THÈSE

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Encapsulation de *Carnobacterium maltaromaticum* LMA28, productrice de composés aromatiques, dans la paraffine d'enrobage du Poro, un fromage artisanal mexicain

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A la vida

A la amistad

Al amor

A la familia

A la alegría

A la esperansa

…

La marca invariable de un anhelo, es saber que si luchamos tarde o temprano se hará realidad.
(Carlos Casanti)

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Nomenclature

3-Mal: 3-methylbutanal

α-KG: alpha ketoglutarate

ANOVA: variance analysis

AOP: Protected Designation of Origin

AraT: specific aminotransferase for aromatic amino acids

AT: specific aminotransferase for branched chain amino acids

Aw: Water activity

BCV^+: blanched chain volatile producing strains

BCV^-: blanched chain volatile not producing strains

BRG: Bioencapsulation Research Group

C: *Carnobacterium*

C: concentration

Ca: calcium

Carno: *Carnobacterium maltaromaticum LMA28*

Cbn: carnobacteriocins

CDs: cyclodextrins

CFU: colony forming unit

CitP: citrate permease

CitL: citrate lyase

CNRZ: National Center for Animal Research (France)

CONACYT: National Council for Science and Technology (Mexico)

CSC: commercial starter cultures

D: desirable

DCA: Completely randomized design

DNA: deoxyribonucleic acid

DO: Mexican equivalent to the French AOP

E: *Enterococcus*

EY: Encapsulation efficienty

EMC: enzyme modified cheese powder

ENSAIA: Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires

EU: European Union

G': storage module

G'': loss module

GA: Gum Arabic

GC: chromatographie en phase gazeuse

GDH: glutamate déshydrogénase

GRAS: generally recognized as safe

HADH: hydroxy acid dehydrogenase

HCl: hydrochloric acid

I: Intensity

KADC: keto acid decarboxylase

KADH: keto acid dehydrogenase

L/Lb: *Lactobacillus*

L: *Listeria*

LAB: lactic acid bacteria

LMA: Food Microbiology Laboratory

MCM: selective culture media for *C. maltaromaticum*

MS: modified starch

MRS: Rogosa and sharpe agar

NaCl: sodium chloride

NAD: nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

ND: non desirable

NPB: non paraffin beads

NR: not reported

O/W: oil-in-water emulsion

W/O: water-in-oil emulsion

O/W/O: oil-in-water-in-oil emulsion

W/O/W: water-in-oil-in-water emulsion

OSA: n-octenyl succinil anydride modified starches

P: probability

PALCAM: specific culture media for *L. monocytogenes*

PB: paraffin beads

PCs: Principal components

PC1: principal component 1

PC2: Principal component 2

PCR: polymerase chain reaction

pH: potential hydrogen

PTA: phosphotransferase

QP1: Poro cheese from factory 1

QP2: Poro cheese from factory 2

QP3: Poro cheese from factory 3

QP4: Poro cheese from factory 4

QP5: Poro cheese from factory 5

RNAse: Ribonuclease

rRNA: acide ribonucléique ribosomique

SPI: soybean protein isolated

t_o: Initial time

TPA: textural profile analysis

TS: tryptone salt

TSA: trypticase soy agar

TSB: soy broth trypcase

TTGE: Time temperature gel electrophoresis

UHT: Ultra-high temperature processing

YE: yeast extract

YI: Yellowing index

Chapitre 1. Introduction Générale

1. Introduction générale

En 2011, au Mexique, 275 411 tonnes de fromage ont été produites et la consommation totale du pays, la même année, était de 311 000 t, soit une consommation par personne et par an de 2,8 kg (SIAP, 2013). Bien que la consommation de fromage par habitant et par an soit faible par rapport à des pays comme la France (26.3 kg) ou la Grèce (23.4 kg) (CDIC, 2011), elle atteste malgré tout d'un marché conséquent. Au Mexique plus de trente types de fromages différents existent, la plupart produits de façon artisanale avec une distribution régionale. S'il existe des entreprises productrices équipées de façon moderne utilisant du lait pasteurisé, de ferments lactiques commerciales, il y a surtout des nombreuses petites et très petites entreprises produisant des fromages au lait cru avec une technologie artisanale. Mais cette industrie fromagère reste une activité économique importante, source d'emplois et de revenus pour les familles des régions productrices (Villegas de Gante, 2004).

Parmi la trentaine de fromages dénombrés, le fromage Poro est l'un des produits les plus typiques de l'artisanat mexicain. Il est fabriqué dans la région de Los Ríos dans l'état de Tabasco, principalement dans la ville de Balancan. Il est obtenu à partir de lait cru de vache.

C'est un fromage doux, à pâte légèrement pressée, présentant une maturation involontaire d'environ un mois qui se produit principalement pendant la période de commercialisation. Sa forme est parallélépipédique de petit format (environ 250 g) et il est traditionnellement emballé dans une enveloppe cellulosique jaune (Cervantes Escoto et al., 2006, Villegas de Gante, 2004).

De ce qui fait la typicité d'un produit, les caractéristiques sensorielles sont essentielles à l'identité d'un fromage et à son acceptabilité par les consommateurs (Van Hekken et al., 2006). Ces caractéristiques organoleptiques, aspect, texture et flaveur par exemple, sont autant de stimuli agréables ou non pour le dégustateur, l'influençant dans sa consommation, pouvant aller du plaisir au rejet et par conséquent dans ses achats (Sancho Valls et al., 2001). Concernant le Poro, il apparaît que son acceptabilité est étroitement liée au caractère frais du fromage juste après sa production. Ainsi, le développement rapide de notes olfactives associées plutôt à des fromages affinés dégrade l'acceptabilité du fromage Poro. Pour cette raison, la période de vente du Poro est très courte (3 semaines) et, en particulier, elle est bien inférieure à la durée de vie du produit. Cela limite la possibilité pour les producteurs d'obtenir de meilleurs avantages économiques et de développer leur marché.

La qualité sanitaire du fromage Poro permet sa consommation au-delà de sa période de vente habituelle, il apparaît intéressant de trouver une stratégie pour améliorer ses propriétés olfactives et prolonger ainsi leur temps de distribution sur les marchés, sans pour autant nuire à la typicité de ce

produit candidat à l'obtention d'une dénomination d'origine (DO) équivalent à l'appellation d'origine protégée française.

Une stratégie employée pour améliorer les propriétés sensorielles d'un fromage est l'aromatisation du produit, c'est-à-dire l'addition de nouvelles molécules olfactives. En fromagerie, il existe différentes molécules volatiles d'intérêt : diacétyle, acide butyrique, methyonal, 2-butanone, 3-methylbutanal entre autres (Hou et al., 2014, Sgarbi et al., 2013, Peralta et al., 2014, Cakmakci et al., 2013, Miks-Krajnik et al., 2013, Pachlova et al., 2013).

Parmi elles, le 3-méthylbutanal est un des principaux composés aromatiques de nombreuses variétés de fromages à pâte dure et mi-dure (Hou et al., 2014, Pachlova et al., 2013). Apportant des notes olfactives caractéristiques (fruite, maltées, chocolatée) (Afzal et al., 2014, Hou et al., 2014).

Les études menées jusqu'à présent se sont plutôt attachées à démontrer le lien entre la présence de ce composé aromatique, les caractéristiques organoleptiques du fromage et sa microflore.

Les bactéries lactiques (LAB) appartenant aux genres *Lactococcus, Lactobacillus, Streptococcus, Carnobacterium* et *Enterococcus,* tout comme certaines levures comme *Debaryomyces hansenii*, *Yarrowia lipolytica* et *Geotrichum candidum,* sont supposées être impliquées dans la production de 3-méthylbutanal dans le lait et le fromage (Helinck et al., 2004, Smit et al., 2005, Afzal et al., 2013a, Afzal et al., 2012, Afzal et al., 2010b, Bergamini et al., 2013, Boutrou and Gueguen, 2005, Gkatzionis et al., 2013).

Cette molécule présente donc un intérêt tout particulier pour développer l'aromatisation d'un produit tel que le fromage Poro et ainsi masquer les « off-flavors » apparaissant lors de sa conservation. Pour cela, il apparaissait comme nécessaire de s'intéresser à des procédés permettant d'introduire cette molécule dans le fromage ou dans son emballage.

L'encapsulation est un des procédés physicochimiques ou mécaniques permettant de piéger une molécule active d'intérêt (micro-organismes, vitamines, composés volatiles…) pour préserver une propriété d'un produit ou pour ajouter une fonctionnalité. Cette technologie a de multiples applications en industrie alimentaire : contrôle des réactions d'oxydation, masquage des odeurs, couleurs ou flaveurs, permettant, entre autres, la libération contrôlée et la prolongation de la durée de vie en rayon (Burgain et al., 2011). Il existe différentes techniques d'encapsulation et une quantité de matériels potentiellement utilisables. Il n'y a donc pas une méthode préconisée par rapport à une autre, mais plutôt un choix à faire selon plusieurs critères tels que l'objectif de l'encapsulation, les caractéristiques du composé à encapsuler, la compatibilité entre les matériaux utilisés, la toxicologie, le coût, ... (de Vos et al., 2010). Chaque procédé d'encapsulation doit être mis au point, adapté pour répondre à une exigence particulière et permettre son application industrielle.

Les hydrogels d'alginate de calcium sont fréquemment utilisés pour le piégeage de micro-organismes. En effet, ils présentent des avantages certains, comme leur innocuité, leur biocompatibilité et leur faible coût. (Burgain et al., 2011, Park and Chang, 2000). L'alginate est un polysaccharide formé d'unités d'acides β-D-manuronique et α-L-glucuronique et extrait de certaines espèces d'algues (*Macrocystis pyrifera, Ascophyllum nodosum*).

L'enrobage de fromage est une technique bien connue et largement utilisée. Le Poro, comme d'autres fromages, est recouvert de paraffine, préservant ainsi le fromage des flux d'eau (pouvant être la cause d'apparition de moisissures ou de dessèchement), évitant aussi au fromage d'exsuder sa matière grasse, le rendant ainsi plus facile à manipuler et plus attrayant pour le consommateur (Kampf and Nussinovitch, 2000). L'incorporation d'agents actifs dans l'emballage/ l'enrobage du fromage est une technique connue pour prévenir ou minimiser les risques de dégradations chimiques, biochimiques ou microbiologiques (Yilmaz and Dagdemir, 2012). Avec l'encapsulation, il est possible d'incorporer des composés aromatiques dans la paraffine d'enrobage du Poro, permettant ainsi potentiellement d'éviter les modifications des caractéristiques intrinsèques du fromage ou de sa technologie de fabrication. C'est donc cette approche qui a été retenue plutôt que l'incorporation du 3-methylbutanal directement dans le fromage.

A ce stade, deux alternatives sont envisageables pour incorporer des composés aromatiques volatiles à l'enrobage de paraffine. La première consiste à réaliser une encapsulation directe de la molécule d'intérêt. La seconde stratégie est d'encapsuler directement une bactérie non pathogène, elle-même capable de produire la molécule d'intérêt. Parmi les bactéries lactiques, *Carnobacterium maltaromaticum*, est une bactérie fréquemment rencontrée dans les aliments (viandes, poissons, et produits laitiers) et qui présente deux fonctionnalités intéressantes. Elle est en effet capable de produire des composés aromatiques tels que le 3-méthylbutanal (Afzal et al., 2013a) mais aussi d'inhiber des souches pathogènes alimentaires (Afzal et al., 2010b) grâce à la production de bactériocines. Ces bactériocines ont une action antibactérienne contre des souches phylogénétiquement proches (Jacquet et al., 2012) telles que *Listeria monocytogenes*, un agent pathogène responsable de l'infection listériose chez l'animal comme chez l'Homme.

Le but de ces travaux est donc d'améliorer les propriétés organoleptiques, notamment aromatiques, du fromage Poro par l'incorporation dans son enrobage de paraffine de billes d'alginates contenant 1) du 3-méthylbutanal directement encapsulé 2) des souches de *Carnobacterium maltaromaticum* LMA28. Par ailleurs, l'activité inhibitrice de *Carnobacterium maltaromaticum* LMA28, isolée à partir d'un fromage à pâte molle, est aussi étudiée vis-à-vis de *L. monocytogenes* pour une application en industrie fromagère.

Ce manuscrit se décline en trois chapitres.

La revue bibliographique présente l'état de l'art sous forme de 2 articles de revue :

- le premier article, accepté pour publication, donne une vue d'ensemble du rôle du 3-méthylbutanal, ainsi que ses voies principales d'élaboration chez les bactéries lactiques et plus particulièrement chez *C. maltaromaticum*.
- le second article présente une vue d'ensemble des technologies et matériaux les plus utilisés pour l'encapsulation d'arômes dans l'industrie alimentaire ainsi que les récentes applications dans le domaine de l'encapsulation de composés aromatiques.

Les résultats sont présentés sous forme de 3 parties comprenant 2 publications scientifiques soumises et un chapitre de résultats :

- la première partie porte sur la caractérisation physicochimique, microbiologique, rhéologique et sensorielle d'un fromage typique Mexicain, le fromage Poro.
- la seconde partie est axée sur l'amélioration des caractéristiques sensorielles des fromages Poro en incorporant dans leur enrobage des microbilles d'alginate contenant des composés volatils directement encapsulés (3-méthylbutanal) ou une souche productrice de ce composé (*Carnobacterium maltaromaticum* LMA28).
- la troisième partie évalue l'effet antimicrobien de *C. maltaromaticum* LMA28 contre *L. monocytogenes* en utilisant un plan d'expériences à 4 facteurs. Une partie de ces résultats est publiée (Afzal et al., 2013b).

Les conclusions générales et les perspectives que laissent entrevoir ce travail sont présentées dans le dernier chapitre de cette thèse.

Chapitre 2. Revue bibliographique

1. Le 3-methylbutanal et l'arôme des fromages

1.1. Introduction

Comme cela a été mentionné précédemment, l'objectif général de cette recherche est l'amélioration des propriétés aromatiques du fromage Poro, un fromage typique du Mexique produit artisanalement à partir de lait cru.

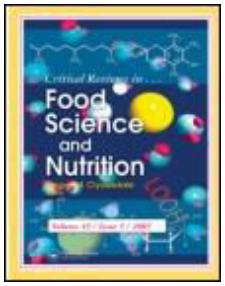
Les fromages possèdent naturellement une énorme variété de molécules aromatiques qui constitue leur profil aromatique. L'une des stratégies visant à modifier ou à améliorer les caractéristiques olfactives d'un produit est l'ajout de nouvelles molécules aromatiques ayant des caractéristiques correspondant aux notes olfactives désirées.

Divers composés volatils d'intérêt (dactylo, acide butyrique, methyonal, 2-butanone, 3-methylbutanal…) contribuent aux notes aromatiques du fromage. Les bactéries lactiques ont souvent été associées à la formation de ces composés dans les produits laitiers.

Parmi ces composés, le 3-methylbutanal est connu pour fournir des notes maltées, chocolatées et des rappels de noix au fromage (Afzal et al., 2014). Ce composé volatil peut être synthétisé par diverses espèces, y compris *C. maltaromaticum* ; une espèce qui a été largement étudiée au laboratoire LIBio.

Dans la perspective d'utiliser *C. maltaromaticum* pour sa production de 3-methylbutanal qui permettrait d'améliorer les caractéristiques sensorielles du fromage Poro, nous nous sommes intéressés à comprendre le rôle des bactéries productrices des composés aromatiques dans le fromage. L'objectif de cette partie bibliographique est, par conséquent, de clarifier et de mettre en évidence le rôle et l'importance du 3-méthylbutanal lié à certains types de fromages et de résumer les connaissances actuelles pour son possible contrôle dans le domaine fromager.

1.2. Biosynthesis and role of 3-methylbutanal in cheese by lactic acid bacteria: Major metabolic pathways, enzymes involved and strategies for control



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Abstract

Branched chain aldehyde, 3-methylbutanal is associated as a key flavor compound to many hard and semi-hard cheese varieties. The presence and impact of this flavor compound in bread, meat and certain beverages has been recently documented, however its presence and consequences regarding cheese flavor were not clearly reported. This paper gives an overview of the role of 3-methylbutanal in cheese, along with the major metabolic pathways and key enzymes leading to its formation. Moreover, different strategies are highlighted for the control of this particular flavor compound in specific cheese types.

Keywords: Cheese, flavor formation, 3-methylbutanal, metabolic pathways, control strategies

1.2.1. Introduction

The presence of branched-chain volatile aldehydes has been reported to be perceived either as a malty/off-flavor or as nutty/chocolate-like aroma among various cheese types associated to both hard and soft categories. Special emphasis has been given to 3-methylbutanal, as its concentration or relative abundance always seemed to be higher as compared to 2-methybutanal and 2-methylpropanal due to the efficient breakdown of leucine by the cheese microbiota (Bosset and Gauch, 1993, Centeno et al., 2002, Ayad et al., 2004b, Deetae et al., 2007, Irigoyen et al., 2007).

Some decades ago, the identification, chemical nature of malty flavor and microbial defects in milk resulting from the use of malty flavor producing strain, *Streptococcus lactis* var. *maltigenes* were dealt with in various works (Jackson and Morgan, 1954). Since then, the researchers, gave attention to the possible consequences of the presence of these flavor compounds while characterising cheese flavor profiles and corresponding cheese microflora. Recently, many studies have highlighted both the desirable and non desirable role of 3-methylbutanal with respect to various cheese types. It is

assumed that the flavor perception might be related to numerous factors including cheese moisture, texture, milk type and more particularly, internal balance of many odorous compounds generated from various sources like proteins, fat and carbohydrates. However, to get a desired flavor from a cheese is yet a very complex and difficult task, a little imbalance between various odorous compounds could impair the final flavor quality (Fox and Wallace, 1997, Moller et al., 2013).

Lactic acid bacteria (LAB) belonging to genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Carnobacterium* and *Enterococcus* are generally supposed to be involved in the formation of 3-methylbutanal in milk and cheese (Helinck et al., 2004, Smit et al., 2005, Afzal et al., 2012, Afzal et al., 2013b, Afzal et al., 2010b, Bergamini et al., 2013). Moreover, some yeasts such as *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Geotrichum candidum* when used as adjunct or starter in soft cheese manufacturing were also found to contribute the formation of 3-methylbutanal (Boutrou and Gueguen, 2005, Gkatzionis et al., 2013).

Extensive studies have been carried out to investigate the possible biosynthetic pathways of 3-methylbutanal in bacteria. The intracellular biosynthesis of 3-methylbutanal from leucine catabolism generally takes place by the two possible metabolic pathways in LAB: either by a direct pathway using α-ketoacid decarboxylase enzyme (KADC) such as proven for *L. delbrueckii* subsp. *lactis* CNRZ 207 (Helinck et al., 2004) or by an indirect pathway comprising α-ketoacid dehydrogenase enzyme (KADH) such as proven for *L. helveticus* CNRZ 32 (Helinck et al., 2004) and *E. faecalis* 10C1 (Ward et al., 1999). Recently, it has been demonstrated that in *Carnobacterium maltaromaticum* both metabolic pathways were present and functional (Afzal et al., 2012).

Due to the importance of this flavor compound in particular cheese types, numerous strategies have been proposed to control its production. Different approaches have been pointed out using exogenous addition of substrate, microbial adjuncts possessing potential and complementary metabolic pathways, bacteriocin induced lysis, and modifying the environmental conditions by changing oxygen or potential redox. Recently, Smit *et al.* (Smit et al., 2009), documented the origin, presence and impact of branched-chain aldehydes such as 3-methylbutanal, 2-methylbutanal and 2-methylpropanal in bread, meat and certain beverages.

The aim of the present review is to clarify and highlight the role and significance of 3-methylbutanal related to particular cheese types and to summarize the current knowledge for its possible control in cheese products.

1.2.2. Key enzymes and metabolic pathways involved in the biosynthesis of 3-methylbutanal from leucine catabolism among LAB

The catabolism of leucine during cheese ripening is mainly initiated by the action of microbial aminotransferases, although chemical degradation (Strecker degradation) can alsooccur (Yvon and Rijnen, 2001, Yvon et al., 1997, Smit et al., 2009, Garcia-Cayuela et al., 2012) (Figure 1). Among LAB, the deamination of glutamate to α-ketoglutarate (α-KG) catalyzed by glutamate dehydrogenase (GDH) is usually linked to a transamination route.

1.2.2.1. Aminotransferase and glutamate dehydrogenase

Aminotransferase activity (AT) was found to be present in a large group of cheese related LAB. Activities varied and diversity existed among the strains (Yvon et al., 1997, Smit et al., 2004, de Palencia et al., 2006, Garcia-Cayuela et al., 2012, Freiding et al., 2012). The major aromatic aminotransferase (AraT) was purified and characterized from *L. lactis* and the role of this enzyme in the initiation of degradation of several amino acids including leucine was demonstrated by Yvon *et al.* (Yvon et al., 1997), which was responsible for the synthesis of precursors of aroma compounds usually found active under cheese ripening conditions. Moreover, it was found to exhibit overlapping substrate specificities towards both branched chain and aromatic amino acids (Yvon et al., 1997, Yvon and Rijnen, 2001). The role of both aromatic and branched chain aminotransferases (AraT/BcaT) was studied in a cheese model (Rijnen et al., 2003) and it was demonstrated that both BcaT and AraT were involved in the degradation of leucine. Leucine ketoisocaproate by aminotransferase enzyme and during this conversion 110 the amino group of leucine is transferred to α-ketoglutarate resulting in the formation of glutamic acid.

The importance of GDH activity as major criterion for the selection of flavor producing LAB strains was pointed to intensify cheese aroma (Tanous et al., 2002). Later on, a beneficial effect on aroma formation was observed by using a combination of GDH positive lactobacilli with *L. lactis* ssp. *cremoris* NCDO763 (Kieronczyk et al., 2004). The biosynthetic pathways for α-KG formation and its impact on cheese aroma development was explored and it has been shown that the citrate-oxaloacetate pathway, that requires citrate permease (CitP), citrate lyase (CitL) and aspartate aminotransferase, was operative for *L. lactis* ssp. *diacetylactis* and hence stimulated the conversion of amino acids (Tanous et al., 2005).

1.2.2.2. Major metabolic pathways for the biosynthesis of 3-methylbutanal

The transamination of leucine results in α-ketoisocaproate, which is the central metabolite in leucine catabolism (Smit et al., 2004) and gives rise to 3-methylbutanal either directly as a result of non-

oxidative decarboxylation by α-ketoacid decarboxylase (KADC) or indirectly via an oxidative decarboxylation by the activity of α-ketoacid dehydrogenase (KADH) (Larrouture-Thiveyrat and Montel, 2003, Helinck et al., 2004). In the literature, the direct pathway for the biosynthesis of 3-methylbutanal is very well documented in LAB. On the contrary, the indirect pathway was not studied extensively (Table 1).

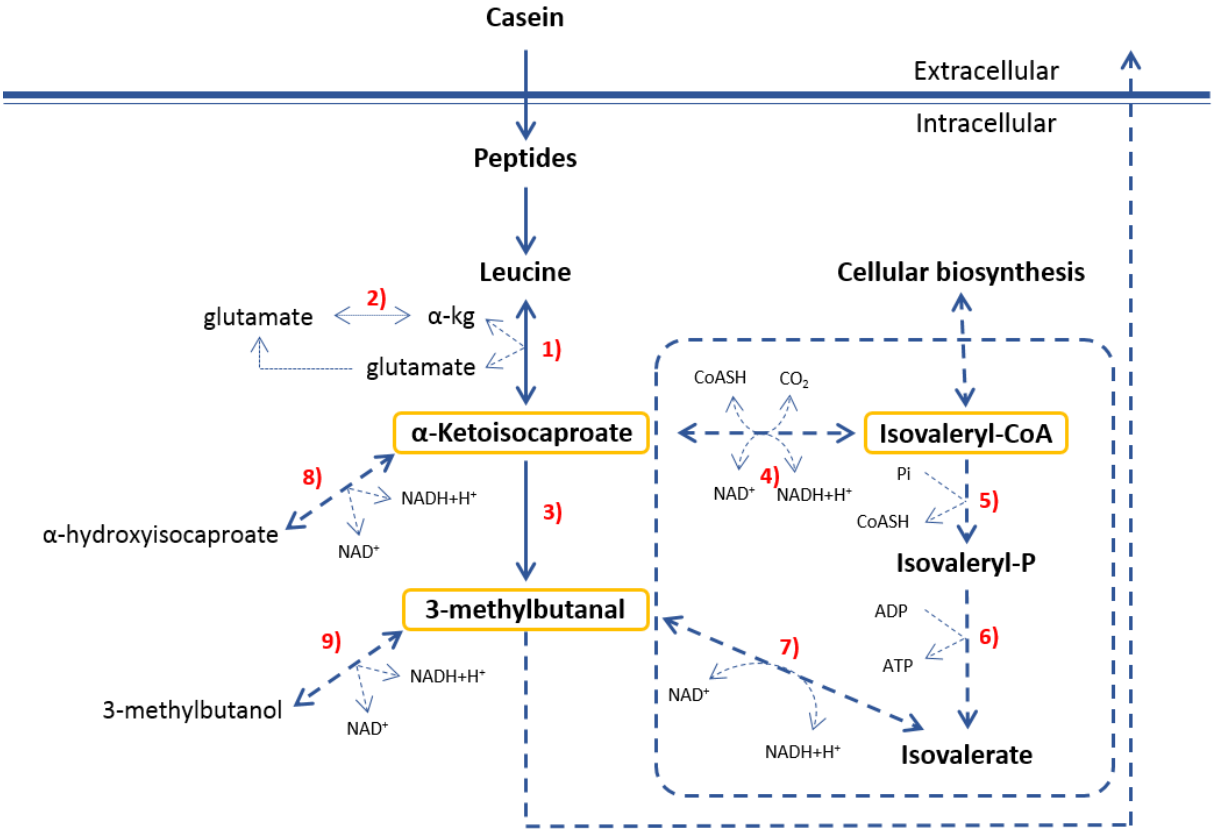


Figure 1. Intracellular metabolic pathways for the biosynthesis of 3-methylbutanal from leucine catabolism. Enzymes of the direct pathway (in the solid line): 1) AT, aminotransferase, 2) GDH, glutamate dehydrogenase, 3) KADC, α-ketoacid decarboxylase. Enzymes of indirect pathway (in the dashed lines): 4) KADH, α-ketoacid dehydrogenase, 5) PTA, phosphotransferase, 6) ACK, acylkinase, 7) AldDH, aldehyde dehydrogenase. Other enzymes include 8) HADH, α-hydroxyacid dehydrogenase, 9) AlcDH, alcohol dehydrogenase.

The direct pathway

The direct pathway appeared to be rare among LAB and KADC activity was found to be highly strain dependant. KADC activity has been reported in *L. lactis* (Smit et al., 2004), some *L. lactis* wild strains (Ayad et al., 1999, Ayad et al., 2001, de la Plaza et al., 2004, de Palencia et al., 2006), *L. delbrueckii* (Helinck et al., 2004) and *C. maltaromaticum* (Afzal et al., 2012). The gene encoding KADC enzyme (*kdcA*) was identified by N-terminal sequencing of the partially purified protein in *L. lactis* (de la Plaza et al., 2004) or by screening a mutant library in *L. lactis* (Smit et al., 2005). In *L. lactis*, KADC activity

would be of prime importance in order to control desired formation of 3-methylbutanal, as most of the aldehydes could be formed through this pathway (Smit et al., 2004).

The indirect pathway

The indirect pathway constituted the oxidative decarboxylation of α-ketoisocaproate to isovaleryl-CoA by the KADH enzyme complex along with several intermediate enzymes including phosphotransferase (PTA), acyl kinase (ACK), and aldehyde dehydrogenase for the biosynthesis of 3-methylbutanal. This pathway has been reported *in vitro* in *Propionibacterium freudenreichii* (Thierry et al., 2002), *L. helveticus* (Helinck et al., 2004), *C. maltaromaticum* (Afzal et al., 2012) and via the gene product in *Enterococcus faecalis* (Ward et al., 1999). The KADH enzyme activity was determined and found to be dependant of both NAD+ and NADP+ in *C. maltaromaticum* (Afzal et al., 2012). The genes encoding KADH complex were found encoded in one operon (*ptb-buk-bkDABC*) in *E. faecalis* (Ward et al., 1999) and in *L. casei* (Liu et al., 2008). In *C. maltaromaticum* LMA 28 strain, these genes encoding KADH enzyme complex (*bkdA, bkdB, bkdC and bkdD*) have been identified by degenerate primer design (Afzal et al., 2012).

Regulation of 3-methylbutanal biosynthesis

The biosynthesis of 3-methylbutanal in bacteria depends on the functionality of intracellular pathways and is mainly regulated by redox environment (NAD+/NADH, H+ yield, presence/absence of oxygen). Indeed, high formation of 3-methylbutanal in *L. lactis* (Kieronczyk et al., 2006), *Proteus vulgaris* (Deetae et al., 2011) and *C. maltaromaticum* (Afzal et al., 2013a) has been attributed to the presence of oxygen or stimulation of KADC enzyme activity.

Table 1. Demonstration (in vitro and via gene product) of direct/indirect pathways for the biosynthesis of 3-methylbutanal in LAB.

Bacterial species	Strain	Study model	Pathways KADC/KADH	Reference
Lactococcus lactis subsp. *cremoris*	NIZO B1157	In vitro/via gene product	KADC	(Ayad et al., 1999, Ayad et al., 2001, Smit et al., 2004, Smit et al., 2005)
Lactococcus lactis	IFPL730	In vitro/via gene product	KADC	(de la Plaza et al., 2004, de Palencia et al., 2006)
Lactobacillus delbrueckii subsp. *lactis*	CNRZ 207	In vitro	KADC	(Helinck et al., 2004)
Lactobacillus helveticus	CNRZ 32	In vitro	KADH	(Helinck et al., 2004)
Enterococcus faecalis	10C1	Via gene product	KADH	(Ward et al., 1999)
Lactobacillus casei	ATCC 334	Via gene product	KADH	(Liu et al., 2008)
Carnobacterium maltaromaticum	LMA 28	In vitro/via gene product	KADC/KADH	(Afzal et al., 2012)

1.2.3. Presence and role of 3-methylbutanal in various varieties of cheeses

An overview of previous studies on flavor characteristics of some cheeses revealed the presence and crucial role of 3-methylbutanal for the unique flavor development in these cheese types (Table 2). The presence of Strecker aldehydes in hard cheddar cheese made from cow's pasteurized milk was reported to be responsible for a nutty/balanced flavor and was considered as desirable (Avsar et al., 2004, Hannon et al., 2006). However, on the contrary, Egyptian Ras and Manchego cheese usually made from either cow/buffalo/sheep's raw milk revealed an unclean/burnt flavor (Centeno et al., 2002, Ayad et al., 2004a) which was considered as non-desirable. This unclean/burnt flavor was attributed to the high levels of aldehydes and alcohols and was related to the poor quality milk used for cheese manufacture. The flavor perception and desirability of aldehydes in Parmigiano Reggiano, Parmesan and Roncal cheese made from either cow/sheep's raw and pasteurized milk were not clearly reported (Bosset and Gauch, 1993, Barbieri et al., 1994, Irigoyen et al., 2007).

The perceived chocolate-like aroma after six weeks of cheese ripening in semi-hard Proosdij-type cheese made from cow's pasteurized milk using a mesophilic strain *L. lactis* ssp. *lactis* B851 with acidifying mesophilic and an adjunct thermophilic culture was attributed to the presence of high concentration of 3-methylbutanal (Ayad et al., 2003). A similar chocolate-like flavor was perceived as well in Gouda/Proosdij type cheese and was considered as desirable (Engels et al., 1997, Van Leuven et al., 2008). During the study of aroma development in reduced-fat semi-hard cheese using culture adjunct of *Lactobacillus paracasei* (CHCC 4256), 4 times higher concentration of aldehydes and alcohols were determined as compared to controls but flavor perception/desirability was not clearly mentioned (Thage et al., 2005). Some wild strains of *L. lactis* isolated from ewes' raw milk cheeses were reported to produce high levels of aldehydes and alcohols, which were considered as responsible for abnormal odours (Morales et al., 2003). Aldehydes and alcohols were generally present and considered as potent odorants in soft cheeses. At low concentrations, they were perceived as a fruity flavor and considered desirable, while at high concentration, they resulted in rather off-flavor and highly non desirable (Sable and Cottenceau, 1999). The impact of various microorganisms on aromatic profiles of soft cheese has started to be elucidated (Bintsis and Robinson, 2004, Massouras et al., 2006, Irlinger et al., 2012). A richer pattern of aroma compounds namely aldehydes, alcohols and esters were achieved using *Lactobacillus paracasei* subsp. *paracasei* and *Debaryomyces hansenii* as adjuncts in the manufacture of Feta-type cheese (Bintsis and Robinson, 2004). During the study of aromatic profile of Teleme cheese made from either sheep's or goat's milk or a combination of both, Massouras *et al.*, (Massouras et al., 2006) found the highest level of volatile compounds in cheese made from sheep's milk.

1.2.4. Strategies for control of 3-methylbutanal concentration in cheese

The significance and impact of 3-methylbutanal in cheese has attracted 209 considerable attention for obtaining desired formation in specific cheese types. A number of strategies have been followed during these recent years to control the formation of 3-methylbutanal in cheese using cheese models at laboratory scale or *in vitro* investigations (Table 3). In this respect, a balanced flavor may be obtained by accelerating cheddar cheese ripening and proteolysis by the addition of free amino acids and enzyme modified cheese powder (EMC), however both strategies seemed to be expensive and not practical at industrial scale use (Fox and Wallace, 1997, Hannon et al., 2006). Most of the volatile compounds are generated through amino acid catabolism, and define final aroma characteristics of particular cheese. To obtain desired flavor formation, the use of particular isolates with either potential enzyme activities (Ayad et al., 2003, Thage et al., 2005, Whetstine et al., 2006, Garde et al., 2007, Sgarbi et al., 2013, Ruyssen et al., 2013, Pappa et al., 2013, Salmeron et al., 2014) or enzymes complementing metabolic pathways seemed to be the most promising approach (Ayad et al., 2001, Amarita et al., 2006, Pedersen et al., 2013). Another interesting strategy for the enhancement of cheese aroma and more particularly, the aldehydes, could be the use of bacteriocin producing strains to induce lysis of the bacteriocin sensitive adjunct cultures and as a consequence, to promote the release of intracellular enzymes and their accessibility towards corresponding substrates (Martinez-Cuesta et al., 2006, de Palencia et al., 2004, Martinez-Cuesta et al., 2002). Many studies have started to investigate the influence of various parameters like oxygen or redox potential on the flavor forming pathways, for the control of desired formation of aldehydes/alcohols in cheese (Kieronczyk et al., 2006, Deetae et al., 2011, Afzal et al., 2012, Caldeo and McSweeney, 2012, Afzal et al., 2013a). Indeed, the presence of oxygen/oxidizing agent was found responsible for the increased formation of 3-methylbutanal (Kieronczyk et al., 2006, Deetae et al., 2011). Recently, it has been demonstrated that the curd washing and addition of milk and cream during cheese manufacture could also significantly influence the sensory and volatile profile of cheese(Broadbent et al., 2013, Kaminarides et al., 2013, Afzal et al., 2013b, Hou et al., 2014). Until now, a chocolate-like flavor due to 3-methylbutanal could not be perceived in soft cheese; instead, main emphasis has been given to obtain a well-balanced flavor.

1.2.5. Conclusions

The branched chain aldehyde, 3-methylbutanal is associated as a key flavor compound to many hard/semi-hard cheese varieties, while, it is considered as potent aromatic compound in soft cheese. This flavor compound arises from leucine catabolism either by the direct pathway or indirect pathway or from both depending upon the functionality of these pathways in cheese related

microorganisms. In some of the hard/semi-hard cheese varieties, the presence and role of 3-methylbutanal is regarded as chocolate-like and highly desirable. Many strategies have been proposed for the control of this flavor compound. The combination of knowledge of flavor forming pathways, control strategies, final flavor perception could lead to a better control of this specific flavor formation before considering its use in industrial applications.

Table 2. Presence and role of 3-methylbutanal in different cheese types.

Cheese type	Cheese variety	Milk type	Raw/pasteurized	Bacteria/yeasts	Flavor/perception	Desirability of flavor	Literature
Hard	Cheddar	Cow	Pasteurized	*L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*	Dark chocolate/malty/nutty	D	(Avsar et al., 2004)
	Cheddar	Cow	Pasteurized	*L. lactis* ssp. *lactis* 303, *L. lactis* ssp. *cremoris* 227 ± EMC powder	balanced	D	(Hannon et al., 2006) 2006)
	Egyptian Ras	Cow/buffalo	Raw	Without addition of starter cultures	unclean	ND	(Ayad et al., 2004a)
	Manchego	Sheep/ewes	Raw	*Lactococcus lactis* (BCV^+, BCV^-, CSC)	Burnt/toasted/unclean/nuts	ND	(Centeno et al., 2002)
	Parmigiano Reggiano	cow	Raw	Without addition of starter cultures instead natural whey culture added	NR	NR	(Bosset and Gauch, 1993)
	Parmesan	cow	Raw	–	NR	NR	(Barbieri et al., 1994)
	Roncal	Sheep/ewes	Pasteurized	*Lactococcus lactis* (CSC), *Lactobacillus paracasei* (adjunct)	NR	NR	(Irigoyen et al., 2007)
Semi-hard	Proosdij type/gouda	Cow	Pasteurized	Mixed strain mesophilic starter culture Bos, Mixed strain thermophilic starter culture APS, *L. lactis* ssp. *lactis* B851	Chocolate-like	D	(Ayad et al., 2003)
	Proosdij type	Cow	Pasteurized	Mixed strain mesophilic starter culture Bos, Mixed strain thermophilic starter culture APS	Chocolate-like/nutty	D	(Engels et al., 1997)

BCV^+ branched chain volatile compounds producing strains; BCV^- branched chain volatile compounds not producing strains; CSC commercial starter cultures; D desirable; ND non desirable; NR not reported

Table 2. Presence and role of 3-methylbutanal in different cheese types (Continuation).

Cheese type	Cheese variety	Milk type	Raw/pasteurized	Bacteria/yeasts	Flavor/perception	Desirability of flavor	Literature
Semi-hard	Gouda-type	Cow	Raw/pasteurized	*L. lactis* ssp. *lactis* biovar *diacetylactis*, *L. lactis* ssp. *cremoris*	Chocolate-like/nutty	D	(Van Leuven et al., 2008)
	Ewes' raw milk	Sheep/ewes	Pasteurized	*Lactococcus lactis* (CSC and wild strains)	Roasted hazel/nuts	ND	(Morales et al., 2003)
	Reduced-fat round-eyed	Cow	Pasteurized	DL-starter (CH-N11, Chr. Hansen A/S), *Lactobacillus paracasei* ssp. *paracasei* CHCC 4256	NR	NR	(Thage et al., 2005)
	Feta-type	Sheep/ewes	Pasteurized	*Lactobacillus paracasei* ssp. *paracasei*, *Debaryomyces hansenii*	NR	NR	(Bintsis and Robinson, 2004)
Soft	Teleme	Sheep/ewes	Pasteurized	*L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*	NR	NR	(Massouras et al., 2006)
	Camembert -type cheese model	Cow	Pasteurized	*Proteus vulgaris* 1M10, *Debaryomyces hansenii* 304	Fruity	NR	(Deetae et al., 2009)
	Smear soft cheese model	Cow	Pasteurized	*L. lactis* ssp. *lactis*, model community (7 bacteria, 4 yeast), ± *Psychrobacter celer* 91, ± *Hafnia alvei* 2920	Fruity	NR	(Irlinger et al., 2012)

BCV^+ branched chain volatile compounds producing strains; BCV^- branched chain volatile compounds not producing strains; CSC commercial starter cultures; D desirable; ND non desirable; NR not reported

Table 3. Proposed strategies for control of 2, 3-methylbutanal concentration in cheese.

Proposed strategies	Cheese variety/model used	Type	Bacteria used	Resulted flavor	Literature
Addition of free amino acids at intermediate level	Cheddar	Hard	*L. lactis* ssp. *cremoris* 223	clean/mature	(Fox and Wallace, 1997)
Addition of enzyme-modified cheese powder for flavor/ripening acceleration	Cheddar	Hard	*L. lactis* ssp. *lactis* 303, *L. lactis* ssp. *cremoris* 227 + EMC powder	Balanced	(Hannon et al., 2006)
Addition of culture adjuncts	Cheddar	Hard	*L. lactis* 850, *L. lactis* ATCC 29146 (adjunct)	Nutty/chocolate-like	(Whetstine et al., 2006)
	Hispànico	Hard	*L. lactis* ssp. *lactis* INIA 639, *L. lactis* ssp. *lactis* INIA 437, *Lactobacillus helveticus* LH 92	Intensed	(Garde et al., 2007)
	Proosdij	Semi-hard	Mixed strain mesophilic starter culture Bos, Mixed strain thermophilic starter culture APS, *L. lactis* ssp. *lactis* B851 (adjunct)	Nutty/chocolate-like	(Ayad et al., 2003)
	Reduced-fat round-eyed	Semi-hard	DL-starter (CH-N11, Chr. Hansen A/S), *Lactobacillus paracasei* ssp. *paracasei* CHCC 4256 (adjunct)	Aromatic/sweet	(Thage et al., 2005)

Table 3. Proposed strategies for control of 2, 3-methylbutanal concentration in cheese (Continuation).

Proposed strategies	Cheese variety/model used	Type	Bacteria used	Resulted flavor	Literature
Strains possessing enzymes complementing metabolic pathways	Gouda/cheddar	Hard	*L. lactis* ssp. *cremoris* SK110, *L. lactis* ssp. *cremoris* NIZO B1157	Chocolate-like	(Ayad et al., 2001)
	Milk	-	*L. lactis* IFPL730, *L. lactis* IFPL326	Ripened cheese	(Amarita et al., 2006)
Bacteriocin induced lysis	*In vitro*	-	*L. lactis* ssp. *lactis* IFLP359, *L. lactis* IFLP105 (lacticin 3147 producer)	Intensed	(Martinez-Cuesta et al., 2002)
	In vitro	-	*L. lactis* ssp. *lactis* IFLP359, *L. lactis* IFLP730, *L. lactis* IFLP105 (lacticin 3147 producer)	Intensed	(Martinez-Cuesta et al., 2006)
	Cheese model	-	*L. lactis* ssp. *lactis* IFLP3593, *L. lactis* IFLP730	Intensed	(de la Plaza et al., 2004)
Environmental modifications by static and shaking conditions	*In vitro*	-	*Proteus vulgaris* 1M10	Off-flavor	(Deetae et al., 2011)
Oxidizing or reducing agents	*In vitro*	-	*L. lactis* ssp. *cremoris* NCDO 763, *L. lactis* ssp. *lactis* NCDO 1867	Off-flavor	(Kieronczyk et al., 2006)

2. L’encapsulation des composés volatils

2.1. Introduction

Après avoir étudié le composé aromatique d’intérêt et ses voies de production chez les bactéries, nous allons nous intéresser à la façon dont ce composé va être ajouté au fromage Poro.

En raison de la nature traditionnelle du produit, un critère important à considérer lors de l’incorporation de nouvelles molécules olfactives dans le produit est la modification de la technologie de production du fromage. En effet, cela peut influencer les caractéristiques responsables de sa typicité.

Par chance, le fromage Poro est enrobé dans la paraffine, ce qui nous permet d’envisager l’incorporation de la molécule olfactive dans le matériel d’enrobage plutôt que dans le fromage.

Nous avons choisi l'encapsulation comme stratégie pour l'intégration du 3-méthylbutanal dans la paraffine et, sachant que cet ajout pourrait être direct ou par l'intermédiaire de C. maltaromaticum, nous nous sommes intéressés à identifier les différentes techniques et matériaux d'encapsulation. La connaissance de ces techniques va nous permettre d’effectuer un choix judicieux des matériaux et de la technique d’encapsulation qui permettraient également une libération ultérieure des notes aromatiques de 3- méthylbutanal.

Dans cette logique, l'objectif de la deuxième et dernière partie de cette bibliographie est de présenter un aperçu des matériaux et des technologies utilisés pour l’encapsulation des arômes alimentaires ainsi que des applications récentes.

2.2. Aromas encapsulation

2.2.1. Introduction

Aromas are complex mixtures of volatile substances and labile components that sensory perception can be changed as a result of oxidation, chemical interactions, or volatilization (Botrel et al., 2012). They are homogeneous organic compounds with a defined chemical formula, a molecular weight of 50 – 300 Da and a high volatility with a boiling point of 20 – 250°C (Uhlemann and Reiß, 2009). Aromas are mostly lipophilic molecules, but some are hydrophilic (Zuidam and Heinrich, 2010). Chemically unstable in presence of air, light, moisture as well as heat (Voilley and Etievant, 2006), they can be classified as hydrocarbons, alcohols, aldehydes, ketones, esters, acids, sulphides,… They are chemical sensations elicited by a vast number of molecules released by food (Voilley and Etievant, 2006) and can also be referred as flavors (Zuidam and Heinrich, 2010).

Aroma plays an important role in consumer satisfaction and influences furthest consumption of foods. Flavor stability in different foods has been of increasing interest because of its relationship with the quality and acceptability of foods, but it is difficult to control it. To limit aroma degradation or loss during processing and storage, it is beneficial to encapsulate volatile ingredients prior to use. Encapsulation offers an effective approach to cover an active compound with a protective wall material and to impart some degree of protection against evaporation, chemical reactions (such as flavor-flavor interactions, light-induced reactions, and oxidation) or migration (Milanovic et al., 2010).

The development of microencapsulation products started in 1950s in the research of pressure-sensitive coatings for the manufacture of carbonless copying paper (Green and Scheicher, 1955). Encapsulation technology is now well developed and accepted within the pharmaceutical, chemical, cosmetic, foods and printing industries (Augustin et al., 2001, Heinzen, 2002). In food products, fats and oils, aroma compounds and oleoresins, vitamins, minerals, colorants, and enzymes have been encapsulated (Dziezak, 1988, Jackson and Lee, 1991, Shahidi and Han, 1993).

Incorporation of small amounts of aroma into foods can greatly influence finished product quality, cost, and consumer satisfaction. The food industry is continuously developing ingredients, processing methods, and packaging materials to improve aroma preservation and delivery (Zeller and Saleeb, 1996). The stability of the matrices is an important condition to preserve the properties of the flavor materials. Many factors such as the kind of wall material (Imagi et al., 1992), ratio of the core material/wall material (Minemoto et al., 1999), encapsulation method (Minemoto et al., 1997), and storage conditions (Minemoto et al., 1997, Yoshii et al., 1997) affect the stability of encapsulated flavor.

Aroma retention and release from food matrix occurs before and after eating. It depends on thermodynamic and kinetic parameters, aroma's physico-chemical properties as well as food ingredients (Zuidam and Heinrich, 2010, Madene et al., 2006).

Aroma losses can be related to the transfer of molecules. Aroma compounds transfer depends on both sorption and diffusion. The sorption mechanism consists of adsorption, absorption and/or desorption of penetrate molecules and depends on the polymer-volatile compound affinity, whereas diffusion is related to their mobility within the polymeric network of the matrix (Marcuzzo et al., 2010). In addition, physicochemical characteristics of volatile compounds influence polymer's permeability. Aroma compounds' shape and size affect its diffusivity; solubility being influenced by compounds nature, polarity, and ability to condense (Reineccius, 2009).

Different techniques and materials have been used to encapsulate aroma compounds. In this review, an overview of the most used aroma encapsulation technologies and materials for food industry as well as some recent applications of aroma encapsulation are described.

2.2.2. Principal technologies used to aroma encapsulation

There is a large number of techniques available for food additives encapsulation (Nedovic et al., 2011), flavor encapsulation being one of the main used of those technologies (Jafari et al., 2008). There are several models that have been proposed to classify encapsulation techniques. Poncelet and Dreffier classify these technologies as a function of the encapsulation phase and explain that encapsulation is usually performed in three phases: 1) an active principle incorporation to the core material (solution, emulsion, suspension, etc), followed by 2) a mechanical treatment (prilling, atomization, emulsification, etc.), and 3) a stabilization (gelificatin, solidification, evaporation) (Poncelet and Dreffier, 2007). From their side, Jafari et al. (2008), classify the food ingredients encapsulation methods in physical (spray drying, extrusion, co-crystallization, etc.), chemical (molecular inclusion, interfacial polymerization, etc.) and/or physicochemical (coacervation, organic phase separation, liposome entrapment…). Concerning flavors encapsulation Madene et al. suggest two classification groups according to the encapsulation process, chemical (coacervation, co-crystallization, molecular inclusion …) and mechanical (spray-dying, spray chilling, extrusion …) (Madene et al., 2006).

In this review, we do not try to classify encapsulation techniques and materials but we summarize the most frequent food aroma encapsulation devices, their principle and some concrete application examples.

2.2.2.1. Spray drying

Frequently considered as a dehydration process, spray drying is the most common technology used for food aroma encapsulation. This technique allows encapsulating aroma as a powder in a short time. Moreover, it is characterized by its low-cost and accessible equipments (Gharsallaoui et al., 2007, Jafari et al., 2008, Desai and Park, 2005).

Spray drying process (Figure 2) consists in preparation of active principle dispersion in a carrier material, dispersion homogenization, and their subsequent atomization in a hot gas to promote dehydration and thus instantaneously obtain a powder (Desai & Park, 2005; Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007) which size is less than 40µM (Zuidam and Heinrich, 2010). The fine powders agglomeration by process such as fluidized bed could be desirable to improve powder flowability and wettability (Jafari et al., 2008).

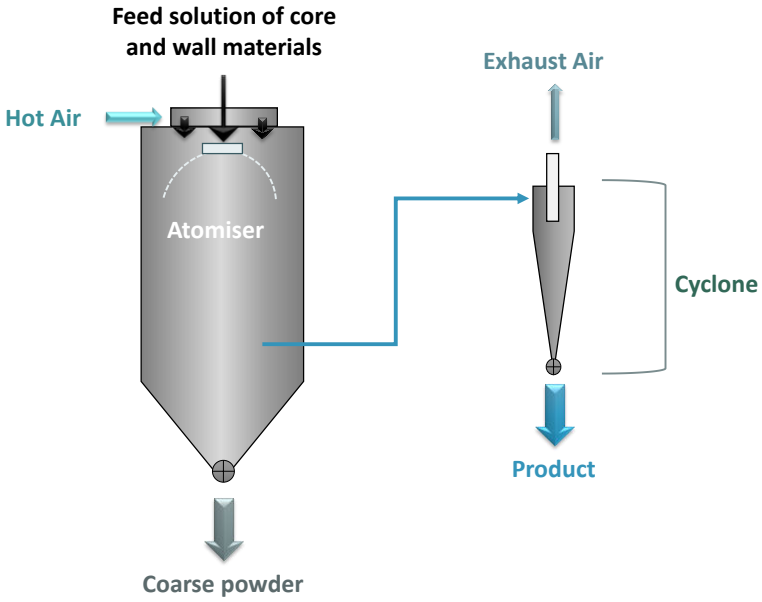


Figure 2. Spray drying encapsulation schematic process (Fang and Bhandari, 2012).

For Reineccius (Reineccius, 2009), a good aroma retention, the stability of the finish product and the possibility of a large scale production are the principal advantages of spray draying encapsulation. (Fernandes et al., 2013) obtained a retention varying from 7.15 % to 47.57 % for rosemary essential oil encapsulated in Gum Arabic. (Belingheri et al., 2013) reported values ranking from 63.42 % to 90.41 % for diacetyl incorporation into porous starch regarding the used solvent. Others authors highlight the economy and flexibility of the process, the availability of the equipment, and the constant quality of the final powders (Zuidam and Heinrich, 2010, Jafari et al., 2008).

As expected spray drying encapsulation have also limitations, the major being materials availability, since must be food grade probed (Dasai & Park et al., 2005). Other disadvantages include the food flavor high volatility and exigent sensibility that may result in losses and changes during the process. Other details such as powders particle size, water solubility and release which might be desirable or not depending on the capsule final purpose should also be considered (Zuidam and Heinrich, 2010, Jafari et al., 2008).

The spray draying process is considered as an art because of the difficulty to control the process, involving factors optimization to obtain a good encapsulation efficiency and quality capsules as well as the complexity of the phenomena (mass and heat transfer) occurring during the process (Jafari et al., 2008). The main process factors that must be considered are: feed temperature, air inlet temperature, air outlet temperature, infeed temperature, atomization type and conditions, drying air flow rate and humidity, and powder particles size (Jafari et al., 2008).

Some recent examples of volatile aroma encapsulation by spray-drying are: Wasabi flavor, menthol and coffee oil.

Encapsulated Wasabi flavor is used in canned tuna spread. Wasabi flavor major volatile component (90%) is 2-propenyl isothiocyanate. Different wasabi concentrations were capsuled in modified starch, maltodextin and the combinations of both, using a spray dryer equipped with a two-fluid nozzle atomizer (0.5 mm diameter, 40 ± 3 $mL \cdot min^{-1}$ flow rate), with a 190±10°C and 100±10°C inlet and outlet air temperature respectively and 4±0.08 MPa nozzle air pressure. It was found that adding 9% (w/w) of wasabi-modified starch-maltodextrine capsules produce the highest amount of retained wasabi flavor (Ratanasiriwat et al., 2013).

Menthol is a cyclic terpene alcohol found in high concentrations in peppermint and corn mint oils extracted from herb plants like *Mentha arvensis* and, commonly used in the food industry. Spray drying encapsulation was studied by Soottitantawat et al. (Soottitantawat et al., 2005b) and Sarkar et al. (Sarkar et al., 2012) using different wall materials including: Gum Arabic (GA), modified starch (MS), guar gum hydrolyze, n-octenyl succinic anhydride (OSA) and some blends of these. L-mentol emulsions were fed through a spray-dryer, equipped with a centrifugal atomizer (180±5°C and 100±5°C inlet and outlet air temperature respectively, 100 $kg \cdot h^{-1}$ airflow rate at outlet temperature and, 30000 rpm rotation speed of atomizer). Mint oil emulsions were spray-dried in a mini spray dryer (0.5 mm nozzle diameter, 2 bar pressure of compressed air, 160°C inlet temperature and 95±2°C outlet temperature). Microcapsules were successfully produced in all cases, however, high surface L-menthol contents were observed when using MS, therefore GA seems to be better to encapsulate L-menthol even if a low retention was reported. Concerning mint oil, GA-OSA blends were demonstrated to be superior to GA as wall material.

Another example of aroma encapsulation for the food industry is coffee oil encapsulation. Coffee is one of the most traded commodities in the world; therefore, there is a great interest to use coffee oil to increase the aromatic potential of soluble coffee, coffee beverages, candies, etc. As we have already said, encapsulation is a good alternative to protect against oxidation and to control flavors release, hence the interest of its use in coffee industry. A coffee oil/gum Arabic emulsion was spray-dried using a laboratory scale spray-dryer equipped with a dual fluid nozzle of 1.2 mm diameter, 36 $m^3 \cdot h^{-1}$ air flow rate, 2.4 $m^3 \cdot h^{-1}$ compressed air flow rate and, 0.8 $L \cdot h^{-1}$ feed flow rate. The microencapsulation process was optimized at 30 % of total solids, 15 % of oil with respect to total solids and inlet air temperature of 170°C (Frascareli et al., 2012).

As the examples above, there are many other aromatic substances which have been encapsulated to use as a food additive, among these: Rosemary essential oil (de Barros et al., 2013, Janiszewska and Witrowa-Rajchert, 2009), oregano essential oil (Botrel et al., 2012), lime essential oil (Bringas-Lantigua et al., 2012), allyl isothiocynate (Ko et al., 2012), mandarin oil (Bringas-Lantigua et al., 2011), rice flavor, sumac (Bayram et al., 2008), cumin (Kanakdande et al., 2007) and d-limonene (Jafari et al., 2007).

2.2.2.2. Spray cooling and Spray chilling

Spray-chilling and spray-cooling are two other important techniques widely used for aroma encapsulation similar to spray-drying (Zuidam and Shimoni, 2010). In spray chilling and spray cooling (Figure 3), flavors are mixed with the wall material and then atomized into a cooled or chilled air occasioning wall solidification (Desai and Park, 2005, Madene et al., 2006), to obtain a dispersion of liquid aroma in fat crystals. Once the lipid wall material reaches its melting point the flavor is then released (Zuidam and Shimoni, 2010).

Contrary to spray drying, those two methods do not involve mass transfer processes because water is not evaporated. The main difference between them is the wall material melting point. Common used materials include fat and methyl esters with melting points of 45-122°C as well as hard mono- an di-acylglycerols with melting points of 45-65°C for spray-cooling, and fractionated or hydrogenated vegetal oils with melting points ranging from 32 to 42°C for spray-chilling (Zuidam and Heinrich, 2010, Nedovic et al., 2011, Desai and Park, 2005).

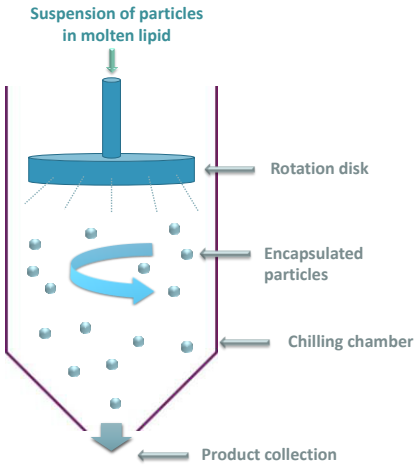


Figure 3. Spray chilling encapsulation schematic process (Fang and Bhandari, 2012)

Concerning the strengths and weaknesses of those methods: these are the least expensive encapsulation processes, recurred in aroma encapsulation to improve heat stability, control release and convert a liquid aroma into solid powders (Gouin, 2004). Those techniques are customizable in both batches and continuous processing modes (Nedovic et al., 2011). On the other hand, produced microcapsules are water insoluble (Nedovic et al., 2011) and lipophilic aromas diffuse easily through fat crystals making these capsules unfit for long shelf life applications (Zuidam and Heinrich, 2010).

Due to this barrier problem, other lyophobic materials are being studied. Anhydrous erythriol, a lineal sugar alcohol with a melting point of 121°C and a thermal and chemical stability, has been used to flavor encapsulation. Factors such as degree of aroma retention as well as thermal and moisture sorption properties of the obtained capsules were described (Sillick and Gregson, 2013). Those capsules were well tested to encapsulate cinnamic aldehyde and limonene for chewing gum applications (Gregson and Sillick, 2011).

2.2.2.3. Extrusion

This method in combination with the good material, is known for offering the advantage of stabilize aroma against oxidation and has been used to encapsulate volatile and unstable compounds (Manojlovic et al., 2008). As spray-drying, extrusion consists on the physical entrapment of an active ingredient in a solid matrix to reduce their mobility (Zeller and Saleeb, 1996). Among extrusion techniques we can find, melt extrusion, dropping and co-extrusion (Zuidam and Heinrich, 2010).

Melt extrusion (Figure 4a) is the third major encapsulation process in aroma industry (Porzio, 2012). It consists to melt the wall material, incorporate the active ingredient, extrude the blend and cool the extrudate. Aromas can be encapsulated in carbohydrate melt by using an extruder with one or

more screws in a continuous process, resulting extrudate could be broken into small pieces. Extruded encapsulated aromas are used as ingredients in food industry (desserts, cakes, biscuits, tea bags, etc.).

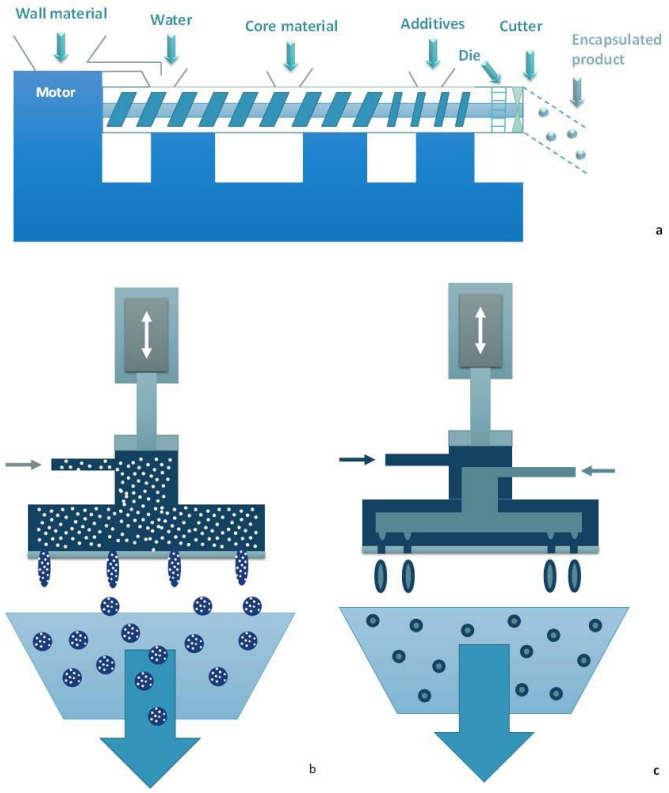


Figure 4. Melt extrusion (a) (Fang and Bhandari, 2012, Uhlemann and Reiß, 2009), simple extrusion or dropping (b) and, co-extrusion (c) (Uhlemann and Reiß, 2009) schematic diagrams.

To prepare microspheres by dropping (Figure 4b), the active ingredient is dissolved in shell material (alginate, k-carrageenan, agarose, gelatin, gellan gum, agar, etc.) and then dropped into a gelling bath (Zuidam and Heinrich, 2010). The primary interest of this technique is the formation of small droplets uniform in size. Different methods to decrease size particles and obtain good flow rates exist, as the application of an electrostatic potential, a vibration frequency or a jet cuter (Poncelet and Dreffier, 2007, Rathore et al., 2013).

Contrary to dropping and melt extrusion, which are matrix-type methods, co-extrusion (Figure 4c) is a dropping variation that can be used to prepare capsules with a core of aroma and a shell produced by interfacial gelling like in dropping (Zuidam and Heinrich, 2010). The technique uses two coaxial nozzles. Core material is extruded from the inner nozzle and the shell material from the outer nozzle, then extrude are dropped into a gelling bath. Above-mentioned size reduction techniques are also applicables (Piazza and Roversi, 2011, Poncelet and Dreffier, 2007).

Dropping majors advantages are the simplicity and economy of the method, and its main weakness are the difficult scaling up of the process (de Vos et al., 2010, Rathore et al., 2013). Melt extrusion products have the advantage to supply larger flavor particles for visual impact in products without losing solubility and control-release. In this process high internal temperatures developed by mechanical friction, result in high internal pressures and exit temperatures cause disadvantages such as loss of moisture and flavor (Porzio, 2004). Concerning co-extrusion advantages and weaknesses, the capsules have an aroma retention performance similar to those made by complex coacervation and dropping, nevertheless the thinness of their shell and their large size made them more sensitive to shear. Moreover, for food industry co-extrusion is an expensive process (Zuidam and Heinrich, 2010).

The application of an electrostatic potential to the dropping alginate-ethyl vanillin beads was studied by Manojlovic et al. (Manojlovic et al., 2008). Ethyl vanillin was entrapped in a 20 $g \cdot L^{-1}$ alginate dispersion, and dropped into a 15 $g \cdot L^{-1}$ calcium chloride solution. The used electrostatic potential was 4.5 kV, distance between needle and gelling solution was 2.5 cm and flow rate 25.2 $cm^3 \cdot h^{-1}$. Obtained microbeads were spherical and with 450±20 µM of diameter and approximately 10 % w/w of final ethyl vanillin. Other food additives such as thym (*Thymus serpyllum L.*) a popular herb and volatile oil, were also encapsulated by electrostatic extrusion in a calcium alginate hydrogel matrix, even if the efficiency and stability of encapsulated compound is good, process modifications are necessaries for large-scale production (Stojanovic et al., 2012).

Octenyl succinic acid anhydride modified starches (OSA starches) are extensively used as carried materials for spray drying and melt extrusion in the food industry. The potential of these for melt extrusion encapsulation of flavors was studied by Zasypkin & Porzio, (Zasypkin and Porzio, 2004). A co-rotating twin-screw extruder equipped with a die containing multiple (0.79 mm) openings was employed to encapsulate butter and lemonade flavors. The extrusion processing parameters were optimized to yield a minimum setting time of the material into the glassy state while ensuring no significant aroma loss. Process parameters were: a steady-state pressure in the die (not exceeding 70 $kg \cdot cm^{-2}$), a product temperature not exceeding 113°C and finally a setting time of the product in a cold airflow of 13°C) no exceeding 4s.

2.2.2.4. Yeast encapsulation

Compared to other techniques, this is a very special method as it uses microorganism cells as a wall material (Fang and Bhandari, 2012). Yeast eukaryotic structure made it a good encapsulating wall material, its natural properties confers benefits over other microencapsulation technology (Shi et al., 2008). *Saccharaomyces cerevisiae* yeast cells have extensively been used since years in food industry

(bread, beer, wine). They are food-grade and low cost food additives (Paramera et al., 2011). Their phospholipid membranes can behave as liposomes and have been used for encapsulation of both hydrophobic and hydrophilic molecules (Shi et al., 2008) (Figure 5).

Yeast encapsulation of essential oils and other aroma compounds are a low cost, high volume technique. In this process, an aqueous suspension of yeasts is mixed with the essential oil under a determined temperature and time. A passive dilution of essential oils across the cells wall takes place and high concentrations of essential oils can be sequestered (Fang and Bhandari, 2012).

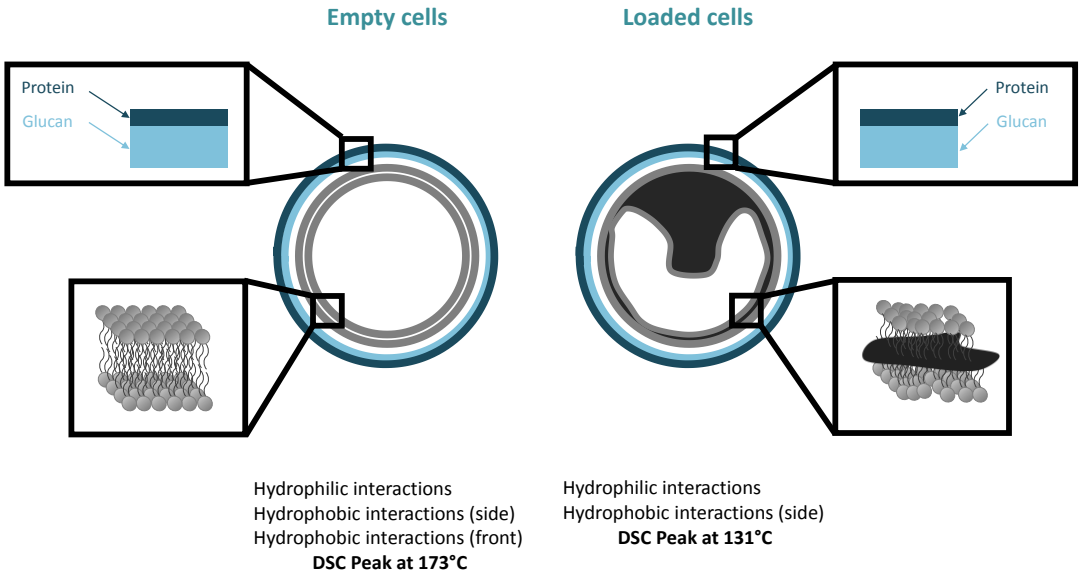


Figure 5. Yeast encapsulation schematic representation (Normant et al. 2005)

The first encapsulation procedure suggested by Shank (1977) was coating dyes by emptying yeast cells for the use in carbonless carbon paper and also for numerous fat-soluble substances like drugs condiments, flavors, fragrances, chemicals, vitamins and adhesives (Pham-Hoang et al., 2013). Since then yeast encapsulation has been frequently contested; Normand et al. (Normand et al., 2005) encapsulated Limonene in *S. cerevisiae* to study their release mechanism by microscopic observation and the physical properties of the empty or loaded yeast. They found that the limonene localized inside the yeast phospholipid bilayer could be released by two possible ways: a thermal treatment exceeding 260°C and a water activity superior to 0.7. They conclude that the exceptional performance of this encapsulation method lies in the fact that in many food products aroma must resist the process temperature and be released when hydration occurs in the mouth.

The flavor retention and release performance of yeast were also investigated by Dardelle et al. (Dardelle et al., 2007). They confirmed once again the role of water and temperature in the diffusion

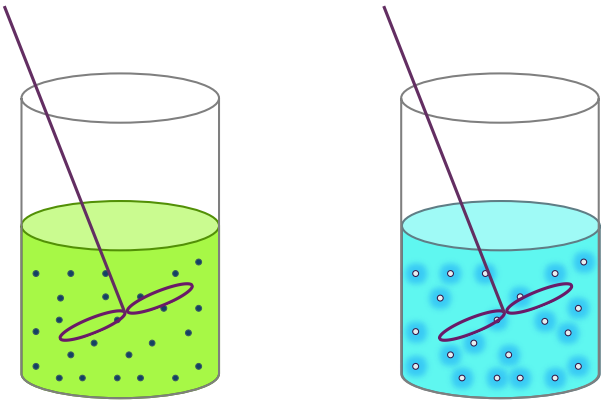
of the encapsulated aroma, as well as the importance of the aroma hydrophobicity. Furthermore, they conducted organoleptic tests using untrained consumers, showing higher scores for yeast encapsulated aroma when compared with conventional spray dried aroma powders.

Other examples of yeast encapsulation applications are: menthol encapsulation, used in chewing gums to increase persistence in mouth (Sasaki et al., 2003b, Sasaki et al., 2003a) and, beef aroma for French fries and crackers to make aroma resistant to thermic process and enhance long-lasting effect (Benczedi et al., 2006).

2.2.2.5. Emulsification

Emulsification is the process of dispersing one liquid in a second immiscible liquid, if active component is included in the first liquid, emulsification can be used as an encapsulation technique (de Vos et al., 2010). Emulsions are usually produced under high shear with or without the aid of an emulsifying agent (Zuidam and Shimoni, 2010) permitting the formation of small spherical droplets that can be used directly in the liquid state, in form of dried powders (Fang and Bhandari, 2012) or of gel (Augustin and Hemar, 2009).

Emulsions can be simple emulsion (Figure 6a) such as, oil-in-water (O/W) or water-in-oil (W/O), or multiple emulsions (Figure 6b) (e.g. O/W/O or W/O/W). The advantages of simple emulsions are their ease preparation and low cost. However, they have certain disadvantages like physical instability, limited controlled release and a limited number of emulsifiers (Fang and Bhandari, 2012). More sophisticated emulsion systems (multiple emulsions) were created to improve the physical stability of emulsions by enhancing the robustness of the interface, to protect sensitive agents against degradation and to better control core release (Augustin and Hemar, 2009).



a b

Figure 6. Simple O/W emulsion (a) and multiple W/O/W emulsion systems.

Active agents such as aroma might be encapsulated in O/W emulsions and are dried to provide a powder to be incorporated in beverages or other food matrix (Zuidam and Shimoni, 2010).

For this reason emulsion technique is considered most likely a part of the encapsulation process (e. g. spray-drying, freeze drying and extrusion) (Fang and Bhandari, 2012).

Marcuzzo et al. (Marcuzzo et al., 2010) used emulsification to encapsulate different aroma compounds (methyl-ketones, ethyl-esters and alcohols) in i-carrageenan films and compare their aroma release with that of a classic lipid matrix. Based on the obtained results, they hypothesize that, in lipid matrix, aroma release is affected by diffusivity, while in carrageenan emulsified films release is influenced by the affinities between volatile compounds and polymers.

2.2.2.6. Melt dispersion

This is another emulsion type also called solid lipid emulsion, consisting in the emulsification of molten fad or wax at a temperature above their melting point followed by cooling during mixing to solidify the fad or wax containing the active core (Augustin and Hemar, 2009, Zuidam and Shimoni, 2010).

Active component loading and stability depends on the type of lipid under, their melting point and the type of crystal network formed on cooling (Augustin and Hemar, 2009). This is a less expensive and simple technique, that in combination with others encapsulation process is relatively easy to scale-up, an important criteria that makes it interesting for the food industry (Milanovic et al., 2010).

Milanovic et al. (Milanovic et al., 2010) and Stojakovic et al. (Stojanovic et al., 2012) encapsulated aroma in carnauba wax. Milanovic et al. (Milanovic et al., 2010) objectives were to develop formulations to produce ethyl vanillin wax microcapsules and to study their size, surface morphology, aroma loading and degradation. They obtain spherical micro beads in the range of 210-360 µm, with encapsulation efficiency of 87 %, and found that their decomposition thermal process occurs at around 200°C, while matrix degradation starts at 250°C, indicating therefore that carnauba wax is an attractive material to be used as a flavor carried. From their side, Stojakovic et al. (Stojanovic et al., 2012) wereinterested in finding a mathematical model to describe the thermal release of ethyl vanillin carnauba wax microcapsules, confirming that release is not a single-step reaction but a complex kinetic process.

2.2.2.7. Freeze-drying

Also known as lyophilisation or cryodesiccation, freeze-dying is a process frequently used for the dehydration of heat-sensitive materials and aromas (Fang and Bhandari, 2012). The active ingredient

is dissolved in water and frozen at temperatures between -90 and -40 °C then; low pressure and temperature are used to sublimate water. After drying, the brittle cake can be broken into smaller pieces (Zuidam and Shimoni, 2010, Fang and Bhandari, 2012).

The key advantage of freeze-drying is that is a simple and easy process; some sensitive active compounds can need a cryprotectant agent. Other disadvantage are a high energy consummation, a long process time and a high cost (Zuidam and Shimoni, 2010, Fang and Bhandari, 2012).

This technique has been used to encapsulate water-soluble essences and natural aromas. Kaushik and Roos (2007) used different matrices containing gum arabic, sucrose and gelatin to encapsulate limonene by freeze-drying; limonene retention was determined by spectrophotometry. Results suggest that gum arabic/sucrose/gelatin (1:1:1) mixture could be used for limonene encapsulation in freeze-drying, obtaining high amount of limonene retention by homogenizing the emulsion at a single stage pressure of 100 MPa.

Kaasgaard and Keller (Kaasgaard and Keller, 2010) demonstrated that a combination of a charged small-molecule emulsifier and an oppositely charged polysaccharide adsorbed to the emulsion droplet surface can substitute the commonly used encapsulation materials for flavor oil emulsions drying. Chen et al. (Chen et al., 2013) compared the properties and stability of fish oil co-encapsulated with phytosterol ester and limonene microcapsules, produced by spray-drying and freeze-drying. The addition of limonene can mask the fish aroma in the microcapsules but in this case freeze-drying did not produce powders with better characteristics than spray-drying ones.

2.2.2.8. Coacervation

Coacervation, often considered as the original encapsulation method (Madene et al., 2006), is a modified emulsification encapsulation technology (de Vos et al., 2010). It consists in a separation phase of one or more hydrocolloids from the initial solution and the successive deposition of the recently formed coacervate phase around the active ingredient emulsified in the same reaction media. Then, if it is necessary, hydrocolloid shell can be crosslinked using an appropriate chemical or enzymatic crosslinking agent (Gouin, 2004).

Generally, the core material used in the coacervation must be compatible with the recipient polymer, and insoluble in the coacervation medium (Madene et al., 2006). Coacervation is classified as simple or complex (Figure 7). Simple coacervation involves only one type of polymer while complex regards two or more types of polymers of opposite charges (Fang and Bhandari, 2012).

In food industry, coacervation is mostly complex (Fang and Bhandari, 2012). The most studied coaservation is the gelatin/gum arabic system (Gouin, 2004) and can be cosslinked with glutaraldehyde or transglutaminase (Zuidam and Heinrich, 2010). However, there are many others

systems including heparin/gelatin, gelatin/carboximethylcelulose, β-lactoglobulin/gum Arabic and guar gum/dextran (Fang and Bhandari, 2012).

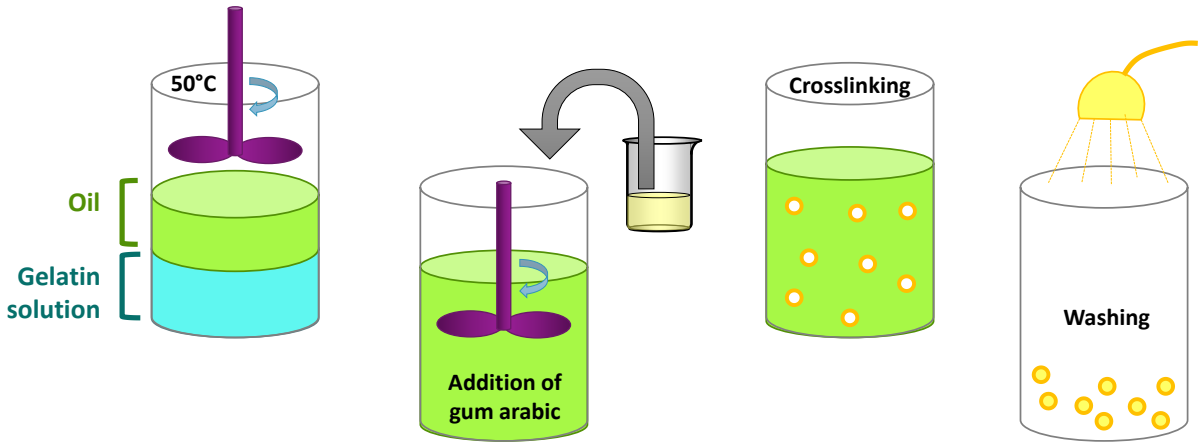


Figure 7. Schematic diagram of encapsulation complex coacervation.

Coacervation is frequently used to encapsulate water-insoluble liquids such as oil and aroma (Fang and Bhandari, 2012). Numerous advantages and properties of coacervation compared to other encapsulation process have been reported, nevertheless, it presents two main disadvantages for food industry: expensive cost and the frequent use of glutaraldehyde, not allowed in Europe for food applications (Gouin, 2004, Zuidam and Heinrich, 2010).

For aroma encapsulation, aroma should be present in the mixture as coacervation proceeds, and the coacervate nuclei are adsorbed onto the surface of the volatile compounds. Aroma may also be added during or after separation phase. Indifferently, the coacervation mixture must be continually stirred and the addition of a droplet stabilizer may be necessary (Madene et al., 2006).

Sweet orange oil was microencapsulated by complex coacervation of soybean protein isolated (SPI) and gum Arabic (GA). It was found that the best pH for SPI/GA coacervation is 4.0 with a 1:1 ratio between SPI/GA for the highest microencapsulation efficiency. SPI was quite compatible with GA for complex coacervation, coacervates shows a good flavor retention, L-limonene, principal volatile component of sweet orange oil, was not lost during the process (Xiao et al., 2011).

Another example is the formation of heat-resistant aroma nanocapsules by gelatin/gum Arabic complex coaservation, studied by Lv et al. (Lv et al., 2014). Jasmine essential oil was used as aroma compound, pH 4.8 and a mixing ratio 1:1 gelatin/gum Arabic was favorable for nanoparticles that were cross-linked by transglutaminase. Resultant nanocapsules resist a water bath of 80°C for 7h; aroma begins to decrease after 5h.

2.2.2.9. Co-crystallization

Also called co-precipitation this less recurred technique offers an economical and flexible alternative since the procedure is relatively simple (Jackson and Lee, 1991, Chen, 1994). In co-crystallization, active ingredient is incorporated in supersaturated sucrose syrup at a temperature above 120°C and low moisture, to attain simultaneous crystallization (Bhandari et al., 1998, Madene et al., 2006, Sardar and Singhal, 2013) (Figure 8). The crystal structure of sucrose is modified from a perfect agglomerate to aggregates of very small crystals providing a porous matrix where aroma are included or entrapped (Chen, 1994, Madene et al., 2006, Sardar and Singhal, 2013).

Co-crystallization improves solubility, wettability, homogeneity, anti-caking and stability of encapsulated molecules; granular product has a low hygroscopicity, good flowability, and dispersion properties (Quellet et al., 2001). Nevertheless, heat sensitive compounds may be degraded during the process (Bhandari et al., 1998).

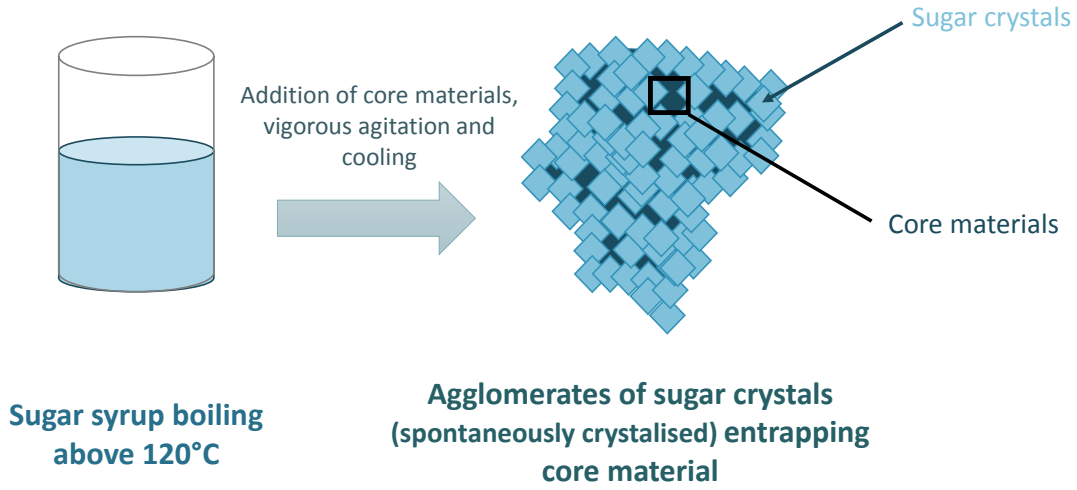


Figure 8. Schematic diagram of a co-crystallisation process (Fang and Bhandari, 2012)

Encapsulation by co-crystallization has been reported for diverse flavor compounds such as Jamaica granules (Bhandari et al., 1998), orange peel oil (Beristain et al., 1996), and more recently cardamom oleoresin (Sardar and Singhal, 2013). This was emulsified with gum arabic and co-crystallized to prepare flavored sucrose cubes. These latters showed lower hydroscopicity at 93% relative humidity, a longer dissolution time, and a decreased cristallinity as compared to crystal sucrose, with an encapsulation efficiency of 35.23 % of 1,8-cineole and 67.18 % α-terpinyl, the active compounds of cardamom oleoresin.

2.2.2.10. Molecular inclusion or complexation

Molecular inclusion is an encapsulation process that takes place at a molecular level using typically cyclodextrines (CDs) as wall materials (Fang and Bhandari, 2012, Desai and Park, 2005). CDs are cyclic oligosaccharides 6-8 glucose molecules, made by the enzymatic action of cyclodextrin glucosyltransferase upon starch (Zuidam and Heinrich, 2010). The external part of the CDs molecule is hydrophilic, while the internal part is hydrophobic permitting the inclusion of aroma molecules (Desai and Park, 2005) (Figure 9).

Desai and Park (Desai and Park, 2005) and Astray et al. (Astray et al., 2009), mention that molecular inclusion of aroma in β-cyclodextrin molecules (the commonly used cyclodextrin) is the most effective microencapsulation technique for protecting aromas. The encapsulation efficacy of CDs inclusion is affected by the core materials; the higher the hydrophobicity and the smaller the encapsulated molecule is, the greater the affinity for the CDs. In addition, other factors such as temperature, time, and water amount control the loading rate and efficiency (Fang and Bhandari, 2012). The principal limitations of this method are the use of β-cyclodextrin for food applications which is subjected to regulatory requirements in several countries (Desai and Park, 2005) and the fact that cyclodextrins are relatively expensive (Madene et al., 2006).

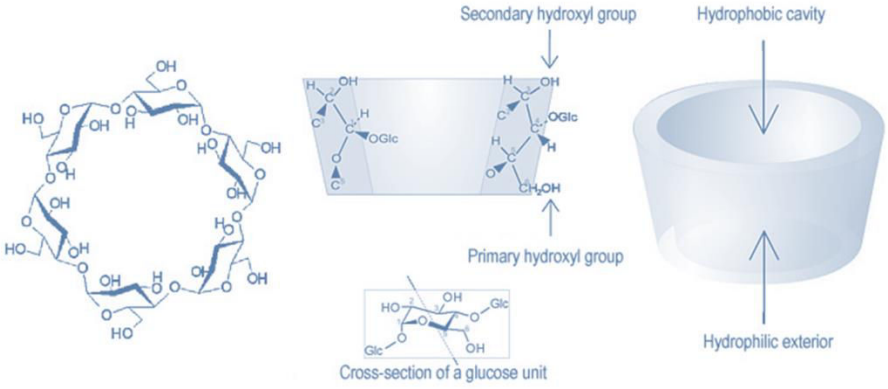


Figure 9. Structure of β-cyclodextrin molecule.

Some aroma used in food industry has been encapsulated within cyclodextrins (Reineccius and Risch, 1986, Loftsson and Kristmundsdottir, 1993, Reineccius et al., 2002). In 2005, Reineccius et al., test the capacity of three common aroma solvents to interfere with the ability of certain CDs to form molecular inclusion complexes with aroma. They found that the smallest and polarest solvent molecule had the least effect on complex formation, in disparity; the intermediate size had the greatest effect.

The complexation efficiency of 6 cyclodextrins for aroma encapsulation was also studied by Ciobanu et al. (Ciobanu et al., 2013) by headspace gas chromatography. Concluding that the static headspace gas chromatography is a successful tool to study the interaction among the cyclodextrins and the 13 tested aroma compounds. They also make emphasis on the importance of determining the stability constant of inclusion complex to take advantage of the complexation potential of CDs.

Less often used than cyclodextrins, starch is also frequently used for molecular inclusion. Ades et al. (Ades et al., 2012) search for the best inclusion mode for the oral release of aroma substances. Therefore, menthone, menthol and limonene were used as model aroma for complexation with starches with different amylose content. They found that complexation increases with amylose content in the starch and aromas were released under simulated saliva conditions indicating that the complex are an efficient platform to control aroma release in the oral cavity.

2.2.3. Common materials used to encapsulate volatile flavors

Lot of substances can be used to encapsulate, entrap or coat active products depending on the encapsulation objectives, the encapsulation technique and the encapsulated product characteristics. However, for the food industry, encapsulation materials must be certified for food applications (GRAS). Materials used in encapsulation for food processes are mostly biomolecules (Nedovic et al., 2011, Wandrey et al., 2010).

In the next paragraphs, we present the principal groups of biomolecules used to encapsulate aroma for food industry, as well as the most frequently materials associated with each technique. Examples of encapsulated molecules are summarized (Table 3).

2.2.3.1. Carbohydrates

Carbohydrates are the principal biomolecules used in encapsulation for food applications. They are natural homo- and copolymers composed of sugar residues and/or their derivatives. There is an enormous variety of native polysaccharides that can also be chemically modified obtaining thus other materials (Wandrey et al., 2010).

Among the most popular carbohydrates used in encapsulation we found starch and their derivatives (amylose, amylopectin, dextrines, maltodextrines, polydextroses, etc.) (Nedovic et al., 2011), cellulose and their derivatives (methyl, ethyl, hydroxypropyl, carboxy methyl, etc.), plant extrudates and extracts (gum arabic, mesquite gum, pectines, etc.), marine extracts (Carragenan, alginate, etc.) and, microbial and animal polysaccharides (xanthan, gellan, dextran, chitosan, etc.) (Wandrey et al., 2010).

The ability to bind aroma to starches, maltodextrins, corn syrup solids and gum arabics, in addition to their low cost and GRAS status, make them the preferred choice for aroma encapsulation (Nedovic et al., 2011, Wandrey et al., 2010).

2.2.3.2. Proteins

Proteins are also used for encapsulation, they are natural macromolecules composed of lineal chains of amino acids, traditionally used in food (Wandrey et al., 2010). Proteins have excellent functional properties such as solubility, viscosity, emulsification, and film-forming properties making them useful for encapsulation (Madene et al., 2006). In addition, they present high blinding properties for aroma compounds (Gharsallaoui et al., 2007).

Principal proteins used in encapsulation process for food industry are gluten, milk or whey proteins, gelatin (Nedovic et al., 2011, Wandrey et al., 2010). Whey proteins and gelatin have been largely used in flavor encapsulation. Polypeptone, soy protein, or gelatin derivatives are also protein-based materials that can form stable emulsions with volatiles compounds. However, their solubility in cold water, potential to react with carbonyls, and high cost, limit their possible applications in food process (Madene et al., 2006).

2.2.3.3. Lipids

We call lipid a large diversity and structural variety of hydrophobic molecules such as oils, fats, waxes, phospholipids, etc., widely distributed in nature (Wandrey et al., 2010). Among lipid materials available for food applications we found fatty acids, fatty alcohols, bees, carnauba and candelilla waxes, glycerides and phospholipids and other inorganic materials such as polyvinylpyrrolidone, paraffin, shellac, among others (Nedovic et al., 2011).

Microstructural characteristics, colloidal stability, rheological and moisture barrier properties of lipids are governed by their chemical and physical properties. Polar lipids are amphiphilic, they can form crystalline structures. This characteristic made it suitable to protect and control the release of sensible molecules like aroma (Augustin and Hemar, 2009).

2.2.3.4. Yeast

As mentioned before, yeast can be used as wall material. Their highly glycosylated mannoprotein outer layer and β-glucan inner layer as well as their plasmic membrane, permit yeast to control the osmotic pressure and exchanges with the environment, making it a potentially excellent encapsulation wall material (Shi et al., 2010, Pham-Hoang et al., 2013).

2.2.4. Conclusions

Numerous developments have been made in the field of encapsulated food aroma. This is because of several favorable properties of the encapsulated form of flavors. The choice of an appropriate technique of encapsulation depends on the properties of the aroma compounds, the degree of stability required during storage and processing, the properties of the food components, the specific release properties required, the maximum obtainable aroma load in the powder, and the production cost. However, each encapsulation process, generally developed to solve a particular problem encountered by a product development, presents advantages and disadvantages. Microencapsulation by spray drying is the most economical and flexible way that the food industry can encapsulate aroma ingredients. Thus, this technology is now becoming available to satisfy the increasingly specialized needs of the market.

Today, the comprehensive technology of encapsulation enables to satisfy all relevant product requirements, such as designable properties, easy product handling, improved shelf life and controlled release. Encapsulation efficiency might be the limiting factor of most of the techniques used for aroma encapsulation. Spray drying is once again among the most performant and well documented. Spray drying encapsulation yields range from 5 to 99 % depending mainly on the encapsulate compound volatility. However, the other encapsulation techniques have not been sufficiently explored and documented.

Understanding the industrial constraints and requirements to make a viable microencapsulation technology, is the most important aspect from transition to full-scale production. In this order of ideas, it is convenient to continue exploring additional alternatives for flavor encapsulation.

Table 3. Recent application examples of aroma encapsulation.

Encapsulation technique	Wall material	Encapsulated flavor	Encapsulation efficiency (%)	Reference
Spray drying	Gum Arabic	Rossemary essential oil	7.15 – 47.53	(de Barros et al., 2013)
Spray drying	Gum Arabic	Mint oil	80.93	(Sarkar et al., 2012)
Spray drying	Porous starch	Diacetyl	63.42 – 90.41	(Belingheri et al., 2013)
Spray drying	Maltodextrin/ Gum Arabic/ modified starch	Oregano essential oil	5.1-33.9	(Botrel et al., 2012)
Spray drying	Gum Arabic	Coffe oil	47.93 – 81.99	(Frascareli et al., 2012)
Spray drying	Maltodextrin/ Gum Arabic	Lime essential oil	99.9	(Bringas-Lantigua et al., 2012)
Spray drying	Gum Arabic	Allyl isothiocyanate	39 -57	(Ko et al., 2012)
Spray drying	Maltodextrin/ Gum Arabic	Mandarin oil	98.40-99.6	(Bringas-Lantigua et al., 2011)
Spray drying	OSAn	L-menthol	95	(Mortenson and Reineccius, 2008)
Spray drying	Modified starch	D-limonene	74 - 94	(Soottitantawat et al., 2005a)
Spray chilling	Anhydrous erythritol	Limonene	90	(Sillick and Gregson, 2013)
Yeast encapsulation	Yeast cells	Curcumin	33	(Paramera et al., 2011)
Yeast encapsulation	Yeast cells	Resveratrol	4.52	(Shi et al., 2008)
Yeast encapsulation	Yeast cells	Limonene	29.35	(Hafner et al., 2011)
Mealt dispersion	Carnouba wax	Ethyl vanillin	87	(Milanovic et al., 2010)
Freeze drying	Gum Arabic	Limonene	60.2 – 75.3	Kaushik et al. 2007
Complex coacervation	Soybean protein isolate/ Gum Arabic	Sweet orange oil	68	(Xiao et al., 2011)
Complex coacervation	Soy protein isolate/ Gum Arabic	Propiolis extract	66.12 – 72.01	(Nori et al., 2011)
Co-cristalization	Gum Arabic	α-terpinyl	67.18	(Sardar & Singhal, 2013)
Complexation	Maize amylose	Hexanal/ (E)-2-Nonenal	75-85	Wulff et al. 2005)

Chapitre 3. Résultats

1. A la recherche des caractéristiques du fromage Poro

1.1. Introduction

Après avoir réuni des informations concernant le 3-methylbutanal et sa production par les bactéries, nous avons désormais une meilleure connaissance de cette molécule volatile que nous souhaitons incorporer dans l'enrobage du fromage. Nous avons aussi une meilleure vue de l'ensemble des techniques et matériels d'encapsulation qui pourront nous aider à incorporer les nouvelles notes aromatiques dans l'enrobage du fromage.

Cependant, avant d'essayer d'incorporer de nouvelles notes olfactives dans l'enrobage de fromage Poro, il faut s'intéresser à connaitre ce fromage. Précédemment, nous avons évoqué le caractère typique de ce produit mexicain qui est en effet candidat à l'obtention d'une appellation d'origine (DO).

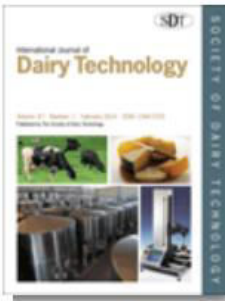
La législation mexicaine demande aux producteurs qui postulent pour l'obtention de cette DO pour leur produit, de possèder des connaissances sur divers aspects (IMPI, 2013), tels que :

- une description détaillée du produit
- les caractéristiques de fabrication
- son origine géographique
- son mode d'emballage
- des éléments prouvant des liens entre le nom, le produit et le territoire

Dans le cadre de la préservation du patrimoine mexicain et dans le but de connaitre les caractéristiques spécifiques définissant la typicité de fromage Poro, l'objectif du prochain article a été d'établir un profil général du fromage Poro en caractérisant ses propriétés physico-chimiques, microbiologiques et sensorielles.

La connaissance de ces caractéristiques a permis de remplir une des exigences pour l'obtention de la DO du fromage Poro. De plus, nous avons obtenu des informations concernant les caractéristiques sensorielles de ce produit et les principales bactéries composant sa flore technologique.

1.2. Mexican Poro Cheese: Physicochemical, textural, rheological, sensorial and microbiological characterization



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Abstract

The physicochemical, microbiological, textural, rheological, and sensory characteristics of Poro cheese were studied. Differences in moisture, fat, NaCl, titrable acidity, pH, color texture and rheology amongst cheese factories were observed and ranges were established. Fifteen descriptors were generated to provide a descriptive analysis, eight of which were significantly different amongst the factories; there were no differences in the global acceptability of cheese. The favorite cheese had the highest scores for aroma attributes. Conventional and molecular methods were used to identify the main microorganisms in the cheese, for which *Lactobacillus plantarum, L. fermentum. L. farciminis and L. rhamnosus* were the main microorganisms found.

Key words: Artisanal cheese, physicochemical characteristics, sensorial characteristics, lactic acid bacteria

1.2.1. Introduction

Poro cheese is a handmade product originally from the region of Los Rios, Tabasco in Mexico. It is produced from raw milk of Zebu cattle or Zebu-Brown Swiss, and is a semi-firm paste slightly-pressed cheese, characterized by an unintentional maturation of less than one month at room temperature (20°C – 30°C and 74 % – 90 % relative humidity), principally during the period while it is being sold. It has a small rectangular prism shape and a 200 g – 350 g weight. It is coated with paraffin wax and packed in yellow cellophane. As in other artisanal Mexican cheese types, the elaboration process has been transmitted from generation to generation. The seven-day Poro cheese manufacturing process is characterized by several stages: milk reception and filtering, temperature verification, acidification and coagulation by whey addition from the previous day, rennin addition, curd cutting, whey

draining, molding and draining, pressing and salting, rest, airing, coating and wrapping for sale, and distribution (Torres de la Cruz, 2009).

Origin appellations designate the region-specific provenance of a product, whose quality and characteristics are due solely to the geographical environment, comprised of natural and human factors. When talking about a product whose history, tradition and tipicity are anchored with a place and a society, surrounded by a knowledge that is transmitted throughout time; the appellation acquires a patrimonial dimension, which gives the products an authentic prestige. Ultimately, this forms part of a common patrimony, which society will strive to protect (Barjolle, Boisseaux & Dufour 1998).

Products with a strong identity have a better market positioning because buyers can identify their quality attributes (Solís-Méndez et al., 2012), which are governed by the know-how of the manufacturer (Solís-Méndez et al., 2012). This situation represents a great challenge for artisanal Mexican and world cheese producers.

There are several criteria for Mexican cheese classification as fat content, consistency, ripening time. However, despite the existence of objective criteria, Mexican cheese have not been given extensive product designations when compared to EU market cheese. Only 13 prestigious product designations for Mexican products are known (IMPI, 2013). The lack of prestigious designations mean that Mexican cheeses have to rely on the history and typicity surrounded by an ancestral expertise. There are at least four known analyses reported for characterizing artisanal cheeses: physicochemical, rheological, sensorial and microbiological. On worldwide scale, several artisanal cheeses have received extensive characterization to establish unique attributes of cheese varieties, enabling the preservations of these products and their attributes. Examples of those are Sepet and Enzine cheese from Turkey, Majorero and Murcia al Vino cheeses from Spain and Añejo, and Ranchero cheese from Mexico (Ercan et al., 2011, Karagul-Yuceer et al., 2007, Fresno and Álvarez, 2012, López et al., 2012, Hernández-Morales et al., 2010, Solís-Méndez et al., 2012).

In the context of preserving the heritage of Poro cheese and protecting the specific characteristics, which define its tipicity *via* a collective brand or an origin designation, the objective of this work was to establish a general profile of Poro cheese by characterizing their physicochemical, microbiological, rheological and sensory properties.

1.2.2. Materials and methods

1.2.2.1. Sampling

Cheese samples were collected from five different artisanal cheese factories in Balancán, Tabasco, Mexico. Factories were chosen by their location, prestige and product availability. Cheese units were taken in triplicates at the end of their seven days elaboration process; they were stored in a cool container at 4°C and transported to the laboratory.

All the analyses were performed on cheese samples from five cheese factories (QP1, QP2, QP3, QP4 and QP5); the microbiological characterization was carried out on cheese samples from only two cheese factories (QP1 and QP3). Factory names were coded for confidentiality reasons.

1.2.2.2. Physicochemical Analysis

Fat content by Gerber-Van Gulik(Ramos, 1976), moisture (method 926.08, AOAC 1995), protein (935 method, AOAC, 1995), ash (method 935.42, AOAC 1995) and NaCl (method 935.43; AOAC 1995) were determined as well as pH with a potentiometer HI 9230 (Hanna Instruments, Italy) and water activity with an Aqualab water activity meter (Decagon, WA, USA) at 25°C.

1.2.2.3. Texture Profile Analysis

A textural profile analysis (TPA) was performed using a TA-Xt2i texture analyzer (Stable Micro Systems, Surrey, UK) and Texture Expert 7.15 H software with a load cell of 5 kg. Cylindrical cheese samples of 15 mm diameter and 10 mm height were compressed uniaxially to 50 % deformation using a 35 mm diameter acrylic disc (A/BE35) at pretest, test, and post-test speeds of 1 $mm \cdot s^{-1}$. Measurements were performed in triplicate and hardness, adhesiveness, cohesiveness, springiness and chewiness were calculated from the double bite curves.

1.2.2.4. Rheological Analysis

Dynamic oscillatory measurements were performed with a Physica MCR 301 (Physica Messtechnik, Stuttgart, Germany), with a parallel plate rough geometry, in which the rotating plate was 50 mm in diameter. Cylinders samples 50 mm in diameter, were cut from the centre of the chesses and they were wrapped in aluminum foil to prevent dehydration and tempered for 1H at room temperature (Lobato-Calleros et al., 2002). The cheese samples were placed in a sanding disc attached to the rheometer peltier to prevent sample slippage. In order to control the sample contact with the geometry, the normal force was adjusted to 0.5 N. The linear viscoelastic region of the chesses being determined by amplitude sweeps, using deformation values of 10^{-3} to 100 % and 1 HZ frequency and

25°C. Frequency sweeps were made from 0.01 to 100 Hz. The storage modulus (G') and the loss modulus (G'') of cheeses samples were obtained from the equipment software by triplicate.

1.2.2.5. Color Measurement

Internal cheese color was measured using a MiniScan 45/0 LAV (HunterLab, Hunter Associates Laboratory, EE.UU.). Hue and chroma were calculated using the CIElab scale with D65 as illuminant and an observer angle of 10°, yellowing index was also measured. Samples were measured in triplicate.

1.2.2.6. Sensorial Analysis

An eight member's trained panel performed a descriptive analysis for Poro cheese. Judges were trained for 30 h. A 15 cm interval scale was used, where zero represented attribute absence and 15 a very high intensity (Table 4). Panelists received monadically, at 22°C, cheese samples of 1cm x 1cm x 1 cm in medicine cups coded with three digits random numbers. Cheese samples were randomly evaluated for each panelist in triplicate.

Overall acceptability of Poro cheese was evaluated using a nine point hedonic scale by a panel of 101 consumers. On the other hand, overall and specific attributes acceptabilities were evaluated by the cheese producers and their families, also using a nine points hedonic scale. Evaluated specific attributes were the same used by the trained panel except for tactile hardness and tactile creaminess that were not used.

1.2.2.7. Microbiological Analysis

Microbiological enumeration

Ten grams of each cheese were aseptically taken and put with 90 mL of sterile 2 % sodium citrate solution into a sterile stomach bag, and homogenized by 2 min; decimal dilutions were prepared in tryptone salt buffer and used for enumeration on agar plates. Culture media and incubation conditions are summarized in Table 5. After incubation, colony forming units (CFU) was counted and means and standard deviations were calculated.

Strains isolation and biochemical identification

For the isolation, purification, characterization and identification of Lactic Acid Bacteria (LAB) isolates, LAB suspected colonies from different origin were randomly selected and subcultured. The identitity of Gram-positive, catalasa-negative cocci and rods was determined by performing biochemical and physiological tests. Rod-shaped bacteria were tested for their ability to produce gaz

and to grow in Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France) at 15°C for seven days and 45°C for two days. Cocci were tested for their ability to produce gaz, to hydrolyse arginine and to grow in presence of 6.5 % NaCl and 10 % of bile, in Elliker broth (Biokar, Beauvais, France) at 10°C for ten days, at 45°C for 48h. Carbohydrate fermentation patterns were determined using API 50 CH test strips (BioMérieux, Marcy l'Etoile, France), according to the manofacturer's instructions.

DNA extraction

Total genomic DNA was extracted from each isolate. Tubes contining 9 mL of Elliker or MRS broth were used to accommodate the isolated strains and incubated for 48h at 30 or 37°C. Two mL of each culture had been used for DNA extraction using the methode described by Serhan et al. (2009).

For cheeses DNA extraction, 5 g of cheese were homogenized by using a stomacher (Interscience, France) in 50 mL of steril 2 % trisodium citrate, 40 mg of pronase and 100 µL of β-mercaptoethanol were added and incubation was done at 37°C overnight. Pelets were recupered by centrifugation at 5000 rpm for 15 min, and resuspended in sterile water, then DNA was extracted using the QIAamp DNA Stool mini Kit (QIAGEN, Germany).

PCR amplification

DNA bacterial and cheese strains were treated with RNAse and then 16S ribosomal genes were amplified using universal bacterial primers fD1 and rD1 (Shannon et al., 2001). The amplification program was: 95°C for 5 min, followed by 35 cycles of 95°C for 2 min, 45°C by 30 s, 72°C for 4 min, then 72°C for 10 min, carried in a thermocycler (Bio-Rad Laboratoiries, Hercules, CA, USA). PCR products were stoked a -20°C until use.

Strains identification

The PCR extracts from bacterial isolates were sequenced by GATC-biotech company (Germany). Partial sequences were compared to those in the GenBank database using the BLAST program.

Time Temperature Gel Electrophoresis (TTGE)

Cheese PCR products were someted to a TTGE analysis using a Dcode universal mutation detection system (Bio-Rad Laboratoires, Hercules, CA, USA) and following the method descibed by Serhan et al (2009).

1.2.2.8. Statistical Analysis

A completely randomized design (DCA) was performed for physicochemical data, color, texture and rheology variables ($P<0.05$). Averages were compared by the least significant difference test (LSD).

For the descriptive analysis, a randomized complete block design (RCB) with a split plot arrangement was used, while for the acceptability tests a RCB was performed. The least significant difference method was used for comparison of means. Principal components analysis was applied to obtain preference maps.

The statistical calculations were performed using the statistical software SAS ® version 9.1 (SAS Institute, Inc., Cary, NC), along with The Unscrambler® version 9.2 (CAMO PROCESS AS, Oslo, Norway).

Table 4. Sensory descriptors, definitions and references for the evaluation of Poro cheese

Descriptor	Definition	Reference and Intensity
Color	Color from white to yellow.	White adhesive paper (Janel) YI (-19.18), I = 1.5 Yellow adhesive paper (Post-it) YI (53.63), I = 11
Layers resolution	Space resolution due to the layers arrangement during cheese pressing.	Craker Habanera (Gamesa), I = 5 Banderilla puff pastry (El globo), I = 11.5
Sour milk aroma	Aromas associated with the fermented sour milk.	Lactic acid, 0.05 % in milk, I = 4.5 Lactic acid, 1 % in milk, I = 9
Butter aroma	Aromas associated with the butter.	Diacetyl, 5 drops in 100 mL of milk, I = 4 Diacetyl, 10 drops in 100 mL of milk, I = 9
Propionic acid aroma	Strong, irritating smell associated with mature cheese.	Propionic acid 5 drops in 100 mL of milk, I = 5 Propionic acid 15 drops in 100 mL of milk, I = 10
Tactile hardness	Force required compressing a sample piece between the fingers.	Cocktail sausage (Swan), I = 4.5 Cut into cubes of 6 mm by 6mm raw carrot, I = 13
Tactile creaminess	Sensation associated with the fat present in the sample at the time of touching.	Cotija like cheese (Esmeralda, Distribuidora de Lácteos Algil), I = 4 Philadelphia Cheese (Kraft Foods Inc.), I = 13
Sandiness	Sensation associated with small particles of sand.	Panela cheese (LALA), I = 5 Parmesan cheese (Kraft Foods Inc.), I = 12
Elasticity	Sample capacity to recover its original shape when compressed with the molars.	Cotija like cheese (Esmeralda, Distribuidora de Lácteos Algil), I = 4 Panela cheese (LALA), I = 10
Humidity	Amount of water perceived in the oral cavity	Cotija like cheese (Esmeralda, Distribuidora de Lácteos Algil), I = 4 Panela cheese (LALA), I = 11
Hardness	Force required penetrating the simple with molars.	Cocktail sausage (Swan), I = 3 Cut into cubes of 6 mm by 6mm raw carrot, I = 11
Saltiness	Basic taste sensation occasioned by salts.	Sodium chloride, 0.35% in water, I = 6 Sodium chloride, 0.5 % in water, I = 11
Sourness	Basic taste sensation occasioned by acids.	Citric acid, 0.2% in water, I = 5 Citric acid, 0.5% in water, I = 11
Global taste intensity	Force of cheese flavor.	Cotija like cheese (Esmeralda, Distribuidora de Lácteos Algil), I = 12.5
Global taste duration	Duration of cheese flavor.	Cotija like cheese (Esmeralda, Distribuidora de Lácteos Algil), I = 12

YI, Yellowing index; I, Intensity.

Table 5. Medium and incubation conditions

Group targeted	Medium	Incubation temperature (°C)	Incubation time	Anaerobic or aerobic conditions
Total count	PCA	30	72 h	A
Total coliforms	VRBA	30	24 h	A
Molds	OGA	25	5 days	A
Yeasts	OGA	25	5 days	A
Enterococcus spp.	BEA	37	48 h	A
Halotolerant flora	BHI + 6.5% NaCl	25	48 h	A
Lactococcus mesophylls	M17	25	48 h	A
Lactobacillus mesophylls	MRS pH 5,7	25	48 h	An
Lactobacillus thermophiles	MRS pH 5,7	42	48 h	An

A, aerobic; An, anaerobic conditions; PCA, plate count agar; VRBA, Violet red bile agar; OGA, Oxitetracycline glucose agar; BEA, Bile esculin agar; BHI, Brain heart infusion agar; M17 agar; MRS, Man, Rogosa and Sharpe agar (Biokar, Beauvais, France).

1.2.3. Results and discussion

1.2.3.1. Physicochemical Analysis

The physicochemical properties of Poro cheese (Table 6) indicate significant differences for all the parameters, and those differences are probably related to the milk composition and cheese elaboration procedures, as was also concluded by Pisano et al. (2006) in Italian Fiore Sardo cheese, by Vasek et al. (2008) in Argentinean Corrientes cheese and by Hernández-Morales et al. (2010) in Mexican Añejo cheese.

Table 6.Chemical composition of Poro cheese

Factory code	Fat (%)	Moisture (%)	Protein (%)	NaCl (%)	Ca (%)	pH	a_w
QP1	29.8^{A}	34.8^{A}	24.5^{A}	3.9^{A}	0.28^{A}	4.9^{A}	$0.947^{A,B}$
QP2	31.3^{B}	31.5^{B}	39.4^{B}	$2.3^{B,C}$	0.12^{B}	4.3^{B}	0.953^{A}
QP3	37.0^{C}	28.1^{B}	26.3^{C}	$2.5^{B,C}$	$0.19^{A,B}$	4.6^{C}	0.930^{B}
QP4	31.2^{B}	33.6^{A}	$27.4^{B,C}$	2.0^{C}	0.12^{B}	$4.7^{C,A}$	0.954^{A}
QP5	$30.6^{A,}$	$37.0^{A,C}$	26.8^{C}	2.7^{B}	0.15^{B}	$4.4^{B,C}$	0.955^{A}

$^{A-C}$ Different superscript letters within same column indicate significant differences (P≤0.05).

In general Poro cheese physicochemical properties range from 29.8 to 37.0 % fat, 28.1 to 37.0 % moisture, 24.5 to 39.4 % protein, 2.0 to 3.9 % NaCl, 0.12 to 0.28 % Ca^{+2}, 4.3 to 4.9 pH, and 0.930 to 0.955 for a_w. These values are similar to those reported for Añejo cheese (Hernández-Morales et al.,

2010) an artisanal semi-hard cheese from Mexico, but different to those reported for Ranchero cheese (Solís-Méndez et al., 2012) another Mexican cheese considered as soft.

1.2.3.2. Texture Profile Analysis

Variations were observed in the mechanical properties of cheese among producers except for cohesiveness (Table 7). The TPA values of Poro cheese ranges from 11.92N to 29.63 N for hardness, from -0.643 Ns to -0.0002 Ns for adhesiveness, from 0.38 to 0.47 for cohesiveness from 0.63 to 0.89 for springiness and from 4.08 to 9.97 for chewiness. Great variations among producers in TPA parameters were also reported in other artisanal cheeses such as Añejo and Ranchero Mexican cheese (Hernández-Morales et al., 2010, Solís-Méndez et al., 2012), and in Chihuahua cheese made with raw milk (Van Hekken et al., 2007). These important variations could be due to the variability in milk composition and the differences in the artisanal process between cheese makers. Similar TPA values were founded in another Mexican artisan cheese called Ranchero cheese except for the adhesiveness value, artisanal Ranchero cheese TPA values are 24.83 ± 12 N for hardness, -0.93 ± 0.4 Ns for adhesiveness, 0.4 ±13 for cohesiveness, 0.76 ± 0.12 for springiness and 6.6 ± 2.03 N for chewiness (Solís-Méndez et al., 2012).

Table 7. Mean texture analyses (TPA) values of Poro cheese

Factory code	Hardness (N)	Adhesiveness (Ns)	Cohesiveness	Springiness	Chewiness (N)
QP1	11.92^{A}	-0.000^{A}	0.42^{A}	0.83^{A}	4.08^{A}
QP2	$14.89^{A,D}$	-0.313^{B}	0.40^{A}	0.63^{B}	3.77^{A}
QP3	29.63^{B}	-0.176^{C}	0.38^{A}	0.80^{A}	9.01^{B}
QP4	23.86^{C}	-0.001^{A}	0.47^{A}	0.89^{A}	9.97^{B}
QP5	16.63^{D}	-0.643^{D}	0.41^{A}	$0.74^{A,B}$	5.10^{A}

$^{A-D}$ Different superscript letters writhing same column indicate significant differences (P≤0.05).

1.2.3.3. Rheological Analysis

The mechanical behavior of Poro cheese was determined by low amplitude oscillatory tests, dynamic measurements were carried out in the linear viscosity range, so cheese structure is preserved. Figure 10 shows the elastic modulus (G') and the viscous modulus (G'') for each cheese. In general all cheeses showed values of G' greater than G'' except for the first point on the QP1 graph. Furthermore, the values of G' and G'' increase with the frequency increment. This is the typical behavior of a viscoelastic material with solid character and has been previously reported in cheese by

several authors as Ustunol, Kawachi & Steffe (1995), Tunick (2000), Brown et al. (2003) and Muliawan & Hatzikiriakos (2007).

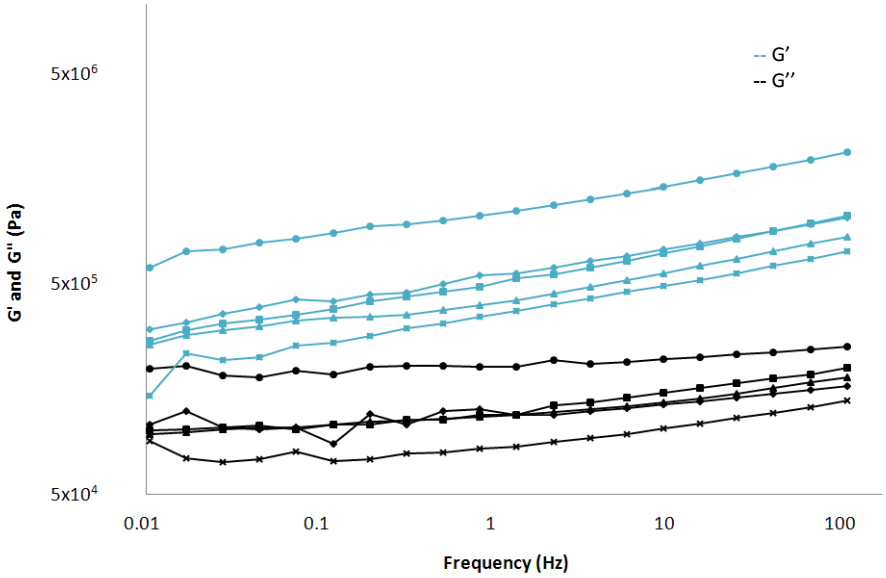

Figure 10. Average graph of storage modules (G') and loss modules (G'') for the five Poro cheese factories: (◆) QP1, (▲) QP2, (●) QP3, (✕) QP4 and (■) QP5.

Values of G' and G'' for each cheese are shows in Table 8. There are significant differences among the cheese factories, particularly for QP3 with the highest values and QP4 with the lowest values. Poro cheese G' and G'' range from 281.91 to 1081.14 and 67.4 to 177.92 kPa, respectively.

Table 8. Rheological characteristics of Poro cheese

Factory code	Storage module G' (kPa)	Loss modules G'' (kPa)
QP1	510.69^{A}	110.62^{A}
QP2	424.15^{A}	117.70^{A}
QP3	1081.14^{B}	177.92^{B}
QP4	281.91^{C}	67.40^{C}
QP5	487.05^{A}	117.80^{A}

$^{A-C}$ Different superscript letters writhing same column indicate significant differences (P≤0.05).

1.2.3.4. Color Measurement

Color is a sensory attribute whose perception is highly variable and dependent on factors such as lighting, observation angle, the observer, and so on. However, it is also a very important feature of cheese, because it is one of the first sensory attributes perceived before purchase or consume a product (Hernández-Morales et al., 2010). Table 9 shows the results of color measurements of Poro

cheese. Cheeses samples present significant differences (P<0.05). The values of Luminosity, Yellowness Index (YI), Chroma and Hue of Poro cheese range from 86.33 to 90.01, 31.89 to 40, 17.37 to 36.3 and 61.45 to 90.08, respectively. The composition of cows feeding has been associated with differences in color due to the higher content of carotenoids (Verdier-Metz et al., 2000).

Table 9. Results of color measurements of Poro cheese

Factory code	Luminosity	YI	Chrome	Hue*
QP1	86.33^{A}	32.42^{A}	17.37^{A}	90.08^{A}
QP2	$87.49^{A,B}$	36.02^{B}	19.70^{BC}	89.59^{A}
QP3	89.06^{B}	37.92^{B}	21.27^{B}	$89.46^{A,B}$
QP4	89.41^{B}	$40.00C^{B}$	22.53^{B}	88.91^{B}
QP5	90.01^{B}	31.89^{D}	36.3^{D}	61.45^{C}

$^{A-D}$ Different superscript letters within same column indicate significant differences (P≤0.05); YI, Yellowing index,*Values expressed as degrees

1.2.3.5. Sensorial Analysis

The panel generated fifteen descriptive attributes to characterize the appearance, aroma, flavor and texture of Poro cheese. Table 10 shows those attributes, their definition and their reference values on the scale. The results of variance analysis and means comparison showed differences among cheese samples for eight of the fifteen attributes (Table 7).

Table 10. Intensity scores for the sensory attributes identified in Poro cheese

Descript	Cheeses factory code				
	QP1	**QP2**	**QP3**	**QP4**	**QP5**
Color	6.73^{A}	7.42^{A}	7.4^{A}	6.01^{B}	7.04^{A}
Layers resolution	7.34^{A}	7.12^{A}	5.49^{B}	5.79^{B}	6.79^{A}
Sour milk aroma	7.71^{A}	8.07^{A}	7.92^{A}	7.80^{A}	7.97^{A}
Butter aroma	7.19^{A}	6.85^{A}	7.17^{A}	7.06^{A}	6.66^{A}
Propionic acid aroma	7.81^{A}	7.75^{A}	8.21^{A}	8.20^{A}	7.77^{A}
Tactile hardness	$9.93^{B,C}$	$9.92^{B,C}$	10.94^{A}	9.52^{C}	$10.33^{A,B}$
Tactile creaminess	$6.39^{A,B}$	$5.34^{D,C}$	5.64^{D}	6.85^{A}	$5.91^{B,C}$
Sandiness	7.50^{A}	6.79^{A}	7.13^{A}	6.86^{A}	7.51^{A}
Elasticity	5.61^{A}	5.60^{A}	5.25^{A}	5.51^{A}	5.56^{A}
Humidity	4.71^{A}	$4.27^{A,B}$	3.73^{C}	4.46^{A}	$3.92^{B,C}$
Hardness	$6.84^{A,B}$	7.11^{A}	6.96^{A}	6.12^{B}	7.27^{A}
Saltiness	7.76^{A}	7.77^{A}	7.37^{A}	7.14^{A}	7.18^{A}
Sourness	7.45^{C}	8.39^{A}	$7.98^{A,B}$	$7.75^{B,C}$	$7.79^{B,C}$
Global taste intensity	9.62^{A}	9.65^{A}	$9.20^{A,B}$	$8.88^{B,C}$	8.68^{C}
Global taste duration	9.02^{A}	8.88^{A}	8.72^{A}	8.62^{A}	7.9^{B}

$^{A-D}$ Different superscript letters within same row indicate significant differences (P≤0.05).

For color, the only difference was found in QP4, whose score was lower, which indicates the least yellow cheese. Instrumentally this cheese was not the least yellow, but cheeses QP2 and QP3 presented YI values above it. Regarding the layers space resolution, QP3 and QP4 had the lower space resolution. The production process of Poro cheese change between cheese factories, for example, the operation affecting the layers (pressing), in QP3 dairy takes one day more than in the others, additionally QP4 is pressed using a Dutch type press, which causes QP3 and QP4 to have a more compact structure and therefore it is not easy to perceive the layers.

No differences were found among the evaluated flavor attributes (sour milk aroma, butter aroma and propionic acid aroma). However, textural attributes (tactile hardness and tactile creaminess) differed between cheeses. QP3 was perceived as the hardest one, this data coincides with instrumental measurements. Cheese perceived as more creamier were QP4 and QP1. There was no significant difference for sandiness and elasticity evaluated in mouth, however, there were differences for humidity and hardnes. The cheeses QP1, QP2 and QP4 were perceived as more humid, while QP1 and QP4 were less firm. There were no differences among cheese samples for saltiness but for sourness, QP2 and QP3 cheese samples were detected as more acid. The cheese global taste intensity of QP1, QP2 and QP3 was higher than the other, while the global taste permanence was lower for QP5.

The principal components analysis of descriptive data showed that the first two principal components (PCs) explained 66 % of the variability of the cheeses descriptors. The first principal component (PC1) explained 36 % of the data variability and correlate positively with the attributes of color, sour milk aroma, hardness and sourness, and negatively with creaminess and humidity. The PC2 explained 30 % of variance and was positively correlated with the attributes layers, elasticity, saltiness and global taste intensity; it is negative correlated to propionic acid aroma (Figure 11).

No significant difference for overall acceptability was found among cheese samples evaluated by habitual consumers. When the overall acceptability of cheeses was evaluated by the producers and their families, significant differences were found, in this case cheese with the highest acceptability was QP4 and the one with the lowest was QP1. Besides the overall acceptability, the producers also rated the acceptability by attribute for each cheese. Producers found significante difference among cheese samples for nine of the fourteen attributes. External preference map of Poro cheese is showed on Figure 12. No finding acceptability difference among cheese samples could be explained by inexperience of the consumers, in comparison with producers.

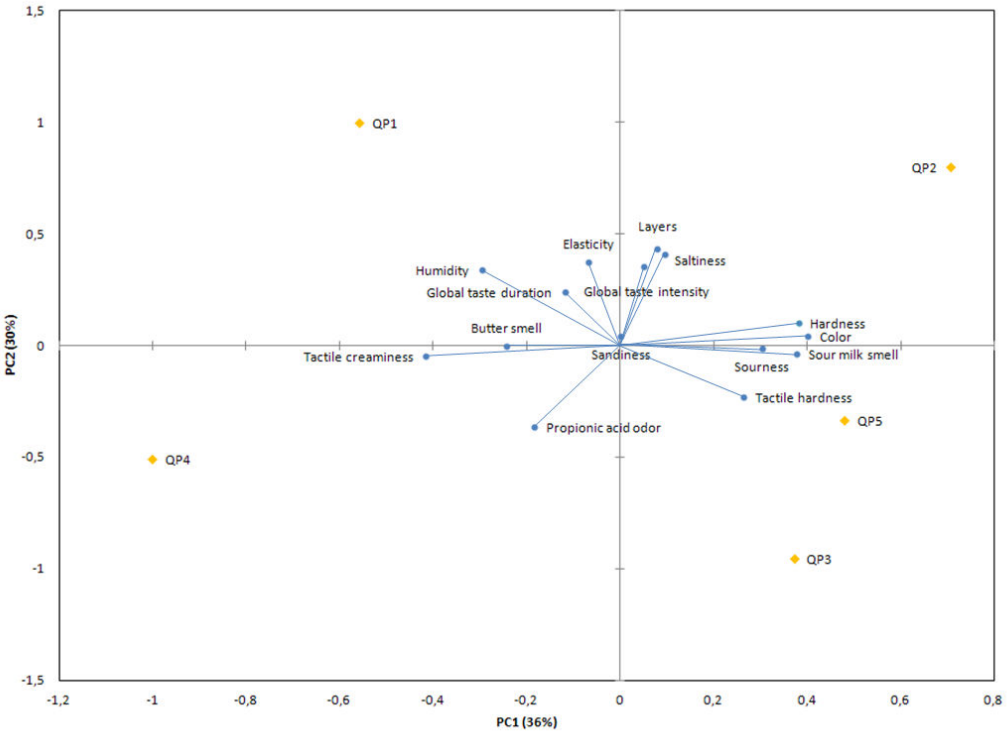

Figure 11. Principal components graphic for Poro cheese sensory attributes (loads) and cheeses (scores).

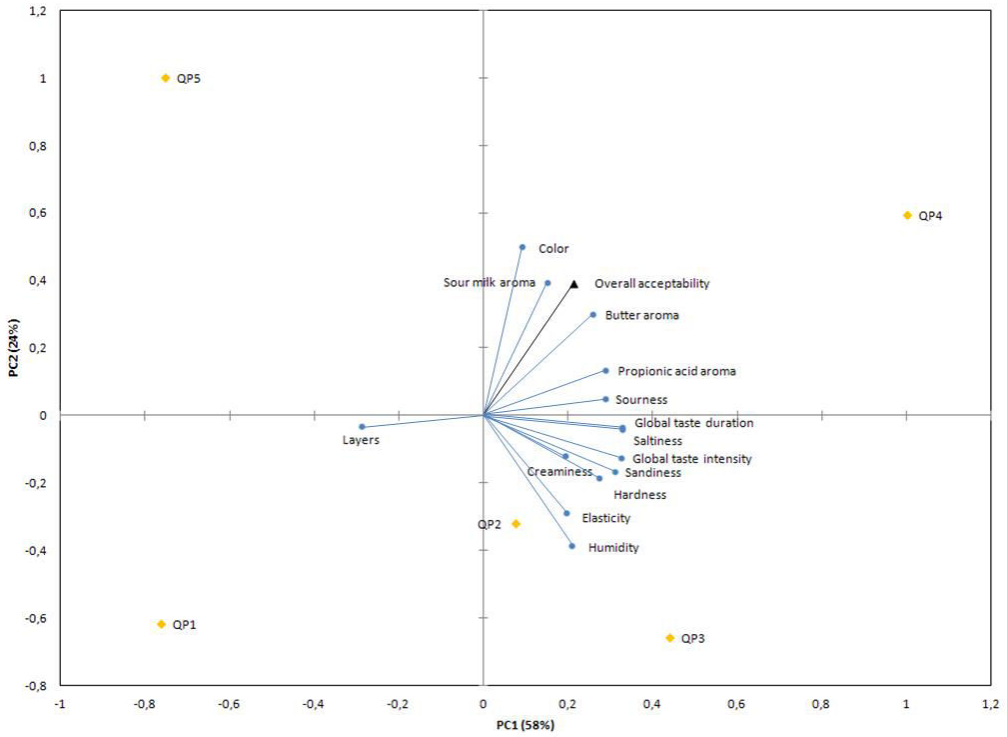

Figure 12. Poro cheese external preference map.

1.2.3.6. Microbiological Analysis

Among the five cheese factories, cheeses from QP1 and QP3 were chosen for microbiological analysis due to their significant difference concerning the physicochemical and sensorial data. The microbial population present in Poro cheese was determined by conventional microbiological analyses using different culture media. Samples from the two different productions (QP3 and QP1) were used to obtain the average bacterial population (Figure 13). It is clear that cheeses from QP1 have higher values in every case, QP3 cheeses factory is smaller and familial so they can better control the hygiene in the production process. Concerning the presence of fecal contamination bacteria, no coliforms were founded in Poro cheese samples. This shows a good sanitary quality of those products using raw milk.

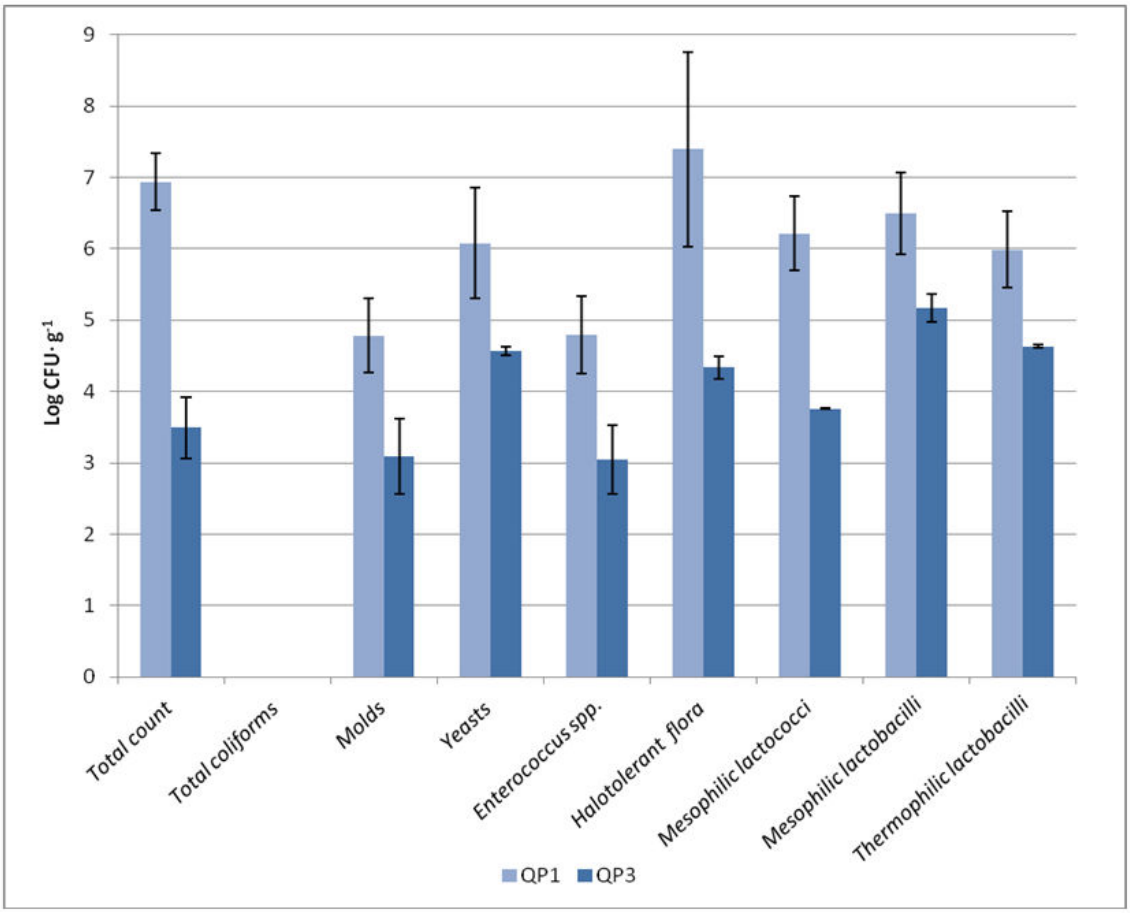

Figure 13. Average bacterial population in Poro cheese (Log CFU·g-1)

DNA extracts were obtained from cheeses and after PCR amplification were migrated on a TTGE, this exercise allows to confirm the homogeneity of Poro cheese microbiological population, because the chesses presents the same finger prints with two major and five minor bands (Figure 14).

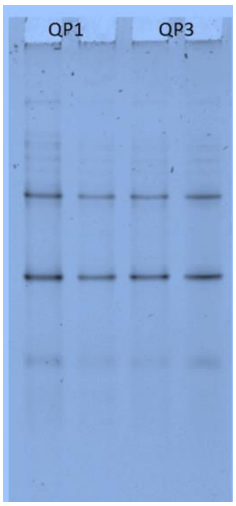

Figure 14. PCR-TTGE analysis of V3 16S rRNA fragments from Poro cheese samples, from 2 productions QP1 and QP3.

After numeration, 112 strains were randomly picked from plates containing lest than 300 colonies, those strains were first characterized by conventional microbiology methods. The genera *Enterococcus* and *Lactobacillus* were majority identified with the species *L. plantarum*, *L. pentosus, L. farciminis, L. rhamnosus*.

By the molecular approach *Lactobacillus* and *Enterococcus* were identified. The principal species identified (Table 11) had been identified previously in other cheese around the world (Serhan et al., 2009, Navidghasemizad et al., 2009, Veljovic et al., 2007, Pisano et al., 2006) ; *L. plantarum, L. rhamnosus and L. pentosus* were founded at least in two other Mexican cheeses (Renye Jr et al., 2011, Morales et al., 2011), while *L. brevis* has been identified only in Poro chees (Table 8).

Table 11. Main identified lactic acid bacteria Poro cheese

		Poro cheese		Other Mexican cheeses		
Accession	**% Identity**	**Closes relative**	**LAB population %**	**Chihuahua[A]**	**Doble Crema[B]**	**Cotija[B]**
HQ449670.1	99	*Lactobacillus fermentum*	15	+		
AB300210.1	100	*Lactobacillus plantarum*	52	+	+	
AB289102.1	99	*Lactobacillus farciminis*	11		+	
JN415185.1	100	*Lactobacillus rhamnosus*	11	+	+	
FR871760.1	99	*Lactobacillus pentosus*	4		+	+
AB548882.1	99	*Lactobacillus brevis*	4			
GU122150.1	100	*Enterococcus faecium*	4	+	+	

[A] Renye Jr et al. (2011), [B] Morales et al. (2011)
+ Identified LAB in the indicated cheese

1.2.4. Conclusion

This study has established the range of physicochemical, color, texture and rheology Poro cheese variables. Poro cheese could be considered as a semi-hard cheese but due to their TPA values it could be also classified as a soft cheese. However, these values can be considered as typical values for this product and they are associated with variations in the cheese making process as well as to the composition of cheese and milk.

Overall acceptability and acceptability by attribute of Poro cheese were evaluated by the producers and their families, allowing the construction of an external preference map for overall acceptability of Poro cheese, where high scores for flavor descriptors are related to a greater acceptability and high scores for elasticity, humidity and layers resolution descriptors are related to a lower acceptability.

Despide the differences found in physicochemical variables and the marked preference of producers for QP4, no significant difference was found between the overall acceptability of the cheeses evaluated by habitual consumers.

Artisanal cheese has additional characteristics that are attributed to the presence of microorganisms. In this research, the main Poro cheese LAB were identified, *L. plantarum* was the most frequently found lactic acid bacteria.

The obtained data constitutes the parameters for characterizing Poro cheese, which will strongly help to support its origin appellation request process.

Acknowledgements

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2. Encapsulation des bactéries et composés volatiles

2.1. Introduction

Ayant établi ou plutôt documenté les caractéristiques du fromage Poro, nous pouvons désormais nous intéresser à l'incorporation du 3-methylbutanal dans la paraffine utilisée pour enrober ce fromage.

Comme nous l'avons appris lors de notre révision bibliographique sur les techniques et les matériels d'encapsulation, l'objectif de l'encapsulation, la compatibilité des matériaux et les facteurs technologiques et économiques, sont les principaux critères à prendre en compte pour choisir une méthode d'encapsulation.

Dans le cas du fromage Poro, les deux facteurs les plus importants à considérer sont les problèmes technologiques et économiques. En effet, ce fromage est fabriqué par des petits producteurs qui travaillent dans des conditions purement artisanales.

Pour cette raison, nous avons décidé d'utiliser l'extrusion simple (goutte à goutte) d'une matrice d'alginate comme technique d'encapsulation. Cette technique reste économiquement abordable et technologiquement simple et peut être facilement reproduite par les producteurs de fromage Poro.

Parmi les bactéries productrices de composés volatils d'intérêt fromager, *C. maltaromaticum*, bien connu par l'équipe de recherche, est capable de produire du 3-methylbutanal et représente une option pour l'incorporation de cette molécule dans l'enrobage de fromage.

Deux alternatives sont donc envisagées pour l'incorporation du 3-methylbutanal dans l'enrobage du fromage. L'encapsulation directe de ce composé volatil et l'encapsulation de *C. maltaromaticum* sensé produire le composé aromatique depuis sa capsule.

L'objectif de ce deuxième article de résultats est donc d'améliorer les caractéristiques sensorielles du fromage Poro en incorporant, dans l'enrobage de paraffine, des billes d'alginate de calcium contenant (1) du 3-méthylbutanal directement encapsulés, (2) des souches de *Carnobacterium maltaromaticum LMA28.* Le but est aussi de tester l'effet de l'incorporation du composé aromatique sur la perception sensorielle d'un fromage modèle (babybel®).

Sachant que *C. maltaromaticum* a également des propriétés antimicrobiennes, nous devons nous assurer que l'impact sur la flore technologique du fromage Poro, identifiée lors de caractérisation de ce fromage, soit minimal.

2.2. Encapsulation of positive bacteria to promote flavor development in paraffin coated cheese

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Abstract

Flavor encapsulation is now an established technique, but the different methods are associated with significant loss of flavor. This work proposes to study an original alternative, the direct encapsulation of a bacteria able to produce useful aromatic compounds. The effect on the sensorial olfactory perception of alginate beads containing 3-methylbutanal or *Carnobacterium maltaromaticum* LMA28 added to a paraffin coated cheese was tested, and, in addition, the possible antimicrobial repercussions on cheese lactic acid flora were examined. Size, shape and encapsulation efficiency of beads were determined. The bacterial ability to produce 3-methylbutanal and both types of bead capability to diffuse the volatile compound with and without the paraffin coating were also tested. Both types of beads were uniform and spherical with a mean diameter of 1.69±11 mm. Entrapped *C. maltaromaticum* LMA28 is able to produce 3-methylbutanal in sufficient amounts to be diffused through the paraffin coating. Results show bacteria encapsulation as a better alternative to incorporate 3-methylbutanal olfactory notes to the cheese in comparison to the volatile compound direct encapsulation.

Key words: Bacteria encapsulation, 3-methylbutanal flavor notes, *C. maltaromaticum* LMA sensorial characteristics, artisanal cheese

Highlights

- Two possibilities were studied to ameliorate the olfactory characteristics of Poro cheese.
- Bacteria encapsulation results are better than the direct encapsulation of volatile compounds to incorporate the olfactory notes into the cheese.
- Trained judges confirmed improvement between the olfactory characteristics of cheeses having or lacking the bacteria capsules.
- The results contribute to solve a significant problem for Poro cheese producers.

2.2.1. Introduction

Poro cheese is a raw milk handmade product with soft slightly pressed paste; coated with paraffin wax and packed in yellow cellophane, involuntarily matured during its sale period (1 month) (Villegas de Gante, 2004). This Mexican cheese is produced in a tropical region (Los Rios, Tabasco) and as many other traditional cheeses is made from raw milk. Tropical cheeses have physicochemical characteristics such as low pH, high salt concentration and low water activity, this due to the necessity of keep them in a difficult ecological environment which can accelerate cheese ripening, microbial multiplication and off flavor development.

Traditionally Poro cheese is paraffin coated, and even if no food born related flora was found in it, this product presents an off-flavor development, which results in shorter sales period than its shelf life and consequent economic losses. Sensorial attributes are critical for a product identity and consumer's acceptability. Appearance, texture, taste and flavor are the main factors affecting acceptance or rejection rates (Van Hekken et al., 2006, Sancho Valls J. et al., 1999). In addition, Poro cheese have a traditional product status that limits the direct incorporation of food additives to avoid off flavor development, this further complicates the issue.

Coating cheeses is a well-known procedure; as Poro cheese, other cheeses are waxed or film-wrapped to create a barrier against mold entry, reducing the rate of moisture loss, preventing oiling off and therefore making the cheese more attractive and easier to handle (Kampf and Nussinovitch, 2000). The incorporation of active agents into packaging and coating materials is now a well known technology to prevent or minimize chemical, biochemical and microbiological deterioration (Yilmaz and Dagdemir, 2012). By encapsulation, it is possible to incorporate aromatics compounds into the Poro cheese paraffin coating, offering an alternative to avoid modifications in cheese characteristics and manufacturing technology.

Encapsulation is a physicochemical or mechanical process to entrap active components into a material (microorganisms, volatile components, etc.) to preserve or add some functional properties. This technology has multiple applications in food industry; controlling oxidative reactions, masking flavors, colors and odors, providing sustained and controlled release, extending shelf life, among others (Burgain et al., 2011). There are different encapsulation techniques and a long list of materials which may be used, none of these can be considered as a universal, the choice depends on diverse factors such as: encapsulation objective, active compound characteristics and requirements, materials compatibility, toxicology, among others (de Vos et al., 2010). Calcium alginate hydrogels are frequently used for the entrapment of microbial cells, moreover, it is non-toxic, biocompatible

and cheap (Burgain et al., 2011, Park and Chang, 2000). Alginate is a polysaccharide of β-D-manuronic and α-L-glucuronic acids extracted from some algae species.

Two alternatives are possible to incorporate volatile flavor into a paraffin coating. First, we could make the direct encapsulation of the aromatic molecule or, second the encapsulation of a safe bacteria able to produce the desired compound. It is well known that microorganisms play a positive role in cheese ripening, as they provide specific cheese flavors (Irlinger and Mounier, 2009). Among the lactic acid bacteria (LAB), *Carnobacterium maltaromaticum* is a bacterium frequently found in food (meat, fish and dairy products) and has two principal functions; flavor compound production and foodborne pathogens inhibition (Afzal et al., 2010a). *C. maltaromaticum* is capable of producing 3-methylbutanal from the leucine catabolism (Afzal et al., 2013a) as well as antimicrobial peptides (bacteriocins) generally active against phylogenetically close bacteria (Jacquet et al., 2012).

The 3-methylbutanal is an aldehyde commonly used as a food additive due to its flavor notes. It is extremely volatile and slightly soluble in water. In cheese, odor descriptors used are malty, chocolate and caramel (Lacroix et al., 2010) with detection and recognitions thresholds of 0.5 and 1.2 μgL^{-1} of water, respectively (Czerny et al., 2008).

The aim of this work is to improve sensorial characteristics of Poro cheese by incorporating, into the paraffin coating, calcium alginate beads containing (1) directly encapsulated 3-methylbutanal, (2) strains of *Carnobacterium maltaromaticum* LMA28. The flavor incorporation effect on cheese sensorial perception was tested with a cheese model by a panel of experts in aroma evaluation.

2.2.2. Material and methods

2.2.2.1. Encapsulation solutions preparation

A 10^4 µM 3-methylbutanal (Sigma-Aldrich Co, Saint-Quentin Fallavier, France) solution was prepared and mixed with a 2.6% (w/v) sodium alginate (Sigma-Aldrich Co, Saint-Quentin Fallavier, France) solution to obtain a $5 \cdot 10^3$ µM 3-methylbutanal, 1.3% (w/v) alginate encapsulation mix (Mix 1).

Carnobacterium maltaromaticum LMA28 was cultivated at 30°C, in Tryptic Soy Borth-Yeast Estract (TSB-YE) (Biomerieux, Craponne, France and BD, Le Pont des Claix, France respectively) adjusted at pH 6.5 for 24h. Culture was added to a 1.3% (w/v) alginate in TSB-YE media enriched with 2.5 $g \cdot L^{-1}$ of supplementary glucose (Merck Millipore, Darmstadt, Germany) to obtain the encapsulation mix (Mix 2) containing 10^8 $UFC \cdot g^{-1}$ cells concentration. The mixtures were prepared under sterile conditions.

2.2.2.2. Encapsulation

Beads were produced using an extrusion system coupled to a peristaltic pump. The mix solutions were pumped through a 0.3 mm diameter needle into 200 mL of a 1.5% (w/v) $CaCl_2$ (Sigma-Aldrich

Co, Saint-Quentin Fallavier, France) sterile solution. Beads remained in the solution for 10 min for hardening, and then they were recovered by filtrations and rinsed with sterile distilled water.

2.2.2.3. Beads morphology characterization and encapsulation efficiency

Bead size distribution was measured by optical microscopy (Olympus AX70 PROVIS, objective 40). Images were taken with a camera (Olympus DP70) coupled with a microscope. Diameter and a shape factor (ratio between the largest and the smallest capsules diameters) were obtained by image analysis (Visilog 7.1, Noesis, Gifsur Yvette, France) on 20 randomly selected beads from each mix solution.

The encapsulation efficiency for, 3-methylbutanal and *C. maltaromaticum LMA28* beads, were calculated using equations 1 and 2 respectively:

Equation 1 $$EY_{3-Mal} = \left(\frac{C}{Ci}\right) \times 100$$

where Ci is the initial concentration of 3-methylbutanal in the encapsulation solution (Mix1) and C is the 3-methylbutanal concentration measured from the head space of vials containing the Mix 1 beads. The use of 3-methylbuthanal remaining in the hardening $CaCl_2$ solutions, as control was impossible due to their high volatility

Equation 2 $$EY_{Carno} = \left(\frac{N}{Ni}\right) \times 100$$

Where Ni is the number of CFU of *C. maltaromaticum LMA28* in the encapsulation solution (Mix 2) and N is the number of viable CFU in fresh beads.

2.2.2.4. Determination of 3-methylbutanal

The amount of 3-methylbutanal released from the beads was measured from the head space of 10 mL vials containing 1 g of beads using a GC Flash electronic nose (Alpha M.O.S. Heracles II, Toulouse, France). After equilibration (60 min at 40°C), 500µL of aroma headspace from each sample were manually injected into the electronic nose equipped with a capillary column (MXT-5; 10m x 0.18 mm ID x 0.20 µM film thickness; Restek Corporation, Bellefonte, PA, USA) at a speed of 300 µL/s. The column temperature program was 40°C (5 s) – 4 °C/s – 270°C (30 s). The temperature of injector and detector was set at 200°C and 270°C respectively. Used carrier gas was hydrogen. Results were analyzed by Alpha Soft version 12.46 software (Alpha M.O.S., Toulouse, France). Samples were tested in triplicate.

Identification of 3-methylbutanal was accomplished by comparing samples retention time to that of 3-methylbutanal standard and quantification was done by comparing the sample peak area to the peak area of the pure component at a known concentration.

2.2.2.5. Survival of *C. maltaromaticum* LMA28 and 3-methylbutanal production essays

After encapsulation 3-methylbutanal release over the time of Mix 1 beads was determined. 10 mL vials containing 1 g of beads were prepared and stored at 30°C, three vials were taken each 0, 24, 48 and 72 h, and analyzed using the methodology described in section 2.2.2.4.

C. maltaromaticum LMA28 survival and 3-methylbutanal production/release kinetics of Mix 2 beads were followed in two different conditions not paraffin beads (NPB) and paraffin beads (PB). For survival test, beads were conditioned in vials containing 1g of NPB or PB and incubated at 30°C, one vial of each treatment was taken each 0, 24, 48, 72 h, beads were suspended in 9 mL of TS (Tryptone Salt Buffer, Biokar, Beauvais, France), and after appropriate dilution, 1 mL of dilution was used to determine the number of viable cells by plate counting on TSB-YE pH 6.5 in quadruplicate and incubated at 30°C for 48h. Cells concentration were expressed as $CFU \cdot g^{-1}$ of capsule mass. For 3-methylbutanal production/release, 10 mL vials containing 1 g of NPB or PB were prepared and stored at 30°C, tree vials of each treatment were taken each 0, 24, 48 and 72 h, and analyzed using the methodology described in section 2.2.2.4.

2.2.2.6. Bacteria inhibition test

Bacterial inhibition assays were conducted to test the *C. maltaromaticum* LMA28 on the Poro cheese lactic acid technological flora. *C. maltaromaticum* LMA28 beads were deposited in TSA-YE (TSB-YE added with agar bacteriologic type A, Biokar, Beauvais, France) plates (1 g per plat) and incubated for 24h at 30°C, 8 strains including a positive control *Listeria monocytogenes EGDe* lux (Table 12) were tested, 150 µL of each culture strain were inoculated in tubes containing 15 mL of TSA-YE, carefully mixed and poured into the plates containing *C. maltaromaticum* LMA28 beads, after solidification, plates were stored for 24h at 4°C and subsequently incubated 48h at 30°C. Tests were made in duplicate. Presence or absence of inhibition was observed. All the culture strains were taken from the LIBio collection.

2.2.2.7. Sensorial effect

In order to test if beads have a real sensorial effect on cheese, mini-babybel® cheeses were coated with paraffin containing or not the *C. maltaromaticum* LMA28 beads (1 g of beads per cheese),

paraffin temperature was 65°C±2 to minimize the effect of temperature on encapsulated bacteria viability.

Thirty-six coated cheeses were incubated in olfactory test flasks for 48h at 30°C to promote the bacteria growth and 3-methylbutanal production, half of these cheeses were immediately used and the others were stored at 30°C for 1 week.

A trained olfactory panel of 10 members performed sensory analysis. A paired-comparison difference test was applied to determine which of coated cheeses, with or without *C. maltaromaticum* LMA28 beads, has more 3-methylbutanal aroma. Before evaluation, samples coating were fractured, and cheeses in their flask were equilibrated at 40°C for 1h. Two different samples were simultaneously presented to each panelist. A judge was asked to determine if samples were the same or not.

2.2.2.8. Statistical analysis

Values were expressed as means ± standard deviation of triplicate determinations for 3-methylbutanal concentrations, of 20 capsules for size and shape determinations, and of duplicate for cells concentrations.

The significant difference between samples was evaluated using a one-tailed binomial test, for a 10 members panel, the difference between samples was significant if the number of correct answers was 9 ($p<0.05$) or 10 ($p<0.01$) (NF V 09-012, 1983 ISO 5495).

2.2.3. Results

2.2.3.1. 3-methylbutanal encapsulation rate and release

The 3-methylbutanal release rate from beads produced with Mix 1 was determined. The initial 3-methylbutanal concentration of Mix 1 was $5 \cdot 10^3$ µM, and after encapsulation 3-methylbutanal from the head space of vials containing Mix 1 beads was measured over time. The t_0 concentration was 75 µM, 24h later the concentration was 58 µM and after 48h only 3 µM were found (Figure 15).

2.2.3.2. Carnobacterium maltaromaticum LMA28 growth and 3-mehylbutanal production kinetics

To confirm the *C. maltaromaticum* LMA28 ability to produce 3-methylbutanal, the growth and 3-methylbutanal production kinetics of non-encapsulated *Carnobacterium maltaromaticum* LMA28 were followed. The growth kinetic shows a stationary phase starting after 8h that persist over the time at least for 40h while 3-methylbutanal production is never stable, this increases to reach a maximal point at 28h (94 µM) and decrease until 34 µM after 48h (Figure 15).

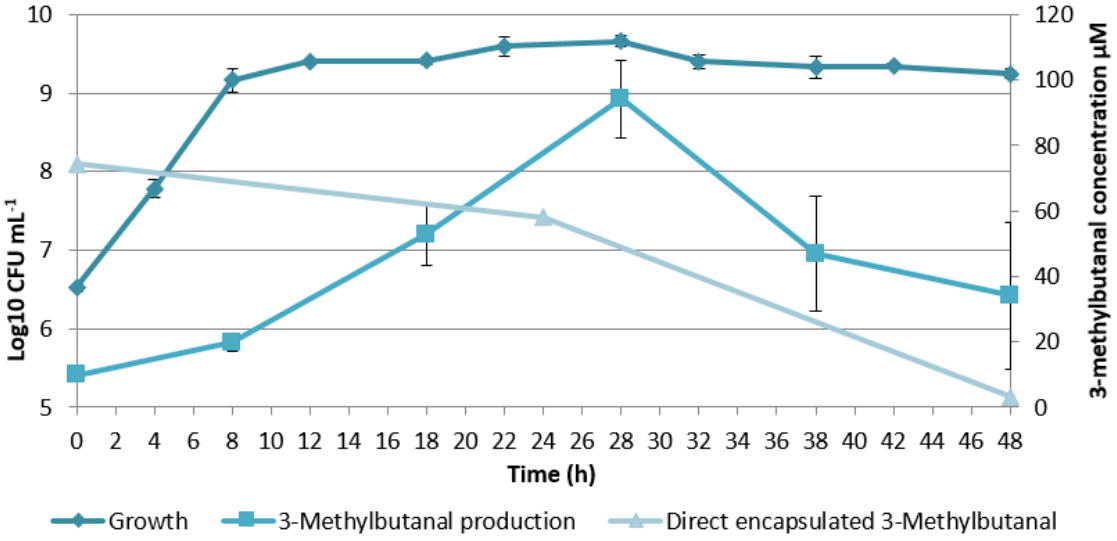

Figure 15. *C. maltaromaticum* LMA28 growing and 3-methylbutanal production kinetics.

2.2.3.3. Encapsulated bacteria growth and 3-mal production/release

Encapsulated *C. maltaromaticum* LMA28 survival and 3-methylbutanal release kinetics were determined with two objectives. First, to determine if the encapsulation could stabilize the bacteria survival. Secondly, to know the effect of alginate encapsulation and paraffin coating in 3-methylbutanal production and release.

Once encapsulated, the cell concentration in 1 gram of *C. maltaromaticum* LMA28 beads was 10^6 UFC. Growth kinetics show a 2.5 log increment in 45 h followed by a stationary phase of at least 25h. Initial 3-methylbutanal beads concentration/release was 24.7 µM, while bacteria is in the exponential phase, 20 to 45h after kinetic initiation, 3-methylbutanal production/release reaches its maximum 52 µM and remains almost constant; after 45h production/release decreases and at 70h is only 26 µM (Figure 15). Even that 3-methylbutanal production/release of encapsulated bacteria seems lower than in free cells, it remains stable for a long period.

As it was mentioned, beads will be used entrapped in the cheese paraffin layer, then 3-methylbutanal production/release of paraffin-coated beads were also determined. The 3-methylbutanal initial concentration was 8 µM and after 8h increase to almost 28 µM attending its maximal level 29.7 µM around 45h of monitoring, at 70h concentration decrease slightly until 23µM (Figure 16).

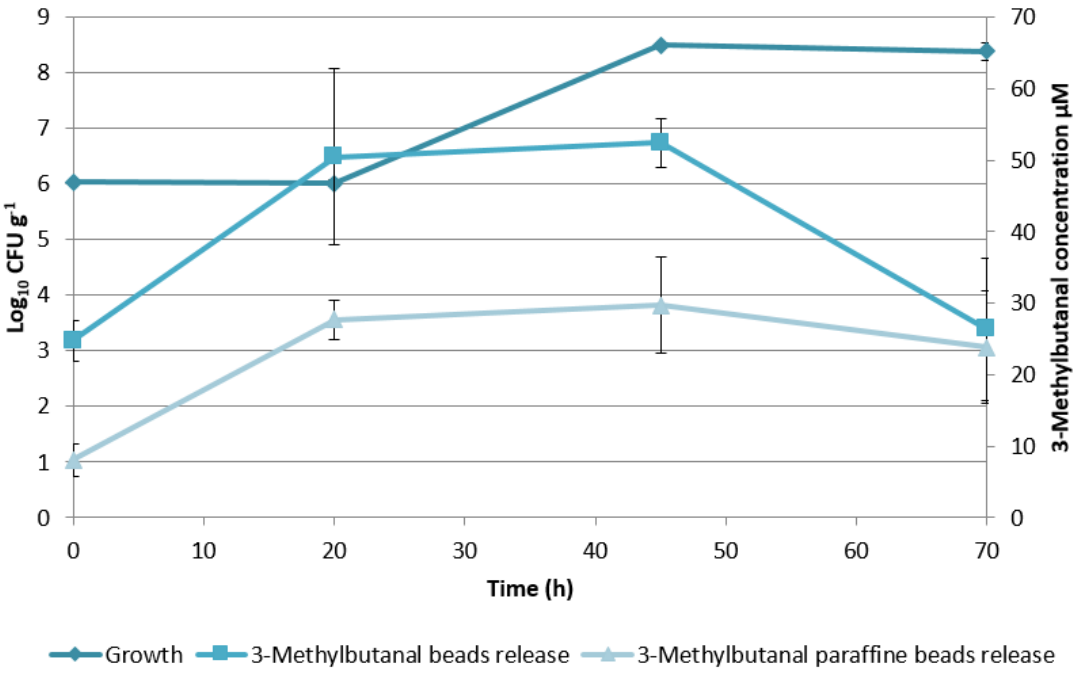

Figure 16. Entrapped *C. maltaromaticum* LMA28 growing and 3-methylbutanal production/release kinetics.

2.2.3.4. Beads characterization and encapsulation efficiency

The 3-methylbutanal concentration in fresh Mix 1 beads was 90 µM·g^{-1} while the Mix 1 initial concentration was 5·10^{3} µM, then the encapsulation efficient of 3-methylbutanal beads was only 1.5%. Furthermore, these losses may be due to the high compound volatility combined with the encapsulation technique. Besides, concerning *C. maltaromaticum* LMA28 encapsulation efficiency, the cells concentration in Mix 2 was 10^{8} UFC·ml^{-1} and the cells concentration in fresh beads was 10^{6} UFC·g^{-1}, at that point encapsulation efficiency was 70.9%.

Optical microscopy image enable us to determine the size and morphology of beads and allowed to confirm the bacteria presence (Figure 17). There is no significant difference between beads ($P<0.05$), both kinds were almost spherical and uniforms, the average diameter and shape factor of these are around 1.69±0.15 mm and 0.86±0.01 respectively.

Figure 17 allows us to observe the bacterial population into the *C. maltaromaticum* LMA28 beads and the absence of cells in 3-methylbutanal once.

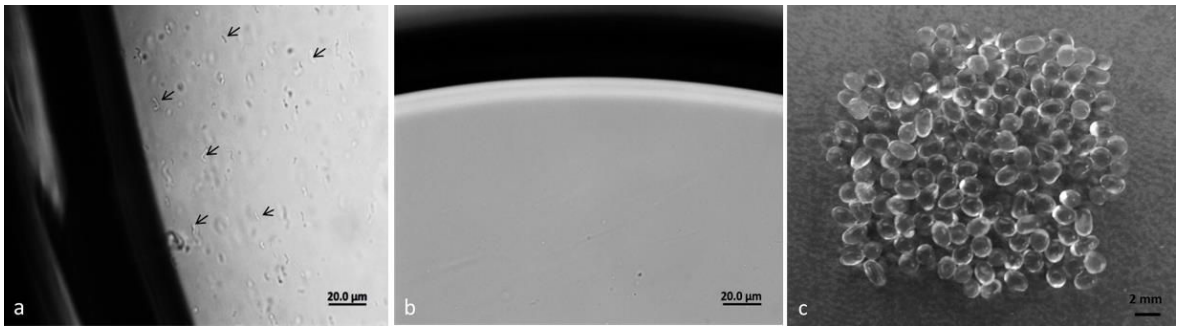

Figure 17. Beads microscope images (a) with bacteria (←), (b) without bacteria (c) whole bead.

2.2.3.5. Effect of C. maltaromaticum LMA28 beads in Poro cheese flora

Due to the Poro cheese tipicity, the *C. maltaromaticum* LMA28 incorporation in Poro cheese coating should not have an important effect in its microflora, and because the known ability of *Carnobacterium* to produce bacteriocins it was important to verify its effect against the principal lactic acid bacteria of Poro cheese, which are responsible of most of its characteristics . Table 12 shows the main LAB isolated from Poro cheese (González Ariceagaa et al., 2014) and antibacterial effect of *C. maltaromaticum* LMA28 beads on each strain. From seven strains, evaluated, only *E. faecium* F1D2 is sensible to the *C. maltaromaticum* LMA28 antimicrobial effect.

Table 12. Tested strains for *C. maltaromaticum* LMA28 antibacterial activity.

Tested LAB	***C. maltaromaticum LMA28* antibacterial activity**
Lactobacillus fermentum F4D10	-
Lactobacillus plantarum F3C2	-
Lactobacillus farciminis F2C1	-
Lactobacillus rhamnosus F2D2	-
Lactobacillus brevis F1C3	-
Lactobacillus pentosus F4D8	-
Enterococcus faecium F1D2	+
Listeria monocytogenes EGDe *lux**	+

* Positive control

2.2.3.6. Sensorial effect

The expert panel did not find significant difference among the paraffin coated cheeses with and without *C. maltaromaticul* LMA28 beads after 48h of incubation. However, after one incubation week at 30°C, 9 out of 10 trained panel members found a difference and chose the cheese whose coating contains the *C. maltaromaticum* LMA28 beads as the one having more 3-methylbutanal

related olfactory notes. It means that there is a significant perception difference ($p<0.05$) attributed to the bacterial beads addition and its 3-methylbutanal production.

2.2.4. Discussion

2.2.4.1. 3-methylbutanal encapsulation rate and release

Flavor encapsulation by extrusion has been used previously (Manojlovic et al., 2008). However, a loss of aromatic molecules was reported. Losses can be related with the molecule diffusion properties and the encapsulating material interaction. In polysaccharide matrix, diffusion increases with the increment in temperature or water content and decrease with the molecular weight decreasing of the volatile compound. Compounds with a higher affinity for the polysaccharide will be better retained (Voilley and Etievant, 2006). Retention is also related to the polarity, such that more polar compounds have lower retention rates; furthermore, compared with other chemical groups, aldehydes are less retained by polysaccharides (Goubet et al., 1998). It is apparent that there is not universal solution, and each new application needs lots of development work.

Considering this information, it is of significant interest to discover another strategy to incorporate the 3-methylbutanal into the beads without losing it during the encapsulation process.

2.2.4.2. *Carnobacterium maltaromaticum* LMA28 growth and 3-methylbutanal production kinetics

It is important to note that the concentration of 3-methylbutanal produced by the bacteria after 48h incubation is even higher than the concentration of direct encapsulated 3-methylbutanal at 0h, this suggests the possibility of encapsulate the bacteria and make them produce the volatile compound once encapsulated.

C. maltaromaticum LMA28 3-methylbutanal production was previously tested by (Afzal et al., 2013a) in other media and conditions. In batch culture with a 90 % of oxygen saturation C. *maltaromaticum LMA28* produce 180 µM of 3-methylbutanal at 15h and after 48h, 3-methylbutanal production also decreases (56 µM), consistent with what we found.

The production of 3-methylbutanal by LAB's is due to their high amino-peptidolytic-activity (Ziadi et al., 2008). This volatile compound is derived from the leucine catabolism. In *C. maltaromaticum LMA28* case, the leucine catabolism can be carried out by two different pathways making these bacteria a better option to the aroma production (Afzal et al., 2012).

2.2.4.3. Encapsulated bacteria growth and 3-methylbutanall production/release

Based on those results, it is posible to infer that, when entrapping bacteria in a nutrient-rich matrix, both kinetics are normalized and the 3-methylbutanal production is guaranteed during a longer time period. This behavior was even valid when beads were paraffin coated. In this case, as expected, 3-methylbutanal release decreased. However, the release continues to be better than when the 3-methylbutanal was direct encapsulated.

The encapsulation objective is usually to protect the active ingredients against the environment. Furthermore this study attempts to provide the entrapped bacteria with nutrients to promote growing and metabolite production. Alginate as other polymers are known to allow throughput of nutrients and metabolite diffusion (Martin et al., 2013). When mixed with the cell culture media, it could be considered like an active packing which provide a stimuling enviroment for cells development (Leonard et al., 2013). Paraffin is one of the best additives to reduce water permeability (Khwaldia, 2010). Besides, it is a good oxygen barrier (Keenan et al., 1999) and in the food industry it has been used as edible film, whitin among other objectives, to retain volatil compounds (Khwaldia, 2010).

2.2.4.4. Beads characterization and encapsulation efficiency

Contrary to what was reported by Kraseakoopt *et al*. (2004), who obtained an efficiency of 99.9% using a pre-existing extrusion technique to entrap *L. acidophilus* in a 2 % alginate matrix, *C. maltaromaticum* LMA28 was less efficient (70.9 %). Other techniques such as emulsification have also been used for bacteria encapsulation with good efficiency rate (Mokarram et al., 2009).

The beads size and shape homogeneity results from the encapsulation protocol were alginate and $CaCl_2$ concentrations as well as the mechanical parameters of the encapsulation system remained constant (Bucko et al., 2005). Success in bacteria immobilization depends on a large number of parameters, material selections being one of the most important (Bucko et al., 2005). Alginate is a linear polysaccharide of D-manuronic and L-glurronic acids that gels in the presence of divalent cations. It has high biocompatibility, low cost and extensively used for cells entrapment (Park and Chang, 2000, Burgain et al., 2011, Rathore et al., 2013). The simplicity of the encapsulation technique and supplies low costs, make this method viable to be used for the artisanal cheese producers.

2.2.4.5. Effect of C. maltaromaticum LMA28 beads in Poro cheese flora

The antibacterial effect of *C. maltaromaticum* results from their bacteriocins production, among these carnobacteriocins (Cbn) B2 and CP5 are active against *E. faecium* but as well as Cbn BM1 these were not active against other LAB such as *L. fermentum* and *L. plantarum* (Jasniewski et al., 2009).

Poro cheese is made exclusively from raw cow's milk. Whey obtained from the previous day cheese production is added to the milk to ensure the presence of a similar microbial population granting Poro cheese its characteristics. Like in other traditional cheeses (Pogacic et al., 2013) LAB microflora in Poro cheese comes from raw milk, whey culture and cheese factory environment. Strains used in this study integrate Poro cheese non-starter lactic acid flora, this type of bacteria generally plays a significant role during cheese ripening (Settanni and Moschetti, 2010) influencing cheese quality, flavor characteristics, peculiarity and authenticity (Steele et al., 2013), thus the importance of minimize the effect of *C. maltaromaticum* LMA28 ant its bacteriocins against Poro cheese technological flora.

C. maltaromaticum LMA28 beads were able to inhibit the *Listeria* strain used as positive control. This is an important advantage since Listeria is one of the most important pathogenic bacteria in relation to raw milk fresh cheeses (Almeida et al., 2013).

2.2.4.6. Sensorial effect

Flavor components contribute to the sensory perception of dairy products, and the quality of these is largely determined by the sensory perception (Smit et al., 2005). Sensory evaluation is a tool to ensure food quality. Sensory discrimination tests permits to demonstrate that samples are perceptibly different, these differences could be overall or attribute differences (Meilgaard et al., 2006). A pair comparison test is an example of discrimination tests that can be used to determine differences between two samples in a particular attribute (NF V 09-012, 1983 ISO 5495). In this research, the studied attributes are those related to 3-methylbutanal sensorial descriptors (malty/chocolate) that allows improving cheese sensorial characteristics.

C. maltaromaticum strains produce a malty/chocolate-like aroma resulted of the 3-methylbutanal production from the leucine catabolism (Afzal et al., 2010a), *C. maltaromaticum* LMA28 was previously identified as an efficient 3-methylbutanal producer (Afzal et al., 2012). The sensorial effect of *C. maltaromaticum LMA28* inoculation on 20-34 ripening days cheese was already tested by Afzal *et al.*(2013b). In this study the panel found a significant difference ($p<0.05$) in taste and odor highlighting the presence of malty and butyric flavor in cheeses. In this research, encapsulation allows the bacteria growing and aroma production, avoiding the direct contact between cheese and bacteria, and the consequent modification of the cheese ecosystem. Furthermore, aroma production

contribute to improve our model cheese olfactory characteristics, this improvement will probably be transposable to Poro cheese.

2.2.5. Conclusion

Off-flavor development is a relevant problem to Poro cheese small producers who could obtain better economic benefits by increasing the sales period of their product. As Poro cheese is a traditional product contesting to obtain a Mexican designation of origin (DO), food additives that prevent off-flavor development cannot be incorporated into the cheese.

The incorporation of desirable olfactory notes into the Poro cheese paraffin coating is a potential solution to the problem. To reach this goal, we decided to use encapsulating technology. 3-methylbutanal was directly encapsulated in alginate by dropping, however, deceptively; encapsulation efficiency and 3-metylbutanal release rates were not sufficiently high. With this concern as a starting point, we found a new alternative to incorporate the volatile compound into the paraffin coating, using the encapsulation of *C. maltaromaticum* LMA28, a strain well known in the research group, and were able to produce 3-methylbutanal.

This study shows that the *C. maltaromaticum LMA28* entrapment is a better alternative to control de 3-methylbutanal release during time in comparison with the 3-methylbutanal direct encapsulation. Due to the molecule volatility, it seems easier to produce it after the encapsulation process. Additionally, this ensures its extended release. Even if the encapsulated bacteria are known for its antibacterial ability, their bacteriocin diffusion has a minimal effect in Poro cheese lactic acid flora. Results also showed that 3-methylbutanal production and diffusion occurs even when beads are paraffin coated, obtaining a significant effect on the sensory characteristics of a cheese; thus satisfy the purpose of this active package.

Calcium alginate microencapsulation is a fast and simple encapsulation technique; the used materials are cheap and easily accessible, making the process viable to be implemented by the artisanal cheese producers.

Besides the advantage solution for Poro cheese producers, the originality of this research finding lies in the bacterial encapsulation, not in its probiotic interest or antibacterial properties, but it attempts to explore its potential as a producer of aromatic compounds.

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3. *C. maltaromaticum* en tant qu'agent anti *L. monocytogenes*

3.1. Introduction

Comme cela a été évoqué dans l'article précédent, *C. maltaromaticum* est aussi connu pour ses propriétés antibactériennes, et notamment anti-Listeria. En effet, certaines bactéries ont la propriété d'inhiber d'autres bactéries phylogénétiquement similaires.

Le fromage mexicain est produit artisanalement et à partir de lait cru, il constitue donc un excellent candidat pour la prolifération de *L. monocytogenes*, une souche pathogénique bien connue et capable de croître même dans des conditions extrêmes. Elle est d'ailleurs responsable de la listériose (méningites, encéphalites, septicémies, avortements spontanés, …) chez l'animal comme chez l'homme.

Les propriétés anti-Listeria de *C. maltaromaticum* peuvent représenter un autre avantage vis-à-vis de son incorporation dans l'enrobage du fromage. Elle pourrait en effet être capable d'inhiber des colonies de Listeria présentes dans le fromage ou bien d'empêcher leur contamination lors de la commercialisation.

L'objectif de cette étude est d'évaluer l'activité inhibitrice de *C. maltaromaticum* LMA28 isolée d'un fromage à pâte molle contre *L. monocytogenes*. Dans un première temps des milieux de culture (TSB-YE et lait écrème) précédemment contaminés avec Listeria ont été utilisés. Pour la suite, nous devrions envisager de recommencer les expériences en utilisant des fromages modèles.

3.2. Effect de *C. maltaromaticum* LMA28 sur l'inhibition de Listeria

3.2.1. Introduction

En technologie fromagère, les bactéries lactiques (LAB) sont impliquées dans un grand nombre de procédés, permettant la production de différents fromages à partir du lait. Ces LAB proviennent généralement de levains commerciaux mais peuvent être également naturellement présentes dans le lait cru. Parmi ces bactéries, l'espèce *Carnobacterium maltaromaticum* a été mise en évidence dans un grand nombre de fromage français (Appellation d'Origine Protégée, AOP) après affinage et stockage au froid sans que cela affecte la qualité du produit fini (Millière et al., 1994).

Ces dernières années, des souches de *Carnobacterium maltaromaticum* ont été utilisées comme agent protecteur contre des bactéries pathogènes telle que *Listeria monocytogenes* dans le poisson ou les produits carnés (Duffes et al., 1999, Vaz-Velho et al., 2005, Brillet et al., 2005, Matamoros et al., 2009, Vescovo et al., 2006).

Listeria monocytogenes est une bactérie ubiquitaire largement présente dans l'environnement et qui peut causer des troubles sévères chez l'homme par l'ingestion de denrées contaminées. (Almeida et al., 2013). C'est une bactérie capable de croître à des températures comprises entre 1 et 44°C, à un pH de 5 ou plus, en fort environnement salin et possède aussi une résistance relative aux procédés de congélation et de séchage. Agent pathogène majeur, responsable de la listériose chez l'animal comme chez l'homme. Elle peut être à l'origine de méningite, encéphalite, septicémie, avortements spontanés ou naissances prématurées. Les personnes à risque sont les groupes classiquement identifiés, c'est-à-dire les prématurés, les personnes âgées, les femmes enceintes ou les patients immunodéprimés (Arslan and Ozdemir, 2008). L'objectif de cette étude est d'évaluer l'activité inhibitrice de *C. maltaromaticum* LMA28 isolée d'un fromage à pâte molle contre *L. monocytogenes* dans l'industrie fromagère.

3.2.2. Matériels et méthodes

3.2.2.1. Détermination des conditions optimales pour l'inhibition de *L. monocytogenes* dans un milieu TSB-YE et lait écrémé

Afin d'étudier les conditions de cultures optimales pour l'inhibition de *L. monocytogenes* par *C. maltaromaticum* LMA 28, un plan d'expérience a été réalisé, basé sur une matrice de Doehlert de 4 facteurs (X) et différents niveaux (Tableau 13).

Table 13. Plan d'expériences (matrice Doehlet) pour 4 facteurs (X) (*C. maltaromaticum* LMA28, NaCl, pH et durée) pour vérifier la réponse de croissance, exprimée en Log10N R de *L. monocytogenes* CIP 82110 dans du milieu TSB-YE et dans du lait écrémé.

	Facteurs			
Expérience No.	***C. maltaromaticum* LMA 28 (log_{10})**	**NaCl (g/L)**	**pH**	**Temps (h)**
1	8.0	4.50	7.00	48
2	0.0	4.50	7.00	48
3	6.0	9.00	7.00	48
4	2.0	0.00	7.00	48
5	6.0	0.00	7.00	48
6	2.0	9.00	7.00	48
7	6.0	6.00	8.50	48
8	2.0	3.00	5.50	48
9	6.0	3.00	5.50	48
10	4.0	7.50	5.50	48
11	2.0	6.00	8.50	48
12	4.0	1.50	8.50	48
13	6.0	6.00	7.38	72
14	2.0	3.00	6.62	24
15	6.0	3.00	6.62	24
16	4.0	7.50	6.62	24
17	4.0	4.50	8.13	24
18	2.0	6.00	7.38	72
19	4.0	1.50	7.38	72
20	4.0	4.50	5.87	72
21	4.0	4.50	7.00	48
22	4.0	4.50	7.00	48
23	4.0	4.50	7.00	48
24	4.0	4.50	7.00	48

Dans un cas, les expériences ont été effectuées en utilisant un milieu de culture classique (TSB- YE), dans l'autre dans du lait écrémé UHT.

Le milieu de culture pour chaque expérience a été inoculé avec une concentration initiale de 10^3 UFC.mL^{-1} de *L. monocytogenes* CIP 82110 et incubé à 25°C pendant différentes durées déterminées par le plan d'expérience. Après la période d'incubation, des milieux de culture sélectifs ont été utilisés pour la numération de *C. maltaromaticum* (MCM, milieu de culture à base de bouillon de soja trypticase et extrait de levure spécialement conçu pour la numération de *C. maltaromaticum* (Edima et al., 2007) et *L. monocytogenes* (PALCAM, milieu formule par Nette et al. 1985, pour la différenciation et l'isolement de *L. monocytogenes* dans les produits alimentaires tels que le lait et le fromage). Le facteur de réponse de croissance de *L. monocytogenes* CPI 82110 est exprimé en Log10N et peut être prédit dans toutes les régions expérimentales du plan d'expérience selon l'équation 3 :

Equation 3

$$R\ (Log_{10}N) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1X_2 + \beta_{13} X_1X_3 + \beta_{14} X_1X_4 + \beta_{23} X_2X_3 + \beta_{24} X_2X_{24} + \beta_{34} X_3X_4$$

Où β_0 est le terme constant, β_1 détermine l'influence de la concentration de *C. maltaromaticum* dans l'inoculum, β2 l'influence de la concentration en NaCl, β_3 l'influence du pH et β_4 l'influence de la durée. β_{12} représente l'interaction entre la concentration en *C. maltaromaticum* et celle en NaCl; β_{13}, l'interaction entre *C. maltaromaticum* et le pH; β_{14} l'interaction entre la concentration en *C. maltaromaticum* et la durée; β_{23} l'effet d'interaction entre la concentration en NaCl et le pH; β_{24} effet d'interaction entre la concentration en NaCl et la durée et enfin, β_{34} l'effet d'interaction entre le pH et la durée. β_{11}, β_{22}, β_{33}, β_{44} sont des paramètres de forme. L'analyse des données, l'analyse de la variance (ANOVA) et les régressions polynomiales ont été réalisées en utilisant le programme NEMROD-W (LPRAI, Marseille, France).

3.2.2.2. Inhibition de *L. monocytogenes* dans un fromage modèle

Du fait de l'intérêt industriel de *C maltaromaticum* dans l'industrie fromagère, il est intéressant de déterminer son potentiel inhibiteur sur *L. monocytogenes* dans le fromage.

Un fromage modèle le Babybel® (24% matière grasse, 23% protéines, 0.7g sodium), fromage à pâte pressée, enrobé de paraffine a été choisi. Les fromages ont été séparés de manière aseptique de leur enrobage de paraffine puis ont été mécaniquement homogénéisés avec une solution tampon citrate (100 ml). Des dilutions décimales ont été utilisées pour inoculer des boîtes de Petri avec un milieu

sélectif MCM (*C. maltaromaticum*) et PALCAM (*L. monocytogenes*). Les boîtes ont été incubées à 30 °C pendant 48 h, avant dénombrement des Unités Formant Colonie (UFC).

Pour étudier l'effet de la couche de paraffine sur la prolifération de *L. monocytogenes* dans le modèle, les fromages ont été séparés de leur couche de paraffine en conditions stériles puis ont été contaminés par *Listeria monocytogenes* EGDe *lux* à une concentration de 10^5 UFC. 50 % des fromages contaminés ont été à nouveau recouvert de paraffine. Les fromages ont ensuite été placés en incubation à 30°C pendant 24h. Enfin, une numération a été effectuée sur chaque fromage comme précédemment indiqué.

3.2.3. Résultats et Discussion

3.2.3.1. Détermination des conditions optimales pour l'inhibition *de L. monocytogenes* dans un milieu TSB-YE et lait écrémé

L'influence de 4 paramètres expérimentaux (i.e. la concentration en *C. maltaromaticum* LMA 28, la concentration en NaCl, le pH and le temps) sur la croissance de *L. monocytogenes* CIP 82110 a été étudiée en utilisant une matrice de Doehlert afin de rechercher les conditions optimales d'inhibition de cette souche dans un milieu TSB-YE et dans du lait. Il en résulte une équation polynomiale présentée en Table 14. Que ce soit dans le milieu TSB-YE ou dans le lait, les coefficients de l'équation polynomiale (β_1 = - 3.62; - 1.547) permettent de conclure que la concentration en *C. maltaromaticum* LMA 28 influence positivement la réponse de la croissance de *L. monocytogenes* CIP 82110 dans les deux milieux de culture testés.

Dans le milieu TSB-YE, les coefficients de l'équation polynomiale correspondant au pH était également significatif (β_3 = - 1.639) montrant une diminution d'UFC soit une action inhibitrice sur la croissance de la souche. En revanche, dans le lait, la concentration en NaCl, le pH et le temps influencent peu la réponse de *L. monocytogenes* CIP 82110, mais que les interactions entre ces facteurs sont significatives et représentées par des coefficients négatifs dans l'équation polynomiale.

Les conditions optimales pour l'inhibition de *L. monocytogenes* CIP 82110 dans le TSB-YE et dans le lait sont présentées sous la forme d'un graphique en 2D en Figure 18. Ainsi, dans le milieu TSB-YE, en maintenant la concentration en NaCl et le temps d'incubation constants, la réponse d'inhibition de croissance la plus importante de *L. monocytogenes* CIP 82110 peut-être prédite par une augmentation de la concentration de *C. maltaromaticum* LMA 28 et du pH (Figure 18b). De plus, dans le lait, à pH et temps constants, la réponse maximale d'inhibition de *L. monocytogenes* CIP 82110 peut être obtenue par l'augmentation de la concentration couplée à une diminution de la concentration en NaCl (Figure 18a').

L'étude de l'influence des 4 paramètres étudiés sur la croissance de *L. monocytogenes* a révélé que la concentration en *C. maltaromaticum* représentait le principal facteur d'inhibition, en résultant la diminution de la réponse de croissance de *L. monocytogenes* que ce soit dans le TSB-YE comme dans le lait. En outre, les autres facteurs montrent également avoir une influence sur la croissance de la souche. Le mécanisme exact d'inhibition n'est toutefois pas encore élucidé et mériterait des études complémentaires. On peut supposer qu'il est relatif à l'effet de compétition entre substrats/nutriments, des changements de composition du milieu nutritif, l'influence du pH, de l'oxygène et à la libération de substances anti-microbiennes ciblant les cellules compétitives (Leroy and De Vuyst, 2001).

L'effet antimicrobien entre bactéries peut être relié à des interactions métaboliques entre eux-mêmes. Ces interactions constituent un ensemble complexe de phénomènes biologiques hétérogènes. Ces phénomènes ont été classés en : compétition, amensalisme, antagonisme, neutralisme, commensalisme et mutualisme ou protocoopération (Monnet et al., 2008). L'effet de *C. maltaromaticum* sur *L. monocytogenes* peut être dû à un antagonisme, lutte réciproque des deux populations par la production de molécules inhibitrices, comme les bactériocines (Monnet et al., 2008).

Différents modèles prédictifs ont été mis au point pour étudier l'influence de bactéries dans le milieu de croissance et dans les systèmes alimentaires comportant des cocktails bactériens (Malakar et al., 1999, Gimenez and Dalgaard, 2004), en mono et co-cultures (Antwi et al., 2008, Antwi et al., 2007, Charlier et al., 2009, Cornu et al., 2011). Par conséquent, la mise au point d'un modèle semble être un outil intéressant pour prédire les phénomènes d'inhibition de *L. monocytogenes* par différents facteurs et dans différents milieux de culture ou systèmes alimentaires.

3.2.3.2. Inhibition de L. monocytogenes dans un fromage modèle

Dans les expériences réalisées sur le fromage modèle, aucune colonie n'a été retrouvée sur les milieux sélectifs de *Listeria* ou de *Carnobacterium* écartant la présence de ces deux souches dans le fromage modèle.

La numération finale sur les fromages contaminés, recouverts ou non de paraffine, a montré une diminution de 2 unités logarithmiques par rapport à la concentration de *Listeria* inoculée initialement. Ce résultat remet en question la pertinence d'utiliser le Babybel comme fromage modèle pour *Listeria* puisqu'il semble présenter naturellement une activité inhibitrice ou de retard de croissance sur la souche pathogène.

Table 14. Estimation des coefficients des différents modèles quadratiques pour les quatre variables, *C. maltaromaticum* LMA 28, NaCl, pH et la durée de la matrice Doehlert pour la réponse de croissance (R) de *L. monocytogenes* CIP 82110 dans TSB-YE et le lait écrémé.

		Milieu de culture	
Facteur	**Termes**	**TSB-YE**	**Lait écrème**
Coefficient constant	β_0	5.78***	5.455***
C. maltaromaticum ($Log_{10}N$)	β_1	-3.62***	-1.547***
NaCl (g/L)	β_2	0.711	0.038
pH	β_3	-0.639**	0.634**
Temps (h)	β_4	0.046	0.318
Carnobacterium^2	β_{11}	0.435	0.390
$NaCl^2$	β_{22}	0.205	-0.323
pH^2	β_{33}	0.680	-O.859*
Temps ^{2}	β_{44}	0.466	0.140
Carnobacterium·NaCl	β_{12}	0.641	-0.185
Carnobacterium·pH	β_{13}	-1.598	-0.418
Carnobacterium· Temps	β_{23}	0.470	1.356*
NaCl·pH	β_{14}	1.576	-0.609
NaCl· Temps	β_{24}	-0.763	-1.72*
pH· Temps	β_{34}	-1.237	-1.341*

Les réponses ont été représentés par l'équation polynomiale : $R\ (Log_{10}N) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1X_2 + \beta_{13} X_1X_3 + \beta_{14} X_1X_4 + \beta_{23} X_2X_3 + \beta_{24} X_2X_{24} + \beta_{34} X_3X_4$

Significativité : ***≤1%, **<1%, * ≤5%

Cao-Hoang et al. 2010 ont étudié l'effet anti-*Listeria* de films de caséinate de sodium enrichis en nisine et ont également utilisé le Babybel® comme fromage témoin. Cette étude semble montrer un potentiel d'application de films antimicrobiens pour protéger le produit de *Listeria*. Toutefois, il apparaît qu'il existe une différence minime dans la numération de population de *Listeria* entre les fromages testés avec ou sans film de caséinate de sodium. Cela pose la question de savoir si l'effet inhibiteur provient du film anti-microbien ou d'une capacité intrinsèque du fromage choisi comme modèle.

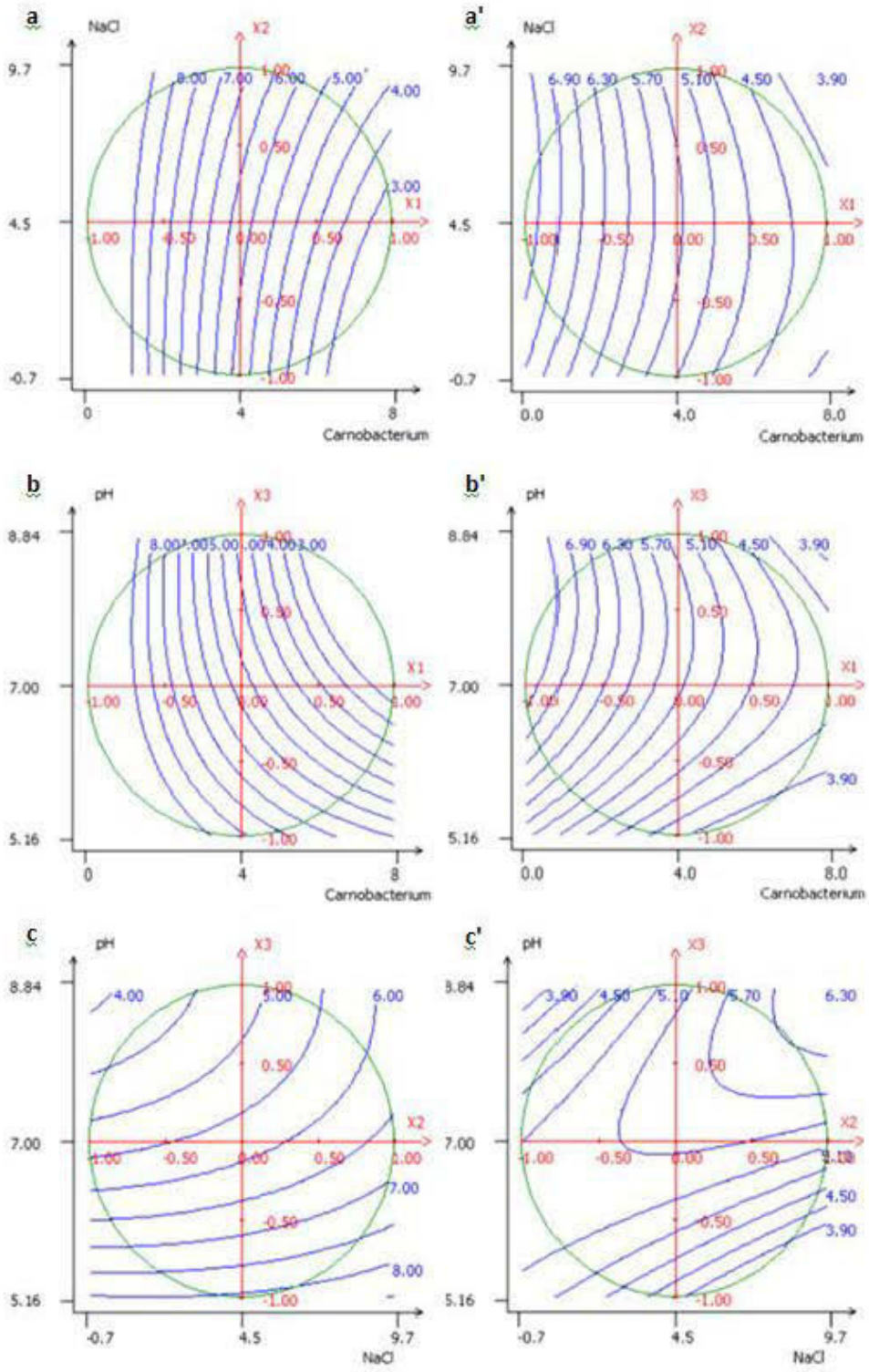

Figure 18. 2D représentation graphique de la réponse optimale de l'inhibition (R) TSB-YE (a, b et c) et le lait (a ', b' et c ') du *L. monocytogenes* CIP 82110. Réglage de facteurs : pH et la durée = (a et a'), NaCl et durée (b et b') et *C. maltaromaticum* LMA2 et durée (c et c'). (pH = 7, NaCl = 4,5 gL^{-1} et longueur = 48h, au centre du domaine).

3.2.4. Conclusions et perspectives

L'effet de quatre facteurs (*C. maltaromaticum*, concentration en NaCl, pH et temps) et de leurs interactions sur la croissance de *L. monocytogenes* inoculée dans deux milieux (TSB-YE et lait écrémé) a été étudié.

La concentration de *C. maltaromaticum* LMA28 s'est révélé être le facteur déterminant pour obtenir une inhibition de *L. monocytogenes* dans les deux milieux de culture testés.

Cependant, le mécanisme exact d'inhibition est encore inconnu et mériterait d'autres investigations Il n'a pas été possible de déterminer l'inhibition de *Listeria* dans le fromage modèle choisi (Babybel), et ceci principalement en raison de l'absence de croissance de *Listeria* même sur le fromage seul.

Le choix d'un nouveau modèle de fromage qui permettrait la prolifération de colonies de *Listeria* serait nécessaire pour obtenir des résultats permettant d'affirmer ou d'infirmer le potentiel inhibiteur de *C. maltaromaticum* LMA28 sur *Listeria* en milieu alimentaire.

Pour chaque nouveau fromage modèle testé, il serait souhaitable de mettre au point un nouveau plan d'expérience pour obtenir plus d'informations sur les facteurs influençant l'inhibition (*C. maltaromaticum* LMA28, la température et la durée de stockage).

Chapitre 4. Conclusions et perspectives

1. Conclusions et perspectives

Ces travaux de thèse portent sur l'étude pluridisciplinaire (biochimique, physicochimique, microbiologique) de l'amélioration des qualités sensorielles et, plus spécifiquement, aromatiques du fromage Poro afin d'en allonger sa durée de commercialisation sur le marché mexicain. Le Poro est un fromage artisanal au lait cru de saveur douce, de forme prismatique et rectangulaire et de petit format (250 g). Pour sa conservation, il est recouvert de paraffine et emballé dans un papier cellophane jaune.

Des études préliminaires ont montré que le principal facteur influençant les propriétés sensorielles du fromage pendant sa période de consommation est le développement rapide de notes olfactives liées à la flore d'affinage du fromage (propionique, acétique). Ces dernières influencent négativement la décision d'achat des consommateurs traditionnellement habitués à la consommation de fromage frais avec des notes olfactives douces (acide lactique).

En nous basant sur ces constatations, nous avons établi une stratégie pour apporter de nouvelles notes olfactives au fromage. Ces nouvelles notes aromatiques devraient améliorer l'acceptabilité du fromage et permettre d'accroitre sa période de vente et de consommation, et ce, tout en veillant à respecter le caractère typique et artisanal du fromage, candidat à l'obtention d'une dénomination d'origine (DO) l'équivalant mexicain de l'AOP française.

Dans un premier temps, nous nous sommes intéressés à l'un des principaux composés volatils associés à des descripteurs olfactifs de fromages à pâte molle. Le 3-methylbutanal, aldéhyde à chaîne ramifiée est, en effet, associé au caractère typique de nombreuses variétés de fromages durs ou semi-durs. Il est souvent associé à des flaveurs maltées, chocolatées ou de noisette. Ce composé provient de la voie de catabolisme de la leucine.

Différentes stratégies permettent l'addition de ces nouvelles notes olfactives dans le fromage. Afin de ne pas intervenir sur le procédé de fabrication, nous avons décidé de modifier la composition de l'enrobage de paraffine, par l'incorporation de capsules contenant le composé volatil d'intérêt.

Des nombreux travaux ont été réalisés sur l'encapsulation des composés aromatiques. L'encapsulation a, en effet, de nombreuses possibilités d'applications industrielles pour permettre d'améliorer les propriétés organoleptiques d'un produit. Le spray-drying est le procédé le plus utilisé pour encapsuler des composés aromatiques dans les aliments. Néanmoins, il est important de tenir compte du fait que les résultats de cette recherche devront être applicables dans les petites fromageries productrices de Poro. De ce fait et compte tenu des facteurs économiques et des difficultés technologiques engendrées par la plupart des méthodes d'encapsulation, nous avons

choisi une technique d'encapsulation plus simple, ne nécessitant aucun appareillage coûteux, l'extrusion au goutte à goutte de billes d'alginate.

Les conclusions et perspectives de cette recherche portent sur les 3 axes de notre étude : La caractérisation du fromage Poro, l'amélioration des caractéristiques olfactives du fromage et enfin, l'utilisation de la souche d'intérêt *C. maltaromaticum* LMA28 pour lutter contre la prolifération de bactéries pathogènes (*L. monocytogenes)*.

Caractérisation de fromage Poro

Concernant la caractérisation de notre fromage, les propriétés physicochimiques montrent que le Poro peut être considéré comme un fromage à pâte molle. Les valeurs obtenues peuvent être considérées comme des valeurs typiques pour ce type de produit. Les écarts mesurés sont associés à des variations dans le processus de fabrication du fromage ainsi que dans sa composition et celle du lait utilisé.

Avec l'aide de l'évaluation sensorielle, l'acceptabilité globale et l'acceptabilité d'attributs spécifiques du fromage Poro ont été évaluées par les producteurs et leurs familles (personnes connaissant particulièrement bien les caractéristiques typiques du produit pouvant être considérées comme des experts) afin de permettre la construction d'une carte des préférences internes pour le fromage Poro. Dans cette cartographie, un score élevé pour un descripteur de flaveur est lié à une plus grande acceptabilité du produit, cette conclusion nous a confortés dans notre choix de développer le caractère aromatique du produit. A l'inverse, des scores élevés pour l'élasticité, l'humidité et la résolution des couches (homogénéité de la pâte du fromage) sont liés à une moindre acceptabilité.

Aucune différence significative n'a été mise en évidence entre les fromages au niveau de l'acceptabilité globale lors de l'évaluation par les consommateurs habituels. Cependant les producteurs du fromage Poro ont relevé une différence dans les caractéristiques sensorielles avec une préférence marquée pour les fromages codés QP4.

Au niveau microbiologique, les fromages artisanaux ne présentent aucune trace de contamination fécale (coliformes) attestant d'une bonne qualité sanitaire du produit. L'identification des principales souches de bactéries lactiques ont permis de mettre en évidence une homogénéité de la population bactérienne en présence (*L. plantarum, L. pentosus, L. farciminis, L. rhamnosus*), la plus représentée étant *L. plantarum*.

Les données obtenues à partir de cette étude constituent une bonne caractérisation du fromage Poro. Ces paramètres seront des critères objectifs qui contribueront à soutenir son processus de demande d'appellation d'origine. Malgré cela, de nombreuses questions restent à élucider. Comme exposé précédemment, le fromage Poro suit un processus pour l'obtention d'une appellation

d'origine (DO). Pour cela, il est nécessaire d'intégrer un dossier contenant une description détaillée du produit (caractéristiques, composants, délimitation de territoire, liens entre produit, territoire et appellation, … (IMPI, 2013)). Plusieurs études se sont attachées à caractériser les facteurs qui apportent la typicité à ce fromage tels que le terroir, les caractéristiques physicochimiques, microbiologiques ou encore sensorielles. Cependant, il y a encore un long chemin à parcourir et beaucoup de paramètres à considérer avant d'obtenir cette appellation d'origine. Il sera, par exemple, nécessaire d'élucider le rôle de la flore microbienne du fromage Poro sur chacune de ses caractéristiques sensorielles (texture, arôme, goût). En effet, les fromages artisanaux présentent des caractéristiques attribuées à la présence de micro-organismes et qui contribuent à leur typicité. Le potentiel de ce produit pour sa commercialisation au-delà de sa région de production et à l'étranger dépend fortement de ses qualités organoleptiques au cours du vieillissement ainsi que de la capacité à garantir la sécurité sanitaire pour le consommateur. La possibilité d'utiliser un cocktail spécifique de bactéries dans le fromage Poro permettrait la création d'une version industrielle du fromage produit à partir de lait pasteurisé.

Amélioration des caractéristiques olfactives du fromage

Les caractéristiques du fromage étant connues, il s'agit dans un second temps d'améliorer l'apparition de défauts olfactifs au cours de sa conservation. Pour cela, deux voies possibles ont été étudiées : l'encapsulation directe de 3-méthylbutanal et l'encapsulation d'une bactérie productrice du composé en question. Le 3-méthylbutanal a été encapsulé dans de l'alginate par extrusion mais, malheureusement, l'efficacité d'encapsulation comme la libération du 3-métylbutanal, se sont avérées insuffisamment performantes. La seconde méthode utilisée a été l'encapsulation de *C. maltaromaticum* LMA28, une souche bien connue de l'équipe du laboratoire et capable de produire du 3-méthylbutanal dans des conditions maîtrisées.

Une fois *C. maltaromaticum* encapsulée, la production / libération de 3-méthylbutanal atteint son maximum après 20h et reste constante jusqu'à 45h. Après ce temps, la production / libération de 3-méthylbutanal commence à diminuer mais reste suffisante. Dans le cas de *C. maltaromaticum* incluse dans l'enrobage de paraffine, la production/libération atteint son maximum après 45h et décroit graduellement en restant aussi supérieure au taux obtenu par encapsulation directe du composé.

Les résultats montrent que l'encapsulation de *C. maltaromaticum* LMA28 est une meilleure alternative pour la libération de 3-méthylbutanal par rapport à l'encapsulation directe du 3-méthylbutanal.

D'après les résultats présentés précédemment, il apparaît qu'en piégeant la bactérie productrice dans un milieu nutritif riche, la production de 3-méthylbutanal se fait sur une plus longue période et

ce, même si les billes sont incluses dans la paraffine d'enrobage. Dans ce cas, toutefois, comme on pourrait s'y attendre, on assiste à une diminution de la libération du composé volatile qui reste néanmoins supérieure aux résultats obtenus par encapsulation directe de 3-méthylbutanal. En effet, du fait de la grande volatilité de cette molécule, il apparaît qu'il est plus facile et efficace de produire la molécule après encapsulation, ce procédé assurant, en outre, sa libération prolongée.

La micro-encapsulation dans l'alginate de calcium est une technique d'encapsulation rapide, simple, économique et facile à mettre un œuvre même pour de petites productions artisanales. Outre le fait d'être avantageuse pour les producteurs de fromage Poro, l'originalité de cette méthode réside dans l'encapsulation de bactéries, non pas en tant que probiotiques ou pour leurs propriétés antimicrobiennes, mais pour explorer leur potentiel en tant que producteur de composés aromatiques.

Les résultats ont montré que la production de 3-méthylbutanal ainsi que sa diffusion se produisent même lorsque les billes sont recouvertes de paraffine, entraînant une modification significative et positive des caractéristiques sensorielles du fromage.

Concernant les expériences sensorielles menées sur le fromage modèle Babybel® qui ont démontré une différence entre la perception sensorielle des fromages enrobés contenant ou pas *C. maltaromaticum* LMA28, il serait intéressant de réitérer les essais sur le fromage Poro pour intégrer les caractéristiques olfactives intrinsèques de ce fromage très typique en comparaison à l'odeur très neutre et peu complexe du Babybel®.

Concernant l'originalité de cette étude, il faut pointer l'aspect innovant d'encapsuler des bactéries productrices de composés volatils plutôt que d'encapsuler le composé directement. Il pourrait être intéressant d'évaluer cette technologie en utilisant d'autres bactéries lactiques produisant des molécules volatiles d'intérêt industriel, *Lactococcus lactis,* par exemple, connue pour sa capacité à produire du diacétyle.

Ayant montré l'intérêt de ces travaux de micro-encapsulation par extrusion à l'échelle pilote, il serait aussi nécessaire et intéressant de projeter l'application possible à plus grande échelle. D'autres méthodes d'encapsulation pourraient être testées permettant la production de plus grands volumes de capsules ou l'utilisation de matrices compatibles avec les matériaux d'enrobage (paraffine) sans pour autant négliger la viabilité des cellules encapsulées. Dans ce contexte, des essais préliminaires ont été réalisés. Des capsules contenant du 3-methylbutanal enrobés dans de la cire (carnauba) ont été produites par coextrusion. Les capsules obtenues ont une forme sphérique et compacte et une taille uniforme (Figure 19).

Se pose également la question du risque sanitaire du fait de la présence de milieu de culture dans la matrice d'encapsulation, ce qui pourrait faciliter le développement d'autres bactéries et la possible contamination du fromage.

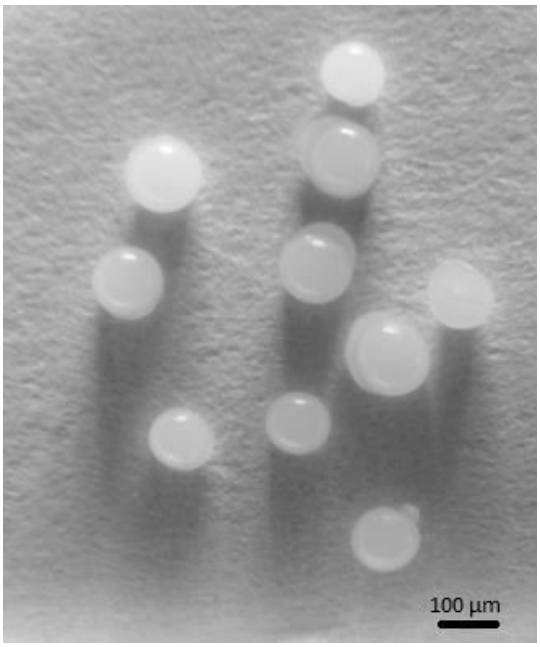

Figure 19. Capsules produites par co-extrusion.

C. maltaromaticum LMA28 & effet antibactérien

Comme nous l'avons évoqué précédemment, *C. maltaromaticum* est capable d'inhiber certaines bactéries phylogénétiquement similaires comme *L. monocytogenes*, un micro-organisme pathogène connu comme étant responsable d'intoxications alimentaires avec des fromages au lait cru. Lutter contre la prolifération de *L. monocytogenes* pourrait donc aussi être un avantage de cette étude.

Même si les bactéries encapsulées sont connues pour leur capacité antibactérienne due à la production de bactériocines, nos résultats ont montré que leur diffusion a un effet minime sur la flore lactique du fromage Poro.

Nous avons étudié l'effet de quatre facteurs (*C. maltaromaticum*, concentration en NaCl, temps et pH) et de leurs interactions sur la croissance de *L. monocytogenes* inoculée dans deux milieux de culture (TSB- YE et lait écrémé).

La concentration de *C. maltaromaticum* LMA28 s'est révélée être le facteur déterminant pour obtenir une inhibition de *L. monocytogenes* dans les deux milieux de culture testés.

Cependant, le mécanisme exact d'inhibition est encore inconnu et mériterait d'autres études. Il n'a pas été possible de déterminer l'inhibition de *Listeria* dans le fromage modèle choisi (Babybel), et ceci principalement en raison de l'absence de croissance de *Listeria* sur le fromage seul.

En ce qui concerne l'inhibition de *L. monocytogenes* par *C. maltaromaticum* dans le fromage, le choix d'un nouveau modèle de fromage qui permettrait la prolifération de colonies de *Listeria* serait donc nécessaire pour obtenir des résultats permettant d'affirmer ou d'infirmer le potentiel inhibiteur de *C. maltaromaticum* LMA28 sur *Listeria*. Pour chaque nouveau fromage modèle testé, il serait souhaitable de mettre au point un nouveau plan d'expérience pour obtenir plus d'informations sur les facteurs d'influence. Le potentiel d'inhibition de *C. maltaromaticum* sur d'autres bactéries désirables et indésirables dans le fromage devrait aussi être élucidé.

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Annexes

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Characterization of *Carnobacterium maltaromaticum* LMA 28 for its positive technological role in soft cheese making

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ABSTRACT

Carnobacterium maltaromaticum is a lactic acid bacterium isolated from soft cheese. The objective of this work was to study its potential positive impact when used in cheese technology. Phenotypic and genotypic characterization of six strains of *C. maltaromaticum* showed that they belong to different phylogenetic groups. Although these strains lacked the ability to coagulate milk quickly, they were acidotolerant. They did not affect the coagulation capacity of starter lactic acid bacteria, *Lactococcus lactis* and *Streptococcus thermophilus*, used in dairy industry. The impact of *C. maltaromaticum* LMA 28 on bacterial flora of cheese revealed a significant decrease of *Psychrobacter* sp. concentration, which might be responsible for cheese aging phenomena. An experimental plan was carried out to unravel the mechanism of inhibition of *Psychrobacter* sp. and *Listeria monocytogenes* and possible interaction between various factors (cell concentration, NaCl, pH and incubation time). Cellular concentration of *C. maltaromaticum* LMA 28 was found to be the main factor involved in the inhibition of *Psychrobacter* sp. and *L. monocytogenes*.

1. Introduction

Cheeses host a complex ecosystem characterized by a succession of different microbial groups emerging during milk fermentation, ripening or storage. Lactic acid bacteria (LAB) are usually involved throughout numerous processes occurring during milk transformation into cheese. Microorganisms generally play a positive role during cheese maturation processes, as they provide specific cheese flavors (Irlinger and Mounier, 2009). They generally come from commercial starter cultures but are also found in raw milk, in the dairy environment and are thus called opportunistic LAB (Fleet, 1999). The presence of an opportunistic psychrotrophic LAB named *Carnobacterium maltaromaticum* has been demonstrated in numerous French cheeses (Appellation d'Origine Protégée, AOP = Protected Designation of Origin, PDO) at the end of ripening and cold storage, without affecting the final product quality (Cailliez-Grimal et al., 2007; Millière et al., 1994). These species frequently predominate in a large range of foods, including non-dairy foods such as fish or meat and are commonly associated with the spoilage of modified-atmosphere-packaged products (Vihavainen et al., 2007).

Many *Carnobacterium* strains have been used previously as protective cultures against pathogenic bacteria (for instance *Listeria monocytogenes*) in fish and meat products (Brillet et al., 2005; Duffes et al., 1999; Matamoros et al., 2009; Vescovo et al., 2006). The bacteriocin-producing ability of *Carnobacterium* strains was found to play a key role in controlling spoilage and pathogenic bacteria in food and in *in vitro* model systems (Dos Reis et al., 2011; Martin-Visscher et al., 2011; Bernardi et al., 2011), while external factors, including pH, salt and storage temperatures, were also shown to have a significant impact (Hwang, 2009; Leroi et al., 2012).

The identification, isolation, ecology and technological aspects of *C. maltaromaticum* in cheese were recently documented (Afzal et al., 2010). The objective of this study was to characterize several *C. maltaromaticum* strains isolated from soft cheese by comparing their technological abilities, acid tolerance and

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inhibitory activities. One selected strain was tested during ripening of soft cheese. An experimental plan was then done to evaluate the inhibition potential of this strain against *L. monocytogenes* and *Psychrobacter* sp.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Strains used in this study, along with culture media and growth conditions, are presented in Table 1. Strains were grown in trypticase soy broth (Biokar, Beauvais, France) supplemented with yeast extract (TSB-YE), except for *Escherichia coli*, which was grown in Lysogeny broth (LB) (Biokar, Beauvais, France) and *Psychrobacter* sp. grown in TSB-YE containing 8% NaCl (Table 1). For selective enumeration, PALCAM agar (Biokar, Beauvais, France) was used for *L. monocytogenes* CIP 82110, MCM (Edima et al., 2007) was used for *C. maltaromaticum* LMA 28 and TSB-YE with NaCl (8%) for *Psychrobacter* sp.

2.2. Identification

2.2.1. Determination of carbon assimilation profiles

Carbohydrate fermentation patterns were determined using API 50 CH test strips (BioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

2.2.2. Molecular typing using pulsed-field gel electrophoresis (PFGE)

Carnobacterium strains were inoculated in 10 mL of TSB-YE broth and grown at 30 °C overnight. The culture (1 mL) was inoculated in 20 mL of TSB-YE broth and incubated at 30 °C until the bacterial suspension absorbance reached 0.4–0.8 (exponential phase). Pulsed-Field Gel Electrophoresis (PFGE) of all *Carnobacterium* strains was performed with the enzyme *SmaI* as described by Haenni et al. (2010).

Table 1
Bacterial strains used in this study.

Bacterial species	Strain designation[a]	Growth medium	Incubation temperature (°C)
Carnobacterium maltaromaticum	LMA 5[b]	TSB-YE	30
C. maltaromaticum	LMA 7[b]	TSB-YE	30
C. maltaromaticum	LMA 14[b]	TSB-YE	30
C. maltaromaticum	LMA 23[b]	TSB-YE	30
C. maltaromaticum	LMA 28[b]	TSB-YE	30
C. maltaromaticum	LMA 32[b]	TSB-YE	30
C. divergens	DSM 20623[T]	TSB-YE	30
C. gallinarum	DSM 4847[T]	TSB-YE	25
C. mobile	DSL 4848[T]	TSB-YE	30
C. viridans	CIP 107728	TSB-YE	25
Listeria grayi	CIP 6818[T]	TSB-YE	30
L. innocua	CIP 12511	TSB-YE	30
L. innocua	CIP 107775	TSB-YE	30
L. ivanovii	CIP 12510	TSB-YE	30
L. ivanovii	CIP 7842	TSB-YE	30
L. monocytogenes	CIP 82110[T]	TSB-YE	37
L. monocytogenes	CIP 7831	TSB-YE	37
Enterococcus faecalis	ATCC 19433	TSB-YE	37
E. faecalis	CIP 78117[T]	TSB-YE	37
E. faecium	CIP 106742	TSB-YE	37
Escherichia coli	XL-1Blue	LB	37
Lactococcus lactis	DSM 20481	TSB-YE	30
Streptococcus thermophilus	INRA 302	TSB-YE	37
Psychrobacter sp.	LMA 1	TSB-YE + NaCl (8%)	30

[a] ATCC: American Type Culture Collection, Mannassas, USA; CIP: Collection de l'Institut Pasteur, Paris, France; DSM: Deutsche Sammlung von Mikro-Organismen und Zellkulturen, Göttingen, Germany; INRA: Institut National de la Recherche Agronomique; LMA: Laboratoire de Microbiologie Alimentaire, ENSAIA-INPL, Nancy, France; SLCC: Special *Listeria* Culture Collection, University of Wurzburg, Germany; T: Type.
[b] Strains isolated by Millière et al. (1994).

2.2.3. Antibacterial assays and primers used for the detection of genes encoding bacteriocins

Antibacterial activity of six strains of *C. maltaromaticum* was determined against *Carnobacterium*, *Listeria* and *Enterococcus* species by the agar well diffusion method (Mathieu et al., 1993) (Table 1). Primers (Eurogenetec, Herstal, Belgium) (Table 2) were used for the detection of genes encoding different bacteriocins in DNA of *C. maltaromaticum* strains. DNA extraction and PCR were conducted as described above (in 2.4. Nucleic acid extraction).

2.2.4. Isolation of Psychrobacter sp.

Cheese sample (5 g) was suspended in 50 mL of citrate buffer (trisodium citrate, 2% w/v and NaCl, 8.5 g/L) and homogenized in stomacher (Interscience, St. Nom-la-gatehouse, France). A volume of 1.5 mL was then inoculated into three different selective media: brilliant green bile broth, lactose broth (BLBVB Biokar, Beauvais, France) and TSB-YE + NaCl (8%). These media were incubated at 4 °C and 30 °C during 48 and 24 h respectively. After enrichment, bacteria were isolated on TSA-YE. Gram staining, oxidase and catalase tests were performed on each isolated colony.

2.3. Technological aptitude

2.3.1. Milk acidification

C. maltaromaticum strains (LMA 5-7-14-23-28-32), *Streptococcus thermophilus* INRA 302 and *Lactococcus lactis* DSM 20481 were inoculated in 150 mL of semi-skimmed milk supplemented with 1 g/L of yeast extract at the initial population of 10^8 cfu/mL. The cultures were incubated in a thermostatic water bath at 30 °C to monitor acidification using an automatic multimeter (Consort D230, Neuilly-sur-Seine, France). The kinetic parameters, maximum acidification rate (V_m) and time corresponding to V_m (T_m) were determined by the method described by Spinnler and Corrieu (1989).

2.3.2. Milk coagulation

The bacterial strains were subcultured twice in TSB-YE at their optimum growth temperatures during 16 h. The cell pellets were obtained as described above. Milk coagulation times were obtained by the use of a rheometer (Stress Tech, Rheologica Instruments AB, Sweden). The cell pellets were used to inoculate 18 mL (placed in a C25 cup) of semi-skimmed UHT milk supplemented with yeast extract (1 g/L) in a tank thermostatically controlled by a water bath at 30 °C. The geometry used to monitor milk coagulation is a paddle system (four blades placed at right angles to each other) specially designed by Rheologica to follow milk coagulation. The shear rate was fixed at 100 s^{-1} and remained constant throughout the analysis. Data are collected automatically every 20 s and all runs are carried out at least in duplicate.

2.3.3. Acid tolerance

The strains were subcultured twice in TSB-YE at 30 °C for 16 h. These precultures were used to inoculate TSB-YE and semi-skimmed UHT milk at 1:10 ratio and incubated at 30 °C. Cultures were stopped in exponential growth phase (OD_{660nm} = 0.4). Volume of 1 mL of the suspension was used to inoculate 9 mL of TSB-YE or milk to a specified value of pH. The pH range selected varied from 6.5 to 3, adjusted using HCl or lactic acid in aseptic conditions. After a contact time of 4 h at 30 °C, spreadings and numerations were

Table 2
Oligonucleotides used in this study.

Target	Target gene	Primer	Sequences (5′–3′)	Reference
Carnobacteriocin BM1 (IIa)	cbnBM1	cbnBM1-for	GCT ATC TCT TAT GGC AAT GGT G	Quadri et al., 1994
		cbnBM1-rev	TAG AAG CCC ATC CAC CGA TA	
Carnobacteriocin B2 (IIa)	cbnB2	cbnB2-for	TGA ATA GCG TAA AAG AAT TAA ACG TG	Quadri et al., 1994
		cbnB2-rev	TTA CGG TCT CCT ACC AAT GGA	
Piscicolin Pi126 (IIa)	pisA	pisA-for	CGG CTC CAC CTG TAG TCA A	Jack et al., 1996
		pisA-rev	TGG CGT TTC CTG TAA TAA AAA TG	
Carnobacteriocin CbnA (IIc)	cbnA	cbnA-for	TGG TGG AGA CCA AAT GTC AG	Worobo et al., 1994
		cbnA-rev	AAC CAG CCT AAA GGA CCT GAA	
Divergicin DvnA (IIc)	dvnA	dvnA-for	GGG GCA ACA TTT TTC TCA AC	Worobo et al., 1995
		dvnA-rev	ACC TCC TGC TAT TGC ACC AC	
Divergicin Dvn750 (IIc)	dvn750	dvn750-for	CAG AAC AAT TTC TTC CCT TGG	Holck et al., 1996
		dvn750-rev	TTT ATT CCA GCC CAC ACT CC	
Carnocyclin CcnA (circular)	ccnA	ccnA-for	GCA TAT GGT ATC GCA CAA GG	Martin-Visscher et al., 2008
		ccnA-rev	GCA ATT GCT GCT TTA ACT GCT	
Total bacterial community	V3 region of 16S rDNA	HDA1-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGCAC	Ogier et al., 2002
		HDA2	GGGGGGACTCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCA	
		2HDA1-EcoR1	CCGGAATTCGACTCCTACGGGAGGCAGCAGT	Serhan et al., 2009
		2HDA2-EcoR1	CCGGAATTCGTATTACCGCGGCTGCTGGCA	

carried out by inclusion method in Petri dishes using 1 mL of sample and 12 mL of TSA-YE.

2.3.4. Analysis of flavor compounds by headspace gas chromatography

Flavor compounds produced by the different strains were investigated in semi-skimmed (UHT) milk and in different reaction mixtures. Strains (5 mL) were inoculated in TSB-YE culture medium at optimum growth temperatures. Cells in late exponential phase were harvested by centrifugation (5000 × g, 4 °C, 10 min) and resuspended in 5 mL of semi-skimmed UHT milk. These suspensions were used to inoculate 45 mL semi-skimmed UHT milk incubated at 30 °C and 4 °C for 24 h. Samples were stored at −25 °C until analysis by headspace gas chromatography (Afzal et al., 2012). Briefly, a 1.0-mL headspace sample was injected (splitless) on the column after 2 min of incubation at 90 °C. The chromatograph (PR 2100, Périchrom, Saulx-lès-Carthusian, France) was equipped with an injector with headspace (Headspace HT 300A, Périchrom) and a flame-ionization detector (Périchrom). The volatile compounds were separated using a capillary column of 30 m × 0.25 mm × 0.2 μm and proportioned by integrator WINILAB III (Périchrom). The carrier gas was nitrogen at a constant flow of 2 mL/min. The temperature of the detector was maintained at 190 °C. The vials were pressurized for 30 s and injection lasted 1 s. The adjustment of the quantity of product by the valve of open escape or "split" was 5:5. The oven temperature was initially kept at 45 °C for 1 min, increased by 5 °C/min to 190 °C, and then maintained at 190 °C for 12 min. Standard curves were carried out with solutions of 3-methylbutanal and 3-methylbutanol with concentrations varying between 1 and 5000 μM.

2.3.5. Cheese manufacture

Among the six laboratory strains tested, only *C. maltaromaticum* LMA 28 was selected to study its impact on microbial community and final quality of soft cheese. Soft cheeses were manufactured in the laboratory (Edima et al., 2007). Milk was inoculated with different initial populations (10^1, 10^4 and 10^7 cfu/mL named 2A, 3B and 4C) of *C. maltaromaticum* LMA 28 and without *C. maltaromaticum* LMA 28 (control named 1T). After ripening for 10 days at 10 °C, the cheeses were stored at 4 °C for 42 days. Cheese samples were taken at different time intervals during 42 days and frozen at −20 °C.

2.3.6. Sensory analysis

Two experiments were carried out. In the first experiment, the panel was composed of seven people. Semi-skimmed UHT milk samples inoculated with *C. maltaromaticum* strains were tested for the presence of aroma and its intensity was numerically expressed in a scale from one (low) to five (high).

In the case of the second experiment, the contribution of *Carnobacterium* to aroma development of soft cheeses was assessed during the ripening period by comparing the aroma of cheeses made with or without adjunct cultures by a triangle aroma test (AFNOR, 1995) as described by Milesi et al. (2007). Samples (5 g) were placed in sealed glass containers and maintained in an oven at 30 °C for 30 min, after which they were provided to the panel. The same containers were re-equilibrated in the oven and reused twice. Three samples were supplied to each panelist; 2 of the samples had been taken from the same cheese, whereas the third was different. The samples were identified with random 3-digit codes. The panel, composed of 12 untrained members, was asked to find the sample that differed and to comment briefly on the differences. The panelists evaluated cheese duplicates but they did not replicate measurements with the same cheeses.

2.3.7. Statistical analysis

Data from chemical composition, microbiological counts, FAA, and organic acid and volatile compound assays were analyzed by one-way ANOVA with a 95% confidence level. All analyses were made in duplicate. All statistical analyses were performed using the SPSS 10.0 software (SPSS Inc., Chicago, IL).

2.4. Nucleic acid extraction

2.4.1. Samples from isolate colonies or broth culture

The bacterial DNA was extracted as previously described (Serhan et al., 2009). gDNA was extracted with DNA stool kit (Qiagen, France) and plasmid DNA using NucleoSpin Plasmid kit (Macherey Nagel, France) according to manufacturer recommendations.

2.4.2. Samples of cheeses

Cheese sample (5 g) was suspended in 50 mL of citrate buffer and homogenized using a stomacher (Interscience, France). For each sample, 40 mg of pronase (Sigma) and 100 μL of β-mercaptoethanol were added and placed at 37 °C overnight. The cells

were washed twice using ultrapure water after centrifugation (5000 rpm, 10 min and 4 °C). DNA was extracted as previously described (Serhan et al., 2009).

2.4.3. PCR amplification of the V3 region of 16S rDNA and analysis of PCR products by TTGE

DNAs extracted from cheeses were subjected to universal PCR targeting the eubacterial 16S rRNA gene. The V3 region of the gene was amplified using the primers HDA1-GC and HDA2 (Ogier et al., 2002) for the TTGE analysis, or the universal primers 2HDA1-EcoR1 and 2HDA2-EcoR1 (Serhan et al., 2009) for the cloning experiments. The PCR products obtained from amplification of the V3 region of 16S rRNA were analyzed by Temporal Temperature Gel Electrophoresis TTGE (Dcode universal mutation detection system, Bio-Rad Laboratories, USA) as described by Serhan et al. (2009) (Table 2).

2.4.4. PCR protocols

The amplification was carried out in a thermal cycler iCyclerTM (Bio-Rad Laboratories, Hercules, CA, USA). For bacteriocin gene detection, the thermal program for amplification was 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 53 °C for 30 s, 72 °C for 30 s with a final step for 5 min. For TTGE, the amplification program was 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min with a final step for 7 min.

2.4.5. Cloning and sequencing of TTGE fragments for bacterial species identification

To identify the bacterial species present in cheese, the amplified 16S rDNA were inserted into a cloning vector, plasmid pUC19, containing the gene for ampicillin resistance as described by Serhan et al. (2009). PCR amplifications were performed with the primers 2HADA1EcoR1 and 2HADA2EcorR1 (Table 2). Each primer contains an *Eco*RI restriction site for subsequent cloning in the plasmid pUC19. The PCR products were digested with *Eco*RI and ligated to pUC19 for cloning into *E. coli*. The *E. coli* recombinant strains were screened by performing colony-PCR with the TTGE primers followed by TTGE analysis of the PCR products. The TTGE analysis was performed along with the PCR product obtained with the DNA matrix from which the recombinant clones originated from. This allowed inferring which band was cloned in the recombinant clones. The insert of suitable recombinant plasmids pUC19 was then sequenced (GATC Biotech), and sequences were used to identify bacterial species using BLAST analysis on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Experimental design and data analysis for investigation of bacterial interactions in milk

In order to study the bacterial interactions in co-cultures and to investigate the optimum culture conditions for the inhibition of *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 in semi skimmed UHT milk, a Doehlert experimental design (Doehlert, 1970) was carried out. Four variable factors (X) of different ranges were: cellular concentration of *C. maltaromaticum* LMA 28 (X_1 = 0–10^8 cfu/mL; 5 levels); NaCl (X_2 = 0–9 g/L; 7 levels); pH (X_3 = 5.5–8.5; 7 levels) and duration of incubation (X_4 = 24–72 h; 3 levels) (Table 3). Each experiment was started with the initial population of *Psychrobacter* sp. LMA 1 and/or *L. monocytogenes* CIP 82110 at about 10^3 cfu/mL and incubated at 25 °C for respective duration. Selective enumerations were carried out after incubation for *C. maltaromaticum* LMA 28, *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 using selective media, MCM (Edima et al., 2007), TSA-YE + NaCl 8% and PALCAM. The factorial growth response (R) of either *Psychrobacter* sp. LMA 1 and/or *L. monocytogenes* CIP 82110 expressed as $Log_{10}N$ can be predicted in all experimental regions according to the following equations

Table 3
Experimental plan (Doehlert matrix) using four variable factors (X) (*Carnobacterium* cells concentration, NaCl, pH and duration) to check the growth response (R expressed as $Log_{10}N$) of *Psychrobacter* sp. and *L. monocytogenes* CIP 82110 in semi skimmed UHT milk.

Experiment no.	Factors			
	C. maltaromaticum LMA 28 (log_{10})	NaCl (g/L)	pH	Duration (h)
1	8.0	4.50	7.00	48
2	0.0	4.50	7.00	48
3	6.0	9.00	7.00	48
4	2.0	0.00	7.00	48
5	6.0	0.00	7.00	48
6	2.0	9.00	7.00	48
7	6.0	6.00	8.50	48
8	2.0	3.00	5.50	48
9	6.0	3.00	5.50	48
10	4.0	7.50	5.50	48
11	2.0	6.00	8.50	48
12	4.0	1.50	8.50	48
13	6.0	6.00	7.38	72
14	2.0	3.00	6.62	24
15	6.0	3.00	6.62	24
16	4.0	7.50	6.62	24
17	4.0	4.50	8.13	24
18	2.0	6.00	7.38	72
19	4.0	1.50	7.38	72
20	4.0	4.50	5.87	72
21	4.0	4.50	7.00	48
22	4.0	4.50	7.00	48
23	4.0	4.50	7.00	48
24	4.0	4.50	7.00	48

$$R(\mathrm{Log}_{10}N) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_{24} + \beta_{34} X_3 X_4$$

Where β_0 is the constant term; β_1 determines the influence of *C. maltaromaticum*; β_2 the influence of NaCl; β_3 the influence of pH; β_4 the influence of duration; β_{12} the interaction effect between *C. maltaromaticum* and NaCl; β_{13} the interaction effect between *C. maltaromaticum* and pH; β_{14} the interaction effect between *C. maltaromaticum* and duration; β_{23} the interaction effect between NaCl and pH; β_{24} the interaction effect between NaCl and duration; β_{34} the interaction effect between pH and duration and β_{11}, β_{22}, β_{33} and β_{44} are "shape" parameters. Data analysis, ANOVA and polynomial regressions were performed using the NEMROD-W software (LPRAI, Marseille, France).

3. Results

3.1. Identification of the strains and comparison of the technological potential

3.1.1. Biochemical and genotypic identifications

The differentiation of the 6 strains of *C. maltaromaticum*, isolated from soft cheeses was performed using biochemical and genotypic tests. A comparison of their biochemical characteristics showed weak differences in their fermentation pattern (data not shown). All isolates were consistent in their ability to produce acid from β-gentiobiose, saccharose, N-acetyl glucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, D-mannose, D-fructose, D-glucose and ribose. All were able to use lactose and hydrolyzed products of lactose (glucose and galactose). Like other

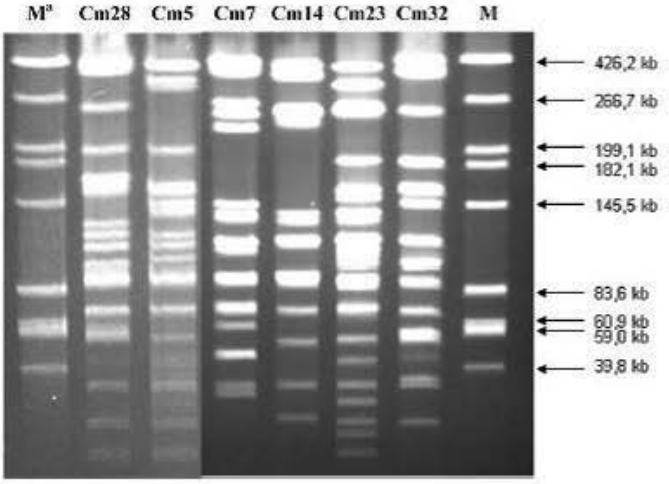

Fig. 1. Pulsed-field gel electrophoretic profiles of gDNA of *C. maltaromaticum* strains digested by *SmaI*. [a]Molecular weight marker from gDNA of *Streptococcus agalactiae* A 909 digested by *SmaI*. *C. maltaromaticum* LMA 28; *C. maltaromaticum* LMA 5; *C. maltaromaticum* LMA 7; *C. maltaromaticum* LMA 14; *C. maltaromaticum* LMA 23; *C. maltaromaticum* LMA 32.

carnobacteria (Cailliez-Grimal et al., 2007), isolates did not produce acid from erythritol, D-arabinose, L-xylose, adonitol, rhamnose, dulcitol, inuline, melezitose, D-raffinose, xylitol, D-lyxose, D-fucose, D-arabitol and L-arabitol.

In the conditions tested, three strains (LMA 7, 14 and 32), among the *C. maltaromaticum* strains tested, exhibited no antibacterial activity against the target strains tested (data not shown). The three other strains (LMA 5, 23, 28) were active against all the *Carnobacterium* and *Listeria* sp. (data not shown). Moreover, *C. maltaromaticum* LMA 5 and 28 were found to possess inhibitory activities against the three strains of *Enterococcus* (data not shown). DNA extracted from all the strains was tested with six pairs of bacteriocin primers. An amplicon was obtained with BM1 primers for all the strains and none with the other primers (pil6, CbnA, Dvn750, CcnA). Only DNA extracted from *C. maltaromaticum* LMA 7 and 14 did not give an amplicon with the B2 primers (data not shown).

The diversity of the *C. maltaromaticum* isolates was examined by the PFGE method. The restriction patterns observed for the isolates were diverse and generated between 12 and 21 bands (Fig. 1). The strains displayed a unique band pattern with seven or more than seven differences indicative of a genetic diversity. So each strain was declared non-associated to each other according to the criteria of Tenover et al. (1995).

Table 5
Coagulation time of semi-skimmed milk in presence of different LAB at 30 °C.

Strains	Coagulation time[a] (h)
C. maltaromaticum LMA 28	7.93 ± 1.69
L. lactis DSM 20481	1.8 ± 0.87
S. thermophilus INRA 302	2.36 ± 1.26
L. lactis DSM 20481 + *C. maltaromaticum* LMA 28	2.25 ± 0.21
S. thermophilus INRA 302 + *C. maltaromaticum* LMA 28	3.26 ± 1.36
S. thermophilus INRA 302 + *L. lactis* DSM 20481	1.76 ± 0.25
S. thermophilus INRA 302 + *L. lactis* DSM 20481 + *C. maltaromaticum* LMA 28	1.93 ± 0.23

[a] Values are mean ± standard deviation of three independent experiments.

3.2. Technological aptitude

Among the six strains of *C. maltaromaticum* cultured in milk supplemented with yeast extract (1 g/L), four strains (*C. maltaromaticum* LMA 5, 14, 23 and 28) showed weak and similar acidification kinetics of milk with a V_m of 0.1 pH/h, T_m of 20 ± 1 h and a final pH of 5.4 compared to *L. lactis* and *S. thermophilus*, starter LABs used in dairy industry (Table 4). In the presence of HCl and lactic acid, the weak acidifying strains, *C. maltaromaticum* LMA 14, 23 and 28 conserved a survival rate of 100% at lower pH in milk compared to non acidifying strains, *C. maltaromaticum* LMA 7 and 32.

In the conditions tested, all the strains were able to produce 3-methylbutanal in milk cultured at 4 °C and at 30 °C after 24 h of incubation (Table 4). Milk bottles tasted and smelled significantly differently ($P < 0.05$) for each *Carnobacterium* species after 48 h. The taste panel could clearly distinguish the milk malty flavor in bottles inoculated with *C. maltaromaticum* LMA 28 and lactic and aromatic flavor with LMA 14. The other strains did not exhibit particular flavors. The strain *C. maltaromaticum* LMA 28 was tested for cheese assay.

The milk coagulation time of *C. maltaromaticum* LMA 28 was significantly different from to the one of starter bacteria (Table 5). However, the coagulation times of *L. lactis* and *S. thermophilus* were not significantly different when used in single or in combination with *C. maltaromaticum* LMA 28 indicating that the presence of *C. maltaromaticum* LMA 28 has no influence on the coagulation capacity or velocity of these starters (Table 5).

3.2.1. Sensorial impact of *C. maltaromaticum* LMA 28 on cheeses

After 20 and 34 days of ripening, cheeses tasted and smelled significantly differently ($P < 0.05$) to the reference without *C. maltaromaticum* LMA 28. The taste panel clearly distinguished the malty and butyric flavor in cheeses inoculated with *C. maltaromaticum* LMA 28.

Table 4
Acidification kinetic parameters, bacterial cells viability in milk at pH 4, detection of 3-methylbutanal and sensory analysis of milk at 30 °C during 24 h of strains tested.

Strains	Acidification kinetics parameters			% Bacterial viability in milk at pH 4		3-Methylbutanal (μM) (SE ≤ 0.5)	Sensory analysis 1(weak) to 5 (high)
	V_m (pH/h)[a] (SE ≤ 0.01)	T_m (h) SE ≤ 0.1	Final pH SE ≤ 0.1	HCl	Lactic acid		
C. maltaromaticum LMA 5	0.11	21.0	5.4	22	70	70.0	2
C. maltaromaticum LMA 7	ND	ND	6.2	10	35	65.0	2
C. maltaromaticum LMA 14	0.10	19.0	5.4	60	100	50.0	3
C. maltaromaticum LMA 23	0.11	19.0	5.4	100	100	50.0	3
C. maltaromaticum LMA 28	0.11	18.0	5.4	80	100	65.0	4
C. maltaromaticum LMA 32	ND	ND	5.9	8	40	20.0	2
S. thermophilus INRA 302	0.57	7.0	4.3	ND	ND	0.0	ND
L. lactis DSM 20481	0.46	12.0	4.4	100	100	70.0	ND

V_m: Maximum acidification rate; T_m: Corresponding time to V_m; ND: Non detected.
[a] Results are expressed as the mean of three independent experiments with the standard error (SE).

3.3. Impact of C. maltaromaticum LMA 28 on bacterial flora of soft cheese

TTGE analysis enabled the dynamics of the microbiota to be visualized by examining fingerprintings of the dominating bacterial groups evolving during ripening (Ogier et al., 2002). The electrophoretic profiles of bacterial populations corresponding to cheese samples after 25 days of ripening (Fig. 2) show that cheese 1T, non-inoculated with *C. maltaromaticum* LMA 28 generated 6 bands and cheeses inoculated with different initial levels of *C. maltaromaticum* LMA 28 generated 5 bands. The additional band (band d) present in cheese non-inoculated with *C. maltaromaticum* LMA 28 was absent in the profiles of other cheeses. A similar analysis was performed on cheeses after 30 and 42 days of ripening and similar profiles were observed (data not shown).

The band d, only present in control cheese, was selected for cloning and sequencing and the sequence was compared by performing blastn alignments. This corresponded to *Psychrobacter* sp. with 100% identity (closest accession number FN433052.1).

After enrichment using different selective media (BLBVB, Lasseur and TSB-YE + NaCl 8%) and isolation at 4 °C and 30 °C on TSA-YE agar, Gram staining, catalase and oxidase tests were performed. Gram-negative colonies with positive catalase and oxidase tests were isolated to obtain pure cultures. The colonies obtained after 10 days at 4 °C or 48 h at 30 °C were round, mucous, whitish with regular edges. The V3 regions of the 16S rRNA of these colonies were amplified by PCR with primers HDA1-GC and HDA 2. The resulting fragments were then sequenced (GATC, Germany) and corresponded to *Psychrobacter* sp.

3.4. Interactions of C. maltaromaticum LMA 28 with Psychrobacter sp. LMA 1 and L. monocytogenes CIP 82110

To study the impact of *C. maltaromaticum* LMA 28 cells concentration on the two target strains *Psychrobacter* sp. LMA 1, isolated previously and *L. monocytogenes* CIP 82110, sensitive to the bacteriocins CbnBM1 and CbnB2, and to search for the optimum conditions of their inhibition in milk (NaCl, pH and duration), a Doehlert experimental design was used and polynomial equations were obtained. Table 6 shows the coefficient estimates and the

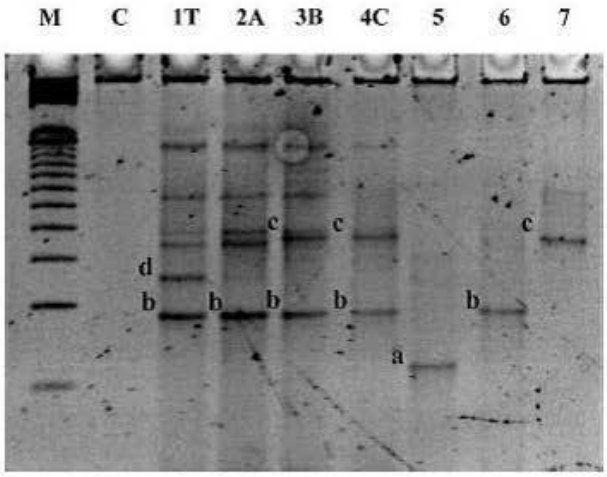

Fig. 2. TTGE of PCR amplification products with primers a) HDA1-GC, b) HDA2. M: marker; C: control; 1T, 2A, 3B, 4C: PCR products of DNA extracted from cheeses 1T, 2A, 3B and 4C; 5: PCR product of gDNA of *S. thermophilus* INRA 302 (band a); 6: PCR product of gDNA of *L. lactis* DSM 20481 (band b); 7: PCR product of gDNA of *C. maltaromaticum* 28 (band c).

Table 6
Coefficient estimation of the different quadratic models for the 4 variables, *C. maltaromaticum* LMA 28, NaCl, pH and duration of the Doehlert's matrix for the growth response (R) of *Psychrobacter* sp. LMA 1 and *Listeria monocytogenes* CIP 82110 in milk.

Factors	Terms	Bacterial strains	
		Psychrobacter sp. LMA 1	*L. monocytogenes* CIP 82110
Constant coefficient	β_0	3.900***	5.455***
C. maltaromaticum ($Log_{10}N$)	β_1	−1.532***	−1.547***
NaCl (g/L)	β_2	0.148**	0.038
pH	β_3	0.349***	0.634**
Duration (h)	β_4	1.006***	0.318
Carnobacterium^2	β_{11}	0.595***	0.390
NaCl^2	β_{22}	0.118*	−0.323
pH^2	β_{33}	−0.221**	−0.859*
Duration^2	β_{44}	−0.735***	0.140
Carnobacterium.NaCl	β_{12}	0.329**	−0.185
Carnobacterium.pH	β_{13}	0.533**	−0.418
Carnobacterium.Duration	β_{23}	−0.451**	1.356*
NaCl.pH	β_{14}	0.318*	−0.609
NaCl.Duration	β_{24}	−0.003	−1.560*
pH.Duration	β_{34}	−0.190*	−1.341*

The response was represented by the polynomial equation: $Log_{10}N = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{44}X_4^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_{24} + \beta_{34}X_3X_4$.
Coefficient significance: *** ≤1%; ** <1%; * ≤5.

determination of model coefficients that were fitted to the experimental data.

The coefficient terms of polynomial equations for *C. maltaromaticum* LMA 28 were the least ($\beta_1 = -1.532$; -1.547) predicted that the cellular concentration of *C. maltaromaticum* LMA 28 influenced the response by decreasing *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 counts cells number. However, NaCl, pH and duration had less influence toward the response of *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110, but the interactions between them had an influence represented by negative coefficient values. Therefore, the increase in the cellular concentration of *C. maltaromaticum* LMA 28 improved the inhibition of both *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110 in milk.

Thus, keeping the pH and NaCl constant, the maximum inhibitory growth responses of *Psychrobacter* sp. LMA 1 could be predicted by an increase in the concentration of *C. maltaromaticum* LMA 28 and decrease in the duration of incubation (Fig. 3a). Moreover, keeping the NaCl and duration constant, the maximum inhibitory growth responses of *L. monocytogenes* CIP 82110 could be predicted by an increase in the concentration of *C. maltaromaticum* LMA 28 and decrease in pH (Fig. 3b).

Maximum inhibition for *Psychrobacter* sp. LMA 1 was obtained at 10^6 cfu/mL cellular concentration of *C. maltaromaticum* LMA 28, 4 g/L of NaCl, and 6.6 of pH during 27 h respectively. Maximum inhibition for *L. monocytogenes* CIP 82110 was obtained at 10^5 cfu/mL cellular concentration of *C. maltaromaticum* LMA 28, 5.6 g/L of NaCl, and 5.6 of pH during 42 h respectively.

4. Discussion

The LABs comprise a wide variety of species which themselves include highly diverse strains. The carbon assimilation profiles of six strains of *C. maltaromaticum* isolated from the same habitat showed only few differences between these strains. All strains were found to possess a gene encoding *cbnBM1* and only two of them (*C. maltaromaticum* LMA 7 and LMA 14) do not possess the gene encoding *cbnB2*. Although all strains of *C. maltaromaticum* have at least one gene encoding a bacteriocin, none of the target bacteria

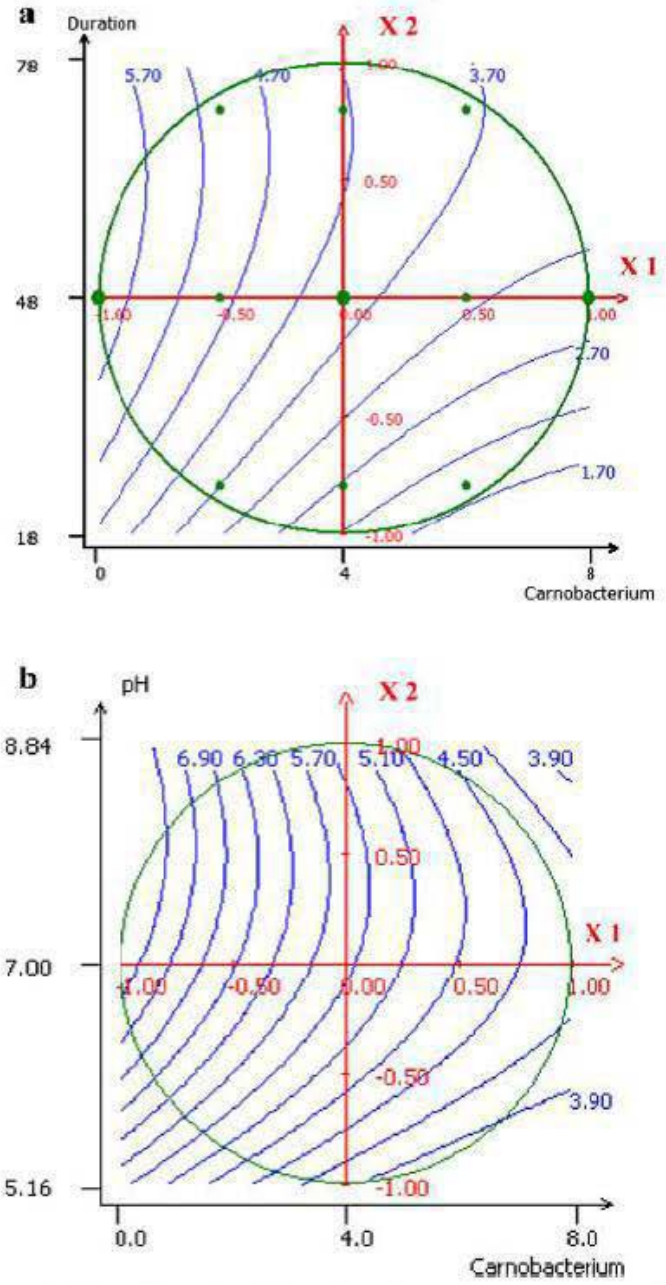

Fig. 3. 3D graphical presentation for the optimum inhibitory response (R) in milk of a) *Psychrobacter* sp. LMA 1 in function of cellular concentration of *C. maltaromaticum* LMA 28 ($\log_{10}$) and duration where, X_1 axis = cellular concentration of *C. maltaromaticum* LMA 28 and X_2 = duration at fixed values of NaCl and pH (4.5 g/L, 7, center of the domain) and of b) *L. monocytogenes* CIP 82110 in function of cellular concentration of *C. maltaromaticum* LMA 28 ($\log_{10}$) and pH where, X_1 axis = cellular concentration of *C. maltaromaticum* LMA 28 and X_2 = pH at fixed values of NaCl and duration (4.5 g/L, 48 h, center of the domain).

tested was inhibited by *C. maltaromaticum* LMA 7 and LMA 14, suggesting that the gene was not expressed in the culture conditions tested. The PFGE analysis showed that all the strains were different from each other according to the criteria of Tenover et al. (1995), showing a diversity of this species in the same biotope.

In cheese industry, two species played a major role in the milk transformation into cheese i.e. *L. lactis* and *S. thermophilus*, which acidify and coagulate milk quickly. Our results revealed that *C. maltaromaticum* has no function during the acidification and coagulation stage of milk. Moreover, despite an optimal growth at alkaline pH, the weak acidifying strains of *C. maltaromaticum* are able to withstand low pH between 3 and 4.5 during acidification of milk. The TTGE profiles of cheeses inoculated with a different initial population of *C. maltaromaticum* LMA 28 showed that this species was present after 42 days of ripening regardless of its initial concentration. Thus, *C. maltaromaticum* was found compatible with starter LAB and its presence did not alter the ability of starter LAB to quickly transform milk into lactic gel and is able to survive during the maturation of cheese. In presence of *C. maltaromaticum* LMA 28, the band corresponding to the genus *Psychrobacter* present in cheese after 25 days does not appear after 35 days in cheese inoculated with an initial population of 10^1 cfu/mL of *C. maltaromaticum* LMA 28 and in other cheeses inoculated with an initial population of 10^4 and 10^7 cfu/mL of *C. maltaromaticum* LMA 28. These bacteria can be able to grow at low temperatures and tolerate high percentage of NaCl between 1 and 6%. Different species of *Psychrobacter* have been isolated from various ecosystems; cold or hot, slightly or heavily salted, frozen sea, fish, refrigerated meat and from clinical samples (Bowman, 2006). In case of cheese, this bacterium could arise either from raw milk or environmental contamination. It belongs to the order *Pseudomonadales*, well known in food microbiology as spoilage flora of dairy products that exhibit high proteolytic activities. It could play a role in accelerating the aging process of cheese. To reduce this process, *C. maltaromaticum* could be use as ripening flora.

The study on the inhibition of the two target strains *Psychrobacter* sp. LMA 1 and *L. monocytogenes* CIP 82110, in milk predicted that the cellular concentration of *C. maltaromaticum* the most inhibiting factor resulted in high inhibitory growth response of *Psychrobacter* sp. and *L. monocytogenes* in milk. However, the combination of other variables had also an impact. The exact mechanism of inhibition is still unknown and deserves further investigations. It could be related to competition effects for substrates/nutrients, food environmental changes like pH, oxygen or redox, and to the release of antimicrobial compounds targeting competing cells (Leroy and De Vuyst, 2001). Various predictive models have been adapted to study the interactions of spoilage bacteria in growth media and food systems including mixed cultures (Gimenez and Dalgaard, 2004; Malakar et al., 1999), in mono and co-cultures (Antwi et al., 2007, 2008; Charlier et al., 2009; Cornu et al., 2011). Therefore, the modeling approach will be an interesting tool to predict the factors responsible for the inhibition of *Psychrobacter* sp. and *L. monocytogenes* in growth media and food systems.

5. Conclusion and perspectives

The use of the strain *C. maltaromaticum* LMA28 in cheese technology and its impact on the microflora of this soft cheese has been demonstrated in this study. It presence caused a decrease in the concentration of *Psychrobacter* sp., which might be responsible for accelerating the aging phenomena of soft cheese. Moreover, the cellular concentration of *C. maltaromaticum* LMA 28 was the main factor involved in the inhibition of *Psychrobacter* sp. and *L. monocytogenes* CIP 82110 in our experimental conditions. However, the exact mechanism of inhibition is still unknown and deserves further investigations. In addition, *C. maltaromaticum* LMA 28 produced malty/chocolate-like aroma due to 3-methylbutanal from the catabolism of leucine (Afzal et al., 2012). However, it is essential to study its impact on proteolysis and lipolysis during cheese ripening. Being psychrotrophic and alkalinophilic, having antibacterial activity and an ability to increase flavor, this species could play a major role as cheese ripening flora with no negative interference on the starters currently used (for instance *S. thermophilus* and *L. lactis*).

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Encapsulation de *Carnobacterium maltaromaticum* LMA28, productrice de composés aromatiques, dans la paraffine d'enrobage du Poro, un fromage artisanal mexicain

Résumé

Ces travaux de thèse portent sur l'étude pluridisciplinaire (biochimique, physicochimique, microbiologique) de l'amélioration des qualités aromatiques du Poro, fromage artisanal mexicain au lait cru, afin d'en allonger sa durée de commercialisation sur le marché mexicain. Pour cela, après une caractérisation physicochimique du fromage, différentes techniques d'encapsulation dans l'enrobage de paraffine du fromage ont été testées. Par ailleurs, l'activité inhibitrice de *Carnobacterium maltaromaticum* LMA28, isolée à partir d'un fromage à pâte molle, a aussi été étudiée vis-à-vis de *L. monocytogenes* pour une application en industrie fromagère. L'évaluation sensorielle a permis de construire une cartographie des préférences internes pour le fromage Poro. Dans un second temps nous nous sommes intéressée à masquer l'apparition de défauts olfactifs dans le Poro : L'encapsulation de *C. maltaromaticum* LMA28 est une meilleure alternative pour la libération de 3-méthylbutanal par rapport à l'encapsulation directe du 3-méthylbutanal. Enfin, d'un point de vue microbiologique, aucune trace de contamination fécale n'a été retrouvée dans les produits. Les principales souches de bactéries lactiques isolées dans le fromage ont été : *L. plantarum, L. pentosus, L. farciminis, L. rhamnosus.* L'effet de quatre facteurs (*C. maltaromaticum*, concentration en NaCl, temps et pH) et de leurs interactions sur la croissance de *L. monocytogenes* inoculée dans deux milieux de culture (TSB- YE et lait écrémé) a été étudié. La concentration de *C. maltaromaticum* LMA28 s'est révélée être le facteur déterminant pour obtenir une inhibition de *L. monocytogenes* dans les deux milieux de culture testés.

Mots clés : Fromage Poro, 3-methylbutanal, *C. maltaromaticum*, encapsulation, activité antibactérienne

Encapsulation of *Carnobacterium maltaromaticum* LMA28, producer of aromatic compounds, in the paraffin coating of Poro, a Mexican artisanal cheese

Abstract

This thesis focuses on the interdisciplinary study (biochemical, physicochemical, microbiological) in order to improve sensory qualities and, more specifically, aromatic profile of Poro cheese in order to extend its commercialization period on the Mexican market. After cheese characterization, several encapsulations techniques were tested into cheese paraffin coating. Moreover, inhibitory activity of *C. maltaromaticum* LMA28 isolated from a soft cheese against *L. monocytogenes* was also studied. Sensory evaluation led to a preferences internal mapping. It appeared that C. *maltaromaticum* LMA28 encapsulation was a better alternative for the 3-methylbutanal release compared with 3-methylbutanal direct encapsulation to improve off-flavor apparition in Poro. Sensory experiments performed on Babybel® (cheese model) showed a difference between the sensory perceptions of cheese coated containing or not C. *maltaromaticum* LMA28. Micro-encapsulation in calcium alginate is a quick encapsulation technique, simple, inexpensive and easy to implement even for a small-scale productions. Finally, from a microbiological point of view, no trace of fecal contamination was found in the products. The main strains of lactic acid bacteria isolated were cheese: L. *plantarum*, L. *pentosus*, L. *farciminis*, and L. *rhamnosus*. The effect of four factors (C. *maltaromaticum*, NaCl concentration, time and pH) on L. monocytogenes growth, inoculated into two culture media (TSB -YE and skim milk). The C. *maltaromaticum* LMA28 concentration appeared to be the determining factor for L. *monocytogenes* inhibition in both media tested.

Keywords: Poro cheese, 3-methylbutanal, *C. maltaromaticum*, encapsulation, antibacterial activity