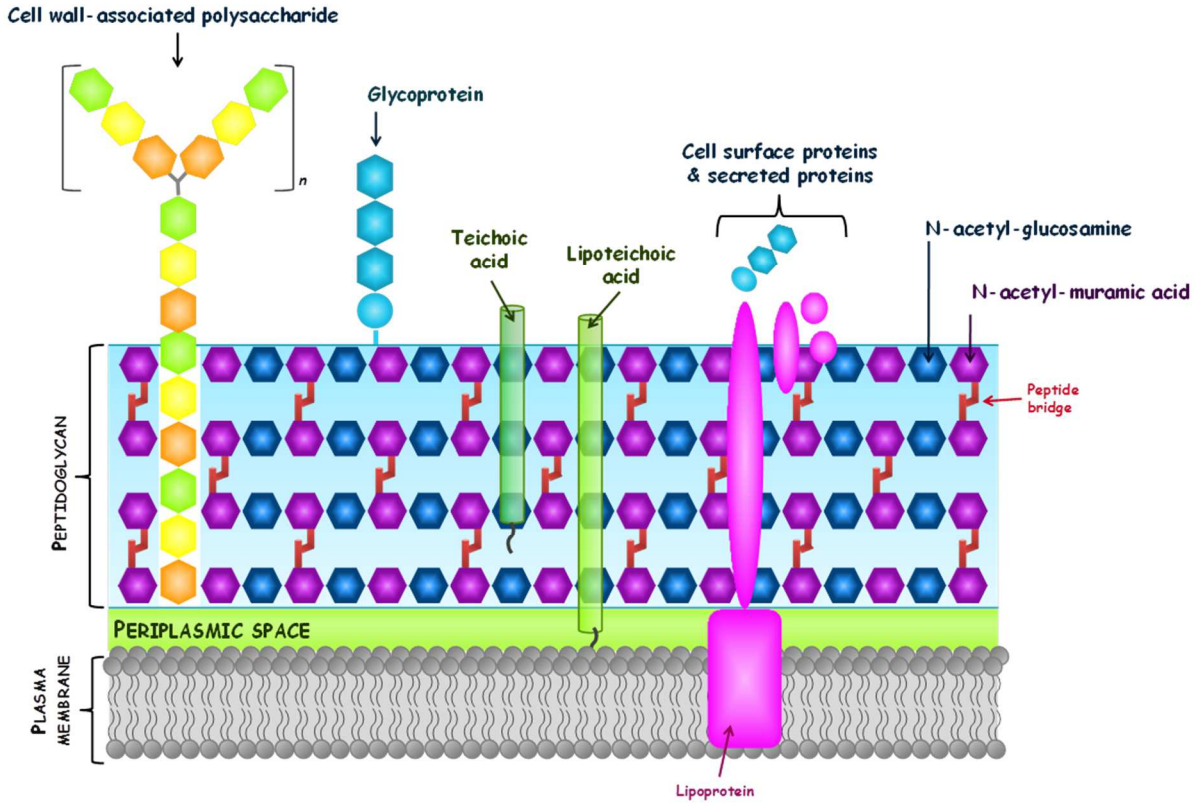


Figure 47 : Bacterial cell-wall architecture of Gram-positive bacteria.

The PG consists of a biochemically cross-linked network of carbohydrates and peptides; it plays a key role in structural integrity thanks to a tough and elastic structure that provide high resistance to hydrostatic and osmotic pressure to the cell (Vollmer and Seligman, 2010). PG is composed of glycan chains themselves by regularly constituted of β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The PG 3D organization is complex and not fully known (Vollmer and Seligman, 2010). In some species, an S-layer of rigidly arrayed protein molecules covers the outside of the cell and provides chemical and physical protection of the cell surface (Sleytr and Beveridge,

1999). TA, polysaccharides, and proteins are anchored covalently and non-covalently to the PG (Delcour et al., 1999).

Microbial cell wall constitutes the frontier between the cell and its environment. Therefore, the cell wall plays crucial role in controlling the cell surface properties and by governing biointerfacial phenomena such as cell adhesion and cell aggregation (Dufrêne et al., 2001). The way the bacteria interact with their environment is directly related to the cell surface structures, since these structures are in direct contact with the other food components. The surface of Gram-positive bacteria is covered by neutral and acidic polysaccharides, different cell wall proteins and TA with a high acidic nature (Beveridge and Graham, 1991) that are determinant for bacterial interaction with its environment.

2.2.4. Bacterial molecular entities involved in interactions

Among the surface molecular structures of the cell wall involved in adhesion, there are proteins, LTA, lipids but also external appendages (lectins) and extracellular polymers such as polysaccharides (Busscher and Weerkamp, 1987).

2.2.4.1. Polysaccharides

Polysaccharides present a significant variation in chemical composition and molecular weight. Generally, these polysaccharides are released in the bacterial environment and for this reason they are called exopolysaccharides (EPS). However, because they often readsorb onto the bacterial surface, they continue to be involved in the bacterial surface properties (Ubbink and Schär-Zammaretti, 2007a). Later, EPS were renamed as 'Extracellular Polymeric Substances' as this matrix also contain proteins, nucleic acids, lipids and other biopolymers (Flemming and Wingender, 2010). EPS have commercially relevant material properties and are exploited for industrial and medical applications (Rehm, 2010).

2.2.4.2. Teichoic and lipoteichoic acids

TA are the second major component of the cell wall of most Gram-positive bacteria. These anionic cell wall polymers are typically composed of polyglycerol phosphate or polyribitol phosphate repeating units covalently anchored to either PG or attached to the cytoplasmic membrane. Wicken and Knox (Wicken and Knox, 1970) showed that TA are covalently linked to peptidoglycan whereas LTA, more widespread among

lactobacilli, were covalently linked to cytoplasmic membrane through a glycolipid pattern. TA represent more than half the weight of the bacterial cell wall. TA have a highly variable structure and their abundance depends on the species or strain of bacteria, stage or rate of growth, pH, carbon source and phosphate availability. LTA are widely present in LAB and are found in enterococci, lactobacilli, lactococci, leuconostoc and streptococci (Fischer, 1994). Their structure is less variable than TA and their production is independent of growth conditions (Fischer, 1988). Due to their polyanionic character and their hydrophobicity, TA and LTA may indirectly influence the adhesion properties of bacteria by modifying the physicochemical properties of the bacterial cell wall.

2.2.4.3. Proteins

Proteins are organic compounds that consist of 20 common amino acids joined by peptide bonds. All amino acids have a central carbon atom to which a hydrogen atom, an amino group (NH_2) and a carboxyl group (COOH) are attached in common. Sequences of amino acids fold to generate compact domains (3D structures; secondary, tertiary and quaternary) from linear chains (primary structure). Electrostatic interactions, van der Waals forces, hydrogen bonds and hydrophobic interactions play an important role in defining and stabilizing the 3D structure and adsorption of protein molecules.

In general, proteins exported to the bacterial surface have a signal peptide which enables to direct the protein to the export way for their migration to surface (Nielsen et al., 1997). Some of these proteins contain in their structure one or more areas that are necessary to anchor them to the cell wall or cell membrane. In the absence of anchoring motif, these proteins will be secreted into the external medium. Therefore, exported proteins to the bacterial surface proteins may be associated with either the plasma membrane or to the cell wall or secreted into the surrounding medium (Sánchez et al., 2008). Recently, another subgroup of related cell wall proteins has been identified. The latter have neither signal peptide nor anchoring motif to the cell wall that would justify their presence at the bacterial surface. They are called "Anchorless proteins" or "moonlighting proteins", and are intracellular proteins mostly involved in metabolic pathways of carbohydrates or protein (Sánchez et al., 2008). Their role in the interaction bacteria/host has been widely reported (Chhatwal, 2002).

2.2.4.3.1. Proteins strongly associated with the bacterial cell wall

Surface proteins strongly associated with the bacterial cell wall can be divided into two categories: protein covalently linked (*e.g.* LPxTG proteins and lipoproteins) and non-covalently associated proteins (Navarre and Schneewind, 1999). The best identified surface proteins are those having a LPxTG motif in the C-terminal part of their amino acid sequence. This pattern is recognized by sortases enzymes or surface peptidases that catalyze the cleavage reaction between threonine and glycine of the LPxTG motif creating a covalent bond between the protein and the peptidoglycan (Navarre and Schneewind, 1999). Another family of covalently bound to the wall proteins is constituted by lipoproteins. These are related to both the wall and the plasma membrane by a pattern "lipobox" at their N-terminal in their amino acid sequence. This pattern allows their covalent anchoring to the lipid bilayer of the cytoplasmic membrane (Sutcliffe and Harrington, 2002).

2.2.4.3.2. S-layer proteins

The S-layer proteins (SLp) form a special class of proteins non-covalently associated to the cell wall. They consist of unique subunit protein with a molecular weight from 40 to 200 kDa, which combine together to form sheets in two-dimensional structure and paracrystalline (Sleytr and Beveridge, 1999). The SLp are organized on the surface of bacterial cells to form a layer which may have a protective function for the microorganism. The SLp bind non-covalently to the bacterial cell wall and can be dissociated by agents altering the hydrogen bonds and by chaotropic agents such as lithium chloride and guanidine chloride (Pum and Sleytr, 1999, Sleytr and Beveridge, 1999). This S-layer was mainly studied by electron microscopy (Sleytr et al., 1999) and also with AFM that is useful to study the molecular organization of the proteins with the S-layer (Dufrêne, 2004). In some probiotic bacteria, the SLp have one or more N-terminal domains, responsible for adhesion to other components of the cell wall such as TA/LTA, or polysaccharides (Sára and Sleytr, 2000).

2.2.4.3.3. Secreted proteins

These proteins are exported to the bacterial surface thanks to a signal peptide. However, due to the absence of anchoring pattern to the cell wall in their structure, they are completely excreted in the environment. In probiotic LAB, two types of secretion

system have been identified: secretion system ATP-dependent and a specific protein export system. In the first system, proteins are exported in the form of pre-proteins with the reported sequences in their N-terminal part. These pre-proteins are translocated through hydrophilic membrane channels under a denatured state and then the signal peptide is proteolysis and protein acquires its native 3D structure thanks to the intervention of chaperone proteins (Tjalsma et al., 2008, van Wely et al., 2001). In the second secretory pathway, most proteins are exported to the surface with a signal peptide and in their native state or even in some cases in an oligomeric form.

2.2.4.3.4. Anchorless proteins

These proteins are a class apart from the proteins present at the cell wall or secreted into the extracellular medium. The latter have neither signal peptide nor anchoring to the cell wall, like LPxTG, lipobox or LysM domain, hence their designation as "anchorless or moonlighting proteins" (Chhatwal, 2002). They are mainly cytoplasmic proteins that play an essential role in the growth and metabolism of microorganisms. When they are exported to the surface of the cell wall of some pathogens belonging to the genera *Streptocococcus, Staphylococcus* and *Listeria monocytogenes*, these proteins once on the surface will be for some of them involved in the phenomena of invasion, virulence and interaction with components of the extracellular matrix of the host (Chhatwal, 2002, Pancholi and Chhatwal, 2003, Schaumburg et al., 2004, Modun and Williams, 1999). In several probiotic LAB, anchorless proteins have recently been described in the literature. Many of these proteins involved in various metabolic pathways in the cytoplasm, were also identified on the surface of the cell wall of many lactobacilli such as *L. crispatus* (Hurmalainen et al., 2007), *L. plantarum* (Kinoshita et al., 2008) and *L. johnsonii* (Granato et al., 2004). They seem to perform a function in the host/bacteria interaction (Wang et al., 2013). Among the anchorless proteins most frequently observed, there are the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase, the elongation factor EF-Tu and the GroEL which is a protein from the family of chaperones Hsp 60 (Sánchez et al., 2008, Wang et al., 2013).

2.2.4.3.5. Pili

Many bacteria express nanoscale filamentous structures known as pili or fimbriae on their surfaces (Telford et al., 2006). Bacterial pili are defined as non-flagellar,

proteinaceous, multi-subunit surface appendages involved in adhesion to other bacteria, host cells or environmental surfaces (Kline et al., 2010). Pili-mediated bacteria-host interactions have been demonstrated in the probiotic Gram-positive bacterium LGG (Kankainen et al., 2009, von Ossowski et al., 2010, Lebeer et al., 2012b, Reunanen et al., 2012). AFM imaging revealed that pili was located all around the cells, and also that they form remarkable star-like structures (Tripathi et al., 2012, Tripathi et al., 2013). This indicates that pili–pili interactions are responsible for mediating bacteria–host interactions and for promoting bacterial aggregation (Telford et al., 2006, Tripathi et al., 2013).

2.3. Composition of dairy components

Cow milk is a complex mix which contains proteins, carbohydrates, lipids, minerals and vitamins.

2.3.1. Proteins

Milk proteins have been very well characterized at the molecular level and are probably the best characterized of all food protein systems.

The main whey proteins (β-lactoglobulin, α-lactalbumin) are highly structured, but the four caseins (α_{s1}, α_{s2}, β, κ) lack stable secondary structures (O'Mahony and Fox, 2013). In fact, physical measurements have demonstrated that the caseins are unstructured, but theoretical considerations indicate that the caseins are highly flexible molecules (Holt and Sawyer, 1993). The caseins are generally considered as hydrophobic proteins, especially the β-casein (Horne, 1998). The high surface hydrophobicity can be explained by their lack of stable secondary and tertiary structures that implies that most of their hydrophobic residues are exposed which is the result of high levels of proline in their primary structure (O'Mahony and Fox, 2013). It should be noted that all the caseins are phosphorylated whereas the principal whey proteins are not. The κ-casein protein is glycosylated and contains galactose, N-acetylgalactosamine and N–acetylneuraminic (sialic) acid (O'Mahony and Fox, 2013).

Other minor proteins include immunoglobulins which is a glycoprotein (Hurley and Theil, 2013), bovine serum albumin (Farrell Jr et al., 2004) and the lactoferrin. This latter is an iron-binding protein which plays a bacteriostotic role (González-Chávez et

al., 2009). Other enzymes are also encountred: lactoperoxydase, alkaline phosphatase, catalase and plasmin (Fox and Kelly, 2006, O'Mahony and Fox, 2013).

2.3.2. Lactose

Lactose is produced by mammary glands and is a disaccharide derived from glucose and galactose. As glucose can be found in either the α-pyranose form or β-pyranose form, the lactose molecule can be referred as α-lactose or β-lactose (Fox, 2011).

2.3.3. Lipids

Milk fat globules are the natural colloidal assemblies secreted by the mammary epithelial cells as small spherical globules ranging from 0.1 to 20 μm in diameter and is surrounded by a complex membrane, the milk fat globule membrane (MFGM) (Lopez, 2011, Singh, 2006). The milk fat globule contains triacylglycerol representing 98 % of the total milk lipids with n-6 and n-3 fatty acids (Lopez, 2011). Other components can be found in these fat globules such as fat soluble nutrients (*e.g.* carotenoids, vitamins) and also active molecules (*e.g.* phospholipids, sphingolipids, cholesterol, MFGM proteins). Moreover, components located in the MFGM are known to have affinity to the bacteria cell surface, this is the case for mucins (Dague et al., 2010), phospholipids, proteins, glycophospholipids and gangliosides.

In dairy products, the bacterial adhesion to milk fat droplets in cream has been reported (Ly et al., 2006a, Jiménez-Flores and Brisson, 2008, Brisson et al., 2010). In addition, the cell surface characteristics of the strain influence the binding properties to lipid globules. For example, hydrophobic one between several *L. reuteri* strains can greater adhere toward the MFGM (Brisson et al., 2010). This association was correlated to the presence of extractable proteins on the bacterial surface suggesting that these biomolecules might play an important role for adhesion to fat globule (Brisson et al., 2010).

2.3.4. Others

Minerals are also found which are calcium, inorganic phosphate, and citrate (Atkinson et al., 1995). Calcium ions are largely associated to citrate and phosphate ions. Other minerals such as magnesium, sodium, potassium, chloride are also encountered in

milk but have a lesser role (Gaucheron, 2004). Two types of vitamins are supplied in milk: fat-soluble vitamins A, D, E, K (Gomis et al., 2000, Jensen, 1995a) and water-soluble vitamins C, B1, B2 (Jensen, 1995b).

2.4. Adhesion factors

Microbial adhesion has long been studied as it is the initial step in biofilm formation. The microbial cell surfaces are of structural complexity and chemical heterogeneity (Dufrêne, 2000), indeed a search has been initiated many years ago into microbial electrophoretic mobility (Schär-Zammaretti and Ubbink, 2003, van Loosdrecht et al., 1987a), contact angles (van der Mei et al., 1998b), cell surface hydrophobicities (van Loosdrecht et al., 1987b, van Loosdrecht et al., 1987a), surface free energies (Absolom et al., 1983, Busscher et al., 1984), and other physico-chemical properties of microbial cell surfaces with the aim of applying surface thermodynamics (Bos et al., 1999, Absolom et al., 1983) or DLVO (Derjaguin-Landau-Verwey-Overbeek) theories (van Oss, 1995, Hermansson, 1999) to explain initial microbial adhesion to surfaces. More recently, atomic AFM (Gaboriaud and Dufrêne, 2007) was used to evaluate bacterial interaction with surface or biomolecules (Burgain et al., 2013b).

Adhesion of bacteria is governed not only by long range forces such as steric and electrostatic interactions, but also by short range forces such as van der Waals, acid-base, hydrogen bonding (Boks et al., 2008) and biospecific interactions (carbohydrate-protein, protein-protein) (Salerno et al., 2004). Bacterial interactions and bacterial adhesion to surfaces are complex phenomena involving non-specific and specific binding events and the balance between them depend on the type of the bacterium, the type and state of the host surface and the environmental conditions (Waar et al., 2005). It is obvious that the understanding of bacterial interactions is a complex matter.

In this part, the description of the different bonds and interaction that can be involved between bacteria and the environment is provided.

2.4.1. Physico-chemical properties

In recent decades, the initial step of microbial adhesion and aggregation processes were described by the classical physicochemical approach, namely the DLVO theory (Boks et al., 2008) developed for macromolecules and particles. The electrostatic and

van der Waals forces have been combined in the DLVO theory of colloid stability, giving the interaction energy as a function of separation distance (Figure 48).

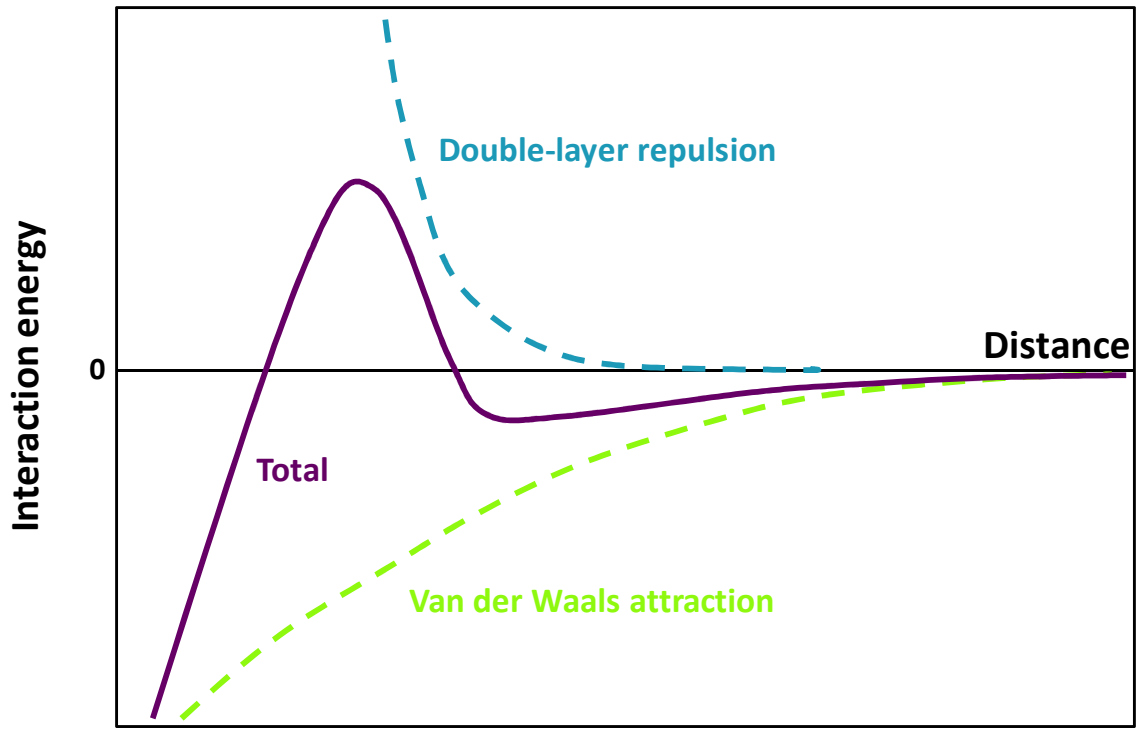


Figure 48 : DLVO theory.

Non-specific interactions involved in the initial adhesion step depend on physic-chemical surface properties of the bacteria and the target surface (Hermansson, 1999). These non-covalent interactions include electrostatic interactions (DL), van der Waals (LW) and Lewis acid-base interactions (AB) (electron donor-electron acceptor). They mainly depend on the nature of the structures on surfaces that will come into contact, the conformation of macromolecules on these surfaces but also micro-environmental factors such as pH, temperature, ionic strength (Hermansson, 1999). Because neither the DLVO nor the thermodynamic approach can fully explain bacterial adhesion (Hermansson, 1999), "extended" DLVO theory (generally known as XDLVO) was proposed (Oss, 1989). Adhesion energy can be expressed as follows (Hermansson, 1999):

$$\Delta G^{adh} = \Delta G^{LW} + \Delta G^{DL} + \Delta G^{AB}$$

where ΔG^{LW} and ΔG^{DL} represents the van der Waals and electric double layer interactions from the "classical" DLVO theory and ΔG^{AB} relates to acid-base interactions. When ΔG^{adh} is negative attraction between bacteria and its target can occur whereas repulsion is observed when ΔG^{adh} is positive (Jacobs et al., 2007). The DLVO and XDLVO

theories are commonly used whereas they are not strictly valid in the case of microorganisms, which are defined as soft colloids and whose separation from the external environment is not a 2D interface but a 3D interphase. Thus, a transition volume exists between the cell and its external environment. This new concept which is more rigorous and perfectly suitable and valid for microorganisms was introduced in the mid 2000s by Duval and Ohshima (Duval and Gaboriaud, 2010, Duval and Ohshima, 2006).

2.4.2. Electrostatic interactions

Electrostatic interactions occurring between the surface of the microorganism and the interacting surface is due to the recovery of the ionic double layer associated with charged groups on the surface of the two entities (Rutter and Vincent, 1984). When the surface of the bacterium, negatively charged comes in contact with the surface of the substrate, also negatively charged, it produces repulsive electrostatic interactions due to the overlapping of the two surface layers loaded.

The free energy due to electrostatic interactions between a sphere of radius R and a flat surface can be obtained by the following equation (Jacobs et al., 2007):

$$\Delta G^{DL} = R\psi_0^2 \varepsilon ln\big(1 + (-\kappa y)\big)$$

Where y is the distance between the particles; ψ_0 the surface potential; ε the dielectric constant of the liquid and 1/κ the Debye length.

The ψ_0 potential (or surface potential of the particle) is not directly measurable, but can be determined by measuring the zeta potential (ζ) by the micro-electrophoresis methods.

With the relation :

$$\psi_0 = \zeta \left(\frac{1 + z}{R}\right) \exp(\kappa z)$$

where 1/κ the Debye length (thickness of the diffuse ionic layer)

z the distance measured from the particle surface to the slipping plate (m)

ε the relative dielectric permittivity

R the radius of the particle

Z the zeta potential (Volt) measured at the slipping plate

ΔG^{DL} being a function of the magnitude 1/κ, this interaction energy gradually decreases with increasing the ionic strength of the suspension liquid (Hori and Matsumoto, 2010). Moreover, it should be noted that ΔG^{DL} decreases exponentially with the distance of separation between the two bodies. Below 20 nm, when the bacterium crosses the energy barrier due to repulsive electrostatic forces, it is at a distance in the nanometer range, where appear short-distance interaction such as Lewis acid-base or hydrophobic interactions. Regarding the bacterial attachment to interfaces, electrostatic interactions are considered as the determinant phenomena (Bellon-Fontaine et al., 1996). From these considerations, bacteria can be associated to a charged colloidal particle. Ly et al. (Ly et al., 2006b) highlighted the fact that electrostatic interactions between bacteria and the food matrix have poorly been studied. Electrostatic forces are usually due to charged groups located on bacterial surface and substrate such as phosphates, lipopolysaccharides or carboxyls.

2.4.3. Lifshitz-van der Waals interactions

These are the forces holding together the molecules and atoms of the two entities and which depend on attractions between atoms and molecules as they come closer. The van der Waals forces are generally of low intensity, they decrease rapidly with distance. These forces result from the interaction between the electrons of a molecule and charges from another. When the bacterium is at a distance between 50 and 20 nm of the substratum, it can only subject to the attractive Van der Waals interactions. To avoid interpenetration of the electron clouds of the two molecules, electrostatic repulsion forces come in. The interaction energy due to van der Waals forces from microbial body (1) support (2) and liquid (3) engaging in the phenomenon of adhesion (Jacobs et al., 2007) is calculated by the following equation:

$$\Delta\, G_{1,2,3}^{LW} = -2\left[\left(\sqrt{\gamma_3^{LW}} - \sqrt{\gamma_2^{LW}}\right)\left(\sqrt{\gamma_3^{LW}} - \sqrt{\gamma_1^{LW}}\right)\right]$$

Where γ_1^{LW}, γ_2^{LW}, γ_3^{LW} are interfacial tensions of the microbial cells, the carrier and the liquid, respectively.

The van der Waals interactions will be repulsive in the case of a free energy $\Delta\, G_{1,2,3}^{LW}$ positive for $\gamma_1^{LW} < \gamma_3^{LW} < \gamma_2^{LW}$, and attractive when $\Delta\, G_{1,2,3}^{LW}$ is negative.

2.4.4. Lewis acid-base

Lewis acid-base interactions (electron acceptor/donnor) enable the formation of hydrogen bonds (Lewis interactions). These are strong electrostatic interactions, short distance, and possible when hydrogen is in contact with an electronegative atom.

The interaction energy linked to the Lewis acid-base strength $\Delta G_{1,2,3}^{AB}$ of microbial body (1), the support (2) and liquid (3) coming into contact is measured by the equation (Jacobs et al., 2007).

$$\Delta G_{1,2,3}^{AB} = 2\left(\sqrt{\gamma_1^+\gamma_3^-} + \sqrt{\gamma_2^+\gamma_3^-} + \sqrt{\gamma_1^-\gamma_3^+} + \sqrt{\gamma_2^-\gamma_3^+} - \sqrt{\gamma_1^+\gamma_2^-} - \sqrt{\gamma_1^-\gamma_2^+} - 2\sqrt{\gamma_3^+\gamma_3^-}\right)$$

Where γ^+ represents the surface tension of the electron acceptor and parameter γ^- the surface tension of the electron donor element. It is important to note that in terms of the values of γ^+ and γ^- of the three bodies involved, $\Delta G_{1,2,3}^{AB}$ can take negative values (attraction) or positive values (repulsion).

2.4.5. Surface hydrophobicity

DLVO theory has been extended by the inclusion of acid-base interactions (XDLVO) which accounts for the hydrophobicity of the surfaces involved.

The hydrophobicity of bacteria is largely due to the nature of the compounds present on the surface, useful for adhesion (Rosenberg and Doyle, 1990). To evaluate the hydrophobicity of a surface, two techniques have been developed: the contact angle and adherence to hydrocarbons.

Measuring the contact angle between a water droplet and the surface under study allows a direct evaluation of hydrophobic/hydrophilic surface properties (Rosenberg and Doyle, 1990). The surface is hydrophobic if the contact angle is greater than 50 ° and hydrophilic when the angle is less than 50 °.

2.5. Environmental conditions influencing the interaction forces

The bacterial surface is highly dynamic depending on environmental conditions (pH, ionic strength and hydrophobicity) by proton dissociation or association (Poortinga et al., 2002).

2.5.1. pH

LAB are negatively charged at pHs encountered in physiologic media. Similarly, most of food substrata possess negative surface characteristics and for this reason, for bacterial adhesion an energy barrier must be overcome.

The bacterial cell surface charge originates from protonation or dissociation of amino acids groups, carboxyl and phosphate groups. Consequently, the bacterial charge depends on pH. The surface charge modification as a function of pH is given in Figure 49.

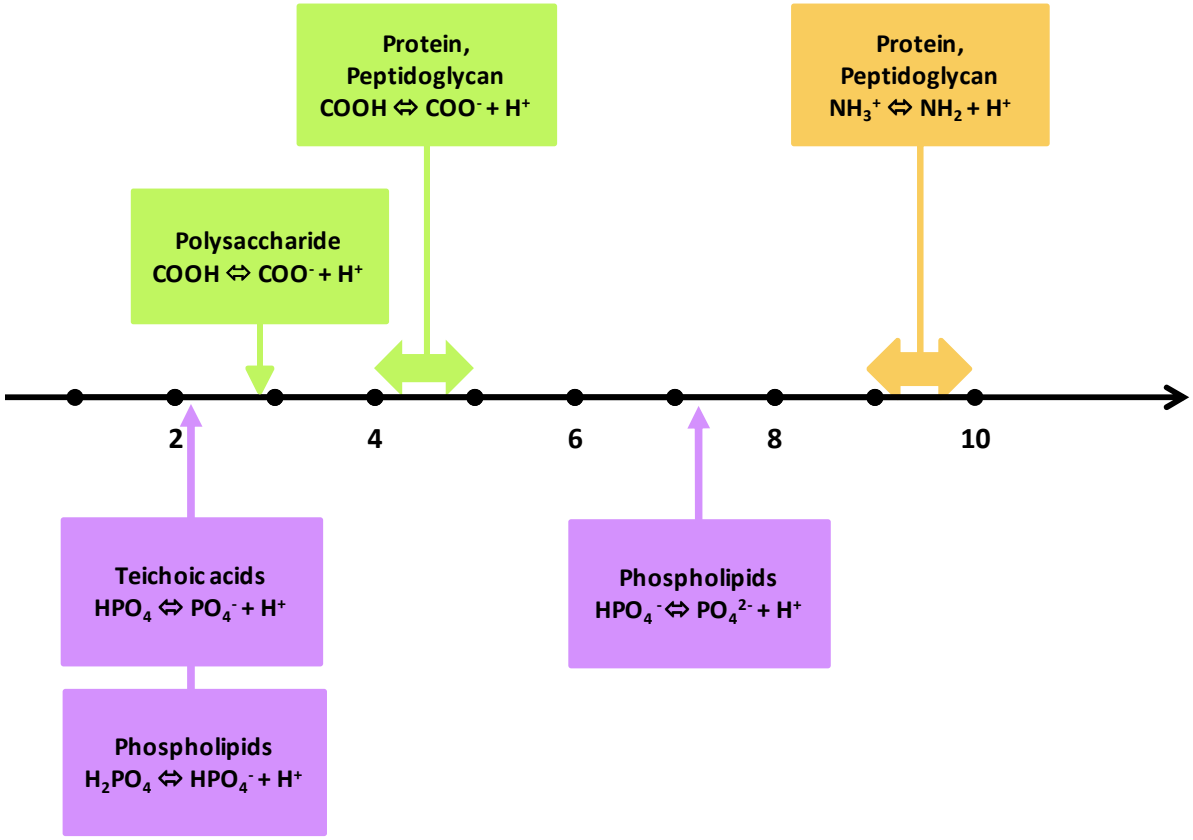


Figure 49 : Groups responsible for the modification of the bacterial surface charge as a function of pH

The number of carboxyl and phosphate groups exceeding the number of amino groups at physiological pH (*i.e.* between 5 and 7) most of bacterial cells are negatively charged (Poortinga et al., 2002).

2.5.2. Ionic force

In the presence of a charged particle in an aqueous solution, an electric double layer is formed by counter ions against the surface charge (Hori and Matsumoto, 2010). As bacteria are commonly negatively charged (Loosdrecht et al., 1989), repulsive electrostatic energy is caused by the overlapping of the electrical double layers (Marshall et al., 1971, Bos et al., 1999, van Loosdrecht et al., 1987a). This repulsive energy increases as the ionic strength decreases due to shielding of surface charges by ions in electrical double layers lessens. On the contrary, at low ionic strength, when a bacteria approaches a surface, there is an energy barrier which bacteria cannot overcome by swimming or Brownian motion (Figure 50) (Marshall et al., 1971, Loosdrecht et al., 1990). In these conditions, a lower secondary energy minimum is observed with a distance from the surface that is usually within several nanometers, depending on the ionic strength.

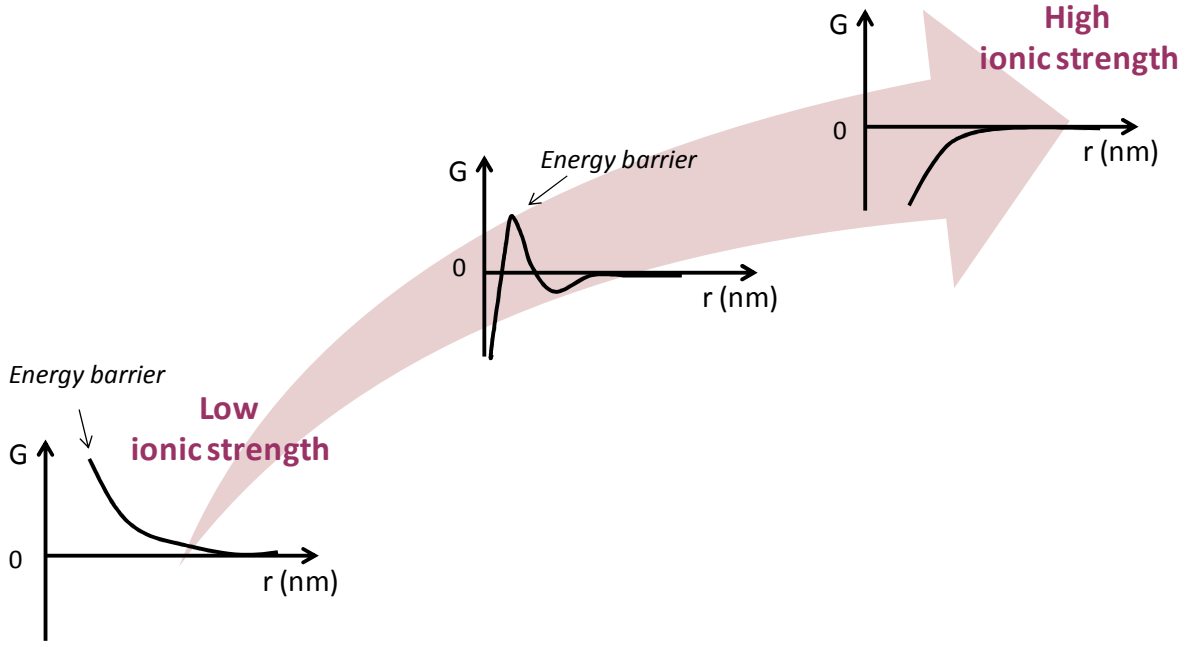


Figure 50 : Influence of ionic strength on the total interaction energy between a bacterial cell and a surface.

For a two steps adhesion procedure, during the first part the bacterium comes to the position by its motility or Brownian motion: at that time adhesion is still reversible. In the second part, the bacterium uses nanofibers (such as pili and flagella, EPS), which can penetrate the energy barrier due to their small radius, for bridging between the cell and the surface. As the energy barrier becomes higher and farther from the substrate at lower ionic strengths, however, it becomes difficult for the nanofibers and EPS to reach

the substrate and bacterial cells become unable to adhere. On the contrary, at high ionic strengths, the energy barrier disappears and bacterial cells can easily attain irreversible adhesion. The link between decreasing bacterial adhesion and decreasing ionic strength was demonstrated and is consistent with the DLVO theory (Loosdrecht et al., 1989, Abu-Lail and Camesano, 2003, Bunt et al., 1995, Zita and Hermansson, 1994).

2.6. Techniques to study microbial adhesive interactions

2.6.1. Surface characterization

The physic-chemical characterization of bacterial surface is crucial to understand how they can interact with other ingredients. Several techniques have been implemented to evaluate the different surface properties, such as micro-electrophoresis for measuring the net charge (Wilson et al., 2001, van Loosdrecht et al., 1987a), measuring the contact angle (van Loosdrecht et al., 1987b, van der Mei et al., 1998b) or the method of microbial adhesion to solvent (MATS) (Bellon-Fontaine et al., 1996, Ly et al., 2006a), infrared spectroscopy (Amiel et al., 2001, Curk et al., 1994) and X-ray photoelectron spectroscopy (XPS) (Dufrêne et al., 1997, Dufrêne and Rouxhet, 1996, Rouxhet and Genet, 2011).

2.6.1.1. Microbial adhesion to hydrocarbon or to solvent

The net surface charge of the bacteria and the presence of lipophilic compounds affect the bacterial partitioning between two immiscible liquids. The microbial adhesion to hydrocarbons test (MATH) is widely employed for partitioning of bacteria between hydrocarbon and aqueous phase (Rosenberg, 1984, Rosenberg et al., 1980). The hydrophobicity of bacterial cells can be defined as the tendency of a microorganism to adhere to a nonpolar material compared to water (Ofek et al., 2003). The MATH test is performed by filing a bacterial suspension with a hydrocarbon (Figure 51) such as hexadecane at a ratio of 1:10 of hexadecane to aqueous solution (*e.g.* 0.1 ml of hexadecane and 1.0 ml of bacterial suspension). The mix is vigorously vortexed during 1 min in order to obtain droplets of hexadecane dispersed in the aqueous phase. The separation between the phases is rendered possible by letting the system at rest for 2 or 3 min. During this time, the hexadecane rise to the top, carrying the hydrophobic bacteria. On the contrary, hydrophilic bacteria remain in the aqueous phase because they do not bind to hydrocarbon. The difference between the initial optical density at

530 nm (OD_{530}) of the aqueous phase and the OD_{530} of the same phase after vortexing give information on the portioning of the bacteria between the two phases. In fact, hydrophilic bacteria remain in the aqueous phase and the OD_{530} is slightly changed whereas hydrophobic bacteria are retained with hexadecane and the OD_{530} of the aqueous phase decrease (Ofek et al., 2003).

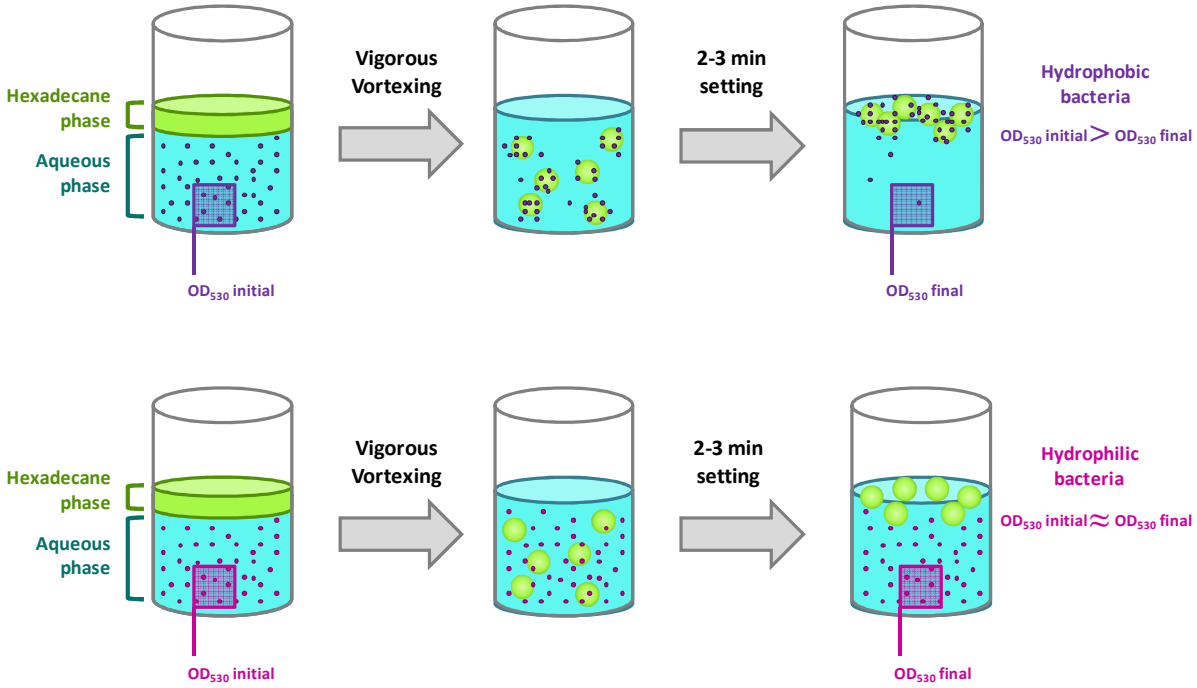


Figure 51 : Description of the principle of the MATH method.

The MATS technique derives from MATH previously described. Bellon-Fontaine et al. (Bellon-Fontaine et al., 1996) and Lee and Yii (Lee and Yii, 1996) developed MATS technique using three solvents : hexadecane (nonpolar solvent), chloroform (polar solvent, acid, electron acceptor) and ethyl acetate (basic solvent, electron donor). The acidity/basicity or ability to develop Lewis acid-base interactions is assessed after separation of bacteria between an aqueous phase and an organic phase with an acidic nature such as chloroform or base such as ethyl acetate. The MATS method consists in putting in contact with the organic solvent, a bacterial suspension in an aqueous medium of known initial optical density (OD_{600}). After vigorous stirring, the solvent micro-droplets are formed, on which bacteria are adsorbed, more or less depending on their surface properties. After standing and separation of the phases, measuring the absorbance of the aqueous phase is carried out. The adhesion ability of the bacteria to the solvent is expressed as a percentage according to the following relation:

$$\%Adhesion = \frac{OD_{600}^{initial\ aqueous\ phase} - OD_{600}^{aqueous\ phase\ after\ mixing}}{OD_{600}^{initial\ aqueous\ phase}}$$

According to Lee and Yii (Lee and Yii, 1996), it is accepted that bacteria is hydrophobic when the percentage of adherence to hexadecane is greater than 50 %, hydrophilic when this percentage is less than 20 % and moderately hydrophobic if the percentage is between these two values.

2.6.1.2. Electrophoretic mobility

The electrostatic potential can be measured by electrokinetic models such as electrophoresis.

The apparatus consists of a micro-electrophoresis microscope equipped with a camera, a measuring chamber, a laser and a computer equipped with a software for analysis. The laser illuminates the bacteria in the measuring cell. Bacteria are visualized by light microscopy and transcribed on the computer using a camera. Thus, it is possible to see the trajectory of bacteria during the application of the electric field. An image analyzer calculates the mobility of the particles using the trajectories according to the equation:

$$\mu = \frac{u_E}{E}$$

Where u_E is the velocity of the bacteria suspended in an aqueous medium and μ the electrophoretic mobility under an electric field (E) ranging from 100 to 1000 V/m.

The measuring chamber has two electrodes (anode and cathode), the two probes and the measuring cell. Probes retrieve information regarding the temperature and the conductivity of the medium used for the measurement. The electric field is uniform in the measuring cell and the charged particles migrate to the electrodes. Positively charged particles migrate towards the cathode (negative electrode) and, conversely, those negatively charged migrate towards the anode (positive electrode). Bacteria will migrate toward the opposite charge at a certain speed according to their charge. More bacteria is charged, the more its electrophoretic mobility is high, and they will move faster towards the electrode (Grare et al., 2007). In the case of hard sphere, the electrophoretic mobility of the particles are directly proportional to the zeta potential from the Smoluchowski equation (Duval and Gaboriaud, 2010). In any case, this equation is valid for biological systems due to their soft, heterogeneous and ion-

permeable characters. The first work taking into account the softness of bacteria to describe their electrophoretic mobility were made by Ohshima (Ohshima, 1995, Ohshima and Kondo, 1991). Recently, a new model for applying the Ohshima model without restrictions on size and charge to biological cells, eukaryotic or prokaryotic has been described (Duval, 2005, Duval and Ohshima, 2006). This theoretical advance has helped highlight the highly heterogeneous nature of interphase bacteria - aqueous solution as a function of ionic strength.

Bacterial electrophoretic mobilities can be measured as a function of pH under constant ionic strength. The pH at which electrophoretic mobility is zero is referred as the isoelectric point (IEP). The IEPs of bacteria provide information on the molecular composition of bacterial surface. For example, an IEP ≤ 2 can result from a wealth of phosphate groups (Van der Mei et al., 1998a) present on TA whereas 2 ≤ IEP ≤ 2.8 can reflect bacteria covered by anionic polysaccharides (phosphate and carboxyl groups negatively charged). An example of electrophoretic mobility for *Lactobacillus rhamnosus* GG and caseins is provided on Figure 52. The bacterium exhibit an IEP at a pH of around 3.7.

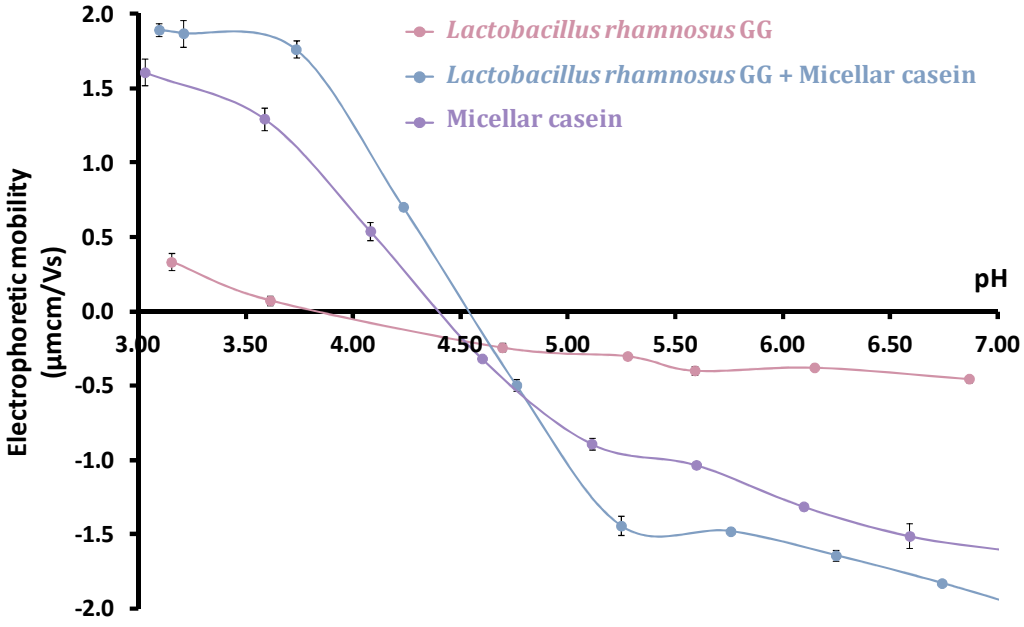


Figure 52 : Electrophoretic mobility of *Lactobacillus rhamnosus* GG, micellar casein, and a mixture of *Lactobacillus rhamnosus* GG and micellar casein.

Bacteria are typical example of soft particles. In this case, the softness is given to the membrane wall which is covered with a fluid-permeable structure. According to

Ohshima (Ohshima, 1995), these particles can be regarded as a hard particle covered with a shell of polyelectrolytes. The model of electric double layer strictly applicable to hard particles (*i.e.* not permeable to fluid) therefore cannot describe the distribution of ions and potential in the interphase (Duval and Gaboriaud, 2010). Indeed, the shell is defined as a gradual transition from physical and chemical properties of the outer membrane of the cell wall into the extracellular medium (Duval and Ohshima, 2006). So there is no Stern layer and diffuse layer of covers not only the polyelectrolyte shell to the external environment, but also right inside the shell.

Other methods such as proton titration (van der Wal et al., 1997) and dielectric spectroscopy (Kijlstra et al., 1994) can also be used to bacterial cell surface charge.

2.6.1.3. AFM – force mode

The approaches included in the DLVO theory are limited by the fact that they consider only the surfaces as smooth, rigid, chemically homogeneous, which is far from reality.

In this case, many bacteria present on their surface appendages that can be fimbriae, pili, fibrils, or flagella. These appendices allow microorganisms to adhere to different types of substrates or to aggregate. In addition, the solvated macromolecules at the cell surface can generate macromolecular interactions, which can be either repulsive (steric repulsion) or attractive (polymer bridging). Therefore, it is necessary to develop methods capable of probing the surface properties of microbial cells at a molecular scale in order to refine the traditional view of the process of microbial adhesion.

AFM has the ability to observe single cells at nanometer resolution resulting in an opportunity to study in depth the structure of cell surfaces. With AFM the monitoring of structural dynamics in response to environmental stimuli is also possible (Müller and Dufrêne, 2011a, Müller and Dufrêne, 2011b).

AFM force-distance curves are obtained by following the cantilever deflection (d) as a function of the vertical displacement of the piezoelectric scanner (z) which ensures three-dimensional positioning with high accuracy. In order to measure the cantilever deflection, a laser beam is focused on the terminal part of the cantilever and the position of the reflected beam is detected thanks to a position sensitive detector (PSD) with piconewton sensitivity.

The raw force-distance curve can be plotted: the PSD voltage versus scanner position. Then, in the part where the tip and the sample are in contact, the slope of the retraction force curve can be determined, which enables the conversion of the PSD voltage into a cantilever deflection. The cantilever deflection is then converted into a force (F) according to the Hooke's law: F = -k * d, where k is the cantilever spring constant. The curve can be corrected by plotting F as a function of (z-d). The zero separation distance is then determined as the position of the vertical linear parts of the curve in the contact region.

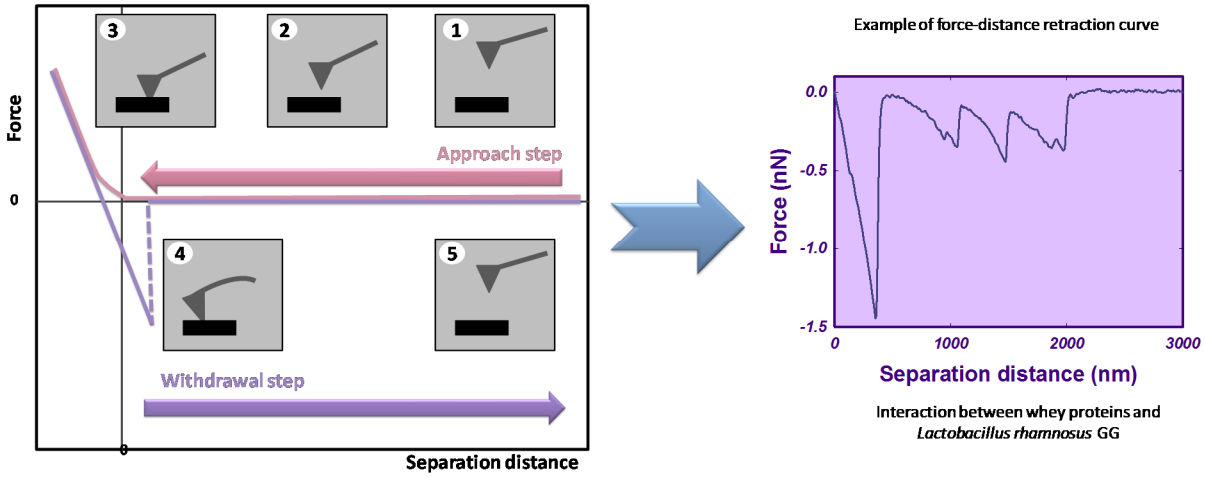


Figure 53 : AFM contact mode : principle and example of a result obtained for the measurement of interaction between LGG wt and whey proteins at pH 6.8.

On the force-distance curve, two different curves are found: the approach and the retraction. By considering the approach, when the tip is far from the sample, the resulting force is null (Figure 53 - step 1). When the tip approaches the surface, the cantilever may bend upwards due to repulsive forces (Figure 53 – step 2). Finally, the probe comes into contact when the gradient of attractive forces exceeds the spring constant plus the gradient of repulsive forces (Figure 53 – step 3). This approach part of the force-distance curve can be used to measure a variety of surface features, such as van der Waals and electrostatic forces but also, solvation, hydration and steric forces. In fact, in the contact an increase of the force can be observed and the shape of the approach curve can provide direct information on the viscoelasticity of the sample.

In a second time, when considering the retraction part, the tip picks up the sample and the force-distance curve exhibits an hysteresis referred to as the adhesion ``pull-off'' force (Figure 53 – step 4). Finally, the probe returns to its original position (Figure 53 – step 5) and the same process will be repeated to another location in the sample.

The retraction curve can be exploited to measure the binding force or stretching associated with single biomolecules. An example of experimental result is provided on Figure 53. It can be seen that adhesion occur between LGG and whey proteins as four peaks can be noted.

AFM can localize and manipulate individual molecules, this technique is known as single-molecule force spectroscopy (SMFS). By labeling the tip with specific antibodies or ligands, single molecule can be detected in complex environments such as living cells (Hinterdorfer and Dufrêne, 2006). A conventional strategy is to covalently anchor proteins on tips via a polyethylene glycol crosslinker.

The combination of AFM imaging and force spectroscopy to probe the surface topography, elasticity and adhesion of probiotic reveals major differences between bacteria having a crystalline like protein surface layer and those without such layers (Schär-Zammaretti and Ubbink, 2003). The wide variety of surface properties observed in these lactobacilli was suggested to have implications for food processing.

AFM enables force measurements on surfaces and consequently the hydrophilic/hydrophobic properties, the specific/non-specific adhesion with bacteria. These investigations can be realised in a physiologic medium providing results more closer to reality than MATS / MATH for example (Alsteens et al., 2007, Dague et al., 2007, Dupres et al., 2010, Francius et al., 2008).

2.6.1.4. XPS

XPS provides a direct chemical analysis of solid surfaces (Dufrêne et al., 1997). The sample is irradiated by an X-ray beam, which induces ejection of photoelectrons. The analyse of the kinetic energy of the emitted electrons and their binding energy in the atom of origin is determined. The technique provide information of the outermost molecular layers of the surface (2 to 5 nm). The recorded spectrum presents different peaks that are characteristic of a given electron energy level of a given element, and their position are influenced by the chemical environment (Boonaert and Rouxhet, 2000). The use of high vacuum for analysis requires that the cells must be freeze-dried before being introduced in the spectrometer.

Informative relationships were frequently observed between the surface composition determined by XPS and other surface properties such as particular

structural features, hydrophobicity, electrical properties or interfacial behaviour (Rouxhet and Genet, 2011).

2.6.2. Display of bacterial location in the matrix

2.6.2.1. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a valuable tool for obtaining high resolution images and 3D reconstructions. The principle of this microscope was developed by Marvin Minsky in 1953, but it took times (mid 1980s) before commercial models became generally available. The confocal principle is now well known (Cox, 2002). A laser is focused by the objective lens and the reflected light or emitted fluorescence is focused by the same lens to a spot at the detector. A pinhole between specimen and detector allows the selection of information from single focal plane. By scanning successive planes, a 3D image of the sample can be created.

CLSM has been used extensively in cell biology (Wright et al., 1993). Conventional epifluorescence microscopy may be used for discriminating viable bacteria in liquid samples such as milk (Pettipher et al., 1980) but, the optical sectioning capability of CLSM has the advantages of increased sensitivity and reduced out-of-focus blur, enabling direct observation in dairy products (Hassan et al., 1995b). The CLSM allows the direct observation of LAB in an hydrated state and living conditions (Hassan et al., 1995a). The use of non-toxic fluorescent probes and image analysis, CLSM allows observation of microorganisms within their physicochemical environment. That observation can be performed dynamically and nondestructively.

Moreover, the selection of adapted fluorescent probes may provide information on bacterial viability; for example dead bacteria will be stained in red and alive bacteria in green by using a live/dead kit based on plasma membrane permeability (consisting of SYTO9 and propidium iodide as fluorescent probes).

2.6.2.2. AFM – Topography mode

AFM topography imaging is a powerful complement to fluorescence and electron microscopies, offering new possibilities for visualizing the supramolecular organization of cell surfaces (Müller and Dufrêne, 2011a). The technique involves scanning a tip over the sample surface, while sensing the interaction forces between the tip and the sample (Figure 54) (Scheuring and Dufrêne, 2010). In other microscopy methods images are created not by using an incident beam whereas for AFM images are created by measuring 'near-field' interactions between the instrument tip and the surface. Different operating modes are encountered: the contact mode and the tapping mode for example. In the first mode, the cantilever deflection is recorded while the sample is scanned at constant height. In the tapping mode, a tip oscillating near its resonance frequency is scanned over the surface and the amplitude and phase of the cantilever are monitored (Dufrêne, 2008).

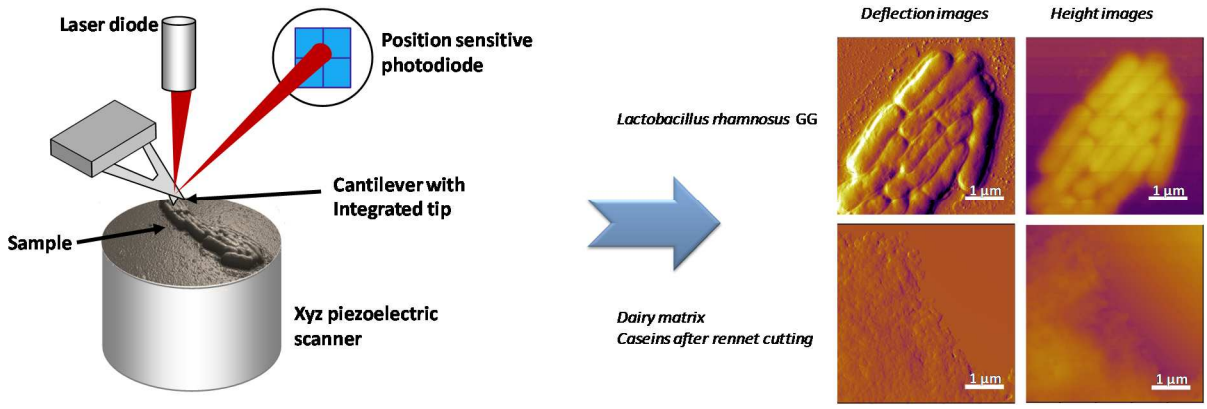


Figure 54 : AFM topography mode : principle and examples of obtained images for LGG wt and a protein matrix made of renneted caseins.

The nanoscale architecture of cell wall PG in living *Lactococcus lactis* bacteria was recently described (Andre et al., 2010) by combining AFM topography and the use of cell wall mutants. AFM has proved to be a valuable tool to reveal the fine ultrastructure of PG in LAB (Firtel et al., 2004, Andre et al., 2010).

2.6.2.3. Electron microscopy

Electron microscopes use electrons instead of light to produce images of the sample. As the resolving power of a microscope being a linear function of the wavelength, the use of electrons (having wavelengths shorter than the photons of visible light) allows for a higher resolution (Erni et al., 2009). For example, light microscopes

are limited to a resolution of about 200 nm when the spatial resolution achieved with multi-purpose TEMs today is generally around 0.2 nm (Klang et al., 2013). For these reasons, much smaller structural details can be visualized. Two major types of electron microscopes are encountered: scanning electron microscopes and transmission electron microscopes. For imaging with electron microscopy, specimens must be solid (*i.e.* powder, particles, etc) because they are viewed under high-vacuum.

2.6.2.3.1. Scanning electron microscopy

The image production with SEM is realized point by point by scanning a focused electron beam across the surface of a solid specimen. The primary electrons penetrate the solid specimen and are deflected by a large number of elastic scattering processes. The energy spectrum of electrons that leave the specimen are collected by the detector system. Topographic contrast arises primarily from the secondary electron signal and the quasi 3D surface topography can be recorded mostly due to these secondary electron (Klang et al., 2013).

SEM is a valuable technique in dairy research by providing information on microstructure of relevant products which can be related to physical properties (Kalab, 1993). However, an extensive sample preparation is required for dairy products prior to microscopic observations due to their high moisture and fat contents (Hassan et al., 1995a).

Environmental scanning microscopy (ESEM) is an electron microscopy technique that allows the direct observation of wet samples in their native state and the possibility to perform dynamic experiments. Moreover, this technique does not require coating as it is necessary for conventional SEM analysis. The principal limitation of ESEM is its low resolution compared to SEM and TEM (Klang et al., 2013).

2.6.2.3.2. Transmission electron microscopy

For image production with TEM, a high voltage electron beam is employed. An electron gun usually fitted with a tungsten filament cathode allows the emission of electrons. The electron beam is accelerated by an anode (typically at 100 keV) with respect to the cathode, is focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam (Klang et al., 2013). Part of electrons collides with the

specimen and electrons move around the nuclei of atoms in the sample. The electrons are collected, focused and magnified by lenses to produce a projected image. The contrast formation in electron microscopic images is usually done by amplitude contrast and phase contrast (Williams and Carter, 2009).

2.7. Influence of bacteria – milk components interactions for dairy products

2.7.1. Location of the bacteria in the matrix

The direct observation of bacteria within the milk gel is a difficult step as the medium is heterogeneous and opaque to light. However, observations can be realized by using electron microscopy (Hassan et al., 2003a, Ayala-Hernandez et al., 2008, Ly-Chatain et al., 2010, Costa et al., 2010) or fluorescent microscopy (Ly-Chatain et al., 2010, Léonard et al., 2013). The bacterial location within the gel network is clearly affected by the bacterial interaction with other milk components when the medium is still liquid. It was shown that, in a first time, bacteria make contact with the fat globule membrane (Laloy et al., 1996) or settle at the casein/fat interface but, after a month, they became embedded in the MFGM or inside the fat globules. LAB have been shown to be preferentially associated with the fat/protein interface in cheese (Lopez et al., 2006) and in emulsions stabilized with milk proteins (Ly et al., 2008). However, the reason for a preferential location within the matrix is not yet fully known while it could be useful for cheese manufacture as they have lipolytic and proteolytic enzyme activities. Particularly, bacteria are located in whey pockets (Lopez et al., 2006) so, the bacteria should have a higher affinity for whey proteins. This was confirmed by AFM force measurements that described specific interactions between the probiotic bacteria LGG or LGR-1 with soluble proteins whereas non-specific interactions were observed with caseins (Burgain et al., 2013b). The environmental conditions and especially the pH was also shown to be determinant for establishment of interactions between bacteria and milk proteins (Burgain et al., 2013b). The spatial distribution of *Lactococcus lactis* colonies in model cheese (Jeanson et al., 2011) was modelled and bacterial colonies were shown to be randomly distributed, fitting Poisson's model. Moreover, it was demonstrated that the initial inoculation level strongly influenced the mean distances between colonies. Anyway, the manner the colonies interact with the matrix at a microscopic scale is far from being understood (Floury et al., 2013).

2.7.2. Texture of yogurts

LAB producing EPS are commonly used to modify the yogurt and cheese texture (Hassan et al., 1996b, Hassan and Frank, 1997, Perry et al., 1997, Perry et al., 1998). In fact, yogurts made with EPS producing cultures are less susceptible to syneresis, more viscous, and had more water holding capacity than the ones made with EPS non-producing cultures (Hassan et al., 1996a, Hassan et al., 1996b). The use of cultures producing high level in EPS allows the obtention of softer cheese curd (Hassan and Frank, 1997) and water retention in Mozzarella cheese is improved (Perry et al., 1997). The way the EPS influences textural and rheological properties of dairy products is not fully known. EPS was previously observed in yogurt and cheese by using SEM (Schellhaass and Morris, 1985, Teggatz and Morris, 1990, Skriver et al., 1995, Bhaskaracharya and Shah, 2000). In these studies, the sample preparation technique cause the appearance of EPS as filaments attached to casein micelles. However, this observation has been recognized to be an artifact resulting from the sample preparation process (Kalab, 1993). Cryo-scanning electron microscopy (cryo-SEM) seems to provide a solution to many of the problems associated with conventional SEM as wet samples are stabilized by rapid freezing and observed in their frozen hydrated state (Hassan et al., 2003a).

In set fermented milks, bacterial strains producing EPS were mainly found in protein-free cavities which was not the case for bacteria not producing EPS (Girard and Schaffer-Lequart, 2007). Other authors demonstrated that fermented milks containing bacterial strains not producing EPS, presented a microstructure of the gel which was homogeneous with small and uniformly distributed pores. On the contrary, the gel structure of fermented milks containing strain producing EPS presented large cavities filled with the bacteria. The authors explained these effects by the likely incompatibility of the EPS with the protein aggregates in the milk (Hassan et al., 2003b).

2.7.3. Microencapsulation of probiotic bacteria

In recent years, the interest for the microencapsulation of probiotic bacteria is growing. Moreover, the use of milk proteins as an encapsulating matrix has demonstrated its great interest to protect microbial cells (Burgain et al., 2011). Gelation properties of milk proteins can be exploited for probiotic encapsulation. For example,

rennet gelation of milk proteins was used to develop a microencapsulation technology for protection of two probiotic strains: *Lactobacillus paracasei* ssp. paracasei F19 and *Bifidobacterium lactis* Bb12 (Heidebach et al., 2009a). This is the case for the heat induced gelation of whey proteins that was exploited to produce microparticles containing probiotic bacteria. In this way, *Bifidobacterium* Bb-12 was microencapsulated by spray-drying with whey proteins and the entrapped cells presented better survival during simulated gastric digestion (De Castro-Cislaghi et al., 2012). Another example is the microencapsulation of LGG with gelled whey protein isolates (Doherty et al., 2011). CLSM and AFM in tapping mode were used to assess the good entrapment of the bacteria in the matrix (Doherty et al., 2010, Doherty et al., 2011). Images proved that bacteria were well encapsulated in microparticles constituted of whey proteins and high encapsulation yield were recovered. In the same way, microencapsulation of LGG into different dairy matrices revealed that the addition of whey proteins to caseins improve the bacterial encapsulation rate (Burgain et al., 2013a). Once again, the preferential interaction between bacteria and whey proteins was highlighted. However, to our knowledge, no studies quantified the interaction forces that occur between dairy proteins and bacteria.

2.8. Conclusion

The understanding of bacterial interaction with milk components is of huge importance as LAB are introduced in many dairy products and have technological role. The way the biomolecule located on bacterial surface can interact with other structures is now clear thanks to the knowledge on bacterial adhesion. Nevertheless, important work remain lacking on defining which molecules are engaged in interactions.

3. Material and methods

3.1. Material

Bacteria that were used in this chapter are the following:

- *L. rhamnosus* GR-1 (LGR-1),
- *L. rhamnosus* GG wild-type (ATCC 53103) (LGG wt).

The three LGG isogenic mutants are:

- the pili-deficient *spaCBA* mutant (CMPG 5357)(Lebeer et al., 2012b),
- the *welE* mutant (CMPG 5351) mutated for long galactose-rich EPS (Lebeer et al., 2011, Lebeer et al., 2009),
- the *dltD* mutant (CMPG 5540) (Vélez et al., 2007) having modified LTA molecules.

Bacterial stock cultures were stored at −80 ◦C in MRS (De Man et al., 1960) broth containing 20 % (v/v) glycerol. Pre-culture was initiated by inoculating 9 ml of MRS broth with 100 µl of bacterial stock. This pre-culture was then used to inoculate fresh MRS broth and the growth was conducted at 37°C until the end of exponential phase when OD_{600} reached around 1.2. It was chosen to harvest the cells at this moment because pili genes are mainly expressed in exponential phase (Laakso et al., 2011). Cells were harvested by gentle centrifugation (3000 g, 10 min, room temperature) to avoid the loss of pili (Tripathi et al., 2012).

Micellar casein powder (Promilk 872B) was obtained from Ingredia IDI (Arras, France). Whey proteins isolates powders (Prolacta 90) were purchased from Lactalis Ingredients (Bourgbarré, France). The solutions were prepared by adding between 1 and 12.5 g of protein powder into 100 g of distilled water. The rehydration was done by stirring for 2 h at room temperature and then overnight at 4 ◦C. The denatured whey proteins were obtained by heating the native whey solution at 78 ◦C for 10 min then cooling it to room temperature. Heat denaturation of whey proteins allows the unfolding of the structure leading to aggregation phenomena.

For microencapsulation, the chymosin enzyme (Chy-Max Plus; 199 IMCU/ml; Chr. Hansen, Hørsholm, Denmark) was prepared by diluting hundred times the initial solution with distilled water. Tween® 80 was purchased from Sigma-Aldrich and sunflower oil in a local store.

3.2. Characterization of bacterial and protein surface properties

3.2.1. Electrophoretic mobility

The bacterial cells were first subcultured at 37 ◦C in MRS medium, followed by a culture until the end of exponential phase. These cells were harvested by centrifugation (1 min, 7000 rpm, room temperature) and resuspended in KNO_3 10 mM. Milk protein solutions were prepared at a final concentration of 1 % (w/w). The electrophoretic mobility of the cells and milk protein solutions were measured in the pH range 3–7 at 20 ◦C using a Malvern Zetasizer nano-ZS. The pH was adjusted by the Malvern MPT-2 autotitrator via addition of NaOH 10 mM and HCl 10 mM in a 10 ml sample. Electrophoretic mobility was derived from velocity under an applied electric field of 150 V. Electrophoretic mobility was evaluated three times on the same sample and this was repeated during three independent studies.

3.2.2. X-ray photoelectron spectroscopy

Bacteria were first subcultured at 37 ◦C in MRS medium. This pre-culture was then used to inoculate 20 mL of MRS broth which was incubated overnight at 37 ◦C until the end of exponential phase. Cells were harvested by centrifugation (1 min, 7000 rpm, room temperature) and washed twice with physiologic water (NaCl 9 $g.L^{-1}$, pH 7.4). After the final washing, the pellet was transferred to cryotubes and quickly cooled in liquid nitrogen and placed on the shelves of a freeze-dryer (Christ alpha 1-2, freeze-dryer, Osterode, Germany) (van der Mei et al., 2000). The microbial powder obtained after freeze-drying was gently crushed with a spatula and used for XPS analysis using a Kratos Axis Ultra (Kratos Analytical, UK) spectrometer. For each bacterial cell, three independent points were studied to evaluate surface composition.

3.3. Atomic force microscopy

3.3.1. Substrate immobilization onto PEI-coated glass slide for topography images

A glass slide was pre-coated with polyethilenenimine (PEI) (0.1 % (w/v) in milli-Q-grade water) with a contact time of 10 min. It was rinsed with milli-Q-grade water and stored in a sterile Petri dish. The cells (1 mL of bacterial suspension with an optical density (OD_{600}) of 1.2) were electrostatically immobilized onto PEI-coated glass slide (contact time: 30 min). The glass was slightly dehydrated under a nitrogen flow to

realize topographic measurements. This method avoids the use of chemical binders between the substrate and the bacterial sample and leads to minimization of any chemical modification of the bacterial cell wall or bacterial surface organization.

Regarding milk proteins, solutions with a final concentration of 1 % were directly deposed on freshly cleaved mica for 30 min.

3.3.2. Preparation of bacteria-coated probes

The preparation of cantilevers and tips for force measurements was realized as followed (Figure 55): they were pre-coated with PEI by immersion for 5 h in the PEI solution, and then washed in milli-Q-grade water. The negatively charged bacteria were attached to the positively charged PEI-coated probes thanks to electrostatic attractions established during 1 h of contact with the cell culture. The bacteria-coated probes were rinsed with milli-Q water before use. The designed probe can be called "lacto-probe" (Dague et al., 2010).

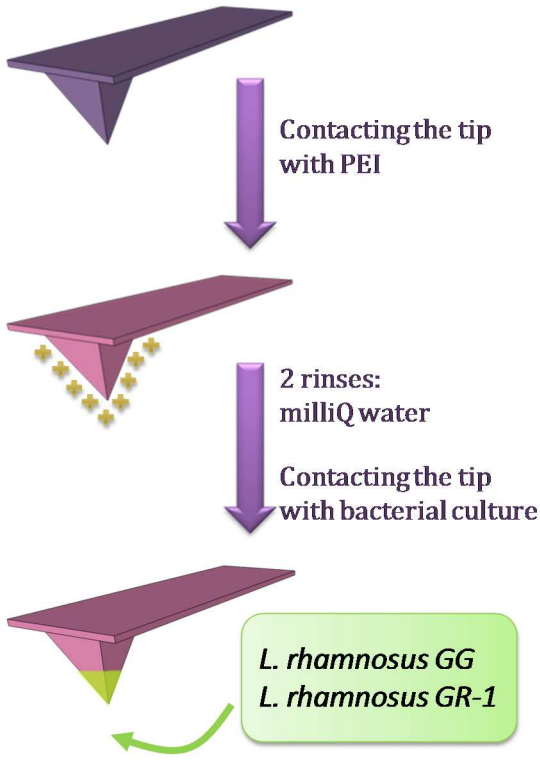


Figure 55 : Preparation of bacteria-coated probes.

3.3.3. Preparation of protein-coated mica

The solutions of milk proteins were used at a final concentration of 1 % and deposed on mica (Figure 56). After 30 min of contact, the mica was disposed into PBS buffer with a controlled pH of 6.8 or 4.8 to perform force measurements in liquid

(Dague et al., 2010). These two pH values were chosen according to the protein electrophoretic mobility profiles. At pH 6.8 (the pH encountered in fresh milk) milk proteins are negatively charged and at pH 4.8 the negative charge of the proteins is less pronounced because of the proximity of the IEP. However, it is not possible to choose a pH below this value because of the acid gelation of caseins below their IEP.

Figure 56 : Preparation of protein-coated mica.

3.3.4. Preparation of protein-modified probes

For force measurements, AFM probes with attached particle (Borosilicate Glass, 2 µm), coated with gold and modified with a NH_3 terminated PEG linker were purchased from Novascan (Ames, Iowa) with a spring constant of 0.01 nN/m. The preparation of cantilevers and tips was realized as follows (Figure 57): they were coated with milk proteins by immersion for 15 h in the solution, and then washed in milli-Q-grade water. The protein-coated probes were rinsed into milli-Q water before their use.

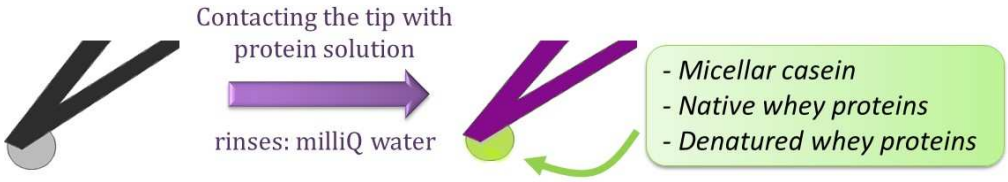


Figure 57 : Preparation of protein-modified probes.

3.3.5. Preparation of bacteria-modified surfaces

Mica functionalized with a NH_3 terminated flexible PEG linker was purchased from Novascan (Ames, Iowa). The bacterial suspension was deposed on mica for 15 h of contact (Figure 58). The mica was then rinsed with mili-Q grade water and disposed into PBS buffer with a controlled pH of 6.8 to perform force measurements in liquid (Dague et al., 2010).

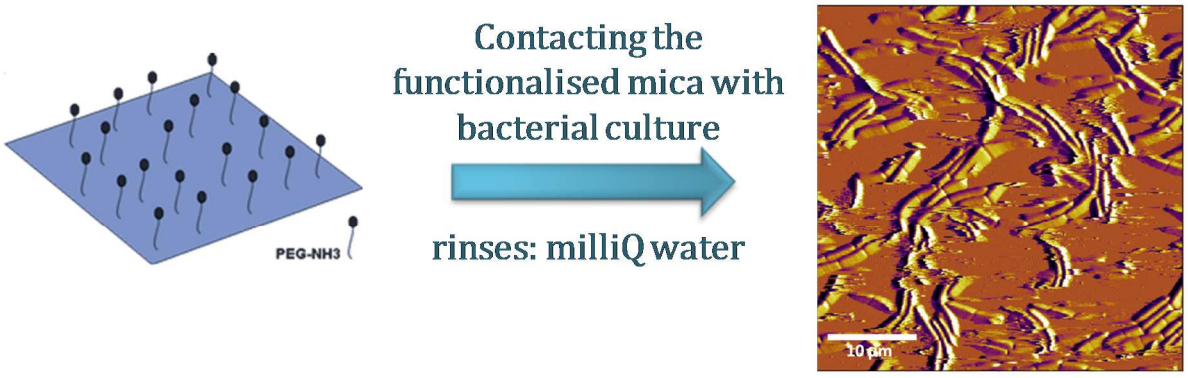


Figure 58 : Preparation of bacteria-modified surfaces.

3.3.6. AFM measurements

For topography images, Olympus micro cantilevers OMCL-TR400 were purchased from Atomic Force (Mannheim, Germany) with a spring constant of 20 pN/nm.

Force measurements and topography images were made at room temperature using an Asylium MFP-3D atomic force microscope (Santa Barbara, CA, USA) with IGOR Pro 6.04 (Wavemetrics, Lake Osewego, OR, USA) as operation software. It was used in contact mode with an applied force lower than 250 pN and images were acquired at a scan rate of 1 Hz and scan size of 30 µm × 30 µm. AFM force–distance curves were obtained by following the cantilever deflection (d) as a function of the vertical displacement of the piezoelectric scanner (z) with a scan speed of 1 µm/s. In order to measure the cantilever deflection, a laser beam was focused on the terminal part of the cantilever and the position of the reflected beam was detected thanks to a position sensitive detector (Quilès et al., 2012, Polyakov et al., 2011). The adhesion force can be deduced from the cantilever spring constant k_c and the deflection by the following relation:

$$F = k_c * d$$

On the force–distance profile, two different curves are encountered: the approach and the retraction but only the retraction is considered for estimation of adhesion forces. Initially, the probe comes into contact with the sample. In a second time, when considering the retraction part, the tip picks up the sample and the force–distance curve can exhibit single or multiple peaks that correspond to adhesive events or interaction forces between the tip and the sample surface. The difference between these peaks and the baseline provides information on adhesion events. Finally, the probe returns to its

original position and the same process will be repeated at another location in the sample (Dufrêne et al., 2001) which allows the performance spatially resolved force-mapping, called force–volume image.

3.3.7. Fitting models: FJC and WLC

The observed peaks on retraction curves can be described by statistical mechanics of ideals chains: WLC (Worm-Like Chain) or FJC (Freely Jointed Chain) models. Regarding the FJC model (Marszalek and Dufrene, 2012), the polymer is considered as a series of rigid, orientationally independent statistical (Kuhn) segments. These segments are connected through flexible joints. The Kuhn length (l_k) is related to the contour length (L_c) by:

$$L_c = n * l_k$$

Where n is the number of monomers. The extension x can be expressed as a function of the pulling force F:

$$x(F) = L_c \left[coth\left(\frac{F l_k}{k_b T}\right) - \frac{k_b T}{F l_k} \right]$$

In AFM studies, polysaccharides behave as series of loosely connected segments and are well described by the FJC model.

The elastic response of proteins is well described by the WLC model (Marszalek and Dufrene, 2012). The polymer is considered as an irregular curved filament, which is linear on the scale of the persistence length (l_p), a parameter that represents the stiffness of the molecule. Extension is limited by the contour length (L_c) of the molecule (the length of the linearly extended molecule without stretching the molecular backbone). The force F versus extension x is given by the following equation:

$$x(F) = L_c \left[coth\left(\frac{F l_k}{k_b T}\right) - \frac{k_b T}{F l_k} \right] \left[1 + \frac{F}{k_s L_c} \right]$$

3.4. Microencapsulation

The formulation consists of 90 % of micellar casein and 10 % of semi-denatured whey proteins. The bacteria were added after mixing the protein solution and before enzymatic incubation. For this, 0.5 g of freeze-dried LGG (wt or mutants) was added to 200 g of protein mixture.

Microparticles were produced by using an emulsification method employing a patented process. The enzymatic incubation was completed in a double-walled, temperature-controlled reactor, made of stainless steel. An amount of 200 mL of protein mixture was added into the first reactor (reactor for enzymatic reaction) held at 5 °C. Then, 18 ml of diluted enzyme preparation was added and mixed with the solution. The mixture was left for 30 min at 5 °C allowing the chymosin enzyme to cut the κ-casein. This solution is then pumped to a second reactor (emulsification reactor) where emulsification can take place. For this purpose, 800 ml of cooled sunflower oil containing 1 % (w/v) of Tween® 80, were placed under stirring at 500 RPM. The enzymatic phase was progressively added and cold emulsification at 5 °C was performed during 10 min. Afterwards, the temperature was raised to 40 °C for 25 min (temperature ramp: 1.4 °Cmin^{-1}) under mechanical agitation.

Microparticles were removed from the reactor and separated by filtration. The harvested particles were washed with distilled water. Subsequently, the microparticles were frozen at -20 °C, then lyophilized (Christ alpha 1-2, freeze-dryer, Osterode, Germany).

3.5. Encapsulation rate

The enumeration of LGG (wt and mutants) living cells was done by serial dilutions. The samples (0.1 mL) were plated in duplicate on MRS agar. CFU were determined after incubation (48 h at 37 °C). The ratio between the number of bacteria added in the protein mixture and the number of bacteria in the final particles was done. The initial number of bacteria introduced in the protein mixture was obtained by determining the CFU in 1 mL of solution. The result was multiplicated by 200, which is the total mixture quantity used for microencapsulation. At the end of encapsulation, the microparticles total weight was measured. The final CFU in 0.2 g of microparticles was also determined and reported to the total quantity of obtained microparticles. The encapsulation rate was calculated by applying:

$$ER\ [\%] = \frac{[\mathrm{CFU/g}]^{\text{Final microparticles}} * [\text{quantity of microparticles (g)}]}{[\mathrm{CFU/g}]^{\text{Initial solution}} * 15} * 100$$

3.6. Transmission Electron Microscopy

Microparticles were fixed during 2 h in glutaraldehyde (2.5 %), then flushed with cacodylate buffer during 3 h. A post fixation with osmium tetroxide during 1 hour was performed at ambient temperature. Successive dehydration in increasing ethanol baths was then done (30, 50, 70, 80, 90, and two times at 100 %). The sample was finally embedded in epoxy resin followed by a resin polymerisation at 56 °C during at least 48 h.

The day after, ultrathin slides (70 nm) were obtained with an Ultramicrotome (Reicher-Yung). Acetate Uranyle was used as a slide contrast agent. Microstructure of particle was examined with a CM12 Philips Scanning Electron Microscope.

4. Study of bacterial interaction (LGG or LGR-1) with milk proteins

In vitro interactions between probiotic bacteria and milk proteins probed by atomic force microscopy.

J. Burgain, C. Gaiani,, G. Francius, A.M. Revol-Junelles, C. Cailliez-Grimal, S. Lebeer, H.L.P. Tytgat, J. Vanderleyden & J. Scher (2013). *Colloids and surfaces B: Biointerfaces*, 104, 153-162.

Abstract

Interactions between microbial cells and milk proteins are important for cell location into dairy matrices. In this study, interactions between two probiotic strains, *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* GR-1, and milk proteins (micellar casein, native and denatured whey proteins) were studied. The bacterial surface characterization was realized with XPS to evaluate surface composition (in terms of proteins, polysaccharides and lipid-like compounds) and electrophoretic mobility that provide information on surface charge of both bacteria and proteins along the 3–7 pH range. In addition, AFM enabled the identification of specific interactions between bacteria and whey proteins, in contrast to the observed non-specific interactions with micellar casein. These specific events appeared to be more important for the GG strain than for the GR-1 strain, showing that matrix interaction is strain-specific. Furthermore, our study highlighted that in addition to the nature of the strains, many other factors influence the bacterial interaction with dairy matrix including the nature of the proteins and the pH of the media.

Résumé

Les interactions entre les bactéries et les protéines laitières sont importantes pour la localisation des cellules dans les matrices. Dans cette étude, les interactions entre deux bactéries probiotiques, *Lactobacillus rhamnosus* GG et *Lactobacillus rhamnosus* GR-1, et les protéines laitières (caséines micellaires, protéines solubles natives ou dénaturées) ont été décrites. La surface des bactéries a été caractérisée par XPS d'une part dans le but de quantifier les protéines, polysaccharides et les composés lipidiques et par mobilité électrophorétique d'autre part afin de connaitre la charge de surface pour des pH compris entre 3 et 7. L'AFM a permis d'identifier des interactions spécifiques entre les bactéries et les protéines solubles à l'opposé des interactions non spécifiques observées avec les caséines. Ces évènements spécifiques sont plus importants pour des interactions avec LGG plutôt qu'avec LGR-1, montrant ainsi que les interactions avec la matrice dépendent de la souche considérée. De plus, cette étude souligne le fait, qu'en plus de la nature de la souche, d'autres facteurs peuvent influencer les interactions bactérie-matrice laitière comme c'est le cas par exemple de la nature des protéines ou du pH.

4.1. Introduction

The study of microbial development in fermented dairy foods is of interest for food manufacturing in order to improve and control some processing steps, like the ripening step (Jeanson et al., 2011). In addition to LAB added as starter cultures, dairy food matrices such as milk, cream, fermented milk, cheese and ice cream can also contain probiotic bacteria. It is obvious that the bacterial surfaces physic-chemically interact with dairy components such as milk proteins, fat globules and/or lactose. The surface characteristics of bacteria are determined by the chemical composition of their cellular surface, in particular by proteins, polypeptides and polysaccharides (Ly et al., 2006a) and thus represent a major key in bacterial re/activity with food components (Ubbink and Schär-Zammaretti, 2007b, Vadillo-Rodríguez et al., 2005, Ubbink and Schär-Zammaretti, 2007a).

Bacterial location in fermented dairy foods has been studied at macroscopic level, particularly in Cheddar cheese (Sheehan et al., 2009), where it was observed that the spatial repartition of bacteria depends on their location at the end of the immobilization step, on the spatial distribution of nutrients (lactose, proteins, etc.) and on interactions between bacteria (Jeanson et al., 2011). At microscopic level, it was observed that the cells were preferentially located around fat globules and more particularly in whey pockets (Lopez et al., 2006). Other authors have suggested that the location of the bacteria may be influenced by the strain itself (Ly-Chatain et al., 2010).

At the microstructure level, bacterial colonization of milk is dependent of the physical state of the matrix (*e.g.* liquid for milk and solid after coagulation). In fact, in liquid milk, the cells can move thanks to attractive or repulsive forces towards milk components that influence their location (Ly et al., 2006a). On the contrary, after gelation, the bacteria are entrapped within the curd and develop as bacterial colonies with a 3D spatial distribution (Jeanson et al., 2011, Hannon et al., 2006). The microbial cell wall constitutes the frontier between the cells and their environment and is thus also a determining factor in this colonization. The cell wall plays several key roles like regulating the cell shape, controlling the cell surface properties and governing biointerfacial phenomena such as cell adhesion and cell aggregation (Dufrêne et al., 2001). Most of the bacterial strains used in dairy products are Gram-positive bacteria that are covered by neutral and acidic polysaccharides, different cell wall proteins and TA with a high acidic nature (Beveridge and Graham, 1991).

During the past three decades, the major focus has shifted towards probiotic dairy products, as the market of functional foods continues to expand (Burgain et al., 2011). The probiotic microorganisms used are generally members of the genera Lactobacillus and *Bifidobacterium*. Probiotic bacteria are defined as "live microorganisms which when administrated in adequate amounts can provide a health benefit on the host" (FAO/WHO, 2002). However, to exert these beneficial effects, probiotic bacteria must maintain their viability first during storage and then during their passage through the upper GI tract in order to arrive in the intestine in a viable state (Burgain et al., 2011). A good alternative for the plain delivery of probiotics into the intestine, is their formulation in cheese (Karimi et al., 2011). The delivery via cheese has several advantages as the cheese creates a buffer against the high acidity encountered in the stomach, forms a dense matrix and protects the cells by the possible presence of fat (Gardiner et al., 1999a). Therefore, many authors focused on the microencapsulation of probiotic bacteria into dairy matrices aiming at protecting the cells for a controlled release in the gut (Heidebach et al., 2009a, Doherty et al., 2011, Picot and Lacroix, 2004). However, these studies only recorded parameters like encapsulation yield or survival rate and differences were observed when changing the dairy matrix or the encapsulation technique. Dealing with these observations, the way the bacteria interact with the matrix was never elucidated in order to understand how encapsulation yield or survival rate could be improved.

The present study aimed to investigate the interaction of probiotic bacterial cells with dairy proteins, in addition to the influence of the environmental pH and the nature of the strains on these interactions. For this purpose, a new approach was developed to estimate the adhesion forces by AFM. AFM allows the probing of interfacial phenomena at the nanoscale, including specific and non-specific interactions. AFM force measurements were correlated to surface characterization of microbial cells and milk proteins by XPS and electrophoretic mobility. The approach was developed using Lactobacillus rhamnosus GG and GR-1 as model probiotic strains. The GI isolate LGG is extensively used as probiotic in dairy products. Its probiotic properties are well documented and include host immunostimulation and reduction of symptoms from GI disorders (Doron et al., 2005, Saxelin, 2008). LGR-1 is a closely related probiotic strain isolated from a female urethra, documented health effects include host immunostimulation and resolution of moderate diarrhea in HIV/AIDS patients (Reid,

2010, Hummelen et al., 2010, Hummelen et al., 2011, Anukam et al., 2008), and prevention of recurrent bacterial vaginosis (MacPhee et al., 2010, McMillan et al., 2011).

4.2. Results and discussion

4.2.1. XPS analysis

The study of bacterial surfaces is an important parameter to study bacterial interactions with the environment. The potential of XPS in providing informations on chemical composition of bacterial cell surfaces was demonstrated (Mozes and Lortal, 1995). This technique involves the irradiation of the sample by an X-ray beam resulting in an ejection of photoelectrons. The kinetic energy of the emitted electrons can be analysed and their binding energy in the atom of origin determined. The obtained information concerns only the outermost molecular layers of the surface (around 2–5 nm). Each peak of the recorded spectrum is characteristic of a given electron energy level of a given element, and its position is influenced by the chemical environment. Consequently, XPS measurements can provide an elemental analysis and a rough functional group analysis of the bacterial surface (Dufrêne et al., 1997).

Tableau 24 presents the surface elemental composition, in terms of mole fraction and atomic concentration ratios with respect to total carbon, determined on each bacterial strain, LGG and LGR-1.

Consistent with the general biochemical composition of lactobacilli cell walls, the main elements detected were C_{1s}, O_{1s} and N_{1s}. The carbon peak is composed of three components: carbon bound only to carbon and hydrogen (binding energy: 284.8 eV), carbon bound to oxygen or nitrogen by a single bond (binding energy: 286.3 eV) including ether, alcohol, amine and amide, and finally carbon making one double bond or two single bonds (binding energy: 532.7 eV) corresponding to carboxylic acid, carboxylate, ester, carbonyl or amide. The nitrogen peak appeared at 399.9 eV corresponding to unprotonated amine or amide functions. The last oxygen peak showed two components with oxygen singly bound to carbon and hydrogen (binding energy: 532.7 eV) that can be attributed to hydroxide, acetal and hemiacetal. Finally, oxygen can make a double bond with oxygen (binding energy: 531.4 eV) referring to carboxylic acid, carboxylate, ester, carbonyl and amide.

Tableau 24: XPS analysis of LGG and LGR-1 (mean ± SD, n=2)

Strain	Obtained from raw spectra				
	% C	**% O**	**% N**	**N/C**	**O/C**
LGG	67.80 ± 0.97	26.58 ± 2.31	5.62 ± 1.07	0.08	0.39
LGR-1	68.39 ± 0.27	27.64 ± 0.09	3.97 ± 0.18	0.06	0.40
	Obtained from C_{1s} peak deconvolution				
	C-C, C-H	**C-O, C-N**	**C=O**	**O-C=O**	
LGG	28.20 ± 1.33	47.07 ± 2.97	19.82 ± 1.07	4.92 ± 0.57	
LGR-1	36.21 ± 1.10	44.56 ± 1.05	14.72 ± 2.43	4.52 ± 0.28	
	Obtained with the matrix formula				
	$\%C_{ps}$	**$\%C_{pr}$**	**$\%C_{lp}$**		
LGG	24 ± 4	20 ± 4	24 ± 1		
LGR-1	28 ± 0	14 ± 1	27 ± 0		

Regarding their carbon, oxygen and nitrogen composition, no significant difference was observed between the two strains, except maybe for nitrogen, which was slightly higher for LGG. The results obtained for LGR-1 in this study are in general similar to those published by other authors (Reid et al., 1999). The biomolecular composition of the bacterial surfaces was deduced from the XPS data. The surface composition was modeled in terms of three classes: polysaccharides (Ps), proteins (Pr) and lipids (Lp). More complex compounds such as lipoproteins, were considered as a combination of lipids and proteins. The molecular composition was computed with the following elemental concentration ratios (Dufrêne et al., 1997, Rouxhet and Genet, 2011):

$$[N/C]_{obs} = 0.279\ (C_{Pr}/C)$$

$$[O/C]_{obs} = 0.325\ (C_{Pr}/C) + 0.833\ (C_{Ps}/C)$$

$$[C/C]_{obs} = (C_{Pr}/C) + (C_{Ps}/C) + \left(C_{Lp}/C\right) = 1$$

The proportions of carbon associated to each molecule can be determined by solving the system. The polysaccharide content was almost the same for both strains, corresponding to the large quantity of EPS produced by the bacteria (Tableau 24). Previous AFM studies revealed the presence of two types of polysaccharides on the cell

wall of LGG: long galactose-rich EPS molecules and shorter glucose-rich polysaccharides (Francius et al., 2008). The protein content was higher for the LGG strain, which could be related to the presence of the recently identified pili on this Gram-positive bacterium (Lebeer et al., 2012b, Kankainen et al., 2009). Importantly, a recent study showed that high centrifugation speeds used during the preparation of the sample can result in the removal of pili (Tripathi et al., 2012).

Regarding results in lipid-like compounds presented in Tableau 24, it seems that LGR-1 has a higher lipid content than LGG. Important lipid-like compounds of lactobacilli include LTA, which are polymers linked to the cytoplasmic membrane by a glycolipid anchor (Lebeer et al., 2008). LTA of LGG were shown to be built out of polyglycerolphosphate and decorated with D-alanyl esters, without glycosyl substituents under standard growth conditions in MRS medium (Vélez et al., 2007). The D-alanylation modulates the charge electromechanical characteristics of the cell wall (Neuhaus and Baddiley, 2003).

4.2.2. Electrophoretic mobility and AFM topography

The electrophoretic mobility of LGG and LGR-1 (Figure 59A) and milk proteins (Figure 59B) were recorded in the 3–7 pH range.

The electrophoretic mobility of LGR-1 was negative over the entire pH range, whereas LGG had an IEP of pH 3.5. Profiles like this indicate that anionic compounds dominate the surface of the cells. This includes strong acids, such as the phosphate based LTA (pKa ≈ 2.1) and weak acids, such as the carboxylate containing acidic polysaccharides and proteins (pKa ≈ 3.5–4.5) (Rijnaarts et al., 1995, Boonaert and Rouxhet, 2000, Schär-Zammaretti and Ubbink, 2003, Deepika et al., 2009).

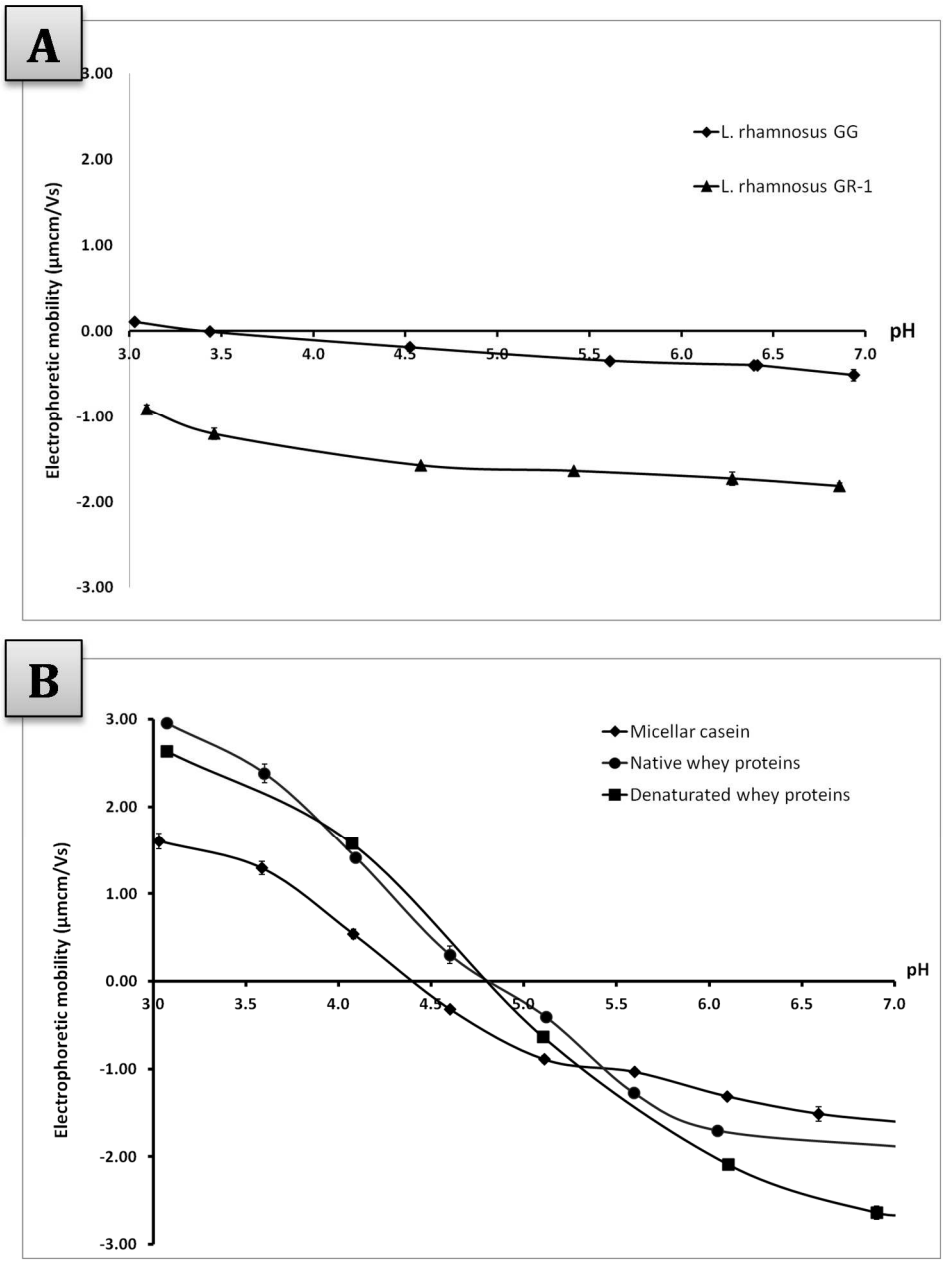


Figure 59: Measured electrophoretic mobility vs. pH for bacterial strains *L. rhamnosus* GG and GR-1 (A) and milk proteins (micellar casein, native and denatured whey proteins) (B) (mean ± SD, n=3).

The profile of electrophoretic mobility of milk proteins always shows different IEPs for each protein. For whey proteins, the IEP is around 4.8, regardless of the protein state (native or denatured) with an electrophoretic mobility range of −1.5 for a neutral pH to 3.0 for an acidic pH of 3. For micellar casein, at neutral pH, the density of charges was lower than for whey proteins and the electrophoretic mobility became positive at pH 4.4 with a final value of around 1.6 at acidic pH. At first, the decrease in the net charge causes morphological and size changes of the casein micelle during acidification

(Deepika et al., 2009) and in a second stage, the protruding ends of the κ-casein totally collapse (Tuinier and de Kruif, 2002). During acidification, the loss of charge is continuous and the micelles shrink to smaller sizes (Moitzi et al., 2010). The neutralisation of charges on casein molecules and particularly the phosphoserine residues after dissolution of the CaP occurs during acidification. The morphological and size changes of the casein micelle during acidification mentioned earlier can be observed on AFM deflection images (Figure 60) for casein micelles at pH 4.8 and 6.8, and also image of whey proteins at pH 6.8. Individual micelles of around 300 nm were distinguished at pH 6.8 whereas smaller ones (100 nm) were visible at pH 4.8.

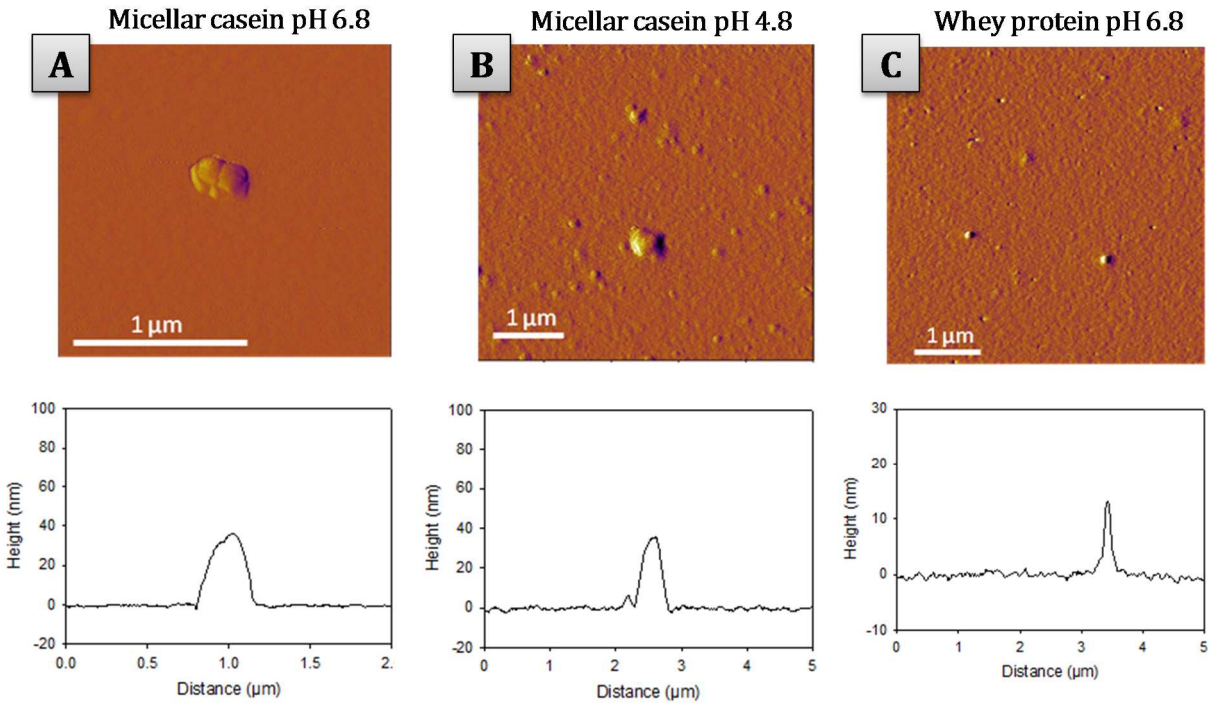


Figure 60 : Deflection images of micellar casein at two pHs (A: pH 6.8 and B: pH 4.8) and whey proteins (C: pH 4.8). Each image corresponds to 512 horizontal lines that describe the outward and return of AFM cantilever tip (1024 scans are made on each image). The graphics below each image correspond to height profiles taken from a cross section on the AFM images.

The casein micelles are polydispersed in size with a reported average of 200 nm (Dalgleish, 2011). The obtained micelles present a softer and smoother surface than at pH 6.8. In this case, hydrophobic attractive bonds overcome electrostatic repulsions. Milk casein micelles are natural association colloids that appear as heterogeneous raspberry-like particles at pH 6.8 (Figure 60). After acidification at pH 4.8, the micelles decrease in size and lose their surface heterogeneities, presenting a smooth surface. This

was first observed by other authors using AFM under liquid conditions (Ouanezar et al., 2012).

In order to visualize cell surface morphology, AFM images were taken of the two strains that were used in the study (Figure 61).

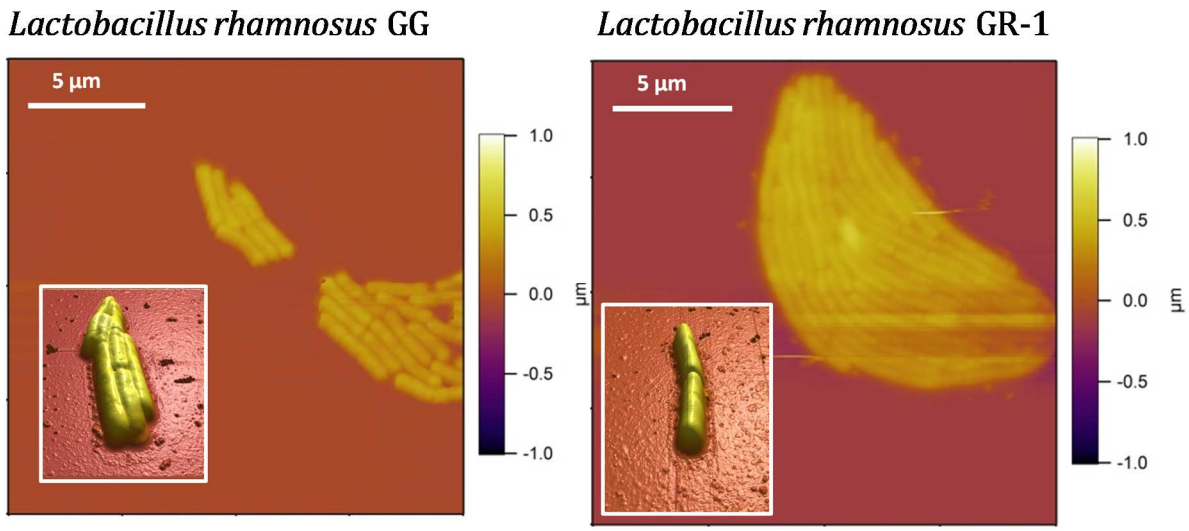


Figure 61: Height images of bacterial strains *L. rhamnosus* GG and *L. rhamnosus* GR-1. Each image corresponds to 512 horizontal lines that describe the outward and return of AFM cantilever tip (1024 scans are made on each image).
Insets: 3D views of bacterial strains.

For both LGG and LGR-1, it can be observed that the bacteria tend to stay side by side, with individual cells having a mean size of around 3 µm. In previous studies (Francius et al., 2008), high resolution images of LGG entrapped in membrane pores and in liquid condition were recorded. These authors succeeded to distinguish the surface morphology of the cells and more in particular, waves were observed.

4.2.3. AFM forces analysis between strains and proteins

The bacterial strains were electrostatically immobilized on the AFM tip for force spectroscopy measurements. Results on interaction forces between the lacto-probe and milk proteins are shown in Figure 62 for LGG and Figure 63 for LGR-1.

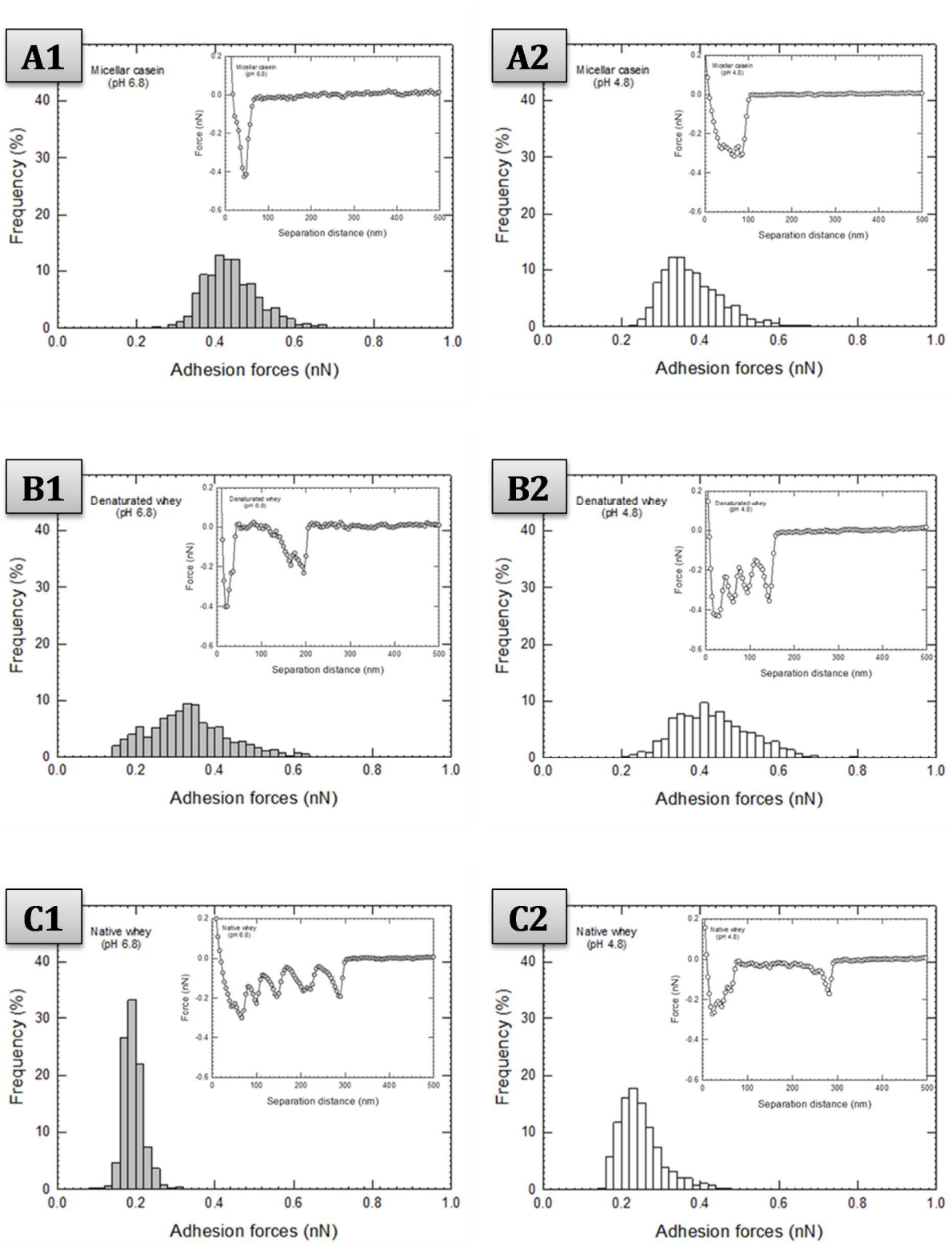


Figure 62: AFM force measurements between milk proteins and *L. rhamnosus* GG: statistical distribution of the adhesion force as a function of pH (grey for pH 6.8 and white for pH 4.8), and of the nature of milk protein (A: micellar casein, B: denatured whey proteins and C: native whey proteins).
Insets: representative force-distance curves.

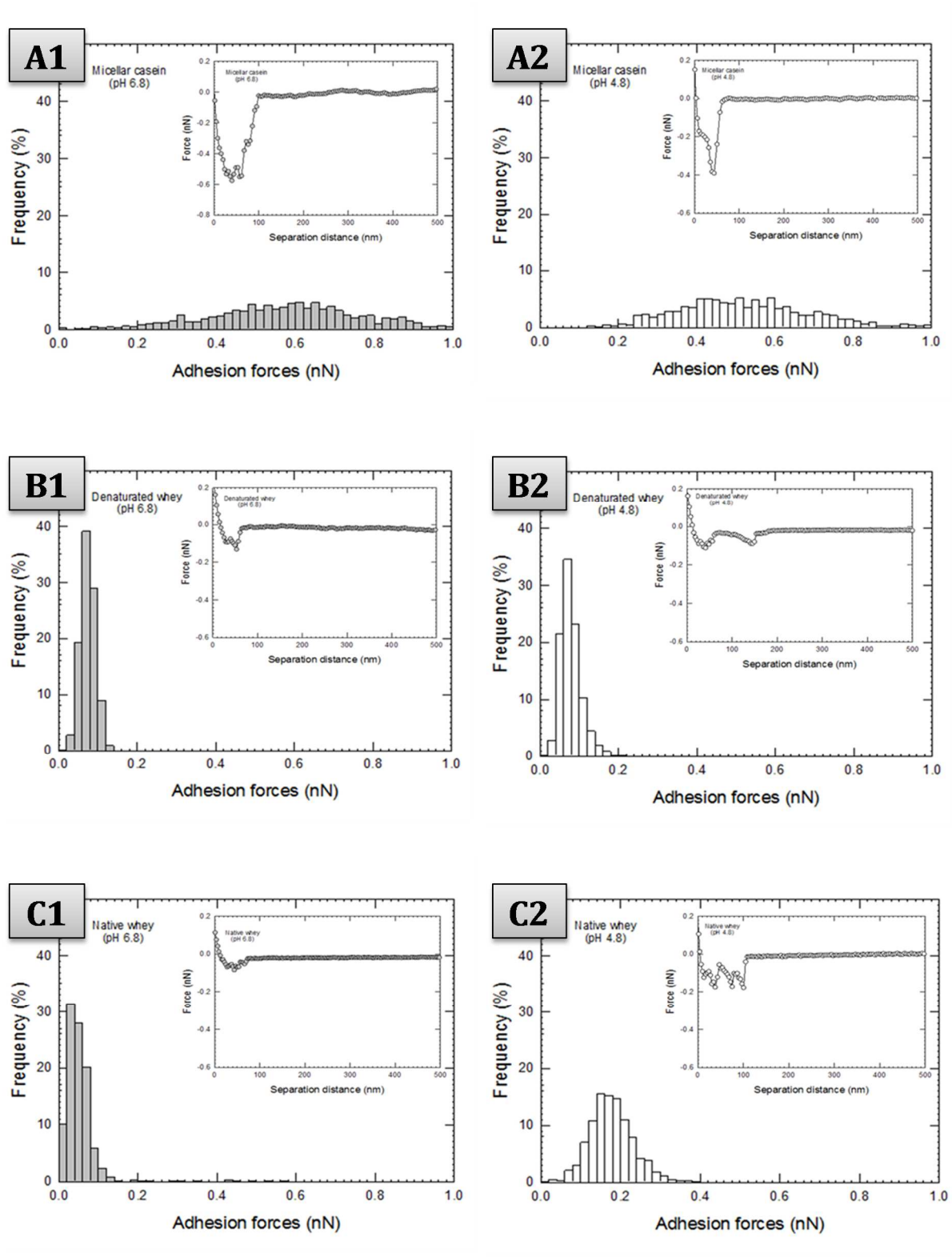


Figure 63: AFM force measurements between milk proteins and *L. rhamnosus* GR-1: statistical distribution of the adhesion force as a function of pH (grey for pH 6.8 and white for pH 4.8), and of the nature of milk protein (A: micellar casein, B: denatured whey proteins and C: native whey proteins).
Insets: representative force-distance curves.

Histograms represent the different interaction force distribution with insets of typical retraction force–distance curves. The shape of the retraction force–distance curve is an important point to be discussed. The profile observed for bacterial interactions with micellar casein (Figure 62 A1 and A2; Figure 63 A1 and A2) is typical for non-specific interactions, including hydrophobic, electrostatic and Van der Waals forces (Dague et al., 2007). However, because force measurements were performed in PBS, electrostatic charges were screened in a way that their contribution to adhesion between bacteria and proteins could not be estimated by AFM. On the contrary, specific interactions occurred between LGG or LGR-1 with whey proteins in a native or in a denatured state (Figure 62 B1 and B2, C1 and C2; Figure 63 B1 and B2, C1 and C2). In these cases, between one and five adhesive events occurred for several nanometres (between 20 and 300 nm) after the contact point. This particular shape of the retraction curve can be attributed to the stretching of molecules present on the tip (and consequently on the bacteria) but also to the milk proteins deposited on mica. However, it can be noticed that adhesion events are more important in number for the LGG strain than for the LGR-1 strain. Furthermore, the pH seems to have an influence on the specific adhesion events, as for interactions between LGG and denatured whey proteins, additional events were observed after acidification. The opposite was observed for the interactions between LGR-1 and native whey proteins with a number of specific adhesive events more important at pH 6.8 than at pH 4.8.

Figure 64 summarizes the adhesion forces that were obtained from AFM force measurements (calculated from Figure 62 and Figure 63).

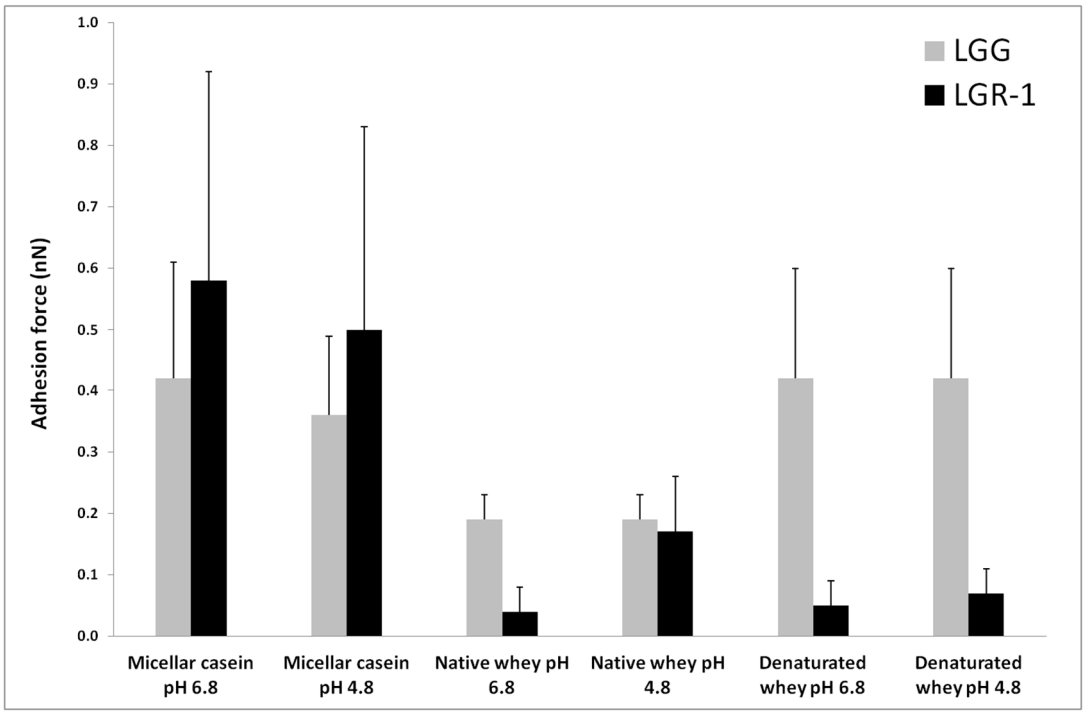


Figure 64: Overview of AFM force measurements between probiotic bacteria and milk proteins: adhesion force measurements (mean ± SD, n=1024 adhesion events).

First of all, it can be concluded that for a given milk protein and strain, pH seems to have no effect on adhesion forces, except for adhesion events between strain GR-1 and native whey proteins, which were more important at pH 4.8 than 6.8. However, it was described earlier that a number of specific adhesive events are more important at pH 6.8. This means that in this study, there may be a high number of ruptures on the retraction curve with weak adhesion force. This is the case for force measurement between *L. rhamnosus* GG and native whey proteins at pH 6.8 where multiple ruptures were observed and an adhesion force of only 0.2 nN. On the contrary, for force measurements between *L. rhamnosus* GG and micellar casein at pH 6.8, only one rupture was observed and the adhesion force was around 0.4 nN. Looking at interactions between *L. rhamnosus* GG and milk proteins, adhesion forces appear more important with micellar casein and denatured proteins, regardless of the pH, and less important with native whey proteins. Concerning the bacterial strain GR-1, it seems that adhesion forces are more pronounced with micellar casein and weaker for native and denatured whey proteins. Finally, the lowest number of interactions was observed for *L. rhamnosus* GR-1 with native whey proteins at pH 6.8 and denaturated whey proteins at both pHs. The highest amount was found for interactions between LGR-1 with micellar casein.

4.2.4. Hypothesis concerning interactions between bacteria and milk proteins

In bovine milk, the four caseins (α_{s1}-α_{s2}-β-κ-caseins) are assembled together to form a structure called micelle. These proteins do not have a well-defined tertiary structure. As previously underscored by other authors (Corredig et al., 2011), interactions between casein micelles and biomolecules are of interest because the interior of the casein micelles is not accessible for large molecules whereas, it is porous enough to provide access to the inner part of the structure for small molecules (e.g. enzymes). On the contrary, whey proteins have a defined tertiary structure accessible to other biomolecules.

This explains why specific interactions can be established between molecules that decorate bacterial surfaces and whey proteins and not for casein micelles. In this study, it was established that interactions between the probiotic strains LGG and LGR-1 with whey proteins are specific in nature, whereas non-specific events were reported for interactions with casein micelles (Figure 65).

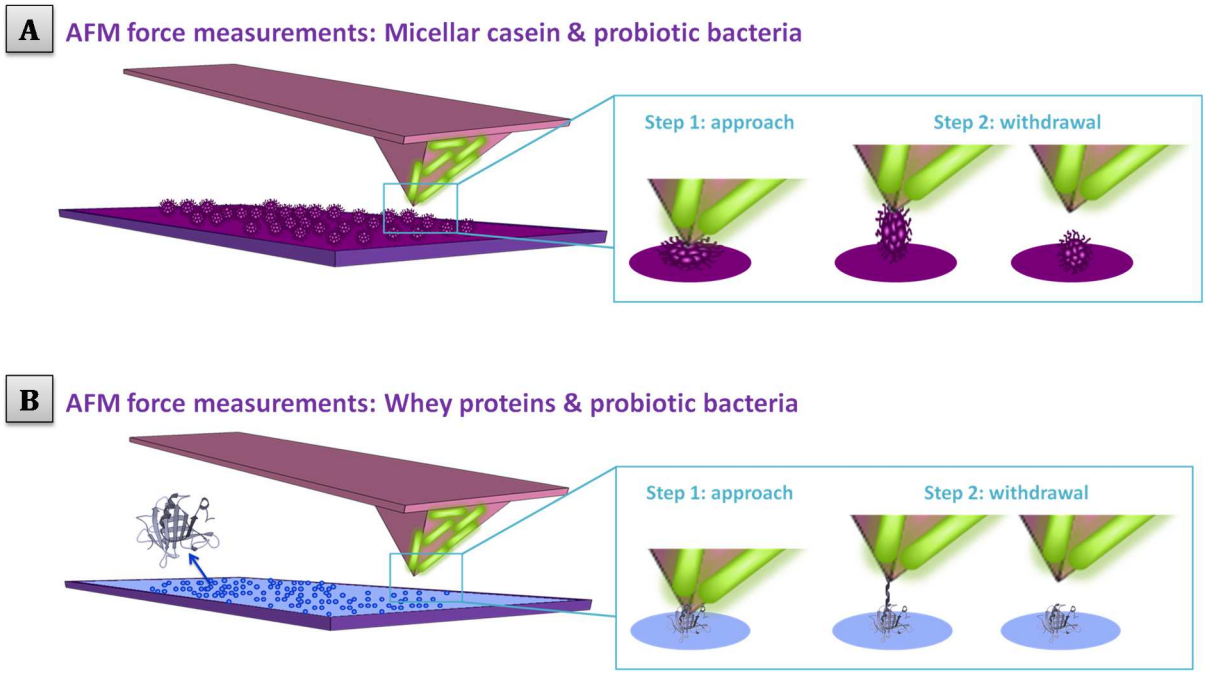


Figure 65: Non-specific interactions between probiotic bacteria and micellar casein (A) and specific interactions between probiotic bacteria and whey proteins (B).

The adhesive nature of bacteria is mainly due to various cell surface features consisting of proteins such as pili, and polysaccharides such as EPS. Adhesion of bacteria is governed not only by long range forces such as steric and electrostatic interactions, but also by short range forces such as Van der Waals, acid–base, hydrogen bonding and biospecific interactions. However, as mentioned above, electrostatic forces were not present for these measurements due to the use of PBS buffer. It is readily conceivable that these forces are important for bacterial interactions but these could not be estimated in this study. Bacterial interactions and bacterial adhesion to surfaces are both complex phenomena involving non-specific and specific binding events and the balance between them depends on the type of bacteria, the type and state of the host surface and environmental conditions. It is obvious that the understanding of bacterial interactions is a complex matter. The association of EPS with casein micelles is mostly electrostatic in nature (Corredig et al., 2011). Because electrostatic charges were no longer present on bacterial surfaces and proteins during this work, only Van der Waals, hydrophobic and steric interactions occurred. Moreover, interactions between anionic EPS and milk proteins depend on structural parameters of EPS such as the charge density, the nature of charged groups, the molecular weight and the stiffness of chains, but also on the protein involved, casein or whey protein (Girard and Schaffer-Lequart, 2008). As proposed by other authors (Lopez et al., 2006), bacteria are preferably organized as colonies in dairy products. Moreover, the bacteria are preferentially located at the fat/protein interface, which might be due to the fact that during the formation of the rennet-induced casein network, the bacteria are forced out of the protein matrix and are repelled in whey pockets that surround fat inclusions. Considering the results of the present study, a better understanding is established why bacteria preferably interact with whey proteins as they can establish specific interactions, which is not the case with casein micelles.

4.3. Conclusion

The use of AFM force spectroscopy, in combination with electrophoretic mobility measurements and XPS provided new insights into the interactions between probiotic bacteria and milk proteins. During the past few years, many authors have observed the preferential location of bacteria in whey pockets. The present work quantifies, for the first time, directly on the nanoscale and compares the adhesion forces between two

related probiotic strains (LGG and LGR-1) and milk proteins (micellar casein, native and denatured whey proteins). The bacterial surface characterization was realized using XPS, which provided information on the surface composition in terms of proteins, polysaccharides and lipid-like compounds. The electrophoretic mobility measurements provided information on surface charges of both bacteria and proteins along the 3–7 pH range. The observed specific adhesion between bacteria and whey proteins and the non-specific interactions with micellar casein, allow the understanding for the preferred interaction with whey proteins. From the results presented in this work, it can be concluded that many factors influence the bacterial interaction with the dairy matrix, including the nature of the proteins, the nature of the strains and the pH of the media. Future studies should identify which biomolecules on the bacteria specifically can interact with milk proteins.

5. Study of bacterial interaction (LGG & 3 isogenic mutants) with milk proteins

Significance of bacterial surface molecules interactions with milk proteins to enhance microencapsulation of *Lactobacillus rhamnosus* GG.

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Abstract

Probiotic bacteria are being increasingly encapsulated to enhance their delivery in an active state at their preferred site of action. In this study, a patented encapsulation process was used to protect the model probiotic strain *Lactobacillus rhamnosus* GG (LGG) into dairy matrices. The mechanism of microencapsulation was studied at the molecular level by comparing the encapsulation rate of LGG wild type and three of its surface mutants with AFM. A significant decrease in the encapsulation rate was observed when the bacteria were depleted for pili, while the pilus also appeared to be crucial for location inside the microparticle. Hereto, the *spaCBA* (mutant lacking pili), the *welE* mutant (lacking long EPS) and the *dltD* mutant having modified LTA were used. AFM enabled the confirmation of specific interactions between bacteria and whey proteins, in contrast to the observed nonspecific interactions with micellar casein. The role of the pili, *i.e.* multimeric appendages of several micrometers, was also modeled using WLC (Worm-Like Chain) or FJC (Freely Jointed Chain) models. This revealed that molecular mechanisms of microencapsulation of probiotic bacteria should ultimately benefit their targeted application.

Résumé

Les bactéries probiotiques sont de plus en plus encapsulées dans le but de les véhiculer dans un état actif au niveau de leur site d'action. Dans cette étude, un procédé d'encapsulation breveté a été utilisé afin de protéger LGG dans des matrices laitières. Le mécanisme de microencapsulation a été étudié à l'échelle moléculaire en comparant les taux d'encapsulation de la souche sauvage et trois de ses mutants par AFM. Une diminution significative du taux d'encapsulation a été observée lorsque la bactérie ne possède pas de pili alors que le pili semble être décisif dans la localisation des bactéries dans les microparticules. Les mutants *spaCBA* (absence de pili), *welE* (absence des longs EPS) et *dltD* (LTA modifiés) ont été utilisés. L'AFM a permis de confirmer l'existence d'interactions spécifiques entre les bactéries et les protéines solubles à l'inverse des interactions non-spécifiques observées avec les caséines. Le rôle du pili a également été décrit en utilisant les modèles WLC et FJC. Ainsi, les mécanismes moléculaires de la microencapsulation des bactéries probiotiques devraient, à terme, permettre leur application ciblée.

5.1. Introduction

The concept that probiotic yoghurt and other fermented milk products can improve digestion, boost immunity and provide other health benefits are long claimed by food companies and modern consumers increasingly adhere to this trend (Burgain et al., 2011). Probiotic microorganisms used in functional foods are generally members of the genera *Lactobacillus* and *Bifidobacterium*. Probiotic bacteria are defined as "live microorganisms, which when administrated in adequate amounts can provide a health benefit on the host" (FAO/WHO, 2002). However, to exert these beneficial effects, probiotic bacteria must maintain their viability first during storage and then during their passage through the upper GI tract in order to arrive in the intestine in a viable state (Burgain et al., 2011). Functional foods containing probiotics are also increasingly subject to regulation by regulatory authorities, such as the EFSA, to ascertain that the probiotic health claims are based on solid scientific evidence, both with respect to the characterization of the probiotic microorganism and human intervention studies to validate health benefits. In this sense, the design of biopolymer based microparticles to encapsulate, protect and release probiotic bacteria at the required site of action is now gaining interest (Matalanis et al., 2011). Milk proteins can be used as an encapsulating carrier in order to produce this kind of delivery system (Doherty et al., 2011, Heidebach et al., 2009a, Livney, 2010). In most cases, microencapsulation with dairy proteins led to an increase in bacterial survival during digestion. However, even if in some cases the bacterial location into the microparticle was observed, the mechanisms behind the interaction between the probiotic bacteria and the matrix were never studied.

To advance the targeted microencapsulation of probiotic bacteria by milk proteins, it is of importance to investigate the specific interaction of bacterial surface molecules with the dairy matrices. It is generally known that the adhesive nature of bacteria is mainly due to cell surface structures consisting of proteins such as pili, and polysaccharides such as EPS and that both long range forces (steric and electrostatic interactions) and short range forces (Van der Waals, acid-base, hydrogen bonding and biospecific interactions) are involved, but –to the best of our knowledge- such adhesion studies have not yet been performed in relation to probiotic microencapsulation.

The present study aimed to investigate how changes in bacterial surface can influence the encapsulation efficiency of probiotics in dairy matrices, by combining detailed genetic methods with surface mutants and AFM. Hereto, LGG was chosen as

model probiotic organism, because it is one of the most intensively studied probiotic strain worldwide. It has most of the characteristics generally desired for a good probiotic bacterium including the ability to colonize the human GI tract (Goldin et al., 1992). LGG has been shown to promote human health by reducing the risk of nosocomial rotavirus-related diarrhea in infants (Szajewska et al., 2001), shortening the duration of acute diarrhea (Szajewska et al., 2007), reducing upper respiratory tract infections among children in day care (Hojsak et al., 2010), decreasing the risk of developing atopic eczema (Kalliomäki et al., 2007), alleviating the symptoms of eczema (Isolauri et al., 2000) and decreasing the risk of dental caries in children (Näse et al., 2001). Recently, various molecular genetic studies have started to unravel the molecular determinants of its peculiar probiotic characteristics (Lebeer et al., 2012b, Lebeer et al., 2011, Lebeer et al., 2009, Lebeer et al., 2008, Claes et al., 2012) and have indicated crucial roles for surface molecules such as *spaCBA*-encoded pili, long galactose-rich EPS molecules and LTA in host interactions.

5.2. Industrial efficiency

Microencapsulation efficiency. Microencapsulation of LGG wt probiotic strain and three well-characterized surface mutants was realized in a dairy protein matrix composed of 90 % micellar casein, 6 % native whey proteins and 4 % denatured whey proteins (Figure 66A). An excellent encapsulation rate of 175 ± 48 % with a final microparticles content of 10^{11} CFU.g^{-1} was obtained for LGG wt. On the contrary, the microencapsulation of the pili-deficient mutant into the same matrix revealed a low encapsulation rate of 1.5 ± 2.6 % with a final concentration of the bacteria in the microparticles of only 10^{9} CFU/g. The EPS-deficient mutant expressed the best encapsulation rate of 559 ± 90 %, while the LTA mutant presented intermediate encapsulation efficiency with 248 ± 102 %. After microencapsulation, the freeze-dried microparticles were observed by TEM (Figure 66B).

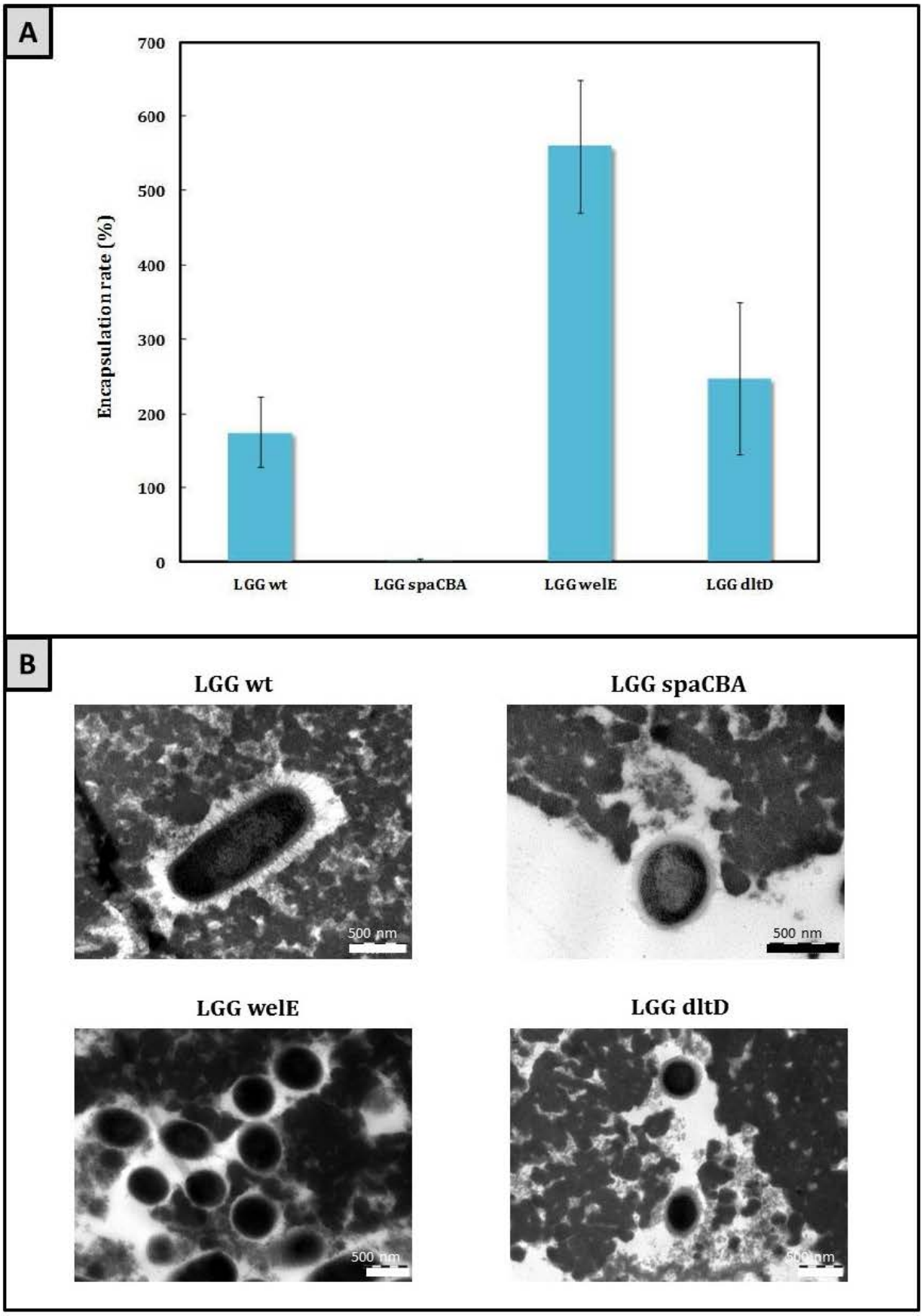


Figure 66 : Encapsulation rate observed for LGG wt and three mutants (mean ± SD, n=3) and microparticle TEM images for each encapsulated bacteria (B).

High encapsulation efficiency was correlated with a good entrapment of the bacteria into the microparticles. For the pili-deficient mutant (lower encapsulation rate), most of bacteria were observed at the microparticles surface or just under. The EPS mutant was particular as almost all bacteria are found as cluster inside the microparticle, in contrast to the wild-type strain or the LTA mutant where individual bacteria were mainly observed. These measurements with isogenic mutants affected in specific surface molecules provided the first indication that the bacterial surface plays a fundamental role in microencapsulation and that the fact whether specific molecular interactions could be established or not may seriously affect the ability of probiotic bacteria to be retained in the matrix.

5.3. General forces measurements

Force measurements between caseins or whey proteins with bacteria were performed in liquid (PBS, pH 6.8). During retraction of the tip from the surface, the force curve often showed a non-linear elongation reflecting stretching of flexible molecules either on the tip or on the mica surface. On Figure 67A, it is clearly observed that only few adhesive events occurred between micellar casein and the bacteria whatever the strain (wild type or its three mutants). On the contrary, a lot of adhesive events were observed between the bacteria and whey proteins. Therefore, bacterial interaction with whey proteins presenting strong adhesive events were further studied into detail. Interestingly, no adhesive event was recorded for the pili mutant even with the whey proteins (Figure 67B). This mutant does not express long flexible adhesive SpaCBA pili on its surface, which was previously also related to a significantly reduced adhesion capacity to human mucus, intestinal epithelial cells and biofilm formation (Lebeer et al., 2012b).

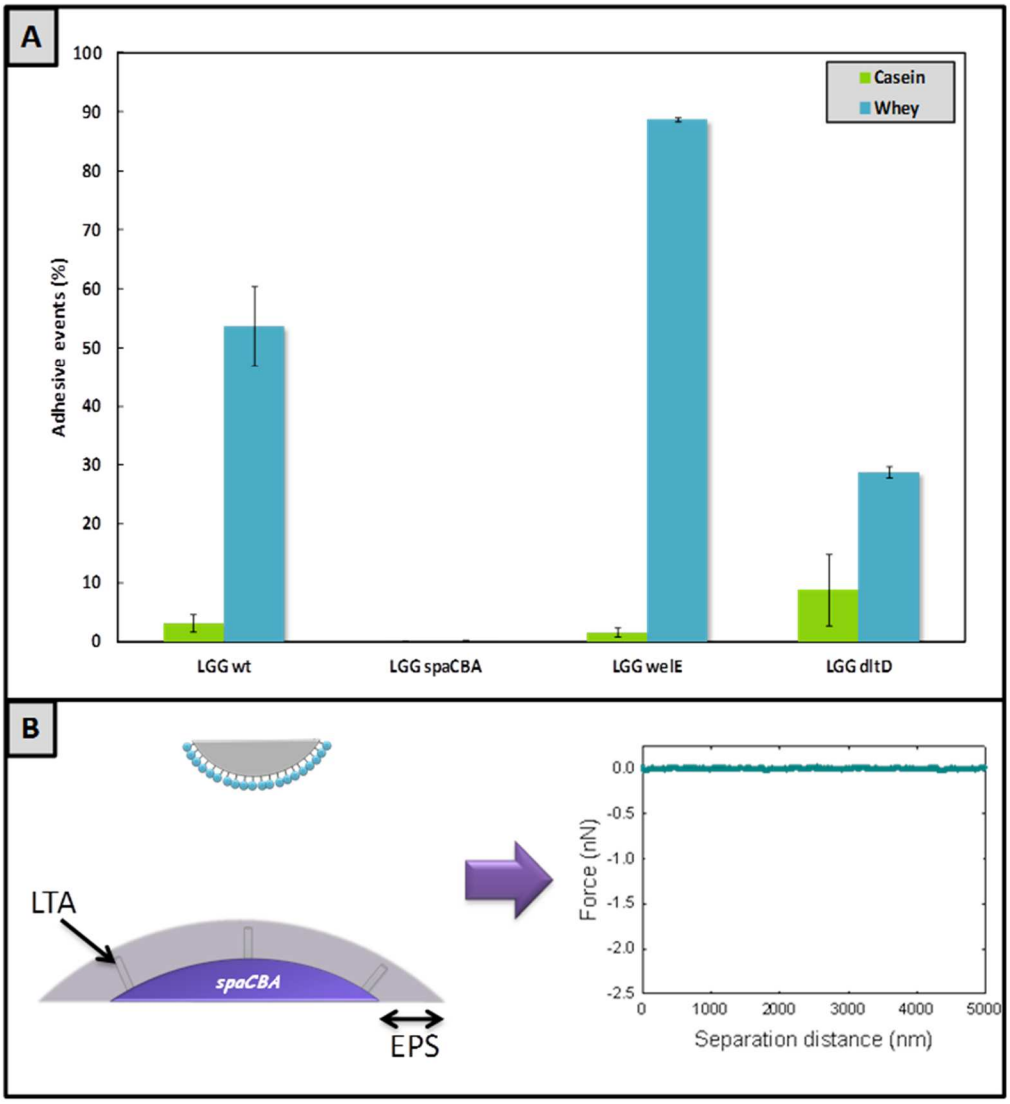


Figure 67 : Adhesive events between dairy proteins (casein or whey proteins) and bacteria: LGG wt and three surface mutants (A); Retraction curve obtained for force measurements between whey protein-coated probe and the spaCBA mutant, including a model for the postulated interaction (B).

5.4. Specific forces exploration between whey proteins and LGG strains

LGG wild type. Analysis of adhesion forces between the whey protein-coated probe and LGG wt was first performed. This strain is known to possess a thick shell constituted by EPS and to express the SpaCBA pilus (Kankainen et al., 2009, Lebeer et al., 2009) as represented in the Figure 68A. The number of ruptures observed during the retraction of the protein-modified tip from the sample varied between 0 and 10 ruptures, with a mean of around 4 ruptures (Figure 68B). A wide distribution was found for both Kuhn and persistence lengths. So, the values were about 0.030 ± 0.001 nm and 0.417 ± 0.024 nm for the FJC model. The second value could be attributed to the size of a

disaccharide molecule (Marszalek and Dufrene, 2012, Li et al., 1999, Marszalek et al., 2002, Rief and Grubmüller, 2002, Rief et al., 1997). Then, three different values of persistence length with the WLC model that describe the behavior of polypeptides molecules under a pulling force were found. The first value was about 0.022 ± 0.001 nm, while 0.193 ± 0.013 nm was found for the second and 0.360 ± 0.023 nm for the last peak (Figure 68C). In this case, the second and third values were found close to amino acids size or α-helix rise per amino acids that are in the range of 0.15 to 0.60 nm (Gräter et al., 2008, Ainavarapu et al., 2007, Tobi and Elber, 2000). For both models, a short length of about 0.02 to 0.03 nm was calculated that probably reflected the high flexibility of the macromolecules. It can be seen that adhesion between LGG wt and whey proteins revealed a contour length of maximum 3 µm with a mean of 430 ± 30 nm (Figure 68D). Rupture forces were determined on the last adhesive event that occurred during the retraction of the tip from the sample (Figure 68E) and was of 0.47 ± 0.01 nN (mean ± SD; total number of force curves = 1024). Representative retraction curves with the fitted model FJC (Figure 68F) or WLC (Figure 68G) are presented. Most of the force curves can be fitted by the two models according to closed values of the fitting parameter. This is a strong indication that most of the detected macromolecules exhibit polypeptidic and polysaccharidic behavior and implies that most of these molecules are glycoproteins (Dupres et al., 2009). A number of ruptures can be observed on these retraction curves that can be the result of multiple contact points between whey proteins and biopolymers located on bacterial surface.

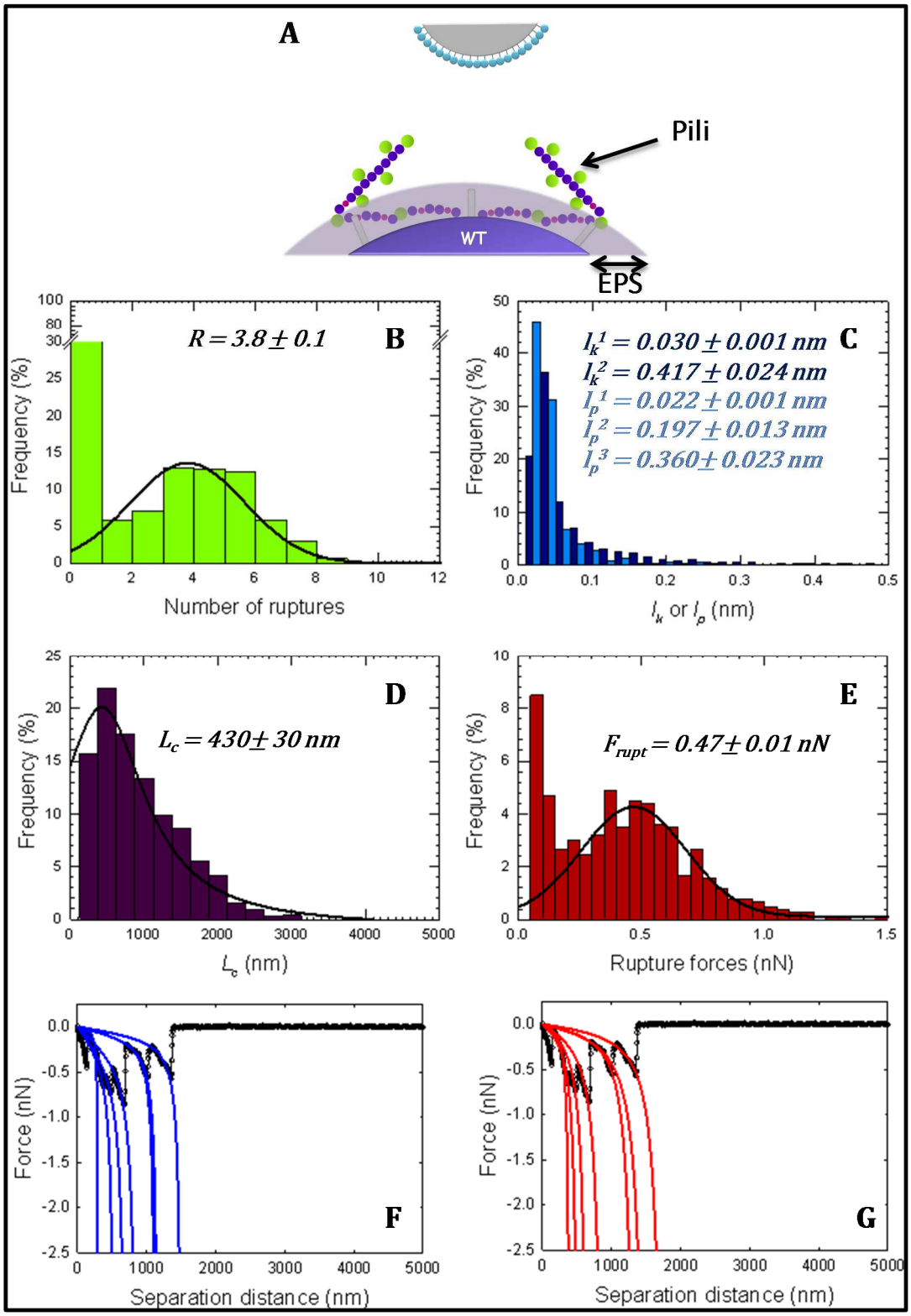


Figure 68 : Interactions were explored by measuring forces between protein coated probe and LGG wt (A). Number of ruptures occurring during retraction step after interaction between whey proteins and the LGG wt strain (B); Kuhn length (lk for FJC) and Persistence length (lp for WLC) as a function of model (C); Contour length (Lc) (D) ; Rupture forces (E); Retraction curve fitted with the FJC model (F), or the WLC model (G).

EPS *mutant.* The analysis of adhesion forces between the whey protein-coated probe and the EPS mutant was then performed. This mutant does not possess long galactose-rich EPS, but express the SpaCBA pilus (Lebeer et al., 2009) as shown in Figure 69A. Since the shell constituted by EPS present for LGG wt is no longer present, the pili are more exposed in this mutant. The number of ruptures observed during the retraction of the protein-modified tip from the sample varied between 0 and 12 ruptures, with a mean of around 6 ruptures (Figure 69B). The Kuhn length was of 0.053 ± 0.002 nm whereas the persistence length was of 0.030 ± 0.001 nm (Figure 69C). It can be seen that adhesion between the EPS mutant and whey proteins revealed a contour length of maximum 5 µm with two maxima, the first at 574 ± 150 nm and the second one at 1577 ± 46 nm (Figure 69D). Rupture forces were determined on the last adhesive event that occurred during the retraction of the tip from the sample (Figure 69E) and was of 0.41 ± 0.03 nN (mean ± SD; total number of force curves = 1024). Representative retraction curves with the fitted model FJC (Figure 69F) or WLC (Figure 69G) are presented. The FJC model presented a better fit below 1000 nm while both models can fit peaks up to 1000 nm.

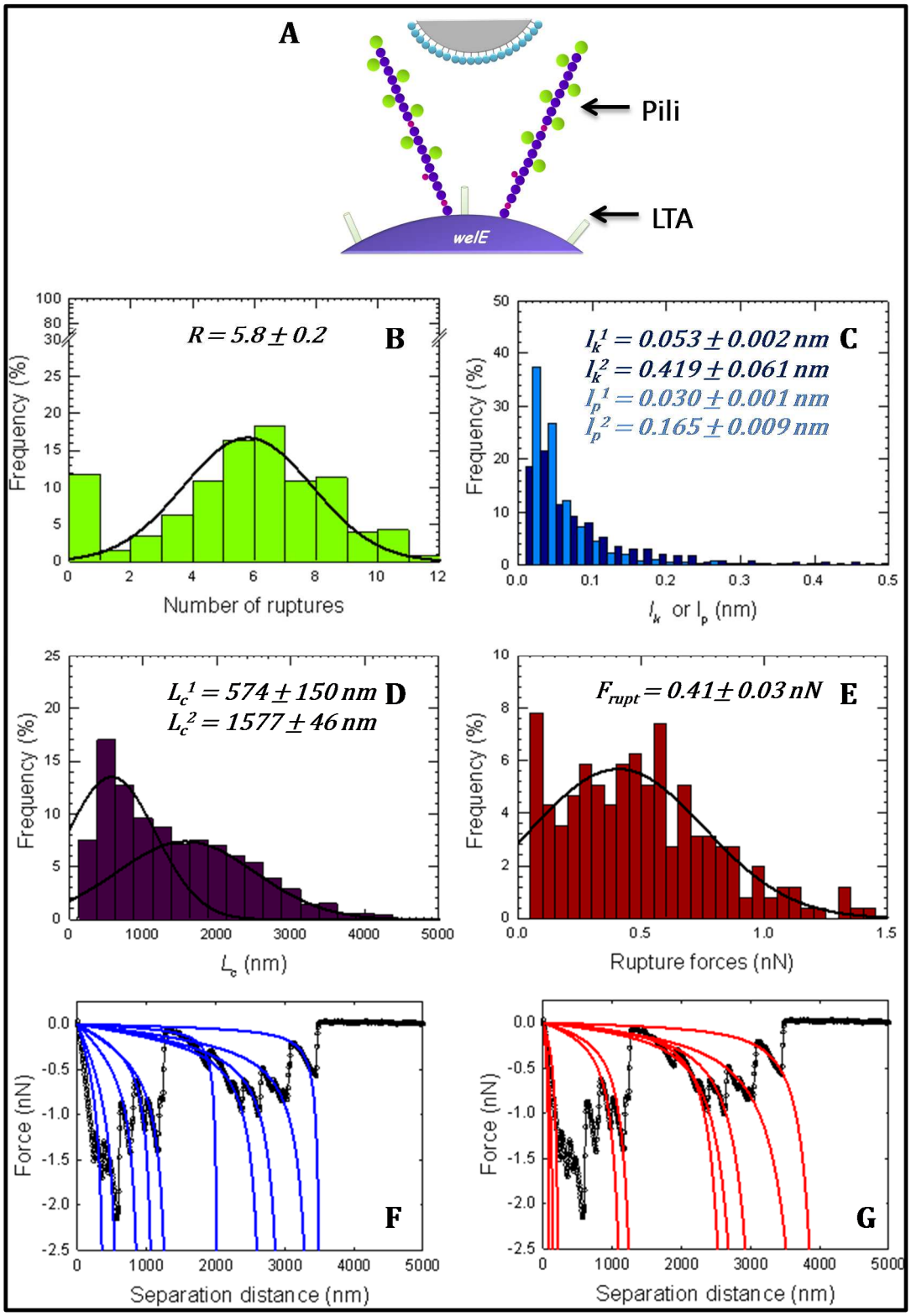


Figure 69 : Interactions were explored by measuring forces between protein coated probe and the welE mutant (A). Number of ruptures occurring during retraction step after interaction between whey proteins and the *welE* mutant (B); Kuhn length (lk for FJC) and Persistence length (lp for WLC) as a function of model (C); Contour length (Lc) (D); Rupture forces (E); Retraction curve fitted with the FJC model (F), or the WLC model (G).

LTA *mutant.* The analysis of adhesion forces between the whey protein-coated probe and the *dltD* mutant, presenting a modified LTA but possessing the EPS shell and expressing the SpaCBA pilus (Vélez et al., 2007), is presented in Figure 70A. The number of ruptures observed during the retraction of the protein-modified tip from the sample varied between 0 and 8 ruptures with a mean of around 3 ruptures (Figure 70B). The Kuhn length was of around 0.032 ± 0.001 nm whereas the Persistence length was of 0.024 ± 0.001 nm (Figure 70C). It can be seen that adhesion between the LTA mutant and whey proteins revealed a contour length of maximum 3µm with two maxima, the first at 593 ± 32 nm and the second one at 1784 ± 6 nm (Figure 70D). Rupture forces were determined on the last adhesive event that occurred during the retraction of the tip from the sample (Figure 70E) and was of 0.49 ± 0.03 nN (mean ± SD; total number of force curves = 1024). Representative retraction curves with the fitted model FJC (Figure 70F) or WLC (Figure 70G) are presented. For this mutant the peaks were well fitted by both models, presenting adhesive events with whey proteins for a maximum of around 1000 nm.

Pili *mutant.* As already mentioned, the analysis of adhesion forces between the whey protein-coated probe and the *spaCBA* mutant, which does not express the adhesive SpaCBA pili on its surface, is presented in Figure 67B. As no adhesive event occur the number of ruptures, Persistence and Kuhn lengths were not determined.

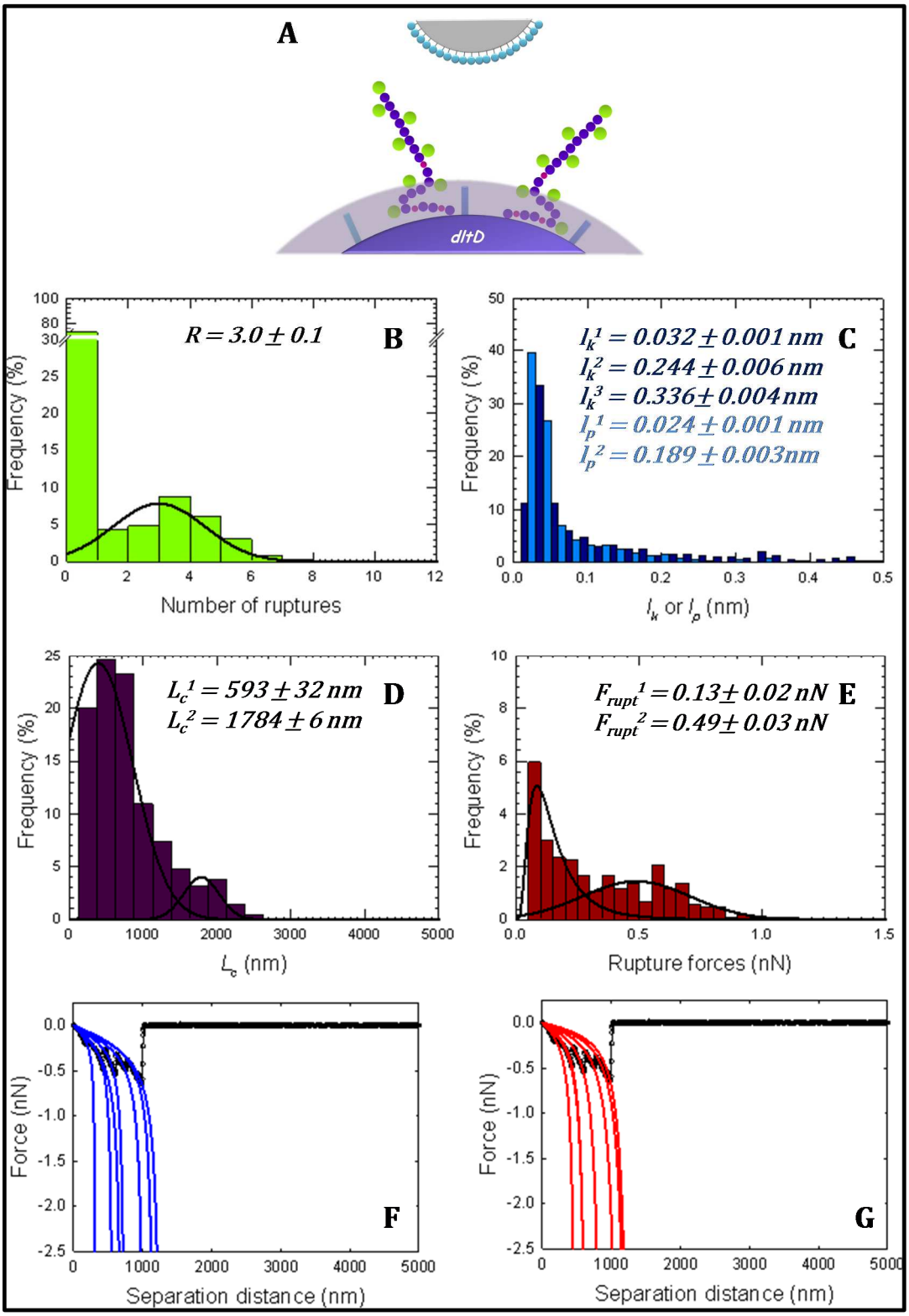


Figure 70 : Interactions were explored by measuring forces between protein coated probe and the dltD mutant (A). Number of ruptures occurring during retraction step after interaction between whey proteins and the *dltD* mutant (B); Kuhn length (lk for FJC) and Persistence length (lp for WLC) as a function of model (C); Contour length (Lc) (D); Rupture forces (E); Retraction curve fitted with the FJC model (F), or the WLC model (G).

5.5. Discussion

Microencapsulation of LGG wt probiotic strain and three of its surface mutants was realized in a dairy matrix composed of 90 % micellar casein and 10 % semi-denatured whey proteins, a formulation that was optimized previously (Burgain et al., 2013a). The whey fraction contains a part of remaining native whey since the applied heating is not enough to totally denature the proteins. This can be explained by the fact that β-lactoglobulin does not unfold entirely upon heating. Even if major unfolding takes place at temperatures around 75 °C, a second unfolding can be observed at approximately 130 °C. In particular, structures stabilized at 75 °C by disulfide bridges are retained and could explain the observed heat resistance (Wit and Fox, 1989).

Encapsulation rates up to 100 % were obtained for LGG wt, the EPS and LTA mutants. Because of their bacterial surface properties, probiotic bacteria tend to form aggregates (25 ± 4 cells per aggregate) *via* homophilic adhesion (Tripathi et al., 2013). During the microencapsulation procedure, shear forces lead to partial separation of aggregates, so that the number of CFU observed was higher after encapsulation than before. This is particularly the case for the EPS mutant where the bacteria remain grouped inside the microparticle (Figure 66B) and that is the reason why an encapsulation rate of 559 % was observed. The breakdown of *Lactobacillus* chains was already mentioned by others for an encapsulation method requiring an homogenization step. Given these differences in terms of encapsulation efficiency, bacterial interactions with dairy proteins were explored in order to attempt to give an explanation on the observed phenomena. In a previous study, the authors demonstrated non-specific adhesion events between LGG strain with micellar casein opposite to specific adhesion that can occur between the bacteria and whey proteins (Burgain et al., 2013b).

However, the bacteria were attached to the tip and proteins immobilized on mica. In the present work, the contrary was done, *i.e.* bacteria were immobilized onto functionalized mica and proteins onto functionalized colloidal tips. With this configuration, the contact surface between bacteria and proteins is increased in order to identify which molecule on the bacterial surface can establish interactions with the proteins. For this purpose, adhesion forces between bacteria and milk proteins (micellar casein and whey proteins) were estimated. The non-specific adhesion between the bacteria and caseins, and the specific one with whey proteins was confirmed. The fact that only few adhesive events can occur between micellar casein and the bacteria was

explained by the casein assembly in bovine milk: the four caseins (α_{s1}- α_{s2}- β- κ-caseins) form a structure called micelle, which has no well-defined tertiary structure. Due to this unique casein micelle structure, the inner part is not accessible for large molecules (Corredig et al., 2011). On the contrary, whey proteins have a defined tertiary structure accessible to biomolecules (Corredig et al., 2011). These differences in the tertiary structure of the proteins probably explain why specific interactions can be established between bacterial surface molecules with whey proteins and not with casein micelles.
Adhesive events that can be established between bacteria and whey proteins are well described by both FJC and WLC models. This behavior reflects that the molecules involved in interactions are glycosylated proteins. Some of already known molecules can be responsible for the observed stretching. For example, glycoproteins (Lebeer et al., 2012a) are secreted by LGG but also the pilus is a putative glycosylated protein.

The *spaCBA* mutant used in this work does not express the SpaCBA pilus (Lebeer et al., 2012b). Here, it was shown for the first time that the absence of that molecule on bacterial surface lead to a lack of interaction with dairy proteins even with whey proteins. The presence of pili on bacterial surface may be involved in adhesion with whey proteins and stretched during the retraction step. The presence of multiple pili on LGG was recently discovered. They are predominantly located near the cell poles and average 10-50 per cell with lengths up to 1 µm (Kankainen et al., 2009). The LGG pili are made of three pilin subunits. The major one is SpaA, this component build up the pilus shaft, and two other minor subunits, SpaB and SpaC, are also found (Kankainen et al., 2009). The SpaC subunit is an 895 residue, protein which has a central role for bacterial adhesion to human mucus and intestinal epithelial cells. The 3D structure of SpaC is not available yet, but some general structural features are known from the pilin structures of Gram-positive bacterial pili. In particular, domains constituted of β-strands decorated with inserted helices, strands and loops are encountered (Kang and Baker, 2012).

The pilus presents a length of 1 ± 0.3 µm (Tripathi et al., 2012), which is three times smaller than the contour length observed for adhesion between LGG wt and whey proteins. This can be explained by the fact that bacterial cells were subjected to centrifugation leading to the formation of bundles stabilized by strong lateral interpili interactions. Because the SpaC subunit is not only located at the pilus tip but also along the pilus shaft, they can establish several contact points with whey proteins leading to various rupture length below 3 µm. These interactions could be the result of zipper-like

adhesion mechanism as it is already known to operate for adherence to cell surfaces (Tripathi et al., 2013). In fact, it seems that SpaC subunit of the pili has broad adhesion specificity (Tripathi et al., 2013).

When analyzing the adhesion forces between the EPS mutant and whey proteins, the same comment as for adhesion with LGG wt may be issued regarding the contour length that is up to the pilus length of 1 µm. Moreover, the contour length for the EPS-deficient mutant is extended compared to the wt strain. It was already observed that LGG bacterial mutant lacking hydrophilic EPS formed bundle-like assemblies that were more tightly packed (Tripathi et al., 2012). This also explains why only few bacteria were recovered as individual inside the microparticle (Figure 66B). The absence of EPS around the bacteria leads to an increased exposure of the pili that can establish higher number of contact points with whey proteins. The key role of pili in interactions with whey proteins was finally confirmed by use of the pili mutant (Figure 71).

Indeed, the absence of pili on the *spaCBA* mutant revealed a lack of adhesive events between the bacteria and whey proteins. On the contrary, when the pilus is expressed, adhesive events were shown to occur between the bacteria and whey proteins. The SpaC subunit may promote long-distance or intimate interaction with its environment (Kankainen et al., 2009). Non-specific interactions like hydrophobic interactions could be involved in pili-protein interactions as pili are known to be hydrophobic (Craig et al., 2004) and particularly, the two pilins SpaB and SpaC are rich in hydrophobic residues (Kankainen et al., 2009). SpaC can bind mucins (Kankainen et al., 2009, Reunanen et al., 2012, von Ossowski et al., 2011) which are the major protein component of the intestinal mucus covering the epithelium of the GI tract.

Of interest, oligosaccharides and glycoproteins from the whey fraction have been shown to possess epitopes that are similar to those on mucosal cells (Schwertmann et al., 1999), suggesting a similar pili-mediated molecular mechanism of adhesion of LGG to mucus and whey proteins.

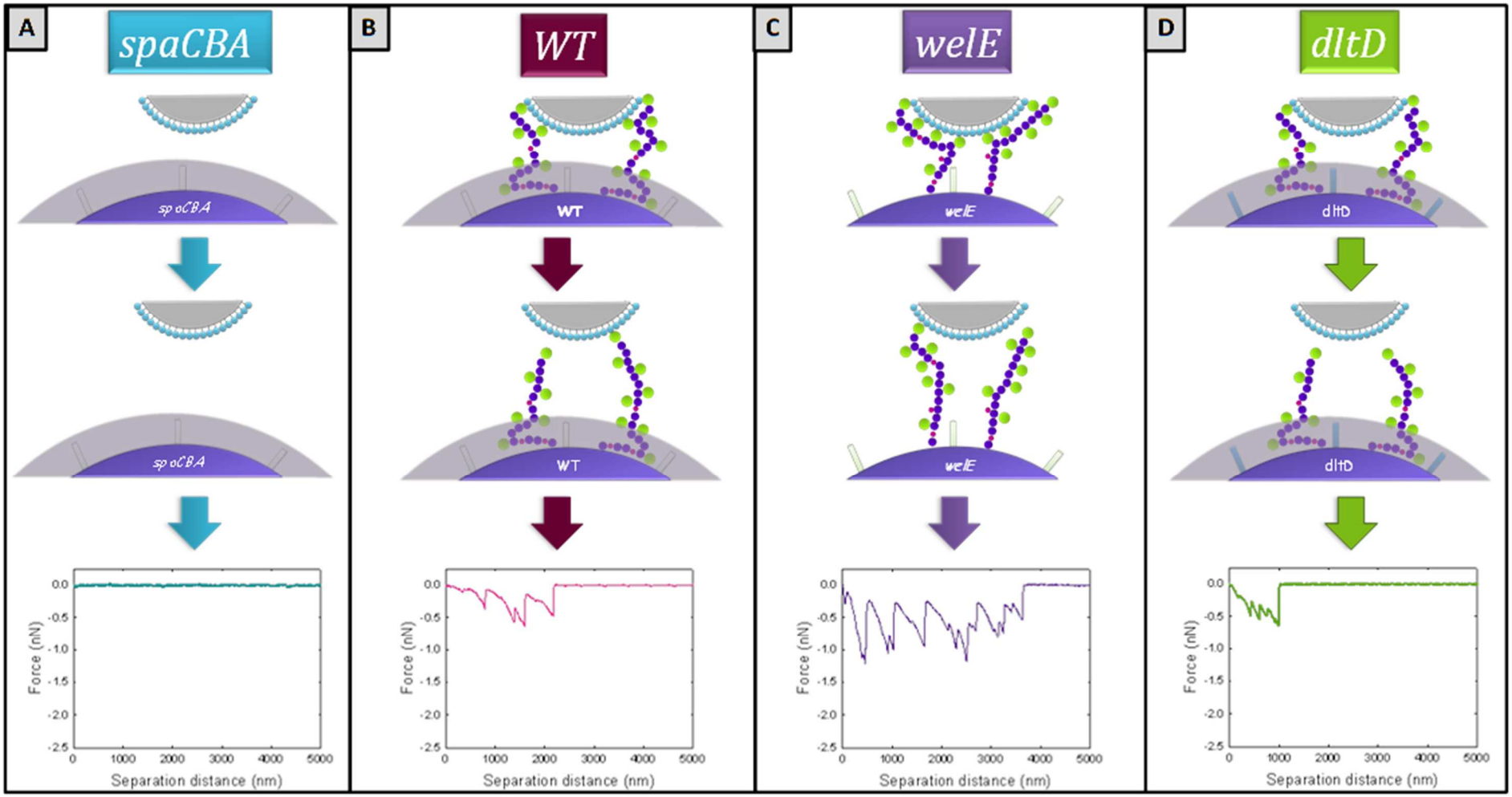


Figure 71 : Proposed mechanism of action: interaction between protein coated probe and *spaCBA* mutant (A), LGG wt (B), *welE* mutant (C) or *dltD* mutant (D).

5.6. Conclusion

The design of functional foods requires a good understanding of how individual components interact with others. For the first time, differences in encapsulation efficiency observed at the macroscale can be explained by adhesion mechanisms observed at the nanoscale. The high adhesion that exists between pili and whey proteins explains why the tested probiotic bacteria are better retained in the matrix during microencapsulation. This finding is of great importance as the microencapsulation of probiotic is increasingly studied and used for food industry. In order to optimize encapsulation efficiencies, the mastery of bacterial interaction with the encapsulation matrix is crucial. Because the use of encapsulated bacteria in the food industry is now slowed by its cost, providing new tools are of wide interest.

6. Conclusion du chapitre

Dans un premier temps, la mise en évidence des interactions non-spécifiques qui existent entre LGG ou LGR-1 et les caséines a pu être expliquée par la structure même de ces protéines (absence de structures secondaires). A l'inverse, les protéines solubles qui possèdent des structures secondaires bien établies peuvent quant à elles interagir spécifiquement avec ces bactéries.

Dans un second temps, la recherche plus précise de la nature des molécules bactériennes impliquées dans ces interactions a permis de mettre en évidence le rôle prépondérant du pili. Ces longs filaments protéiques possèdent des motifs qui sont habituellement engagés dans des phénomènes de reconnaissance spécifique au niveau des cellules intestinales. Dans notre cas, il semblerait qu'un tel motif permette une interaction spécifique avec les protéines dont l'énergie est supérieure à celle qui maintient les structures secondaires des biomolécules. En effet, lors du retrait de la pointe AFM, on a pu constater que des structures secondaires (hélices α par exemple) sont dans un premier temps rompues jusqu'à ce que le lien entre les bactéries et les protéines soit lui-même affecté.

Chapitre 5 :

Conclusion générale et perspectives

La mise au point d'un système d'encapsulation par émulsification à l'échelle laboratoire a permis d'étudier les différentes étapes inhérentes à ce type de procédé. Dans le processus de coagulation des caséines, le découplage de la phase enzymatique primaire et de la phase non-enzymatique secondaire a permis la production d'une émulsion stable sous agitation. La mise en œuvre d'une rampe de température permet de figer cette émulsion, c'est-à-dire de coaguler les gouttelettes constituées lors de l'émulsification. Il est ainsi obtenu des microparticules de protéines laitières coagulées contenant les bactéries probiotiques.

Les microparticules produites grâce à ce dispositif sont de type matriciel c'est-à-dire que les bactéries probiotiques sont dispersées dans une matrice qui est dans ce cas, uniquement composée de protéines laitières. Le dimensionnement de ce procédé à l'échelle pilote a permis une plus grande production de microparticules d'une part, mais surtout la possibilité d'une extrapolation à l'échelle industrielle. Le choix d'un matériel adapté et la sélection des conditions de fonctionnement idéales ont permis la microencapsulation de bactéries probiotiques de façon maitrisée sans endommager les cellules par le procédé : forts taux de cisaillement, température trop élevée... Toute cette mise au point a fait l'objet de nombreux essais et des compromis ont dû être formulés afin que le résultat soit au plus proche de nos attentes en termes de taille et de forme des microparticules, de résistance dans des environnements hostiles...

Que ce soit à l'échelle laboratoire ou à l'échelle pilote, la distribution de taille des microparticules couvre une large gamme du fait de la méthode par émulsification employée. Toutefois, le diamètre moyen est inférieur à 100 µm aux deux échelles. Les microparticules sont molles et de forme sphérique ce qui est en faveur d'une diminution de la perceptibilité par le consommateur.

L'extrapolation du procédé vers l'échelle industrielle a été initiée dans le cadre d'une similitude géométrique. Néanmoins, il est probable que d'autres similitudes nécessitent une nouvelle expérimentation. Ce serait le cas, par exemple, d'un industriel souhaitant réaliser le procédé dans une cuve qu'il possède déjà.

La recherche d'une formulation optimale pour encapsuler LGG a permis de clarifier l'importance de la nature et des quantités de protéines utilisées dans la matrice. Il apparait ainsi que la nature et les proportions des protéines utilisées influencent aussi

bien le taux d'encapsulation que le taux de survie des bactéries dans des conditions gastriques.

A ce propos, une matrice composée uniquement de caséines ne semble pas être optimale pour l'encapsulation de la souche LGG. A l'inverse, un mélange de 90 % de caséines et 10 % de protéines solubles dénaturées semble être une excellente solution. Ceci est expliqué par la nature du gel produit : le gel issu d'un mélange caséines-protéines solubles dénaturées est plus dense que le gel produit uniquement à partir de caséines. Plus le gel sera dense, plus il sera protecteur de la substance encapsulée lors d'une digestion gastrique.

Il a été observé une localisation préférentielle des bactéries à la surface des microparticules lorsque seules des caséines sont utilisées. Lorsque des protéines solubles sont ajoutées aux caséines, les bactéries sont bien localisées à l'intérieur des matrices. Ces observations mettent en évidence l'importance d'une étape clé lors de l'encapsulation. Il s'agit du moment où les bactéries et les protéines sont mises en contact.

L'analyse des interactions entre bactéries et protéines a été possible par la mise au point d'une étude utilisant la mesure de forces par AFM. Des pointes, ainsi que des micas, ont été fonctionnalisés et utilisés pour l'étude des interactions. Il est apparu d'abord que des interactions spécifiques pouvaient s'établir entre les bactéries et les protéines solubles alors que ce n'était pas le cas avec les caséines. Ces premières informations permettent d'expliquer la localisation préférentielle des bactéries dans les microparticules mais également les différences des taux d'encapsulation. Ces derniers sont significativement plus élevés après l'ajout de 10 % de protéines solubles dénaturées. Dans l'objectif d'approfondir l'étude des interactions, trois mutants de surface de LGG ont été utilisés et la mesure des forces par AFM a de nouveau été menée. La présence de pili à la surface de la bactérie apparait comme un élément déterminant. Ce dernier établit des interactions fortes et sur de longues distances avec les protéines solubles. Cette information a pu être reliée à l'échelle microscopique avec la localisation des bactéries dans des microparticules constituées de caséines et de protéines solubles. L'absence de pili a dans ce cas entrainé la localisation de nombreuses bactéries à la surface de la microparticule, mais c'est surtout son impact sur le taux d'encapsulation qui a été démontré. La majorité des bactéries (sans pili) n'ont pas du tout été retenue dans la matrice d'encapsulation.

Dans ce travail, les informations obtenues à l'échelle macroscopique (taux d'encapsulation et taux de survie dans l'estomac par exemple) ont pu être reliées à des données microscopiques telles que la localisation des bactéries dans les microparticules. Tout ceci a finalement pu être expliqué par une étude à l'échelle moléculaire. On peut donc conclure qu'une étude multi-échelle permet la description et la compréhension des phénomènes observés au cours de l'encapsulation ().

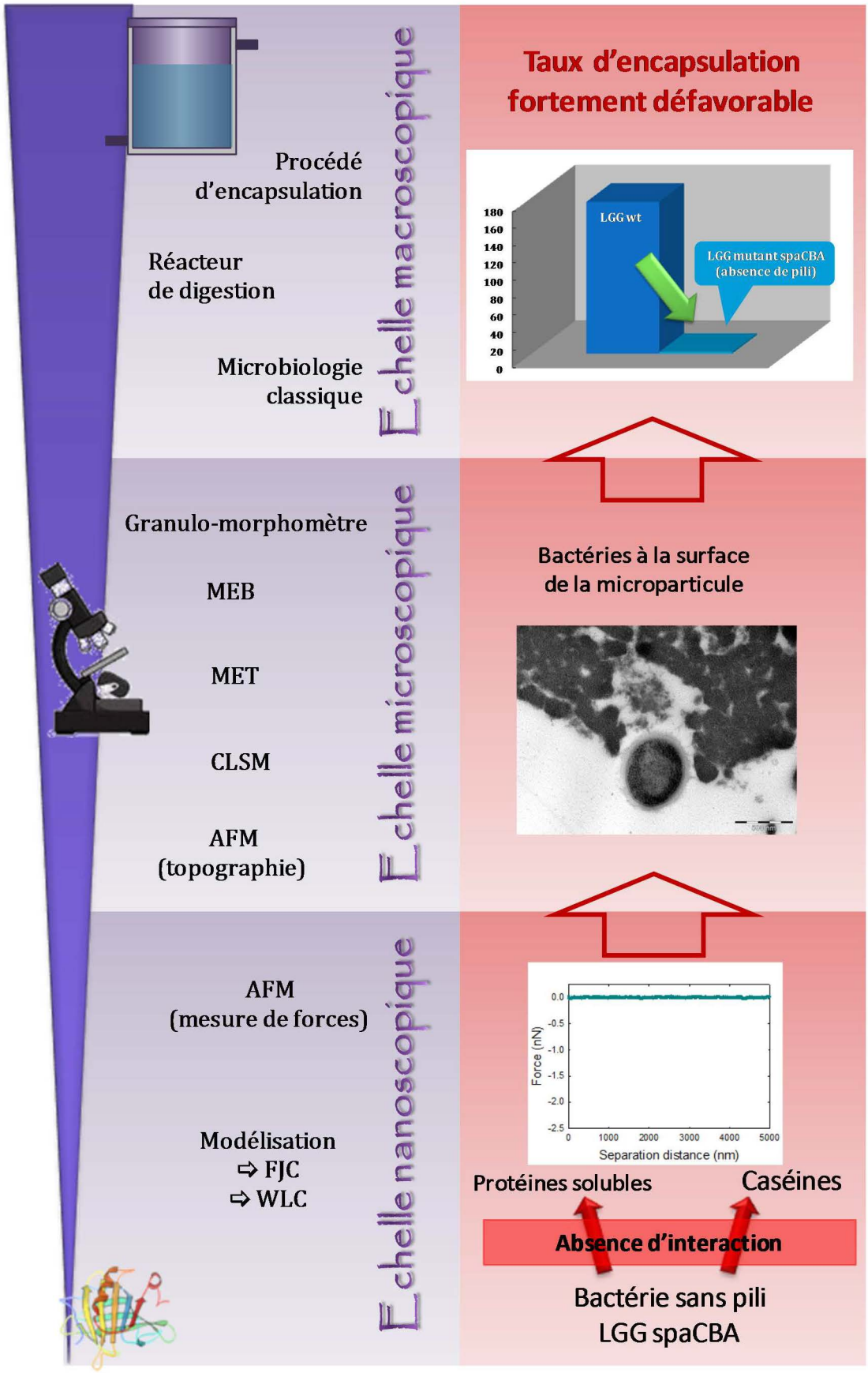
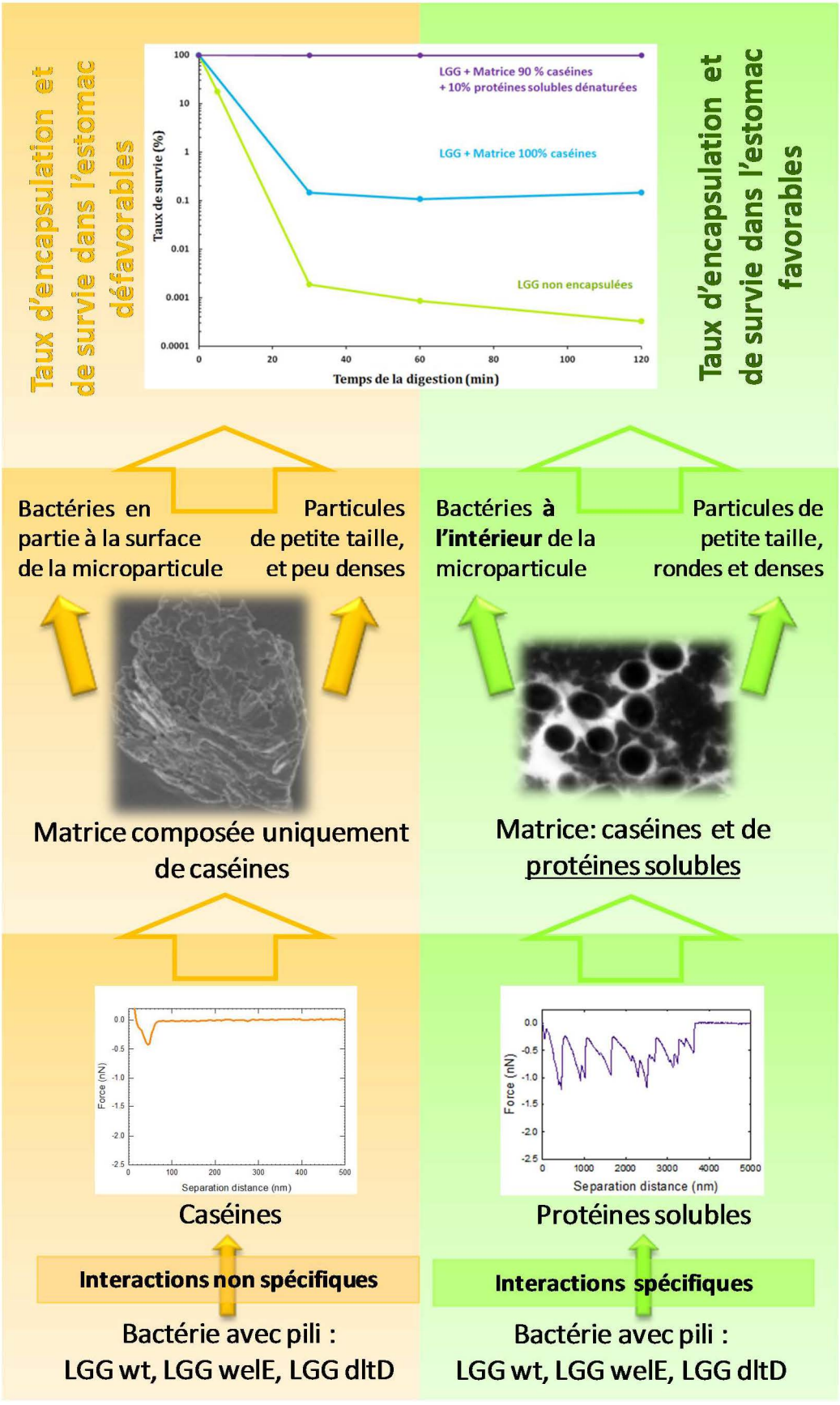


Figure 72 : Description des phénomènes observes au cours de l'encapsulation par une approche multi-échelle.


Taux d'encapsulation et de survie dans l'estomac défavorables
Taux de survie (%)
Temps de la digestion (min)
LGG + Matrice 90 % caséines
+ 10% protéines solubles dénaturées
LGG + Matrice 100% caséines
LGG non encapsulées
Taux d'encapsulation et de survie dans l'estomac favorables
Bactéries en partie à la surface de la microparticule
Particules de petite taille, et peu denses
Bactéries à l'intérieur de la microparticule
Particules de petite taille, rondes et denses
Matrice composée uniquement de caséines
Matrice: caséines et de protéines solubles
Force (nN)
Separation distance (nm)
Caséines
Protéines solubles
Interactions non spécifiques
Interactions spécifiques
Bactérie avec pili :
LGG wt, LGG welE, LGG dltD
Bactérie avec pili :
LGG wt, LGG welE, LGG dltD

Les perspectives de ce travail sont multiples et certaines d'entre elles vont être décrites par la suite.

(1) L'encapsulation par émulsification est une technique intéressante mais elle a l'inconvénient de produire une gamme de tailles de particules large ce qui n'est pas en faveur d'une libération ciblée. De même, la couche résiduelle d'huile à la surface des microparticules peut certes être un avantage dans la protection des bactéries envers des conditions défavorables mais elle peut également être nuisible que ce soit d'un point de vue organoleptique (oxydation des lipides) que d'un point de vue nutritionnel (profils nutritionnels pour l'obtention d'allégations). Il parait donc intéressant d'adapter le procédé à d'autres techniques comme la fluidisation par exemple.

Au cours de ce travail, seule l'étude de la microencapsulation de bactéries probiotiques a été présentée mais d'autres biomolécules méritent elles aussi d'être protégées (Tableau 25). Les matrices laitières produites ici sont intéressantes dans le sens où les microparticules produites sont insolubles dans des milieux aqueux. Elles permettent donc de véhiculer des composés bioactifs sensibles dans des milieux où la teneur en eau est élevée.

Tableau 25 : Exemples de composés bioactifs.

Composés bioactifs	Exemples
Composés phytochimiques	Flavonoïdes Catéchines Phytoestrogènes (isoflavones/lignanes) Anthocyanidines Tanins (proanthocyanidines) Lycopène (caroténoïde) Phytostérols (sitosterol/ stanol ester) Fibres (insolubles et solubles) β-Glucane (fibre) Lutéine (caroténoïde)
Lipides bioactifs	Acides gras ω-3 Acide linoléique conjugué
Protéines et peptides bioactifs	Lactoferrine Peptides bioactifs Enzymes (lactase…)
Prébiotiques	Fructo-oligosaccharides Sucres alcools
Bactéries probiotiques	Bactéries lactiques Bifidobactéries
Vitamines	Acide folique Tocophérols α-carotène, β-carotène
Minéraux	Calcium

Le procédé d'encapsulation présenté dans ce manuscrit a été également testé sur une biomolécule, la quercétine. La quercétine est un flavonoïde possédant des propriétés anti-oxydantes. Les taux d'encapsulation observés pour cette biomolécules ont varié entre 78 % et 86 %. Les microparticules produites sont de très petite taille (Figure 73).

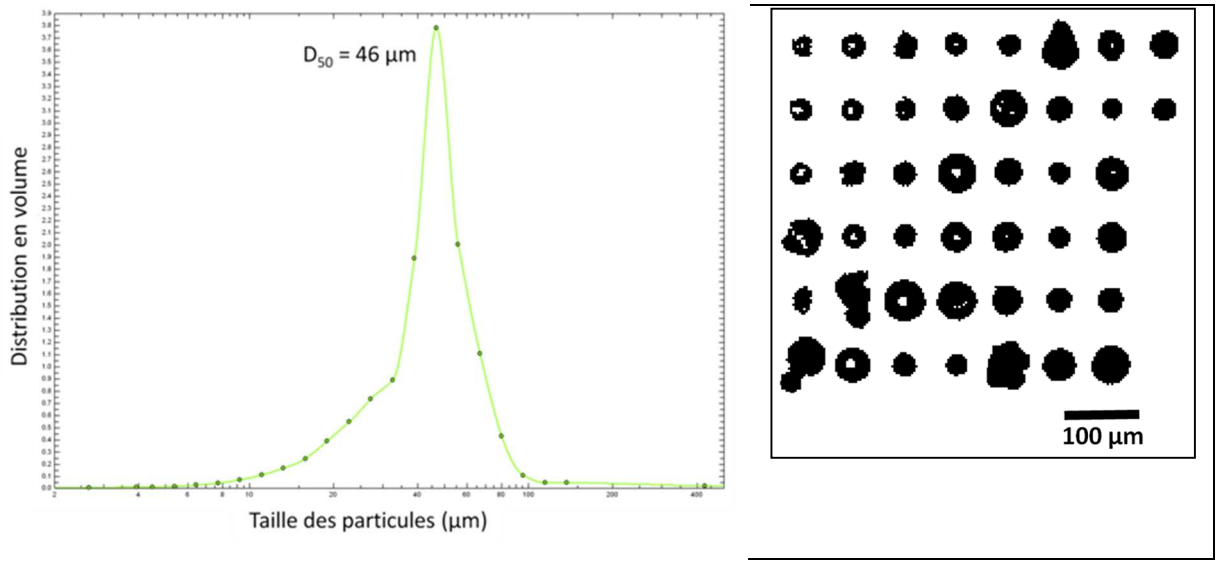


Figure 73 : Résultats pour la microencapsulation de quercétine.

On constate donc que le procédé développé est applicable à d'autres biomolécules mais qu'il nécessite tout de même une certaine adaptation, que ce soit dans la prise en compte de la nature de la molécule et donc du cisaillement imposé, de la taille des microparticules souhaitée ou encore de la formulation de la matrice encapsulante.

(2) La résistance des microparticules dans des conditions simulant celles de l'estomac a été prouvée. Toutefois, l'objectif final est la libération des bactéries dans un état viable et fonctionnel au niveau de l'intestin. Cette étape de passage des conditions stomacales vers les conditions intestinales a été testée montrant ainsi une dissociation des microparticules lors de la remontée du pH (pH de 6.8 pour l'intestin). La viabilité des bactéries a pu être vérifiée mais pas leur fonctionnalité. En effet, pour réaliser cette étude il est nécessaire de mettre en place une collaboration avec un laboratoire équipé pour ce type de manipulation. Vu l'importance des autres parties de la thèse, cet aspect n'a pas été abordé, faute de temps.

(3) L'influence de facteurs environnementaux comme le pH au cours de l'encapsulation peut également être étudié. En effet, en diminuant le pH, la charge de surface des bactéries et des protéines va être modifiée entrainant sans doute une modification dans leurs interactions. A ce propos, l'étude des forces par AFM a été initiée. Elle révèle que les interactions à un pH plus acide (pH 4.8) impliquent toujours le pili mais la longueur qui peut être étirée avant rupture de la liaison est plus faible. Le pH a donc probablement une influence sur la structure du pili.

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Annexes

PROCEDE D'ENCAPSULATION DE MOLECULES BIOACTIVES DANS DES MATRICES LAITIERES ET MICROPARTICULES OBTENUES

La présente invention a pour objet un procédé d'encapsulation de molécules bioactives dans des matrices laitières, les microparticules obtenues par ledit procédé et leur utilisation dans les domaines cosmétiques, alimentaires et pharmaceutiques.

Aujourd'hui les consommateurs s'intéressent de plus en plus aux aliments pouvant apporter un bénéfice sur leur santé. C'est pourquoi il y a un intérêt grandissant pour les produits à base de probiotiques.

En 2001, l'Organisation mondiale de la Santé (OMS) et l'Organisation des Nations unies pour l'alimentation et l'agriculture (FAO) ont donné une définition officielle des probiotiques qui sont des « micro-organismes vivants qui, lorsqu'ils sont ingérés en quantité suffisante, exercent des effets positifs sur la santé, au-delà des effets nutritionnels traditionnels ».

Ces micro-organismes vivants, bactéries ou levures, aident à la digestion des fibres, stimulent le système immunitaire, préviennent ou traitent la diarrhée, diminuent le cholestérol, améliorent la tolérance au lactose ou préviennent certains cancers. Toutefois ces effets sont fonction des souches utilisées.

Parmi les microorganismes utilisés comme probiotiques, on retrouve souvent

- des bactéries lactiques (LAB), notamment "Bifidobacterium spp." plus particulièrement les espèces *Bifidobacterium bifidum (bifidus), Bifidobacterium lactis, Bifidobacterium longum, Bifidobacterium breve, Lactobacillus spp.* plus particulièrement *Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum* et *Lactobacillus rhamnosus,*
- des bactéries non lactiques notamment *Escherichia coli* et
- des levures comme par exemple *Saccharomyces cerevisiae et Saccharomyces boulardii*.

Pour que les probiotiques aient un effet bénéfique sur la santé, il faut que plusieurs conditions soient réunies :

- qu'ils soient vivants (ou lyophilisés),
- que les bonnes souches soient sélectionnées pour l'effet recherché,
- que les souches aient montré leur résistance à l'acidité gastrique et à la bile,
- que les cures soient d'au moins 10 jours par mois et
- que la démonstration de leur bénéfice ait été faite tant chez l'être humain sain que chez le malade.

Surtout, pour être efficaces les probiotiques doivent impérativement parvenir vivants dans le côlon et en nombre suffisant. Ils ne doivent en effet pas être dégradés suite à leur passage dans l'estomac et doivent être capables de résister à l'acidité gastrique et au suc du pancréas.

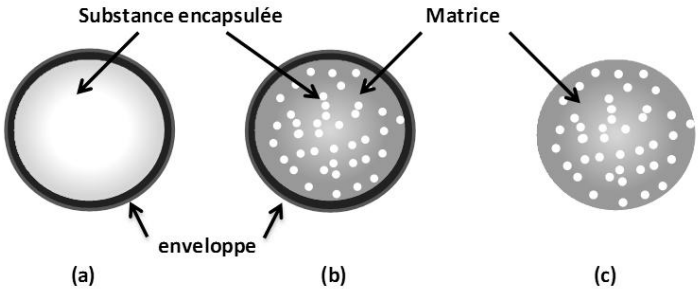
Des concentrations efficaces de probiotiques dans les aliments ont été définies, qui sont non seulement fonction du type de probiotique et du type d'aliment, mais aussi fonction du pays concerné. Ainsi un minimum de 10^6 UFC/g (Unité formant colonies/g) a été fixé pour les bifidobactéries dans les produits laitiers pour les pays de MERCOSUR (Argentine, Bolivie, Brésil, Chili, Paraguay et Uruguay), alors qu'au Japon un minimum de 10^7 UFC/g a été recommandé dans les produits laitiers frais. Au niveau européen, la règlementation a fixé des concentrations minimales de 10^9 UFC/g pour certains microorganismes utilisés en tant qu'additifs alimentaires.

Le maintien de la viabilité et de la fonctionnalité des bactéries probiotiques jusqu'à ce qu'elles atteignent le tube digestif est donc primordial.

Or, la survie des bactéries probiotiques libres introduites dans les produits alimentaires est faible en raison des conditions défavorables rencontrées pendant le stockage et le passage dans l'estomac.

C'est pourquoi la microencapsulation est depuis longtemps utilisée car c'est une technologie puissante qui permet de protéger les cellules bactériennes et d'augmenter la survie des bactéries probiotiques durant le stockage puis le transit gastrique.

C'est un processus physicochimique qui permet de piéger une substance ou une cellule vivante dans un matériau, afin de produire des microparticules ou des microcapsules dont le diamètre peut aller de quelques nanomètres à quelques millimètres. Ce procédé permet de former une barrière protectrice pour composés encapsulés comme les probiotiques. Les systèmes encapsulés peuvent être de différents types, soit de type réservoir (a), soit de type matrice enrobée (b), soit de type matriciel (c),



Cette technique est applicable non seulement aux probiotiques mais aussi à d'autres molécules bioactives fragiles qui doivent être protégées de l'environnement ou véhiculées vers une cible spécifique et ce, dans des domaines aussi diverses que, notamment, le domaine pharmaceutique, le domaine cosmétique, le domaine alimentaire et le domaine textile.

Dans le cas des probiotiques, les matériaux utilisés doivent être de qualité alimentaire. Il s'agit le plus souvent de polymères naturels, tels que l'alginate, les carraghénanes, la gomme gellane, le chitosan, la gélatine, l'amidon ou des protéines (notamment celles du lait). Les technologies utilisées pour l'encapsulation sont par exemple l'atomisation, l'émulsification ou l'extrusion (Burgain J. *et al.* Journal of Food Engineering, (2011), **104**, 467-483).

Parmi les matériaux utilisés pour encapsuler les probiotiques, les produits laitiers ont démontré un avantage qui pourrait être attribué au pouvoir tampon des protéines laitières et à leur capacité à former un réseau suite à la coagulation (Livney Y.D. in *Colloid & Interface Science,* 2010, **15**, 73-83). Par ailleurs, ces protéines sont autorisées, sans restriction par la législation, dans les produits alimentaires.

Heidebach T. *et al. (International Dairy Journal,* 2009, **19**, 77-84) décrivent une méthode d'encapsulation de cellules probiotiques dans des microcapsules de caséine de qualité alimentaire. Cette méthode, en circuit ouvert, est basée sur la gélification catalysée par une transaminase, de suspensions de caséine contenant des probiotiques. Seule l'huile de tournesol est utilisée comme matière grasse et c'est une encapsulation à l'échelle laboratoire, sur de petites quantités. Toutefois, cette méthode ne permet de produire que de très faibles quantités de microparticules à une échelle laboratoire.

Le brevet EP 1876905 décrit une méthode d'encapsulation d'acides gras sensibles à l'oxydation à l'aide d'une émulsion huile dans eau (l'huile étant la molécule bioactive et la phase aqueuse contenant des caséinates de sodium, la solution protéique) à des températures supérieures à 70 °C ; cette émulsion est ensuite séchée pour donner une matrice.

Les inventeurs ont également décrit un procédé à l'échelle du laboratoire qui met en œuvre des protéines laitières et montré qu'il était possible de réaliser une encapsulation de probiotiques avec de bons rendements ; les microcapsules obtenues permettent d'augmenter la survie des probiotiques durant le stockage ainsi que le transit gastrique (présentation orale au XIX International Conference on Bioencapsulation, Amboise, France, 5-8 octobre 2011). Toutefois ce procédé est réalisé en circuit ouvert, et en l'absence d'agitation de la phase enzymatique et n'est pas transposable à l'échelle industrielle.

En général, alors qu'au niveau du laboratoire les résultats sont prometteurs, les technologies mises en œuvre posent souvent des difficultés de mise à l'échelle et sont difficilement transposables à l'échelle industrielle.

En outre, réduire la taille des microparticules reste un défi majeur, car il faut éviter que les microparticules affectent la texture et les propriétés sensorielles d'un produit alimentaire, cosmétique ou pharmaceutique dans lequel elles sont incorporées.

Aussi la présente invention a-t-elle pour objectif de fournir un procédé d'encapsulation de molécules bioactives permettant une résistance à l'environnement gastrique, et une libération contrôlée au niveau d'une cible spécifique, notamment au niveau intestinal et permettant de disposer de particules dont la structure (taille, forme..) et les propriétés rhéologiques font qu'elles n'affectent pas la texture, ni les propriétés sensorielles des produits qui les contiennent. Ce procédé est utilisable à l'échelle industrielle, permet d'encapsuler tout type de molécules bioactives et met en œuvre une matrice composée de protéines de lait et de composants de qualité alimentaire.

La présente invention a également pour but de fournir des microparticules permettant d'augmenter la survie durant le stockage puis le transit gastrique des molécules bioactives qu'elles contiennent.

Aussi la présente invention a pour objet un procédé d'encapsulation de molécules bioactives par des protéines laitières choisies dans le groupe comprenant :

- de la caséine micellaire seule,

- de la caséine micellaire associée à des protéines laitières solubles natives,
- de la caséine micellaire associée à des protéines laitières solubles dénaturées, et
- de la caséine micellaire associée à des protéines laitières solubles natives et à des protéines laitières solubles dénaturées,

le dit procédé étant mis en œuvre en circuit fermé et comprenant les étapes suivantes :

a. la mise en contact d'une solution desdites protéines laitières présentant un pH compris entre 4,8 et 8 et contenant au moins une molécule bioactive à encapsuler, avec la chymosine, à une température comprise entre 1 et 10 °C, avantageusement 3 et 5 °C, jusqu'à ce qu'au moins 85 % du caséinomacropeptide soit clivé,

b. l'addition progressive de la solution protéique obtenue à l'étape a) à une matière grasse en présence d'un agent tensioactif à une température comprise entre 1 et 10 °C, avantageusement entre 3 et 5°C sous agitation comprise entre 100 et 1400 rpm, jusqu' à formation de gouttelettes et obtention d'une émulsion. La vitesse d'agitation à appliquer est fonction de la taille des particules souhaitée,

c. l'application d'une rampe de température allant de 1 à 60 °C, avantageusement de 5 à 40 °C, et

d. une phase de séparation des microparticules formées.

Au sens de la présente invention, on entend par caséine micellaire un assemblage de caséines α, β, et κ dont le diamètre moyen est de 120 nm. L'obtention des caséines micellaires se fait par des techniques de filtration membranaire du lait écrémé : microfiltration (pour purifier et ensuite séparer les caséines micellaires des protéines solubles, du lactose et des minéraux) et diafiltration (pour concentrer les caséines micellaires). Ces techniques sont connues de l'homme du métier.

Au sens de la présente invention, on entend par protéines laitières solubles natives: la β-lactoglobuline, l'α-lactalbumine, les immunoglobulines (10%), l'albumine de sérum bovin et la lactoferrine. L'obtention des protéines solubles se fait par des techniques de filtration membranaires du lait écrémé : microfiltration (pour purifier et ensuite séparer les protéines solubles, le lactose et les minéraux des caséines micellaires), une ultrafiltration tangentielle (pour éliminer les minéraux et le lactose) et finalement une diafiltration (pour concentrer les protéines solubles). Ces techniques sont connues de l'homme du métier.

Au sens de la présente invention, on entend par protéines laitières solubles dénaturées, les protéines obtenues par dénaturation des protéines laitières solubles natives, notamment par chauffage.

Au sens de la présente invention on entend par « circuit fermé » un système complètement isolé de l'extérieur, ce qui empêche toute contamination externe.

Selon la présente invention, la matière grasse peut être toute matière grasse classiquement utilisée pour des procédés d'encapsulation sous réserve qu'elle soit fluide aux températures auxquelles le procédé est mis en œuvre, c'est-à-dire entre 1 et 60 °C. Elle peut être de nature alimentaire pour des applications alimentaires ou de nature non alimentaire pour d'autres types d'applications (notamment textile).

La réaction de coagulation du lait peut être divisée en trois processus cinétiques principaux. Le premier est la réaction enzymatique qui consiste en une hydrolyse de la caséine-κ, le second est un processus non-enzymatique de floculation des micelles de caséine et le troisième consiste en la formation de liaisons au sein du gel.

La chymosine est une enzyme utilisée en fromagerie et est capable de libérer le caséinomacropeptide (CMP) par clivage de la liaison Phe105-Met106 de la caséine-K, située à la surface des micelles de caséine dans le lait. La libération de ce peptide entraîne une diminution de la charge nette négative et une augmentation de l'hydrophobicité de la para-K-caséine, ce qui va conduire à l'agrégation des micelles de caséine.

Toutefois, si la chymosine est mise au contact du lait à faible température, la caséine-κ est clivée mais les micelles ne coagulent pas ou très peu, et ce n'est qu'en augmentant la température que la formation du gel sera rendue possible. C'est ce découplage de la phase primaire enzymatique et de la phase secondaire non-enzymatique qui a été exploité pour la mise au point d'un système d'encapsulation où la coagulation a été déclenchée au moment adéquat par une augmentation de la température.

Conformément à l'invention, lors de l'étape a), le CMP est libéré de la partie C-terminale de κ-caséine suite à l'action de la chymosine. En effet, il est nécessaire que l'étape a) ait atteint ce stade pour que l'étape c), c'est-à-dire la coagulation des protéines suite à l'augmentation de la température, puisse débuter. L'étape a) est appelée phase enzymatique.

Conformément à l'invention, l'agitation minimale à l'étape b) doit permettre un cisaillement suffisant pour la formation de gouttelettes. En revanche il est impératif d'éviter la dénaturation des molécules bioactives à encapsuler suite à un trop fort cisaillement. Aussi, l'agitation maximale tient compte de la vulnérabilité des molécules bioactives face au cisaillement, de la viscosité de la matière grasse (elle-même dépendante de la température) et de la nature du mobile d'agitation. Une vitesse d'agitation comprise entre 100 rpm et 1400 rpm satisfait à ces exigences. L'homme du métier saura adapter la vitesse d'agitation à ces différents paramètres à la lumière de ses connaissances générales.

La durée de l'étape a) dépend du rapport enzyme/substrat mais également de paramètres tels que la température et le pH. L'homme du métier, saura, à la lumière de ses connaissances générales, choisir la durée en fonction de ces différents paramètres.

La durée de l'addition de la solution protéique, obtenue lors de la phase enzymatique, à l'étape a) doit être suffisamment courte pour permettre la formation de gouttelettes au fur et à mesure de l'addition de la phase enzymatique à une matière grasse. Avantageusement la durée sera de 4 à 16 L/heure, notamment comprise entre 5 et 10 L/heure.

Le type d'agent tensioactif sera choisi parmi ceux couramment utilisés par l'homme du métier dans le domaine. On peut citer, à titre d'exemple, le Tween® 80 et le Span® 20, à une concentration de 1 % p/v par rapport à l'huile de tournesol utilisée comme matière grasse. Il devra être de grade alimentaire ou pharmaceutique en fonction de l'application.

Les rapports entre la quantité d'enzyme et la quantité de protéines laitières dépendent de la structure voulue du produit final ; l'homme du métier saura les adapter à la lumière de ses connaissances générales. A titre d'exemple on peut utiliser un rapport de 0,05 à 0,4 IMCU/g de protéines laitières pour l'enzyme Naturen® de chez Hansen.

Conformément à l'invention, la rampe de température va de la température d'incubation enzymatique (entre 1 et 10°C) jusqu'à 60°C, la température d'action optimale de la chymosine étant de 40°C. Une température trop élevée peut entrainer :

- une dénaturation de la chymosine, ou
- une destruction de la molécule bioactive ou
- une dénaturation des protéines.

La durée de cette rampe peut varier en fonction du produit final souhaité et du matériel utilisé. Si le transfert de chaleur est efficace au sein de l'installation la durée peut être réduite. Toutes ces adaptations sont à la portée de l'homme du métier.

L'étape d) de séparation, peut être réalisée par toute technique connue de l'homme du métier, notamment par filtration ou centrifugation.

Dans un mode de réalisation avantageux de l'invention, les molécules bioactives s'entendent comme des molécules biologiquement actives comme par exemple des médicaments, des produits cosmétiques et des compléments alimentaires, et sont choisies dans le groupe comprenant notamment :

- les composés phytochimiques : flavonoïdes, catéchines, phytoestrogènes (isoflavones/lignanes), anthocyanidines, tanins (proanthocyanidines), lycopène (caroténoïde), phytostérols (sitosterol/ stanol ester), fibres (insolubles et solubles), β-Glucane (fibre), lutéine (caroténoïde)
- les lipides bioactifs : acides gras ω-3, acide linoléique conjugué,
- les protéines et peptides bioactifs : lactoferrine, peptides bioactifs, enzymes (lactase…),
- les prébiotiques : fructo-oligosaccharides (FOS), sucres alcools,
- les bactéries probiotiques : bactéries lactiques, bifidobactéries,
- les vitamines : acide folique, tocophérols, α-carotène, β-carotène,
- les minéraux : calcium.

Le procédé selon l'invention est particulièrement intéressant pour l'encapsulation des molécules bioactives alimentaires, hydrophiles, amphiphiles ou hydrophobes, notamment pour l'encapsulation des probiotiques.

Conformément au procédé de l'invention, toutes ces molécules bioactives peuvent être encapsulées seules ou en mélange d'au moins deux d'entre elles. On peut encapsuler, par exemple, un prébiotique avec un probiotique ou une enzyme et une vitamine ou un probiotique avec une vitamine sous réserve qu'il n'y ait pas d'interactions négatives entre les différentes molécules.

Dans un mode de réalisation avantageux de l'invention, le procédé comprend après l'étape d), une étape de récupération des microparticules formées et éventuellement une étape de séchage desdites microparticules pour faire une poudre.

La récupération des microparticules est réalisée par filtration, centrifugation ou toute autre technique de séparation connue de l'homme du métier.

Le séchage peut être réalisé par n'importe quelle technique connue de l'homme du métier, notamment par une étape de lyophilisation.

Dans un mode de réalisation avantageux du procédé selon l'invention, la matière grasse est choisie dans le groupe comprenant :

- les huiles fluides entre 1 et 60 °C, en particulier des huiles végétales, notamment l'huile de tournesol,
- la matière grasse laitière fractionnée fluide entre 1 et 60 °C et
- les matières grasses bénéfiques pour la santé, notamment celles contenant des acides gras polyinsaturés, comme par exemple les huiles riches en ω3.

Dans un mode de réalisation avantageux de l'invention, cette matière grasse peut également avoir un rôle de stabilisateur vis-à-vis des molécules à encapsuler. En effet, une fine couche de matière grasse reste adsorbée à la surface des microparticules. Cette couche lipidique peut ainsi s'opposer à la migration de composé de l'intérieur de la microparticule vers l'extérieur mais également du milieu environnant vers l'intérieur de la microparticule.

Dans un mode de réalisation avantageux du procédé selon la présente invention, la chymosine est utilisée à une concentration comprise entre 0,01 et 0,5 IMCU/g de protéines laitières, avantageusement entre 0,05 à 0,4 IMCU/g de protéines laitières (IMCU = International Milk Clotting Unit).

Dans un autre mode de réalisation avantageux de l'invention, dans la solution de protéines laitières, la caséine micellaire représente entre 70 et 100 % en poids de la solution, les protéines laitières solubles natives représentent entre 0 et 30 % en poids de la solution et les protéines laitières solubles dénaturées représentent entre 0 et 30 % en poids de la solution.

Ainsi conformément à l'invention la solution de protéines laitières peut contenir soit uniquement de la caséine micellaire, soit de la caséine micellaire et des protéines laitières solubles natives, soit de la caséine micellaire et des protéines laitières solubles dénaturées, soit de la caséine micellaire, des protéines laitières solubles natives et des protéines laitières solubles dénaturées.

Dans un mode de réalisation avantageux de l'invention, la fraction volumique (φ), qui représente le rapport de la phase dispersée/phase totale,

$$\varphi = \frac{\text{Volume de protéine laitière}}{\text{Volume de protéine laitière + volume de matière grasse}}$$

est inférieur ou égal à 0,2.

Une fraction volumique supérieure à 0,2 entraine la formation d'un système concentré qui ne permet pas de produire efficacement des microparticules.

Le procédé de l'invention peut être mis en œuvre par tout dispositif permettant de travailler en circuit fermé sous agitation. La figure 1 est une représentation schématique d'un exemple de dispositif fermé apte à mettre en œuvre l'invention. Ce dispositif comprend :

- une enceinte fermée (101) sous agitation, dite enceinte pour la réaction enzymatique, dans laquelle est réalisée la réaction enzymatique, c'est-à-dire l'étape a),
- une enceinte fermée (102) sous agitation dite enceinte d'émulsification, dans laquelle est réalisée l'étape d'émulsification, c'est-à-dire les étapes b) et c),
- des moyens (103), qui permettent d'amener la solution de protéines obtenues à l'étape a) dans l'enceinte (102) contenant la matière grasse,
- des moyens de régulation de température (104), des échangeurs efficaces munis des systèmes de régulation de température ou tout équipement connu de l'homme de l'art permettant le contrôle de la température à un point consigne ou réaliser un profil de profil programmable, pour, dans un premier temps, réfrigérer le système (enceinte pour la réaction enzymatique et enceinte d'émulsification) à une température d'environ 5°C puis dans un second temps, pour chauffer la cuve d'émulsification grâce à la programmation de la rampe de température.

La présente invention a également pour objet des microparticules susceptibles d'être obtenues par le procédé décrit précédemment. Les microparticules obtenues sont de type matriciel, c'est-à-dire que les molécules bioactives sont dispersées dans une matrice de protéines laitières. Leur réseau protéique est compact et bien structuré.

Les microparticules obtenues selon le procédé de l'invention sont des microparticules non hydrosolubles et ne se réhydratent pas quand elles sont placées dans un milieu aqueux.

La taille, mais également la forme et la texture des microparticules sont des facteurs qui affectent la perception par un consommateur. En effet, des microparticules petites, sphériques et molles seront moins facilement détectées que des microparticules dures, rugueuses et de grande taille. La nature de la matrice alimentaire dans laquelle elles sont introduites est

également déterminante. Par exemple, les mêmes microparticules seront plus facilement détectables par le consommateur si elles sont introduites dans un yaourt brassé plutôt que dans un yaourt ferme.
Conformément à l'invention, les microparticules peuvent être utilisées dans des compositions cosmétiques, pharmaceutiques ou alimentaires. A cette fin, elles peuvent être utilisées soit directement, soit sous forme d'une poudre obtenue après séchage desdites microparticules. Cette poudre et les compositions cosmétiques, pharmaceutiques et alimentaires qui les contiennent font également partie de l'invention.
Elles peuvent également être utilisées comme véhicule des molécules bioactives qu'elles contiennent.
En fonction du type d'application, c'est-à-dire selon la nature du produit dans lequel on souhaite ajouter les microparticules, la présente invention permet, en modifiant soit la vitesse d'agitation ou la nature du mobile d'agitation :

- de moduler la quantité de molécule bioactive encapsulée,
- d'ajuster la taille des microparticules et
- d'ajuster la forme des microparticules.

L'homme du métier, saura, à la lumière de ses connaissances générales adapter ces différents paramètres.
Les figures 1 à 4 et les exemples 1 et 2 qui suivent illustrent l'invention.
La figure 1 est une représentation schématique d'un exemple de dispositif apte à mettre en œuvre le procédé selon l'invention. (101) enceinte fermée pour la réaction enzymatique, (102) enceinte fermée dite d'émulsification, (103) moyens qui permettent d'amener la solution de protéines obtenues à l'étape a) dans l'enceinte (102), (104) moyens de régulation de température.
La figure 2 représente le nombre d'UFC (Unité formant colonie) contenu dans 1g de microparticules pour l'encapsulation de *L. rhamnosus* GG obtenu conformément à l'exemple 1.1.5. A : solution de caséine micellaire seule ; B : solution de caséine micellaire 90 % + protéines laitières solubles natives 10 % ; C : solution de caséine micellaire 90 % + protéines laitières solubles dénaturées 10 % ;

La figure 3A illustre la distribution des tailles de microparticules contenant *L. rhamnosus* GG obtenues selon le procédé de l'exemple 1.1.5 ; la figure 3B représente la sphéricité des microparticules en fonction de leur taille ; la figure 3C représente les microparticules obtenues selon le procédé de l'exemple 1.1.5 (image obtenue grâce à la camera présente dans le granulomorphomètre). La matrice utilisée pour l'encapsulation de *L. rhamnosus* GG présentée dans cet exemple est composée de 90% de caséines micelllaires et 10% de protéines laitières solubles dénaturées (Formulation C de la figure 2).
La figure 4A illustre la distribution des tailles de microparticules obtenues pour l'encapsulation de la quercétine selon le procédé de l'exemple 2 ; la figure 4B représente la sphéricité des microparticules en fonction de leur taille ; la figure 4C représente les microparticules obtenues selon le procédé de l'exemple 2. La matrice utilisée pour l'encapsulation de la quercétine est composée uniquement de caséines micellaires (Formulation A de la figure 2).

Exemple 1 Microencapsulation de *L. rhamnosus* GG *dans différentes matrices protéiques d'origine laitière*

1.1 Matériel et méthodes

1.1.1. Préparation de la caséine micellaire

De la caséine micellaire est réhydratée dans l'eau pendant 2 heures à 25 °C sous agitation et puis laissée une nuit sous agitation pour donner une solution finale à une concentration de 12,5 % en caséine micellaire.

1.1.2. Préparation de la solution de protéines laitières solubles natives

Les protéines laitières solubles natives sont réhydratées dans de l'eau pendant 2 heures à 25 °C sous agitation et puis laissées une nuit sous agitation pour donner une solution finale à 12,5 % en protéines natives.

1.1.3. Préparation de la solution de protéines laitières solubles dénaturées :

La solution obtenue à l'étape précédente est chauffée pendant 10 minutes à 78 °C. La solution passe de translucide à opaque, signe de dénaturation protéique. Cette température de 78°C pendant 10 minutes pour un volume de solution de 200 ml permet d'obtenir une diminution de plus de 50% de structures secondaires (hélices alpha, boucles de grande taille (large loop), coudes (turn)).

1.1.4. Préparation des matrices protéiques

200 ml de solutions de protéines laitières sont préparés selon le tableau suivant :

Formulations	Caséine micellaire (%)	Protéines solubles natives (%)	Protéines solubles dénaturées (%)	*L. rhamnosus* GG (ATCC 53103) (g pour 200 ml de solution protéique)
A	100	0	0	0,50
B	90	10	0	0,50
C	90	0	10	0,50

1.1.5. Encapsulation

200 ml de formulations A, B ou C, telles que préparées à l'étape précédente, sont mis en contact, pendant 30 minutes, sous une agitation de 150 rpm (Lightin® LabMaster), avec la chymosine (CHY-MAX® de CHR Hansen) à raison de 0,18 IMCU/mL de lait, dans une enceinte fermée thermostatée (figure 1 (101)) réfrigérée à 5 °C grâce à un réfrigérant circulant dans la double paroi (figure 1 (104)).

La solution ainsi obtenue est ajoutée à un débit de 8,2 L/h à 800 ml d'huile de tournesol contenant 8 g de tween 80®, maintenu à une température de 5 °C grâce à un réfrigérant circulant dans la double paroi de la cuve d'émulsification (figure 1 (102)) d'un volume de 1500 ml et sous une agitation de 500 rpm (Heidolph Digital 2000 Bioblock Scientific). La fraction volumique φ est de 0,2. L'agitation est maintenue pendant 10 minutes, de manière à former des gouttelettes, puis une rampe de température de 5 à 40 °C est appliquée en 25 minutes. Les gouttelettes liquides commencent à devenir solides autour de 21 °C. On obtient un système où des microparticules solides sont dispersées dans de la matière grasse.

L'huile excédentaire est éliminée par filtration. Les microparticules sont récupérées, lavées à l'eau distillée et séchées par lyophilisation.

Le nombre d'UFC (Unité Formant Colonie) contenu dans 1g de microparticules est déterminé par dénombrement microbiologique sur un milieu gélosé spécifique aux lactobacilles : le milieu MRS (de Man Rogosa and Sharpe).

1.1.6. Structures et distribution des tailles de microparticules

La distribution en taille et forme des microparticules est mesurée grâce à un granulo-morphomètre laser. La présence d'une caméra sur cet appareil permet également de visualiser le profil des microparticules.

La dispersion de taille de l'échantillon est évaluée en mesurant le span (s) qui norme la largeur de la distribution par rapport à la valeur médiane :

$$s = \frac{d_{90} - d_{10}}{d_{50}}$$

où

d_{10} diamètre correspond à la ligne des 10% sur la courbe de distribution cumulée,

d_{50} diamètre correspond à la ligne des 50% sur la courbe de distribution cumulée, et

d_{90} diamètre correspond à la ligne des 90% sur la courbe de distribution cumulée,

Ainsi, les valeurs des diamètres d_{10}, d_{50} et d_{90} reflètent le fait que 10%, 50% et 90% de la population ont un diamètre inférieur à cette valeur.

La forme des microparticules étant un paramètre clé dans la sensorialité des produits contenant des microparticules, la sphéricité a été déterminée. Cette valeur peut varier entre 0 et 1, une valeur de 1 décrivant une microparticule parfaitement sphérique.

1.2 Résultats

1.2.1. Taux d'encapsulation

Les résultats sont donnés dans le tableau de la figure 2.

Pour toutes les formulations testées, le taux d'encapsulation est très bon.

Les meilleurs résultats sont obtenus avec la formulation C avec 1,8 x 10^7 UFC/g.

1.2.2. Caractérisation des microparticules et distribution de la taille des microparticules

Les résultats sont donnés dans les figures 3A, 3B et 3C

Les microparticules se présentent sous forme matricielle, c'est-à-dire qu'elles contiennent les bactéries dispersées dans la matrice protéique.

Elles sont bien individualisées sans agrégats et de forme sphériques (figure 3B) avec une sphéricité qui varie entre 0,7 et 0,9 en fonction de la taille des microparticules.

La distribution de la taille des microparticules est donnée dans la figure 3A. Les microparticules présentent un diamètre moyen de 129 µm. La valeur *s* est de 1,28 ± 0,02.

Exemple 2 : Microencapsulation de la quercétine dans différentes matrices protéiques d'origine laitière

2.1 Matériel et méthodes

2.2.1. Encapsulation

Le procédé d'encapsulation est le même que celui décrit à l'exemple 1. La quercétine est ajoutée au même moment que les bactéries probiotiques. La matrice utilisée est la suivante : 100% de caséines micellaires (formulation A de la figure 2). La quantité de quercétine est la suivante : 1% en poids/poids total de la formulation.

Les températures, vitesses d'agitation et temps utilisés sont exactement les mêmes que dans l'exemple 1.

2.2 Résultats

2.2.1. <u>Taux d'encapsulation</u>

Le taux d'encapsulation de cette biomolécule varie entre 78% et 86% selon la quantité initiale de quercétine rajoutée au départ dans le lait.

2.2.2. <u>Caractérisation des microparticules et distribution de la taille des microparticules</u>

Ils sont donnés dans la figure 4.

Les microparticules se présentent sous forme matricielle. Elles sont bien individualisées sans agrégats et de forme sphériques (figure 4B) avec une sphéricité comprise entre 0,5 et 0,9.

La distribution de la taille des microparticules est donnée dans la figure 4A. Les microparticules présentent un diamètre moyen de 46 µm et une valeur de *s* de 0,97±0,15.

Le procédé selon l'invention est transposable à l'échelle industrielle et les différents paramètres (vitesse d'agitation, nature du mobile d'agitation,…) peuvent être adaptés non seulement pour moduler la taille et la forme des microparticules mais également la quantité de molécule bioactive encapsulée.

REVENDICATIONS

1. Procédé d'encapsulation de molécules bioactives par des protéines laitières choisies dans le groupe comprenant :

- de la caséine micellaire seule,
- de la caséine micellaire associée à des protéines laitières solubles natives,
- de la caséine micellaire associée à des protéines laitières solubles dénaturées, et
- de la caséine micellaire associée à des protéines laitières solubles natives et à des protéines laitières solubles dénaturées,

le dit procédé étant mis en œuvre en circuit fermé et comprenant les étapes suivantes :

a. la mise en contact d'une solution desdites protéines laitières présentant un pH compris entre 4,8 et 8 et contenant au moins une molécule bioactive à encapsuler, avec la chymosine, à une température comprise entre 1 et 10 °C, avantageusement 3 et 5 °C, jusqu'à ce qu'au moins 85 % du caséinomacropeptide soit clivé,

b. l'addition progressive de la solution protéique obtenue à l'étape a) à une matière grasse en présence d'un agent tensioactif à une température comprise entre 1 et 10 °C, avantageusement entre 3 et 5°C sous agitation comprise entre 100 et 1400 rpm, jusqu' à formation de gouttelettes et obtention d'une émulsion,

c. l'application d'une rampe de température allant de 1 à 60 °C, avantageusement de 5 à 40 °C, et

d. une phase de séparation des microparticules formées.

2. Procédé selon la revendication 1, caractérisé en ce qu'il comprend après l'étape d), une étape de récupération des microparticules formées et éventuellement une étape de séchage desdites microparticules, notamment une étape de lyophilisation.

3. Procédé selon l'une quelconque des revendications précédentes caractérisé en ce que la matière grasse est choisie dans le groupe comprenant :

- les huiles fluides entre 1 et 60 °C, en particulier des huiles végétales, notamment l'huile de tournesol,
- la matière grasse laitière fractionnée liquide entre 1 et 60 °C et
- les matières grasses bénéfiques pour la santé, notamment celles contenant des acides gras polyinsaturés.

4. Procédé selon l'une quelconque des revendications précédentes caractérisé en ce que la chymosine est utilisée à une concentration comprise entre 0,01 et 0,5 IMCU/g de protéines laitières, avantageusement entre 0,05 à 0,4 IMCU/g de protéines laitières.

5. Procédé selon l'une quelconque des revendications précédentes caractérisé en ce que dans la solution de protéines laitières, la caséine représente entre 70 et 100 % en poids de la solution, les protéines laitières solubles natives représentent entre 0 et 30 % en poids de la solution et les protéines laitières solubles dénaturées représentent entre 0 et 30 % en poids de la solution.

6. Procédé selon l'une quelconque des revendications précédentes caractérisé en ce que la fraction volumique (φ)

$$\varphi = \frac{\text{Volume de protéine laitière}}{\text{Volume de protéine laitière + volume de matière grasse}}$$

est inférieure à 0,2.

7. Microparticules susceptibles d'être obtenues par le procédé selon l'une quelconque des revendications précédentes.

8. Utilisation des microparticules selon la revendication 7 dans des compositions cosmétiques, pharmaceutiques ou alimentaires.

9. Utilisation des microparticules selon la revendication 7 comme véhicule des molécules bioactives qu'elles contiennent.

ABREGE

PROCEDE D'ENCAPSULATION DE MOLECULES BIOACTIVES DANS DES MATRICES LAITIERES ET MICROPARTICULES OBTENUES

Procédé d'encapsulation de molécules bioactives par des protéines laitières choisies dans le groupe comprenant de la caséine micellaire éventuellement associée à des protéines laitières du lactosérum natives et/ou dénaturées, le dit procédé étant mis en œuvre en circuit fermé et microparticules non hydrosolubles obtenues par ledit procédé.

UNIVERSITÉ DE LORRAINE

DIRECTION DE LA RECHERCHE ET DE LA VALORISATION

OFFRE DE COMPÉTENCE

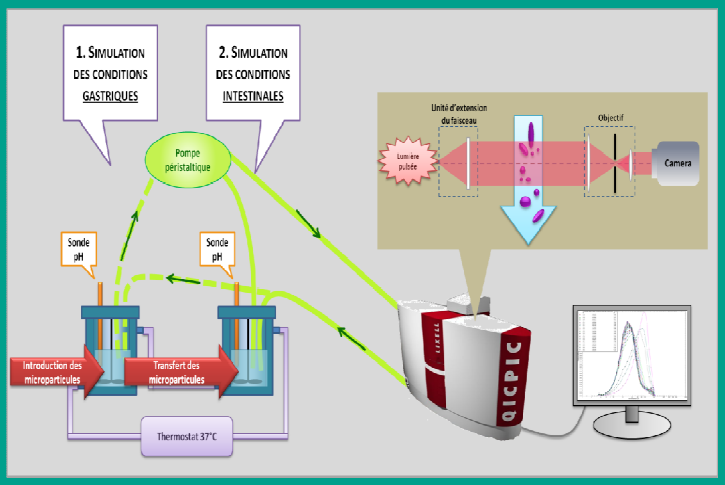
SIMULATION DU TRACTUS DIGESTIF

SYSTÈME DE SIMULATION DES CONDITIONS GASTRO-INTESTINALES AVEC SUIVI EN TEMPS RÉEL DES PHÉNOMÈNES OBSERVABLES

Le LIBio a développé un système pour visualiser les mécanismes inhérents à la digestion. Il s'agit d'un équipement original permettant de suivre in situ l'évolution de la taille (d'environ 1 à 800µm) et de la forme de microparticules ingérées par l'homme, lors de leur parcours dans l'appareil digestif. Deux réacteurs sont utilisés pour simuler les conditions gastriques puis intestinales, et un système de mesure et d'acquisition d'images vidéo permet d'obtenir des données quantitatives et qualitatives de manière immédiate. Cet équipement fournit donc des informations complexes sur les phénomènes de digestion grâce à des descripteurs simples qui retracent l'évolution de la taille, de la forme et du nombre des particules. Les réactions de digestion sont suivies en continu et en temps réel, ce qui est notamment particulièrement adapté aux études de suivi de libération contrôlée de composés bioactifs. De plus, une nouvelle réglementation européenne (n° 1924/2006 du 1 er juillet 2007) oblige les industriels à démontrer la libération contrôlée de leur biomolécules. Ce système permettrait de lever facilement cette contrainte imposée par les critères de dépôt d'allégation.

APPLICATIONS

- ► Industrie alimentaire et nutraceutique
- ► Industrie pharmaceutique
- ► Industrie cosmétique



LABORATOIRE / EQUIPE DE RECHERCHE

Le laboratoire d'Ingénierie des Biomolécules (LIBio) mène des travaux de recherche dans le domaine de la valorisation d'agro-ressources à des fins alimentaires et non alimentaires.
Les domaines de compétences sont:

- Substrats et familles de biomolécules: Composés phénoliques, Huiles et corps gras, Peptides bioactifs, Poudres alimentaires, Polysaccharides, Protéines.
- Physicochimie - Microbiologie - Bioconversion Objectif : après production, les molécules d'intérêt sont fonctionnalisées, vectorisées ou assemblées en structure supramoléculaire. Leurs propriétés structurales et fonctionnelles sont déterminées en vue de leur application dans le domaine des aliments et de la santé.
- Fonctionnalités cibles : Activité antioxydante, activité antibactérienne, Conception de nouveaux emballages (étude des transferts, emballages actifs, écoconception),Vectorisation, Hydratation et réhydratation, et Sensorialité (couleur, goût, arôme, texture)

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MOTS CLES

- ► Digestion, Libération contrôlée
- ► Gastrique, Intestinal
- ► Particules, Biomolécules, Bioactif

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Review

Encapsulation of probiotic living cells: From laboratory scale to industrial applications

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ABSTRACT

In the recent past, there has been a rising interest in producing functional foods containing encapsulated probiotic bacteria. According to their perceived health benefits, probiotics have been incorporated into a range of dairy products but the major current challenge is to market new probiotic foods. In the research sector, many studies have been reported using dairy products like cheese, yogurt and ice cream as food carrier, and non-dairy products like meat, fruits, cereals, chocolate, etc. However, in the commercial sector only few products containing encapsulated probiotic cells can be found. Nutraceuticals are another important vector for probiotics already developed by several companies in a capsule or a tablet form. The review compiles the technologies used to encapsulate the cells in order to keep them alive and the food matrices used in the research and commercial sector for delivery to the consumer.

Contents

Abbreviations: LAB, lactic acid bacteria; ME, microencapsulation; GI, gastrointestinal.

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1. Introduction

Modern consumers expect their food to be healthy and to prevent illness as they are increasingly interested in their personal health (Kailasapathy, 2009). This explains the reason for a rising interest in probiotic health-based products. Probiotic is a term that means "for life" and defined as "live microorganisms that beneficially affect the host's health by improving its microbial balance" (Fuller, 1989). More recently, probiotics have been defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). In fact, probiotic products are important functional foods as they represent about 65% of the world functional food market (Agrawal, 2005) and the market for probiotic products continues to expand (Jankovic et al., 2010). Probiotic bacteria have been incorporated into a wide range of foods, including dairy products (such as yogurt, cheese, ice cream, dairy desserts) but also in non-dairy dairy products (such as chocolate, cereals, juices) (Anal and Singh, 2007). The viability of probiotic cells is of paramount importance because to have their beneficial effects on the host's health they must stay alive as far as their site of action. Many reports indicated that there is poor survival of probiotic bacteria in products containing free probiotic cells (De Vos et al., 2010). Providing probiotic living cells with a physical barrier to resist adverse environmental conditions is therefore an approach currently receiving considerable interest (Kailasapathy, 2009). Microencapsulation (ME) is a powerful technology which has been developed for use in the food industry and allows the protection of bacterial cells (Borgogna et al., 2010).

However, ME of probiotic cells requires some specific processing steps which complicate the manufacture of the food product and increase its cost. Many challenges exist when considering ME of probiotic living cells like, probiotic strain selection for its health benefits and quantity require to have positives effects, but also stability of the cells during the processing steps and storage and finally, effects on sensory properties of the food (Champagne and Fustier, 2007b).

Currently, the probiotic market is affected by global regulatory requirements which have become stricter in recent years. In fact, manufacturers have to take into account cell viability and probiotic function in order to make a health claim (Jankovic et al., 2010). In Europe, the European Food Safety Authority (EFSA) is responsible for judging the health claims suggested by industrialists. In December 2006, EU makers adopted a regulation on the use of health claims and since then, many of them have been rejected. The main reason unveiled by EFSA is a lack of probiotic strain characterisation and that strains referenced are different from those present in the food products for which the claims were made. EFSA has confirmed that it does not plan to issue probiotic health claim guidance as has been done by the Canadian regulatory authority. In fact, Health Canada recently published a guidance document for the use of probiotic bacteria in food and the use of health claims associated with these products (Health Canada, 2009). In the United States there are no such government standards for probiotics and it is essential to have scientific substantiation to make a health claim. The FDA (Food and Drug Administration) expects manufacturers to provide scientific justification for use of any health claim. In 2009, guidance for Industry on "Evidence-based review system for the scientific evaluation of health claims" was published and describes some recommendations to manufacturer to substantiate a claim (FDA, 2009).

In this report, the probiotic term will be firstly defined and its beneficial effects for the human health will be mentioned. Then, a definition of ME and the technologies used to encapsulate probiotic cells will be developed. The last part will focus on the use of encapsulated probiotic cells in food on a laboratory scale. Finally, probiotic foods already available on the market will be listed and the tendency of development at an industrial scale will be approache.

2. Probiotics

2.1. Definition

Probiotics are feed and food supplements that beneficially affect the host's health. Strain identity is important in order to link a strain to a specific health effect and to enable accurate surveillance and epidemiological studies (Pineiro and Stanton, 2007).

The term "probiotic" includes a large range of microorganisms, mainly bacteria but also yeasts. Because they can stay alive until the intestine and provide beneficial effects on the host health, lactic acid bacteria (LAB), non-lactic acid bacteria and yeasts can be considered as probiotics. LAB are the most important probiotic known to have beneficial effects on the human gastro-intestinal (GI) tract. These bacteria are Gram-positive and usually live in a non-aerobic environment but they also can support aerobic conditions (Holzapfel et al., 2001; Anal and Singh, 2007). Bifidobacteria are also Gram-positive and can grow at a pH range of 4.5–8.5 but the most important characteristic is the fact that they are strictly anaerobic (Holzapfel et al., 2001; Anal and Singh, 2007). Other LAB (e.g. *Lactococcus lactis*, *Enterococcus faecium*, etc.) and non-lactic acid bacteria (e.g. *Escherichia coli* strain nissle) but also some yeasts (e.g. *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, etc.) are also considered as probiotics. It has been mentioned that dead bacteria, products derived from bacteria or end products of bacterial growth could provide some health benefits. However, because they are not alive when administrated they cannot be considered as probiotics (Sanders et al., 2007).

The effects of probiotics are strain-specific (Luyer et al., 2005; Canani et al., 2007; Kekkonen et al., 2007) and that is the reason why it is important to specify the genus and the species of probiotic bacteria when proclaiming health benefits. Each species covers various strains with varied benefits for health. The probiotic health benefits may be due to the production of acid and/or bacteriocins, competition with pathogens and an enhancement of the immune system (Chen and Chen, 2007). Dose levels of probiotics depend

on the considered strain (Sanders, 2008), but 10^6–10^7 CFU/g of product per day is generally accepted (Krasaekoopt et al., 2003).

Overall, probiotics are orally administrated and are available in various forms such as food products, capsules, sachets or tablets. The advantage of food products such as dairy products is that they may additionally provide essential nutrients (e.g. calcium, proteins) and the addition of probiotics to these products is a natural way to enhance their functionality (Weichselbaum, 2009). Orally ingested probiotics have to survive adverse conditions during their passage through the GI tract to be able to influence the human gut microflora. The intestinal flora is made up of harmless microorganisms, present in appropriate proportions that are essential for its normal functioning. Ingested probiotic strains do not become established members of the normal intestinal flora but generally persist only for the period of consumption and for a relatively short period thereafter (Corthésy et al., 2007).

2.2. Health benefits

2.2.1. The mechanism of action of probiotic bacteria

There is evidence that probiotics have the potential to be beneficial for our health (Weichselbaum, 2009). Probiotics have been reported to play a therapeutic role by modulating immunity, lowering cholesterol, improving lactose tolerance and preventing some cancers (Kailasapathy and Chin, 2000; Sanders et al., 2007). The effects of probiotics can be classified in three modes of action.

The first is related with the modulation of the host's defences which is most likely important for the prevention and treatment of infectious disease and also for treatment of intestinal inflammation (Collado et al., 2009). Probiotics may influence the immune system by means of products such as metabolites, cell wall components or DNA. In fact, these products can be recognised by the host cells sensitive for these because of the presence of a specific receptor (Cummings et al., 2004). In this context, the main target cells are generally the gut epithelial and the gut-associated immune cells. Finally, the interaction between probiotics and the host's immune cells by adhesion might be the triggering signalling cascade leading to immune modulation (Corthésy et al., 2007).

The second mechanism of action can be described by a direct effect on other microorganisms which can be commensal and/or pathogenic. In this case, the therapy and the treatment of infections are concerned but restoration of the microbial balance in the gut is an important factor too (Kaur et al., 2002). Probiotics have the ability to be competitive with pathogens and therefore allow for preventing their adhesion to the intestine (Tuomola et al., 1999).

Eventually, probiotics have the ability to affect some microbial products such as toxins and host products like bile salts and food ingredients (Patel et al., 2010).

However, it is important to know that these three mechanisms of action are strain-dependent, and to date the modes of action of probiotic bacteria are not yet fully known (Oelschlaeger, 2010).

2.2.2. Examples of published health benefits: the gut and the immune system

In Europe, gut health has been shown to be the key sector for marketing functional foods (Mattila-Sandholm et al., 2002) and probiotics have a considerable potential for preventive or therapeutic effects on GI disorders (Wohlgemuth et al., 2010). The details of probiotics' health benefits related to the gut and the immune system are developed in the following paragraphs.

Inflammatory bowel disease is a chronic recurrent pathology, which mainly consists in ulcerative colitis and Crohn's disease. Recent studies have shown that some probiotic strains (*E. coli* Nissle 1917 and *Lactobacillus rhamnosus* GG) can prevent relapses of inflammatory bowel diseases and are able to decrease the recurrence of ulcerative colitis (Rembacken et al., 1999; Kruis et al., 2004; Zocco et al., 2006; Henker et al., 2008). Nevertheless, in the case of Crohn's disease current evidences suggest that probiotic are ineffective at treating patient with this pathology (Schutlz et al., 2004; Bousvaros et al., 2005; Lomax and Calder, 2009). Evidence on the efficacy of probiotics on constipation is limited but it seems that some strains could bring relief to patients suffering from this pathology (Chmielewska and Szajewska, 2010). A number of probiotic strains are effective in preventing antibiotic-associated diarrhoea (Fitton and Thomas, 2009) and there is also promising evidence of a preventive effect of probiotics in *Clostridium difficile* associated diarrhoea (Parkes et al., 2009). Acute diarrhoea is a health problem which is well studied, particularly in children and studies have shown that selected probiotic strains seem to be effective in reducing the duration of acute diarrhoea (Lomax and Calder, 2009). Studies investigating the preventive effect of probiotics in the context of the common cold and flu infections show that the studied strains failed to lower the incidence of episodes but that they have the potential to decrease the duration of episodes, which suggests that the immune system may be more efficient in fighting off common cold or flu infections after consuming these strains (Weichselbaum, 2009).

Finally, there is no evidence so far proving that probiotics are effective in preventing or treating eczema and allergies. Some probiotic strains seem to lower the risk of developing eczema if taken by pregnant women and their infants in early life (Weichselbaum, 2009).

2.3. Synbiotics: a combination of the probiotics and prebiotics positives effects

Overall, products available on the market that positively influence the intestinal microflora are probiotics and prebiotics. The properties of probiotics have been shown in the previous section. Prebiotics can be defined as 'non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits' (Ouwehand et al., 2007). When considering probiotics, viability and dose level are important parameters for their efficacy and prebiotics have the potential to improve probiotic's viability and vitality, its survival in the GI tract and its further attachment and growth in the intestine. Inulin and fructo-oligosaccharides are the most common prebiotics used, because of their resistance against gastric acid and pancreatic enzymes (Ramchandran and Shah, 2010). Thereby, the synbiotic concept can be defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the GI tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health promoting bacteria, and thus improving host welfare'. Synbiotics are not only a mixture of probiotics and prebiotics but a synergy between the two components (Ouwehand et al., 2007).

3. Encapsulation

3.1. Definition and goals of encapsulation

Encapsulation is a physicochemical or mechanical process to entrap a substance in a material in order to produce particles with diameters of a few nanometres to a few millimetres (Chen and Chen, 2007). Encapsulation of bioactive components can be used in many applications in the food industry: controlling oxidative reaction, masking flavours, colours and odours, providing sustained and controlled release, extending shelf life, etc. Probiotic

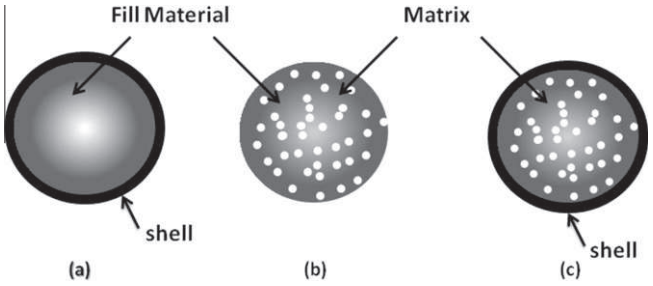

Fig. 1. Schematic representation of encapsulation systems: (a) reservoir type, (b) matrix type, and (c) coated matrix type.

encapsulation is used to protect the cells against an adverse environment more than controlled release (Champagne and Kailasapathy, 2008; Zuidam and Shimoni, 2009). The encapsulated substance called the core material is dispersed in a matrix also named coating or shell. This carrier material must be food grade if used in food industry, and able to form a barrier to protect the encapsulated substance.

As can be seen in Fig. 1, different types of encapsulates can be found, the reservoir type and the matrix type. The reservoir type has a shell around the core material and this is why it can also be called a capsule. In the case of matrix type, the active agent is dispersed over the carrier material and can also be found on the surface. A combination of these two types gives a third type of capsule: the matrix where the active agent is recovered by a coating (Zuidam and Shimoni, 2009).

Finally, encapsulation gives a structure and allows creating new function or innovative systems (Poncelet et al., 2007) for probiotic products. The technology of encapsulation of probiotic living cells evolved from the immobilised cell culture technology used in the biotechnological industry. Probiotics present two sets of problems when considering encapsulation: their size (typically between 1 and 5 μm diameter), which immediately excludes nanotechnologies, and the fact that they must be kept alive. This latter aspect has been crucial in selecting the appropriate ME technology (Champagne and Fustier, 2007a; Zuidam and Shimoni, 2009). Several technologies can be applied to probiotic encapsulation and each of them provides microcapsules with different characteristics in terms of range size of particles and of type of capsule (Fig. 2). For example, emulsification allows the production of a wide particle size range from 0.2 to 5000 μm whereas, extrusion gives a smaller range size but it does not provide particles under 300 μm. In Fig. 2 it can be seen the different types of particles obtained (matrix or reservoir type) by each method.

The ability of microorganisms to survive and multiply in the host strongly influences their probiotic benefits. Studies have reported low viability of probiotics in dairy products such as yogurt and frozen dairy desserts due to the concentration of lactic acid and acetic acid, low pH, the presence of hydrogen peroxide, and the high oxygen content (De Vos et al., 2010). Encapsulation has been investigated for improving the viability of microorganisms in both dairy products and the GI tract (Krasaekoopt et al., 2003; Picot and Lacroix, 2004). The viability of encapsulated probiotic cells depend on the physico-chemical properties of the capsules. In fact, the type and the concentration of the coating material, particle size, initial cell numbers and bacterial strains are some parameters which are important to master (Chen and Chen, 2007). In the case of probiotic encapsulation, the objective is not only to protect the cells against adverse environment, but also to allow their release in a viable and metabolically active state in the intestine (Picot and Lacroix, 2004). The obtained microparticles have to be water-insoluble to maintain their integrity in the food matrix and in the upper part of the GI tract and finally, particle properties should allow progressive liberation of the cells during the intestinal phase (Picot and Lacroix, 2004; Ding and Shah, 2007).

As shown in Fig. 3, encapsulation technology is usually held in three stages. The first step consists in incorporating the bioactive component in a matrix which can be liquid or solid. In case of the core is liquid, incorporation will be a dissolution or a dispersion in the matrix whereas if the core is solid the incorporation will be an agglomeration or an adsorption. For the second step, the liquid matrix is dispersed while a solution is pulverised on the solid matrix. The last step consists in stabilisation by a chemical (polymerisation), a physicochemical (gelification) or a physical (evaporation, solidification, coalescence) process (Poncelet and Dreffier, 2007).

In the next part, techniques used to encapsulate probiotic living cells will be described. However, other techniques can provide microparticles and we can quote liposome, coacervation, co-crystallisation, molecular inclusion, but their use is limited because of their cost, or the large size of bacteria for example (Champagne and Kailasapathy, 2008).

3.2. Materials used to encapsulate probiotic cells

3.2.1. Alginate

Alginate is a naturally derived polysaccharide extracted from various species of algae and composed of β-D-mannuronic and α-L-guluronic acids. The composition of the polymer chain varies in amount and in sequential distribution according to the source of the alginate and this influences functional properties of alginate as supporting material. Alginate hydrogels are extensively used in cell encapsulation (Rowley et al., 1999) and calcium alginate is preferred for encapsulating probiotics because of its simplicity, non-toxicity, biocompatibility and low cost (Krasaekoopt et al.,

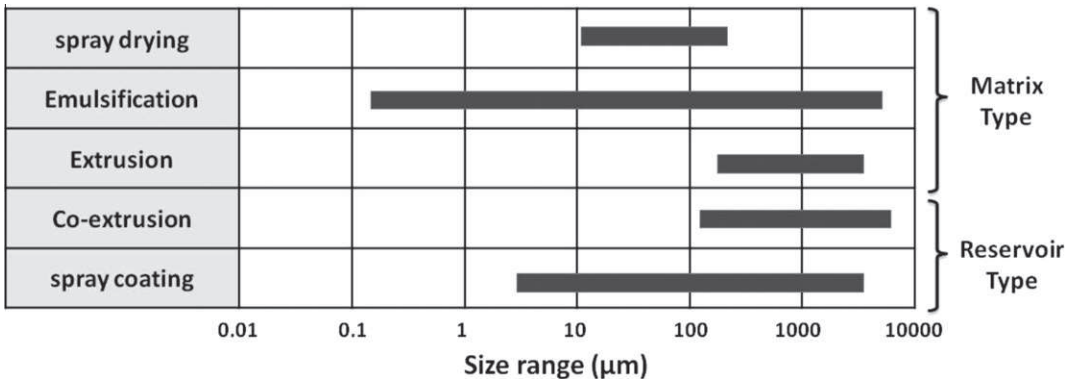

Fig. 2. Probiotic encapsulation technologies: size range provided by each technique.

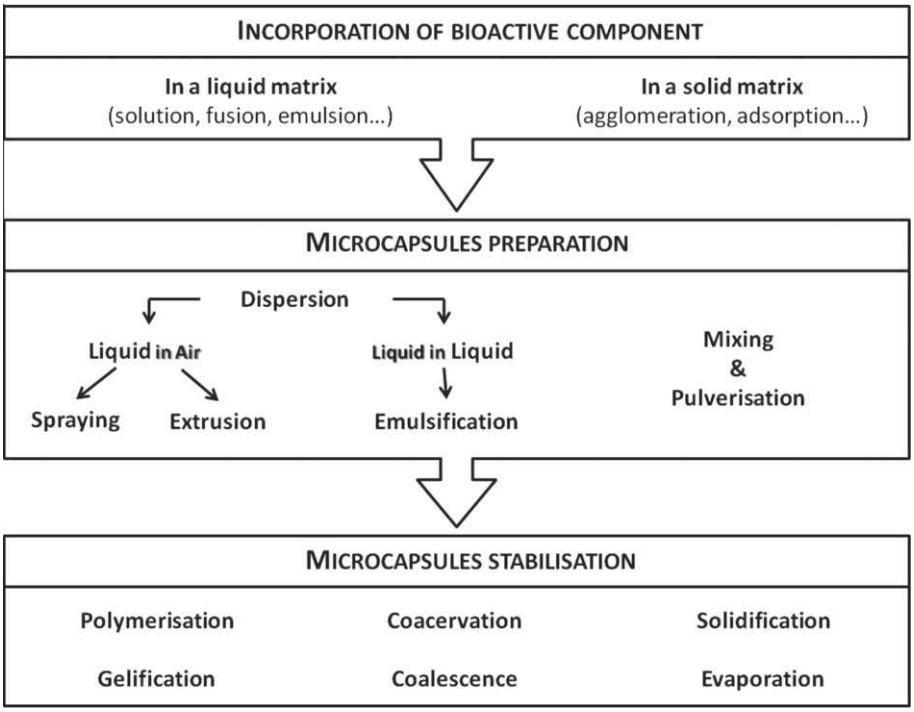

Fig. 3. General plan describing steps to produce microcapsules.

2003). However, some disadvantages are attributed to the use of alginate. For example, alginate beads are sensitive to the acidic environment (Mortazavian et al., 2008) which is not compatible for the resistance of the microparticles in the stomach conditions. Others disadvantages concern the scaling-up of the process that is very difficult. In addition, the microparticles obtained are very porous which is a drawback when the aim is to protect the cells from its environment (Gouin, 2004).

Nevertheless, the defects can be compensated by mixing alginates with other polymer compounds, coating the capsules by another compound or applying structural modification of the alginate by using different additives (Krasaekoopt et al., 2003). For example, mixing alginate with starch is commonly used and it has been shown that this method results in an improvement of probiotic encapsulation effectiveness (Sultana et al., 2000; Sun and Griffiths, 2000; Truelstrup-Hansen et al., 2002; Krasaekoopt et al., 2003).

3.2.2. Gellan gum and xanthan gum

Gellan gum is a microbial polysaccharide derived from *Pseudomonas elodea* which is constituted of a repeating unit of four monomers that are glucose, glucuronic acid, glucose and rhamnose (Chen and Chen, 2007). A mixture of xanthan–gelan gum has been used to encapsulate probiotic cells (Sultana et al., 2000; Sun and Griffiths, 2000) and contrary to alginate, the mixture presents high resistance towards acid conditions.

3.2.3. κ-Carrageenan

κ-Carrageenan is a natural polymer which is commonly used in the food industry. The technology using the compound requires a temperature comprised between 40 and 50 °C at which the cells are added to the polymer solution. By cooling the mixture to room temperature, the gelation occurs and then, the microparticles are stabilised by adding potassium ions (Krasaekoopt et al., 2003). The encapsulation of probiotic cells in κ-carrageenan beads keeps the bacteria in a viable state (Dinakar and Mistry, 1994) but the produced gels are brittle and are not able to withstand stresses (Chen and Chen, 2007).

3.2.4. Cellulose acetate phthalate

Because of having a safe nature, cellulose acetate phthalate is used for controlling drug release in the intestine (Mortazavian et al., 2008). The advantage of this component is that it is not soluble at acidic pH (less than 5) but it is soluble at pH higher than 6. The encapsulation of probiotic bacteria using cellulose acetate phthalate provides good protection for microorganisms in simulated GI conditions (Fávaro-Trindade and Grosso, 2002).

3.2.5. Chitosan

Chitosan is a linear polysaccharide composed of glucosamine units which can polymerise by means of a cross-link formation in the presence of anions and polyanions. This component has not shown a good efficiency for increasing cell viability by encapsulation and it is preferably use as a coat but not as a capsule (Mortazavian et al., 2008). In fact, encapsulation of probiotic bacteria with alginate and a chitosan coating provides protection in simulated GI conditions and therefore, it is a good way of delivery of viable bacterial cells to the colon (Chávarri et al., 2010). However, chitosan has some disadvantages and it seems to have inhibitory effects on LAB for example (Groboillot et al., 1993).

3.2.6. Starch

Starch is a polysaccharide consisting of a large number of glucose units joined together by glucosidic bonds. Starch consists mainly of amylose, a linear polymer of D-glucopyranose joined by α-1-4 glucosidic bond and amylopectin, a branched polymer of glucose joined by α-1-4 glucosidic bond and α-1-6 glycosidic bond for ramification (Sajilata et al., 2006). Resistant starch is the starch which is not digested by pancreatic enzymes (amylases) in the small intestine. Resistant starch can reach the colon where it will be fermented (Sajilata et al., 2006; Anal and Singh, 2007). This specificity provides good enteric delivery characteristic that is a

better release of the bacterial cells in the large intestine. Moreover, by its prebiotic functionality, resistant starch can be use by probiotic bacteria in the large intestine (Mortazavian et al., 2008). Finally, resistant starch is an ideal surface for the adherence of the probiotic cells to the starch granules (Anal and Singh, 2007) and this can enhance probiotic delivery in a viable and a metabolically active state to the intestine (Crittenden et al., 2001).

3.2.7. Gelatin

Gelatin is a protein gum, which makes a thermoreversible gel and was used for probiotic encapsulation, alone or in combination with other compounds. Due to its amphoteric nature, it is an excellent candidate for cooperation with anionic polysaccharides such as gellan gum. These hydrocolloids are miscible at a pH higher than 6, because they both carry net negatives charges and repel each other. However, the net charge of gelatin becomes positive when the pH is adjusted below the isoelectric point and this causes the formation of a strong interaction with the negatively charged gellan gum (Krasaekoopt et al., 2003; Anal and Singh, 2007).

3.2.8. Milk proteins

Milk proteins are natural vehicles for probiotics cells and owing to their structural and physico-chemical properties, they can be used as a delivery system (Livney, 2010). For example, the proteins have excellent gelation properties and this specificity has been recently exploited by Heidebach et al. (2009a,b) to encapsulate probiotic cells. The results of these studies are promising and using milk proteins is an interesting way because of their biocompatibility (Livney, 2010).

3.3. Dispersion methods

3.3.1. Atomization

Spray drying (Fig. 4). A solution containing the probiotic living cells and the dissolved polymer matrix is prepared. The polymer matrices are generally gum arabic and starches because they tend to form spherical microparticles during the drying process (Chen and Chen, 2007; Kailasapathy, 2009; De Vos et al., 2010). The advantages of spray drying are the rapidity and the relatively low cost of the procedure. The technique is highly reproducible and the most important is that it is suitable for industrial applications. One disadvantage of spray drying is the fact that the technique has a small field of application but the main problem is the use of high temperature which is not compatible with the survival of bacteria. In order to improve probiotic survival, protectants can be added to the media prior to drying. For example, granular starch improves culture viability during drying and storage, soluble fibre increase probiotic viability during storage and trehalose is a thermoprotectant. Moreover, spray-dried capsules can be coated by an additional layer in order to give a protection against acidic environment of the stomach or to reduce the deleterious effect of bile salts (Semyonov et al., 2010).

Spray Freeze drying. Spray freeze drying method combines processing steps that are common to freeze-drying and to spray-drying. Probiotic cells are in a solution which is atomized into a cold vapour phase of a cryogenic liquid such as liquid nitrogen. This step generates a dispersion of frozen droplets. Frozen droplets are then dried in a freeze dryer (Wang et al., 2006; Kailasapathy, 2009; De Vos et al., 2010; Semyonov et al., 2010). Spray freeze drying presents various advantages, like providing controlled size, larger specific surface area than spray-dried capsules. The technique also has some disadvantages including the use of high energy, the long processing time and the cost which is 30–50 times more expensive than spray-drying (Zuidam and Shimoni, 2009). Capsules can be coated by an additional shell to give protection against adverse environmental conditions (Semyonov et al., 2010).

3.3.2. Emulsification

Emulsification and ionic gelification. Emulsification is a chemical technique to encapsulate probiotic living cells and use hydrocolloids (alginate, carrageenan and pectin) as encapsulating materials (Fig. 5). The principle of this technique is based on the relationship between the discontinuous and the continuous phases. For encapsulation in an emulsion, an emulsifier and a surfactant are needed. A solidifying agent (calcium chloride) is then added to the emulsion (Chen and Chen, 2007; Kailasapathy, 2009; De Vos et al., 2010).

The emulsion technique is easy to scale-up and gives a high survival rate of the bacteria (Chen and Chen, 2007). The obtained capsules have a small diameter but the main disadvantage of this method is that it provides large size range and shape. The emulsion procedure enables the production of the targeted microcapsules size by variation of agitation speed and the water/oil ratio (Kailasapathy, 2009). The gel beads can be introduced into a second polymer solution to create a coating layer that provides added

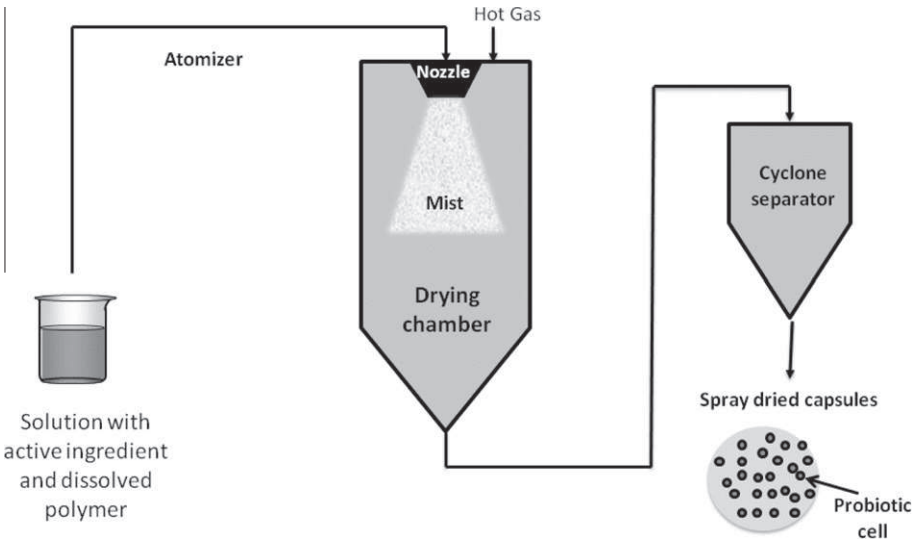

Fig. 4. Schematic presentation of the spray-drying procedure. The solution is pressured and then atomized to form a "mist" into the drying chamber. The hot gas (air or nitrogen) is blown in the drying chamber too. This hot gas allows the evaporation of the solvent. The capsules are then transported to a cyclone separator for recovery.

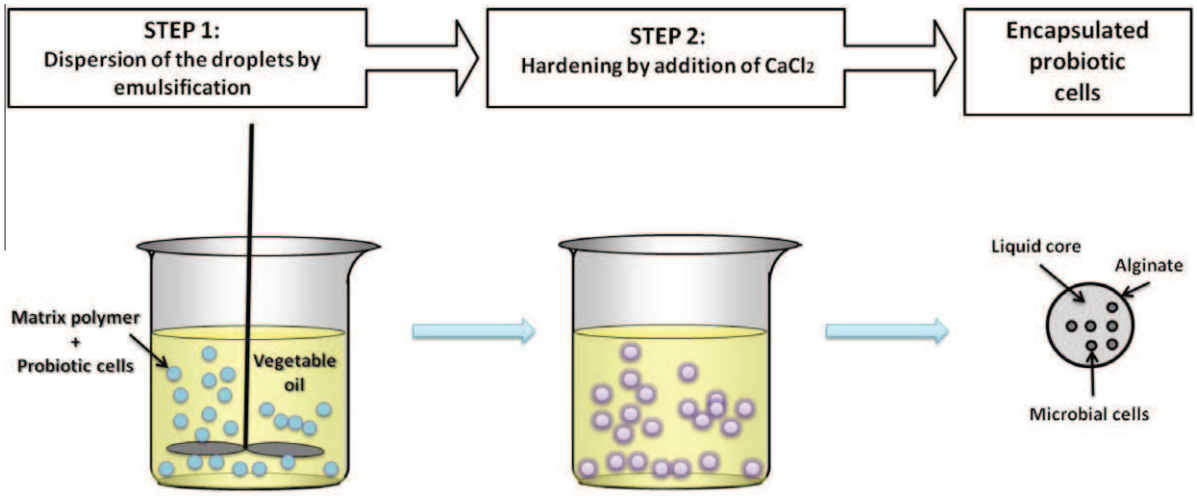

Fig. 5. Schematic presentation of the emulsification procedure. A small volume of the cell-polymer suspension (i.e., the discontinuous phase) is added to a large volume of vegetable oil (i.e., the continuous phase). The mixture is then homogenized to form a water-in-oil emulsion. Once the water-in-oil emulsion is formed, the water-soluble polymer must be insolubilised to form tiny gel particles within the oil phase.

protection to the cell or maybe give improved organoleptic properties (Kailasapathy, 2009).

Emulsification and enzymatic gelification. One problem with classical encapsulation technologies is the use of coatings such alginate, κ-carrageenan, gellan-gum or xanthan which are not allowed in dairy products in some countries (Picot and Lacroix, 2004). The solution can be the use of milk proteins in which probiotics will be encapsulated by means of an enzymatic induced gelation (Heidebach et al., 2009a,b). Milk proteins have excellent gelation properties and they are natural vehicles for probiotics (Livney, 2010).

This method gives water insoluble and spherical particles. Heidebach et al. (2009a) detailed an example of encapsulation by means of rennet gelation (Fig. 6).

Emulsification and interfacial polymerisation. Interfacial polymerisation is an alternative technique which is performed in a single step. The technique requires the formation of an emulsion: the discontinuous phase contains an aqueous suspension with the probiotic cells and the continuous phase is an organic solvent. To initiate the polymerisation reaction, a biocompatible agent which is soluble in the continuous phase, is added. The droplets obtained containing probiotic cells are enveloped in a thin and strong

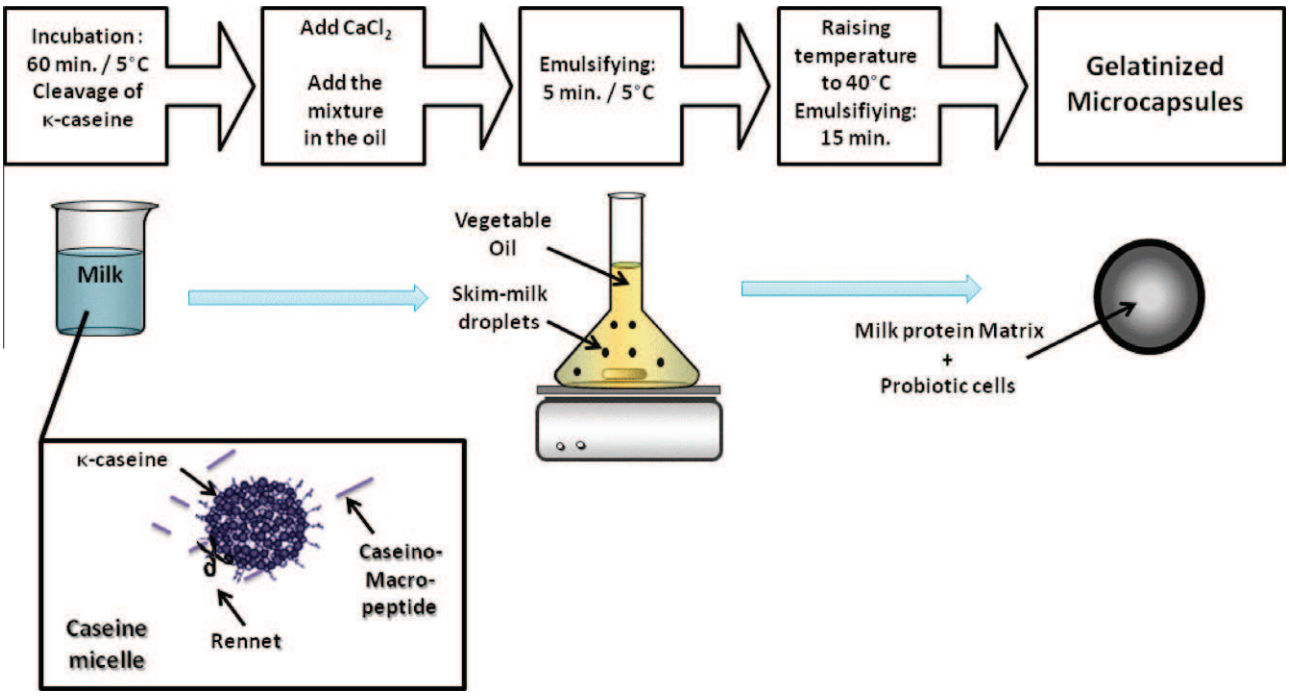

Fig. 6. Schematic presentation of the microencapsulation of probiotic cells by means of rennet-gelation of milk proteins. The principle of the technique is base on using dairy proteins which have been put in contact with rennet at low temperature. This allows keeping a liquid system where κ-casein is cleaved by the enzyme. After that, dairy proteins have been emulsified in a cold oil to form water in oil emulsion. Thermal induction of enzymatic coagulation allows proteins flocculation and provides microparticles where probiotics are dispersed in coagulated dairy proteins.

membrane (Kailasapathy, 2002). Interfacial polymerisation is used to encapsulate microorganisms in order to improve their productivity in fermentation (Yanez-Fernandez et al., 2008).

3.3.3. Extrusion method

Extrusion is a physical technique to encapsulate probiotic living cells and uses hydrocolloids (alginate and carrageenan) as encapsulating materials. The ME of probiotic cells by extrusion consists in projecting the solution containing the cells through a nozzle at high pressure. If the formation of droplets occurs in a controlled environment way (as opposed to spray-drying), the technique is known as prilling. This is preferably done by the pulsation or vibration of the jet nozzle. The use of coaxial flow or an electrostatic field is the other common technique to form droplets (Kailasapathy, 2002). The principle of the technique is explained in Fig. 7 (Krasaekoopt et al., 2003; Chen and Chen, 2007; Kailasapathy, 2009; De Vos et al., 2010). Extrusion is a simple and cheap method that uses a gentle operation which causes no damage to probiotic cells and gives a high probiotic viability (Krasaekoopt et al., 2003). The technology does not involve deleterious solvents and can be done under aerobic and anaerobic conditions. The most important disadvantage of this method is that it is difficult to use in large scale productions due to the slow formation of the microbeads.

3.4. Encapsulation by coating and agglomeration

In spray-coating, the core material needs to be in a solid form and is kept in motion in a specially designed vessel as can be seen in Fig. 8 (Champagne and Fustier, 2007a; De Vos et al., 2010). The advantage of spray coating is that it is easy to scale up, which is why it is mostly use to encapsulate probiotics for nutraceuticals for example. Spray coating is particularly adapted to give multilayer coatings. However, it is important to highlight that spray coating is a technology which is difficult to master.

Institut Rosell and Lal'food are part of the Lallemand group, a private Canadian company which develops products containing bacteria and particularly probiotic products. The company has developed and has patented a ME technology known as Probiocap® (Durand and Panes, 2003). The process is based on coating freeze-dried LAB with fatty acids. The technology allows strains to resist harsh effects of temperature, gastric acidity and compression. Probiocap® offers a number of opportunities to develop new food products and supplements.

Cell Biotech is a successful Danish-Korean company dedicated to probiotics which has developed and patented a dual coating technology for LAB, that are marketed under the brand name Duaolac®. The first layer of coating is made of soy peptides and the second layer is made of cellulose and gum. The technology allows an increase in probiotic viability during processing shelf life and during their passage through the GI tract. The coating system is based on a pH-dependant release mechanism which protects the cells against acidic environment in the stomach and release the coating in the pH-neutral environment of the intestines.

4. Encapsulated probiotics in food products

ME is important for the survival of probiotics during storage and its passage through the digestive tract. Addition of microcapsules should not affect the sensory properties of food products (Champagne and Fustier, 2007a). To avoid negative sensorial im-

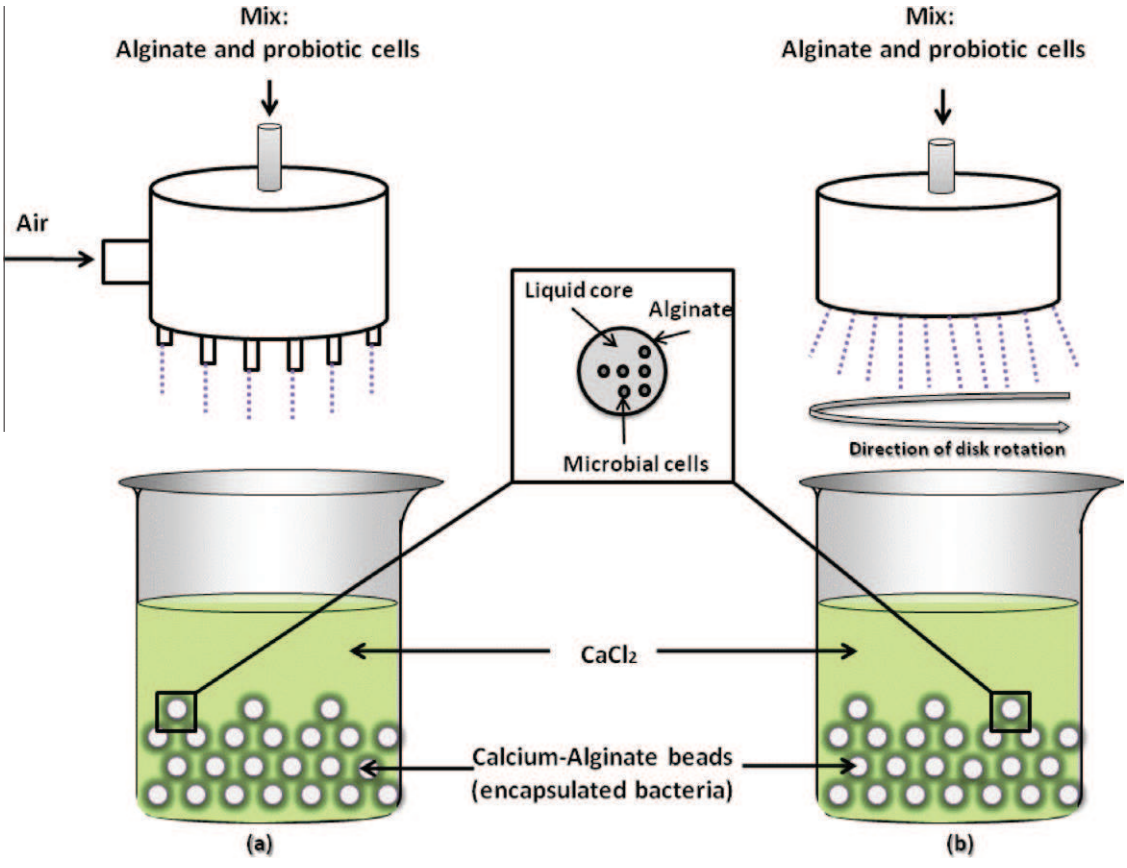

Fig. 7. Extrusion technologies: simple needle droplet-generator that usually is air driven (a) and pinning disk device (b). The probiotic cells are added to the hydrocolloid solution and dripped through a syringe needle or a nozzle spray machine in the form of droplets which are allowed to free-fall into a hardening solution such as calcium chloride.

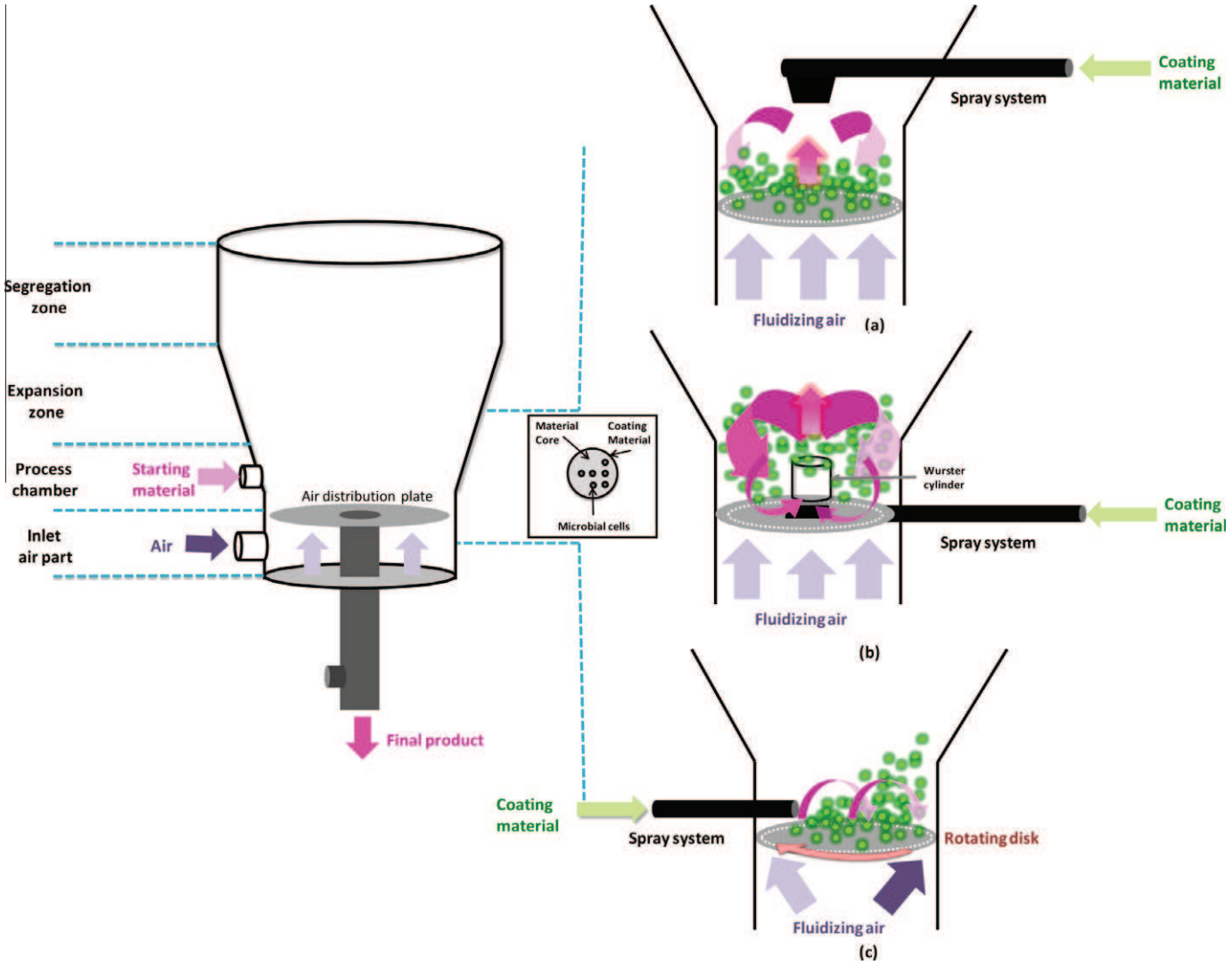

Fig. 8. Schematic presentation of the spray coating technology. A liquid coating material is sprayed over the core material and solidifies to form a layer at the surface. The liquid coating material can be injected from many angles over the core material: fluid-bed top spray coating (a), fluid-bed bottom spray coating with the Wurster device (b), and fluid bed tangential spray coating (c)

pacts of microcapsules in food; it is desirable to obtain a size below 100 μm (Truelstrup-Hansen et al., 2002).

4.1. Recent developments in the research sector

4.1.1. Cheese

Many studies have reported the use of encapsulated probiotic cells (Table 1) and particularly in Cheddar cheese.

Due to a relative high pH (pH 5.5), Cheddar cheese presents the advantage of being a good carrier of probiotic microorganisms. In addition, its good buffering capacity and its relatively high fat content may offer a protection to probiotic bacteria against enzymatic degradation and acidic environment of the GI tract (Gardiner et al., 1998; Stanton et al., 1998). Dinakar and Mistry (1994) immobilised *Bifidobacterium bifidum* with an emulsification technique and the obtained gel beads were frozen and lyophilised. The addition of the immobilised cells in the cheese was not uniform, but the survival of bifidobacteria in the cheese was not affected. In fact, the cells remained viable until 24 weeks and did not affect the flavour and the flavour intensity, texture and appearance of the cheese. These observations can be explained by a lack of bifidobacteria metabolism. To produce acetic and lactic acid, bifidobacteria need substrates such as lactose but in this case it was not available. The low temperature ripening (between 6 and 7 °C) avoids bifidobacterial growth, whose optimal growth temperature is 37 °C, but they remain viable. The composition of the cheese was not affected by probiotic incorporation and the addition of bifidobacteria in Cheddar is valuable, since the bacteria can stay alive for at least 6 months. Others authors have also made the observation that incorporation of bifidobacteria into Cheddar cheese does not negatively impact cheese quality in aroma, flavour and texture (Stanton et al., 1998). Moreover addition of *E. faecium* strain may have a positive influence on cheese properties (Gardiner et al., 1999).

Spray-drying is another technology used to encapsulate probiotics before their incorporation into Cheddar cheese. The spray-dried culture was stable for 7 weeks while it was kept at room temperature and during refrigeration as well (Gardiner et al., 2002). Advantages are the cost-effectiveness and the applicability to large scale production compared to traditional methods like freezing or freeze-drying. However, 7 weeks does not seem to be sufficient when considering cheese ripening. Indeed, 5 weeks was defined as the minimum ripening time by the Codex Alimentarius. Overall, ripening time is longer than 5 weeks in order to develop cheese aroma for example.

Table 1
Examples of encapsulated probiotics and their applications in cheese.

	Probiotic strain	Technology	Materials	References
Fresh	*L. bulgaricus* *S. thermophilus*	Extrusion	Ca-alginate	Prevost and Divies (1987)
Cheddar	*B. bifidum*	Emulsification	κ-Carrageenan	Dinakar and Mistry (1994)
Fresh	*L. lactis spp. lactis*	Emulsification	κ-Carrageenan	Sodini et al. (1997)
Crescenza	*B. bifidum* *B. infantis* *B. longum*	Freeze drying	Ca-alginate	Gobetti et al. (1998)
Cheddar	*L. paracasei*	Spray-drying	Skim milk	Gardiner et al. (2002)
Cheddar	*L. acidophilus* *B. infantis*	Emulsification	Alginate/starch	Godward and Kailasapathy (2003a)
Feta	*L. acidophilus* *B. lactis*		Alginate	Kailasapathy and Masondole (2005)
Kasar	*L. acidophilus* *B. bifidum*	Extrusion and emulsification	Alginate	Özer et al. (2008)
White Brined	*L. acidophilus* *B. bifidum*	Extrusion and emulsification	Alginate	Özer et al. (2009)

Otherwise, it seems that the ME is not necessary for increasing the probiotic viability in Cheddar cheese but in the case of fresh cheese. For example, the ME is a good way of enhancing probiotic viability because of the low pH value of the product (Kailasapathy, 2002). This assertion was resumed in a study in which a comparison of viability was carried out between free and encapsulated probiotic cells in Cheddar cheese. Cheddar cheese was found as an ideal carrier for probiotics but the ME does not increase survival of probiotics. Moreover, the physical barrier inhibits the release of acids (produced by bacteria) and their accumulation in the bacterial surrounding leads to cell death (Godward and Kailasapathy, 2003a). Contradictory results were observed by Dinakar and Mistry (1994). Nevertheless, different probiotic strains were used and may not react in a similar way during the ripening period.

Encapsulated probiotic bacteria have also been incorporated in Crescenza cheese. This soft Italian cheese requires a brief ripening period. In this case, probiotic incorporation in the cheese did not require any change in flavour, appearance, and microbial and physico-chemical properties (Gobetti et al., 1998). Two probiotic strains (*L. acidophilus* and *B. Bifidum*) have been incorporated into Kasar cheese using extrusion or emulsification technology to encapsulate the cells. No difference was noticed between the two techniques when considering bacterial counts, proteolysis and organoleptical properties of the final product. Özer et al. (2008) concluded that ME can be a good way to enhance probiotic viability in Kasar cheese. In another study, Özer et al. (2009) introduced the same strains by the same techniques but in white-brined cheese. The use of probiotic cells in an encapsulated form enhances their viability and does not affect sensory properties of the cheese even though the ME has induced the formation of acetaldehyde and diacetyl.

Prevost and Divies (1987) described a process to continuously produce pre-fermented milk by means of entrapped cells in Ca-alginate particles. The final product had constant characteristics which were not the case with batch wise processing applied in the industry. In addition, the incubation time was reduced by 50% compared with standard starter culture fermentation. The feasibility of a continuous milk pre-fermentation process on an industrial scale was confirmed by Sodini et al. (1997) and also added that it could be easily automated.

4.1.2. Yogurt

The incorporation of probiotic living cells in yogurt enhances its therapeutic value (Chen and Chen, 2007; Weichselbaum, 2009). However, there is poor level of probiotic viability in yogurt because of the low pH (from 4.2 to 4.6). Studies have shown that the use of encapsulated probiotic bacteria was better for their survival. Furthermore, the incorporation of probiotic cells into yogurts could be carried out without making many modifications from the traditional process (Kailasapathy, 2009). Many authors used encapsulated probiotic cells to incorporate into yogurts (Table 2).

Firstly, the encapsulation of probiotic cells in beads comprised of gellan–xanthan gum mixtures is a way to increase their tolerance to acidic environments (Sun and Griffiths, 2000). The introduction of encapsulated probiotic cells in set yogurts appears to increase probiotic survival in the product. However, bacteria have low metabolic activity and there is poor acetic acid produced (Adhikari et al., 2000). This compound gives a sour sensory property to yogurt and the lack of it is considered as a defect. In another study, Adhikari et al. (2003) encapsulated bifidobacteria and incorporated them into stirred yogurt. However, consumers detected a grainy texture in these yogurts (size range particles about 22–50 μm). Even though the ME gives a protection to bifidobacteria in yogurt, the sensory quality affected which is a major problem for consumer acceptance.

Probiotic cells can be encapsulated with prebiotic ingredients (e.g. resistant starch) or cryoprotectants (e.g. glycerol) to improve their viability (Sultana et al., 2000; Capela et al., 2006). It has been shown that this technique enhances probiotic survival in the product but not under simulated GI conditions (Sultana et al., 2000).

The co-encapsulation is another way to enhance probiotic viability and consists in encapsulating two different probiotic bacterial strains together (Godward and Kailasapathy, 2003c). A comparison was made between survival of free and encapsulated cells. Encapsulation and co-encapsulation were found to increase survival and in particular, freeze-dried cells after encapsulation survived better in the yogurt. Thus, yogurt can be a good probiotic carrier if the cells are encapsulated. The protected bacteria which are in a viable state at the moment of consumption, will survive through the GI tract and arrive in the intestine in a viable state (Godward and Kailasapathy, 2003c).

Other studies have demonstrated the use of co-encapsulating two probiotic strains with prebiotics such as raftilose® in order to stimulate bacterial growth and to protect the cells against adverse environmental conditions. The co-encapsulation concept allows an increase of functional food efficiency thanks to the synergy between probiotic and prebiotic. The use of prebiotic ingredients is also a factor to enhance probiotic viability. It was

Table 2
Examples of encapsulated probiotics and their applications in yogurt.

Probiotic strain	ME technology	Materials	References
L. casei	Extrusion		Lacroix et al. (1990)
B. infantis	Extrusion	Gellan/xanthan gum	Sun and Griffiths (2000)
B. longum	Emulsification	κ-Carrageenan	Adhikari et al. (2000)
L. acidophilus	Emulsification	Alginate–starch	Sultana et al. (2000)
B. adolescentis	Emulsification	Alginate	Truelstrup-Hansen et al. (2002)
B. longum	Emulsification	κ-Carrageenan	Adhikari et al. (2003)
L. acidophilus *B. infantis*	Emulsification	Alginate/starch	Godward and Kailasapathy (2003c)
B. breve *B. longum*	Emulsification Spray-drying	Milk fat and whey protein	Picot and Lacroix (2004)
L. acidophilus	Extrusion	Ca-alginate	Krasaekoopt et al. (2004)
L. acidophilus *B. infantis*	Extrusion	Raftilose, raftiline and starch	Anjani et al. (2004)
L. acidophilus	Extrusion	Ca-alginate Chitosan	Iyer and Kailasapathy (2005)
L. acidophilus *L. casei* *L. rhamnosus* *B. infantis*	Emulsification	Alginate	Capela et al. (2006)
L. acidophilus *B. lactis*	Emulsification	Alginate/starch	Kailasapathy (2006)
L. acidophilus *B. bifidum* *L. casei*	Extrusion	Alginate–chitosan	Krasaekoopt et al. (2006)
L. acidophilus *B. longum*	Spray-drying	Maltodextrin/gum arabic	Su et al. (2007)
L. acidophilus	Extrusion	Alginate–chitosan	Urbanska et al. (2007)
L. acidophilus *B. lactis*	Extrusion		Kailasapathy et al. (2008)
L. acidophilus *B. lactis*	Extrusion		Mortazavian et al. (2008)
L. casei	Extrusion	Alginate/pectin	Sandoval-Castilla et al. (2010)

also shown that coating the capsule with chitosan gives better protection to probiotic cells than alginate when considering survival in yogurt and in simulated GI conditions (Anjani et al., 2004; Iyer and Kailasapathy, 2005).

Whey proteins have been used to encapsulate bifidobacteria and it was shown that this technique could be useful for the delivery of viable probiotic cells to the GI tract, when incorporated into dairy fermented products. However, spray-drying technology used in the study requires high temperature so, it is important to consider strain properties for heat resistance (Picot and Lacroix, 2004).

Kailasapathy (2006) explained that the incorporation of capsules containing probiotic cells did not significantly alter yogurt's properties such as appearance, colour, flavour, taste and acidity. In contrast, by incorporating probiotic cells, textural properties were affected, in particular smoothness and consumers have detected grittiness in yogurts. Concerning acidity, it was shown that the addition of probiotic cultures slows down the post-acidification during the storage of yogurt. In another study, Kailasapathy et al. (2008) demonstrated a correlation between post-storage pH and the survival probiotic bacteria which is negatively affected by the presence of fruit pulp. However, all the obtained yogurts contained the recommended levels of probiotic bacteria even after 35-day shelf life.

The ME allows the delivery of a high number of bacteria to desired targets in the GI tract and the capsules containing probiotic cells seems to be good for oral administration by cell therapy (Urbanska et al., 2007). However, the technology used to encapsulate the cells and the probiotic strain, are two important parameters which significantly influence encapsulation yield (Picot and Lacroix, 2004). Talwalkar and Kailasapathy (2004a) observed that a beneficial effect of ME on probiotic cell survival occurs when there is oxygen in the medium, and the microenvironment in this case has a lower oxygen level. Champagne and Fustier (2007b) hypothesised that discrepancies in cell viability between some studies could be due to various oxygen sensitivities between the strains or to different oxygen levels in yogurts. Another reason could be the type of coating material (e.g. chitosan) or the addition of starch in alginate core which improves the viability of the cells. Finally, ME of probiotics for addition into yogurts appears to prevent losses of oxygen-sensitive strains more than protecting the cells against acidic environment (Talwalkar and Kailasapathy, 2004b).

To conclude this part, the addition of encapsulated probiotic cells into yogurts is clearly detected in the mouth by the consumer. However, according to Champagne and Fustier (2007b), the effects on sensory properties can become desirable if the consumer is forewarned and expects the presence of the particles.

4.1.3. Frozen dairy dessert

It is not easy to incorporate probiotic microorganisms into frozen desserts because of high acidity in the product, high osmotic pressure, freeze injury and exposure to the incorporated air during freezing (Chen and Chen, 2007). The introduction of probiotic bacteria in an encapsulated form into frozen desserts (Table 3) may overcome these difficulties and could produce useful markets and health benefits (Chen and Chen, 2007). Entrapment of lactobacilli in Ca-alginate provides a higher survival rate (40%) compared to free cells, when freezing ice cream (Sheu and Marshall, 1993;

Table 3
Examples of encapsulated probiotics and their applications in frozen dairy dessert.

Probiotic strain	ME technology	Materials	References
L. bulgaricus	Emulsification	Alginate	Sheu and Marshall (1993)
L. casei *B. lactis*	Emulsification	Alginate	Sheu et al. (1993)
L. acidophilus *B. infantis*	Emulsification	Alginate/starch	Godward and Kailasapathy (2003b)
L. acidophilus *B. lactis*	Emulsification		Kailasapathy and Sultana (2003)
L. casei *B. lactis*	Emulsification	Ca-alginate	Homayouni et al. (2008)

Sheu et al., 1993). Godward and Kailasapathy (2003b) studied the incorporation of probiotic cells in ice cream in different states. In fact, the cells can be free, freshly encapsulated, encapsulated and freeze-dried and finally co-encapsulated and freeze-dried. The results have shown that free cells survive better than encapsulated cells. Freshly encapsulated probiotic cells had greater survival than those which were freeze-dried after encapsulation and co-encapsulation of *L. acidophilus* and *B. bifidum* enhances the survival of both strains. Finally, addition of probiotics does not affect air incorporation into ice cream.

Another study demonstrated that there was no significant difference between the survival of encapsulated probiotic bacteria and the free cells. The protection of free cells in ice cream may be due to the high total solids making ice cream a suitable food for delivering probiotic living cells to the consumer (Kailasapathy and Sultana, 2003).

The encapsulation of probiotic cells enhances their viability when incorporated into ice cream and this addition had no effect on the sensory properties of the product. The high rate of total solid encountered in ice cream, and resistant starch added as prebiotic, provide further protection for probiotics in this case (Homayouni et al., 2008). The number of viable probiotic cells was between 10^8 and 10^9 CFU/g after three months of storage while the International Dairy Federation (IDF) recommended a viable number of 10^7 CFU/g in food product at the time of consumption (Homayouni et al., 2008).

4.1.4. Other food products

Most of the products containing probiotic cells are dairy products and it is necessary to develop other food carrier for probiotics owing to lactose intolerance in certain populations (Ranadheera et al., 2010). Efforts have been made to identify new food carriers (Table 4).

For example, good quality mayonnaise was obtained when incorporating encapsulated bifidobacteria. Calcium alginate provides protection for bifidobacteria against the bactericidal effects of vinegar. Other advantages can be quoted when considering the use of encapsulated probiotic cells such as growth inhibition of yeasts over 10 weeks (probably due to the antibacterial effect of the probiotics) and the improvement of mayonnaise's sensory

Table 4
Examples of encapsulated probiotics and their applications in various food systems.

	Probiotic strain	ME technology	Materials	References
Cream	*L. lactis*	Extrusion	Ca-alginate	Prevost and Divies (1992)
Mayonnaise	*B. bifidum* *B. infantis*	Emulsification	Alginate	Khalil and Mansour (1998)
Dry beverage	*Bifidobacterium* PL1	Spray-drying	Starch	O'Riordan et al. (2001)
Banana	*L. acidophilus*	Extrusion	κ-Carrageenan	Tsen et al. (2004)
Soft foods	*B. lactis*	Extrusion	Gellan/xanthan gum	McMaster et al. (2005)
Tomato juice	*L. acidophilus*		Ca-alginate	An-Erl King et al. (2007)
Sausages	*L. reuteri*	Extrusion Emulsion	Alginate	Muthukumarasamy and Holley (2006)
Sausages	*L. reuteri* *B. Longum*	Extrusion	Alginate	Muthukumarasamy and Holley (2007)
Biscuits Cranberry and vegetable juices	*L. rhamnosus*	Extrusion	Whey protein	Ainsley Reid et al. (2007)
Orange and apple juices	*L. rhamnosus* *L. salivarius* *B. longum* *L. plantarum* *L. acidophilus* *L. paracasei* *B. lactis*	Emulsification		Ding and Shah (2008)
Chocolate	*L. helveticus* *B. longum*	Spray-coating	Fatty acids	Maillard and Landuyt (2008)
Swine feeding	LAB	Extrusion	Ca-alginate	Ross et al. (2008)
Tomato Juice	*L. acidophilus*	Extrusion		Tsen et al. (2008)
Chocolate	*L. helveticus* *B. longum*	Spray-coating		Possemiers et al. (2010)

properties (Khalil and Mansour, 1998). McMaster et al. (2005) produced beverages and soft foods containing viable encapsulated probiotic cells with a range size of 20–2200 μm by an extrusion method.

The fermentation of banana media by using encapsulated probiotic cells was carried and the obtained product is proposed to be a synbiotic (Tsen et al., 2004). The possibility to use encapsulated probiotic cells in the fermentation of tomato juice was demonstrated. ME allows probiotic cells to survive against the unfavourable pH encountered in tomato juice. Furthermore, the sensory quality of the product has been improved upon incorporation of encapsulated cells compared to free cells (An-Erl King et al., 2007; Tsen et al., 2008).

The potential use of ME to protect the cells in meat products was investigated (Muthukumarasamy and Holley, 2006, 2007). The introduction of encapsulated probiotic cells into dry fermented sausages did not affect sensory properties of the product. Moreover, the viability of encapsulated probiotic cells was improved compared to free cells. In addition, it was shown that probiotics could reduce *E. coli* O157:H7 in number but ME decreased this potential.

The incorporation of probiotic cells encapsulated by spray-coating technology, has been carried out in chocolate (Maillard and Landuyt, 2008). According to these authors, probiotic viability in the small intestine was three times higher when incorporated in chocolate than in dairy product. In this case, probiotic chocolate process was transposed to a larger scale but the challenge here was to obtain a process which is compatible with probiotic survival because high temperatures are required in the usual process. Possemiers et al. (2010) also incorporated encapsulated probiotic cells in chocolate. Results have shown that the introduction of encapsulated probiotic strains into chocolate can be an excellent solution to protect them from environmental stress conditions. In chocolate, the lipid fraction of cocoa butter was shown to be protective for bifidobacteria (Lahtinen et al., 2007).

The encapsulation of probiotic cells in whey protein gel particles could offer protection during processing and storage, as well as extending the food applications to biscuits, vegetable and frozen cranberry juice. Proteins have a protective effect on probiotics and ME is beneficial because it creates a microenvironment appropriate to survival of the cells against adverse conditions (e.g. low pH in cranberry juice) (Ainsley Reid et al., 2007).

Using protein-based technology to encapsulate probiotic cells is an alternative to ME with alginate-type gels or spray-coating which are the two most common encapsulation methods.

4.1.5. Importance of food carrier

Various product factors can affect probiotic growth and survival, such as concentration of proteins, sugar and fat, and pH levels (Ranadheera et al., 2010).

Godward and Kailasapathy (2003a,b,c) applied the same condition to encapsulate probiotic cells but they introduced them into various food matrices: Cheddar cheese, yogurt and ice cream. The conclusion of each study was different. In Cheddar cheese and ice cream encapsulation is not necessary for the survival of the cells whereas it is important when considering yogurt. This showed that the food carrier is important for probiotic survival.

Cheddar cheese and yogurt are both able to protect probiotic cells by giving a favourable environment during manufacturing and storage (Sharp et al., 2008). However, Cheddar cheese seems to be better than yogurt as a delivery food for probiotic because cells were better able to resist the low pH in the stomach.

A potential breakthrough in probiotic encapsulation was reported by investigating the capabilities of various prebiotic fibres to protect the stability and viability of probiotic *L. rhamnosus* strains. In fact, this protection has been revealed during freeze-drying, storage in freeze-dried form and after formulation into apple juice and chocolate-coated breakfast cereals (Saarela et al., 2006). In this case, fibres are considered as prebiotics because they are non-digestible components and they stimulate probiotic viability (Gibson and Roberfroid, 1995). This study highlighted the fact that the optimal carrier for probiotic might depend, in part on how it is eventually used.

Rößle et al. (2010) dipped apple wedges into a solution containing probiotic cells. The resulted product was acceptable in terms of quality and the number of probiotics was sufficient to have a beneficial effect. However, the authors concluded that the use of encapsulation would be useful to enhance probiotic resistance.

The importance of choosing the matrix has been discussed in the study of Klayraung et al. (2009). These authors described how the production of a tablet with lyophilised bacterial cells is carried out. In fact, the matrix influences the survival of the probiotic cells in the stomach. For example, sodium alginate allows higher cell survival in simulated GI conditions and Ross et al.

Table 5
Examples of the use of encapsulated probiotics for industrial applications.

Food product	Company	Observations
Probiotic chocolate	Institut Rosell & Lal'food	Using the Probiocap® ME technology
Probio'stick		
Innovance Probiotique	Ysonut Laboratories	
Yogurt	Balchem Encapsulates & Institut Rosell	
Nutrient Bars		
Tablets		
Chewing gum	Cell Biotech	
Cernivet® LBC ME10	Cerbios-Pharma SA	Using for animal food
Bio-*tract*® tablets	Nutraceutix	
Probio-Tec® capsules	Chr Hansen	
Probiotic whey drink		German market
Orange juice "Dawn"	Chr Hansen & Kerry Group	Irish market
Probiotic ice cream	Dos Pinos	Central American Industry
Doctor-Capsule (Yogurt)	Bingrae Co.Kyunggi-do	Korean market
Attune (Chocolate)	DMS Food Specialities	American market
Yogurt	Jinta Capsule Technology	
Bifina-constipation		Pharmaceutical product
Geneflora™	BioPlus Corporation	Symbiotic product
Granio + reducys®	EA Pharma®	Combination of probiotic strain and Cranberry
ThreeLac™	GHT™ Global Health Trax	Powder sprinkled into the mouth

(2008) highlighted advantages of this technique as its ease of treatment, nontoxic nature and low cost. Efforts have been made to innovate in term of food matrices to incorporate probiotics.

4.2. Recent developments in the industrial field

During the past few years, food products containing encapsulated probiotic cells have been introduced on the market (Table 5).

Institut Rosell and Lal'food with the Probiocap® technology have developed new food products like probiotic chocolate and Probio'Stick. Probio'Stick is an orodispersible powder which contains *Bifidobacterium* and *Lactobacillus* strains. The product allows a reduction of physical symptoms related to stress (Diop et al., 2008), particularly abdominal pain, nausea and vomiting.

In 2007, Barry Callebaut developed a process to produce chocolate containing encapsulated probiotic cells with the Probiocap® technology in partnership with Lal'food. According to Barry Callebaut, the addition of encapsulated probiotic cells has no influence on chocolate taste, texture and mouth feel. A consumption of 13.5 g per day of probiotic chocolate seems to be sufficient to ensure the balance of the intestinal microflora.

Chocolate has also been used by DSM Food Specialities which produces a bar called Attune launched in the United States in January 2007. The product also contains the prebiotic inulin which supports a healthy digestive function. Attune's innovative product line is found in the refrigerated yogurt section and advertising of this product highlights more input in calcium, fibre and less sugar than in most yogurts (www.attunefoods.com).

After two years of collaboration, Balchem Encapsulates and Institut Rosell, have developed a stabilised form of encapsulated probiotics (Balchem newsletter, August 16, 2001). Institut Rosell has incorporated encapsulated probiotic cells into yogurt-covered raisins, nutrient bars, chocolate bars and tablets (Siuta-Cruce and Goulet, 2001). According to information available on the website of Balchem, the clinical testing has revealed "an unprecedented 100% delivery rate". The tests carried out on chocolate bars also revealed a high recovery rate (www.balchem.com).

Chr Hansen distributes Probio-Tec® capsules for dietary supplement, infant formula and pharmaceutical industry (www.chr-hansen.com). With the Kerry Group in Ireland, they have developed the first probiotic orange juice "Dawn" for the Irish market. According to these companies, the probiotic cells remain viable throughout the product's shelf life. The use of encapsulated probiotic cells can be better suited to survive harsh conditions in juices (www.chr-hansen.com).

In Latin America, Chr Hansen and Dos Pinos, one of the top players in Central American ice cream industry, have developed a probiotic ice cream. The product is described as an innovative yogurt ice cream with a number of health benefits (www.chr-hansen.com). However, a probiotic ice cream has already been launched by Unilever in 1999 but at that time, consumers were unwilling to accept this innovation. Thus, taking into consideration the expectations of consumers is an important factor in creating new probiotic food products.

In Korea, yogurts containing encapsulated LAB are available on the market under the brand name Doctor-Capsule (Bingrae Co.Kyunggi-do) as described by Lee and Heo (2000). Jinta Capsule Technology markets a range of products containing encapsulated probiotic cells. For example, yogurt containing encapsulated bifidobacteria and bifina-constipation a pharmaceutical product which contains encapsulated bifidobacteria available in a capsule form.

Most of the products containing encapsulated probiotic cells are available in a tablet/capsule form (Forever Active Probiotic®, Probiotic 7, Multi-probiotic) or in a powder form (PureBaby Probiotic, ThreeLac™). ThreeLac™ proposed by GHT™ Global Health Trax, is a powder containing encapsulated probiotic cells, and which has to be sprinkle into the mouth. Encapsulation technology used here ensure the probiotic cells to get through the hostile stomach acids to reach the intestine (www.ghthealth.com).

Ysonut Laboratories markets Innovance Probiotiques, a nutraceutical with probiotic cells encapsulated by the Probiocap® technology. This product contains a mix of three probiotic strains produced by Institut Rosell (www.ysonut.com).

Cernivet® LBC ME10 distributed by Cerbios-Pharma SA is a pelletable microbial feed for the stabilisation of intestinal microflora and containing the encapsulated probiotic strain *E. faecium* SF68®. The product is registered for use in animal food, for example for chicken and pig fattening. The shelf life of Cernivet® LBC ME10 covers a period of 24 months if stored at refrigerated temperature (www.cerbios.ch).

Nutraceutix is an American company which provides probiotic cells in a variety of forms, from bulk powder to capsules and advanced tablets. Patented Bio-*tract*® tablets gives protection for probiotic living cells against adverse conditions in the stomach (low pH) (www.nutraceutix.net).

America's BioPlus Corporation proposed Geneflora™, a synbiotic containing encapsulated *Lactobacillus sporogenes* as a probiotic and a fructo-oligosaccharide as a prebiotic (www.yeastbuster.com).

Capsules containing cranberry (Granio + reducys®) were marketed by EA Pharma® and present recognised effects on urinary disorders as cystitis. In this product, encapsulated probiotic cells have been incorporated and the chosen strain has positive effects on urinary flora. The combination of these two ingredients makes this product particularly useful in preventing cystitis (www.ea-pharma.com).

Nowadays, encapsulated probiotic cells are essentially introduced in nutraceutical products, but efforts have been made to develop novel food as an ideal carrier for the bacteria.

5. Futures trends

The ME technology has been explored by many companies as a way of enhancing the resistance of probiotic cells in the GI tract and for prolonging the shelf-life of bacterial strains in food products.

In most cases, to encapsulate probiotic living cells, natural biopolymer such as alginate, κ-carrageenan or gellan gum have been used. However, although the results are promising on a laboratory scale, the technologies used present difficulties for scaling-up. For example, in the case of extrusion methods, low production capacities and large particle sizes have been reported. Regarding the emulsion method, large-size dispersions have been obtained. Another major problem is that the addition of some polysaccharides is not allowed in dairy products in some European countries (Picot and Lacroix, 2004).

Most of the foods containing probiotic microorganisms are found in the refrigerated section of supermarkets this being due to the fact that the bacteria are sensitive and can be destroyed by heat. Thus, the dairy sector has a major advantage in probiotic foods. Nevertheless, the researches focus actually on expanding the food categories currently available.

ME has to face many challenges for its application on an industrial scale. On one hand, technological challenges to obtain microcapsules with the best properties must be enhanced. On the other hand, consumer behaviour towards novel foods should be taken into account. ME can achieve a wide variety of functionalities according to the development of the technology and nowadays, encapsulated probiotic cells can be incorporated in many types of food products. In fact, probiotics can be found not only in dairy products, but also in chocolate or cereals too.

An important challenge for probiotic encapsulation is to reduce the particle size because it can negatively affect the textural and the sensorial properties of the product. In laboratory applications, the chosen method is generally emulsification but this technique presents disadvantages for food applications for many reasons. The presence of residual oil on capsule surface is detrimental to the texture and the organoleptic properties of the product. The presence of residual oil hampers capsules incorporation in diet products and the residual oil, surfactant, or emulsifier can be toxic for probiotic cells.

The incorporation of probiotic cells into a wider range food product is limited by an unsuitable food matrix (e.g. low pH or competing microbes) and the non-optimal storage conditions. During the past few years, the use of prebiotics and fibre as protectant for probiotic cells has gained increasing interest.

The development of novel functional foods is a major challenge to address the expectation of consumers who expect healthy and beneficial food products for their health. Industries and laboratories have now to provide possible technologies to an industrial scale with adequate cost. The last decade, efforts were done to improve relations between these two entities. Nevertheless, further researches have to be carried out to optimise the use of encapsulated probiotic cells while considering numerous factors as safety and ecological production. In conclusion, it is evident that the probiotic market has a strong future as the consumers demand is increasing. As benefits provided by probiotics are now well documented, consumer requirements for food, beverage and supplement products enriched with these ingredients will increase.

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MÉDECINE ET NUTRITION

Maldigestion du lactose : formes cliniques et solutions thérapeutiques

Lactose maldigestion: Clinical presentation and treatment

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MOTS CLÉS
β-galactosidase ;
Lactose ;
Bactéries probiotiques

Résumé Le lactose des produits laitiers est digéré au niveau de l'intestin grêle par une enzyme membranaire, la lactase. Pour une grande partie de la population adulte mondiale, il existe un déclin de l'activité lactase et qui conduit à une malabsorption partielle du lactose ingéré. Pour continuer à consommer des produits laitiers et ne pas s'exposer à des carences en calcium ou vitamine D, plusieurs solutions peuvent être envisagées. C'est le cas principalement des yaourts dont les ferments ont prouvé leur efficacité sur la digestion du lactose. Il existe également des auxiliaires technologiques qui vont permettre d'hydrolyser ou supprimer le lactose présent dans les produits alimentaires. Pour finir, les compléments alimentaires peuvent avoir un rôle positif sur la digestion du lactose à condition que leur prise coïncide avec la consommation des aliments contenant du lactose. Toutes ces solutions visent donc à maintenir la consommation des produits laitiers chez des personnes présentant une maldigestion du lactose.

KEYWORDS
β-galactosidase;
Lactose;
Probiotic bacteria

Summary The lactose of dairy products is digested in the small intestine thanks to a membrane enzyme named lactase. For a large part of the world adult population, there is a decline in lactase activity and this leads to a partial malabsorption of the ingested lactose. In order to continue to consume dairy products and to avoid deficiency in calcium and vitamin D, several solutions can be considered. This is the case of yogurt that contains bacteria which have proven effective in lactose digestion. There are also processing aids that allow lactose hydrolysation or lactose remove from food products. Finally, dietary supplements can have a positive effect on lactose digestion if they are consumed at the same time of food products that contain lactose.

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All of these solutions are therefore intended to maintain the consumption of dairy products in people with lactose maldigestion.

- L'intolérance au lactose est fréquente, est facile à traiter et peut être gérée.
- Il est possible de consommer des produits laitiers même en cas de maldigestion ou d'intolérance au lactose.
- Les individus qui ne consomment pas suffisamment de produits laitiers se privent de nutriments importants comme le calcium, la vitamine D ou le potassium, ce qui augmente leur risque face à certaines maladies chroniques comme l'ostéoporose.

Introduction

Le lactose est le principal glucide du lait. Dans la vie du nouveau-né, il constitue une source essentielle de calories. Plus tard, dans la vie de l'enfant et de l'adulte, il est présent dans l'alimentation quotidienne dans le lait mais aussi dans les produits laitiers (Tableau 1).

Le lactose, qui possède des propriétés technofonctionnelles intéressantes, peut également être retrouvé dans certaines préparations industrielles comme les confiseries, les biscuits, les soupes et sauces, ou encore dans certains médicaments.

Pour les personnes présentant une malabsorption du lactose, cela signifie une vigilance accrue sur la composition des aliments. Or, la présence de lactose dans l'aliment n'est pas toujours explicitement mentionnée sur l'emballage. C'est le cas par exemple du caramel. De même, la législation n'impose pas de déclarer la présence de lactose en deçà d'un certain pourcentage.

La consommation de produits contenant du lactose, chez des personnes présentant une non-persistance de la lactase, peut provoquer des troubles variables, principalement digestifs [3]. Ces troubles peuvent avoir différentes origines [4] :

- la « malabsorption primaire » est liée au déclin physiologique de l'activité lactasique des entérocytes. Ce déclin est naturel ; l'activité lactasique étant maximale à la naissance, elle décroît naturellement quand l'enfant grandit. Selon les personnes, ce déficit peut être plus ou moins important à l'âge adulte. On parle alors d'hypolactasie ;
- la « malabsorption secondaire » est liée à des situations pathologiques qui peuvent être réversibles ou chroniques.

Tableau 1 Composition moyenne en lactose de quelques produits laitiers [1,2].

Aliments contenant du lactose	Lactose (g.100/g)
Lait liquide	4—5
Yaourt nature	5
Crème	3
Glace aux fruits	5—6
Fromage frais	3,5
Fromage fondu	~3
Beurre	0,5
Fromage pâte molle	Traces

De nombreuses personnes se définissent actuellement comme « intolérantes au lait ». Pourtant, nous développerons dans la première partie de ce rapport une différence importante entre maldigestion et intolérance clinique au lactose. Dans tous les cas, le nombre de personnes concernées par le problème de malabsorption du lactose est extrêmement élevé. Ce taux varie en fonction des pays mais représente plus de 70 % de la population mondiale [5]. Le problème est que cette population a tendance à supprimer les produits laitiers qui sont pourtant source de calcium et de vitamine D. Ces individus ont donc tendance à être en deçà des doses recommandées en calcium et vitamine D et sont donc exposés au risque d'une diminution de la densité minérale osseuse. Dans la suite de ce rapport, nous présenterons les solutions disponibles, qu'il s'agisse de l'ingestion de bactéries probiotiques ou de lactase exogène et qui permettent une amélioration de la digestion du lactose.

La malabsorption du lactose

Définition du lactose et principes régulant sa digestion

Le lactose est un disaccharide (sucre) comprenant une molécule de glucose et une molécule de galactose. Il n'est pas absorbable tel quel par l'intestin et doit donc d'abord être digéré dans l'intestin grêle. La β-galactosidase est l'enzyme responsable de la digestion du lactose, aussi nommée lactase. Il s'agit d'une enzyme tétramérique composée de quatre sous-unités identiques et inactives séparément. Elle hydrolyse le lactose en libérant du glucose et du galactose de façon équimolaire. Si cette enzyme n'est pas active ou moins active, il s'ensuit des troubles divers mais principalement digestifs. Il est important de différencier la malabsorption primaire, la malabsorption secondaire et l'intolérance au lactose. En effet, l'amalgame est courant chez la plupart des sujets. Ainsi, de nombreuses personnes pensent être intolérantes au lactose alors que ce n'est pas le cas. Ces sujets vont alors consommer moins de produits laitiers et s'exposer à un risque accru de désordres osseux [6].

Pour être absorbé au niveau de l'intestin, le lactose doit d'abord être hydrolysé en glucose et galactose par la lactase. L'enzyme est localisée dans la bordure en brosse des entérocytes, les cellules qui tapissent la muqueuse intestinale. Son activité dépend de l'âge des cellules. Ainsi elle est fortement active dans les cellules les plus âgées et peu active dans les plus jeunes. Cela explique pourquoi les personnes atteintes d'une inflammation locale (une gastroentérite par exemple) et ayant un renouvellement cellulaire accéléré présentent souvent une hypolactasie. Sa répartition est également variable le long de l'intestin grêle.

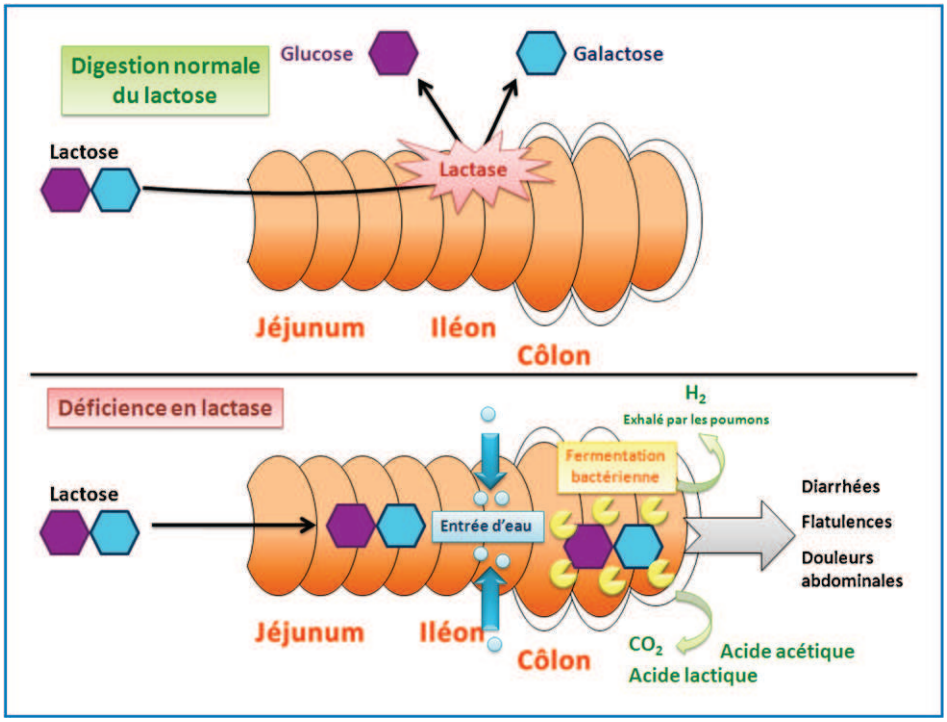

Figure 1. Physiologie de l'intolérance au lactose.

Elle est maximale dans le jéjunum et faible dans le duodénum et l'iléon [7].

Quand l'activité de la lactase est moindre ou absente, une proportion du lactose ingéré est alors non digérée. Cette fraction va parvenir au niveau du côlon où elle sera fermentée par le microbiote intestinal. La fermentation du lactose va entraîner la formation de lactate, d'acides gras volatils (propionate, acétate, butyrate), de gaz (hydrogène, gaz carbonique, méthane) ainsi qu'une acidification du milieu (Fig. 1). En parallèle, le lactose provoque une augmentation du volume liquidien (par transfert d'eau de la muqueuse vers la lumière intestinale) qui conduit à une dilatation de l'intestin et une accélération du transit avec perte d'eau et d'électrolytes [2].

Les symptômes cliniques sont très variés. Cela peut aller de simples maux de ventre à des vomissements. Le Tableau 2 répertorie les symptômes digestifs et extradigestifs associés à une malabsorption du lactose.

Place de l'évolution génétique dans la non-persistance de la lactase

La malabsorption du lactose concerne 10 à 50 % des adultes en France, ces chiffres varient d'une étude à l'autre [3,5,7]. De fortes disparités sont également à noter entre le Nord et le Sud de notre pays. Dans certaines populations humaines, la lactase ne disparaît pas à l'âge adulte. Ainsi, ces populations peuvent continuer de digérer normalement le lait frais toute leur vie. Cette capacité est liée à une mutation sur le chromosome 2 (survenue 4000 ans avant J.-C.). L'influence de cette mutation est visible chez la plupart des Européens (qui peuvent continuer à ingérer du lait à l'âge

Tableau 2 Symptômes cliniques pouvant être rencontrés lors de la malabsorption du lactose.

	Symptômes les plus fréquemment rencontrés	% de patients présentant le symptôme
Symptômes digestifs	Douleurs abdominales	100
	Crampes	100
	Borborygmes	100
	Flatulence	100
	Diarrhées	70
	Constipation	30
	Nausées - vomissement	78
Symptômes extradigestifs	Maux de tête	86
	Problèmes de concentration	82
	Fatigue chronique	63
	Douleurs musculaires	71
	Douleurs articulaires	71
	Arythmie cardiaque	24
	Ulcérations buccales	30
	Signes allergiques	40

Tableau 3 Prévalence du déficit primaire en lactase dans différentes populations [2].

Pays	Prévalence (%)
Pays nordiques (Suède...)	2
Angleterre	25
France (Nord)	12
France (Sud)	50
Italie, Grèce	70
États-Unis (population blanche)	6
États-Unis (population noire)	70
Japon, Thaïlande, Afrique	100

adulte) alors que certaines populations deviennent totalement intolérantes au lactose (Tableau 3).

Le gène de la lactase (*Lct*) est localisé sur le bras long du chromosome 2 [8], il comporte 17 exons et s'étend sur 55 kb [9]. Son ARN messager code pour un peptide immature de 210—220 kDa [10] dont le peptide précurseur est glycosylé puis clivé au moment de son insertion dans la membrane plasmique en tant que peptide mature de 160 kDA [11]. La comparaison de séquences ADN chez des sujets adultes présentant une forte ou une faible activité lactasique a révélé une similitude dans la séquence, à l'exception de quelques mutations ponctuelles mais qui n'affectent pas la séquence des acides aminés [9]. La variation d'activité lactasique a été attribuée à une différence dans la régulation transcriptionnelle du gène. En particulier, l'hypolactasie a été associée au polymorphisme d'un nucléotide situé en amont du gène de la lactase et qui est transmise selon le mode autosomique récessif. C'est le cas du variant ADN (C/T $_{-13910}$) pour lequel une cytosine (C) est remplacée par une thymidine (T) à cette position et qui conduit à une persistance de la lactase. Un génotype CC est associé à une non-persistance de la lactase alors que les génotypes CT et TT correspondent plutôt à une persistance de la lactase. Un second polymorphisme nucléotidique impliquant le changement d'une adénine (A) par une guanine (G), G/A $_{-22018}$, est également associé dans la non-persistance de la lactase. Le phénotype GG correspond à une non-persistance de la lactase alors que les phénotypes AA sont associés à une persistance de la lactase. La plupart des individus avec un génotype hétérozygote GA présentent également une persistance de la lactase. La mutation T $_{-13910}$ semble être le variant en cause pour la persistance de la lactase chez les Européens mais cette mutation n'a pas été observée chez des populations africaines qui présentent pourtant une forte prévalence dans la persistance de la lactase. C'est le cas par exemple des peuples Dinka et Nuer du sud du Soudan ou Maasai du Kenya et de Tanzanie [12—15]. Ainsi, trois nouveaux variants ont été associés à la persistance de la lactase pour ces populations. L'indépendance de l'origine des variants nucléotidiques associés à la persistance de la lactase chez les Européens et les Africains est un exemple d'évolution parallèle due à une forte pression sélective résultant de traits culturels communs comprenant la domestication des animaux et la consommation de produits laitiers à l'âge adulte [15].

Malabsorption primaire

Trois formes de malabsorption primaire du lactose existent :
- la non-persistance de la lactase liée à l'ethnie ;
- la déficience en lactase congénitale ;
- le développement d'une déficience en lactase [4].

Tableau 4 Âge du déclin naturel de la lactase intestinale [2].

Pays	Âge (ans)
Pays nordiques	10—15
Japon	6—8
Afrique	1—6
Thaïlande	Avant 2

La première forme est la plus courante et varie en fonction de l'âge, du sexe, de l'origine ethnique (Tableau 3). L'âge du déclin naturel de la lactase intestinale est également variable selon les pays (Tableau 4). La non-persistance de l'activité lactasique est donc plus ou moins rapide et plus ou moins importante [7].

Pour terminer, la troisième forme de malabsorption rencontrée résulte d'une activité lactasique amoindrie chez des bébés prématurés (moins de 32 mois de gestation). Dans un premier temps, ces enfants vont compenser ce problème par leur microbiote intestinal (ß-galactosidase +) et ne présenter les troubles que tardivement [16].

À ce jour, aucun moyen physiologique ou thérapeutique ne permet d'augmenter l'expression de la lactase intestinale chez l'homme.

Malabsorption secondaire

La malabsorption secondaire résulte d'une maladie intestinale avec lésions histologiques pouvant relever d'étiologies variables (maladie de Crohn, infections bactériennes, traitements par irradiation, syndrome du côlon irritable...) [17]. Le cas le plus courant d'intolérance secondaire concerne les diarrhées infectieuses, principalement les diarrhées virales (adénovirus, rotavirus...). Ce type de malabsorption peut donc être passager ou irréversible [7].

Dans toutes ces situations, la muqueuse intestinale est partiellement lésée, ce qui entraîne un renouvellement cellulaire important [4]. Nous avons vu que seuls les entérocytes les plus âgés pouvaient sécréter la lactase. Par conséquent, les sujets présenteront une hypolactasie plus ou moins prononcée. Curieusement, il a été mis en évidence que la malabsorption pouvait persister des mois alors même que le sujet était guéri, d'où l'intérêt d'une supplémentation en lactase dans ces cas là [16].

Intolérance au lactose

L'intolérance résulte de la malabsorption du lactose et est directement liée à la dose de lactose absorbé. Ainsi, certains sujets intolérants au lactose pourront ressentir les symptômes même en cas d'ingestion d'une faible dose de lactose (par exemple 3 g) alors que d'autres ne les ressentiront qu'à partir d'une dose de 96 g [18]. Quand la quantité de lactose dépasse la capacité de digestion de la lactase, les symptômes apparaissent. L'intolérance vraie au lactose est relativement rare et variable selon les études de 6 à 10 % de la population [1]. Pour mesurer l'intolérance au lactose, les études se basent sur l'ingestion de 12 g de lactose, soit 250 mL de lait. Ainsi, la plupart des intolérants au lactose peuvent absorber jusqu'à 7 g de lactose sans développer de symptômes d'intolérance.

Allergies au lait

Les symptômes liés aux allergies et à l'intolérance peuvent souvent se confondre. Toutefois, les mécanismes sont totalement différents. L'allergie fait intervenir un mécanisme immunitaire alors que l'intolérance est due à un déficit enzymatique. Une allergie au lait est bien plus grave qu'une intolérance au lactose. L'allergie au lait est en fait associée à l'allergie aux protéines laitières et non directement au lactose. L'allergie aux protéines de lait de vache concerne 3 % des jeunes enfants et guérit avant l'âge de six ans dans 90 % des cas. Cette allergie est donc rare chez l'adulte, positionnant le lait en 30e position dans la liste des allergènes alimentaires, bien loin derrière les prunoïdées, ombellifères, fruits à coques et céréales [1].

Méthodes de diagnotic de la malabsorption du lactose

Le diagnostic de la malabsorption peut être réalisé avec un test au lactose et une mesure de l'hydrogène expiré [19]. Ce test évalue la présence d'hydrogène dans l'air expiré par le patient avant et après ingestion de 20 g de lactose (Fig. 2).

En effet, une partie du lactose non digéré est transformé en gaz. L'hydrogène va traverser la paroi colique, puis rejoindre les poumons via la circulation sanguine. Il sera finalement exhalé. Ainsi, l'hydrogène présent dans l'air expiré est retrouvé en quantité proportionnelle au lactose arrivant dans le côlon et donc inversement proportionnelle au degré de digestion du lactose dans l'intestin grêle.

Le test respiratoire au lactose marqué ou C^{13} est moins répandu et plus coûteux. Sa sensibilité et sa spécificité sont supérieures au test à l'hydrogène.

Le test de tolérance au lactose mesure la glycémie à jeun puis 30 minutes après ingestion de 50 g de lactose. Une digestion normale entraîne une élévation d'au moins 0,18 g/L de la glycémie alors qu'une malabsorption va entraîner des taux inférieurs [3].

Amélioration des symptômes liés à la malabsorption du lactose

Le traitement de la malabsorption/intolérance au lactose comprend quatre principes généraux [20] (Fig. 3). Le premier consiste en la réduction du lactose dans l'alimentation. Il faut toutefois noter qu'une restriction dans la durée n'est pas recommandée. De toute façon, une restriction totale n'est généralement nécessaire que pour une courte période. Il est alors important de pallier les carences par un apport en calcium et vitamine D [4]. Ainsi, 30 enfants présentant une intolérance au lactose et une allergie au lait ont réduit leur consommation de produits laitiers. Deux ans après, 15 ont présenté une densité osseuse anormale [21]. Il est donc important de trouver une alternative permettant de maintenir la consommation de produits laitiers lors du traitement de la malabsorption/intolérance au lactose. La présence de certaines bactéries probiotiques dans les produits laitiers est de ce fait une piste envisageable.

Les principaux bénéfices santé des probiotiques

Définition des probiotiques

Les bactéries probiotiques sont des micro-organismes vivants qui, lorsqu'ils sont administrés en quantité adéquate, exercent un effet bénéfique sur la santé de l'hôte [22]. Les bactéries lactiques comptent parmi les principaux probiotiques ; certaines d'entre elles produisent une lactase.

Chez l'adulte déficient en lactase, la consommation de yaourt contenant *Lactobacillus bulgaricus* et *Streptococcus thermophilus* permet d'améliorer la digestion du lactose

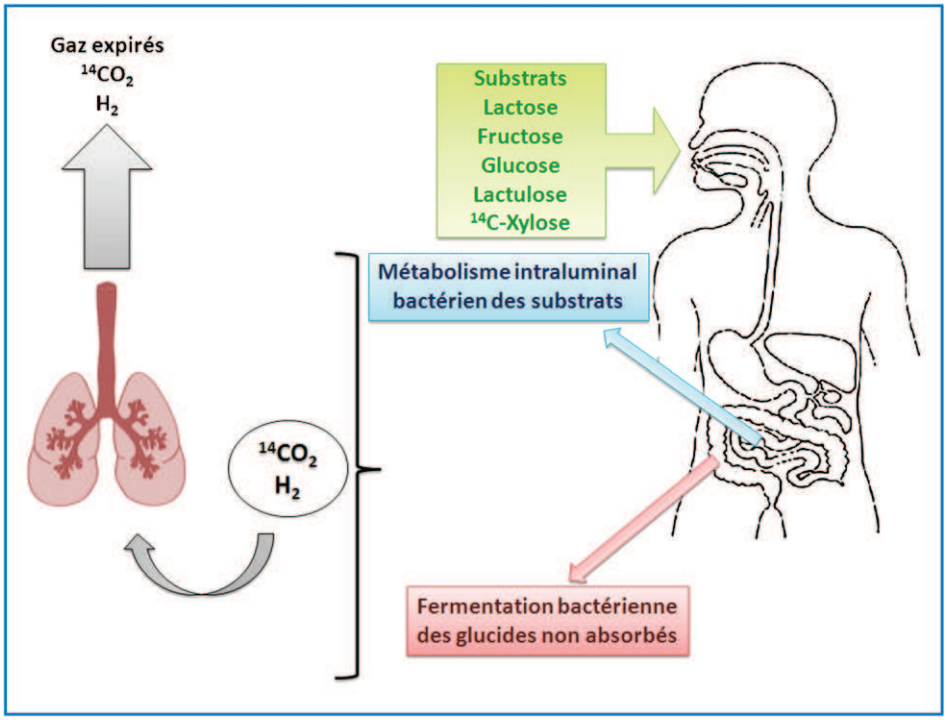

Figure 2. Diagnostic de la maldigestion du lactose — Mesure des gaz expirés.

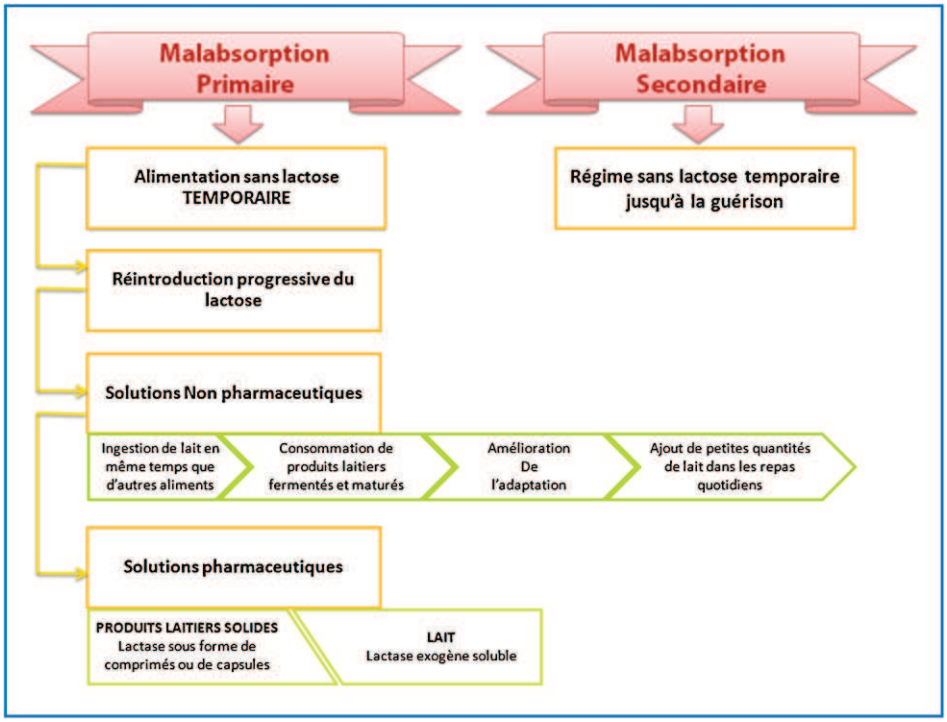

Figure 3. Prise en charge thérapeutique des patients souffrant de malabsorption primaire et secondaire du lactose.

dans l'intestin grêle. La digestibilité du lactose du yaourt peut aller jusqu'à 90 % de la charge [23]. La lactase des micro-organismes du yaourt serait protégée de l'acidité gastrique puis libérée dans le duodénum sous l'action des sels biliaires.

De nombreux travaux ont montré que le traitement thermique appliqué aux yaourts diminue la viabilité des micro-organismes et neutralise l'activité lactasique bactérienne [24,25]. La consommation de yaourt améliore également la tolérance clinique au lactose, sans que celle-ci soit en stricte corrélation avec le degré de malabsorption du disaccharide [26].

La survie des bactéries du yaourt, *L. bulgaricus* et *S. thermophilus*, est faible dans les parties hautes du tractus digestif [27] mais une activité lactasique importante a pu être observée au niveau du duodénum.

D'autres souches probiotiques présentent également un effet positif quant à l'amélioration de la digestion du lactose mais celui-ci semble moins prononcé, c'est le cas de *Lactobacillus acidophilus* [27] et *Saccharomyces boulardii* [28].

Action des probiotiques sur la digestion du lactose

Les mécanismes par lesquels les bactéries probiotiques apportent un effet bénéfique sur la digestion du lactose sont :

- apport intraluminal de la lactase d'origine bactérienne qui s'explique par la libération de la lactase bactérienne par lyse cellulaire dans l'intestin grêle. La lyse cellulaire est notamment provoquée par l'acidité gastrique et la présence de sels biliaires mais la lactase résisterait à l'hydrolyse enzymatique intraluminale [23] ;
- conservation au moins partielle de l'intégrité de la bactérie, ce qui permettrait une entrée du lactose dans la cellule via la perméase bactérienne où son hydrolyse lactasique serait réalisée [29] ;
- la consommation de produits laitiers aurait pour effet de ralentir la vidange gastrique [7], donc de ralentir le transit intestinal et ainsi de rallonger l'action de la lactase résiduelle dans l'intestin. Il en résulte une diminution de la charge osmotique due au lactose ;
- la consommation régulière de produits laitiers contenant des probiotiques permettrait de modifier le pH intestinal et d'autres variables concernant le microbiote intestinal. Cela aurait également pour conséquence de diminuer les désordres digestifs [7]. Par exemple, le lait fermenté (Kéfir) a prouvé son action sur la digestion du lactose [30].

Ainsi, la meilleure absorption et tolérance du lactose dans les yaourts est due au fait que la paroi cellulaire bactérienne de *S. thermophilus* et *L. bulgaricus* offre une protection mécanique à la lactase bactérienne envers l'acidité gastrique. La paroi est ensuite dégradée par les sels biliaires dans l'intestin grêle permettant ainsi d'augmenter l'hydrolyse enzymatique du lactose [7]. Le facteur « paroi cellulaire » semble être essentiel dans la digestibilité du lactose présent dans l'aliment, ce qui explique pourquoi d'autres bactéries probiotiques telles que *L. acidophilus* ne sont pas aussi efficaces dans la digestion du lactose [24]. La paroi cellulaire de ces souches est résistante aux sels biliaires [7] mais une amélioration des symptômes est tout de même observée. En effet, il est bien connu que les lactobacilles se comportent différemment en fonction de l'espèce concernée [20], ce qui est bien illustré ici par le fait que *L. bulgaricus* et *L. acidophilus* ne présentent pas les mêmes capacités à améliorer la digestion du lactose. Des études complémentaires sont nécessaires afin de clarifier le rôle des bactéries probiotiques dans le traitement de l'intolérance au lactose en prenant en compte le taux de survie de chaque souche et leurs effets bénéfiques connus sur les fonctions intestinales [20].

Allégations santé et législation

Actuellement la majorité des dossiers de demande d'allégation santé par addition des bactéries probiotiques font état de refus de la part de l'EFSA. La première cause de refus dans le cas de ces aliments est un manque de caractérisation de la souche. Une fois cette première barrière dépassée, les justifications scientifiques ne sont pas toujours suffisantes. Ainsi des études cliniques sont nécessaires et pas seulement une étude du maintient de la viabilité de la souche in vitro. Les industriels doivent donc dorénavant mettre en place des études scientifiques adaptées s'ils veulent prétendre à une allégation santé.

Il a été clairement démontré que les ferments du yaourt, *L. bulgaricus* et *S. thermophilus*, permettent d'améliorer la digestion du lactose. En effet, ces micro-organismes vont participer à l'hydrolyse du lactose, d'une part, grâce à un processus fermentaire qui va permettre de réduire la teneur en lactose de 25 à 50 % et, d'autre part, grâce à l'action d'une lactase endogène [20]. Ainsi, grâce à l'analyse de 13 études cliniques réalisées entre 1984 et 2001 permis de valider l'allégation relative à l'ingestion de cultures de yaourt vivantes qui permettent d'améliorer la digestion du lactose [31,32]. À ce jour, il s'agit de la seule allégation fonctionnelle relative à l'ingestion de bactéries qui ait été jugée recevable. Les études concluent toutes à une diminution de l'excrétion d'hydrogène après ingestion d'un véritable yaourt contenant au minimum 10^8 UFC/g des deux espèces *L. delbrueckii* subsp. *bulgaricus* et *S. thermophilus*. Grâce à leur survie lors du transit gastro-intestinal [33] (ce qui permet le fonctionnement de la perméase) [34], les bactéries ingérées peuvent continuer à exercer leur effet bénéfique sur la digestion du lactose. Finalement, il a été mis en évidence l'intérêt de l'apport en lactose dans la diète pour stimuler la production de β-galactosidase par *S. thermophilus* et cela sans multiplication de la souche lors du transit [35].

Utilisation de préparations enzymatiques dans les produits laitiers

Définition des préparations enzymatiques

Les préparations enzymatques présentant une activité β-galactosidase sont produites à partir des souches *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Bacillus circulans*, *Aspergillus oryzae* et *Aspergillus niger*. Ces auxiliaires technologiques permettent l'hydrolyse du lactose contenu dans le produit, on distingue les β-galactosidases « acides » et « neutres ». Les β-galactosidases « acides » sont issues de *A. niger* et *A. oryzae* avec une activité optimale à pH 3—5 et une température comprise entre 30 et 60 °C. Les β-galactosidases « neutres » sont quant à elles isolées de *K. lactis* et *K. fragilis* avec un pH optimal compris entre 6,5 et 7,5 et une température de 25—50 °C ou de *B. circulans* dont le pH optimal est de 6—6,5 et une température de 60—65 °C.

D'un point de vue industriel, le choix des paramètres pH, temps et température se fait de façon à limiter le niveau de contamination microbienne du produit plutôt que de favoriser une activité enzymatique maximale. Finalement, l'activité enzymatique est supprimée par application d'un traitement thermique [36].

Il est possible de préparer des produits laitiers dont le lactose a été en grande partie éliminé soit grâce à un processus chimique (enzymatique), soit par un processus physique (ultrafiltration). L'intérêt de ces méthodes est le maintient de l'apport calcique et la conservation des protéines laitières. En revanche, ce sont des techniques qui sont difficiles à mettre en place et très coûteuses [32].

Le délactosage obtenu par l'action d'une préparation enzymatique correspond à la conversion du lactose en glucose et galactose. Une activité secondaire appelée transgalactosylation conduit à la formation de galacto-oligosaccharides. Le délactosage opéré ici peut être complet ou partiel selon l'objectif recherché.

Une alternative a été développée par Valio Finlande. Il s'agit d'une méthode exclusive qui permet d'extraire complètement le lactose de manière non chimique. Cette méthode brevetée est basée sur de la filtration membranaire et permet de garantir une absence totale de lactose. Ainsi, toute une gamme de produits laitiers Zero Lactose™ a pu être déclinée comprenant une boisson lactée, des fromages (Gouda, Emmental), des yaourts, du beurre ou encore de la crème entière.

Association de préparations enzymatiques et de bactéries probiotiques

Les bactéries lactiques génèrent de l'énergie par la transformation d'un sucre en acide lactique soit par la voie de la glycolyse, soit par la voie des pentoses phosphates. Les oses fermentescibles concernés sont les hexoses (glucose, galactose), les pentoses (xylose, ribose, arabinose) ou encore des disaccharides (lactose, saccharose). Le fait d'hydrolyser le lactose du lait avec une β-galactosidase exogène préalablement à la fermentation lactique n'affecte pas les concentrations en produits de fermentation [37]. De plus, la formation de galacto-oligosaccharides par la β-galactosidase exogène permettrait de stimuler la croissance de bactéries probiotiques. En particulier, la croissance des bifidobactéries est stimulée, ce qui permettrait un enrichissement du microbiote intestinal par des bifidobactéries aux dépens de bactéries pathogènes [38].

Utilisation de compléments alimentaires

Pour terminer, des substituts commerciaux contenant de la lactase (d'origine bactérienne ou fongique) sont commercialisés. Toutefois ces suppléments doivent être pris avant chaque repas et surtout ne permettent pas une hydrolyse totale du lactose. De plus, l'efficacité varie considérablement d'un sujet à un autre et la qualité des préparations n'est pas équivalente [14]. Par exemple, l'origine de l'enzyme joue un rôle important. Ainsi, la lactase de *K. lactis* possède une efficacité supérieure à celle d'*A. niger*. Cependant, il faut également prendre en considération le pH de l'estomac et la concentration en sels biliaires qui vont influencer l'efficacité de lactase exogène [12]. Il est donc évident que l'activité de l'enzyme exogène peut être affectée par de nombreux paramètres qui doivent tous être pris en considération si l'on souhaite comparer deux études entre elles.

Il est précisé que la lactase présente dans les souches probiotiques pourrait améliorer le confort digestif des sujets souffrant de malabsorption. Toutefois, l'effet étant dépendant de la souche considérée, des études complémentaires semblent nécessaires avant d'affirmer un réel impact [4].

La lactase peut être vendue sous forme liquide. Son utilisation consiste à ajouter quelques gouttes de lactase à du lait ordinaire. Le tout est placé au réfrigérateur pendant environ 24 heures puis, le lait peut être consommé normalement. On remarque facilement que cette solution

est peu pratique et nécessite une anticipation certaine. L'inconvénient de l'utilisation d'une enzyme exogène est le fait que sa prise doit coïncider avec la prise d'aliments contenant du lactose. Cette solution semble imparfaite puisque l'activité enzymatique varie en fonction de son origine et que la protéine va être rapidement inactivée dans l'estomac puis l'intestin.

Conclusion

À l'échelle mondiale, 70 % de la population sont touchés par un problème d'assimilation du lactose (maldigestion et/ou intolérance). Globalement l'incidence augmente au fur et à mesure que l'on se déplace du nord au sud. La présence de lactose dans les aliments et préparations industrielles est extrêmement fréquente. En effet, de part ses propriétés technologiques (pouvoir sucrant, fermentation, cristallisation...), il est employé dans de nombreux produits alimentaires et très utilisé en industrie pharmaceutique comme excipient (plus de 20 % des médicaments en contiennent).

Les solutions diététiques et thérapeutiques actuellement proposées semblent encore insuffisantes. Nous pouvons les résumer en trois points :

- avoir un « régime strict pauvre en lactose » : cette solution reste difficile dans les pays occidentaux en raison d'une large utilisation du lait en poudre dans de nombreuses préparations commerciales. Toutefois, un marché se développe pour des produits « lactose free » [39]. L'inconvénient est que ces préparations à base de lactose hydrolysé ont un goût particulier qui déplaît ;
- avoir un « apport de lactase » : cette solution permet d'assouplir le régime pauvre en lactose et autorise la consommation plus libre de préparations commerciales. Toutefois, cette utilisation est très contraignante car cet apport doit être systématique avant chaque repas, la lactase perdant rapidement son activité ;
- avoir un apport de probiotiques synthétisant naturellement la lactase.

Cette dernière solution semble être la moins contraignante et est soutenue par l'allégation de santé récemment formulée par l'EFSA concernant l'amélioration de la digestion du lactose par les souches vivantes du yaourt.

Déclaration d'intérêts

Les auteurs déclarent ne pas avoir de conflits d'intérêts en relation avec cet article.

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14

Surface composition of food powders

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Abstract: As food powder production increases it is time to make use of new methodologies to gain a detailed understand of these powders and their functional properties. A growing number of interesting methods have been used to characterize the powder surface; others are in development (pharmaceuticals, cosmetics fields, etc.). Up until now, a major problem facing researchers and manufacturers was the lack of a central source of information that provides practical knowledge focused solely on food powders surfaces.

The first section of this chapter outlines recent methodologies used to characterize the surface of food powders, before factors affecting the surface composition. Finally, relationships between powder surfaces and functional properties are highlighted.

Key words: surface composition, powder surfaces, surface analysis, functional properties.

AQ1

14.1 Introduction

A large number of raw ingredients from various sources (such as animals, vegetables and inorganics) in many food industries can be processed in order to obtain a dry form. Examples of such processes include comminution, agglomeration, spray-drying, dry-mixing, coating and encapsulation. Because these ingredients are difficult to transport, store or even formulate under a fluid form, they are often dried for ease of handling, transportation and storage. In addition to these processes, the packaging, storage and transport conditions may influence the surface composition of a powder particle. It is now generally accepted that particle surface composition in its turn has a strong impact on the functional properties of a powder, such as rehydration, caking, flowability and sticking.

Table 14.1 Summary of the main analysis methods for the characterization of food powder surfaces

Method of analysis	Technique	Example of information obtained	Main problems or disadvantages of the technique	Contribution and interest (reviews and research results)
Microscopy Techniques	LM	Size, shape, color	Relations with surfaces difficult to perform	Du and Sun (2004), Saad *et al*. (2011b)
	CLSM	Identification of several components simultaneously, three-dimensional reconstruction	Necessity of a specialist for image treatments, necessity to find specific probes	Auty *et al*. (2001), Soottitantawat *et al*. (2007)
	SEM	Topography	Mainly qualitative information, gold coating, under vacuum	Haque and Roos (2006), Murrieta-Pazos *et al*. (2011)
	TEM	Gradients in the particle (spatial information)	Not directly linked to the surface, long sample preparation	McKenna *et al*. (1999), Vignolles *et al*. (2009)
	AFM	By imaging: topography, coverage, atomic structure, chemical information (use of derivatized tips) By force measurements: chemical, conformational structural	Necessity of a specialist (artifact identification, data treatments …), sensitive to contamination, limited chemical information	Kingshott *et al*. (2011), Prime *et al*. (2011)

Spectroscopy Techniques	ToF-SIM	Chemical composition, structural information by use of molecular fragments	Very sensitive to surface contamination, no volatiles in the sample	Dupont-Gillain *et al.* (2010), Kingshott *et al.* (2011)
	XPS	Elemental composition, chemical composition, components composition (by using matrices)	Under vacuum, sensitive to surface contamination	Rouxhet and Genet (2011), Rouxhet *et al.* (2008)
	ESEM-EDX	Elemental composition coupled with surface topography	Few data and results on food powders, impossible to scan a surface (dot of measures)	Kingshott *et al.* (2011), Murrieta Pazos *et al.* (2012)
Surface Sorption Techniques	Vapor and gas sorption	Thermodynamic, structural and technological information	Direct relations with surfaces difficult to perform	Gaiani *et al.* (2009), Saad *et al.* (2009)
	IGC	Surface energy, thermal transitions, crystallinity, specific surface area,	Technique well developed on mineral powders but difficult to performed on food powders due to powder rehydration	Brum and Burnett (2011), Boutboul *et al.* (2002)
	Contact angles	Surface free energy, surface hydrophobicity	Difficult to performed on food powders due to powder rehydration	Forny *et al.* (2011), Kiesvaara *et al.* (1993)
Surface Extraction Techniques	Fat extraction coupled with other techniques	Gradients in the particle	Artisanal techniques of extraction (poor reproducibility, long to perform …)	Buma (1971), Drusch and Berg (2008), Kim *et al.* (2005b), Vignolles *et al.* (2007)

Recently, a number of methodologies have been developed in order to better characterize powder particle surfaces (looking at aspects such as composition, energy, structure, shape and rugosity). Some of these are now well developed and are in use by researchers and in industry. Others are less well known and may not be used for food powders at present; but are very promising for the future. It can be seen that strong links exist between the surfaces of powder particles and their functional properties. This is the reason why measuring only the bulk and physical properties of a food powder is not enough. Many food attributes can now be attributed to inhomogeneities between the powder surface and the core or the particle bulk composition. This chapter will examine these issues.

In recent years years significant advances in the analysis of food surfaces at variable levels have been achieved by various techniques, including Microscopy, Spectroscopy, Surface Sorption, Surface Extraction (Table 14.1). Among the most important developments in microscopy is the increasing use of atomic force microscopy (AFM) and confocal laser scanning microscopy (CLSM) in the analysis of powder surfaces. These two techniques complement light and electron microscopy methods and are likely to play an important role in future analysis of food surfaces. Recent developments in Spectroscopy techniques will also be reported, as well as data from the use of Sorption and Extraction surface techniques.

14.2 Microscopy techniques for analyzing the surface of food powders

In this chapter some usual microscopy approaches will be described such as light microscopy and electron microscopy (SEM, TEM) techniques. Then, applications in the field of AFM, rarely used on food powders, will be developed. Finally, future promising tools never used in the food fields will be presented.

14.2.1 Light analysis and light microscopy techniques

Color analyses of food powders are quick and straightforward and are generally carried out using a tristimulus colorimeter. Color is expressed in L (darkness/whiteness), A (greenness/redness), B (blueness/yellowness), Hunter scale and/or international commission on illumination (CIE) parameters. White and black calibrating tiles are used as reference. Powder color is often related to Maillard reactions, for example a strong link was noticed between elevated inlet air temperature during spray-drying and modification of the L (decrease) and B (increase) values for tea powders (Sahin Nadeem *et al.*, 2011). This suggests that powder color darkens at higher drying temperatures. The same tests were carried out on lentil protein powders (Joshi *et al.*, 2011) and linked the

drying method (such as spray, vacuum or freeze) to the final color of the powder. However, a direct relationship between the powder surface (composition and structure) and the powder color has rarely been found. Very few authors have attempted it, although (Gaiani *et al*., 2011) observed links between powder color and functional properties such as rehydration.

The first microscopes developed for powder surface analysis used light and light microscopies (LM) and were first used for milk powders; however these techniques appear to provide only qualitative information about the surface (Buchheim *et al*., 1974). Recent developments in laser diffraction systems and image analysis permit on-line measuring of the size and shape of a large number of particles (Du and Sun, 2004). Very little research has been carried out for food powders on particle morphology (Gaiani *et al*., 2011) whereas there is much research in minerals powders (Chau *et al*., 2009; Ulusoy, 2008). Some qualitative terms are used to give indications of particle shapes (Saad *et al*., 2011b); for example, the sphericity is defined as the ratio of the perimeter to the circumference of a disk with the same area. The convexity is the ratio of the value obtained by projecting a particle area filling concave zones to the real area. This describes the compactness of a particle (Gaiani *et al*., 2011; Saad *et al*., 2011b). Other shape factors, including elongation, convexity, sphericity, compactness, straightness and circularity have also been evaluated.

Confocal laser scanning microscopy was recently shown to be a helpful technique for the characterization of nondestructive microparticles (Arltoft *et al*., 2007; Soottitantawat *et al*., 2007). There has been limited research into analysis of food powder surface by CLSM, and only a few studies have been published; however these preliminary results are very promising. Firstly, CLSM permits the simultaneous identification of several components, including lipids, proteins and polysaccharides, at the surface of the particles through the use of specific fluorescence probes emitting light at different wavelengths (Auty *et al*., 2001; Vignolles *et al*., 2009, 2010). Figure 14.1c represents an example of particle protein and fat labeling. By taking into account the total area, the holes area and the area of each component, the fat and protein surface coverage can be estimated (by image analysis). Secondly, the internal morphology of the particles, such as vacuoles, cracks, cavities and pores, can also be observed (Paramita *et al*., 2010a, b). It can be seen that CLSM allows the visualization and characterization of structures not only on the surface but also inside the particles, and by collecting coplanar cross-sections a three-dimensional reconstruction of the inspected objects is possible (Auty *et al*., 2001; McKenna, 1997; McKenna *et al*., 1999). The acquisition of structural and composition data concerning both the surface and the inner area of particles will be of great interest in the future to track variable phenomena such as composition evolution during storage or during processes such as spray-drying and encapsulation efficiency.

The preparation of the sample is generally easy for food powders. These materials are examined in their original form by dispersing them in either aqueous or non-aqueous media, depending on their solubility (Auty *et al*.,

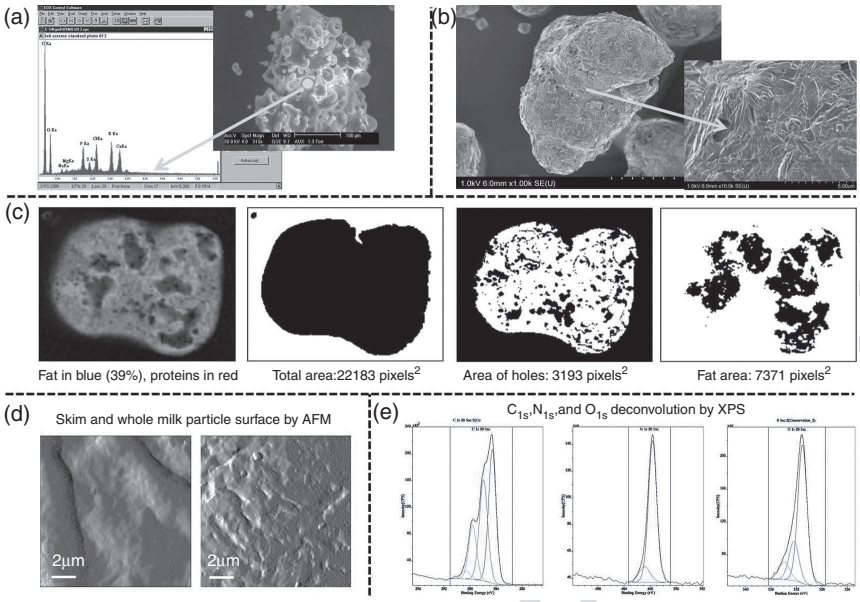

Fig. 14.1 Example of raw results obtained by (a) ESEM-EDX analysis of an agglomerated whole milk powder with the area and percentages of each element present at the surface; (b) SEM image of a particle of aged skim-milk powder with a zoom on lactose crystals; (c) CLSM image of a casein powder demonstrating the presence of fat (in blue) at the particle surface with the image analysis treatment associated; (d) imaging of skim and whole milk particle surface by AFM allowing the determination of surface roughness; (e) C, N and O elements déconvolution obtained by XPS.

2001). The media include stains to increase the contrast of individual components for structural observation or discrimination on the basis of chemical and morphological characteristics.

14.2.2 Scanning and transmission electron microscopy

Electron microscopies are important viewing techniques in the study of food powder surfaces. The two major electron microscopy modes that will be presented here are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Both are useful tools for surface and ultrastructural observations and both use a beam of electrons, instead of light, to form an image of the sample. The main differences between SEM and TEM lie in the placement of the sample in the electron beam path. For TEM the sample is placed in the path of the electron beam, and for SEM it is placed at the end of the focused electron beam path. As a result, little sample preparation is required for SEM, whereas TEM observations usually require different steps, including embedding, cutting into thin sections and staining.

In order to obtain SEM images, it is sometimes necessary to recover the powders with a thin layer of carbon or gold to provide them with conductive properties. Before SEM analysis, the powders are prepared by placing a carbon tape, containing a small amount of powder, on an aluminum disc. The sample is then given a standard gold layer (around 20–40 nm) to produce a conductive surface. SEM images reveal information about food powders' particle shape, size and surface aspect. The suprastructure and organization of milk powders have been widely studied (McKenna *et al*., 1999), as have milk–wheat flour mixtures (Al Mahdi *et al*., 2006). Surface dents and smooth surfaces have been registered in milk powders (Gaiani *et al*., 2006a; Haque and Roos, 2006; Kim *et al*., 2002). Lactose mixtures and milk models obtained by freeze-drying (Marabi *et al*., 2007, 2008), present the same structure described by Haque and Roos (2006), who also noted more brilliant surfaces in higher surface fat content powders. SEM was also used to characterize powders' surfaces during storage, and the apparition of pores was noticed at the surface of casein powders (Gaiani *et al*., 2009). The lactose state at the powder surface was also easily identified using SEM (Yazdanpanah and Langrish, 2011a), and Fig. 14.1b presents an SEM image focused on lactose crystallization which occurred due to inappropriate storage. The surface of fresh milk powder appears to be untextured and amorphous with no crystals apparent on the surface (Murrieta-Pazos *et al*., 2011; Yazdanpanah and Langrish, 2011a), yet the surface of processed milk powder has a heavily textured appearance, suggesting that the surface is crystalline (Yazdanpanah and Langrish, 2011a). The aw increase was also responsible for the apparition of uniform and continuous crystals (Murrieta-Pazos *et al*., 2011). SEM analysis also revealed that starch granules possess different surface topologies (Baldwin *et al*., 1997a). Analysis of potato starch revealed many surface protrusions above a flatter surface, whereas wheat starch had fewer protrusions and a smoother surface. The protrusions are believed to be carbohydrate, which could represent the end of amylopectin side-chain clusters at the granule surface.

Ultrathin samples are absolutely necessary for TEM images, which is one of the reasons its use in food powder analysis has been limited. In addition, this technique is not directly linked to the surface of the powder. Nevertheless, it is still possible to examine gradients in the particle. In order to examine powders, samples are chemically fixed and embedded in resins such as Epon or Araldite. Unlike dense foods such as cheese, dough and comminuted meat products, which require long fixation and impregnation times, powders and other porous foods require less time. Specific details about protein–protein and protein–fat interactions have been observed in milk powders (Vignolles *et al*., 2009), and by negative staining and metal shadowing, macromolecules such as proteins and polysaccharides and assemblies such as micelles can be observed. McKenna *et al*. (1999) examined the microstructure of four commercial whole milk powders and collected the insoluble materials that resulted from a series of functional tests. TEM provided insight into the formation of insoluble material during reconstitution. Even though the TEM technique

was not developed enough to characterize the surface of food powders, it can be envisaged for the future. For example, if information on chemical composition or structural and spatial relationships are required, a comparison between different slides from the surface to the bulk of the powder could be used.

14.2.3 Atomic force microscopy

For food powders, the AFM applications are recent and rare. Use of the technique is important in gaining greater understanding of the particles and their properties. However, investigations performed by SEM present some disadvantages: information about components is difficult to obtain if not used in conjunction with energy dispersive X-ray spectroscopy (EDX), samples have to be coated with a conductive material (creation of artifacts), and vacuum conditions could alter the powders.

Recently, (Murrieta-Pazos *et al*., 2011) demonstrated the use of AFM to characterize the surface topography of two dairy powders presenting low and high surface fat coverage. The resulting images were in agreement with those observed by SEM and the surface rugosity was also determined (Fig. 14.1d). As with food powders, AFM highlights the need to investigate the surface topology of wheat starch granules at the nanoscale (Baldwin *et al*., 1997a; Gallant *et al*., 1997). Atomic force microscopy was used for direct imaging of the inner surface topography of food objects such as starch grains and granules, but also cellulose and/or chitosan (Laity *et al*., 2010; Mathew *et al*., 2006). Atomic force microscopy revealed hierarchical structures with dimensions ranging from nanometers to a few micrometers in both materials. In addition, residual fragments of plant cell walls were observed. These interpretations were correlated to SEM, TEM and small-angle X-ray scattering (SAXS) results. The ability to deform these structures was also clearly shown in the irregular force-displacement curves recorded by AFM on the granule surfaces.

These studies, however, were focused only on surface topography. Prime *et al*. (2011a, b) attempted to take it further and used different modes of AFM to characterize the surface of model spray-dried powders such as maltodextrin, soya oil and sodium caseinate. In coupling phase imaging, nanoindentation and force modulation microscopy, the authors discovered a regular dispersion of a soft circular area (made of oil) distributed across the particle surface. In addition, they investigated the effect of humidity and temperature cycling on the caking behavior of the particles. In cutting the particle wall, nonuniform material was also discovered across the wall, with softer areas surrounding some of the voids present in the cross section (Prime *et al*., 2011a).

Up until now the AFM technique has mostly been used in the study of medical or pharmaceutical powders; but some of these investigations are very similar to those in food applications. For example, novel iron-heparin

complexes presenting nano-scaled wall thickness have been deposited onto the surface of latex particles. AFM images prove that the complexes keep spherical shapes in solution even after drying (Yu *et al*., 2008). An AFM-based approach was also developed in order to study the adhesive forces between tabletting punches and model formulation ingredients (particularly those containing lactose) that can ultimately be used to understand and predict issues that occur during tabletting compression, such as sticking (Bunker *et al*., 2011; Masterson and Cao, 2008). In conclusion, these recent works demonstrated the advantages and the additional information that the use of AFM can bring to studying the properties of powdered materials, particularly food powders.

14.2.4 Future promising microscopy techniques

Nuclear magnetic resonance (NMR) micro-imaging experiments were used in the medical field to examine water penetration in powders and the associated drug release kinetics. The obtained data, combined with morphological information (SEM), revealed a complex process that led to the release of the drug into the liquid phase, meaning the rate of water ingress had no direct influence on release kinetics (Dahlberg *et al*., 2010). This technique was also successfully applied to a study of water transport in porous silica (Aristov *et al*., 2002) and water distribution during the drying process of gelatin gel (Ruiz-Cabrera *et al*., 2005). The NMR micro-imaging technique was sufficient in measuring water concentration profiles inside a granulated bed with correct spatial and temporal resolutions. From mathematical treatment of the obtained profiles the water diffusivity (from the powder surface to the bulk) was related to the powder size and porosity.

In a similar experiment, also in the medical field, a nondestructive tool was employed to determine the uniformity and the repartition of a drug compound in an inhalable powder by confocal raman microscopy (Schoenherr *et al*., 2009). In this instance, quantitative raman spectroscopy was used to find an adequate substitute (mannitol) for lactose as carrier (Maas *et al*., 2011) and the effect of the spray-drying temperature was investigated in order to rend mannitol most suitable for the use in dry powder inhalers. Fischer and Jauss (2007) investigated the use of this microscopy in the general food field but not specifically within the food powder field.

14.3 Spectroscopy techniques for analyzing the surface of food powders

The first technique presented (XPS) was often successfully used to characterize the surface composition of food powders during the last decades. In contradiction, ESEM-EDX was rarely performed and seems to be a promising

tool to investigate the surface of food powders. Finally TOF-SIM and other future promising techniques (mainly tomography) are also presented and may request attention.

14.3.1 X-ray photoelectron spectroscopy

During the last 20 years the X-ray photoelectron spectroscopy (XPS) technique has also been known as electron spectroscopy for chemical analysis (ESCA). This analytical technique was first developed in the mid-1960s at the University of Uppsala (Sweden) with the primary use of material surface analysis. Since then, XPS has been widely used to characterize the surfaces of a variety of solid materials, including ceramics, fibers, glass, metals, minerals, wood and polymers, but not food powders. The first use of the equipment in the field of food powders was by Fäldt (1995) on dairy powders. The reason for this delay was due to the complexity of the chemical composition of dairy powders in comparison with materials. In the mid-1960s the use of XPS was regularly reported in the determination of the surface composition of dairy powders (Gaiani *et al*., 2006a; 2007, 2010, 2011; Kim *et al*., 2002, 2009a, b, c; Millqvist-Fureby and Smith, 2007; Millqvist-Fureby *et al*., 2001; Shrestha *et al*., 2007). Work has since been published on powders containing surface-active proteins (Jayasundera *et al*., 2009) and on the general food field (Rouxhet and Genet, 2011; Rouxhet *et al*., 2008).

XPS provides elemental and chemical state data from the first nanometers of a sample's surface. The sample is irradiated with photons from a soft X-ray source with a well-defined energy. This method is based on an irradiation of the surface that causes a complete transfer of photon energy to atomic electron. When the electron binding energy (E_b) is lower than the photon energy ($h\upsilon$), the electron is emitted from the atom with a kinetic energy (E_k) equal to the difference between the photon energy and the binding energy minus the spectrometer work function Φ:

$$E_k = h\upsilon - E_b - \Phi \qquad [14.1]$$

A numerical method based on matrix inversion can be used to determine the surface coverage of individual components. In the case of milk powders, from the C, O and N percentages the fraction of the area covered by protein, lactose and lipids was calculated by solving the matrix formula. In this instance, the sample's elemental composition is assumed to be a linear combination of pure components (Faldt and Bergenstahl, 1994; Gaiani *et al*., 2006a; Kim *et al*., 2005a; Nijdam and Langrish, 2006). Recently, this technique has been successfully used to investigate links between particle surface chemical composition and functional properties (see Chapter 4). The use of XPS has also been reported in cereal powders, however studies were more limited and poor databases are actually accessible. Despite this, the outer 5–10 nm of the starch granule surface was analyzed by XPS (Baldwin, 2001) and the authors

deduced the protein surface content from the nitrogen peaks area and concluded that this content varies according to the starch botanical source. The principal studies using XPS on cereal powders were undertaken by Saad *et al*. (2009, 2011a) on wheat flour. In these cases, the matrix formula developed for dairy powders was derived from wheat powders. The use of XPS in the field of other food powder is yet to be commonly used.

Thanks to this technique it is now possible to quantify the relative coverage of different components on the surface of organic powders. The majority of results are the same, as the surface composition of a food powder is very different to the global composition. Depending on the process, proteins and lipids can be observed at the powder surface, whereas carbohydrates and minerals are more encapsulated in the particle. In the case of dairy powders, the proposed procedure is that protein adsorbs preferentially to the air-liquid interface during spray-drying and appears on the powder surface after the spray-drying is completed. The hypothesis for cereal powders is different and may be linked to the localization of fracture in the grain during processing.

14.3.2 Environmental scanning electron microscopy (ESEM) and energy dispersive X-ray spectroscopy (EDX)

Recently, environmental scanning electron microscopy (ESEM) images have been combined with EDX analyses. This technique has been used to identify the elemental composition of the sample, or an area of interest thereof. The EDX analysis system works as an integrated feature of ESEM equipment and cannot operate on its own. A variant of X-ray fluorescence spectroscopy, EDX analysis systems rely on the investigation of a sample through interactions between electromagnetic radiation and the sample. Its characterization capabilities are largely due to the fundamental principle that each element has a unique atomic structure allowing X-rays, which are characteristic of an element's atomic structure, to be identified uniquely from one another. To stimulate the emission of characteristic X-rays from a specimen, a high-energy beam of charged particles is focused into the sample, from which an EDX spectrum is obtained. This provides a map of how frequently an X-ray is received for each energy level. The higher a peak in a spectrum, the more concentrated the element is in the sample.

Murrieta-Pazos *et al*. (2011, 2012) are the only ones to have used EDX to investigate the chemical composition of food powders at a depth of approximately 1 μm (Fig. 14.1a). When making comparisons between the surface composition obtained by XPS (a few nanometers) and data collected by EDX, these authors proposed models of powder gradients for skim and whole milk powders. Despite these promising early results, they are still the first available and the area needs to be further developed. For example, in biomaterials applications this technique has rarely been used due to the fact that the probe depth exceeds the thickness of the surface layers that determine biocompatibility (Kingshott *et al*., 2011).

14.3.3 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has proved to be a valuable tool for the analysis of surfaces. This technique uses a pulsed primary ion beam to desorb and ionize species from the sample surface. The resulting secondary ions are accelerated into a mass spectrometer and are then mass analyzed by measuring their time-of-flight from the sample surface to the detector. Baldwin *et al*. (1997b) recorded ToF-SIMS spectra from different starch samples and revealed significant information regarding the types of lipids present at the starch granule surface and the identities of their acyl-chains. Up to now, no work has been published on food powders, however interesting papers appear in the pharmaceutical (lactose powders) and medical (chitosan/hyaluronic acid and chitosan/lipid nanoparticles) domains, whose components are very similar to those used in food (Al-Qadi *et al*., 2011; Grenha *et al*., 2008; Zhou *et al*., 2011a, b). In conclusion, ToF-SIMS appears to provide more detail than XPS, with more selective information available on individual compounds or moieties after adequate data processing. However, ToF-SIMS does not provide a whole quantitative composition (Dupont-Gillain *et al*., 2010). As a result, this technique (coupled with XPS) may become a strong tool for the quantitative and qualitative characterization of food powders.

14.3.4 Future promising spectroscopy techniques

Perfetti *et al*. (2009) demonstrate the potential of X-ray micro tomography (XMT) as a powerful tool for morphological and surface characterization of dry particles and, in particular, their surface layer. XMT provides a high level of detail at both micro- and macro-scale. It was, in this work, used to determine density, porosity, surface/volume ratio, and thickness of the coating layer. In addition, from the segmented images, 3D models were created. Another tomographic technique, the tomographic atom probe (TAP) was used by Larde *et al*. (2009) to provide, at the atomic scale, the spatial distribution of atoms in the analyzed specimen. Due to the difficulties linked to atom probe specimen preparation, this technique had rarely been used to characterize powder materials. More recently, the development of new specimen preparation methods has allowed a systematic characterization of powder materials at the atomic scale.

In recent years Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) has been adapted for the analysis of biomaterials surfaces. This tool presents unique capabilities that complement existing surface analysis methods such as XPS and ToF-SIMS (Griesser *et al*., 2004). These new methods, which are known as Surface-MALDI-MS, are capable of desorbing adsorbed macromolecules from surfaces, even at low levels, and detecting their molecular ions with high mass resolution. This technique could offer unique means of addressing food powder surface analysis needs, such as identifying the proteins and lipids adsorbed, studying interactions between powder surfaces, and identifying surface-enriched additives and contaminants.

14.4 Surface sorption and extraction techniques for analyzing the surface of food powders

Three sorption methods (dynamic vapour or gas sorption, IGC and contact angle) are detailed in this chapter; all of them could give information about the powder surface (composition, hydrophobicity …). Surface extraction techniques are often empirical and mainly focalized on the surface fat. Nevertheless, these techniques could give quick and cheap information concerning the presence of surface fat.

14.4.1 Vapor and gas sorption

In recent years, measurements of gravimetric moisture uptake of food powders were taken by the microclimate method. In this process, the powders are stored over saturated salt solutions which present different relative humidities; however problems with this method include stability of saturated salt solutions, accuracy of water content and stability of temperature and time of equilibration. More recently, dynamic vapor sorption (DVS) equipment has provided automated, rapid and accurate measurements in a large temperature range using dynamic environment control and ultrasensitive recording microbalance. The small quantity of samples necessary for analysis and the dynamic airflow around the samples enables the generation of complete isotherms in under a week, which has led to the equipment being now more widespread. With these methods a water vapor sorption isotherm is obtained, which could describe the relationships between water content and water activity (aw) (Gaiani *et al.*, 2009; Mathlouthi and Roge, 2003; Murrieta-Pazos *et al.*, 2011; Shrestha *et al.*, 2007; Silalai and Roos, 2010; Szulc and Lenart, 2012). With the same equipment it is also possible to acquire data on gas/powder interactions by physical adsorption of a gas on the surface of the powder and by calculating the amount of adsorbed gas corresponding to a monomolecular layer on the surface. Structural information, such as surface area, of the powder is then determined.

Depending on the chemical, physical and structural properties of different food powders, the shape of the isotherm can vary, including the BET-S shape multilayer, Langmuir one layer, Flory Huggins, Brunauer, Emmett, and Teller (BET) isotherm capillary and multilayer. As a result, a mathematical description of the isotherm is required in order to extract data. The initial model recommended for food powders is the two-parameter BET model because of its simplicity of application. This model is based on a multilayer sorption, however it is used only for the low water activity range. The usefulness of the three-parameter Guggenheim, Andersen, and de Boer (GAB) model was also demonstrated. The GAB model is based on multilayers and condensation and covers a wide range of water activity until 0.8. The result of recent developments, a third stage sorption (TSS) model with a four-parameter equation extends the GAB isotherm model to water activity ranges approaching 1. It

is based on the premise that once a certain number of moisture layers exist, the moisture behaves as liquid water, which subsequently has a dilution effect. Overall there are more than 200 models, including the Langmuir, Harkings, Smith, Henderson and Oswin models.

Variable constants are obtained from the application of these models to isotherms, which provides information of variable aspects including thermodynamic (enthalpies of sorption and desorption, water activity and heats of crystallization), structural (specific area, pore size and volume and amorphous state) and technological (drying condition, stability, handling, storage and packaging). Some of these data were, in turn, associated with the particle surface characteristics (Gaiani *et al.*, 2009; Saad *et al.*, 2009). For example, the value of Xm (quantity of monolayer water at the powder surface) was related to the surface hydrophobicity determined by XPS. Powders presenting elevated ratio of C/O by XPS (surface mostly hydrophobe) were characterized by a low Xm. At the opposite end, powders presenting low C/O by XPS (surface less hydrophobe) were linked to an elevated Xm. These values were in turn related to the powder wetting properties (Gaiani *et al.*, 2009).

14.4.2 Inverse gas chromatography

In contrast to conventional gas chromatography, where a well-known stationary phase separates and identifies various components, inverse gas chromatography (IGC) uses probes (identified molecules) to determine the surface properties of a solid packed into a column. IGC can provide different physical chemistry parameters of the solid surface, such as surface energy, thermal transitions, crystallinity, specific surface area, and thermodynamic properties of the probe-solid system (Conder and Young, 1979). As such, IGC could appear as an efficient tool to investigate the surface of food powders, even if only a few studies have so far been reported in this field. Two techniques of IGC can be distinguished: the first is at infinite dilution, consisting of the injection of a small amount of probe vapor, and the second technique is at finite concentration, with the injection of larger quantities of liquid probe. The first technique is more specific to the beginning of the isotherm, whereas the second covers the surface of the solid with one monolayer and permits the plotting of sorption isotherms for different organic probes or water molecules (Conder and Young, 1979). So far, food powders such as sugars (Brum and Burnett, 2011; Newell and Buckton, 2004), dry bakery products, wheat, corn or potato starch, flours (Riganakos *et al.*, 1989, 1994; Lagoudaki and Demertzis, 1994) have been investigated in this way.

In the food field, IGC was used for several years in order to study the influence of moisture on surface properties of food products, such as water sorption by wheat flour (Riganakos *et al.*, 1989). In this study the authors identified two different mechanisms: at low moisture content the water molecules are adsorbed on the active sites of high binding energies (C=O,

COO^- or NH_4^+), whereas at high moisture contents water–water interactions are favored (water cluster formation at the surface).

Techniques such as IGC can also quantify the amorphous content on a powder surface such as lactose. In this instance, Newell and Buckton (2004) investigated relationships between IGC parameters and amorphous content on the solid surface, and three samples of lactose (crystalline, spray-dried and milled) were compared (Newell *et al.*, 2001). The milled lactose was noted to have a similar dispersive surface energy to the amorphous lactose, indicating the preferential localization of the amorphous lactose on the surface. Different lactose surfaces (mixture of crystalline and amorphous solids) were also analyzed by IGC (Brum and Burnett, 2011) to determine the effective surface energy of the mixture.

IGC can also be used in the food field to study the interactions between flavor compounds and food components. IGC is a valuable tool in the investigation of the adsorption of flavor compounds on solid supports such as proteins, starch and sugars. McMullin *et al.* (1975) demonstrated that lactose has a great ability to adsorb aromas compared to the heats of adsorption of a large variety of organic compounds. For a given number of carbon atoms, alcohols have the highest heats of adsorption, hydrocarbons have the lowest, and other functions present intermediate behaviors. The hydrogen bonds formed between the lactose and the functional groups of organic compounds, such as hydroxyl for alcohols, was supposed to be the major factor involved in the strength of adsorption. The interactions between aromas and starch were investigated by IGC (Boutboul *et al.*, 2000, 2002), where the retention of aroma was found to be higher under humid conditions than it was under dry conditions, especially for alcohols. Different hypotheses were then proposed: a predominant adsorption phenomenon involving hydrogen bonds between aroma compounds (between alcohols and glucose residues of the starch) and/or a solvation of aroma compounds by water molecules with diffusion through the starch matrix.

14.4.3 Contact angle

The contact angle of a powder can be determined by various techniques, the most common of these being the sessile drop methods and the sorption methods. With sessile drop methods, powder tablets are produced by the application of a compression force, before a drop of water is carefully placed onto the upper surface of the tablet (Chander *et al.*, 2007). The angle formed by the drop of liquid on the compacted powder is then determined. The second method is a sorption method, which uses an instrument such as a tensiometer. In this instance, the powder is packed into a glass cylinder with a porous glass base. The cylinder is then brought into contact with the liquid, and the increase in the liquid weight (due to the liquid's penetration into the cylinder) is measured over time. Finally, the modified Washburn equation is applied in order to obtain the contact angle calculation (Chibowski and Perea-Carpio,

2002; Forny *et al*., 2011). The porous layer imbibitions techniques are most commonly used, however these procedures lead to overestimated contact angle values in comparison to those measured directly on smooth surfaces of the same solid (Chibowski and Perea-Carpio, 2002; Pepin *et al*., 1999). Also reported in the findings were various indirect methods of porous layer imbibitions, including film flotation, bubble pick-up, induction time, immersion/sink time, and wetting rate (Chander *et al*., 2007).

Contact angles can also be used more generally to characterize the degree of wetting of a food powder, as a powder's wetting ability is greater when the contact angle is lower. The following generality is often used: contact angles above 90° indicate that the liquid does not wet the solid, whereas contact angles under 90° indicate a wetting of the solid (Kiesvaara *et al*., 1993).

14.4.4 Surface extraction techniques

The extraction of fat from the surface of dairy powders was first reported in the early 1970s (Buma, 1971; Buchheim *et al*., 1974). According to these authors, extractable fats consist of different fractions comprising the surface fat, the outer layer of fat in the surface layer of the particle, fat that can be extracted by the solvent through capillary forces and fat that can be reached by solvent through holes left by already-extracted fat.

More recently, the technique developed by Buma (1971) has been enhanced by several authors (Drusch and Berg, 2008; Kim *et al*., 2005b, 2009a; Murrieta-Pazos *et al*., 2012; Vignolles *et al*., 2007, 2009) and more fat fractions are now determined, including inner free fat, encapsulated fat and total fat. The principle of the extraction remains the same, but a great number of variations have been tested in regard to the hydrophobicity of the solvent, the number of extractions, the solvent/powder ratio, the agitation mode and intensity, and also the extraction time. Recent publications suggest that a huge number of extraction methods have since been tested for food powders (Murrieta Pazos *et al*., 2012; Vega and Roos, 2006).

The extraction procedure is only the beginning of the study, as once fat fractions are obtained it is interesting to collect information about these extracted fractions. differential scanning calorimetry (DSC) and high performance liquid chromatography (HPLC) are the most used in the characterization of these fat fractions (Kim *et al*., 2005b, 2009c; Murrieta-Pazos *et al*., 2012). By DSC, the crystallization and melting profiles obtained for various fractions were attributed to a difference in fat suprastructure organization and fat composition (Vignolles *et al*., 2009). Kim *et al*. (2009c) observed lower melting points for surface free fat extracted from powders during long-term storage, which indicates a modification of the surface fat composition. Similarly, a release of low-melting triglycerides towards the surface of the powder during long-term storage was observed by HPLC and, in addition, a fractionation among the different fat fractions during powder spray-drying was observed

(Kim *et al.*, 2005b). In this instance, the high-melting triglyceride species were slightly more concentrated in the free fat fractions and the low-melting triglyceride species were more present in the encapsulated fat. Additionally, HPLC uses revealed no significant differences between fat fractions obtained from standard and agglomerated powder, meaning that functional differences observed between these powders were not due to variable fat compositions (Murrieta-Pazos *et al.*, 2011).

14.5 Factors affecting food powder surface composition

A number of powder surface properties are known to be determined by the characteristics and composition of the liquid feed (the concentrate before it is spray-dried), including the manufacturing processes, processing conditions and storage conditions (as summarized in Fig. 14.2). These processes can be divided into the processes that create the powder properties and those that influence the powder properties by degrading them (by handling, transport or storage). These two processes have a great influence on the powder's final applications (Fitzpatrick and Ahrné, 2005).

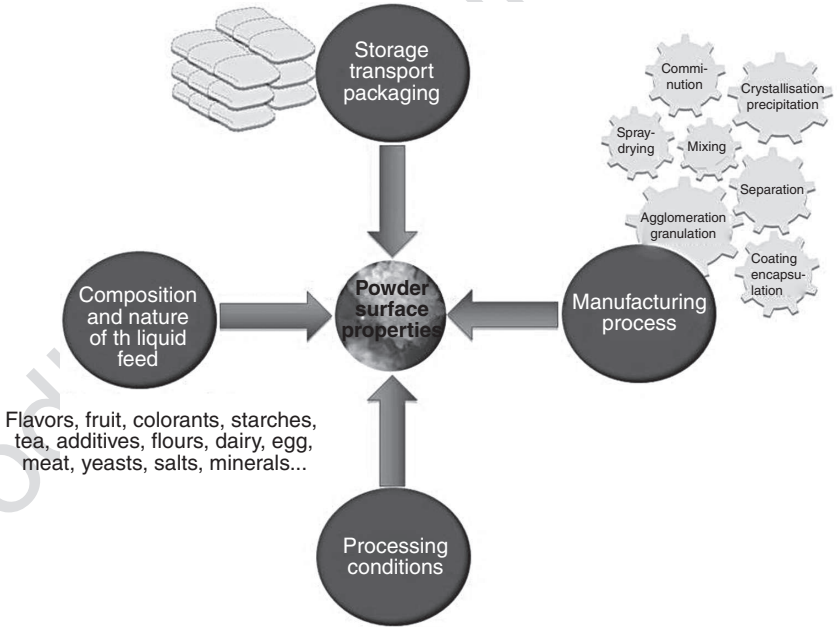

Fig. 14.2 Summary of main factors affecting the surface composition of food powders.

14.5.1 Powder manufacture

Composition of the liquid feed

The initial composition of the liquid feed is of great importance in powder manufacture, especially as the presence of crystalline substances in the liquid phase (prior to drying), instead of including them after drying, was found to decrease the crystallinity of the final product (Palzer *et al*., 2012). The importance of the feed solids content for dairy powders was tested, revealing that, at high feed solid contents, less fat and protein (i.e. more lactose) appear on the surface of the powder. One hypothesis of this could be that fewer components were redistributed within the drying droplet due to high viscosity and rapid crust formation (Kim *et al*., 2003, 2009a). In cocoa beverage powders it was demonstrated that increasing the amounts of carbohydrate (which is a sensitive ingredient) could negatively affect the storage stability of the powder (Montes *et al*., 2011). As these findings show, certain modifications generate entirely different powder structures and surfaces with different powder properties.

Process parameter

The surface composition of industrial spray-dried powders was found to be strongly determined by the spray-drying process and the spray-drying conditions employed, including the drying temperature and degree of homogenization. During drying a number of chemical reactions are accelerated, primarily due to the use of elevated temperatures. As a consequence, the powder structure could change (Palzer *et al*., 2012), particularly during the concentration, drying (temperature) and homogenization of the powder.

During drying, Maillard reactions are strongly accelerated and various components, such as aldehydes, aromatic substances and CO_2, are released. Depending on the application, these reactions can be desired (flavor notes, brown color) or undesired (loss of essential amino acids, flavor and colors out of the expected sensorial profile). For milk powders, (Gaiani *et al*., 2010; Kim *et al*., 2009a, b) demonstrated that, at higher drying temperatures, less fat and protein (i.e. more lactose) appear on the surface of the powder because the surface solidifies sooner. In addition, (Kim *et al*., 2009b) demonstrated that, at lower drying temperatures, an important amount of small fat globules could migrate to the surface, particularly during the first drying period (i.e. when the moisture content in the drying droplet is still high enough and the temperature in the droplet low enough). By the use of CLSM, it was also demonstrated that the fat supramolecular structure in fat-rich dairy powders was connected to the drying air temperatures; inlet air temperature and, consequently, the temperature of the drying droplet, had the most significant influence (Vignolles *et al*., 2010). Similarly, the release and oxidation stability of encapsulated limonene in powders were measured during storage. The powders spray-dried at a high temperature of feed presented higher stability for release and oxidation than those at a lower feed temperature (Paramita *et al*., 2010a, b). The result was attributed to the increase of the shelf thickness of

the particle observed by CLSM and SEM at a higher feed temperature, with the thicker shell wall acting as a barrier to flavor release and diffusion of oxygen and moisture from the surrounding environment.

At the same time, in increasing the number of homogenization passes it is possible to reduce the fat globule size and, consequently, the amount of fat present on the powder surface as the larger fat droplets migrate easily and quickly to the surface prior to the surface formation, or are present at the surface of droplets leaving the atomizer (Kim *et al*., 2009b; Vignolles *et al*., 2009).

Also investigated were dryer types, including industrial-, pilot- and laboratory-scale dryers. From these studies it was recorded that the final surface composition of powders was significantly different depending on the dryer type used. These results show that careful comparison between different studies is required, as milk powder produced by small-scale dryers does not accurately represent powder produced by industrial dryers, both in surface composition and morphology (Fyfe *et al*., 2011a). Recently, stabilized dairy powders for longer-term storage at ambient temperatures by partial crystallization of amorphous lactose were performed in vibrated fluidized beds (Yazdanpanah and Langrish, 2011b). For this purpose, various combinations of temperatures, humidities, and processing times were used and the powders were less sticky, but links with the powder surface composition were not fully investigated. Nevertheless, it is now well known that the amorphous-lactose fraction could be treated in a crystallization facility after spray-drying to crystallize lactose-containing powders in order to limit the caking tendency of the powder (Yazdanpanah and Langrish, 2011a).

Powder storage

Aw and glass transition (Tg) concepts are the two most successful theoretical foundations that have been developed in order to determine powder stability for storage and, more generally, food stability (Montes *et al*., 2011; Rahman, 2012).

From the start, the Tg of the powder is a key influencing factor on the powder structure and composition. Below this temperature, amorphous substances are in a glassy state (under a thermodynamically meta-stable state), whereas above this temperature substances are in a rubbery state (under a thermodynamically unstable state). For a number of food powders (particularly fruit powders), understanding the relationships between Tg and aw could aid in predicting storage temperatures at different relative humidities (Jaya and Das, 2009). Unfortunately, Tg cannot be determined for all food systems, especially cocoa (Montes *et al*., 2011). The temperature of storage under dry conditions was investigated in whole milk powders presenting variable fractions of fats, including hardened rapeseed oil: β-stable and hardened palm oil: β′-stable. During storage, fats may crystallize into forms β′ and β, with the latter being thermodynamically stable. Strong links between the fat compositions in the bulk and storage conditions were made with the fat

surface composition (Millqvist-Fureby, 2003). For Partanen *et al*. (2008), aw modifications during storage were also found to be the reason for a higher rate of oxygen transport in whey protein matrices, allowing quicker oxidation of the encapsulated material, in this instance flaxseed oil. Prime *et al*. (2011a, b) attempted to take this further by using different modes of AFM to characterize the surface of model spray-dried powders maltodextrin, soya oil and sodium caseinate. By combining the use of phase imaging, nanoindentation and force modulation microscopy, these authors differentiated various surface material properties and revealed a regular dispersion of soft area (made of oil) distributed across the particle surface. In addition, they investigated the effect of humidity and temperature cycling on the caking behavior of the particles.

Others observed some surface modifications during storage; however links with theoretical foundations are complicated to make due to the fact that many parameters are concerned and need to be controlled. In Faldt and Bergenstahl (1996)'s research, fat migration was observed at the surface of dairy powders during storage under humid conditions. Lactose crystallization at the powder surface was found to be the cause of the formation of cracks and canals which allowed lipid migration from the bulk to the surface. This phenomenon was also noticed in high protein powders (Gaiani *et al*., 2009; Haque and Roos, 2006; Vega and Roos, 2006). With each of these powders, the apparition of surface pores was observed at the surface by SEM and could therefore be responsible for fat release.

14.6 Impact of powder surface composition on powder functionality

Not many food powders are directly used by the consumer in the powder form. Generally, most powders are included as ingredients of formulation before being finally ingested. Consequently, a major area of research concerns investigating particle/water interactions (how powder surface properties influence their roles in forming wet formulations.) Properties such as particle/air interactions and particle/particle interactions are also of importance and are more likely to occur during processing or storage. Some interesting examples of functional properties linked with the surface of various powders are listed in Table 14.2.

14.6.1 Particle/water interaction

Particle/water interactions for food powders have been largely investigated during the last ten years. These interactions are directly linked to the powder dissolution properties, which are essential quality attributes of a powder as a food ingredient (Gaiani *et al*., 2005, 2006b, 2007). Some common concerns surround dissolution, particularly depending on the different steps used: the

Table 14.2 Examples of some functional properties linked with surface composition for various powders

Powder family	Type of powder	Method of analysis	Composition	Functional properties investigated	References
Dairy powders	Variable from literature	Mathematical	Variable	Solute segregation that occurs during the solid surface formation during the spray drying process	Fu *et al.* (2011)
	Dairy protein powders	XPS	Mix of casein and whey proteins	Influence of the drying temperature Links between lipids coverage and wetting properties	Gaiani *et al.* (2010, 2011b)
		AFM, SEM, XPS	MPC 80	Surface hydrophobicity increase during storage linked with solubility	Fyfe *et al.* (2011b)
		Dynamic surface tension, pendant drop	Milk proteins	Proteins weight profile and surface segregation	Drusch *et al.* (2011)
		XPS	NMC, WPI	Residual surface lipids and wetting properties	Gaiani *et al.* (2007, 2009)
	SMP, WMP, SSMP, Cas, WPI	Size, shape, SEM	Lactose, lipids, milk proteins	Links between particle shape and rehydration properties (solubility, wettability and dispersibility)	Gaiani *et al.* (2011a)
		XPS	Lactose, lipids, whey, caseins	Links between surface composition and wetting properties	Gaiani *et al.* (2006), Kim *et al.* (2002)
	Baby powders	DVS		Vapor sorption decreased due to milk powder content and structure This was related to the process of agglomeration	Szulc and Lenart (2010)
	Model powders systems	Various AFM-based techniques	NaC, maltodextrin, soya oil	Changes in powder topography as a function of humidity and temperature cycling	Prime *et al.* (2011a, b)
Beverage powder		Sorption isotherm	Cocoa carbohydrate	Risk of powder caking during storage	Montes *et al.* (2011)

(*Continued*)

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Table 14.2 Continued

Powder family	Type of powder	Method of analysis	Composition	Functional properties investigated	References
Cereal powder	Cereal flours	Sorption isotherm XPS	Starch, arabinoxylan, lipids, proteins	Links between powder grinding and surface development of XPS on cereal powders	Saad *et al.* (2009, 2011)
		SEM, CLSM	Amaranth, quinoa…	Links between baking properties and variable cereal flour formulations	Alvarez-Jubete *et al.* (2010)
	Starch granules	SEM, ToF-SIMS, AFM		Level of starch granule organization and starch resistance of to enzyme attack	Baldwin *et al.* (1997a, b), Gallant *et al.* (1997)
Other powders	Sugar rich food powders	XPS, surface tension, interfacial viscosity	WPI, sucrose	Surface stickiness and competitive surface migration of proteins	Adhikari *et al.* (2009), Jayasundera *et al.* (2011a, b)
	Sugar	IGC	Sucrose, lecithin	Coating the sugar surface with emulsifiers induced an increase in the lipophilicity of the sugar particles (decrease in the acidity of the surface)	Rousset *et al.* (2002)
	Medical powders	XPS, TOF-SIMS, NMR, XPS, CLSM	Chitosan, mannitol	Optimization of pulmonary administration and lung delivery	Al-Qadi *et al.* (2011)
		XPS, dynamic surface tension	HPMC, BSA, trehalose…	Dissolution, flowability	Elversson and Millqvist-Fureby (2006)
		XPS, DVS, X-ray diffraction	Albumin, phospholipids, protein	An elevated ratio of fine particles is good for an aerodynamic behavior	Bosquillon *et al.* (2004)
	Encapsulated food powders	CLSM, SEM	Gum arabic, maltodextrin	Powders spray-dried at high temperatures show the larger vacuole size and lower density Links with optimum packaging and good flowability	Soottitantawat *et al.* (2007)

wettability (the ability to absorb water); the sinkability (the ability to sink into the water); the dispersibility (the ability to disperse in single particles throughout the water); and the solubility (the ability to dissolve in water). It is generally accepted that some of these steps can be governed by the surface composition of the powder.

More recent studies have concerned the overrepresentation of some components at the powder surface in comparison with the bulk composition (Jayasundera *et al*., 2009). Lipids and proteins (surface-active components) were systematically found to be overrepresented at the surface, whereas lactose and minerals were more localized within the particle (Kim *et al*., 2002; Gaiani *et al*., 2006; Shrestha *et al*., 2007; Vignolles *et al*., 2009). Different mechanisms of powder surface formation were proposed and discussed from these results (Gaiani *et al*., 2010, 2011; Jayasundera *et al*., 2009). In addition, the shortest wetting times were correlated with the highest lactose surface content by comparing dairy powders containing variable combinations of hygroscopic material (Gaiani *et al*., 2006; Kim *et al*., 2002). In order to improve the wetting properties of milk powders, Millqvist-Fureby and Smith (2007) added lecithin. Using XPS, it was possible to detect the lecithin at the surface of the particles and find links between surface lecithin and the improvement of powder wetting properties. However, these wetting properties were also negatively related to the presence of surface fat (Kim *et al*., 2005b; Millqvist-Fureby *et al*., 2001; Vega and Roos, 2006; Vignolles *et al*., 2007).

Investigations of the surface composition in relation to the powder solubility were also undertaken, and pea protein isolate and sodium caseinate powders were spray-dried in the presence of sugar and surfactants. The solubility of the sodium caseinate powder increased, whereas the solubility of the pea protein isolate was found to have decreased. Regardless, clear links with the powder surface were not made (Jayasundera *et al*., 2011). These links were made by Sansone *et al*. (2011) for flavonoïd powders, and it is well known that powders' low solubility could limit their use as components for functional foods, nutraceuticals and pharmaceutical agents. A combination of solubility enhancers, including coating gastroresistant polymer and swelling or surfactant agents, was also added during the spray-drying. *In vitro* dissolution tests were carried out in order to investigate the influence of these polymers on flavonoïd releases from the microparticles, and solubility decreases during 90 days of storage (at 25 and 40°C) at various humidities were observed in milk protein concentrate (MPC) powder. Nevertheless, this degradation was not related to the powder surface composition; instead it was related to the powder surface microstructure. A thin layer of fused casein micelles (SEM) and a concurrent increase of the surface hydrophobicity (XPS) were believed to be responsible (Fyfe *et al*., 2011b).

In order to increase the rehydration properties, skim-milk particles presenting a crystalline surface with an amorphous core were developed, creating an eggshell-like structure. These new architectures allowed for the improvement of surface properties (wetting) with a concurrent enhancement of desirable

bulk properties such as solubility and dispersion. The resulting powders presented good rehydration properties and stability of storage (Yazdanpanah and Langrish, 2011a).

14.6.2 Particle/air interaction

The principal functional property affected by particle/air interactions is the oxidative stability. Microencapsulation and spray-drying are two expanding and interesting technologies for packaging sensible materials, such as volatiles, oils and flavors, in the form of powders. The successful application should achieve a high retention of the sensible materials in the core, with a minimum amount on the powder surface, during processing and storage (Jafari *et al*., 2008). However, a number of authors have observed oxidative problems with negative consequences.

First of all, the presence of fat at the food powder surface rends the powder susceptible to oxidation (Kim *et al*., 2002). These authors observed an oxidation 'signature' using XPS by comparison of the oxygen peak (after deconvolution) of various dairy powders. For example, the Fig. 14.1e represents three XPS spectra for C_{1s}, N_{1s} and O_{1s} peaks after deconvolution. After two days of storage at 40°C, the O_{1s} peak was significantly modified in all cases. The highest modifications were observed in cream powder and the smallest in skim-milk powder, which indicated, as expected, that the cream powder was covered in more fat than skim-milk powder. In addition, the nature of the fat and/or oil-like melting properties (high, medium and/or low melting temperatures) were found to be important (Kim *et al*., 2005b). Three milk powders with different oil phases (high-melting pure, industrial tristerarins and liquid triolein) were prepared and stored for six months at room temperature. The highest fat coverage (by XPS) and the largest increase in cholesterol oxides gas chromatography (GC) were obtained using industrial tristearin as the oil phase in comparison with the high-melting pure oil. The third powder with liquid triolein was in between the two tristearin powders (Granelli *et al*., 1996).

The oxidative stability was mainly studied in oil and/or flavor encapsulated powders. At various relative humidities, the oxidation of flaxseed oil dispersed in a whey protein matrix was slower than the bulk oil in the same matrix (Partanen *et al*., 2008). An in-depth study of the matrix revealed its glassy state (by DSC) at all storage conditions, but it also revealed considerable structural changes (SEM). These results were used to suggest possible mechanisms for oxygen transport in the whey protein matrix (Partanen *et al*., 2008). Maltodextrin, combined with a surface-active biopolymer such as modified starch or a whey protein concentrate, was used as the wall material for d-limonene or fish oil protection. Results revealed that not only the surface composition and surface structure of the powder were of importance, but also the nature of the core material (particularly its volatility) and the powder size. For instance, larger particles retain more volatiles than smaller

ones, but at the same time there is more un-encapsulated oil at the surface of these larger particles (Jafari *et al*., 2007).

In order to effectively protect the active substance, the surface properties of the wall as well as information about the core materials and the particle size are required.

14.6.3 Particle/particle interaction and particle/wall equipment

The flowability of a food powder is important for the powder's easy handling, processing (storage, transportation, formulation, mixing, compression and packaging) and final application. The chemical and physical state of the components in the powder will influence the cohesive nature, stickiness and caking characteristics of the powder, which will in turn influence the flow characteristics (Chen and Ozkan, 2007; Chuy and Labuza, 1994). More specifically, the powder surface state of the components has been found to influence these properties (Forny *et al*., 2011; Kim *et al*., 2005a). The flowability and flow properties of a food powder are often determined by the angle of response, and bad flow properties were observed in powders presenting surfaces largely covered by fat, such as whole milk and cream powders. In addition, a regular flow was registered for skim-milk powder, which has low surface fat. In order to observer whether flow difficulties came from the presence of lipids at the surface, free surface fat was extracted and powders that did not flow before extraction flowed correctly after the procedure (Kim *et al*., 2009c).

Caking is a prevalent situation that can cause problems in operation, equipment surfaces or product yield. In milk powders with different fat contents, Nijdam and Langrish (2006) related the degree of caking to the surface composition. The results indicated that the degree of caking was high for powders containing between 5% and 30% of surface fat and that the caking was significantly reduced when surface fat was less than 5%. Hartmann and Palzer (2011) recently studied caking kinetics of water-soluble amorphous powders, and the sinter bridges created in the powders were measured. The calculated sinter bridge diameters were correlated with the strength of the measured caked powder bulk, however, these investigations did not characterize the powders' surface chemical composition. The conditions of 'decaking' (recovering a flowing sugar after caking) were also established for crystalline and amorphous food powders, and the presence of a thin film of saturated solution at the surface of the crystal was linked to the particle/particle interaction at the level of a laboratory and a pilot silo. In the case of noncrystalline powders, aw, together with Tg, is important in determining whether it is necessary to interpret the origin of the formation of bridges between food powder particles and the caking phenomenon (Mathlouthi and Roge, 2003).

The adhesion of powders to processing equipment surfaces (also called 'stickiness') is a common problem encountered in food handling, processing, and consumption. This serious problem occurs predominantly during drying, and as a result it is not surprising that large efforts are made to control

the temperature and humidity of the production environment to avoid it (Hartmann and Palzer, 2010). The presence of some components, such as high sugar and high fat powders, can make a powder more susceptible to stickiness. The importance of their viscous and glass transition properties were discussed with the fundamentals of adhesion and cohesion mechanisms, without directly taking into account the powder surface (Bhandari and Howes, 2005). During milk powder spray-drying others observed a high wall deposition flux when the outlet particle temperature was above the sticky-point temperature. No significant effect on the wall deposition flux was observed when using a nonstick material, and grounding the spray dryer also had no significant effect on the wall deposition flux (Ozmen and Langrish, 2003). As the results of these investigations into sticking problems show, there is an obvious need to periodically clean the equipment. Wet cleaning may not desirable as it can introduce an opportunity for microbial growth, however there is research potential in the use of wet cleaning followed by efficient drying.

Segregation during dry-mixing is also a fundamental problem for food ingredient mixes. This problem is particularly serious when there is a significant difference in particle size between the ingredients, as differences in particle sizes cause different mobilities of the particles (Manickam *et al*., 2010) and, in addition, particles with different densities and shapes can cause segregation of mixed particles. Nevertheless, up until now, no studies have related directly to the surface composition, even though strategies to overcome segregation mechanisms exist. For example, the addition of oil to the mix can make the mix more cohesive and, as a result, can inhibit segregation. However, this increased cohesion might greatly reduce flowability and result in increased stickiness of the powder, causing it to stick to the equipment.

Direct links between particle/particle interactions and surfaces have often been studied in the field of pharmaceutical powders. Modifications of a powder surface's physical and chemical properties have been correlated with a reduction in powder cohesion and an improvement in powder flow by Carr index and shear cell testing (Zhou *et al*., 2011b). In addition, variations in powder surface coverage (determined by XPS and ToF-SIMS) obtained by the addition of variable magnesium stearate levels suggested that changes in the surface composition exerted a direct impact on the powder cohesion and flow characteristics, whereas changes in the surface morphological properties were less pronounced (Zhou *et al*., 2011a).

14.7 Food industry examples

Food powders are used in industry for their convenience in applications such as transportation, handling and processing and product formulation. Beverage powders (milk, cocoa, coffee, tea, etc.), spice powders, flours and additives are among the most common. The handling and processing characteristics of these products have to be well mastered in order to ensure that a high quality

product reaches the consumer. Some processing steps, such as agglomeration, compaction, instantization and encapsulation, are applied to modify the particle surface in order to produce products with specific purposes and also for convenience (Dhanalakshmi *et al*., 2011).

14.7.1 Maintaining the ingredient stability and functionality (coating and agglomeration)

A number of physical and chemical methods have been used to improve the functional properties of powdered food solids. Agglomeration has been cited as the most effective method for improving the rehydration properties of food powders by modifying the surface (Dhanalakshmi *et al*., 2011).

Fluidized bed agglomeration (Fig. 14.3a) is an encapsulation technology which involves spraying a liquid (which can be a binder solution or water) onto particles. The sprayed liquid droplets wet the particle surface and make the surface sticky, either by the formation of a film of binder solution or by the modification of the particle surface viscosity. This can also be observed in food powders containing carbohydrates, as the amorphous components reach the rubbery state with a decrease in the glass transition temperature (Dhanalakshmi *et al*., 2011). This adhesion between wet sticky particles is the result of collisions, and the agglomeration is primarily a physical phenomenon which results in the bonding of solid particles. Physical or chemical modifications of the surface of the solid are achieved by short-range physical and chemical forces between the particles, and the surface modifications by specific treatments, such as the use of binders and substances that chemically or physically adhere to solid surfaces, can form bridges between particles (Pietsch, 2003). The consolidation of the new structure is achieved by drying, and the repetition of these steps leads to growth of the overall structure. The obtained dry agglomerates have a structure that allows for identification of the initial particles, and in increasing the porosity the product presents good properties for application in instant foods. More generally, agglomeration standardizes the particle size distribution and bulk density, as well as enhancing wetting by improving the penetration of liquid into the porous system (due to faster capillary action) (Shittu and Lawal, 2007).

Dry particle coating (Fig. 14.3b) is another encapsulation procedure that can be used to change the surface properties and/or functionalities of powders and is a very important process for many industries (Teunou and Poncelet, 2002). Typical applications include the modification of flowability, wettability (hydrophobic/hydrophilic properties), solubility, dispersibility, flavor, and particle properties. Coating produces dense particles with one or more layers, and the layers are created by spraying a solution onto the particle. This provides a new surface composition with modified characteristics, and the technique allows for the production of particles with a narrow size distribution. Typical features include high particle sphericity and good solubility, and the obtained product is dust-free and can be easily dispersed. Coated particles provide a

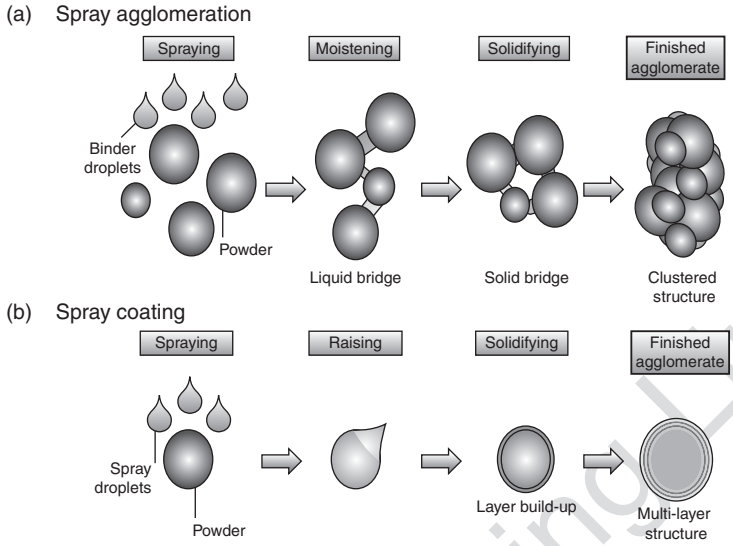

Fig. 14.3 Description of the two main technologies used to modify food powders at industrial scale. Spray agglomeration (a) allows the production of bigger particles bridged together by a binder solution and spray granulation (b) provides spherical particles with one or more layers that protect the core and give specific properties to the particle.

surface layer that can be dissolved at a desired time, depending on temperature and pH, and are produced to allow for the controlled release of the encapsulated substance. In the event that encapsulated ingredients are unstable, the coating provides protection from degradation by heat, moisture or light. The materials that make up the coatings used in food industries mainly consist of water-insoluble biopolymers such as lipids, milk or corn proteins, gums (such as locust bean gum), sodium alginate, κ-carrageenan and gelatin (Burgain *et al.*, 2011). The coatings used for food powders provide a surface composition suitable for protecting the inner area of the particle or to control the efficiency of solubility.

14.7.2 Managing dust formation

Agglomeration of solid particles is used in the food industry to produce dust-free powders with good handling properties such as flowability, mechanical resistance and wettability. This can be achieved by modifying physical properties of the particles such as the particle size and shape, density and porosity (Turchiuli *et al.*, 2012). As such, agglomeration is a process where primary particles are joined together to form larger and porous secondary particles (Palzer, 2005). Based on this definition, the caking of hygroscopic raw materials that can occur during storage can be assimilated as a form of undesirable agglomeration.

Viscosity and elasticity are influencing factors on the agglomeration properties of food particles (Palzer, 2009). Process conditions such as temperature, humidity and strain rate or frequency strongly influence these mechanical properties, as well as the supra-molecular and microscopic structure of the particles (Palzer, 2009). With the aggregation of food particles, it is important to distinguish water-soluble and water-insoluble substances, as well as the amorphous or crystalline nature of these substances (Palzer, 2009).

14.7.3 Improving the functional performance of formulated food powders (instantization and reduction in fat uptake)

In recent years there has been a growing interest in food powders that are instantly soluble in cold liquids (Table 14.3). This modification of the surface composition can also be used to reduce fat uptake of doughnuts in frying by coating wheat flour with microparticulated soybean hulls. In this instance, the sensory properties of the product were not affected in terms of the product's appearance, flavor, crispiness, taste and general liking (Lee *et al*., 2008).

The agglomeration of powder particles has been the subject of much recent interest, and by controlling the porosity and density of food powders the rehydration properties are improved (Dhanalakshmi *et al*., 2011; Forny *et al*., 2011). Agglomerates have coarse and open structures, varying from 0.1

Table 14.3 Examples of food powders requiring a processing step in order to improve their functional properties by modifying the surface

	Product	Function	References
Agglomeration	Coffee	Improving solubility	Sienkiewicz *et al*. (1971)
	Instant soymilk	Improving wettability	Jinapong *et al*. (2008)
	Mixture: cocoa, sugar, maltodextrin, milk powder	Improving reconstitution properties	Kowalska *et al*. (2004)
	Skim-milk powder	Producing dust-free powder with good handling properties	Turchiuili *et al*. (2012)
Coating	Milk based powders	Instantization in cold water	Gonus and Rosse (2011)
	Fat-containing powders coated with lecithin	Improving wetting and solubility	Millqvist-Fureby and Smith (2007)
	Fat-containing powders coated with fatty acids	Improving wetting and solubility	Asano and Mori (1988)
	Flour	Low fat uptake	Lee *et al*. (2008)

to 3mm, that improve the dispersibility of the powder that will be uniformly wetted when put into hot or cold water. In this case, agglomeration is referred to as instantizing because the rehydration and reconstitution of the food powder is improved. This benefit can be exploited either by industrial sectors or by the consumer directly.

The agglomeration of powder particles for instantization has been used for dairy products, such as hot chocolate and milk powder for ice-creams; other beverages, such as coffee and tea; and for starch-based products, such as soups, sauces and baby foods. Enzymes and yeasts, which are ingredients with a wide variety of applications in industrial bakery, can also be agglomerated, as well as maltodextrin, milk proteins and acacia gum, amongst others.

The majority of currently-available cocoa beverage powders are formulated with granulated sugar (Vissotto *et al.*, 2010) (which can be in a crystallized or granulated form), as well as maltodextrin and cocoa powder. The powder can also contain skimmed milk powder and milk whey. In these formulations, the percentage of cocoa powder generally varies from 5% to 20%. This amount is sufficient to make the powders insoluble and difficult to reconstitute in liquid, water or milk. The natural cocoa powder (with a pH comprised between 5.0 and 5.9) shows poorer dispersibility compared to the selected alkaline type of cocoa powder (with a pH between 6.2 and 7.5), and this last type is most commonly used in cocoa beverage powders. However, the presence of, on average, between 10% and 12% cocoa butter, compromises the reconstitution of cocoa beverage powders formulated with this ingredient.

One of the proposed solutions to this problem is a form of agglomeration that can be used to improve the reconstitution properties of cocoa beverage powders in liquids. This technique, however, seems to be insufficient for similar products that contain fatty acids because they tend to form a layer on the surface of the particles, which makes them repellent in cold water. Because the surface composition is mainly represented by fatty acids, the particle hydrophobicity is increased. This then renders the powder less wettable and less soluble (Gonus and Rosse, 2011). In order to meet these challenges, the use of surface-active agents (surfactants) such as lecithin (Millqvist-Fureby and Smith, 2007) provides a way of modifying the surface properties of these powders. Because the use of surfactants can also have negative effects, such as lowering flowability and affecting sensorial properties, another proposed solution is the coating of fat-containing powders by mono- and/or diglycerides of medium-chain fatty acids (Asano and Mori, 1988). In this patent, they also mentioned a chocolate drink that can readily be dispersed. This can be achieved by coating a sugar core with finely-divided dry cocoa powder that has been agglomerated with an edible vegetable fat (such as molten cocoa butter, sunflower oil, soya-bean oil or groundnut oil). Instantization of cocoa powders has since been widely studied and it appears that modification of the surface composition is the best way to improve particle characteristics.

Attention has also been paid to the use of instant soymilk powder. The production of this powder is possible thanks to an agglomeration of the spray-dried powders with maltodextrin as an aqueous binder solution using fluidized bed agglomeration. When this technique was implemented, the handling and reconstitution properties of the powders were improved (Jinapong *et al.*, 2008).

The applications of agglomeration are largely included in products such as dispersible milk powders, soup mixes and easy-flow table salt. With regard to instantizing, the improvement of reconstitutability concerns products such as flour, cocoa powder, instant coffee, dried milk powder, sweetener, fruit beverage powder, instant soup and spice (Dhanalakshmi *et al.*, 2011). In each of these cases, the surface composition is modified in order to achieve the desired properties. Research is still ongoing to help gain an understanding of how surface composition influences particle behavior and how the changes in constituents can achieve the desired functions.

14.8 Conclusion

In order to gain a better understanding of the relationships between surface properties and functional properties it is absolutely necessary to characterize the powder surface in detail. For this purpose, specific analytical methods (physico-chemistry and surface physics) are required in order to evaluate the surface properties of the food powders. Some techniques, such as XPS and SEM, are now well developed in the food powder fields; however others may need more attention from the scientific community. Techniques such as ToF-SIMS, particle morphology and SEM-EDX have rarely been used on food powders and may be an efficient tool in evaluating the modifications of surface properties of a powder. The coupling of atomic, molecular, microstructural and physical approaches might provide an interesting opportunity to better understand food powder surface reactivity.

At the same time, much research is needed to bind basic approaches with technological applications, while integrating the contribution of particle surface and process parameters that occur during powder manufacture to end products. The main functional properties of a food powder may be linked to water, thermal and/or mechanical constraints.

14.9 Future trends

In the future, it might be interesting to follow different orientations. First of all, the development of specific analytical methods that are able to evaluate surface properties needs to continue, with the help of the knowledge already developed in fields like pharmaceutics, chemistry and soil. Research into how

composition and location of components in the particle, as well as the physical state of components coupled with storage conditions, affects powder functional properties needs to be investigated in depth. Subsequently, the development of appropriate designs needs to be investigated in order to permit the development of desired changes on the powder structure and surface composition. This science, also called particle or powder 'engineering design', leads to the concept of producing and preserving desirable powder particle structures which can maintain or enhance stability and functionality. The objective is to increase the powder's suitability for handling and transport and to enhance its ability to rehydrate while delivering the desired functionality.

14.10 References

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14.11 Appendix: Abbreviations

Water activity (aw); Brunauer, Emmett, and Teller (BET); Bovine Serum Albumin (BSA); Confocal Laser Scanning Microscopy (CLSM); Energy Dispersive X-ray spectroscopy (EDX); Differential Scanning Calorimetry (DSC); Dynamic Vapor Sorption (DVS); ESCA (Electron Spectroscopy for Chemical Analysis); Environmental Scanning Electron Microscopy (ESEM); Guggenheim, Andersen, and de Boer (GAB) ; Gas Chromatography (GC); High Performance Liquid Chromatography (HPLC); HydroxyPropyl MethylCellulose (HPMC); Inverse Gas Chromatography (IGC); Light Microscopy (LM); Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS); Milk Protein Concentrate (MPC); Sodium Caseinate (NaC); Native Micellar Casein (NMC); Nuclear Magnetic Resonance (NMR); Small-Angle X-ray Scattering (SAXS); Scanning Electron Microscopy (SEM); Tomographic Atom Probe (TAP); Transmission Electron Microscopy (TEM); Glass Transition Temperature (Tg); Time-of-Flight Secondary Ion Mass Spectroscopy (ToF-SIMS); Third Stage Sorption (TSS) ; Whey Protein Isolate (WPI); X-ray Micro Tomography (XMT); X-ray Photoelectron Spectroscopy (XPS).

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In vitro interactions between probiotic bacteria and milk proteins probed by atomic force microscopy

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ABSTRACT

Interactions between microbial cells and milk proteins are important for cell location into dairy matrices. In this study, interactions between two probiotic strains, *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* GR-1, and milk proteins (micellar casein, native and denatured whey proteins) were studied. The bacterial surface characterization was realized with X-ray photoelectron spectroscopy (XPS) to evaluate surface composition (in terms of proteins, polysaccharides and lipid-like compounds) and electrophoretic mobility that provide information on surface charge of both bacteria and proteins along the 3–7 pH range. In addition, atomic force microscopy (AFM) enabled the identification of specific interactions between bacteria and whey proteins, in contrast to the observed nonspecific interactions with micellar casein. These specific events appeared to be more important for the GG strain than for the GR-1 strain, showing that matrix interaction is strain-specific. Furthermore, our study highlighted that in addition to the nature of the strains, many other factors influence the bacterial interaction with dairy matrix including the nature of the proteins and the pH of the media.

1. Introduction

The study of microbial development in fermented dairy foods is of interest for food manufacturing in order to improve and control some processing steps, like the ripening step [1]. In addition to lactic acid bacteria added as starter cultures, dairy food matrices such as milk, cream, fermented milk, cheese and ice cream can also contain probiotic bacteria. It is obvious that the bacterial surfaces physico-chemically interact with dairy components such as milk proteins, fat globules and/or lactose. The surface characteristics of bacteria are determined by the chemical composition of their cellular surface, in particular by proteins, polypeptides and polysaccharides [2] and thus represent a major key in bacterial re/activity with food components [3,4].

Bacterial location in fermented dairy foods has been studied at macroscopic level, particularly in cheddar cheese [5], where it was observed that the spatial repartition of bacteria depends on their location at the end of the immobilization step, on the spatial distribution of nutrients (lactose, proteins, etc.) and on interactions between bacteria [1]. At microscopic level, it was observed that the cells were preferentially located around fat globules and more particularly in whey pockets [6]. Other authors have suggested that the location of the bacteria may be influenced by the strain itself [7].

At the microstructure level, bacterial colonization of milk is dependent of the physical state of the matrix (e.g. liquid for milk and solid after coagulation). In fact, in liquid milk, the cells can move thanks to attractive or repulsive forces towards milk components that influence their location [2]. On the contrary, after gelation, the bacteria are entrapped within the curd and develop as bacterial colonies with a three dimensional spatial distribution [1,8]. The microbial cell wall constitutes the frontier between the cells and their environment and is thus also a determining factor in this colonization. The cell wall plays several key roles like regulating the cell shape, controlling the cell surface properties and governing biointerfacial phenomena such as cell adhesion and cell aggregation [9]. Most of the bacterial strains used in dairy products are Gram-positive bacteria that are covered by neutral and acidic polysaccharides, different cell wall proteins and teichoic acids with a high acidic nature [10].

Abbreviations: AFM, atomic force microscopy; EPS, exopolysaccharides; IEP, isoelectric point; LGG, *Lactobacillus rhamnosus* GG; LGR-1, *Lactobacillus rhamnosus* GR-1; XPS, X-ray photoelectron spectroscopy.

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During the past three decades, the major focus has shifted towards probiotic dairy products, as the market of functional foods continues to expand [11]. The probiotic microorganisms used are generally members of the genera *Lactobacillus* and *Bifidobacterium*. Probiotic bacteria are defined as "live microorganisms which when administrated in adequate amounts can provide a health benefit on the host" [12]. However, to exert these beneficial effects, probiotic bacteria must maintain their viability first during storage and then during their passage through the upper gastro-intestinal tract in order to arrive in the intestine in a viable state [11]. A good alternative for the plain delivery of probiotics into the intestine, is their formulation in cheese [13]. The delivery via cheese has several advantages as the cheese creates a buffer against the high acidity encountered in the stomach, forms a dense matrix and protects the cells by the possible presence of fat [14]. Therefore, many authors focused on the microencapsulation of probiotic bacteria into dairy matrices aiming at protecting the cells for a controlled release in the gut [15–17]. However, these studies only recorded parameters like encapsulation yield or survival rate and differences were observed when changing the dairy matrix or the encapsulation technique. Dealing with these observations, the way the bacteria interact with the matrix was never elucidated in order to understand how encapsulation yield or survival rate could be improved.

The present study aimed to investigate the interaction of probiotic bacterial cells with dairy proteins, in addition to the influence of the environmental pH and the nature of the strains on these interactions. For this purpose, a new approach was developed to estimate the adhesion forces by atomic force microscopy (AFM). AFM allows the probing of interfacial phenomena at the nanoscale, including specific and non-specific interactions. AFM force measurements were correlated to surface characterization of microbial cells and milk proteins by X-ray photoelectron spectroscopy (XPS) and electrophoretic mobility. The approach was developed using *Lactobacillus rhamnosus* GG and GR-1 as model probiotic strains. The gastro-intestinal isolate *L. rhamnosus* GG is extensively used as probiotic in dairy products. Its probiotic properties are well documented and include host immunostimulation and reduction of symptoms from gastrointestinal disorders [18,19]. *Lactobacillus rhamnosus* GR-1 is a closely related probiotic strain isolated from a female urethra, documented health effects include host immunostimulation and resolution of moderate diarrhoea in HIV/AIDS patients [20–23], and prevention of recurrent bacterial vaginosis [24,25].

2. Materials and methods

2.1. Material

L. rhamnosus GG (LGG) and *L. rhamnosus* GR-1 (LGR-1) were used throughout this study. Bacterial stock cultures were stored at −20 °C in MRS [26] broth containing 20% (v/v) glycerol.

Micellar casein powder (Promilk 872B) was obtained from Ingredia IDI (Arras, France). Whey proteins isolates powders (Prolacta 90) were purchased from Lactalis Ingredients (Bourgbarré, France). The solutions were prepared by adding 1 g of protein powder into 100 g of distilled water. The rehydration was done by stirring for 2 h at room temperature and then overnight at 4 °C. The denatured whey proteins were obtained by heating the native whey solution at 78 °C for 10 min then cooling it to room temperature. Heat denaturation of whey proteins allows the unfolding of the structure leading to aggregation phenomena.

2.2. Characterization of bacterial and protein surface properties

2.2.1. Electrophoretic mobility

The bacterial cells were first subcultured at 37 °C in MRS medium, followed by a culture until the end of exponential phase. These cells were harvested by centrifugation (1 min, 7000 rpm, room temperature) and resuspended in KNO_3 10 mM. Milk protein solutions were prepared at a final concentration of 1% (w/w). The electrophoretic mobility of the cells and milk protein solutions were measured in the pH range 3–7 at 20 °C using a Malvern Zetasizer nano-ZS. The pH was adjusted by the Malvern MPT-2 autotitrator via addition of NaOH 10 mM and HCl 10 mM in a 10 ml sample. Electrophoretic mobility was derived from velocity under an applied electric field of 150 V. Electrophoretic mobility was evaluated three times on the same sample and this was repeated during three independent studies.

2.2.2. X-ray photoelectron spectroscopy

Bacteria were first subcultured at 37 °C in MRS medium. This preculture was then used to inoculate 20 mL of MRS broth which was incubated overnight at 37 °C until the end of exponential phase. Cells were harvested by centrifugation (1 min, 7000 rpm, room temperature) and washed twice with physiologic water (NaCl 9 g/L, pH 7.4). After the final washing, the pellet was transferred to cryotubes and quickly cooled in liquid nitrogen and placed on the shelves of a freeze dryer (Christ alpha 1-2, freeze-dryer, Osterode, Germany) [27]. The microbial powder obtained after freeze drying was gently crushed with a spatula and used for XPS analysis using a Kratos Axis Ultra (Kratos Analytical, UK) spectrometer. For each bacterial cell, three independent points were studied to evaluate surface composition.

2.3. Atomic force microscopy

2.3.1. Substrate preparation

All bacterial cells were used at the end of exponential growth phase. For topographic images, a glass slide was precoated with polyethilenenimine (PEI) (0.1% (w/v) in milli-Q-grade water) with a contact time of 10 min. It was rinsed with milli-Q-grade water and stored in a sterile Petri dish. The cells (1 mL of bacterial suspension with an optical density ($OD_{600\ nm}$) of 1.2) were electrostatically immobilized onto PEI-coated glass slide (contact time: 30 min). The glass was slightly dehydrated under a nitrogen flow to realize topographic measurements. This method avoids the use of chemical binders between the substrate and the bacterial sample and leads to minimization of any chemical modification of the bacterial cell wall or bacterial surface organization.

Regarding milk proteins, solutions with a final concentration of 1% were directly deposed on freshly cleaved mica for 30 min.

For topographic images and force measurements, Olympus micro cantilevers OMCL-TR400 were purchased from Atomic Force (Mannheim, Germany) with a spring constant of 20 pN/nm. The preparation of cantilevers and tips for force measurements was realized as followed: they were precoated with PEI by immersion for 5 h in the PEI solution, and then washed in milli-Q-grade water. The negatively charged bacteria were attached to the positively charged PEI-coated probes thanks to electrostatic attractions established during 1 h of contact with the cell culture. The bacteria-coated probes were rinsed with milli-Q water before use. The designed probe can be called "lacto-probe" [28]. For the milk proteins, solutions were used at a final concentration of 1% and deposed on mica. After 30 min of contact, the mica was disposed into PBS buffer with a controlled pH of 6.8 or 4.8 to perform force measurements in liquid [28]. These two pH values were chosen according to the protein electrophoretic mobility profiles. At pH 6.8 (the pH encountered in fresh milk) milk proteins are negatively charged

Table 1
XPS analysis of *L. rhamnosus* GG and GR-1 (mean ± SD, $n = 2$).

Strain	Obtained from raw spectra					Obtained from C_{1s} peak deconvolution				Obtained with the matrix formula		
	%C	%O	%N	N/C	O/C	C—C, C—H	C—O, C—N	C=O	O—C=O	$\%C_{ps}$	$\%C_{pr}$	$\%C_{lp}$
LGG	67.80 ± 0.97	26.58 ± 2.31	5.62 ± 1.07	0.08	0.39	28.20 ± 1.33	47.07 ± 2.97	19.82 ± 1.07	4.92 ± 0.57	24 ± 4	20 ± 4	24 ± 1
LGR-1	68.39 ± 0.27	27.64 ± 0.09	3.97 ± 0.18	0.06	0.40	36.21 ± 1.10	44.56 ± 1.05	14.72 ± 2.43	4.52 ± 0.28	28 ± 0	14 ± 1	27 ± 0

and at pH 4.8 the negative charge of the proteins is less pronounced because of the proximity of the isolelectric point (IEP). However, it is not possible to choose a pH below this value because of the acid gelation of caseins below their IEP.

2.3.2. AFM measurements

Force measurements and topography images were made at room temperature using an Asylium MFP-3D atomic force microscope (Santa Barbara, CA, USA) with IGOR Pro 6.04 (Wavemetrics, Lake Osewego, OR, USA) as operation software. It was used in contact mode with an applied force lower than 250 pN and images were acquired at a scan rate of 1 Hz and scan size of 30 μm × 30 μm. AFM force–distance curves were obtained by following the cantilever deflection (d) as a function of the vertical displacement of the piezoelectric scanner (z) with a scan speed of 1 μm/s. In order to measure the cantilever deflection, a laser beam was focused on the terminal part of the cantilever and the position of the reflected beam was detected thanks to a position sensitive detector. The adhesion force can be deduced from the cantilever spring constant k_c and the deflection by the following relation:

$$F = k_c \cdot d$$

On the force–distance profile, two different curves are encountered: the approach and the retraction but only the retraction is considered for estimation of adhesion forces. Initially, the probe comes into contact with the sample. In a second time, when considering the retraction part, the tip picks up the sample and the force–distance curve can exhibit single or multiple peaks that

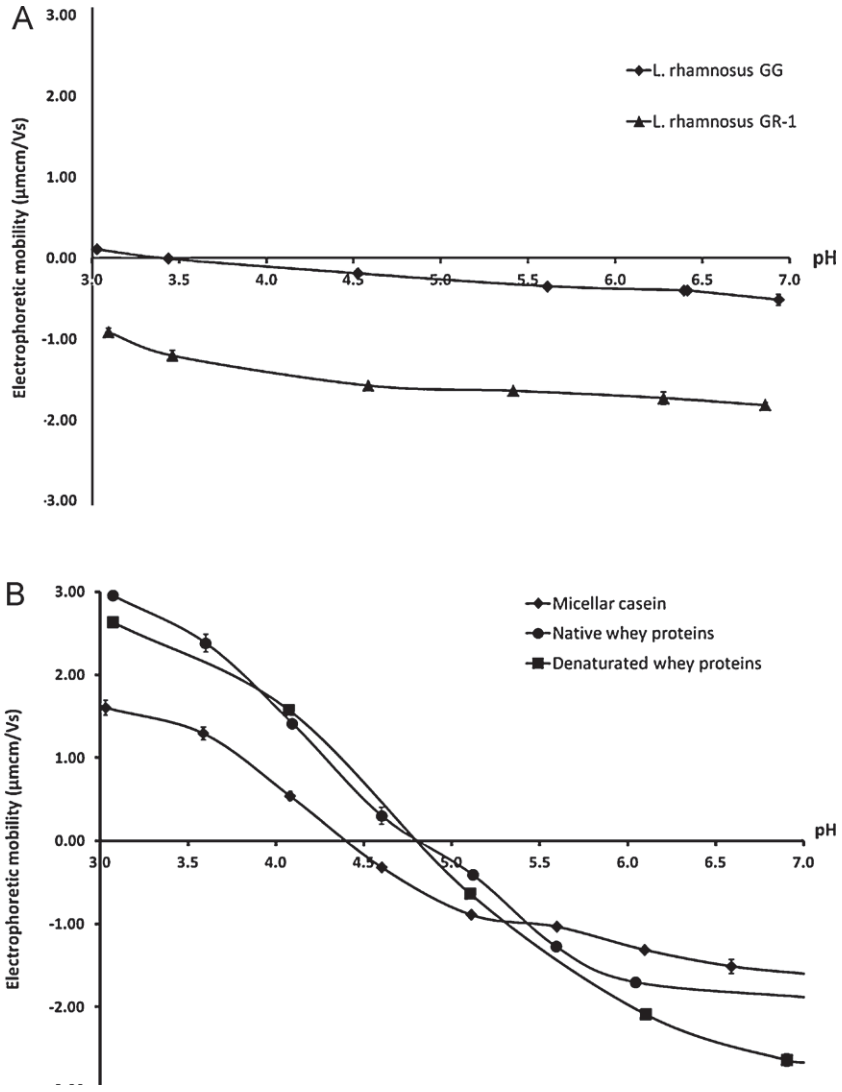

Fig. 1. Measured electrophoretic mobility vs. pH for bacterial strains *L. rhamnosus* GG and GR-1 (A) and milk proteins (micellar casein, native and denatured whey proteins) (B) (mean ± SD, $n = 3$).

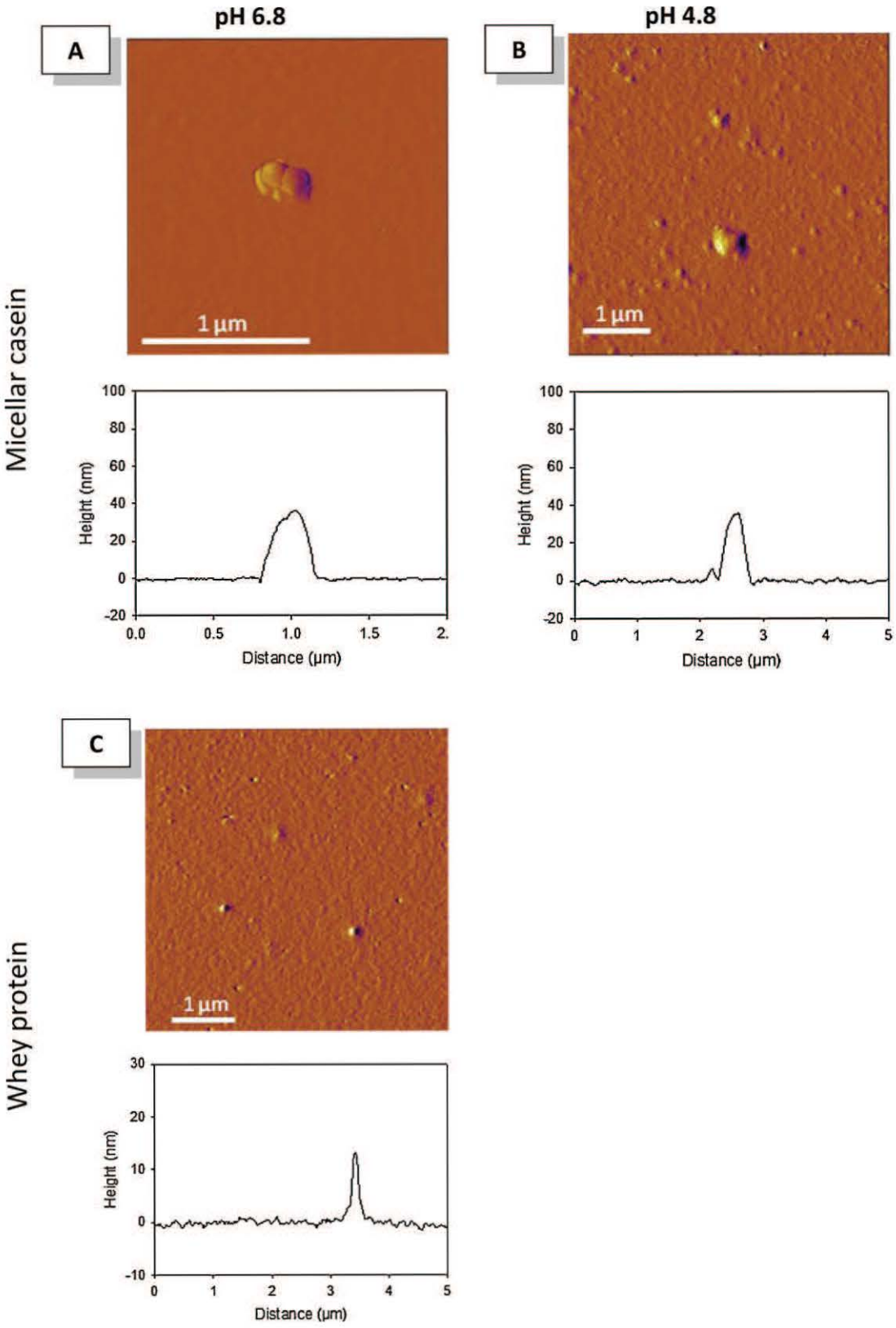

Fig. 2. Deflection images of micellar casein at two pHs ((A) pH 6.8 and (B) pH 4.8) and whey proteins ((C) pH 4.8). Each image corresponds to 512 horizontal lines that describe the outward and return of AFM cantilever tip (1024 scans are made on each image). The graphics below each image correspond to height profiles taken from a cross section on the AFM images.

correspond to adhesive events or interaction forces between the tip and the sample surface. The difference between these peaks and the baseline provides information on adhesion events. Finally, the probe returns to its original position and the same process will be repeated at another location in the sample [9] which allows the performance spatially resolved force-mapping, called force–volume image.

3. Results and discussion

3.1. XPS analysis

The study of bacterial surfaces is an important parameter to study bacterial interactions with the environment. The potential of XPS in providing informations on chemical composition of bacterial cell surfaces was demonstrated [29]. This technique involves the irradiation of the sample by an X-ray beam resulting in an ejection of photoelectrons. The kinetic energy of the emitted electrons can be analysed and their binding energy in the atom of origin determined. The obtained information concerns only the outermost molecular layers of the surface (around 2–5 nm). Each peak of the recorded spectrum is characteristic of a given electron energy level of a given element, and its position is influenced by the chemical environment. Consequently, XPS measurements can provide an elemental analysis and a rough functional group analysis of the bacterial surface [30]. Table 1 presents the surface elemental composition, in terms of mole fraction and atomic concentration ratios with respect to total carbon, determined on each bacterial strain, *L. rhamnosus* GG and GR-1. Consistent with the general biochemical composition of Lactobacilli cell walls, the main elements detected were C_{1s}, O_{1s} and N_{1s}. The carbon peak is composed of three components: carbon bound only to carbon and hydrogen (binding energy: 284.8 eV), carbon bound to oxygen or nitrogen by a single bond (binding energy: 286.3 eV) including ether, alcohol, amine and amide, and finally carbon making one double bond or two single bonds (binding energy: 532.7 eV) corresponding to carboxylic acid, carboxylate, ester, carbonyl or amide. The nitrogen peak appeared at 399.9 eV corresponding to unprotonated amine or amide functions. The last oxygen peak showed two components with oxygen singly bound to carbon and hydrogen (binding energy: 532.7 eV) that can be attributed to hydroxide, acetal and hemiacetal. Finally, oxygen can make a double bond with oxygen (binding energy: 531.4 eV) referring to carboxylic acid, carboxylate, ester, carbonyl and amide. Regarding their carbon, oxygen and nitrogen composition, no significant difference was observed between the two strains, except maybe for nitrogen, which was slightly higher for *L. rhamnosus* GG. The results obtained for *L. rhamnosus* GR-1 in this study are in general similar to those published by other authors [31]. The biomolecular composition of the bacterial surfaces was deduced from the XPS data. The surface composition was modelled in terms of three classes: polysaccharides (Ps), proteins (Pr) and lipids (Lp). More complex compounds such as lipoproteins, were considered as a combination of lipids and proteins. The molecular composition was computed with the following elemental concentration ratios [30,32]:

$$\left[\frac{N}{C}\right]_{obs} = 0.279\left(\frac{C_{Pr}}{C}\right)$$

$$\left[\frac{O}{C}\right]_{obs} = 0.325\left(\frac{C_{Pr}}{C}\right) + 0.833\left(\frac{C_{Ps}}{C}\right)$$

$$\left[\frac{C}{C}\right]_{obs} = \left(\frac{C_{Pr}}{C}\right) + \left(\frac{C_{Ps}}{C}\right) + \left(\frac{C_{Lp}}{C}\right) = 1$$

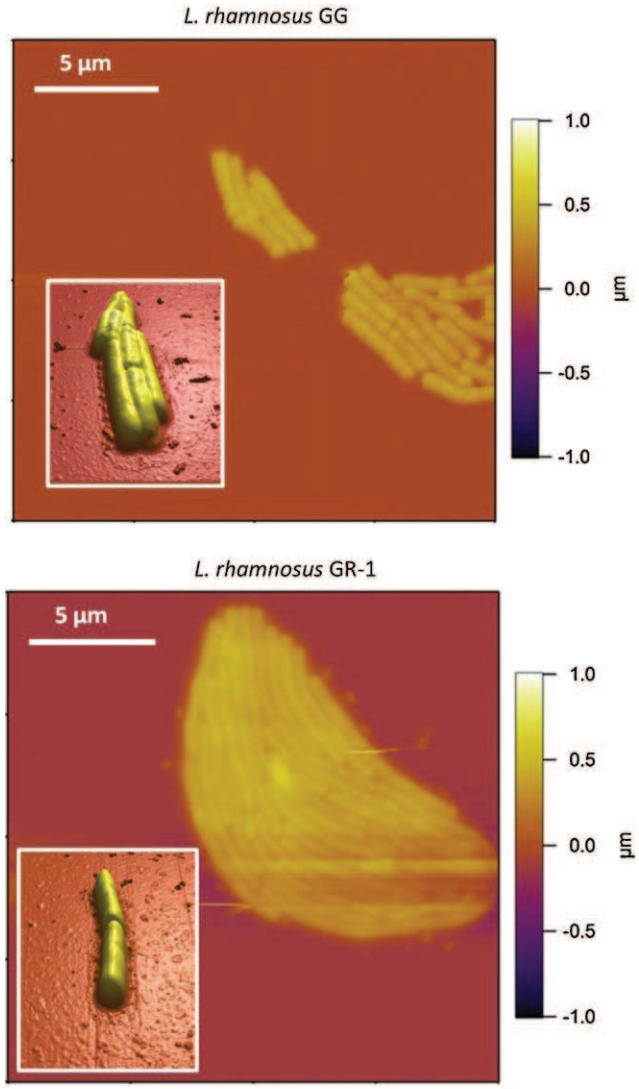

Fig. 3. Height images of bacterial strains *L. rhamnosus* GG and *L. rhamnosus* GR-1. Each image corresponds to 512 horizontal lines that describe the outward and return of AFM cantilever tip (1024 scans are made on each image) insets: 3D views of bacterial strains.

The proportions of carbon associated to each molecule can be determined by solving the system. The polysaccharide content was almost the same for both strains, corresponding to the large quantity of exopolysaccharides (EPS) produced by the bacteria (Table 1). Previous AFM studies revealed the presence of two types of polysaccharides on the cell wall of *L. rhamnosus* GG: long galactose-rich EPS molecules and shorter glucose-rich polysaccharides [33]. The protein content was higher for the *L. rhamnosus* GG strain, which could be related to the presence of the recently identified pili on this Gram-positive bacterium [34,35]. Importantly, a recent study showed that high centrifugation speeds used during the preparation of the sample can result in the removal of pili [36].

Regarding results in lipid-like compounds presented in Table 1, it seems that *L. rhamnosus* GR-1 has a higher lipid content than LGG. Important lipid-like compounds of lactobacilli include lipoteichoic acids, which are polymers linked to the cytoplasmic membrane by a glycolipid anchor [37]. Lipoteichoic acids of *L. rhamnosus* GG were shown to be built out of polyglycerolphosphate and decorated with D-alanyl esters, without glycosyl substituents under standard growth conditions in MRS medium [38]. The D-alanylation

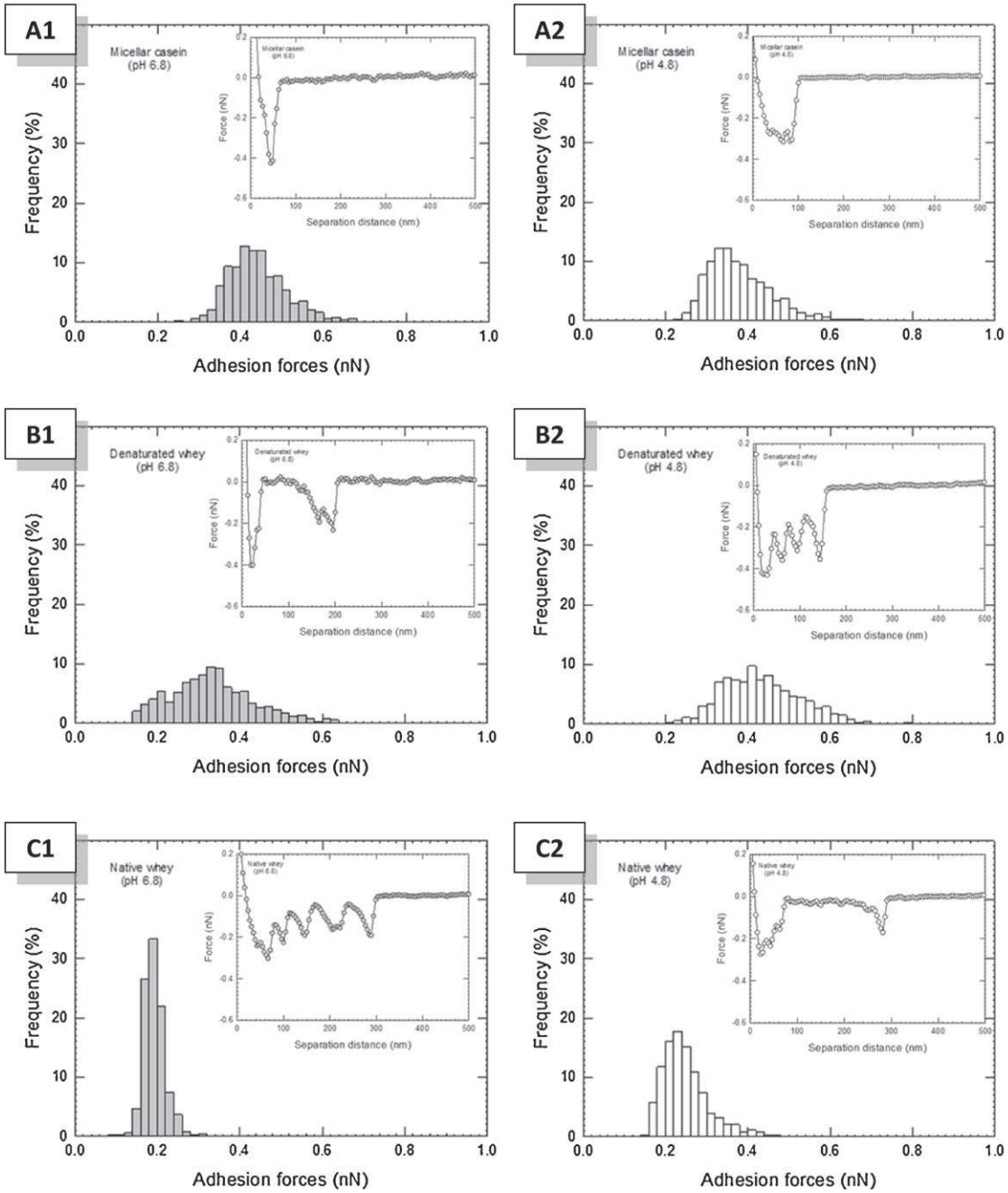

Fig. 4. AFM force measurements between milk proteins and *L. rhamnosus* GG: statistical distribution of the adhesion force as a function of pH (grey for pH 6.8 and white for pH 4.8), and of the nature of milk protein ((A) micellar casein, (B) denatured whey proteins and (C) native whey proteins). Insets: representative force–distance curves.

modulates the charge electromechanical characteristics of the cell wall [39].

3.2. Electrophoretic mobility and AFM topography

The electrophoretic mobility of *L. rhamnosus* GG and GR-1 (Fig. 1A) and milk proteins (Fig. 1B) were recorded in the 3–7 pH range. The electrophoretic mobility of *L. rhamnosus* GR-1 was negative over the entire pH range, whereas LGG had an isoelectric point (IEP, pH where the electrophoretic mobility is zero) of pH 3.5. Profiles like this indicate that anionic compounds dominate the surface of the cells. This includes strong acids, such as the phosphate based lipoteichoic acids (pKa ≈ 2.1) and weak acids, such as the carboxylate containing acidic polysaccharides and proteins (pKa ≈ 3.5–4.5) [40–43].

The profile of electrophoretic mobility of milk proteins always shows different IEPs for each protein. For whey proteins, the IEP is around 4.8, regardless of the protein state (native or denatured) with an electrophoretic mobility range of −1.5 for a neutral pH to 3.0 for an acidic pH of 3.0. For micellar casein, at neutral pH, the density of charges was lower than for whey proteins and the electrophoretic mobility became positive at pH 4.4 with a final value of around 1.6 at acidic pH. At first, the decrease in the net charge causes morphological and size changes of the casein micelle during acidification [44] and in a second stage, the protruding ends of the κ-casein totally collapse [45]. During acidification, the loss of charge is continuous and the micelles shrink to smaller sizes [46].

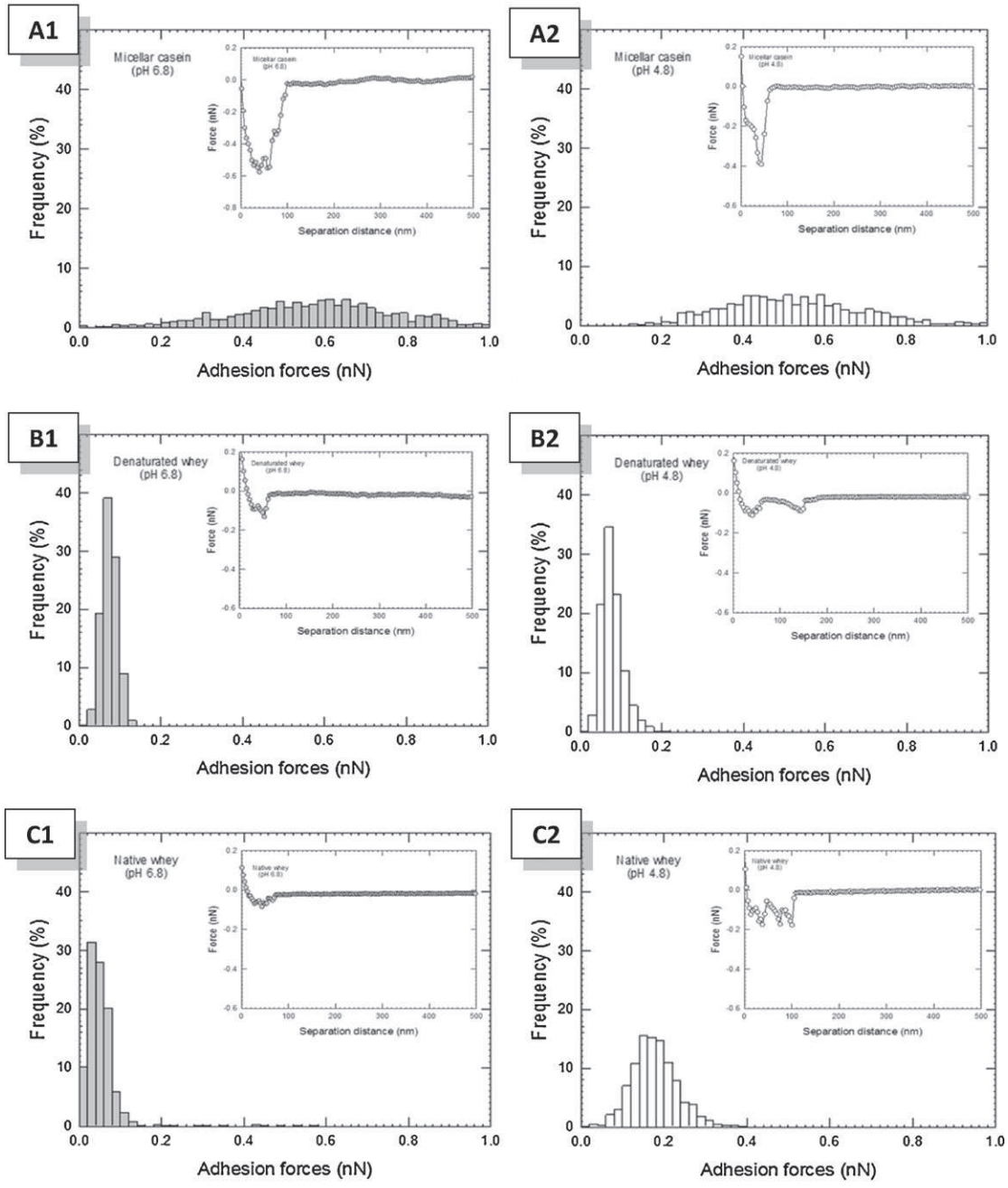

Fig. 5. AFM force measurements between milk proteins and *L. rhamnosus* GR-1: statistical distribution of the adhesion force as a function of pH (grey for pH 6.8 and white for pH 4.8), and of the nature of milk protein ((A) micellar casein, (B) denaturated whey proteins and (C) native whey proteins). Insets: representative force–distance curves.

The neutralisation of charges on casein molecules and particularly the phosphoserine residues after dissolution of the CaP occurs during acidification. The morphological and size changes of the casein micelle during acidification mentioned earlier can be observed on AFM deflection images (Fig. 2) for casein micelles at pH 4.8 and 6.8, and also image of whey proteins at pH 6.8. Individual micelles of around 300 nm were distinguished at pH 6.8 whereas smaller ones (100 nm) were visible at pH 4.8. The casein micelles are polydispersed in size with a reported average of 200 nm [47]. The obtained micelles present a softer and smoother surface than at pH 6.8. In this case, hydrophobic attractive bonds overcome electrostatic repulsions [48]. Milk casein micelles are natural association colloids that appear as heterogeneous raspberry-like particles at pH 6.8 (Fig. 2). After acidification at pH 4.8, the micelles decrease in size and lose their surface heterogeneities, presenting a smooth surface. This was first observed by other authors using AFM under liquid conditions [48].

In order to visualize cell surface morphology, AFM images were taken of the two strains that were used in the study (Fig. 3). For both *L. rhamnosus* GG and GR-1, it can be observed that the bacteria tend to stay side by side, with individual cells having a mean size of around 3 μm. In previous studies [33], high resolution images of *L. rhamnosus* GG entrapped in membrane pores and in liquid condition were recorded. These authors succeeded to distinguish the surface morphology of the cells and more in particular, waves were observed.

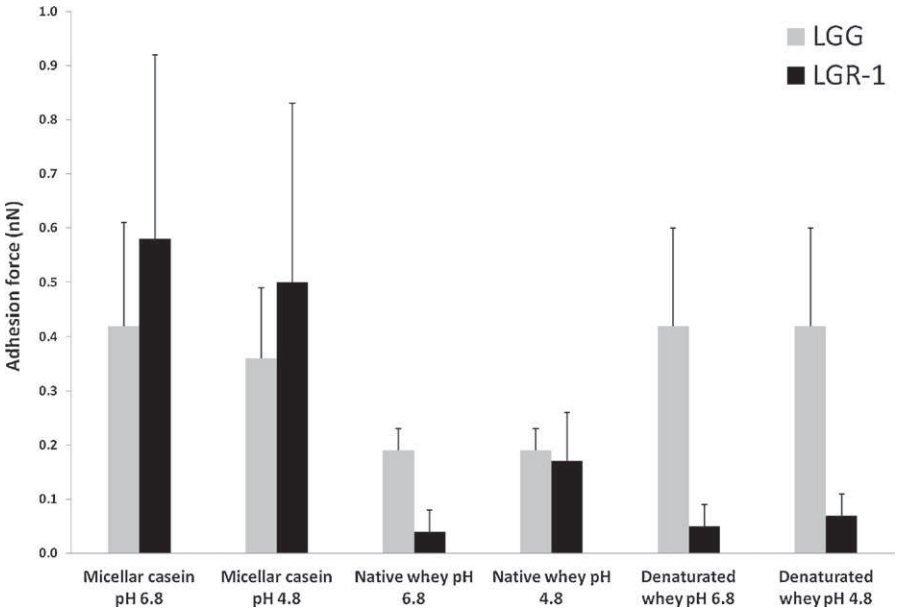

Fig. 6. Overview of AFM force measurements between probiotic bacteria and milk proteins: adhesion force measurements (mean ± SD, $n = 1024$ adhesion events).

3.3. AFM forces analysis between strains and proteins

The bacterial strains were electrostatically immobilized on the AFM tip for force spectroscopy measurements. Results on interaction forces between the lacto-probe and milk proteins are shown in Fig. 4 for *L. rhamnosus* GG and Fig. 5 for *L. rhamnosus* GR-1. Histograms represent the different interaction force distribution with insets of typical retraction force–distance curves.

The shape of the retraction force–distance curve is an important point to be discussed. The profile observed for bacterial interactions with micellar casein (Fig. 4A1 and A2; Fig. 5A1 and A2) is typical for non-specific interactions, including hydrophobic, electrostatic and Van der Waals forces [49]. However, because force measurements were performed in PBS, electrostatic charges were screened in a way that their contribution to adhesion between bacteria and proteins could not be estimated by AFM. On the contrary, specific interactions occurred between *L. rhamnosus* GG or GR-1 with whey proteins in a native or in a denatured state (Fig. 4B1 and B2, C1 and C2; Fig. 5B1 and B2, C1 and C2). In these cases, between one and five adhesive events occurred for several nanometres (between 20 and 300 nm) after the contact point. This particular shape of the retraction curve can be attributed to the stretching of molecules present on the tip (and consequently on the bacteria) but also to the milk proteins deposited on mica. However, it can be noticed that adhesion events are more important in number for the *L. rhamnosus* GG strain than for the GR-1 strain. Furthermore, the pH seems to have an influence on the specific adhesion events, as for interactions between *L. rhamnosus* GG and denatured whey proteins, additional events were observed after acidification. The opposite was observed for the interactions between GR-1 and native whey proteins with a number of specific adhesive events more important at pH 6.8 than at pH 4.8.

Fig. 6 summarizes the adhesion forces that were obtained from AFM force measurements (calculated from Figs. 4 and 5). First of all, it can be concluded that for a given milk protein and strain, pH seems to have no effect on adhesion forces, except for adhesion events between strain GR-1 and native whey proteins, which were more important at pH 4.8 than 6.8. However, it was described

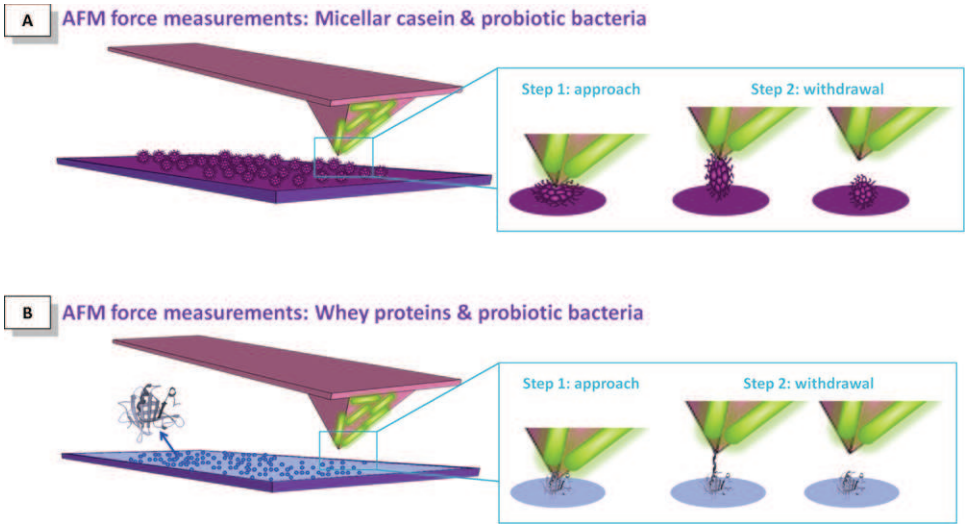

Fig. 7. Non-specific interactions between probiotic bacteria and micellar casein (A) and specific interactions between probiotic bacteria and whey proteins (B).

earlier that a number of specific adhesive events are more important at pH 6.8. This means that in this study, there may be a high number of ruptures on the retraction curve with weak adhesion force. This is the case for force measurement between *L. rhamnosus* GG and native whey proteins at pH 6.8 where multiple ruptures were observed and an adhesion force of only 0.2 nN. On the contrary, for force measurements between *L. rhamnosus* GG and micellar casein at pH 6.8, only one rupture was observed and the adhesion force was around 0.4 nN. Looking at interactions between *L. rhamnosus* GG and milk proteins, adhesion forces appear more important with micellar casein and denaturated proteins, regardless of the pH, and less important with native whey proteins. Concerning the bacterial strain GR-1, it seems that adhesion forces are more pronounced with micellar casein and weaker for native and denaturated whey proteins. Finally, the lowest number of interactions was observed for *L. rhamnosus* GR-1 with native whey proteins at pH 6.8 and denaturated whey proteins at both pHs. The highest amount was found for interactions between *L. rhamnosus* GR-1 with micellar casein.

3.4. Hypothesis concerning interactions between bacteria and milk proteins

In bovine milk, the four caseins (α_{s1}- α_{s2}- β- κ-caseins) are assembled together to form a structure called micelle. These proteins do not have a well-defined tertiary structure. As previously underscored by other authors [50], interactions between casein micelles and biomolecules are of interest because the interior of the casein micelles is not accessible for large molecules whereas, it is porous enough to provide access to the inner part of the structure for small molecules (e.g. enzymes). On the contrary, whey proteins have a defined tertiary structure accessible to other biomolecules. This explains why specific interactions can be established between molecules that decorate bacterial surfaces and whey proteins and not for casein micelles. In this study, it was established that interactions between the probiotic strains *L. rhamnosus* GG and GR-1 with whey proteins are specific in nature, whereas non-specific events were reported for interactions with casein micelles (Fig. 7).

The adhesive nature of bacteria is mainly due to various cell surface features consisting of proteins such as pili, and polysaccharides such as EPS. Adhesion of bacteria is governed not only by long range forces such as steric and electrostatic interactions, but also by short range forces such as Van der Waals, acid–base, hydrogen bonding and biospecific interactions. However, as mentioned above, electrostatic forces were not present for these measurements due to the use of PBS buffer. It is readily conceivable that these forces are important for bacterial interactions but these could not be estimated in this study. Bacterial interactions and bacterial adhesion to surfaces are both complex phenomena involving non-specific and specific binding events and the balance between them depends on the type of bacteria, the type and state of the host surface and environmental conditions. It is obvious that the understanding of bacterial interactions is a complex matter. The association of EPS with casein micelles is mostly electrostatic in nature [50]. Because electrostatic charges were no longer present on bacterial surfaces and proteins during this work, only Van der Waals, hydrophobic and steric interactions occurred. Moreover, interactions between anionic EPS and milk proteins depend on structural parameters of EPS such as the charge density, the nature of charged groups, the molecular weight and the stiffness of chains, but also on the protein involved, casein or whey protein [51]. As proposed by other authors [6], bacteria are preferably organized as colonies in dairy products. Moreover, the bacteria are preferentially located at the fat/protein interface, which might be due to the fact that during the formation of the rennet-induced casein network, the bacteria are forced out of the protein matrix and are repelled in whey pockets that surround fat inclusions. Considering the results of the present study, a better understanding is established why bacteria preferably interact with whey proteins as they can establish specific interactions, which is not the case with casein micelles.

4. Conclusion

The use of AFM force spectroscopy, in combination with electrophoretic mobility measurements and XPS provided new insights into the interactions between probiotic bacteria and milk proteins. During the past few years, many authors have observed the preferential location of bacteria in whey pockets. The present work quantifies, for the first time, directly on the nanoscale and compares the adhesion forces between two related probiotic strains *(L. rhamnosus* GG and *L. rhamnosus* GR-1) and milk proteins (micellar casein, native and denaturated whey proteins). The bacterial surface characterization was realized using XPS, which provided information on the surface composition in terms of proteins, polysaccharides and lipid-like compounds. The electrophoretic mobility measurements provided information on surface charges of both bacteria and proteins along the 3–7 pH range. The observed specific adhesion between bacteria and whey proteins and the nonspecific interactions with micellar casein, allow the understanding for the preferred interaction with whey proteins. From the results presented in this work, it can be concluded that many factors influence the bacterial interaction with the dairy matrix, including the nature of the proteins, the nature of the strains and the pH of the media. Future studies should identify which biomolecules on the bacteria specifically can interact with milk proteins.

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Encapsulation of *Lactobacillus rhamnosus* GG in microparticles: Influence of casein to whey protein ratio on bacterial survival during digestion

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ABSTRACT

Encapsulation of *Lactobacillus rhamnosus* GG in various microparticles made of only milk proteins (casein, native whey and/or denatured whey proteins) was done. The microparticles obtained were rather similar in shape (mostly round) and size (around 60 μm) whatever the formulation but the obtained gel presented different elasticity (varying between 61 and 96 Pa). An original equipment involving a granulo-morphometer coupled to a thermostated reactor was developed and validated to visualize in situ the microparticles during digestion. Although the initial particles were similar, their disintegration in simulated gastric media was totally different and characterized by two stages. An initial decrease in particle size more or less quick depending on the protein composition was followed by a stable phase characterized by the particle size and shape retention. At the end of gastric digestion, a significant amount of intact particles was still noticeable for each formulation. Nevertheless, the formulation containing a mix of casein and denatured whey presented the best bacterial survival (99%) and encapsulation rate (97%) in comparison with formulations containing either only casein or casein and native whey or casein in mixture with native and denatured whey proteins.

Industrial relevance: This paper is part of a global project entitled "Structured dairy matrices to enhance probiotic efficiency". The entire project will provide milk structured matrices allowing the stabilization and the vectorization of *Lactobacillus rhamnosus* GG (LGG). This project will consist of four main axes: milk constituent's interactions with LGG, stabilization process implementation, and structural and functional characterization of the matrices obtained. The scientific objective is to propose models connecting process parameters, matrix structure (from an atomic, molecular to a macro scale) and their functionality. This implies the in-depth study of interactions between milk components and probiotic strain. For this purpose, the use of genetically modified strain of LGG will allow the identification of biomolecules interacting with milk matrices. The industrial aims are to optimize and control the processes to suit the needs of industrial criterions: encapsulation rate, gastric resistance, intestinal release, storage in the final food….

1. Introduction

Modern consumers are increasingly buying into the concept that probiotic yogurt and other fermented milk products improve digestion, boost immunity and provide other health benefits long claimed by food companies (Burgain, Gaiani, Linder, & Scher, 2011). However, regulatory authorities, particularly in Europe, have not supported probiotic health claims on the grounds that the provided data were not sufficient. This year, the European Food Safety Authority (EFSA) has rejected almost all health claims put forward by the probiotic industry. Several reasons are invoked to justify the refusal: microbes have not been sufficiently characterized, the claimed effect was not considered beneficial or because human studies to support the claims were not provided (Schmidt, 2013). The design of biopolymer based microparticles to encapsulate, protect and release a specific bioactive component believed to benefit human health is now gaining interest (Matalanis, Jones, & McClements, 2011). In most cases, the encapsulated substance needs to be released at a specific site in the body. Thus, developing a model identifying the physico-chemical mechanism leading to the release could be a useful tool to predict microparticles' future (Matalanis et al., 2011).

In the past few years, the use of bioactive ingredients derived from the dairy sector has gained interest. Dairy ingredients are widely used in the food industry because of their technological properties. In fact, they are recognized for their surface-active and colloid stabilizing characteristics (Horne, 2009). Moreover, their health benefits are increasingly promoted (Mackie & Macierzanka, 2010). Milk proteins can be used as a carrier for health-promoting delivery system. For example,

Abbreviations: Bdi, Breadth of the distribution; CFU, Colony forming units; EQPC, Diameter of a circle of equal projection area; EFSA, European Food Safety Authority; ER, Encapsulation rate; IMCU, International Milk Clotting Units; LGG, *Lactobacillus rhamnosus* GG; SR, Survival rate.

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nanoparticles are used to solubilize and protect hydrophobic nutraceuticals. In this case, the natural digestibility of caseins is exploited (Livney, 2010). Milk contains two major protein groups: casein and soluble proteins. These proteins differ in their physico-chemical properties and more particularly in their amino acid composition. Caseins and whey proteins make up respectively around 80% and 20% of the proteins in milk. Due to their emulsifying properties and amino-acid composition, caseins play an important role in human nutrition. These characteristics explain their wide use as additives in food. Casein micelles are composed of α_{s1}-, α_{s2}-, β- and κ-casein proteins. Rennet coagulation of milk results from κ-casein proteolysis by chymosin enzyme. In fact, by releasing hydrophilic fragments, repulsive forces disappear allowing thereby micelle aggregation via calcium bond formation (Horne, 2009). Gelation properties of milk proteins can be exploited for probiotic encapsulation. Many health promoting claims attributed to probiotic bacteria are dependent on the cells being both viable and sufficiently numerous in the intestinal tract (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). Nevertheless, the passage through the stomach of most bacteria results in an important loss of viability which lowers the efficacy of the administered supplement. Formulation of probiotics into microcapsules is an interesting method to reduce cell death during the gastrointestinal passage (Burgain et al., 2011). The use of milk protein based microparticles was poorly exploited whereas it was demonstrated that dairy matrices such as cheeses can protect bacterial cells (Stanton et al., 1998) thanks to the good buffering capacity of milk proteins for example (Livney, 2010).

Rennet gelation of milk proteins was used to develop a microencapsulation technology for the protection of two strains: *Lactobacillus paracasei* ssp. *paracasei* F19 and *Bifidobacterium lactis* Bb12 (Heidebach, Först, & Kulozik, 2009a). The obtained microparticles were spherical and water insoluble due to the use of an emulsification process. This technique allows a high encapsulation rate and a good microparticle resistance in simulated gastric conditions leading hence to an important survival rate of bacteria. By using this technique, the authors claimed that probiotic cells can resist to adverse conditions encountered in the stomach. Another enzyme (transglutaminase) was also tested to produce microparticles where milk protein gelation was exploited (Heidebach, Först, & Kulozik, 2009b). The heat induced gelation of whey proteins can be used to produce microparticles containing probiotic bacteria. In this way, *Bifidobacterium* Bb-12 was microencapsulated by spray drying with whey proteins and the entrapped cells presented better survival during simulated gastric digestion (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012). Another example is the microencapsulation of *Lactobacillus rhamnosus* GG (LGG) with gelled whey protein isolates. The produced matrix was able to protect the cells during in vitro stomach incubation (Doherty et al., 2011) and also during ex-vivo digestion (Doherty et al., 2012). Finally, all of these trials for microencapsulation with dairy proteins led to an increase in bacterial survival during digestion.

In this context, the first objective of this study was to compare the resistance of four matrices (without bacteria) constituted by only milk proteins in simulated gastric environment. For this purpose, an original equipment allowing the in situ determination of particle size and shape during digestion was developed. Then, the same matrices were used to encapsulate LGG. Survival rate during gastric digestion was followed during the 2 h. The selection of the best formulation should be done by the confrontation of results: particle size, shape and Bdi, encapsulation and gastric resistance rates.

2. Materials and methods

2.1. Material used

Micellar casein powder (Promilk 872B) was obtained from Ingredia IDI (Arras, France). Whey protein isolate powders (Prolacta 90) were purchased from Lactalis Ingredients (Bourgbarré, France). The chemical characterization of the powders has been already studied (Gaiani et al., 2011). Micellar casein and whey proteins present respectively a protein content around 87% and 90% (w/w).

The rennet preparation (Naturen™) was provided by CHR Hansen (Arpajon, France) and presented an activity of 140 IMCU mL^{-1}. The rennet solution was prepared before being used by diluting 3 g of rennet preparation in 12 g of distilled water (28 IMCU mL^{-1}). The sunflower oil was purchased from a local store.

LGG (ATCC 53103) was used throughout this study. Bacteria were first subcultured at 37 °C in MRS medium. This preculture was then used to inoculate 500 mL of MRS broth which was incubated overnight at 37 °C until an early stationary phase. Cells were centrifugated (15 min., 3500 *g*, room temperature), washed with physiologic water (pH 7.4) and finally harvested by centrifugation (15 min., 3500 *g*, room temperature). The pellet was then frozen and placed on the shelves of a freeze dryer (Christ alpha 1–2, freeze-dryer, Osterode, Germany). The microbial powder obtained after freeze drying has a content of 10^{11} CFU/g.

2.2. Microparticulation procedure

2.2.1. Preparation of the carrier material

All the solutions were prepared by adding 12.5 g of protein powder into 100 g of distilled water (Fig. 1). The rehydration was done by stirring for 2 h at room temperature and then overnight at 4 °C. The denatured whey proteins were obtained by heating the native whey solution at 78 °C for 10 min then cooling it to room temperature. Four formulations were used to produce variable microparticles with different milk protein composition (micellar casein and whey proteins in a native or denatured state). The amount of each solution used to prepare the initial mix of protein solution is detailed in Fig. 1.

The four formulations were tested for the microencapsulation of LGG. The strain was added after mixing the protein solution and before enzymatic incubation (Fig. 1). 0.03 g of freeze dried LGG was added to 15 g of protein mixture.

2.2.2. Microparticle production

Microparticles were produced by using an emulsification method (Heidebach et al., 2009a) and the procedure is detailed in Fig. 1. The encapsulation procedure was completed in a double-walled, temperature-controlled reactor, made of stainless steel. An amount of 15 g of protein mixture was added into the reactor held at 9 °C. Then, 445 μL of diluted rennet preparation was added and mixed with the solution. The mixture was left for 30 min at 9 °C allowing the rennet enzyme to cut the κ-casein. This step was followed by an emulsification. For this purpose, 150 g of cooled sunflower oil was added and stirred with the mixture at 500 RPM (Rotation Per Minute) for 5 min. Afterward, the temperature was raised to 40 °C for 15 min and under mechanical agitation.

Microparticles were removed from the reactor and separated by centrifugation (500 *g*, 1 min). The harvested particles were washed with distilled water. Afterwards, the microparticles were stripped from the container and shaken for 12 s. Another centrifugation following the same conditions was then performed. Finally, the microparticles were removed, re-suspended in distilled water and stored at 4 °C under stirring until their use the next day.

2.2.3. Encapsulation rate (ER)

The enumeration of LGG living cells was done by serial dilutions. The samples (0.1 mL) were plated in duplicate on MRS agar. Colony forming units (CFU) were determined after incubation (48 h at 37 °C). To enumerate the entrapped probiotic cells, the microparticles were mechanically broken with a spatula.

The ratio between the number of bacteria added in the protein mixture and the number of bacteria in the final particles was done. The initial number of bacteria introduced in the protein mixture was obtained by determining the CFU in 1 g of solution. The result

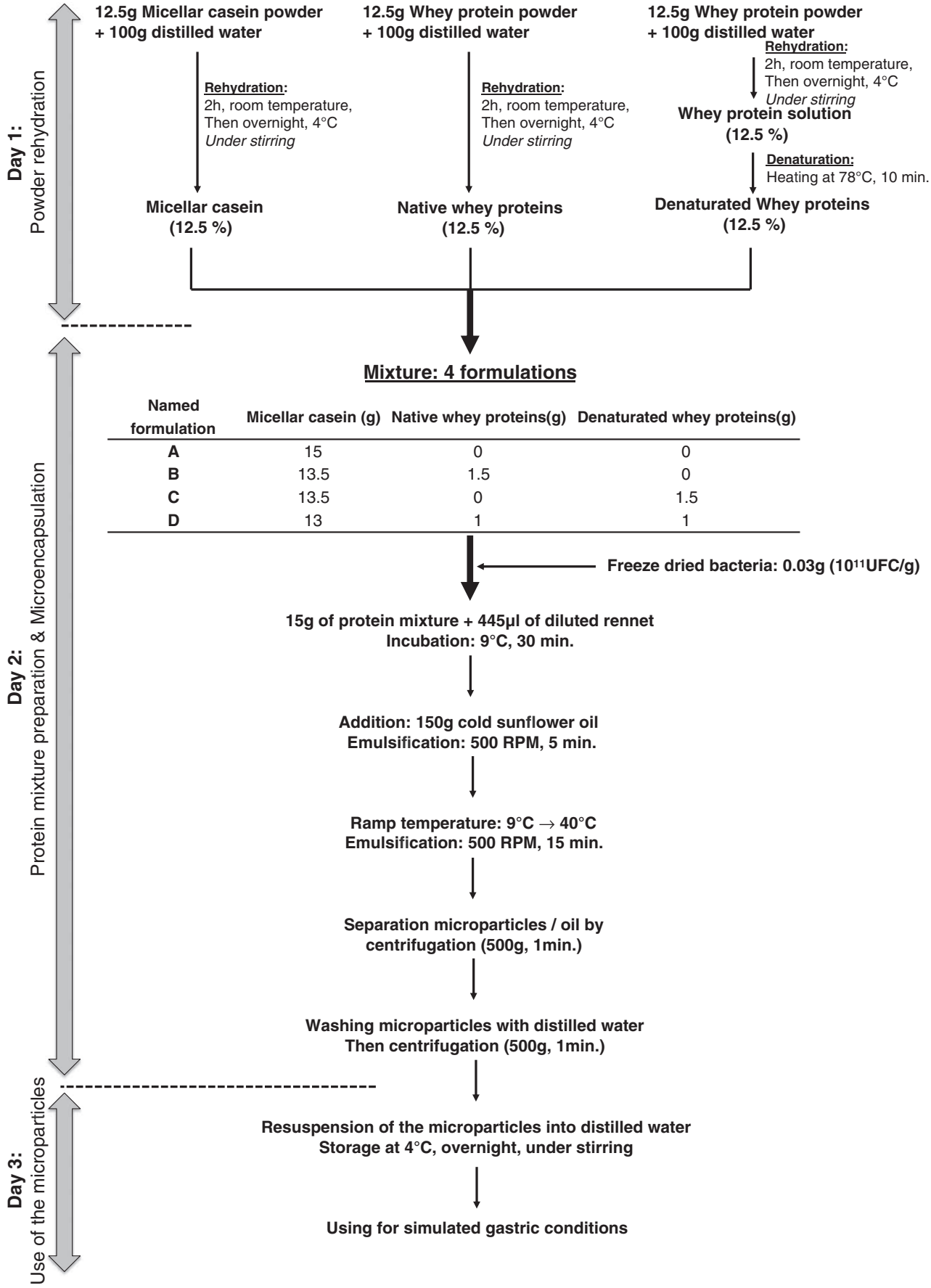

Fig. 1. Flowchart for the production of microparticles.

was multiplicated by 15 which is the total mixture quantity used for microencapsulation. At the end of encapsulation the microparticle total weight was measured. The final CFU in 0.2 g of microparticle was also determined and multiplicated by the total quantity of obtained microparticles. The encapsulation rate was calculated by applying Eq. (1):

$$ER\ [\%] = \frac{[\mathrm{CFU/g}]^{\text{Final microparticles}} * [\text{quantity of microparticles (g)}]}{[\mathrm{CFU/g}]^{\text{Initial solution}} * 15} * 100. \quad (1)$$

2.3. Simulated gastric digestion

Simulated gastric fluid was formulated with sodium chloride (34 mM) and hydrochloric acid with a final pH of 2.5 (USP31-NF26, 2008). This pH value was chosen according to Gbassi, Vandamme, Yolou, and Marchioni (2011) and corresponds to the encountered pH in stomach after consuming a classical meal. Finally, 0.64 mg of pepsin was added to 200 mL of gastric juice. The simulation of the stomach digestion was done in a temperature-controlled reactor, made of glass, with a magnetic stirrer (150 RPM). The particles (2 g) were introduced in 200 mL of gastric solution and left for 2 h at 37 °C to imitate human body temperature. During particle digestion, the reactor was directly connected to a particle size and shape analyzer (Fig. 2).

In order to determine the survival rate (SR) of free or encapsulated LGG during simulated gastric digestion, aliquots of 1.5 mL were collected at different times: 5, 30, 60 and 120 min. The SR was calculated by Eq. (2):

$$SR\ [\%] = \frac{\mathrm{CFU/g}^{(t=t_i)}}{\mathrm{CFU/g}^{(t=0)}} * 100 \quad (2)$$

where $\mathrm{CFU/g}^{(t=0)}$ is the cell count before incubation in the simulated gastric juice and $\mathrm{CFU/g}^{(t=ti)}$ is the cell count at various incubation times t_i.

2.4. Size and morphology analysis

Particle size and shape were determined by using a QICPIC™ analyzer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) equipped with a module specific for dispersions. This equipment allows instantly evaluating and characterizing particle sizes and shapes even in acid and corrosive media like gastric mimic media. Here, the analyzer was directly connected to the reactor of digestion (Fig. 2) and planned to take measurements every 5 min during the 120 min of digestion. The gastric liquid was pumped into the reactor and passed through the measuring cell. Concurrently, images captured were recorded. The analysis of these images enabled size and shape data collection.

Image analysis provides 2D image of a complex 3D particle then determines several size and shape parameters from the 2D image. The diameter of a circle of equal projection area (EQPC) was calculated. It corresponds to the diameter of a circle with the same area as the 2D image of the particle (Fig. 3A). Because different shaped particles may have the same EQPC, other parameters were used to describe the particles. The first shape parameter measured was the sphericity (Fig. 3B) defined by the ratio between the EQPC perimeter with the real particle perimeter. The sphericity values comprised between 0 and 1. For example, a particle with a sphericity of 1 is a perfect circle whereas a particle with a sphericity close to 0 is irregular. Thus, sphericity is a good way to describe the particle shape deviation from a perfect circle. The second shape parameter determined was the convexity (Fig. 3B). This measure provides information about the roughness of the particle. Convexity values also comprised between 0 and 1. A particle with smooth edges has a convexity value of 1 whereas a particle with irregular ones has a lower convexity (Gaiani et al., 2011).

The size distribution was characterized by the breadth of the distribution (Bdi) calculated by the following Eq. (3):

$$Bdi = (d_{90} - d_{10})/d_{50}. \quad (3)$$

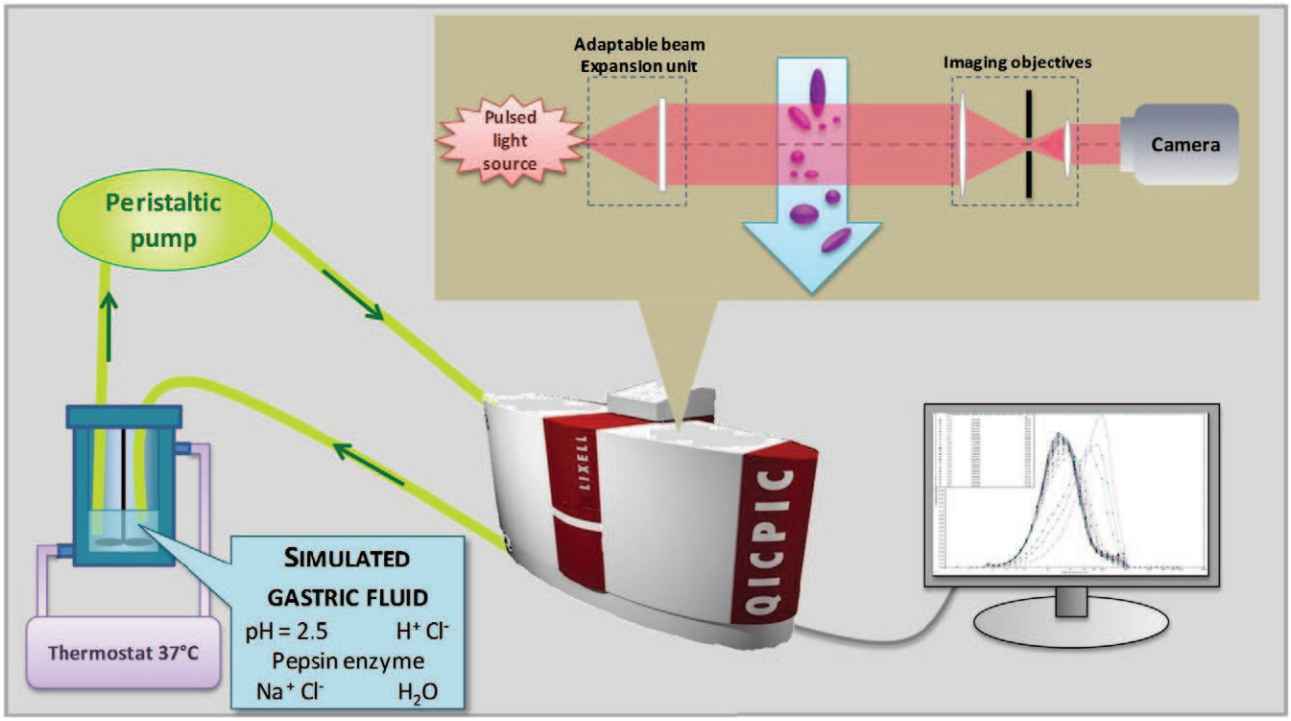

Fig. 2. Installation allowing the monitoring of "in situ" microparticle digestion.

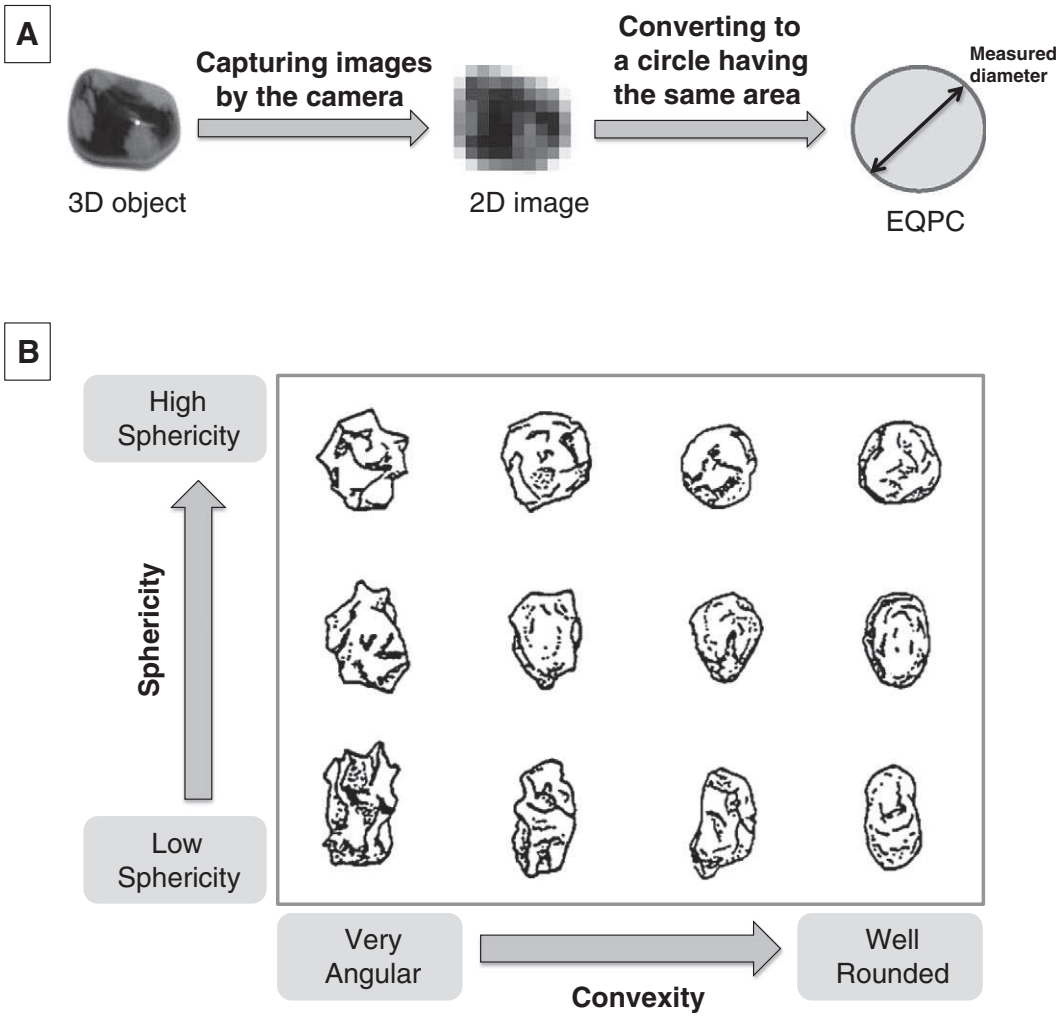

Fig. 3. Particle size and shape descriptors provided by the QICPIC™ analyzer. A — representation of how the EQPC is calculated from a 3D object. B — representation of the sphericity and the convexity variations as a function of real particle morphology.

The diameter values for d_{10}, d_{50} and d_{90} reflect that 10%, 50% or 90% of the population has a diameter below the value. The calculation of the Bdi is a criterion to describe the population polydispersity.

During digestion, the particle sizes were recorded continuously. The d_{50} evolution was normalized and represented as a function of digestion time according to Eq. (4).

$$y_t = \frac{d_{50}(t = t_i)}{d_{50}(t = 0)} \tag{4}$$

where d_{50} $(t = 0)$ is the first particle size measurement during simulated gastric digestion and d_{50} $(t = t_i)$ is the measurement between 5 and 120 min of digestion.

A linear-exponential equation (Eq. (5)) was used to fit the model (Kong & Singh, 2008):

$$y_t = (1 + k\beta t)e^{-\beta t} \tag{5}$$

k, is dimensionless and represents the lag phase. Thus, a large k means that a delayed-sigmoïdal disintegration occurred whereas a small k represents an exponential decay. β ($L\ min^{-1}$) allows the measurement of the gastric emptying of the particles as a function of digestion time ($\beta > 0$).

The k and β values can be obtained with a regression analysis. When the parameters k and β are determined, the half time ($t_{1/2}$) can be calculated using $y_t = 0.5$. The half time corresponds to the time when size was reduced by 50%.

2.5. Dairy gel rheological properties

Rheological properties of the gel (viscous and elastic components as well as the final value of the storage modulus and the phase angle) were measured with a Kinexus rotational rheometer (Malvern Instruments, Orsay, France). The rheometer was equipped with a cup and bob geometry consisting of coaxial cylinders. The oscillatory mode was used with a shear strain of 6% and a frequency of 1 Hz. The kinetics of rennet coagulation were followed in situ for each formulation. Incubation of the protein mixture with the rennet enzyme was realized at 9 °C for 30 min and then, a temperature ramp (2 °C/min) from 9 °C to 40 °C was applied.

2.6. Statistical analysis

The evolution of d_{50} during digestion (y_t) was analyzed and fitted into an empirical model. Modeling of data was performed with XLStat™ 2010.5.06 software (AddinSoft, France) as an add-in to Microsoft Excel™ 2007. Estimates for parameters were obtained by minimizing the mean square error (MSE) according to Eq. (6):

$$MSE = \frac{\sum_{i=1}^{n}\left(y_{exp} - y_{fit}\right)^2}{n} \tag{6}$$

where y_{exp} represents the experimental value, y_{fit} is the fitted value, and n is the number of experimental points. The value of MSE provides information of the goodness of fit. Additionally, the correlation coefficient (r^2) is also a criterion for assessing the performance of the model. A value closer to 1 indicates that the prediction model is effective.

All measurements presented in this paper were performed on three independent samples (excepted survival rate). The KyPlot software version 2.0 was used and a parametric multiple comparison test (Tukey test) was performed. The significance levels were: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{NS}P > 0.05$.

3. Results and discussion

3.1. Microparticle characterization

3.1.1. Size, shape and breadth of the distribution

Different initial sizes depending on the formulation were observed (Fig. 3, black column). The biggest average particles were obtained from formulation A (69 μm) and the smallest from B (56 μm). Formulations C and D provided intermediate microparticles of 59 and 65 μm respectively. Significant differences between A and B ($P < 0.01$) and between A and C formulations ($P < 0.05$) were measured. The convexity and sphericity values of all microparticles whatever the formulation were comprised between 0.80–0.85 and 0.68–0.81 respectively. These values reflected that the microparticles were initially relatively well rounded (Table 1).

The breadth of the distribution (Bdi, calculated by Eq. (3)) provided information on the polydispersity of the produced microparticles (Fig. 4). Formulation C presented the lowest polydispersity (1.08) whereas formulation B had the highest one (1.50). These two formulations had the same amount of casein however whey proteins were in a native form in formulation B and denatured in formulation C. The polydispersity of the two other formulations A and D comprised between these two extremes with values of 1.22 and 1.28 respectively.

3.1.2. Influence of microparticle composition

One goal of this study was to produce microparticles with a dense protein matrix in order to assess their resistance to gastric conditions. To achieve a high network density, milk protein solutions with a concentration of 12.5% were used (Fig. 1). Indeed, this concentration of milk proteins was already found as optimal (data not shown). As presented in Fig. 5, the matrix density was measured indirectly by the gel elasticity (G′). Significant differences were observed between the formulations. Formulation C presents the highest elasticity. Formulation D was slightly less elastic whereas formulations A and B were similar with lower G′ values. It was already demonstrated that the development of microparticles with a dense matrix offers resistance against proteolytic degradation since it is difficult for the enzymes to penetrate into the particles (Gunasekaran, Ko, & Xiao, 2007). Creation of new links between proteins (Zoon, 1988) was found to increase the gel network and was reflected in the storage modulus (G′) (Walstra & Vliet, 1986). These may be related to a lower tendency to breakdown (Mellema, Walstra, van Opheusden, & van Vliet, 2002) and a better resistance.

The protein nature seems to influence the particle size, the Bdi and the gel elasticity. It was observed that a higher amount of micellar casein led to significant bigger particles and lower gel elasticity. The presence of denatured whey proteins was beneficial for producing microparticles with a small polydispersity and a high elasticity. The shape of the microparticles was not significantly modified by the proteins used in the different formulations. However, the protein composition can also influence other factors like porosity, surface and internal microstructure (Matalanis et al., 2011). Indeed, the nature of the proteins presenting variable physical properties (density, refractive index), size, charge, and stability may be of influence (LaClair & Etzel, 2010). By modifying these properties a more or less porous structure may be obtained. And consequently a porous structure should allow an easier access and release of an encapsulated bioactive while a dense structure would limit access and release.

Table 1
Particle shape measurements recorded at different times of digestion. Before digestion means that the size measurement was done in water before adding the particles in the acidic fluid. The beginning of digestion represents the first measurement done during digestion and the end corresponds to the one realized after 120 min of contact with the gastric fluid.

Micro-particle composition	Time of digestion	Convexity			Sphericity		
		x_{10}	x_{50}	x_{90}	x_{10}	x_{50}	x_{90}
A	Before	0.82	0.85	0.84	0.81	0.76	0.72
	Beginning	0.72	0.72	0.68	0.63	0.54	0.46
	End	0.77	0.74	0.74	0.78	0.70	0.64
B	Before	0.82	0.84	0.82	0.81	0.76	0.70
	Beginning	0.75	0.73	0.75	0.76	0.66	0.63
	End	0.77	0.77	0.79	0.78	0.72	0.70
C	Before	0.81	0.83	0.81	0.78	0.74	0.68
	Beginning	0.67	0.61	0.62	0.58	0.42	0.37
	End	0.77	0.71	0.72	0.80	0.67	0.61
D	Before	0.80	0.83	0.82	0.80	0.75	0.71
	Beginning	0.67	0.64	0.56	0.58	0.46	0.34
	End	0.77	0.69	0.64	0.78	0.63	0.51

3.2. Microparticles during digestion

Microparticle size, shape and Bdi were recorded continuously during the entire process of digestion: before their addition into the simulated gastric medium (previously discuss), at the very beginning of digestion and at the end of digestion (Fig. 4).

3.2.1. Beginning of digestion

When the microparticles were added to the gastric environment, a particle diameter increase was systematically observed (Fig. 4, gray column). For experiment A, the particles had an initial d_{50} of 69 μm and after their addition into acid the d_{50} was increased up to 79 μm corresponding to a size increase of 14%. A similar behavior was observed for experiments B, C and D with size increases of 52%, 32%, and 18% respectively. The size increase was only significant for formulations B, C ($P < 0.001$) and D ($P < 0.05$). For formulations A and C, a Bdi increase was observed once the particles approach the acidic environment. Relying on the above Bdi value provided by the images, it can be seen that bigger particles were produced just after their addition into the gastric liquid. These new particles were the result of physical interactions between existing particles explaining thus the increase in size observed. In the case of formulation A the interactions were very important whereas for formulations C and D, initial particles retained their identity and the interactions were less important.

The particle shape parameters (convexity and sphericity) tended to decrease and microparticles became more irregular (Table 1). The most important decrease in convexity value was observed for formulation C with a reduction from 0.83 to 0.61. A comparable decline in sphericity value from 0.74 to 0.42 was also observed for this formulation. It has already been noticed with recorded images (Fig. 4, gray column) that the microparticles from formulation C presented a high deformation (the physical interactions between initial particles being less important than in other formulations). These are particles that most deviate from a spherical shape. On the contrary, sphericity and convexity in formulation B were higher after microparticle addition in the gastric medium. As previously stated, these particles already stood out from the others with their higher compacity in the visual analysis of images in Fig. 4. The creation of aggregates for formulations A, C and D caused a reduction in sphericity as the

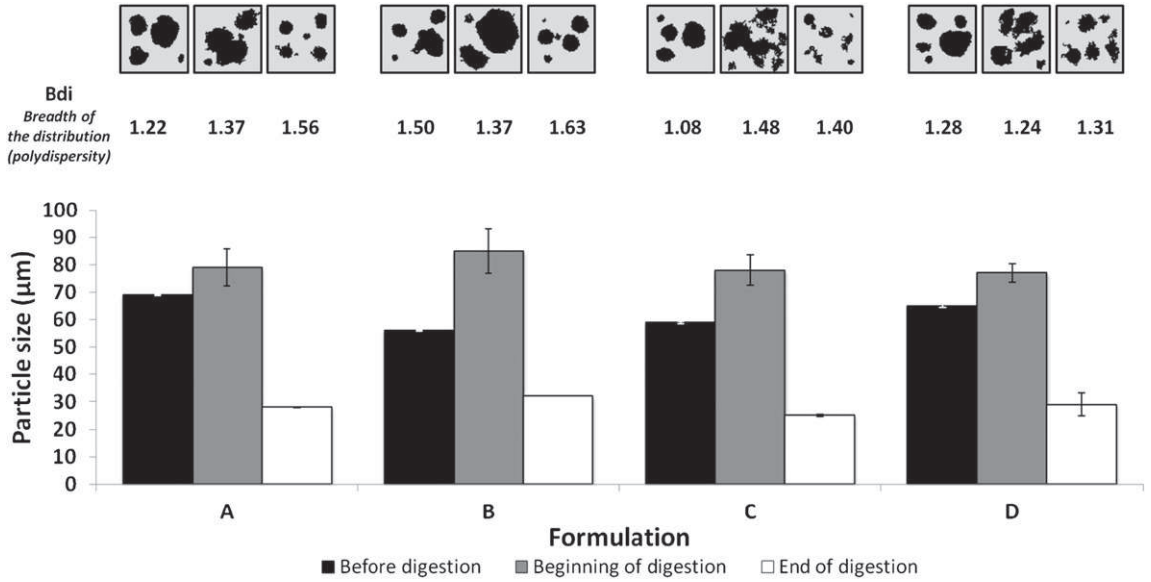

Fig. 4. Particle size measurements (d_{50}) realized at different times (mean ± SD, n = 3) for the four formulations (A, B, C & D): before gastric digestion (in black) means that the size was recorded for a particle dispersion in water, beginning of digestion (in gray) means that the measurement was realized just after the particle introduction in the gastric medium and end of digestion (in white) for a recording after the 120 min of digestion. For each measurement, the breadth of the distribution (Bdi) is indicated and reflects the polydispersity of the produced particles. For each record, associated particle images are provided. The digestion was realized in a gastric medium containing the pepsin enzyme.

microparticles were placed into the gastric solution. Nevertheless, the lowest sphericity for the big particles in each experiment was obtained for formulation D. In fact, at the beginning of digestion, the large particles reflected the generated aggregates. With particle images (Fig. 4), it can be seen that aggregates for this formulation seem to be more flocked than fused particles. In contrast, it seems that for the microparticles from formulation B, the swelling phenomenon was responsible for the increase in size as no aggregates can be noted.

3.2.2. During digestion and end of digestion

The size distributions were recorded every 10 min during the digestion (Fig. 6). Whatever the formulation, an instantaneous particle size decrease is observed during the first minutes. This was noticed by a curve shift from the right to the left corresponding to smaller sizes. After a while, the particle size reached a plateau. This was observed by a superposition of the curves principally for experiments A, B and D. Formulation C presents a slower size decrease but continue without reaching a plateau. At the end of digestion, particles recovered higher convexity and sphericity in all cases because of the individualization of microparticles (Table 1). However, the initial sphericity and convexity values were not reclaimed. The highest value in these both shape parameters was obtained by the particles produced with formulation B. Conversely, microparticles that have suffered greater deterioration are the ones produced with formulation D. This information is reinforced by the particle images in Fig. 4, at the end of digestion the microparticles were spherical and compact for formulation B and the opposite for formulation D.

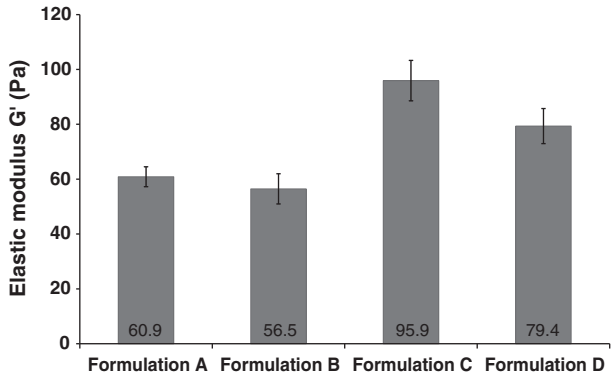

Fig. 5. The elastic modulus (G′) was determined by rheology for the four formulations (A, B, C & D) (mean ± SD, n = 3).

3.2.3. Data modeling of the entire process of digestion

In order to obtain a general view of the entire process of digestion, the particle size retention (y_t) was plotted (Fig. 7) with the calculated values (Eq. (4)) and experimental data were fitted (Eq. (5)). Correlation coefficient (r^2) and MSE were also reported (Table 2). All the curves presented an exponential disintegration profile with digestion parameters k and β that are nearly the same for the four formulations as shown in Fig. 7 and Table 2. These profiles are typical of soft foods due to their fast advancing erosion front that dominates the reactions of food during gastric digestion (Ferrua, Kong, & Singh, 2011). Nevertheless, profiles of disintegration (sigmoïdal, exponential, and delayed exponential…) during food digestion are strongly linked to the force applied. From the four formulations studied here, differences were evident and related to the particle size distribution evolution during digestion. For experiment C, the decline was slow with a $t_{1/2}$ of around 37 min in contrary to experiment D which presented a rapid decrease and a steady particle size with a smaller $t_{1/2}$ of around 8 min. This half time can be used as an indicator of the food disintegration rate. The two other formulations (A and B) presented intermediate behaviors. Nevertheless, the size of the particles produced in this study was small (around 60 µm); thus they cannot be assimilated to the digestion of a solid meal and could be related to a liquid meal. Liquid meals are emptied faster than solid meals, which generally present a lag phase prior to gastric emptying (Fox et al., 2004). Gastric emptying is governed by physical and chemical characteristics of the ingested meal. For example, whey proteins are digested faster than micellar casein (Calbet & Holst, 2004).

(1) The *initial increase in size* observed (formulations A, C and D) at the beginning of digestion could be explained by particle aggregation. Aggregation was confirmed on recorded images during digestion. On the contrary, a swelling phenomenon

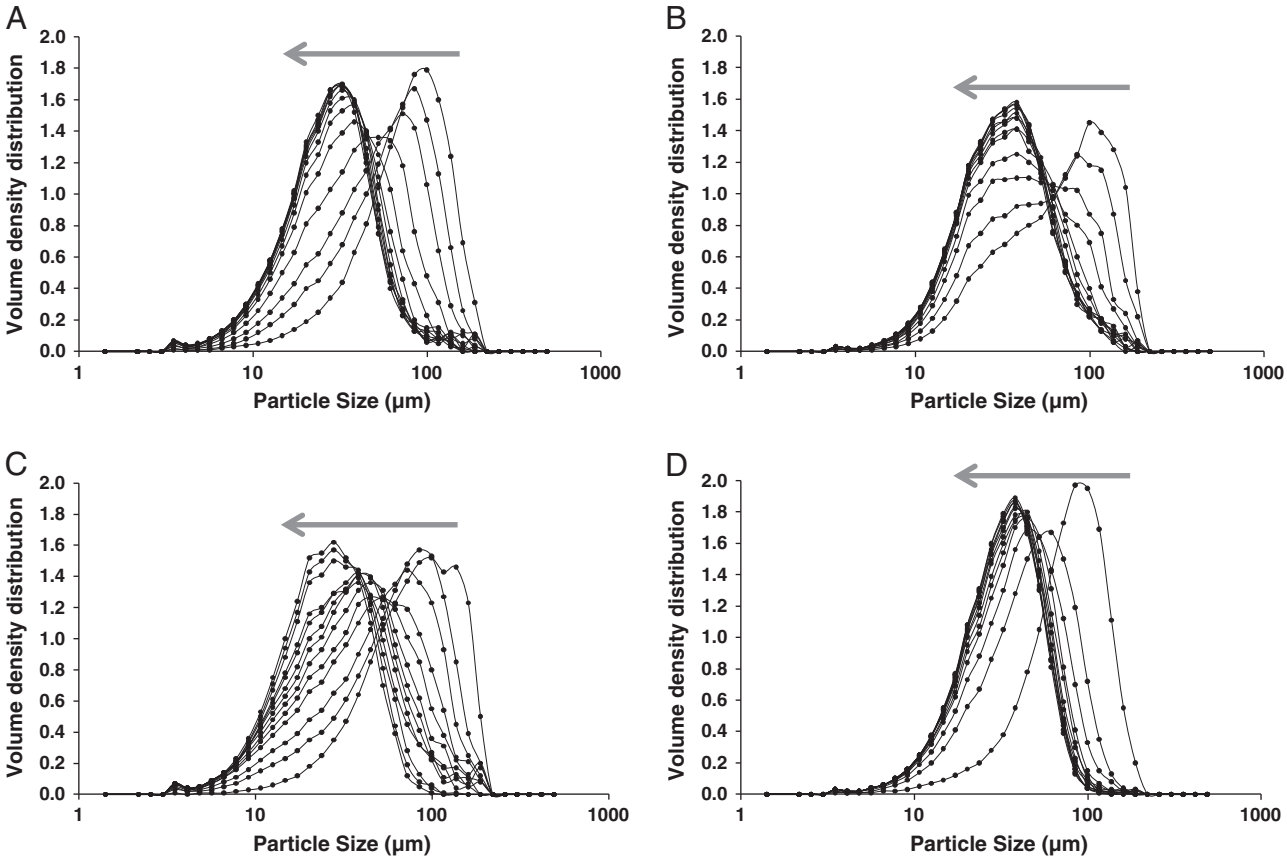

Fig. 6. Particle size distribution recorded as a function of digestion time. The curves are plotted each 10 min. The arrow indicates the direction of evolution from the beginning to the end of digestion for the four formulations (A, B, C and D). The digestion was realized in a gastric medium containing the pepsin enzyme.

was observed for microparticles produced by formulation B. The high presence of hydrogen ions in simulated gastric solution was already found to modify steric protein structure, in this case native whey proteins. Consequently, the particle porosity could increase and the microparticles could uptake the solution (Matalanis et al., 2011).

(2) Then a *decrease in size* occurred along the digestion. Indeed, cohesive forces of food matrices are known to be different as a function of the protein composition (Lundin, Golding, & Wooster, 2008). During digestion the particles could be influenced as follows: hydrolyzation by acid, proteolysis by pepsin, shearing forces by peristaltic stomach movements and finally body temperature (Lundin et al., 2008). Gastric pepsin enzyme may cause the protein hydrolysis into polypeptides, oligopeptides and some free amino acids. Nevertheless, the determining factor enabling hydrolysis is cleavage site accessibility to the enzyme. One of the reasons explaining the good resistance of milk microparticles could be that the cleavage sites were partially hidden in the structure. Indeed, casein resistance under simulated gastric digestion has already been shown (Dupont et al., 2010). The high resistance of κ- and α_{s2}-casein was primarily highlighted. It has been demonstrated that hydrophobic and post-translational modified (phosphorylated and glycosylated) fragments could maintain the protein integrity.

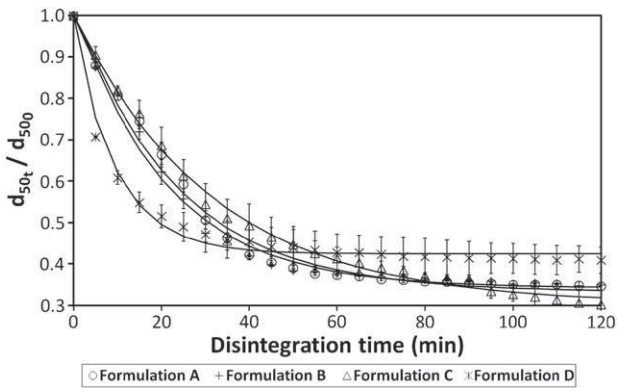

Fig. 7. Normalized diameter (mean ± SD, n = 3) decrease as a function of digestion time. The experimental points are fitted with an empirical model.

3.3. Selection of the better matrices for the encapsulation of LGG

3.3.1. Selection of the better matrices from microbiological measures

The formulations previously studied without probiotic bacteria were finally tested as an encapsulation material for LGG. The best formulation for bacterial survival during gastric digestion was formulation C followed by D. Formulations A and B presented the worse results (Fig. 8). The curve of non encapsulated bacteria is also

Table 2
Determination of the kinetic parameters of gastric disintegration of microparticles for each formulation (A, B, C and D) using the linear exponential equation proposed by Kong and Singh (2008).

Formulation	k	β	MSE × 10^3	r^2	$t_{1/2}$ (min)
A	0.630	0.043	0.477	0.988	25.6
B	0.640	0.048	0.377	0.990	22.4
C	0.590	0.032	0.199	0.995	37.3
D	0.642	0.103	0.314	0.983	8.3

interesting as it is a proof of the efficiency of encapsulation (the population decline from 100 to 0.0005%). When looking at the encapsulation rates, formulation C was again the best one with an excellent rate around 97% (Table 3). It is worth noting that even the worse encapsulation rate obtained for formulation A around 64% was not so bad compared to the other authors that microencapsulated bifidobacteria with whey protein and obtained an encapsulation rate comprised between 0.03% and 25% (Picot & Lacroix, 2004). The improvement of encapsulation efficiency when LGG is encapsulated in a matrix not only composed of micellar casein but also of whey proteins can be explained by the specific interaction settling between the strain and whey proteins (Burgain et al., 2013). Thanks to these adhesion forces, probiotic bacteria can be better retained in the gel explaining why the encapsulation rate was higher with the addition of whey proteins.

3.3.2. Selection of the better matrices from morphology and physical concerns

Microparticle sizes are an important consideration since the microparticles must i) have a high volume-to-surface ratio for increasing the protective effect and ii) be sufficiently small to avoid a negative sensory impact (Anal & Singh, 2007). The particle detection by the consumer will be assimilated as a defect and the sensory perception is affected by the food matrix in which the microparticles are introduced (van Vliet, van Aken, de Jongh, & Hamer, 2009). In the study, the sizes were about the same order and comprised between 56 μm and 70 μm so, formulations cannot be discriminated by the size criterion. The shape is also important for consumer acceptance because a round particle is less detectable than an irregular particle (Imai, Saito, Hatakeyama, Hatae, & Shimada, 1999). However, the produced microparticles have almost the same sphericity and convexity values relying that they were well rounded and spherical.

According to Table 2, it was measured that microparticles from formulation D were the most quickly affected by the digestion (small $t_{1/2}$), and for this reason it cannot be selected for future probiotic microencapsulation. It was explained in this study that at the beginning of digestion the microparticles tend to form aggregates but in the case of formulation B it seems that swelling was responsible for microparticle size increase. The tendency of swelling depends on the type of bonds holding the matrix (Matalanis et al., 2011) and the risk with this phenomena is the diffusion of the encapsulated substance out of the particle (Matalanis et al., 2011). Even if for some delivery system this effect can be exploited for controlled release, in the case of probiotic encapsulation it is not desired since the gastric liquid will have access to the interior of the particle and will be in contact with the cells. For this reason, formulation B will not be kept for probiotic microencapsulation.

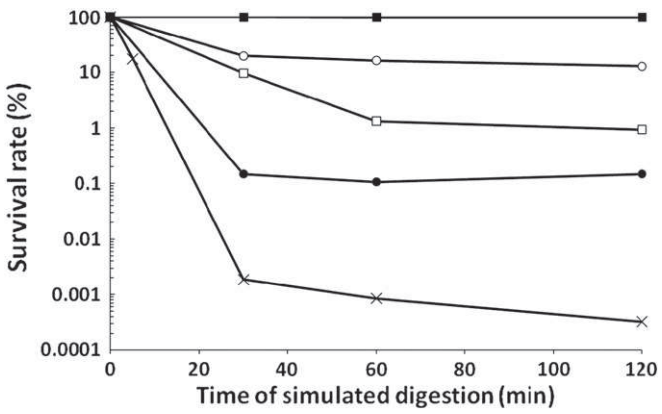

Fig. 8. Bacterial survival (calculated with Eq. (2)) during simulated gastric digestion: non encapsulated bacteria ×; formulation A ●; formulation B □; formulation C ■; formulation D ○).

Table 3
Encapsulation rate (calculated with Eq. (1)) for the probiotic strain *L. rhamnosus* GG encapsulated into different milk protein matrices.

Formulation	Encapsulation rate (%)	Standard deviation (%)
A	64	4
B	88	6
C	97	2
D	84	2

Microparticles prepared by this emulsification method are shown to be polydisperse in size, which is not desirable for controlled release (Cheng, Liu, & He, 2010). However, microparticles produced with formulation C have the lowest polydispersity so they can be selected from others. Moreover, according to the half-time obtained during digestion, formulation C had the longest one meaning that it can better resist gastric environment and also has the strongest gel elasticity (G′). For all of these reasons, formulation C, composed of micellar casein and denatured whey proteins seems to be the more effective to protect probiotic bacteria.

4. Conclusion

The different parameters observed in this study enable discriminating the best formulation to produce microparticles able to protect LGG into simulated gastric conditions. Clearly, formulation C consisting of micellar casein and denatured whey proteins was the best one: particle size, Bdi, G′, half time of gastric digestion, rates of encapsulation and survival.

In addition and for the first time, an original equipment was used to follow in situ the microparticle size and shape evolution during digestion in the stomach. This equipment could be use in the future to improve the target delivery of bioactives by a simple, direct and easy method.

The next step of this work is to use the microparticles for the protection of other sensitive bioactives against adverse environmental conditions encountered in the stomach. Studies are under progress for polyphenolic compounds and other probiotic bacteria.

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Résumé :

Ce travail de thèse a permis la mise au point d'un procédé d'encapsulation de bactéries probiotiques dans des matrices ne contenant que des ingrédients laitiers. L'étude à l'échelle laboratoire puis, le dimensionnement du procédé à l'échelle pilote ont permis la production de microparticules stables dans des milieux aqueux et résistantes à des conditions simulant l'estomac. La nature des protéines présentes mais également leurs proportions ont influencé la localisation des bactéries dans les microparticules : à la surface pour une matrice uniquement composée de caséine et à l'intérieur pour une matrice composée à la fois de caséines et de protéines solubles. L'interprétation de ces résultats par une étude à l'échelle moléculaire des interactions qui s'établissent entre les bactéries probiotiques et les protéines laitières a été possible grâce à l'utilisation de la microscopie à force atomique. Les protéines solubles interagissent de façon spécifique avec les bactéries alors que les caséines interagissent de façon non spécifique. De même, la présence de pili à la surface de la bactérie est favorable à la l'établissement d'interactions fortes entre LGG et les protéines solubles.

Ce travail a donc permis d'expliquer des résultats obtenus à l'échelle macroscopique (taux d'encapsulation et de survie dans des conditions gastriques) grâce à des observations microscopiques et une étude à l'échelle nanoscopique des interactions entre les différents composants. Cette approche multi-échelle a élucidé certains mécanismes régissant l'encapsulation de bactéries probiotiques dans des matrices laitières.

Mots clés : encapsulation, probiotiques, protéines laitières, AFM, interactions.

Abstract :

In this work thesis, an encapsulation process for probiotic bacteria using only milk proteins is developed. The laboratory scale followed by a pilot and an industrial scale development of the process allow the production of stable and resistant microparticles both in aqueous and gastric media. The nature and quantities of proteins added is found to influence the bacterial location in the microparticles. The bacteria are located mostly at the surface when only caseins are present. Oppositely, well encapsulated bacteria are observed when addition of whey proteins are performed. A molecular study of interactions established between milk proteins and bacteria is possible with the use of atomic force microscopy. Whey proteins are found to specifically interact with bacteria whereas the caseins establish non-specific links. In addition, the presence of piliated bacteria is found favorable to establish strong and long interactions between proteins and bacteria.

This work permits the interpretation of results obtained at a macroscale (encapsulation rate and bacterial survival in stomach conditions) thanks to microscopic observations and nanoscopic interactions study. This multiscale approach permits the elucidation of mechanisms driving the probiotic encapsulation in milk matrices.

Keywords : encapsulation, probiotics, milk proteins, AFM, interactions.