UNIVERSITE DE LORRAINE

Ecole doctorale Sciences et Ingénierie des Ressources, Procédés, Produits, Environnement

THESE

Présentée pour l'obtention du grade de

Docteur de l'Université de Lorraine

Discipline : Sciences Agronomiques

Soutenue publiquement le 19 mars 2013

par

Maïra JUNJUA

Exploration du potentiel probiotique de la bactérie lactique *Streptococcus thermophilus* :

- **Évaluation du potentiel probiotique et de sa variabilité**
- **Mise au point et validation de l'outil R-IVET (Recombinase based *In vivo* Expression Technology) pour l'étude de l'état physiologique de la bactérie dans le tractus gastro-intestinal**

Membres du jury

Rapporteurs

Mme Monique ALRIC, Professeur, Université de Clermont Ferrand

Mme Françoise RUL, Chargée de recherche, HDR, INRA, Jouy en Josas

Examinateurs

Mr. Jean-Marc CHATEL, Chargé de recherche, HDR, INRA, Jouy en Josas

Mr. Bruno POT, Directeur de recherche, Institut Pasteur de Lille

Mme Annie DARY-MOUROT, Professeur, Université de Lorraine, (Directrice de thèse)

Mme Yvonne ROUSSEL, Maître de conférences, Université de Lorraine, (Co-directrice de thèse)

Unité de Recherche Animal et Fonctionnalités des Produits Animaux

Equipe Protéolyse et Biofonctionnalités des Protéines et des Peptides

Faculté des Sciences et Technologies, Université de Lorraine, 54500 Vandœuvre-lès-Nancy

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To my beloved husband, Shaheryar

To our love, Shazil

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Abréviations

A	Adénine
ADN	Acide Désoxyribonucléique
ADNc	ADN complémentaire
ARN	Acide Ribonucléique
ARNm	Acide Ribonucléique messager
ARNr 16S	Acide Ribonucléique ribosomial de coefficient de sédimentation 16 Svedberg
ARNr 23S	Acide Ribonucléique ribosomial de coefficient de sédimentation 23 Svedberg
BL	Bactérie Lactique
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
CFU	Colony Forming Units
CG%	Pourcentage en cytosine et guanine
CNRZ	Centre National de recherche zootechnique
$D.O._{650\,nm}$	Densité optique à une longueur d'onde de 650 nanomètre
DFI	Differential Fluorescence Induction
DNA	Deoxyribonucleic Acid
dNTP	Désoxyribonucléotide triphosphate
EDTA	Acide éthylène diamine tétraacétique
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EPS	Exopolysaccharides
FAO	Food and Agriculture Organisation
G	Guanine
GIT	Gastro-intestinal tract
GRAS	Generally Recognized As Safe
h	Heure/hour
IBS	Irritable Bowel Syndrome
IL-8, 10,12	Interleukin
ITS	Internal Transcribed Sequence
IVET	*In vivo* Expression Technology

kb	Kilo base
kDa	Kilo Dalton
LAB	Lactic Acid Bacteria
LB	Lauria Bertani
MCD	Milieu Chimiquement Défini
MLST	Multi Locus Sequence Typing
Mpb	Méga paire de base
ms	Milliseconde
NCBI	National Center for Biological Sequence Analysis
ORF	Phase ouverte de lecture (Open Reading Frame)
PAGE	Electrophorèse en gel de polyacrylamide (polyacrylamide gel electrophoresis)
pb	Paire de bases
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RBS	Ribosome Binding Site
R-IVET	Recombinase Based *In vivo* Expression Technology
RNA	Ribonucleic Acid
SCFAs	Short Chain Fatty Acids
SCOTS	Selective Capture Of Tanscribed Sequence
SDS	Dodécylsulfate de sodium
ssp	Sous-espèce
STM	Signature Tagged Mutagenesis
T	Thymine
TAE	Tris acétate
TE	Tris EDTA
TGI	Tractus Gastro-Intestinal
Th cells	Helper T cells
UFC	Unité Formant Colonie
WHO	World Health Organisation

Introduction générale, Objectifs & Stratégies

Streptococcus thermophilus est la bactérie lactique la plus utilisée après *Lactococcus lactis* dans l'industrie laitière pour la fabrication de yaourts et de fromages à pâte cuite (Emmental, gruyère), filée (Mozarella) ou pressée (Cheddar). En convertissant le lactose en acide lactique, elle permet la formation du coagulat du fait de la précipitation des caséines à pH acide. *S. thermophilus* appartient au genre *Streptococcus* lui-même membre du phylum des firmicutes. A l'exception de *S. thermophilus*, ce genre ne comporte que des espèces commensales ou pathogènes rassemblées en 7 groupes (*Mitis*, *Angionus*, *Bovis*, *Mutans*, *Salivarius*, *Pyogenes* et un groupe composé d'espèces non classées) organisés en deux divisions, *viridans* et *pyogenes* (Farrow et Collins, 1984). *S. thermophilus*, membre du groupe *Salivarius,* qui comporte 3 espèces (*S. salivarius, S. vestibularis et S. thermophilus*), (Kawamura *et al.*, 1995), appartient à la division *viridans* qui est constituée d'espèces commensales voire pathogènes de la cavité buccale et des tractus génitaux des mammifères.

S. thermophilus est le seul streptocoque à avoir le statut de bactérie GRAS (Generally Recognized As Safe). Le séquençage des premiers génomes de cette espèce a confirmé ce statut en montrant que l'espèce en s'adaptant au lait a perdu la grande majorité des gènes de virulence habituellement rencontrés chez d'autres streptocoques pathogènes. Ils ont soit été perdus ou inactivés, le génome *S. thermophilus* comportant une forte proportion de pseudogènes (10% selon Bolotin *et al.*, 2004). Ainsi, des déterminants principaux de la virulence tels que les adhésines habituellement présents à la surface des streptocoques pathogènes sont soit absents soit présents sous forme de pseudogènes (Hols *et al.*, 2005). Néanmoins, *S. thermophilus* a conservé ou acquis des gènes qui, tout en lui ayant permis de s'adapter au lait en tant que nouvelle niche écologique, peuvent être assimilés à des facteurs de virulence. Il s'agit notamment de l'uréase, qui lui permet de résister à l'acidité du milieu, du système de transport d'oligopeptides Ami et de la protéase PrtS, qui lui permettent de générer des peptides à partir des caséines qui seront ensuite internalisés pour être utilisés, ou de la capsule de nature polysaccharidique. Ces deux derniers facteurs, PrtS et déterminants de la capsule, ont été acquis par transfert horizontal (Bourgoin *et al.*, 1996; Delorme *et al.*, 2010).

Malgré son utilisation et sa consommation massive, la physiologie de *S. thermophilus* dans le tractus digestif demeure encore peu connue. Pourtant, les effets bénéfiques provoqués par la consommation régulière de yaourt contenant des bactéries vivantes (donc *S. thermophilus et Lactobacillus bulgaricus*) sont reconnus, faisant de ce produit un aliment fonctionnel de par les ingrédients biotiques (bactéries) et abiotiques (molécules issus de

l'activité bactérienne) qu'il contient. Par exemple, il a été clairement établi que la consommation de yaourt contenant des bactéries vivantes améliore la digestion du lactose et élimine les symptômes de l'intolérance au lactose (Guarner *et al.*, 2005). Elle permet également chez des enfants atteins de diarrhée aqueuse aigüe de diminuer la fréquence des selles et la durée des épisodes de diarrhée (Boudraa *et al.*, 2001). Enfin, le rôle joué par une consommation de yaourt contenant des bactéries vivantes dans la modulation du système immunitaire a été clairement établie (Van de Water *et al.*, 1999). La relative méconnaissance de la physiologie de *S. thermophilus* dans le tractus digestif résulte de l'établissement relativement récent de sa capacité à survivre au passage dans le tube digestif. En effet, si certaines études avaient conclu quant à son incapacité à survivre dans cet environnement (Del Campo *et al.*, 2005; Pedrosa *et al.*, 1995), d'autres ont montré que *S. thermophilus* pouvait être isolée à partir de fèces (Brigidi *et al.*, 2003; Elli *et al.*, 2006; Mater *et al.*, 2005). Ces résultats ont été confirmés par l'analyse de la microflore fécale d'européens qui a révélé que *S. thermophilus* était retrouvé dans les fèces de 90 % des individus (Qin *et al.*, 2010).

Le travail réalisé durant cette thèse a été mené dans l'équipe PB2P de l'URAFPA dont le thème de recherche concerne les aliments fonctionnels et tout particulièrement les laits fermentés. L'équipe PB2P s'intéresse notamment aux capacités de *S. thermophilus*, via son système protéolytique, à produire des peptides bioactifs à partir des caséines du lait et aux propriétés probiotiques de cette bactérie. C'est sur ce dernier point que se sont focalisées mes recherches, les objectifs étant :

- d'explorer les potentialités probiotiques de souches de *S. thermophilus* afin d'identifier des candidats potentiels pour une utilisation en tant que probiotique ;

- de développer un outil basé sur la technologie R-IVET (Recombinase based *In vivo* Expression Technology) afin d'identifier les gènes qui sont exprimés par *S. thermophilus* dans le tractus digestif.

Pour atteindre ces objectifs, des approches utilisant la microbiologie, la biologie moléculaire et la culture cellulaire ont été employées.

Pour ce qui concerne le premier objectif, la résistance aux stress gastriques (faible pH, sels biliaires et H_2O_2) de 30 souches de *S. thermophilus* a été évaluée et leur capacité à adhérer aux cellules de la muqueuse intestinale déterminée en utilisant la lignée cellulaire HT29-MTX. Enfin, leurs propriétés immunomodulantes ont été étudiées. Cette partie a été

réalisée grâce à une collaboration avec l'équipe « Interaction des bactéries commensales et probiotiques avec l'hôte » de l'unité MICALIS (INRA, à Jouy-en-Josas). Suite à cette étude, sur la base de sa résistance aux stress gastriques, de ses capacités d'adhésion, et sachant que la séquence de son génome est disponible, la souche LMD-9 a été sélectionnée pour la deuxième partie de ce travail, à savoir la construction d'un outil basé sur la technologie R-IVET pour étudier l'état physiologique de *S. thermophilus* dans le tractus digestif. La technologie R-IVET est une approche de transcriptomique ciblée qui permet l'identification de fonctions spécifiquement exprimées dans des environnements complexes. Les travaux de biologie moléculaire et les études *in vivo* ont été réalisées dans l'équipe PB2P de l'UR AFPA.

Le manuscrit de thèse se compose de quatre parties:

1. Une introduction en deux parties :

- la première partie présentant une synthèse sur l'utilisation de bactéries lactiques habituellement employées dans l'industrie alimentaire, dont *S. thermophilus,* dans des produits probiotiques/aliments fonctionnels. Elle est rédigée sous la forme d'une revue qui n'est pas encore dans sa forme définitive pour être soumise ;

- la deuxième partie se concentre sur les technologies génétiques utilisées pour étudier l'expression de gènes qui sont importants pour la survie et la physiologie des bactéries dans des environnements complexes tels que le tractus digestif.

2. Une partie Matériel et Méthodes

3. Une partie résultats rédigée sous la forme de deux articles :

- premier article intitulé "*A High throughput in vitro screening of Streptococcus thermophilus strains revealed a high in vitro anti-inflammatory potential of three strains"* pour être soumis à "Applied and Environmental Microbiology" ;

- deuxième article intitulé "Development of the Recombinase-based *In vivo* Expression Technology (R-IVET) in *Streptococcus thermophilus* and

validation in the gastrointestinal tract of mice using the lactose operon promoter" soumis à "Journal of Applied Microbiology".

3. Une quatrième partie qui présente les conclusions et perspectives de ce travail.

Synthèse bibliographique

1. New trends in probiotics: exploring the probiotic potential of *Streptococcus thermophilus*, a lactic acid bacterium usually employed in food/dairy industry

Abstract

Probiotics are live microorganisms that when administered in adequate amount confer a health benefit to the host. They should be present up to 10^7 cells at the time of consumption per gram of the product. Some of the main health related benefits of probiotic consumption are: relief in lactose intolerance and constipation, prevention and cure of diarrhea, antimicrobial, anticarcinogenic, antimutagenic activities, stimulation of immune system and alleviation of urogenital health incidences. They function by influencing the intestinal luminal environment, epithelial and mucosal barrier function or immune system. A probiotic should be capable of resisting the acids and bile salts in gastrointestinal tract, should transit through the gut in viable form and be recovered in feces, resist the degradation by digestive enzymes and oxygen stress, must be safe with demonstrable efficacy and should be able to reach the distal tract in viable form. Nowadays, neutraceutical products with encapsulated probiotic bacteria are gaining interest, but efforts are being made to develop some foods as probiotic carriers. Consumer's interest for functional foods is directly related to their interest to maintain a good health. Among different functional foods marketed so far, yogurt is of wide importance, due to high scale consumption and likeness by consumers. It has been successfully used in therapeutic studies against diarrhea, hypertension and lactose intolerance and it has been used for maintaining intestinal microbial balance. *Streptococcus thermophilus* is one of the basic starter bacteria of yogurt and the second most important species of industrial lactic acid bacteria after *Lactococcus lactis*. It could have great probiotic potential as it has been shown to survive gastro-intestinal transit and moderately adhere to the intestinal epithelial cells. It has been found to confer different health benefits either by producing different metabolites or by directly affecting the immune system. It is also capable of producing β-galactosidase, thus playing a vital role in reducing lactose intolerance. Besides, it is capable of producing antimicrobial substances like bacteriocins and exopolysaccharides which not only confer rheological, textural and sensory properties to yogurts, but also increase the adhesion of the bacterium to enterocytes and stimulate the immune system. It possesses many peptidolytic and proteolytic enzymes capable of generation bioactive peptides and

enhancing mucin secretions. Thus, the health benefits of yogurt combined with the functional properties of *S. thermophilus* could represent an advantage over other probiotic functional foods and could be enhanced by increasing the research done on its properties of survival in the host and itsother probiotic properties.

Keywords: Probiotics, mechanism of action, functional foods, yogurt, *Streptococcus thermophiles*

1.1. Concept of Probiotics: History and Evolution

The word probiotic is derived from the Greek word probios which means "for life". The concept evolved from a theory of Metchnikoff who believed that the long and healthy life span of Bulgarian peasants came from their regular daily consumption of fermented milks. Therefore, he wrote in 1907 that *"A reader who has little knowledge about such matters may be surprised by my recommendation to absorb large quantities of microbes, as a general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have honorable place"*.

From Metchnikoff till date, the probiotic concept has drastically evolved and different definitions of probiotics have been proposed (Figure 1). For example, in 1953 the word "*probiotika*" was used by Werner Kollath to designate organic and inorganic supplements necessary to restore health to patients suffering from a form of malnutrition caused by the consumption of highly refined food (Kollath, 1953). One year later, Ferdinand Vergin proposed that probiotics were the opposite of antibiotics as antibiotics affect the microorganisms living with us in biotic community or even in a symbiosis thus deprive us from essential probiotics (Vergin, 1954).

In 1965, the work of Lilly and Stillwell (Lilly and Stillwell, 1965) gave a new dimension to the probiotic's definition. Indeed, they had observed that certain secretion of *Colpidium campylum* increased consistently the growth of *Tetrahymena pyriformis* by extending its logarithmic phase of growth. Thus, probiotic was defined by these authors as "the secretions of one microorganism which stimulated the growth of another microorganism". Similarly, in 1971, Sperti used the term probiotics to refer to tissue extracts capable of stimulating microbial growth (Sperti, 1971).

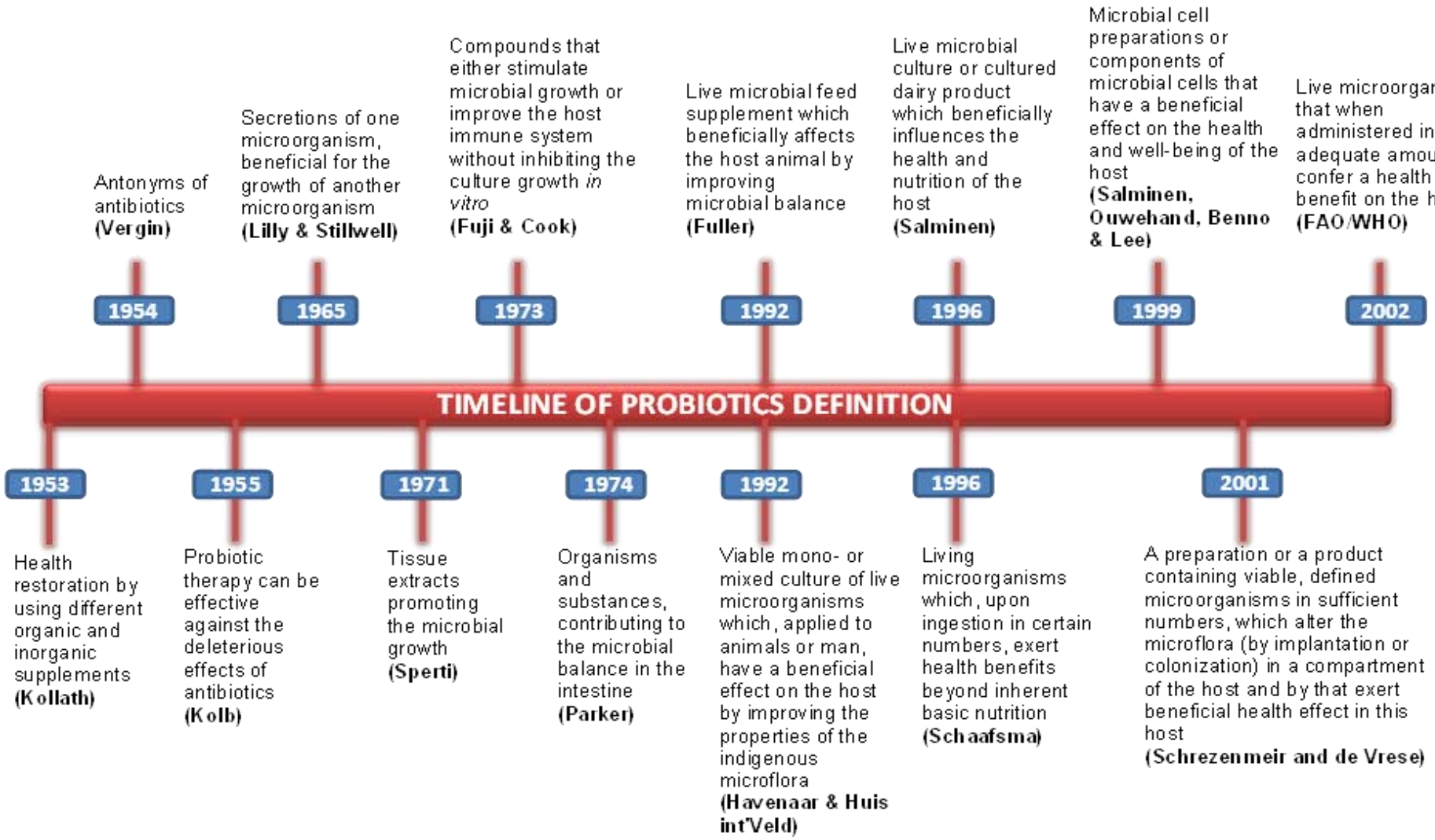

Figure 1. Evolutionary stages of probiotics definition.

In 1974, the word probiotic was used for the first time to describe a microbial feed/food supplement and defined as "organisms and substances contributing to the intestinal microbial balance" (Parker, 1974). In 1992, Fuller removed the word "substances" from the probiotic's definition as it could very well include antibiotics, emphasized on the viability of probiotic preparations and defined probiotics as live microbial feed supplement which beneficially affects the host animal by improving microbial balance (Fuller, 1989). Considering that this definition was not wide enough to cover the entire indigenous microflora, another definition was proposed by Havenaar and Huis in't Veld (Havenaar and Huis in't Veld, 1992). According to them, "probiotics are the viable mono- or mixed microbial cultures which when applied to animal or man have beneficial effects on their host by improving the properties of indigenous microflora". In 1996, the cultured dairy products were also added in the definition of the probiotics and Schaafsma defined probiotics as living microorganisms which when ingested in certain numbers exert health benefits beyond inherent basic nutrition. This definition was confirmed in 2002 by a FAO/WHO working group (FAO/WHO, 2002) stating that "Probiotics are live microorganisms that when administered in adequate amount confer a health benefit to the host", and reconfirmed by the FAO in 2006.

1.2. Probiotic microorganisms and recommended dose

Most of the probiotic microbes studied to date belong to the genera *Lactobacillus* and *Bifidobacterium*, some species of fungi (e.g., *Saccharomyces cerevisiae*), and enterococci (e.g., *Enterococcus faecalis*) and many have been commercialized worldwide (Table 1). The most commonly used genera in probiotic preparations are lactobacilli and bifidobacteria. The first belongs to the group of lactic acid bacteria, and the second is often used with LAB in food. Both bacteria are present in the human gastro intestinal tract (GIT), *Bifidobacterium* spp. being the first colonizers of newborns. This explains why both of these genera are especially used in probiotic products. Among non-commercial probiotic strains the most important are *Streptococcus thermophilus*, *Lactococcus lactis* subsp *lactis*, *Lactococcus lactis* subsp *cremoris*, *Enterococcus faecium*, *Leuconostoc mesenteroides* subsp *dextranium*, *Propionibacterium freudenreichii* and *Pediococcus acidilactici* (Collins *et al.*, 1998). *S. thermophilus* or *L. delbrueckii* subsp. *bulgaricus* are traditionally used in yogurt production and have not been considered as probiotics during a long time by most scientists as they were not expected to survive in the GIT (Senok *et al.*, 2005).

Table 1. Characterized commercially available probiotic microbial strains (partial list), their source and their effects as observed in human clinical trials.

Strain (Source)	Effects on human health	Reference
Bifidobacterium ssp		
B. adolescentis	Potential effects on cytokines production profile when used in combination with *L. rhamnosus.*	(Koyama *et al.*, 2010)
B. animalis DN173010 (Bioactiva)	ND	
B. animalis ssp. *lactis* Bb-12 (Chr. Hansen, Inc)	In combination with *L. acidophilus* NCFM reduction in fever incidence and duration, coughing and rhinorrhea in healthy children. Decrease the occurrence of respiratory infections but not in the individuals with gastro-intestinal symptoms and use of antibiotics.	(Leyer *et al.*, 2009), (Taipale *et al.*, 2011)
B. bifidum (MIMBb75)	Decrease the symptoms of irritable bowel syndrome, improve the quality of life, reduction in parental-reported eczema during initial three months when used in mixture with *B. animalis* subsp. *lactis* and *Lactococcus lactis.*	(Guglielmetti *et al.*, 2011)(Niers *et al.*, 2009)
B. breve strain Yakult (Yakult)	Decrease the occurrence of Necrotizing enterocolitis in premature babies when used in combination with *L. casei* as supplement in human milk, effective against asthma like symptoms when used alone or in combination with galacto/fructo-oligo-saccharide mixture.	(Braga *et al.*, 2011)(Van der Aa *et al.*, 2011)
B. infantis	When 10^8 CFU dose is used it significantly decrease the symptoms of irritable bowel syndrome.	(Whorwell *et al.*, 2006)
B. lactis HN019 (DR10) (Foneterra)	ND	
B. lactis HOWARUTM/Bl	ND	
B. lactis LAFTIs B94	ND	
*B. longum (*BL46)(BL2C)	Normalizes the bowel movements.	(Pitkala *et al.*, 2007)
B. longum BB536 (Morinaga Milk Industry Co.Ltd)	In case of allergic inflammations significant decreases in rhinorrhea and nasal blockage.	(Xiao *et al.*, 2006)
B. longum SBT-2928 (Snow Brand Milk Products Co., Ltd)	ND	

Table 1. continued

Bacillus coagulans GB-30, 6086 (GanedenBC^{30})	Reduce the gastro-intestinal symptoms in adults.	(Kalman *et al.*, 2009)
Escherichia coli Nissle 1917	Significant reduction in daily stool frequency after 14 days use in infants with persistent diarrhea.	(Henker *et al.*, 2008)
Lactobacillus ssp.		
L. acidophilus DDS-1 (Nebraska Cultures, Inc)	ND	
L. acidophilus LA1/LA5 (Chr. Hansen, Inc)	ND	
L. acidophilus LAFTIs L10 (DSM Food Specialties)	ND	
L. acidophilus LB (Lacteol Laboratory)	ND	
L. acidophilus NCDO 1748	ND	
L. acidophilus NCFM® (Danisco, Rhodia, Inc)	Preserve insulin sensitivity versus placebo in type II diabetes patients, alone or in combination with *B. animalis* reduction in fever incidence and duration, coughing and rhinorrhea in healthy children.	(Andreasen *et al.*, 2010)(Leyer *et al.*, 2009)
L. acidophilus R0052	ND	
L. acidophilus SBT-2062 (Snow Brand Milk Products Co., Ltd)	ND	
L. casei Immunitas (Danone)	ND	
L. casei DN-114 001	Improve the incidences of allergic rhinitis.	(Giovannini *et al.*, 2007)
L. casei Shirota (Yakult)	Effective against aspirin-associated small bowel injuries.	(Endo *et al.*, 2011)
L. delbrueckii ssp. *bulgaricus* Lb12	Increase the natural killer cell activity and reduce the risk of catching common cold in adults.	(Makino *et al.*, 2010)
L. fermentum RC-14 (Urex Biotech)	ND	
L. fermentum VRI-003	Reduction in duration and severity of the respiratory illness.	(Cox *et al.*, 2010)
L. johnsonii LA1 (NCC533)	Accelerated recovery of skin immune homeostasis after UV-induced immunosuppression.	(Peguet-Navarro *et al.*, 2008)

Table 1. continued

L. johnsonii LA1/Lj1 (Nestlé)	ND	
L. lactis L1A (Essum AB)	ND	
L. paracasei CRL431	ND	
L. paracasei F19 (Medipharm)	ND	
L. paracasei LAFTIs L26	ND	
L. paracasei Lpc	ND	
L. paracasei ssp paracasei, CRL 431 (Chr. Hansen, Inc)	ND	
L. paracasei ssp. paracasei, F19	Decrease the incidences of eczema and enhance the ratio of Th1/Th2.	(West *et al.*, 2009)
L. plantarum 299V (Probi AB)	ND	
L. reuteri ATCC 55730	Decrease the antibiotic-associated diarrhea.	(Cimperman *et al.*, 2011)
L. reuteri DSM 17938	Positive effects on infantile colic.	(Savino *et al.*, 2010)
L. reuteri RC- 14	ND	
L. reuteri SD2112/MM2 (Biogaia)	ND	
L. rhamnosus 271 (Probi AB)	ND	
L. rhamnosus GG (LGG)(Valio Dairy)	Reduce the frequency of eczema during first seven years of life, mild effect on allergy sensitization.	(Rose *et al.*, 2010)(Kalliomaki *et al.*, 2007)
L. rhamnosus GG (ATCC 53103)	Mild effect on allergy sensitization.	(Kalliomaki *et al.*, 2007)
L. rhamnosus GR-1(Urex Biotech)	ND	
L. rhamnosus HN001 (DR20)	ND	
L. rhamnosus LB21 (Essum AB)	ND	
L. rhamnosus R0011 (Institute Rosell)	ND	
L. salivarius UCC118 (University College, Cork)	ND	
Lactococcus lactis L1A	ND	
Saccharomyces cerevisiae subsp. *Boulardii* (Merc)	Improve intestinal permeability.	(Garcia Vilela *et al.*, 2008)
Streptococcus thermophilus (VSL#3)	Improvement in the symptoms of irritable bowel syndrome in children, and improves the quality of life when used in mixture with seven other probiotic strains (VSL#3).	(Guandalini *et al.*, 2010)

ND : not determined

According to Kailasapathy (Kailasapathy and Chin, 2000) the minimum therapeutic dose should be almost 10^8 to 10^{10} CFU/day, others claim that at least 10^9 to 10^{10} viable organisms should reach the intestine to exert their beneficial effects (Canganella *et al.*, 1997; Sanders, 1998). According to International dairy federation the presence of minimum 10^7 bacterial cells at the time of consumption per gram of the product are necessary for a probiotic product to exert beneficial health effects. It is not possible to state a general dose that is needed for probiotics; it has to be based on human studies showing a health benefit. It varies from strain to strain and from product to product.

1.3. Health properties and mechanism of probiotic action

Numerous health benefits possibly resulting from the ingestion of probiotics have been claimed. Some of them have been observed in animal models and expected to occur in humans as well while others have been proved especially through human trials (Table 1). Some of the main health related benefits of probiotic consumption are: relief in lactose intolerance and constipation, prevention and cure of diarrhea, antimicrobial, anticarcinogenic, antimutagenic activities, stimulation of immune system and alleviation of urogenital health incidences (Table 2) (Bomba *et al.*, 2002; Nagpal *et al.*, 2012; Shah, 2007). In addition to this, certain probiotic bacteria have been recommended for the treatment of atopic dermatitis, chronic liver diseases, allergies, colitis and necrotizing enterocolitis (Boyle and Tang, 2006; Candy *et al.*, 2008). Also, probiotic bacteria can be used to treat irritable bowel syndrome (Santosa *et al.*, 2006) and to reduce serum cholesterol (Shah, 2007). At this stage, it is essential to note that these health effects are highly strain-dependent and there is not a single probiotic strain which can confer all the benefits previously reported (Shah, 2007).

Table 2. Health benefits of probiotic bacteria to the host and their proposed mechanisms of action involved (adapted from Tsai *et al.*, 2012).

Health Benefits	Proposed possible mechanisms
Resistance to enteric pathogens	Antagonism activity Adjuvant effect increasing antibody production Systemic immune effect Colonization resistance Limiting access of enteric,pathogens (pH, bacteriocins/defensins, antimicrobial peptides, lactic acid production, and toxic oxygen metabolites)
Aid in lactose digestion	Bacterial lactase acts on lactose in the small intestine
Small bowel bacterial overgrowth	Lactobacilli influence the activity of overgrowth flora, decreasing toxic metabolite production Normalization of a small bowel microbial community Antibacterial characteristics
Immune system modulation	Strengthening of nonspecific and antigen-specific defense against infection and tumors Adjuvant effect in antigen-specific immune responses Regulating/influencing Th1/Th2 cells, production of anti-inflammatory cytokines
Anticolon cancer effect	Antimutagenic activity Detoxification of carcinogenic metabolites Alteration in pro-cancerous enzymatic activity of colonic microorganisms Stimulation of immune function Influence on bile salt concentration
Decreased detoxification/ Excretion of toxic microbial metabolites	Increased bifidobacterial cell counts and shift from a preferable protein to carbohydrate-metabolizing microbial community, less toxic and for putrefactive metabolites, improvements of hepatic encephalopathy after the administration of bifidobacteria and lactulose
Allergy	Prevention of antigen translocation into blood stream Prevent excessive immunologic responses to increased amount of antigen stimulation of the gut
Blood lipids, heart disease	Assimilation of cholesterol by bacterial cell Alteration in the activity of BSH enzyme Antioxidative effect
Antihypertensive effect	Bacterial peptidase action on milk protein results in antihypertensive tripeptides Cell wall components act as ACE inhibitors
Urogenital Infections	Adhesion to urinary and vaginal tract cells Competitive exclusion Inhibitor production (H_2O_2, biosurfactants)
Infection caused by *Helicobacter pylori*	Competitive colonization Inhibition of growth and adhesion to mucosal cells, decrease in gastric *H. pylori* concentration
Hepatic encephalopathy	Competitive exclusion or inhibition of urease producing gut flora
Neutralization of dietary carcinogens	Production of butyric acid neutralizes the activity of dietary carcinogens

Table 2. continued

NEC(necrotic inflammation of the distal small intestine)	Decrease in TLRs and signaling molecules and increase in negative regulations Reduction in the IL-8 response
Rotaviral gastroenteritis	Increased IgA response to the virus
Inflammatory bowel diseases, type I diabetes	Enhancement of mucosal barrier function
Crohn's disease	Reduction in proinflammatory cytokines including TNFa, reduction in the number of CD4 cells as well as TNFa expression among intraepithelial lymphocytes
Caries gingivitis	Reduction in gingivitis by *L. reuteri*, affects on streptococcus mutants, colonization of the teeth surface by lactobacilli Less carries after the ingestion of living or oral vaccination with heat-killed lactobacilli
Enhanced nutrient value	Vitamin and cofactor production
Adult atopic dermatitis	Adjuvant effect
Human inflammations	Direct competition between pathogenic bacteria in the gut and/or immune modulation
Visceral hyperalgesia	Activation of receptors in the enteric nervous system
Influenza immunization	Increased IgG response
Collagen induced arthritis	Production of anticollagen antibodies
Antibiotic-associated diarrhea (AAD)	By maintaining the gut microflora and ongoing carbohydrate fermentation; and/or by competitively inhibiting the growth of pathogens

Although all of the mechanisms of action of probiotics are far from to be elucidated, it is known that after their consumption probiotics exert their beneficial action by influencing the intestinal luminal environment, epithelial and mucosal barrier function and immune system.

1.3.1. Immunomodulation

An immune system serves to protect the host from pathogens. The site of action and the type of pathogen determines that which type of immune response will be effective. This immune response involves recognition of the pathogen or any other foreign material and the mounting of a reaction to eliminate it. These immune reactions can either be innate immune responses (nonspecific) or acquired (specific) immune responses. Lymphocytes and cells like macrophages, antigen presenting cells and sometimes epithelial cells are the mediators in these responses. The first exposure to a foreign pathogen affects the innate immune response, it is non-specific. Here mediators like cytokines can be released. However in case of acquired

or specific immunity lymphocytes with specific antigen receptors as well as helper T cells (Th) are involved (Erickson and Hubbard, 2000). Intestinal microbiota (a term which has today, replaced the old denomination of "microflora") (Quigley, 2012) represents an ecosystem constituted of a variety of ecological niches, occupied by several bacterial species and a very large amount of strains (Aureli *et al.*, 2011). Human intestinal microbiota is composed of 10^{13} to 10^{14} microorganisms (Gill *et al.*, 2006) belonging to several hundreds of distinct bacterial species and thousands of strains which were identified by using anaerobic culture-based and molecular techniques.

The knowledge accumulated during these last thirty years revealed that the human intestinal microbiota constitutes an essential "organ" as nominated by Eckburg (Eckburg *et al.*, 2005). It contains both beneficial (e.g. Bifidobacteria) and harmful members (e.g. Clostridia), playing vital role in maintaining human health and wellbeing. They are involved in stimulation of non-specific immune system (Olszak *et al.*, 2012), combat against the pathogens, detoxify the carcinogenic compounds, releases variety of metabolites (like short chain fatty acids (SCFAs), Trimethyl amine-N-oxide (TMAO) and hippurate) by the degradation of indigestible food ingredients (Yatsunenko *et al.*, 2012), and interacts with intestinal epithelial cells through these metabolites (Corthier and Dore, 2010). In addition to this, gut bacteria are involved in vitamin synthesis (especially vitamins B and K) and metabolism of xenobiotics. The intestinal microbiota regulates both systemic and local immune responses through the development of gut associated lymphoid tissue (GALT) at an early age (Dugas *et al.*, 1999). Maturation of humoral immune, especially circulation of IgA and IgM-secreting cells results from microbial colonization of the gut.

Immunomodulating capacity of probiotics affects the innate as well as the adaptive immunity. They affect numerous cell types involved in the innate and acquired immune responses like epithelial cells, dendritic cells, monocytes/macrophages, B cells, T cells including those with regulatory properties and NK cells. They may act through different mechanisms such as production of metabolites, increase in production of phagocytic activity of granulocytes or cytokine excretion or immunoglobulin-secreting cell proliferation.

Thus the presence of D-alanines in teichoic acids, an important component of the cell wall of gram positive bacterium *L. plantarum* provokes the production of pro-inflammatory cytokines from peripheral blood mononuclear cells (PBMC) (Grangette *et al.*, 2005).

When a bacterium comes in contact with its host it is internalized by M cells, interacts with dendritic cells and follicle associated epithelial cells triggering macrophages, T and B lymphocytes mediated responses (Winkler *et al.*, 2007). Number of host's signaling pathways are involved in these immune responses. The immunomodulatory effects of probiotics are mainly species and strain specific (Christensen *et al.*, 2002; Lin *et al.*, 2008; Thomas and Versalovic, 2010). Some probiotic *Lactobacillus* strains increase production of tumor necrosis factor through activation of transcription factors nuclear factor-κB and STAT (Miettinen *et al.*, 2000) whereas others suppress tumor necrosis factor production by inactivating nuclear factor-κB (Iyer *et al.*, 2008) or mitogen-activated protein kinase and c-Jun signaling (Lin *et al.*, 2008).

When a pathogen comes in contact with intestinal epithelial cells it influences the immune system by inducing the secretion of certain cytokines. On the other hand, when probiotics are administered, they may cancel the effect of pathogen by decreasing the production of these cytokines. For example, when pathogens *E. coli, Salmonella dublin, Lysteria monocytogenes and Shigella dysenteriae* infect the host cells they induce the secretion of IL-8 (pro-inflammatory cytokine) leading to the inflammation of intestinal epithelium. The probiotics like those present in VSL#3 preparation, when administered in these conditions inhibit/down-regulate the secretion of IL-8 hence, counter effecting the inflammation of intestinal mucosa (Otte and Podolsky, 2004).

1.3.2. Antimicrobial properties

Probiotics may create antagonistic environments for food borne pathogens and spoilage organisms. This is achieved either (i) by secretion of antimicrobial substances like bacteriocins and the deconjugated bile acids, (ii) by competing for limited available resources, (iii) by their anti-adhesive capacities, (iv) by anti-invasive effects, and (v) by antitoxin effects. For example, *L. salivarius* UCC118 produces a broad spectrum bacteriocin which acts as a protective agent against the systemic spread of infection with *Listeria monocytogenes* by killing it in the lumen of gastro-intestinal tract (Corr *et al.*, 2007). *Lactobacillus reuteri* produces a bacteriocin, known as reuterin, which is also a broad spectrum bacteriocin effective not only against Gram-positive and Gram-negative bacteria but also against yeasts, fungi, protozoa and viruses (Cleusix *et al.*, 2008). Bifidobacteria strains also produce certain bacteriocins effective against both Gram-positive and Gram-negative (Cheikhyoussef *et al.*, 2008). Deconjugated bile acids produced by probiotics are the derivatives of bile salts and

have stronger antimicrobial activity as compared to the bile salts synthesized by the host cells (Oelschlaeger, 2010).

With the exception of lactobacilli, iron is an essential element for almost all bacteria (Weinberg, 1997) and competition for iron among microorganisms can be a crucial advantage. *L. acidophilus* and *L. delbreuckii* are for example capable of binding ferric hydroxide at their cell surface, thus making it unavailable for pathogenic microorganisms (Elli *et al.*, 2000). In contrary to this, the probiotic *Escherichia coli* strain Nissle 1917 (EcN) is totally dependent on iron and express iron uptake systems to transport iron into its cells. This property is equally present in certain pathogenic bacteria but EcN has the advantage over these bacteria as it encodes at least seven different iron uptake systems (Grozdanov *et al.*, 2004).

By adhering to the intestinal epithelial cells probiotics block the adhesion of pathogenic bacteria. Evidences have been given for the reduction in the adhesion of pathogenic *Salmonella*, *Clostridium* and *E. coli* to the intestinal mucus in the presence of probiotic strains *Bifidobacterium lactis* Bb12 and/or *lactobacillus rhamnosus* LGG in porcine animal models (Collado *et al.*, 2007). This might be due to the competition for same receptor between probiotics and pathogen or from an increase mucin production induced by probiotics. Finally, *L. acidophilus* suppresses the transcription of *E. coli* O157:H7 genes involved in adherence leading to its reduced adhesion to intestinal mucosa (Medellin-Pena and Griffiths, 2009).

Probiotics can also interfere with production of toxic substances to protect their host against such toxins. In an experiment, all animals treated with *B. breve* strain Yakult survived whereas 90% of the mice in the control group died after infected by *E. coli* (STEC) O157:H7. *In vitro* studies have shown that supression of Shiga toxin is the result of high concentrations of acetic acid produced by strain Yakult (Asahara *et al.*, 2004). The toxin A produced by *Clostridium difficile* responsible for causing diarrhea and its receptor, are hydrolyzed by a 54 kDa serine protease produced by *Saccharomyces cerevisiae* var. *boulardii* present in intestinal brush border (McFarland, 2010; Pothoulakis *et al.*, 1993).

Exclusion is a term used for probiotic mode of action in which they make the gastro-intestinal environment less suitable for the pathogens. Exclusion encompasses the mechanisms like changing the resident microbiota composition, decreasing the luminal pH by

producing acetic and/or lactic acid, synthesizing hydrogenperoxyde or diacetyl, enhancement of the epithelial barrier function, down-regulation of specific host receptors, stimulating the production of mucins and defensins.

Certain probiotics reduce the luminal pH as described in a study (Venturi *et al.*, 1999) where probiotic preparation VSL#3 was used in the patients with ulcerative colitis (Asahara *et al.*, 2004).

Probiotic bacteria can also stimulate defensins (e.g. human beta defensin 2, hBD-2) which are intestinal antimicrobial peptides produced by epithelial cells and are involved in the defense mechanism. Their production is stimulated by butyrate produced by enteric microflora (Ohland and MacNaughton, 2010) which participate in the improvement of mucosal barrier hence limiting the access of enteric pathogens (Wehkamp *et al.*, 2004).

1.3.3. Anticarcinogenic properties

The reputed anticarcinogenic effect of probiotics is supported by *in vitro* studies with carcinoma cell lines and anti-mutagenicity assays and has been confirmed in animal and to a limited extent in human. This effect can be attributable to a combination of events including anti-genotoxic effect, inhibition of colonic enzymes or production of physiologically active metabolites like short chain fatty acids (Commane *et al.*, 2005).

The conversion of different procarcinogens into carcinogens by microbial enzymes like azoreductase, β-glucuronidase and nitroreductase, may be the cause of sporadic colon cancer (Goldin, 1990). The diminution of the activity of such enzymes decreases the risk of colon cancer due to this activity. Further, the reduction of luminal pH, through metabolic activities of LAB, could inhibit the growth of harmful putrefactive bacteria which generated genotoxic agents and thus prevent large bowel cancer (Modler *et al.*, 1990). *L. acidophilus* has been shown to decrease nitroreductase, azoreductase and β-glucuronidase activities in carnivorous animals (Goldin and Gorbach 1977), and later *L. rhamnosus* GG was shown to lower bacterial β-glucuronidase activity in the large intestine (Goldin *et al.*, 1992). Experimental animal and human studies show that probiotic supplements can also modify bacterial enzyme activities. For example, nine subjects (male and female), who consumed 100 g of this product, three times a day for 3 weeks, showed lower faecal concentrations of nitroreductase, azoreductase and β- glucuronidase (microbial enzymes that are associated with carcinogen production in the gut) (Burns and Rowland, 2000), than they exhibited during

control periods, in which they consumed no fermented milk products. Finally, *Lactobacillus* GG positively affected the initiation or promotion of DMH-induced tumours in rats on a high-fat diet. Orally administered strains of *L. casei* were proved to be effective in preventing the recurrence of superficial bladder cancer (Aso *et al.*, 1995). It is suggested that bile salts have been implicated in the initiation of colon carcinogens (Lewis and Gorbach, 1972). *L. acidophilus* reduced the biotransformation of primary to secondary bile salts, thus reducing the possible initiation of cancer (Fernandes and Shahani, 1990). Finally, LAB and other probiotic microorganisms are capable of inhibiting certain toxic material like aflatoxins, produced by moulds which are known to cause cancer. Aflatoxin B1, the best studied of all, causes liver cancer in humans. The inhibition of mould growth and aflatoxin production by LAB has been reported (Gourama and Bullerman, 1995). For example, *L. casei* subsp. *pseudoplantarum* inhibits the biosynthesis of aflatoxins B1 and G1 (Gourama and Bullerman, 1997) and *L. rhamnosus* GG binds aflatoxin B1, and to a lesser extent aflatoxins B2 and G1 (El-Nezami *et al.*, 1996).

1.3.4. Enhancement/ Production of SCFA

Gut microbiota may be involved in metabolic diseases by two major mechanisms: (1) the innate immune response to the structural components of bacteria (e.g., lipopolysaccharide) resulting in inflammation and (2) bacterial metabolites of dietary compounds (e.g., short chain fatty acids (SCFA) from fiber), which display biological activities that regulate host functions (Harris *et al.*, 2001). The short chain fatty acids (SCFA) are acetate, butyrate and propionate. Probiotic bacteria which compete with other bacteria in the colonic lumen for fermentation substrates, which may include non-digestible carbohydrates, proteins and lipids from shed epithelial cells, mucins from GI secretions and recyclable components of dead bacteria, may increase the SCFA production. Thus, Sakata *et al*. (2003) observed an increase in the net SCFA production in pigs fed probiotics, they argue that the increased yield is due to the ability of the probiotic strains to ferment those carbohydrates resistant to the actions of the normal flora. It must be noted that the primary metabolites of the LAB are acetoacetate, acetate and lactate (Macfarlane *et al.,*1998), and that the acetateis the major SCFA isolate found in human faeces. It is not only absorbed and utilized by peripheral tissues (Pomare *et al.*, 1985) but also used by bacteria isolated from the human intestine for the production of butyrate in the colon (Duncan *et al.*, 2002). Lactate and acetoacetate may form substrates for other members of the flora and may be degraded into other SCFA.

1.3.5. Cholesterol lowering

Several probiotic strains could be used to prevent coronary and cardiovascular deseases through their capacity to lower cholesterol. Thus, hypercholesterolemia in mice was shown to be prevented by 7 days regular administration of 10^4 CFU/day of *L. reuteri* CRL 1098 (Taranto *et al.,*2000). 22 and 33% decrease of total serum cholesterol and triglyceride levels, respectively, was observed in these mice. Recently, in a study significant decrease in total plasma cholesterol was observed by using yogurt supplement containing *Bifidobacterium pseudocatenulatum* G4 or *Bifidobacterium longum* BB536 in rats fed with cholesterol-enriched diet (Al-Sheraji *et al.*, 2012). This beneficial effect can be achieved through the deconjugation bile salt reaction catalyzed by the bile salt hydrolase (BSH) of probiotics, cholesterol assimilation, binding of cholesterol to bacterial cell walls and reduction of its biosynthesis (Nagpal *et al.*, 2012). For example, it has been shown that the introduction in the diet of a *L. acidophilus* strain resistant to bile salt and able to assimilate cholesterol prevents dietary elevation of plasma cholesterol levels (Gilliland *et al.*, 1989). Deconjugation reaction results in free bile acids which are not so easily reabsorbed and are excreted in the faeces (De Smet *et al.,* 1994). This bile salt loss leads to an increase of the catabolism of cholesterol into bile acids, leading to lower of cholesterol levels (De Rodas *et al.,* 1996; Driessen and De Boer, 1989).BSH activity has been shown in *Lactobacillus, Enterococcus, Peptostreptococcus, Bifidobacterium, Clostridium* and *Bacteroides* spp. (Bateup *et al.,* 1995; Grill *et al.,* 1995).

Klaver and Van der Meer (1993) proposed that the reduction of cholesterol might be due to the co-precipitation of cholesterol with deconjugated bile salts at pH values below 6.0. This would not explain reduction of cholesterol *in vivo* as the pH of the lower GIT is neutral to alkaline. Marshall and Taylor (1995) also observed co-precipitation of cholesterol with deconjugated bile salts, but also reported cholesterol removal in the absence of bile. They suggested a physical association between cholesterol and the cell surface. Finally, Pereira and Gibson (2002) have shown the capacity of certain probiotics to assimilate cholesterol into their cellular membranes in presence of bile salt.

1.3.6. Regulation in stool transit

Probiotics play an important role in regulation of stool transit i.e. either during the condition of diarrhea or in constipation. Acute diarrhea is a serious cause of infant mortality

that can be caused by viral (rotavirus) or bacterial infections in the gut. Saavedra *et al.* showed that formula combining *Bifidobacterium bifidum* and *S. thermophilus* reduced the incidence of rotavirus-associated diarrhea (Saavedra *et al.*, 1994). In a subsequent study, it was shown that supplementing infants with live *Bifidobacterium lactis* and *S. thermophilus* rendered these infants less susceptible to develop colitis (Saavedra *et al.*, 2004). The recovery of acute watery diarrhoea in young children was observed to be accelerated by *Lactobacillus* GG (Colombel *et al.,* 1987). In another study (Siitonen *et al.,* 1990) volunteers with antibiotic-associated diarrhea (under erythromycin treatment) reacted positively when they received *Lactobacillus* GG. Relief in symptoms like diarrhoea, stomach pain, abdominal pain and nausea was observed. Similar findings have been reported by Isolauri *et al.* (1991) and Majamaa and Isolauri (1997). However, a placebo randomized study failed to demonstrate that yogurt had a positive effect on antibiotic-associated diarrhea in children (Conway *et al.*, 2007). *E. faecium* SF68 was also proved to be effective in children with paediatric diarrhea (Bellomo *et al.,* 1980) and in adults it was found to reduce the duration of diarrhea (Buydens and Debeuckelaere, 1996).

As far as constipation is concerned, a study conducted by Koebnick *et al.* (2003) has shown that *L. casei* strain Shirota (LcS) of Yakult resulted in a significant improvement in severity of constipation and stool consistency, from the second week of treatment onwards. Eighty-nine percent of the patients that received LcS versus 56% of the placebo group reacted positively to the treatment. Marteau *et al.* (2002) have shown that a fermented milk product containing *Bifidobacterium animalis* strain DN-173 010 of Danone shortened the colonic transit time in healthy women.

1.3.7. Relief in lactose intolerance

Lactose intolerance comprises symptoms that are caused by the inability to digest the milk sugar, lactose. Digestion of lactose requires the action of the β-galactosidase (lactase) present in the intestinal mucosa. Individuals with a β-galactosidase deficiency or suffering from a reduction in lactase activity caused by intestinal infection (e.g. rotavirus gastroenteritis) suffer from lactose intolerance, which can cause abdominal pains and diarrhea. The use of probiotic bacteria producing high level of β-galactosidase like *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* allow the alleviation of this intolerance (see below).

1.3.8. Obesity and diabetes

Obesity is a major public health issue as it is causally related to several chronic disorders, including type-2 diabetes, cardiovascular deseasesD and cancer. Indigenous gut microbes may regulate body weight by influencing metabolic, neuro-endocrine and immune functions of the host. The intestinal microbiota, as a whole, provides additional metabolic functions and regulates the host's gene expression, improving the ability to extract and store energy from the diet and contributing to body-weight gain. Imbalances in the gut microbiota and increases in plasma lipopolysaccharide may also act as inflammatory factors related to the development of atherosclerosis, insulin resistance and body-weight gain. In contrast, specific probiotics and related metabolites might exert beneficial effects on lipid and glucose metabolism, the production of satiety peptides and the inflammatory tone related to obesity and associated metabolic disorders (Sanz *et al.,*2010).

The connection between gut microbiota, probiotics, energy homeostasis and inflammation and its role in the pathogenesis of obesity-related disorders are increasingly recognized. The possible mechanisms can be an increased energy harvest from the diet, altered fatty acid metabolism and composition in adipose tissue and liver, modulation of gut peptide YY and glucagon-like peptide (GLP)-1 secretion, activation of the lipopolysaccharide toll-like receptor-4 axis, and modulation of intestinal barrier integrity by GLP-2 (Musso *et al.*, 2010). Several short-term randomized controlled trials showed the benefit of probiotics on insulin sensitivity, inflammatory markers, postprandial incretins, and glucose tolerance.

1.4. Criteria for probiotic selection

Since viable and biologically active microorganisms are usually required at the target site to produce a given health benefit, probiotic cultures after ingestion in humans should first survive the gastric stresses and finally the host's natural barriers against ingested bacteria (Minelli and Benini, 2008) While their passage through the gastrointestinal tract they encounter harsh acidic conditions in stomach, bile acids, salts and enzymes in the intestine and the oxidative stresses. Large majority of bacterial viability is lost during gastric exposure. That is why acid and bile tolerance are among the most important criteria for the selection of probiotics (Dunne *et al.*, 2001). Furthermore, it should be capable of exerting a beneficial effect on the host, e.g. increase in disease resistance (Fuller, 1989), transit through the gut in viable form, resistance to degradation by digestive enzymes like lysozymes residing in the

intestine, resistance to oxygen, must be safe with demonstrable efficacy (Kailasapathy and Chin, 2000) and should be able to reach the distal tract in a viable form and can be recovered from the feces (Elli *et al.*, 2006).

Besides the properties of survival cited above, other criteria can be used to select the appropriate probiotic as shown in Figure 2. Among them can be cited the capability of probiotic to adhere to intestinal epithelium, immune stimulating capacity, anatagonistic activity against pathogen such as *Helicobacter pylori, Salmonella* spp., *Listeria monocytogenes* and *Clostridium difficile,* antimutagenic properties and anticarcinogenic properties (Mattila-Sandholm *et al.*, 2002).

As far as the technological aspects are concerned, a probiotic also need to possess the ability to survive and be viable in the products during food production and storage (Ljungh and Wadström, 2006), should not disrupt the organoleptic properties (color, taste, aroma, texture) of the product, must be phage resistant and should be genetically stable.

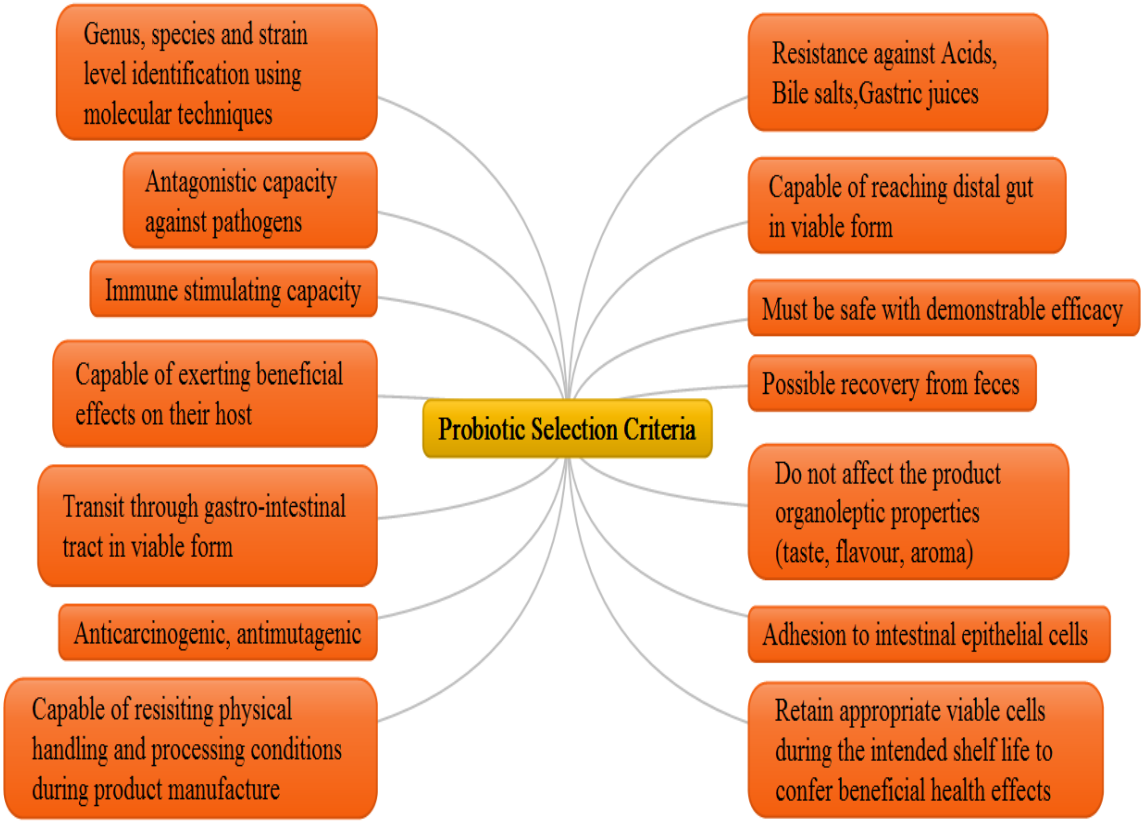

Figure 2. Important considerations for the selection of a probiotic microorganism.

1.5. Mode of administration/ Mode of delivery of probiotics

The viability of probiotic microorganisms until they reach their site of action depends upon the mode of administration or the carrier system used to protect them from adverse gastro-intestinal conditions.

In most of the clinical studies probiotics are used as freeze dried (Rochet *et al.*, 2008), powdered bacteria in form of tablets or packed in capsules (Andreasen *et al.*, 2010). Common examples of probiotic capsule are Mutaflor containing *Escherichia coli* Nissle 1917, which is proved to reduce the incidences of diarrhea when ingested orally (Henker *et al.*, 2008) and Lacteol containing Lactobacillus species, which reduced the daily stool frequency in 50% patients suffering from functional diarrhea and decrease the symptoms associated with it like pain and bloating after its four weeks daily intake (Andresen *et al.*, 2012). However, now they are commercially available in different food matrices as well, which highly affect their persistence in the gastro-intestinal tract.

Food not only serves as carrier for probiotics but through its buffering capacity protects in the GIT and regulates their colonization capacity. Its fat content, type of proteins and their concentration, sugars and pH are some major factors affecting the probiotic growth and survival in food products. Food contains other functional and bioactive ingredients which can enhance the functionality and efficacy of probiotics (Ranadheera *et al.*, 2010). Fermented foods especially dairy products are the main vectors of administration of probiotics to human gastro-intestinal tract (Ross *et al.*, 2002). Lactobacillus and Bifidobacteria are among the known health promoting probiotic microorganisms used in dairy industry. A wide variety of probiotic dairy products are available in different markets. Some common examples include pasteurized milk, fermented milks, ice creams, cheeses and baby feed milk powders (Tamime *et al.*, 2007) beside this soymilk, mayonnaise, meats, baby foods, confectionary, edible spreads, sweets, cakes and chewing gums containing probiotics are also available (Arihara *et al.*, 1998; Champagne *et al.*, 2005; Khalil and Mansour, 1998; Mattila-Sandholm *et al.*, 2002; Stokke *et al.*, 1993). Raw materials such as cereals, fruits and vegetables have recently been investigated to determine their suitability to design new nondairy probiotic foods (Rivera-Espinoza and Gallardo-Navarro, 2010).

Researchers face technological challenges while developing new probiotic products due to aggressive environments within the food matrices like high salts, acid or oxygen

concentration and low storage temperature (Cruz *et al.*, 2010). Viability of probiotics can be highly affected by these factors. Microencapsulation is an effective technique to overcome this problem. Microencapsulation is a physicochemical or mechanical process in which live probiotic bacteria or other therapeutic live cells are entrapped in some edible material to produce particles with diameters of few nanometers to few millimeters to protect them from harsh gastric conditions and to deliver them at suitable sites with improved survival (Chang, 2005; Chen and Chen, 2007). It protects the microorganism from product' s intrinsic properties as well as from gastro-intestinal tract conditions (Pimentel-Gonzalez *et al.*, 2009). The survival of encapsulated tested cultures in simulated gastric environment was considerably better than those of non-encapsulated strains with survival percentage of 34.15% to 57.71% for encapsulated bacteria as compared 17.15% to 43.20% for non-encapsulated bacteria (Khater *et al.*, 2010).

Coating material used in encapsulation is a very important factor for bacterial survival. The most commonly used coating materials are alginate, gellan gum and xanthan gum, κ-carrageenan, cellulose acetate phthalate, chitosans, gelatin, starch and milk proteins (Burgain *et al.*, 2011). The viability of encapsulated probiotic cells depend on the type and concentration of coating material, particle size, initial cell numbers and bacterial strain (Chen and Chen, 2007). The probiotic microcapsules should be water in-soluble to maintain their integrity in food matrix and in the upper part of the gastrointestinal tract and be progressively released in intestine (Picot and Lacroix, 2004). The choice of coating material is dependent upon the product in which the microcapsules will be used. Recently calcium alginate was proved to be a suitable coating material for probiotic viability in food products that require storage below freezing temperatures. Four probiotic bacteria, *Lactobacillus paracasei* L26, *L. casei*-01, *L. acidophilus* Ki and *Bifidobacterium animalis* BB-12 encapsulated in alginate supplemented with L-cysteine.HCl were analyzed for their survivability at temperatures below the freezing point. Best protective effect was obtained at ultrafreezing temperatures (-80°C) and even after 180 days viable cell number in encapsulates were above 6-7 Log (cfu/ml) (Sousa *et al.*, 2012).

Nowadays, neutraceutical (nutrition and pharmaceuticals) products with encapsulated probiotic bacteria are gaining center stage, but efforts are being made to develop novel food as an ideal probiotic carrier system.

1.6. Probiotic functional foods

With the increasing cost of health care, especially in developing countries, prevention approaches aimed at maintaining the health of an individual received special attention. Knowing the role that diet plays in maintaining health of consumers, a whole field of research aiming to use food as means of prevention is being explored, with the development of functional food (Stanton *et al.*, 2005). The term functional foods, introduced for the first time in 1993 (Swinbanks and O'Brien, 1993) covers a wide range of products with typical example of yogurt, cholesterol-lowering spreads and oligosaccharide-added foods (Williamson, 2009). Although there is no consensus about the exact definition of functional foods, but they are generally considered as foods that provide health benefits beyond basic nutrition due to certain physiologically active components, which may or may not have been manipulated or modified to enhance their bioactivity. It is also accepted that these foods should come from natural sources, and their nutritional characteristics should also be able to provide physiological benefits by improving health and wellbeing (Bhat and Bhat, 2011) or to reduce the risks of chronic diseases (e.g., cardiovascular diseases, overweight or obesity, dyslipidemia, hypertension, osteoporosis and diabetes) (Bhat and Bhat, 2011; Siró *et al.*, 2008). According to Jankovic *et al.* (Jankovic *et al.*, 2010), the main role of functional food is to help an individual to recover from temporary illnesses. Dairy products are key products in the worldwide functional food market and account for an important fraction of this market (Özer and Kirmaci, 2010). Besides dairy products, new product categories have been introduced in functional food market by using raw material like cereals, vegetables, fruits etc. Examples of some commercially available functional foods and their respective functional factors are given in Table 3.

Table 3. Some commercially available functional food forms and their functional ingredients.

Functional ingredient	Product forms	Product category
Lactosucrose	Soft drink, yogurt, biscuit, cookie, table sugar	Prebiotics
Monosaccharide : Arabinose	Table sugar	
Disaccharide : Lactulose	Soft drink	
Trisaccharide : Raffinose	Powdered soup	
Oligosaccharides (fructo, xylo, soybeans etc)	Table sugar, tablet candy, pudding, soyabean curd, Soft drink, vinegar, chocolate and tablet candy	
Lactobacillus ssp. Bifidobacterium ssp.	Lactic acid bacterium drink, yogurt and fermented milk	Probiotics
Indigestible (resistant) dextrin	Sausage, cookie, drink, miso soup, curd	Dietary fibres
Psyllium husks	Powdered drink, noodle, cereal	
Wheat bran	Cereal	
Galactomannan	jelly	
Partially hydrolysed guar gum	Powdered drink and boiled rice	
Agar	Noodle and jelly	
Beer yeast	Fermented milk	
Soya protein and peptide	Soft drink, meat ball, sausage, soya milk	Protein and peptides
Low-molecular-weight sodium alginate	Soft drink, soup	
Chitosan	Biscuit	
Sitosterol ester	margarine	
Sardine peptides (containing VY)	Soft drink, soup	
Lacto-tripeptides (VPP and IPP)	Lactic acid bacterium drink	
Dried bonito oligopeptides (containing IKP)	Fermented soyabean soup	
Casein dodecapeptides (FFVAPEPEVFGK)	Soft drinks	
Eucommia leaf glycoside (geniposidic acid)		
Diacylglycerol	Cooking oil	Others
Diacylglycerol and sitosterol		
Casein phosphopeptide	Soft drink, chewing gum, soyabean curd	
Calcium citrate malate	Soft drink	
Heme	Soft drink, jelly	
Menaquinone-7 producing Bacillus subtilis	Fermented soyabean	
Soya isoflavone	Tea drink	
Manitol, palatinose and tea polyphenols	Chocolate, chewing gum	
Xylitol	Chewing gum	
Wheat albumin	Powdered soup	
Globin digest	Soft drink	
Guava polyphenols	Soft drink	

Functional foods can be produced by addition of health-promoting component(s), by reducing/removing harmful components, and/or by modifying the nature or the bioavailability of specific components. Initial development in the area of functional foods started with the fortification of foods with vitamins and/or minerals such as vitamin C, vitamin E, folic acid, zinc, iron, and calcium (Sloan, 2000). Thereafter, the focus shifted to the development of foods fortified with various micronutrients such as omega-3 fatty acid, phytosterol, and soluble fiber (Gupta and Abu-Ghannam, 2012).

Despite of having shorter shelf life, yogurt and cheese remain the most commonly consumed probiotic containing functional foods. Improving the functional properties of these products is gaining scientific attention because incorporation of functional ingredients without changing the organoleptic properties of these products can be easily achieved because of their high consumer acceptance. Among different probiotic functional foods today, yogurt is the most widely consumed product (more than 90% of the French house-hold consumes yogurt at least once a day). Along with other fermented milks it is considered as the main vehicle for probiotic delivery. It is prepared usually by fermenting milk with cultures of *S. thermophilus* and with *L. bulgaricus* (Lourens-Hattingh and Viljoen, 2001). Other probiotic microorganisms are also being used for the production of yogurts like *Lactobacillus acidophilus, Bifidobacterium* etc. Both basic bacteria are present in a concentration of 10^8 cells/ml of yogurt. Yogurt has long been recognized as healthy milk product with higher nutritional value and significant health beneficial effects (Woods, 1992). It is one of the best known foods that contain a huge amount of calcium, 40% of the daily value and considered as staple food everywhere (Adolfsson *et al.*, 2004). A typical commercial yogurt contains fat (0-3.5%), milk solids, non fat (8.25-14%), sugar (0-10%) and stabilizer (0-2%).

The popularity of yogurt results from its perceived health benefits. Its functional properties are consistent with what have been observed during its long consumption and confirmed metabolic activity of yogurt bacteria in both the animal and human digestive tract (Martini *et al.* 1987; Pochart *et al.* 1989; Marteau *et al.* 1990), as well as in *in vivo* animal models (Lick *et al.* 2001; Drouault *et al.* 2002). Yogurt bacteria can also be detected in faeces of human subjects consuming yogurt (Brigidi *et al.* 2003; Callegari *et al.* 2004).Thus, the functional properties of yogurt concerns for example, the improvement of lactose digestion and alleviation of lactose intolerance, the decrease of stool frequency and duration of diarrheal episodes in children displaying an acute watery diarrhea, the enhancement of

immune response, decrease in allergy symptoms (Guarner *et al*., 2005), regulation of hypertension or increase of mucus production by intestinal barrier.

It is well recognized that yogurt consumption improves lactose digestion and eliminates symptoms of lactose intolerance. The improvement of lactose digestion has been demonstrated in humans having or not lactose maldigestion (Guarner *et al.,* 2005*,* Rizkalla *et al.*, 2000). It results from high lactase activity of *S. thermophilus* and most *L. bulgaricus* strains (Sanders *et al*. 1996) which allows the decrease of lactose concentration in yogurt but is also active in GIT.

Yogurt also have proved effect on intestinal microbial balance as reduced intestinal microbial imbalance was observed in patients suffering from chronic liver disease when they consumed yogurt 3 times a day during two weeks. Their intestinal microbial balance was not only maintained but after the intervention, patients displayed significant improvement in debilitation, food intake, appetite, abdominal distension, and ascitic fluid (Liu *et al.*, 2010). Yogurt has an impact on diabetes also. It was observed that probiotic-supplemented fermented milk product called dahi (yogurt) dramatically suppressed diet-induced insulin resistance and protect from streptozotocin induced diabetes in animal models (Yadav and Sinha, 2008; Yadav *et al.*, 2007). It was also observed that probiotic dahi suppressed the diabetes progression and its complications through enhancing the antioxidant system (Yadav and Sinha, 2008).

Presence of bioactive peptides in yogurt is of great importance as it has been shown that enzymatic degradation of bovine β-casein may lead to the formation of biologically active peptides which have high physiological significance (Meisel, 1997). Peptide sequences of different biological activities have been found to occur in all classes of milk proteins. In addition to β-casomorphins, opioid peptides derived from as1–casein, α-lactalbumin and β-lactoglobulin have been described (Teschemacher *et al.*, 1997). For example, antihypertensive effects of yogurt were observed *in vitro* where peptide fractions present in yogurt inhibited the activity of angiotensin-converting enzyme. Yogurts in this study were prepared either using a sole yogurt culture including *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb1466 and *Streptococcus thermophilus* St1342, or *L. acidophilus* L10, *L. casei* L26 and *Bifidobacterium lactis* B94 in addition to yogurt culture. (Donkor *et al.,*2007). Same effect was observed when whey obtained from milk fermentation with *S. thermophilus* and *L. bulgaricus* (danisco) was orally administered to rats (Tsai *et al.,*2008).

Most recently, it was demonstrated that proteolysis of caseins by lactic bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus*) as in yogurt leads to the formation of many peptides including precursors of β-casomorphins or of neocasomorphin-6. It was also observed that total peptide pool obtained from marketed yogurts induces mucin secretion and the expression of *MUC2* and *MUC4* by directly acting on human mucus-producing cell, HT29-MTX. Thus, it was suggested that peptide β-CN (94-123) present in yogurt may maintain or restore intestinal homeostasis and could play an important role in protection against damaging agents of intestinal lumen (Plaisancié *et al.*, 2013).

To be daily consumed, functional food should not be a source of dietary imbalance causing the consumer other health problems and must be considered as a part of his diet. For this point of view, yogurt has an advantage over other fermented products like cheese often containing high salt concentration which has the negative effect on health as individuals suffering from hypertension. Besides, yogurt is a good carrier of probiotics. It not only protects them but also enhances their efficacy (Ranadheera *et al.*, 2010).

Recently a study conducted on Italian markets suggested that the health related consumer characteristics play an important role in shaping the demand for conventional and functional yogurts (Bonanno, 2012), emphasizing on the fact that there is a great potential for probiotic functional yogurts in the fermented food industry. Consumers show higher willingness to pay for foods with health-enhancing features. As it is almost a routine component of meal, it is easy to integrate functional ingredients in yogurt to confer health benefits to its consumers without changing its organoleptic properties.

1.7. Justification for the use of *S. thermophilus* in probiotic functional food

As mentioned before, *Streptococcus thermophilus* is one of the basic starter bacteria of yogurt and is the second most important species of industrial lactic acid bacteria after *Lactococcus lactis*, with a market value of more than 40 billion US dollars. On average 10^{21} live *S. thermophilus* cells are annually ingested by human population (Hols *et al.*, 2005). It is the only safe member among the 40 *Streptococcus* species. The genomic information available for this species supports the idea that the large scale consumption of this bacterium by human beings and its wide use in industry results from the absence of the virulence genes which might be due to genomic evolution after its massive use in milk since centuries (Bolotin *et al.*, 2004; Hols *et al.*, 2005). Besides the traditional use of *S.thermophilus* in

preparation of yogurt, it is used in production of several cheese varieties such as Swiss cheese, Brick cheese, Mozzarella and Parmesan (Parente and Cogan, 2004). The role of *S. thermophilus* in fermentation of milk is not only related to production of lactic acid but also to other technological properties such as sugar metabolism (Vaillancourt *et al.*, 2008), proteolytic activity (Hols *et al.*, 2005) and urease activity (Arioli *et al.*, 2007).

As said before, it has long been believed that *S. thermophilus* died in the GIT so there was no interest to know the functions potentially activated in this environment. Hence, the great majority of the physiological studies had been concentrated on its general physiology in culture medium or when the bacterial cells are placed in dairy fermentation conditions (Arioli *et al.*, 2009; Herve-Jimenez *et al.*, 2008; Hols *et al.*, 2005). At the beginning of the 21st century, its capacity to survive during the digestive transit was clearly established (Brigidi *et al.*, 2003; Mater at al., 2005 ; Elli *et al.* 2006). Living *S. thermophilus* were detected at a magnitude of 10^6–10^7 per gram of intestinal contents (wet weight) of minipig (Lick *et al.*, 2001). Similarly, Brigidi *et al.* (Brigidi *et al.*, 2003) identified and enumerated *S. thermophilus* from fecal samples of individuals fed on the pharmaceutical preparation VSL-3 by a culture-independent Polymerase Chain Reaction assay using specific primers for selective detection of *S. thermophilus*. Later, Mater *et al.* (Mater *et al.* 2005) investigated survival of both bacterial species in human feces by culture on selective media and indicated that substantial numbers of yogurt bacteria can survive human GI transit. Finally, Elli *et al.* (Elli *et al.*, 2006) were able to recover *S. thermophilus* from the feces after passage through the human gut by feeding 20 healthy volunteers commercial yogurt, suggesting that its cells can survive transit through the GI tract.

Recently, Iyer *et al.*, (Iyer *et al.*, 2010) showed that two *S. thermophilus* strains (RD102 and RD104) isolated from Indian fermented milk products were able to survive at low pH (2.5) and to resist to a bile concentration of 2% . These strains were also found to colonize the gut more efficiently in the large intestine of probiotic-fed groups as compared to control and skim milk groups. We also studied the survival capacity of 30 different strains of *S. thermophilus* against different gastric stresses (Junjua *et al.,* article submitted). Six different classes were obtained on the basis of their resistance to acidic pH, bile salts and oxidative stress as well as for their adhesion capacities. Great variability was observed between the classes, further comforting the idea that these capacities are highly strain dependent. The survival capacity of *S. thermophilus* shows that it has more chance to resist the processing

conditions during the product manufacture and more viable cells could be present in the end product to exert their beneficial effects (Junjua *et al*., submitted).

Besides, the positive health properties that yogurt displays, which results at least in part from *S. thermophilus,* several studies have reported a positive effect of this bacterium as well. It was thus found to confer different health benefits either by producing different metabolites or by directly affecting the immune system. Two strains of *S. thermophilus* (RD102 and RD104) are capable of producing folate, which is involved in DNA synthesis and repair. It methylates the DNA and act as a cofactor in certain biological reactions (Iyer *et al.*, 2010). *S. thermophilus* is also capable of producing β-galactosidase. By using gnotobiotic rat model, it was shown that *S. thermophilus* strain FB13 was able to produce a β-galactosidase which remains active during its transit through the GIT. The production of β-galactosidase was detected in the second half of the small intestine and in its active form, it could hydrolyze the lactose in the small intestine explaining the role of *S. thermophilus* in alleviating lactose intolerance (Mater *et al*. 2006). *S. thermophilus* improves condition of diarrhea in younger population (Saavedra *et al.*, 1994), enterocolitis in preterm infants, (Delorme, 2008), reduce symptoms of irritable bowel syndrome in children (Guandalini *et al.*, 2010) and act as an antioxidant in colon possibly by reducing lipid per-oxidation (Ito *et al.*, 2003). Several probiotic bacteria, including the strain *S. thermophilus* St065, release metabolites which exert anti-tumour necrosis factor α (anti-inflammatory) effect. These metabolites were found to be resistant to digestive enzymes and were capable of crossing the intestinal barrier (Ménard *et al.*, 2004). *S. thermophilus* is able to synthesize thermophilins which are bacteriocins, small peptides proteins able to inhibit the growth or kill closely related bacteria (Dortu and Thonart, 2009). Thermophilins have been shown to have *in vitro* inhibitory activities against lactic acid bacteria but also against Gram positive pathogenic strains such as *Enterococcus faecalis*, *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria monocytogenes* (Fontaine and Hols, 2008). For example, thermophilin 1277, which was produced by *S. thermophilus* SBT1277, showed an antimicrobial activity against several lactic acid bacteria and food spoilage bacteria including *Clostridium butylicum*, *C. sporogenes* and *Bacillus cereus.* Production of thermophilins represents a significant interest for conservation in food industry because it limits the proliferation of pathogenic bacteria in dairy products fermented by *S. thermophilus*. As a part of probiotic criteria, it would be interesting to test whether thermophilins could be produced in the GIT and be effective against pathogenic bacteria in the intestinal sites.

Antioxidant properties have been demonstrated in *S. thermophilus*. Among the 49 strains of lactic acid bacteria tested, it was observed that the strain YIT 2001 of *S. thermophilus* exerted the highest antioxydation capacity in the mucus of mice colon which were fed with high doses of iron (Ito *et al.*, 2003). Strong inhibitory activity against lipid peroxidation was also observed in liposomes, but the mechanism of action remains to be identified.

S. thermophilus can also exert antimutagenic effect as observed by carrying out a *Salmonella typhimurium* mutagenicity assay using different fermented fresh yogurts containing viable bacteria, probably *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* or *Lactobacillus acidophilus* and Bifidobacteria (Pool-Zobel *et al.*, 1993).

S thermophilus could actively participate in immune regulatory processes of its host. Most studies on the immunomodulatory properties of *S. thermophilus* have been performed *in vitro* using cellular cultures. Using human peripheral blood mononuclear cells (PBMC) as model, *S. thermophilus* was found to be the one inducing highest production of cytokines such as TNF-α, IL-12 and IFN-γ (Kekkonen, 2008). Moreover, in human monocyte-derived dentritic cells (moDCs) culture, *S. thermophilus* THS has the ability to induce the production of the pro-inflammatory cytokines TNF-α, IL-12, IL-6 and CCL20 and the cytokine Th1 IL-12 and IFN-γ. *S. thermophilus* has also the capacity to induce the expression of moDCs maturation marker HLA class II and CD86 as efficiently as pathogenic bacteria (Latvala *et al.*, 2008). In our study, we analyzed the effect of 30 different strains of *S. thermophilus* on the production of the anti/pro-inflammatory cytokines (IL-8, IL-10 and IL-12) using HT29 cell lines for IL-8 and PBMC for IL-10 and IL-12. The immunomodulatory profile obtained for these strains showed a great variability. Six out of the 30 strains dramatically decreased the IL-8 production. We used the ratio IL-10/IL-12 to evaluate the anti-inflammatory potential of our 30 strains and observed that 3 of them had high anti-inflammatory potential (Junjua *et al.*, submitted). In the study of Rodríguez *et al.* (Rodriguez *et al.*, 2010), milk fermented by *S. thermophilus* CRL1190 and CRL804 given for seven days was found to protect mice against gastritis induced by the administration of acetylsalicylic acid, an anti-inflammatory steroid molecule. It was also observed that these bacterial strains produce exopolysaccharide (EPS) that could stimulate the immune system and have an inhibitory effect on ulcer in the host.

A wide variety of *S. thermophilus* strains has been reported to show the capacity to produce EPS when grown in natural (milk, whey, whey permeate) (Ricciardi *et al.*, 2002; Vaningelgem *et al.*, 2004) or chemically defined media (Degeest and Vuyst, 1999). It is well known that EPS-producing starter cultures may help to improve rheology, texture and sensory characteristics of yogurt (Duboc and Mollet, 2001; Folkenberg *et al.*, 2005). Production of EPS has also an impact on the adhesion capacity of bacteria as observed by Lebeer *et al.,*(Lebeer *et al.*, 2011). It was suggested that EPS production is modulated in the host to fine-tune the balance between adhesion and immune evasion. In our work, when 30 different strains of *S. thermophilus* were subjected to adhesion assays using HT29-MTX (mucous secreting) cell lines. Varying degrees of adhesion were obtained for different strains ranging from 0.6% to 16.2%. The variability of type quantity of produced EPS might be a contributing factor in the variability observed.

S. thermophilus can also be used in co-cultures to obtain its positive impacts. Live *S. thermophilus* ATCC19258 in combination with *Lactobacillus acidophilus* ATCC4356 improve epithelial barrier properties (proved *in vitro* with HT29 and caco-2 cell lines), a potential mechanism contributing to their beneficial effect *in vivo* (Resta-Lenert and Barrett, 2003). Exposure of cell monolayers to live *S. thermophilus* ATCC19258 and *Lactobacillus acidophilus* ATCC4356 significantly limited adhesion, invasion and physiological dysfunction induced by entero-invasive *Escherichia coli* (EIEC 029:NM). Furthermore, live cells alone increased trans-epithelial resistance, contrasting markedly with the fall in resistance evoked by EIEC infection, which could also be blocked by these cells.

S. thermophilus showed competitive growth advantage over *L. bulgaricus* both *in vivo* and *in vitro* (Ben-Yahia *et al.*, 2012). It was observed that GIT of germfree mice when not supplemented with lactose were only colonized by *S. thermophilus,* and when supplemented with lactose it was colonized by both the bacteria when tested separately. However when supplemented with lactose and treated with mixture of both bacteria *S. thermophilus* rapidly colonized the tract of germfree mice 10^{10} cfu/g of feces at the expense of *L. bulgaricus* (< 10^2 cfu/g of feces).

Considering the probiotic potential of *S. thermophilus*, it is becoming important to study the physiological state and the activity of this bacterium in the GIT. In this regard, several studies have already been conducted. The initial studies focused on the activity of lactose operon that encodes β-galactosidase enzyme degradation of lactose. These

experiments were conducted in murine model colonized with *S. thermophilus* or with human flora. The methodology used was a transcriptional fusion between the promoter of the lac operon (p*lac*) and the luciferase gene. In mono-colonised mouse model associated with *S. thermophilus* carrying the transcriptional fusion with *plac*, it was shown that the bacterium produced a ß-galactosidase active in stool (Drouault *et al.*, 2002). The activity was increased 10-fold when the mice drank lactose supplemented water (4.5%). Degradation of lactose in the GIT of mice was also verified by measuring a decrease of 50% of the lactose in stool. Using the same methodology, the *plac* promoter activity was measured in the different compartments of the GIT of mice and gnotobiotic mice colonized by human flora (Mater *et al.*, 2006). These results show that *S. thermophilus* survives transit and it expresses ß-galactosidase active especially in the second part of the small intestine. The physiological state of *S. thermophilus* was also investigated by a proteomic approach, but this time in models of gnotobiotic rats. For this, the protein recovered from the feces of rats whose GIT was colonized by *S. thermophilus* LMD-9, were identified by two-dimensional electrophoresis coupled with liquid chromatography-mass spectrometry (Rul *et al.*, 2011). These results showed that the major proteins expressed in the GIT are involved in basic cellular functions such as translation, transport and metabolism of carbon, nitrogen and nucleic bases. This indicates that *S. thermophilus* was metabolically active in the GIT of rats and even after 30 days of inoculation. By proteomic analysis, the proteins synthesized by *S. thermophilus* during growth in milk were compared with those synthesized in the feces of rats. Among the proteins overproduced in feces, are the proteins involved in the stress response (such as SodA, superoxide dismutase, GroEL, EF-Tu) and carbon metabolism, particularly in the glycolytic pathway. Instead, the 41 regulated proteins down-expressed in stool, are involved in protein synthesis, cell division, biosynthesis of nucleotides, repair and supply of energy. Similar results were obtained with the strain LMG18311. It was also demonstrated that the presence of lactose in drinking water of *S. thermophilus* LMD-9 mono-associated rats allowed rapid colonization and large compared to a control without lactose. These studies have confirmed the importance of glycolytic enzymes and lactose transporter in the ability of *S. thermophilus* to colonize the GIT of rats (Thomas *et al.*, 2011).

1.8. Conclusion

Results obtained on the health benefits of the probiotics are encouraging they fully justify the increase of scientific efforts in this area. They have to be consumed regularly and in higher levels of viable microorganisms are recommended to exert their beneficial effects. Their daily consumption could be facilitated by their incorporation in a food frequently consumed and being a part of consumer's normal diet. However, the composition of food such as fat concentration, protein type and concentration or sugars and pH may affect probiotic growth and survival in food (Ranadheera *et al.*, 2010). Further, during the fabrication of products, probiotic microorganisms are subjected to different processing stresses and additional efforts like microencapsulation, freeze drying etc are often required to protect them from adverse conditions and to keep them viable until the end product. Finally, the nature of food matrix may affect their efficacy. Thus, the exploration of probiotic properties of bacteria already employed in food manufacturing is an interesting potential track to go on further research.

Yogurt has been shown to exert several health benefits and represents a good carrier for probiotics. However, without knowing the probiotic potential of *S. thermophilus,* we continue to incorporate probiotic bacteria in yogurt to obtain their additional health benefits whereas different strains of *S. thermophilus* which already resist to the processing stresses could alone exhibit similar properties. Its use in yogurt as a probiotic not only contributes to the positive effect of yogurt either through its proteolytic activity or by production of different metabolites but also exerts its own probiotic capacities by interacting with the host within the GIT.

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2. Méthodes d'identification des fonctions bactériennes activées dans un environnement complexe

Malgré leur petite taille et leur organisation cellulaire simple, les bactéries sont des systèmes très complexes et dynamiques qui au cours de leur longue évolution se sont adaptées à pratiquement voire tous les écosystèmes terrestres. Cela a été possible grâce à l'acquisition d'un répertoire de séquences génétiques (gènes et régions régulatrices) qui leur permet une adaptation rapide à un nouvel environnement. Une partie de cette adaptation provient de la capacité qu'ont les bactéries de modifier leur métabolisme en réponse à un changement du milieu extérieur, donc en produisant de nouvelles protéines.

L'étude de la physiologie des bactéries a été initialement réalisée *in vitro* et les méthodes utilisées ont largement contribué à la compréhension du métabolisme bactérien. Cependant, l'analyse des génomes bactériens, dont ceux de bactéries très étudiées par les microbiologistes telles qu'*Escherichia coli* ou *Bacillus subtilis*, a révélé les limites de telles études puisqu'une proportion non négligeable des gènes détectés demeure de fonction inconnue. L'élucidation du rôle de ces gènes représente un enjeu majeur et requiert l'étude de la physiologie bactérienne, non plus en conditions de laboratoire mais dans l'environnement naturel de la bactérie, c'est-à-dire dans un environnement complexe tel que l'hôte.

Pendant les deux dernières décennies, des technologies sophistiquées ont été développées pour analyser les fonctions préférentiellement activées lorsque les bactéries sont présentes dans l'hôte ou dans un contexte complexe. Il s'agit de méthodes de transcriptomique ou de protéomique. Les études transcriptomiques décrivant l'état physiologique des bactéries dans leur environnement complexe sont soit basées sur la détection de promoteurs induits ou sur la détection des changements d'expression génique par la mise en évidence des transcrits (ARNm). Compte tenu des travaux réalisés durant ma thèse, je me focaliserai sur la description des approches de transcriptomique qui peuvent être divisées en deux catégories : celle basée sur l'hybridation et celle basée sur des constructions génétiques (An et Grewal, 2012). Les méthodologies basées sur l'hybridation comprennent les puces à ADN (cDNA microarrays) et la technique SCOTS (Selective Capture Of Transcribed Sequences). Parmi les méthodes impliquant des constructions génétiques, je décrirai la STM (Signature-Tagged Mutagenesis) et les techniques de capture de promoteurs comprenant la DFI (Differential Fluorescence Induction) et les techniques IVET (*In vivo* Expression Technology).

2.1. Méthodes d'études basées sur l'hybridation

2.1.1. Technologie des puces à ADN

Une des techniques les plus couramment utilisées pour identifier les gènes différemment exprimés est la puce à ADN (biopuce ou DNA microarrays). Elle est constituée de fragments d'ADN (ADNc ou oligonucléotides) qui serviront de sondes et qui sont immobilisés sur un support solide. L'ADN fixé représente tout ou partie du génome étudié ou de l'ADN codant des éléments de régulation tels que les petits ARN (Gomez *et al.*, 2011). Le principe des puces est l'hybridation compétitive entre les sondes immobilisées et les ADNc cibles, produits à partir des ARNm extraits du microorganisme étudié dans deux conditions différentes et marqués avec des fluorochromes différents (Figure 1). Il est ainsi possible de comparer les niveaux d'ARNm produits par une bactérie cultivée dans un contexte *in vivo* par rapport à un contexte *in vitro*. Contrairement à la technologie du Northern blot qui repose sur le même principe d'hybridation, l'utilisation d'une puce à ADN permet de mesurer en même temps les niveaux d'expression d'un grand nombre de gènes, en utilisant de faible quantité d'ADNc (Cao *et al.*, 2011). Cette technologie a été appliquée pour identifier des gènes de virulence dans différents modèles animaux (Boyce *et al.*, 2002; Cao *et al.*, 2011).

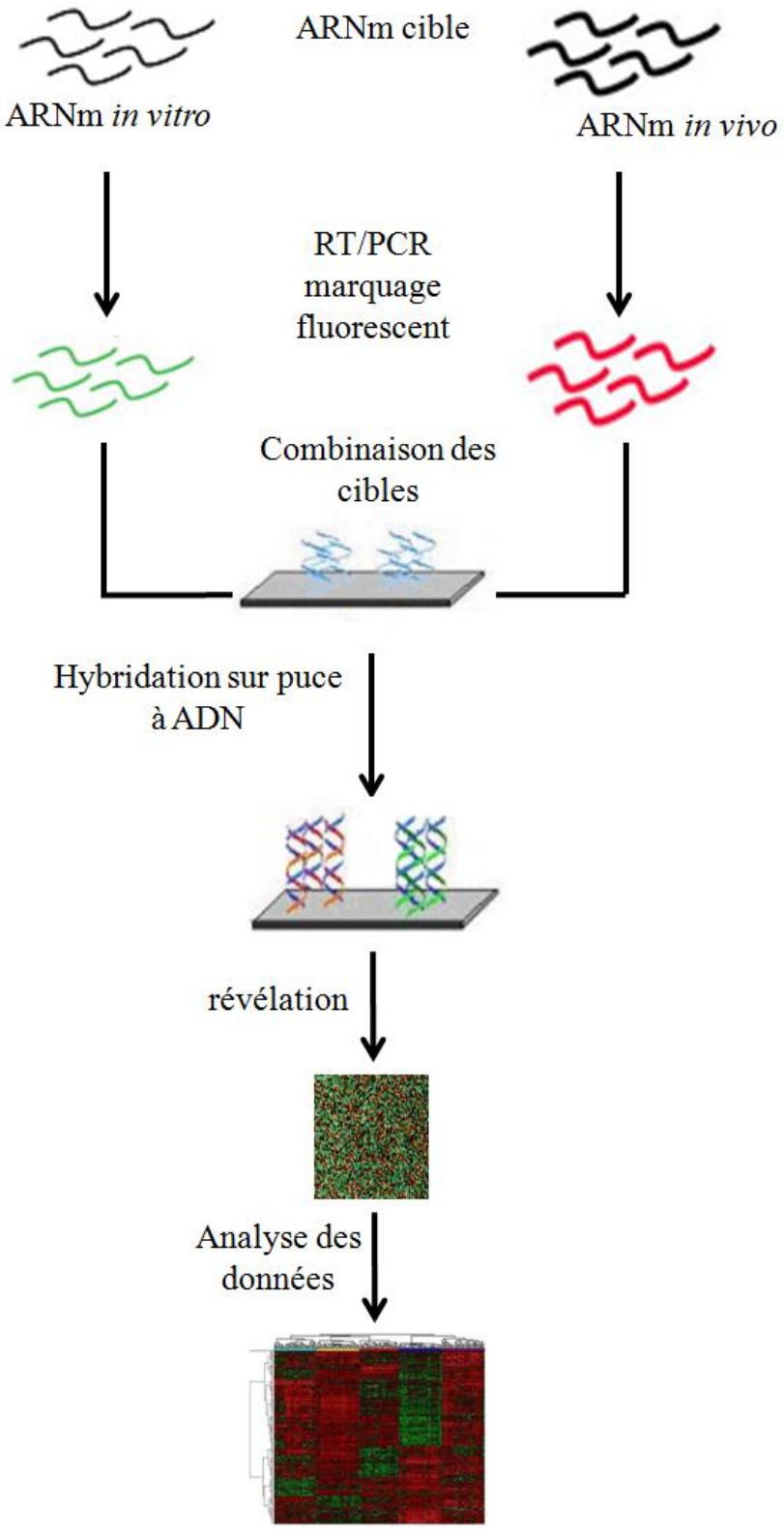

Figure 1. Principe de la technique des puces à ADN.

Les ARN sont extraits de deux échantillons, convertis en ADNc, marqués avec 2 fluorochromes différents puis hybridés sur une puce qui comprend un grand nombre d'oligonucléotides ou d'ADNc du génome de la bactérie étudiée. Après hybridation, les fluorochromes sont excités par un laser et le signal recueilli. Pour chaque sonde, ce signal est proportionnel à la quantité d'ADNc cible fixé.

Pourtant, en dépit du fait que l'établissement du profil d'expression par les puces à ADN peut être réalisé de manière rapide et à haut débit à l'échelle d'un génome complet, relativement peu d'études publiées ont utilisé les puces pour analyser la physiologie bactérienne *in vivo* (Gomez *et al.*, 2011). En effet, cette technique est limitée par le faible nombre de bactéries présentes dans le tissu de l'hôte ainsi que par la difficulté à récupérer suffisamment d'ARNm bactérien de bonne qualité à partir du tissu eucaryote, sachant par ailleurs que l'ARNm bactérien a une demi-vie courte et qu'il est présent à côté d'une grande quantité d'ARNr et d'ARN provenant de l'hôte. En outre, cette approche mesure un niveau d'expression global de la population bactérienne dans l'environnement complexe, alors que celui-ci peut varier d'une cellule à une autre, et ce, d'autant plus que le milieu environnant est hétérogène. Aussi, les biopuces apparaissent peu adaptées à l'étude de l'état physiologique des bactéries présentes dans un système *in vivo* et sont par contre très utilisées pour établir le profil global d'expression génique dans des conditions *in vitro* qui miment les systèmes *in vivo* (Boyce *et al.*, 2004).

2.1.2. Selective Capture Of Transcribed Sequence (SCOTS)

Les facteurs limitant l'utilisation des biopuces dans les systèmes *in vivo* sont minimisés dans la technique SCOTS (Selected Capture Of Transcribed Sequences). Cette méthode s'affranchit en effet de la faible quantité de cellules bactériennes présentes dans le contexte eucaryote-hôte en amplifiant de manière spécifique le transcriptome bactérien. Dans cette approche, les gènes bactériens préférentiellement exprimés dans une condition de croissance particulière sont capturés par une combinaison d'hybridation soustractive et de PCR avec des amorces « taggées » (Graham et Clark-Curtiss, 1999). Comme cela est représenté à la Figure 2, l'ARN total est extrait des cellules bactériennes placées dans deux conditions différentes (*in vitro* et *in vivo*) et converti en ADNc grâce à l'utilisation de 2 types d'amorces. L'amorce utilisée côté 3' est de séquence aléatoire tandis que celle utilisée côté 5' contient un tag. Afin de ne retenir que l'ADNc provenant d'ARNm bactérien, les ADNc obtenus à partir des deux conditions de croissance de la bactérie subissent plusieurs cycles dits de normalisation. Ces cycles consistent à hybrider les ADNc avec de l'ADN bactérien biotinylé et initialement bloqué par de l'ARN ribosomique, puis à capturer les hybrides grâce à l'utilisation de billes recouvertes de streptavidine, à éluer les ADNc hybridés et enfin, à les amplifier par PCR en utilisant les amorces « taggées ». Après cette phase de normalisation, une hybridation soustractive entre les ADNc provenant des deux conditions est alors réalisée, les ADNc élués puis amplifiés sont alors clonés dans un vecteur pour construire une banque

de gènes activés ou réprimés chez l'hôte. Ils pourront être par la suite utilisés comme sonde afin de mesurer l'expression des gènes correspondants dans les deux pools d'ADNc « normalisés » et issus des deux conditions testées.

La technique SCOTS, initialement développée en 1999 pour l'identification des gènes exprimés par *Mycobacterium tuberculosis* dans les macrophages (Graham et Clark-Curtiss, 1999), a été utilisée avec succès chez de nombreuses bactéries Gram négatives telles que *Escherichia coli* (Dozois *et al.*, 2003), *Haemophilus parasuis* (Jin *et al.*, 2008), *Actinobacillus pleuropneumoniae* (Baltes et Gerlach, 2004), *Riemerella anatipestifer* (Zhou *et al.*, 2009) ou *Salmonella enterica* serovar Typhimurium (Daigle *et al.*, 2001; Faucher *et al.*, 2005), ainsi que chez des bactéries Gram positive telles que *Mycobacterium avium* (Hou *et al.*, 2002) et *Streptococcus suis* (Fittipaldi *et al.*, 2007). La technique SCOTS peut être appliquée à n'importe quelle bactérie sans besoin d'un modèle animal, d'une grande quantité et qualité d'ARNm et d'informations génétiques préliminaires. Elle présente également l'avantage de détecter les gènes exprimés « *in vivo* » à partir d'une petite quantité de cellules bactériennes. Cependant, ce n'est pas une méthode quantitative. En combinaison avec les puces à ADN, elle permet d'obtenir des résultats plus fiables et plus quantitatifs (An et Grewal, 2012).

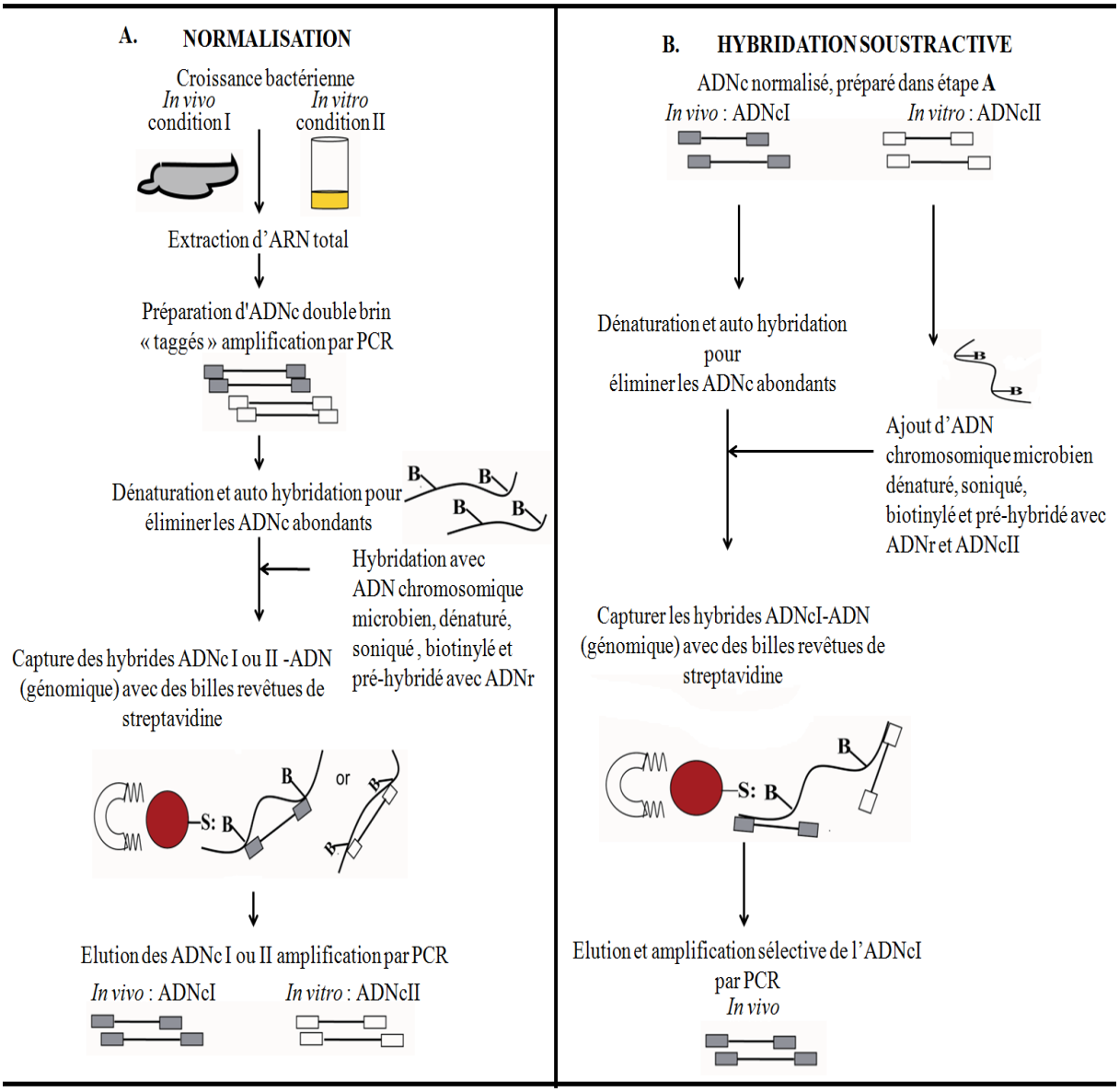

Figure 2. Représentation schématique de la technique SCOTS (Selective Capture Of Transcribed Sequence) (d'après An et Grewal, 2012).

A. Les ARN exprimés dans des conditions de croissance différentes de la bactérie d'intérêt sont convertis en ADNc double brin et « taggés » grâce à l'utilisation d'une amorce qui contient une séquence unique en 5'. Les ADNc sont alors normalisés de manière à ne recueillir que les ADNc provenant d'ARNm initiaux. **B.** Etape d'hybridation soustractive afin de recueillir les ADNc correspondant à des gènes exprimés différemment dans l'une des conditions testées.

2.2. Méthodes basées sur des constructions génétiques

2.2.1. Méthodes de sélection négative : technique STM

Le principe de base des méthodes génétiques basées sur la sélection négative repose sur le fait qu'une bactérie possédant une mutation dans un gène essentiel à sa survie dans l'hôte ne sera pas retrouvée en fin d'expérience après passage chez celui-ci.

Afin d'avoir une approche globale pour identifier les gènes essentiels à une « infection » ou en système *in vivo*, des méthodes ont été développées pour cribler directement les phénotypes de survie dans l'hôte. Les techniques les plus courantes sont la STM (Signature Tagged Mutagenesis) et la traSH (transposon Site Hybridization). Ces méthodes consistent à construire une banque de mutants par insertion de transposon au hasard dans le génome de la bactérie, générant ainsi un grand nombre de mutants. Dans le cas de la méthode STM, les transposons portent chacun une étiquette « tag » différente. Chaque « tag » est constitué d'une séquence oligonucléotidique unique flanquée de deux étiquettes de fixation pour des amorces de séquence conservée, permettant ainsi une amplification par PCR et une reconnaissance individuelle du mutant (Hensel *et al.,* 1995). Les mutants sont alors administrés à l'hôte puis ré-isolés sur milieu solide, comme la banque initiale. La présence de chaque tag est alors recherchée par amplification suivie d'une hybridation sur les deux pools de mutants (ceux administrés initialement et ceux récupérés de l'hôte). L'absence d'un tag dans le pool de mutant récupéré indique que le gène muté est important pour la survie de la bactérie chez l'hôte (Figure 3). Cette technique validée chez *Salmonella typhimurium* (Hensel *et al.*, 1995) a permis de mettre en évidence de nombreux facteurs de virulence chez les bactéries Gram négatif, Gram positif et même chez des virus. Cependant, la méthode est limitée techniquement par le besoin d'un transposon utilisable dans la souche et par la taille de la banque de mutants en rapport avec le nombre d'étiquettes uniques. Cette technologie nécessite de posséder un modèle d'infection approprié qui permette de récupérer un nombre suffisamment important de bactéries pour obtenir une vision non biaisée des résultats. En outre, la STM peut négliger des mutations pertinentes, qui peuvent être complémentées dans une population complexe (Autret et Charbit, 2005; Saenz et Dehio, 2005). Récemment, cette technique a néanmoins permis de montrer que le gène codant la listeriolysin O (LLO) était impliqué dans la survie de *Listeria monocytogenes* chez l'hôte (Melton-Witt *et al.*, 2012).

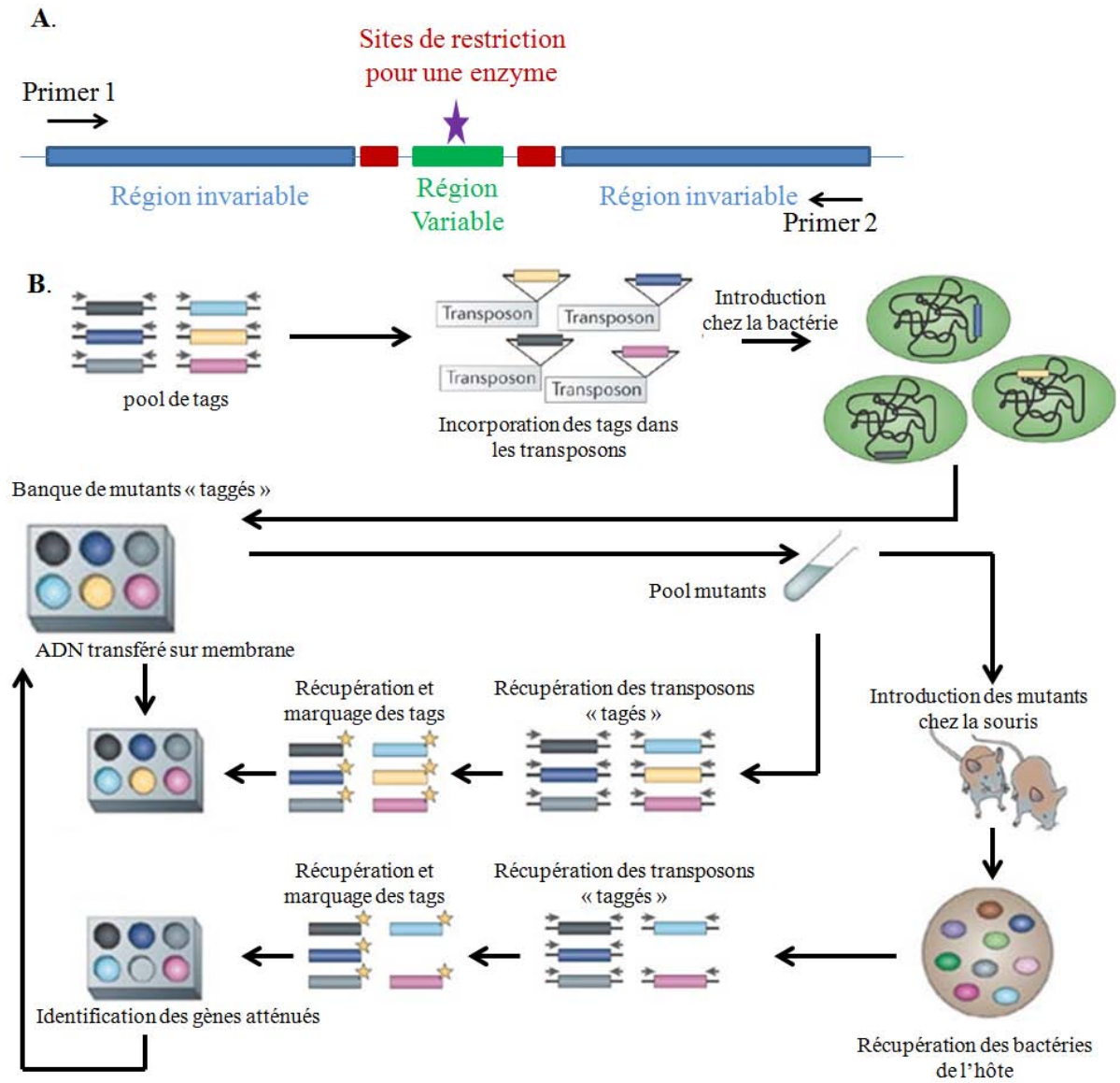

Figure 3. Représentation schématique de la technologie STM (Signature Tagged Mutagenesis) (d'après West *et al.*, 2003).

A. Représentation d'un tag. Chaque tag comporte une région centrale de 40 pb composée d'une séquence de 20 pb unique à chaque tag, flanquée de 2 sites de restriction. Cette région est elle-même flanquée de 2 séquences invariables de 20 pb chacune. Elles permettront d'amplifier tous les tags puis de les marquer en utilisant les mêmes amorces. Après marquage, la partie centrale de chacun des tags sera libérée grâce à l'action de l'enzyme de restriction reconnaissant les sites situés de part et d'autre de la séquence unique. **B.** Une banque de mutants est produite par introduction d'un tag dans chaque mutant *via* un transposon. Ces mutants sont ensuite inoculés à l'animal puis récupérés sur milieu solide. En parallèle, le pool de mutants avant administration à l'animal est également cultivé sur milieu solide. L'ADN génomique des deux pools de mutants (administrés à l'animal ou non) est transféré sur filtres. Les tags issus des mutants récupérés après administration à l'animal sont amplifiés, marqués puis digérés. Ils sont ensuite hybridés avec l'ADN génomique des mutants administrés à l'animal ou non. L'absence de signal d'hybridation pour certains mutants ayant été administrés à l'animal permet d'identifier les gènes atténués *in vivo*.

2.2.2. Méthodes de sélection positive : techniques IVET, R-IVET et DFI

A l'inverse des méthodes de sélection négative, les stratégies de sélection positive de promoteurs (promoter-trap) ont été développées pour identifier les promoteurs bactériens induits *in vivo* (ivi promoteurs pour *in vivo* induced promoters). Ces techniques permettent de révéler les gènes qui sont actifs dans les différents compartiments de l'hôte. Initialement développées pour identifier les gènes de virulence des bactéries pathogènes, ces techniques peuvent également être utilisées chez des bactéries non pathogènes dans n'importe quel système complexe pour mettre en évidence les gènes exprimés dans ces conditions.

Parmi ces techniques peuvent être citées les technologies IVET (*In vivo* Expression Technology) et R-IVET (Recombinase *In vivo* Expression Technology) et la DFI (Differential Fluorescence Induction). Ces méthodes sont basées sur la construction d'une banque de fragments génomiques clonés en amont d'un gène rapporteur. Si ce fragment contient un promoteur, le gène rapporteur est exprimé dans les conditions où le promoteur est activé. Le principal avantage de ces techniques de transcriptomique ciblée est qu'elles s'affranchissent de l'étape d'acquisition des ARNm. Elles permettent d'identifier les gènes spécifiquement exprimés en milieu complexe au stade de la bactérie individuelle (Bron *et al.*, 2004; Mahan *et al.*, 2000).

2.2.2.1. Technologie IVET

L'approche IVET a beaucoup été utilisée pour identifier des promoteurs induits *in vivo*, en particulier ceux activés durant des infections (Angelichio et Camilli, 2002; Rediers *et al.*, 2005). Cette technique a été utilisée pour la première fois en 1987 afin d'identifier chez *Xanthomonas campestris* des gènes induits chez cette bactérie lors de l'infection du navet (Osbourn *et al.*, 1987). Cependant la dénomination IVET revient à Mahan et ses collaborateurs (Mahan *et al.*, 1993) qui ont appliqué cette méthode à *Salmonella typhimurium*. Dans sa version initiale cette technologie comporte deux composants. Le premier correspond à la souche d'intérêt porteuse d'une mutation dans un gène codant un facteur essentiel de croissance (*egf* pour essential growth factor). Le second correspond à un plasmide portant le gène *egf* dépourvu de son promoteur. Ce gène est lié à un gène rapporteur (gène *rep*). Une banque de fragments d'ADN génomique insérés en amont du gène *egf* est alors construite. Seuls les clones qui possèdent en amont du gène *egf* un fragment ayant une activité promotrice peuvent se développer en absence du facteur de croissance dans les conditions

testées. Le gène *rep,* quant à lui, est utilisé afin de repérer les clones porteurs de promoteurs constitutifs (Rainey et Preston, 2000; Rediers *et al.*, 2005). Plusieurs gènes essentiels ont été utilisés (Figure 4) tels que le gène *purA* qui code une protéine essentielle pour la synthèse de purine (Mahan *et al.*, 1993), *panB* impliqué dans la biosynthèse de pantothénate (Rainey, 1999), *dap* ou *asd* impliqués dans la biosynthèse de diaminopimélate (Handfield *et al.*, 2000; Rediers *et al.*, 2005) ou *trpEG* nécessaire à la biosynthèse du tryptophane (Brown et Allen, 2004).

Pour s'affranchir du besoin d'une souche auxotrophe à complémenter par une fusion transcriptionnelle, le système IVET a été par la suite simplifié en utilisant un gène de résistance à un antibiotique dépourvu de son promoteur. L'utilisation de l'antibiotique comme pression de sélection permet ainsi de recouvrer les clones qui ont un promoteur actif en amont du gène de résistance utilisé. Ainsi, des promoteurs induits *in vivo* ont été identifiés en utilisant le gène de résistance au chloramphénicol chez *Helicobacter pylori* (Angelini *et al.*, 2004), *Streptococcus gordonii* (Kiliç *et al.*, 1999) et *E. coli* (Khan et Isaacson, 2002). Des gènes de résistance à d'autres antibiotiques tels que la tétracycline (Wu *et al.*, 2002), l'érythromycine (Walter *et al.*, 2003) ou la kanamycine (Hunt *et al.*, 2001) ont également été utilisés avec succès dans des approches IVET. Cependant, l'utilisation d'un antibiotique comme pression de sélection n'est fonctionnelle que si cet antibiotique peut pénétrer le tissu ou le compartiment où la bactérie est présente, pour ne sélectionner que les clones qui portent un promoteur actif. La dose d'antibiotique utilisée doit, en outre, être subtilement évaluée par rapport au niveau d'activité des promoteurs à sélectionner. En effet, si la pression de sélection est forte, les promoteurs induits de manière faible ou transitoire ne permettront pas aux clones de produire suffisamment d'enzyme d'inactivation de l'antibiotique et seront par conséquent contre-sélectionnés (Gomez *et al.*, 2011).

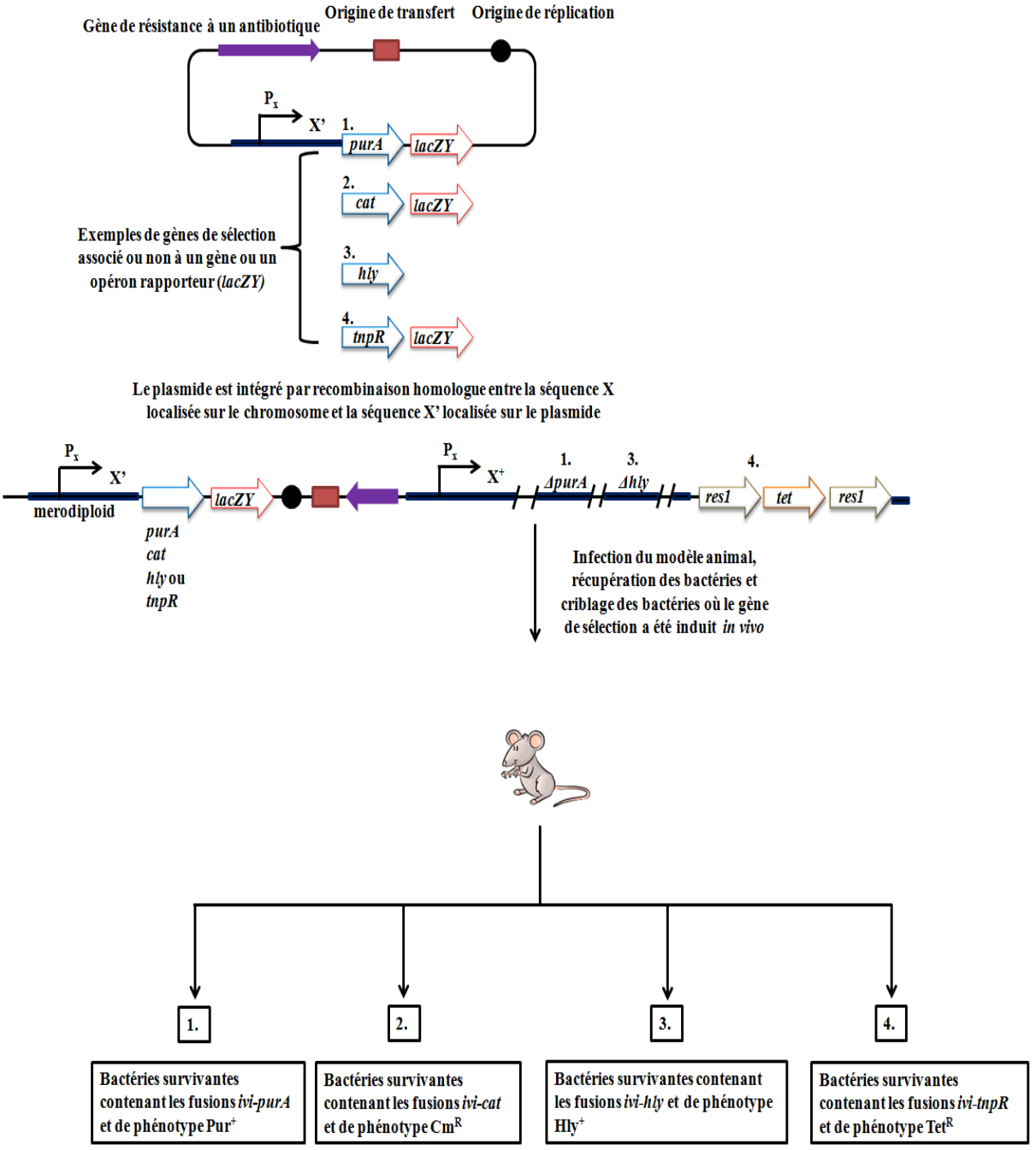

Figure 4. Représentation schématique de 4 variantes de la technologie IVET (*In vivo* Expression Technology).

2.2.2.2. Technologie R-IVET

Pour écarter les inconvénients de la technique IVET, Lee et ses collaborateurs ont développé une technique R-IVET (Lee *et al.*, 1999) qui utilise une fusion transcriptionnelle avec un gène de recombinase site-spécifique capable de reconnaitre et de déléter un marqueur situé entre les deux séquences qu'elle reconnait (Figures 5 et 6). Dans ce cas, le système comporte deux éléments : (1) la fusion transcriptionnelle au gène de recombinase (*cre* par exemple) localisée sur un plasmide et (2) le gène marqueur (le plus souvent, un gène de résistance à un antibiotique) introduit dans le chromosome de la souche entre deux sites de recombinaison (ex : sites *lox*, substrats de la recombinase Cre). Par rapport à la technologie IVET, l'intérêt principal de la technologie R-IVET est que la sélection des clones n'est pas réalisée durant l'infection de l'hôte par les bactéries mais après récupération des bactéries de l'hôte. Les clones sont criblés pour la présence ou l'absence du marqueur de résistance à l'antibiotique. Un clone qui est devenu sensible à l'antibiotique est porteur d'un promoteur qui a été activé *in vivo*. La technologie R-IVET permet la contre-sélection des clones qui ont été activés *in vitro* puisque les clones qui sont actifs dans ces conditions voient leur cassette de résistance à l'antibiotique excisée rendant leur croissance en présence de cet antibiotique impossible.

Le deuxième avantage de la technologie R-IVET est que les promoteurs faibles ou transitoirement activés peuvent être détectés. Finalement, des systèmes R-IVET plus sophistiqués ont été développés en incorporant un marqueur sélectionnable à l'intérieur de la cassette qui permet d'identifier les clones porteurs de promoteurs actifs après sélection plutôt qu'après criblage (Osorio *et al.*, 2005). Les gènes marqueurs que la résolution de la cassette élimine sont par exemple *sacB*/sucrose (Osorio *et al.*, 2005), *melA*/α-galactosidase (Bachmann *et al.*, 2008), *rpsL*/Streptomycin (Tuntufye *et al.*, 2012) ou *upp*/5-Fluorouracil. Il existe également des gènes marqueurs pour lesquels la résolution de la cassette régénère la fonction en plaçant un promoteur à la position adéquate, ce système a été nommé S-IVET (Selectionable *In vivo* Expression Technology) par les auteurs (Livny et Friedman, 2004) et utilisé dans plusieurs systèmes R-IVET (Hanin *et al.*, 2010; Galia *et al.,* communication personnelle). L'irréversibilité de la résolution de la cassette du R-IVET est un avantage certain par rapport à la technologie IVET puisqu'il permet de marquer définitivement le clone, ce qui permet son analyse postérieure. Cette particularité représente cependant un désavantage s'il est question d'observer un changement dans l'activation de l'expression des gènes (Hsiao et Zhu, 2009)

L'utilisation de la technologie R-IVET a permis d'identifier des gènes induits *in vivo* chez une variété de microorganismes tels que *Vibrio cholerae, Lactobacillus plantarum, Staphylococcus aureus, Mycobacterium tuberculosis* ou *Bordetella pertussis* (Bron *et al.*, 2004; Camilli et Mekalanos, 1995; Lowe *et al.*, 1998; Saviola *et al.*, 2003; Veal-Carr et Stibitz, 2005).

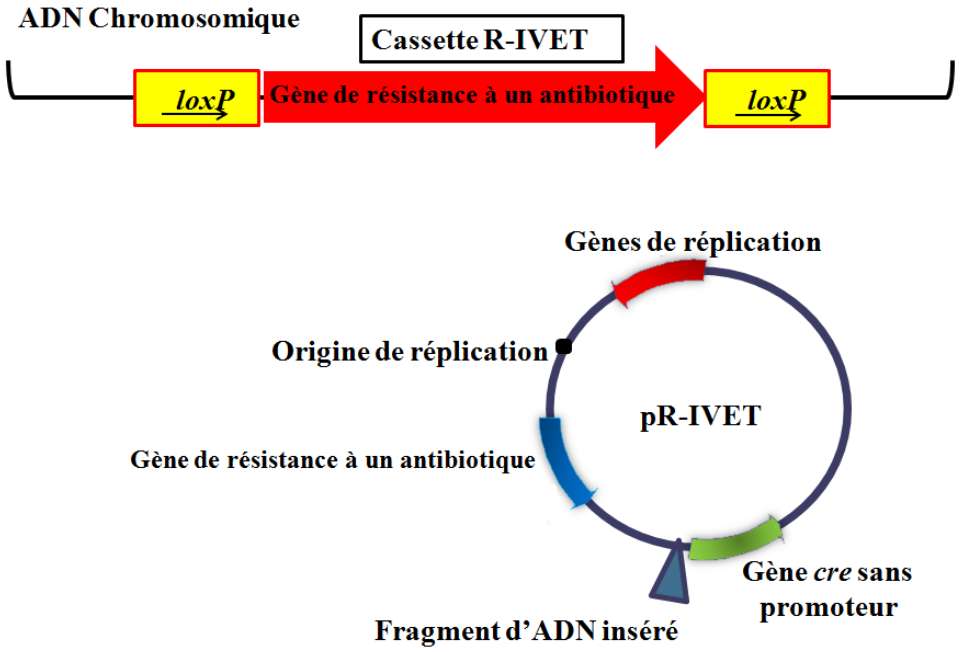

Figure 5. Représentation schématique de la technologie R-IVET (Recombination based *In vivo* Expression Technology).

Présentation des deux composants de la technologie R-IVET : la cassette chromsomique R-IVET contenant un gène de résistance à un antibiotique flanqué par les sites *loxP* et le plasmide porteur du gène *cre* dépourvu de son promoteur.

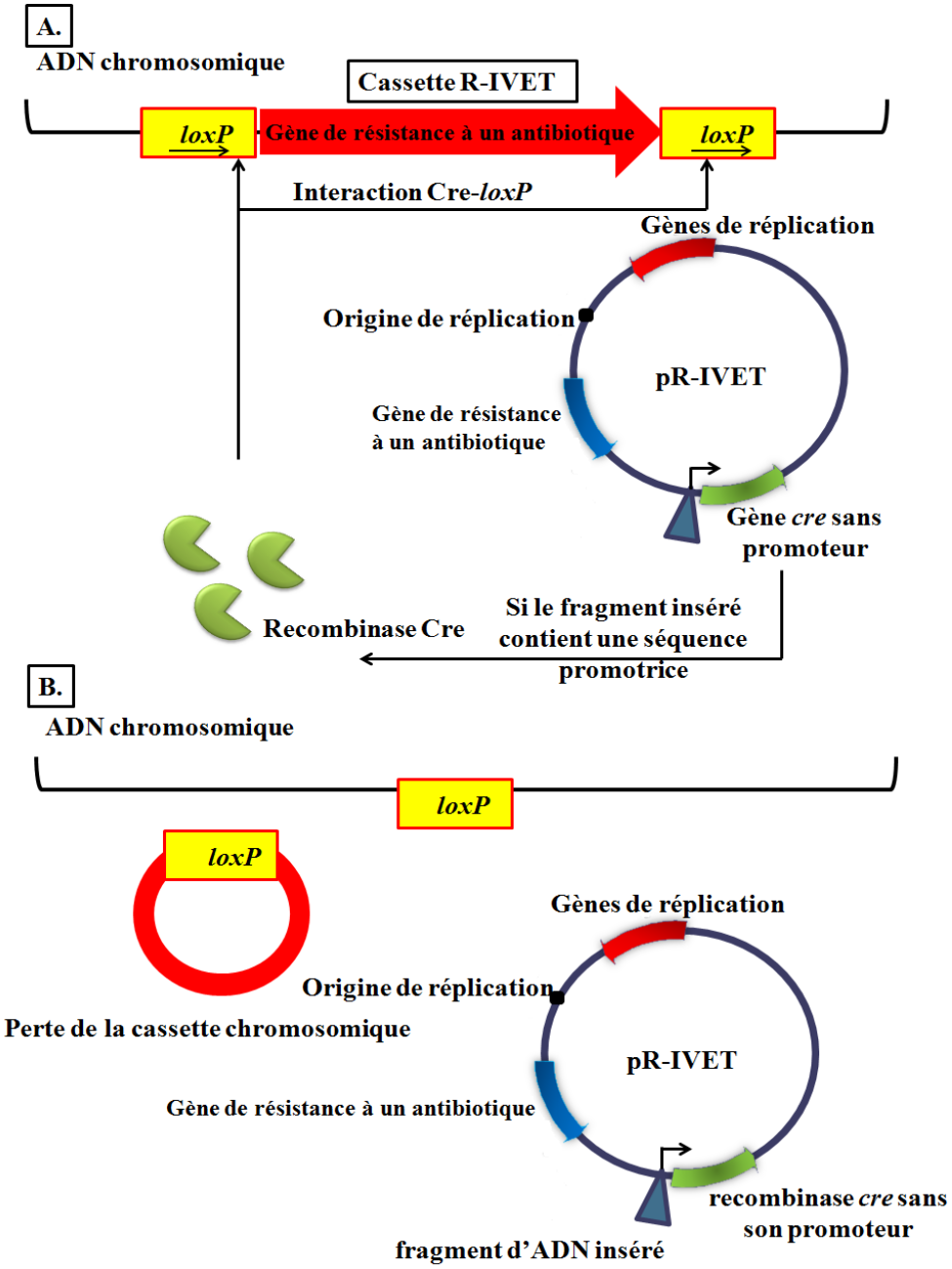

Figure 6. Représentation schématique du principe de la technologie R-IVET (Recombination based *In vivo* Expression Technology).

A. Expression de la recombinase Cre. La présence d'une séquence promotrice dans un fragment cloné en amont du gène *cre* aboutit à la transcription du gène *cre* et à la synthèse de la recombinase qui peut alors interagir avec les sites *loxP*. **B.** Excision de la cassette chromosomique. La fixation de la recombinase Cre sur les sites *loxP* aboutit à l'excision de la cassette chromosomique et donc à la perte de la résistance à l'antibiotique que conférait la présence de cette cassette dans le chromosome bactérien.

2.2.2.3. Technologie DFI

C'est pour lever la limitation de marquage irréversible que Valdivia et ses collaborateurs ont développé un système IVET pour suivre l'activation transcriptionnelle de gènes bactériens en utilisant la GFP (Green Fluorescent Protein) comme fonction rapporteur (Valdivia et Falkow, 1996). Cette technique nommée DFI « Différential Fluorescence Induction » permet de suivre en temps réel l'induction du promoteur en mesurant la fluorescence (Figure 7). Lorsque la technique est couplée au tri cellulaire par cryométrie en flux (FACS), un criblage à haut débit des clones peut être réalisé de façon semi-automatique. Cette stratégie utilisée par Bumann et Valdivia pour *Salmonella enterica* a permis d'identifier des promoteurs bactériens induits dans des sites anatomiques distincts et à des stades différents de l'infection (Bumann et Valdivia, 2007). La technique DFI permet ainsi de suivre l'expression des gènes au niveau d'une seule cellule, avec une localisation spatio-temporelle chez les animaux infectés (Bongaerts *et al.*, 2002). Comme pour les techniques (R) IVET, la DFI nécessite de construire une banque d'ADN génomique dans le plasmide portant le gène de la GFP, ce qui requiert de nombreuses manipulations génétiques. La DFI présente des contraintes supplémentaires comme le besoin en matériel lié à la technologie FACS et à l'utilisation de la GFP qui doit être optimisée pour une utilisation dans la bactérie d'intérêt. Cette technologie a néanmoins été appliquée à différentes bactéries telles que *Staphylococcus. aureus*, *Streptococcus*. *pneumoniae* (Bartilson *et al.*, 2001; Marra *et al.*, 2002) ou *L. plantarum* (Bron *et al.*, 2006).

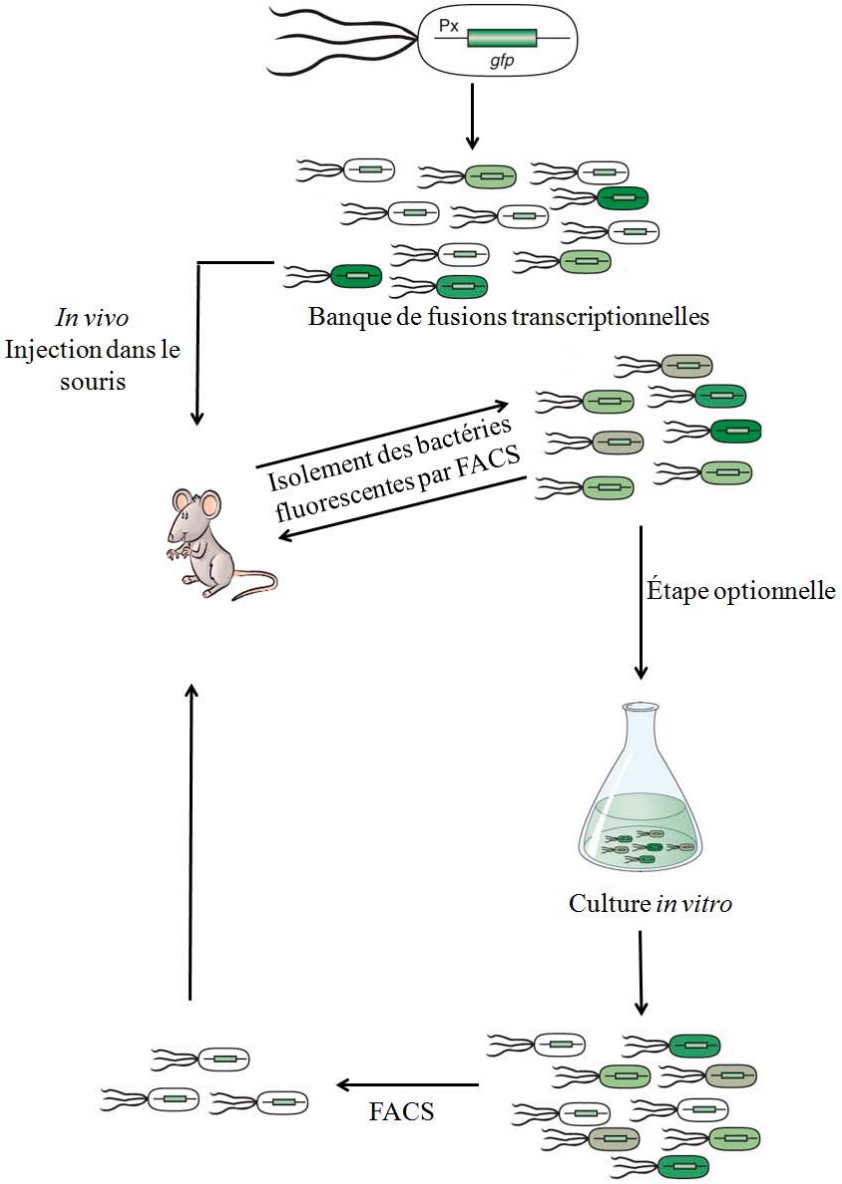

Figure 7. Représentation schématique de la technologie DFI (Différential Fluorescence Induction).

Une banque de fusions transcriptionnelles est réalisée puis introduite chez la bactérie d'intérêt. Les clones recombinants sont alors administrés à l'animal puis récupérés et triés par FACS suivant le degré de fluorescence émis par la fusion transcriptionnelle qu'ils portent.

2.2.3. Avantages et inconvénients des technologies IVET et R-IVET

Le principal avantage des techniques « promoter trapping » que sont l'IVET, R-IVET et DFI repose sur la stratégie de sélection positive, ce qui n'est pas possible avec la technique STM, par exemple.

Alors que la majeure partie des études utilisant la stratégie « promoter trapping » ont été menées sur des bactéries pathogènes en situation d'infection de leur hôte, ces technologies sont applicables et ont été appliquées à de nombreux autres microorganismes non pathogènes tels que des bactéries lactiques (Bachmann *et al.*, 2008; Bron *et al.*, 2004; Walter *et al.*, 2003). En fait, les technologies IVET/R-IVET peuvent être appliquées à n'importe quel microorganisme se trouvant dans un habitat particulier susceptible d'induire des gènes spécifiques.

Alors que les technologies IVET et R-IVET ne sollicitent aucun équipement spécialisé contrairement aux techniques de puces et du DFI, elles requièrent des systèmes de manipulations génétiques élaborés avec des gènes rapporteurs efficaces et des techniques performantes de biologie moléculaire incluant le clonage, la mutagénèse et la transformation bactérienne. La méthode est assez fastidieuse car elle nécessite tout d'abord de construire le plasmide de fusion transcriptionnelle puis de créer la banque d'ADN génomique avec un nombre important de clones pour recouvrir la totalité du génome de la souche.

Afin de sélectionner les promoteurs qui sont spécifiquement actifs dans les conditions choisies, les méthodes IVET possèdent une étape qui permet d'éliminer les promoteurs actifs *in vitro*. Le choix de la condition *in vitro* est de grande importance car elle va déterminer ce que sont les gènes induits *in vivo*.

Pour l'étude de l'état physiologique de *S. thermophilus* lors de son transit dans le tractus gastro-intestinal, nous avons choisi d'utiliser la technique R-IVET, certes fastidieuse à mettre en place, mais d'une grande puissance parce qu'elle permet de détecter n'importe quel gène actif dans n'importe quelle condition même très complexe en présence d'autres microorganismes.

Matériels & Méthodes

1. Souches, milieux et conditions de culture

Les souches bactériennes utilisées dans ce travail sont présentées dans le Tableau 1. Elles proviennent d'isolements réalisés au laboratoire à partir de produits laitiers d'origines différentes (yaourt ou fromages) (P. Bracquart, communications personnelle) ou de la collection CNRZ (Centre National de Recherches Zootechniques, INRA Jouy-en-Josas, France) ou de la collection ATCC (American Type Culture Collection, Manassas, VA, USA). Les précultures de *S. thermophilus* ont été réalisées en lait écrémé reconstitué (10%, m/v) et incubées une nuit à 42°C. La croissance a été réalisée à 37 ou 42°C en milieu M17 (Terzaghi et Sandine, 1975) supplémenté avec du lactose (20 g/L, milieu LM17) ou du glucose (20 g/L, milieu GM17). Pour déclencher la compétence chez *S. thermophilus*, le milieu MCD (Milieu Chimiquement Défini) a été utilisé (Letort et Juillard, 2001). Il s'agit d'un milieu synthétique ne contenant que des acides aminés comme source azotée. Les souches sont stockées à – 80°C en lait écrémé reconstitué à 10% (m/v). *Lactobacillus acidophilus* NCFM est une souche commerciale d'origine humaine qui a été cultivée sous aération limitée à 37°C en milieu MRS (Difco). *Escherichia coli* a été cultivée en milieu LB (Sambrook et Russell, 2001).

La composition des milieux est présentée dans le Tableau 2. Quand nécessaire, les antibiotiques suivants ont été ajoutés aux milieux de culture : érythromycine (Sigma) à 5 µg/mL pour *S. thermophilus* ou à 400 µg/mL pour *E. coli* ; spectinomycine (Sigma) à 300 µg/mL pour *S. thermophilus* et streptomycine à 20 µg/mL.

Tableau 1. Liste des souches utilisées dans ce travail.

	Souches	**Origine**	**Sources**
Streptococcus thermophilus	CNRZ455	Fromage	URAFPA, UL
	WG19258M	Lait Pasteurisé	ATCC
	ATE11PB6MJ	Yaourt	URAFPA, UL
	EBL1066	Yaourt	CNRZ
	CNRZ160	Yaourt	CNRZ
	CNRZ21	Yaourt	CNRZ
	CNRZ302	Fromage	CNRZ
	EBL307	Fromage	CNRZ
	EBL308	Fromage	CNRZ
	EBL385	Fromage	CNRZ
	CNRZ391	Yaourt	CNRZ
	EBL404	Yaourt	CNRZ
	EBL407	Yaourt	CNRZ
	EBL445	Fromage	CNRZ
	4F44	Fromage	CNRZ
	EBLHAD17	Yaourt	URAFPA, UL
	EBLHAD8α	Yaourt	URAFPA, UL
	LMD9	Yaourt	ATCC
	LMG18311	Yaourt	
	PB18	Fromage	URAFPA, UL
	PB18O	Fromage	URAFPA, UL
	PB2(B17)	Non déterminée	URAFPA, UL
	PB302	Yaourt	URAFPA, UL
	PB385	Yaourt	URAFPA, UL
	PB5MJ	Yaourt	URAFPA, UL
	ST14	Yaourt	URAFPA, UL
	EBLST19	Yaourt	URAFPA, UL
	EBLST20	Yaourt	URAFPA, UL
	ST88	Fromage	URAFPA, UL
	EBLY4	Yaourt	URAFPA, UL
***Streptococcus thermophilus* mutant**	STUL5001	Dérive de la souche LMD-9	Ce travail
Escherichia coli	TOP10		Invitrogen
Lactobacillus acidophilus	NCFM	origine humaine	

Tableau 2. Composition des milieux de culture utilisés pour la croissance bactérienne.

Milieux et conditions de stérilisation	**Composition**		**Utilisation**
	Constituant	**Concentration g/L**	
Lait Autoclave 110°C, 15 min	Lait en poudre	100	Conservation des souches à -80°C Préculture à 42°C
LB Autoclave 120°C, 20 min	Tryptone enzymatique	10	Cultures d'*E. coli*
	Extrait de levure	5	
	NaCl	10	
	Agar (pour milieu solide)	20	
	Eau qsp	1L	
M17 (Terzaghi et Sandine, 1975) Autoclave 120°C, 20 min	Bacto-tryptone	2,5	Croissance de *Streptococcus thermophilus*
	Extrait de levure	2,5	
	Sulfate de magnésium	0,25	
	Acide ascorbique	0,5	
	Extrait de viande	3	
	Soyase	5	
	Lactose* ou glucose*	20	
	Bacto-peptone	2,5	
	β-glycérophosphate de sodium	19	
	Agarose Pour milieu solide	15	
	Eau qsp	1L	

*les solutions de lactose et de glucose ont été préparées et stérilisées séparément et ajoutées ensuite dans le milieu M17.

2. Lignées cellulaires et les configurations de culture

La lignée cellulaire HT29 (issue d'un adénocarcinome colorectal humain) provient de l'European Collection of Cell Cultures (ECACC) et a été soit stockée à -190°C ou cultivée dans du milieu DMEM (Dulbecco's Modified Eagle's Medium) (Lonza, Bâle, Suisse) supplémenté avec 1 g/L de glucose, 10% (v/v) de sérum de veau fœtal (SVF, Lonza, Bâle, Suisse) décomplémenté (56°C pendant 1 h), 1% d'acides aminés non essentiels et 2 mM de glutamine (Lonza, Bâle, Suisse). De la pénicilline et de la streptomycine (PS) (Lonza, Bâle, Suisse) ont été ajoutées (0,1%, m/v) au milieu de croissance lorsque cela était nécessaire. Les cellules ont été cultivées à 37°C soit dans des flacons (Becton Dickinson, Franklin Lake, États-Unis) ou en plaques de 24 puits, dans une atmosphère humide avec 10% de CO_2 et 90% d'air.

La lignée cellulaire HT29-MTX (INSERM UMR S 938, Paris, France), capable de sécréter du mucus a été utilisée pour les tests d'adhésion. Les cellules ont été cultivées à 37°C pendant 21 jours dans du DMEM supplémenté avec 10% de SVF et 2 mM de glutamine. Les cellules de la lignée PBMC (cellules mononucléaires du sang périphérique) ont été achetées auprès de « Stem Cells » et cultivées dans des plaques de 24 puits, en RPMI-1640 (Rosewell Park Memorial Institute ; Sigma-Aldrich) selon les instructions du fournisseur, à 37°C et sous atmosphère humide comportant 10% de CO_2 et 90% d'air.

3. Souris utilisées

Les expériences utilisant la souris ont été réalisées en respectant la législation française (N°86/609/CEE). Deux types de souris ont été utilisés : des souris âgées de 6 semaines de la lignée C57BL/6J et des souris âgées de 18 semaines de la lignée SWISS. Durant toute l'expérience elles ont eu accès à de la nourriture standard et à de l'eau. Dans le cas de la validation *in vivo* de l'outil R-IVET par utilisation du promoteur P*lac*, l'eau de boisson proposée 20 h avant l'expérience contenait du lactose (5%, m/v). Avant utilisation les fèces de chaque souris ont été prélevées, suspendues et les bactéries de la flore intestinale étalées sur milieu LM17 supplémenté en spectinomycine ou érythromycine afin de s'assurer de l'absence de clones bactériens résistants à ces deux antibiotiques dans la flore intestinale des souris. Après une culture d'une nuit en milieu M17, *S. thermophilus* a été récupéré par centrifugation et re-suspendu dans du milieu M17 de façon à obtenir une concentration cellulaire de 5.10^9 UFC par mL. 200 µL de cette suspension ont été utilisés pour le gavage de

chaque souris. Les échantillons de fèces ont été récupérés à différents temps, re-suspendus puis dilués dans du tampon PBS et les dilutions appropriées ont été étalées sur le milieu sélectif approprié.

4. Vecteurs bactériens et amorces d'amplification

Les plasmides et les amorces utilisés dans ce travail sont présentés dans les Tableaux 3 et 4. Les amorces ont été conçues au moyen du logiciel Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) et synthétisées par la société EUROGENTEC.

Tableau 3. Plasmides utilisés dans ce travail.

Plasmides	Caractéristiques	References
pSET4s	$Spec^R$; origine de réplication de pG^+host3 et de pUC19, *lacZ*' Spc^r	(Takamatsu *et al.*, 2001)
pNZ5520	Cm^R; dérivé de pNZ18 et contenant un multi site de clonage en amont du gène *cre*	(Bachmann *et al.*, 2008)
pG^+host9	Em^R; dérivé de pWV01	(Maguin *et al.*, 1996)
pULNcreA	Em^r; dérivé de $pG^+host9^{(TR)}$ contenant le gène *cre* dépourvu de son promoteur et le terminateur transcriptionnel T*las* localisé en amont du gène *cre*	Ce travail
pULNcreB	pULNcreA avec le terminateur transcriptionnel *pepN* localisé en aval du gène *cre*	Ce travail
pULNcreB-s*hsp*	pULNcreB contenant le promoteur Ps*hsp* cloné dans le site *Bgl*II en amont du gène *cre*	Ce travail
pULNcreB-*lac*	pULNcreB contenant le promoteur P*lac* cloné dans le site *Bgl*II en amont du gène *cre*	Ce travail
pULNcreB-*prtS*	pULNcreB contenant le promoteur P*prtS* cloné dans le site *Bgl*II en amont du gène *cre*	Ce travail

Cm^R : résistance au chloramphénicol ; Em^R : résistance à l'érythromycine ; (TR) : thermorésistance.

Tableau 4. Amorces utilisées au cours de ce travail.

#	Sequences 5' to 3'	Caractéristiques
1	ATCCGTAAAACCATCAAATCTTAATT	Construction de la cassette *specR* flanqué de 2 sites *loxP* 3616-pb amplicon avec #4 pour la cassette non supprimé ou 2382 pb pour la cassette supprimé
2	TGACTCCCCGTCGTGTAGATAACTAG	Amplification de gène de résistance à la spectinomycine pSET4S avec #3 (1201 pb)
3	CGCTACGATAACGCCTGTTT	Utilisé avec #2
4	TTAAACCAATCTGTCCTCGGG	Utilisé avec #1
5	ATAATAACTAGTGAGAGGCCGCCACGGCGATGTGC	*Spe*I, construction du pULNcreA, 1457-pb amplicon avec #6
6	ATAATAGTCGACGCTTCTGGAGCTCAATTGAAAG	*Sal*I, utilisé avec #5
7	ATATATGGTACCTCGCTTTGATTGTTCTATCG	*Kpn*I, construction du pULNcreB, 159-pb amplicon avec #8
8	ATAATAGTCGACAAGCAGCAGTTGATAAAGC	*Sal*I, Utilisé avec #7
9	CCATTTTGAACGATGACCTC	Confirmation de la *creB* presence, 2041-pb amplicon avec #10
10	GACAGCTTCCAAGGAGCTAA	Utilisé avec #9
11	ATAATAAGATCTTCATGAATGTCTTGCTCACTCC	*Bgl*II, construction du pULNcreB-P*prtS*, 211-pb amplicon avec #12
12	ATAATAAGATCTTCCTCCTAGTTTTTCTCCTT	*Bgl*II, utilisé avec #11
13	ATAATAAGATCTGGACGAACTAAAATAGTGACG	*Bgl*II, construction du pULNcreB-P*shsp*, 171-pb amplicon avec #14
14	ATAATAAGATCTCATAATAATCACTCCTTTC	*Bgl*II utilisé avec #13
15	ATAATAAGATCTACGATGGATGATATCGAAG	*Bgl*II, construction du pULNcreB-P*lac*, 324-pb amplicon avec #16
16	ATAATAAGATCTTTCGGAAACCTCCTATTATTTG	*Bgl*II utilisé avec #15
17	ACACAAAGTCGCTGTTCTCG	Séquençage de la cassette R-IVET
18	TGCAAGTAAAATTGCACCTGTT	Séquençage de la cassette R-IVET
19	ATCTTCCGCTTTAGTTTTTGGC	Séquençage de la cassette R-IVET
20	CCATGGCAATTATTTGGTTACG	Séquençage de la cassette R-IVET
21	TGGTACCGTGGAATCATCCT	Confirmation du *specR* presence, 361-pb amplicon avec #22
22	GGAGAAGATTCAGCCACTGC	Utilisé avec #21
23	AAACAGGCGTTATCGTAGCG***ATAACTTCGTATAGCATACATTATACGAAGTTAT***TAATGTTTGGTGTAGGTAATA	
24	CTAGTTATCTACACGACGGGGAGTCA***ATAACTTCGTATAATGTATGCTATACGAAGTTAT***TCCCCAAGCAAAAATTGGA	

Les séquences soulignées représentent les sites de restriction utilisés pour les clonages. Les séquences indiquées en caractères gras et italiques représentent les sites *loxP*.

5. Enzymes utilisées dans ce travail

Les enzymes de restriction (New England Biolabs) ont été utilisées dans les conditions recommandées par le constructeur. Les *Taq* polymérases (Fermentas ou Invitrogen) ont été utilisées pour vérifier la présence du gène *shsp* dans les souches de *S. thermophilus* et des séquences dans les clones transformants obtenus chez *E. coli* ou *S. thermophilus*. L'*AccuTaq* (Sigma) a été utilisée pour l'amplification des fragments destinés à être clonés et/ou séquencés et l'ADN polymérase *Phusion* (Finnzymes ; Thermo Scientific, Finland) a été utilisée pour les réactions d'overlapping PCR lors de la construction de la cassette chromosomique. La Calf Intestinal alkaline Phosphatase (CIP) (New England Biolabs), la T4DNA ligase (New England Biolabs), la RNase A (Roche), le lysozyme (Eurobio), la protéinase K (Roche), la catalase (Sigma) et le cocktail antiprotéasique de Roche ont été utilisés suivant les instructions de leurs fournisseurs respectifs.

6. Suivi de croissance de *Streptococcus thermophilus*

La croissance des souches de *S. thermophilus* a été suivie soit par mesure de densité optique (DO) à 600 nm avec le spectrophotomètre (UVIKON® 810, KONTRON Spectrophotometer) ou en BIOSCREEN CTM ou par un lecteur ultra polyvalent de microplaques (Tecan), lors des expériences de stress.

La croissance en Bioscreen a été utilisée afin de déterminer l'impact de l'insertion de la cassette chromosomique au locus choisi, la croissance de la souche mutante a été étudiée. Dans ce but, un triplicat de précultures réalisées en lait (10%, m/v) et provenant de 3 clones différents a servi à réaliser des précultures secondaires en milieu LM17 et GM17 contenant ou non de la spectinomycine (300 µg/mL). Après 2 h d'incubation, des cultures ont été réalisées après avoir été ajustées à une DO de 0,05. Un volume de 350 µL de chaque culture a été déposé en triplicat dans les puits de la microplaque puis recouvert de 50 µL d'huile pour maintenir *S. thermophilus* en anaérobiose. La croissance des souches a été suivie pendant 12 h par mesure de la densité optique à 600 nm toutes les 15 min. Les microplaques thermostatées à 42°C ont été agitées pendant 20 s avant chaque prise de D.O.

7. Extraction d'ADN génomique de *S. thermophilus*

Pour extraire l'ADN génomique de *S. thermophilus*, la bactérie a été cultivée pendant 24 h dans 50 mL de milieu LM17. Les cellules bactériennes ont été récupérées par centrifugation 10 min à 3900 *g* à 4°C, lavées 2 fois avec 5 mL de tampon TE et remises en suspension dans 5 mL de TE. Afin d'hydrolyser les parois bactériennes, les cellules ont été mises en présence de 250 µL de lysozyme (Roche) 5% (m/v) pendant une heure à 37°C. 600 µL d'EDTA (0,5 M, pH 7) puis 50 µL d'une solution de protéinase K (Roche) à 2% (m/v) ont été ajoutés. Après 15 min d'incubation à 37°C, 660 µL d'une solution de SDS (10% m/v) ont été ajoutés et l'ensemble incubé à 50°C jusqu'à ce que les cellules bactériennes soient lysées. Le lysat a ensuite été aliquoté à raison de 1 mL par tube et 350 µL d'acétate de potassium froid ont été ajoutés à chaque aliquot de lysat cellulaire. Après 15 min d'incubation sur la glace et centrifugation à 12000 *g* pendant 15 min à 4°C, le surnageant contenant les acides nucléiques a été récupéré. Les ARNs ont ensuite été hydrolysés par ajout de 2,5µL d'une solution à 1% (m/v) de RNase (Roche) et incubation une heure à 37°C. Après une centrifugation de 10 min à 12000 *g* à 4°C, la phase aqueuse a été récupérée et un volume d'isopropanol froid ajouté afin de précipiter l'ADN génomique. Le culot d'ADN a été récupéré par centrifugation à 12000 *g* pendant 10 min puis lavé à l'éthanol à 70% (v/v), séché à température ambiante et dissout dans 50 µL d'eau ultra pure. Les compositions de différentes solutions utilisées sont présentées dans le Tableau 5.

Tableau 5. Composition de différentes solutions utilisées pour l'extraction d'ADN génomique.

Tampon	**Constituant**	**Concentration**
TE (pH 8)	Tris-HCl	10 mM
	EDTA	8 mM
Acétate de potassium	Acide acétique	5 M
	Acétate de potassium	3 M
EDTA (pH 8)	EDTA	0,5 M

8. Extraction d'ADN plasmidique d'*E. coli*

L'ADN plasmidique a été extrait soit en utilisant le « Miniprep kit » de la société Fermentas, dans les conditions recommandées par le fournisseur, soit en utilisant le protocole détaillé ci-après.

A partir de 4 mL de culture réalisée en milieu LB en présence d'érythromycine à 400 µg/mL, les cellules bactériennes ont été récupérées par centrifugation 10 min à 4500 *g* à température ambiante. Le culot bactérien a été repris dans 100 µL de solution I (Tableau 6). Après addition de 200 µL de solution II (Tableau 6) et 5 min d'incubation dans la glace, 150 µL de solution III (Tableau 6) ont été ajoutés. Après incubation sur la glace pendant 10 min, l'ensemble a été centrifugé 10 min à 12000 *g* à 4°C et le surnageant incubé en présence de RNase A (100 µg/mL final) pendant une heure à 37°C. L'ADN a ensuite été précipité par ajout d'un volume d'isopropanol froid et récupéré après une centrifugation de 20 min à 12000 *g*. Le culot d'ADN plasmidique a ensuite été lavé dans deux volumes d'éthanol à 70% (v/v), séché à température ambiante et dissout dans 50 µL d'eau ultra pure.

Les ADN plasmidiques ont été dosés par détermination de l'absorbance de la solution à la longueur d'onde de 260 nm ou par électrophorèse en gel d'agarose, en comparant l'intensité de la bande plasmidique avec l'intensité des différentes bandes d'un marqueur de poids moléculaire High Range DNA Ladder (EUROMEDEX).

Tableau 6. Composition de solutions utilisées pour l'extraction d'ADN plasmidique.

Solutions	Composants	Concentrations
Solution I	Glucose	0,05 M
	Tris-HCl (pH 8)	0,025 M
	EDTA (pH 8)	0,01 M
Solution II	NaOH	0,2 M
	SDS	1%
Solution III	Acétate de potassium	3 M
	Acide acétique	5 M

9. Réactions de PCR standard et séquençage

Les PCR ont été réalisées dans un « My Cycler » (BioRad) ou un « Mastercycler pro thermocycler » (Eppendorf) à partir d'ADN en solution ou de lysat bactérien. Dans ce cas, la colonie a été reprise dans 10 µL d'eau ultra pure et les cellules lysées au four à micro-ondes (puissance maximale pendant 2 à 3 min). Deux microlitres de ce lysat ont été utilisés pour une réaction PCR. Le mélange réactionnel d'amplification contenait pour 20 µL de volume final, 2 µL de tampon de *Taq* polymérase concentré 10X, du $MgCl_2$ (1,5 mM), chacun des dNTP (200 µM chacun), chacune des amorces (0,8 µM chacune), 1,25 unités de *Taq* Polymérase et de l'ADN cible (environ 200 ng). Les conditions de PCR standard utilisées ont été les suivantes : étape initiale de dénaturation de 3 min à 95°C, 30 cycles de 3 étapes (dénaturation 30 s à 95°C, hybridation 30s à 54°C et extension 30s à 72°C), puis une étape d'extension de 10 min à 72°C.

Lors de clonage ou de séquençage de produit de PCR, l'*AccuTaq* a été utilisée. Dans ce cas, le volume réactionnel utilisé était de 50 µL et contenait 2,5 unités d'*AccuTaq*. Les conditions de PCR énoncées précédemment ont été utilisées mais avec une température d'élongation de 68°C.

Les produits obtenus ont été purifiés à l'aide du kit High pure DNA purification (Roche). Les séquençages ont été réalisés sur les deux brins par la compagnie «Beckman Coulter Cogenics». La comparaison des séquences et les alignements ont été réalisés à l'aide du logiciel libre Bioedit (http://www.mbio.ncsu.edu/BioEdit/page2.html) en utilisant l'algorithme Clustal W.

10. Electrophorèses d'ADN

Un volume d'échantillon à analyser a été ajouté à 0,2 volume de tampon de dépôt (composé de 0,25% (m/v) de bleu de bromophénol, de 70% (m/v) de saccharose et de 0,1 M d'EDTA à pH 8) et soumis à une migration électrophorétique horizontale dans du tampon TAE (Tris acétate 40 mM, EDTA 1 mM). Le pourcentage en agarose des gels a été choisi en fonction de la taille des fragments d'ADN à séparer. Les fragments d'ADN ont été ensuite visualisés, après révélation des gels dans une solution de bromure d'éthidium à 0,5 µg/mL, sous UV à 254 nm grâce à l'utilisation du GelDoc (BioRad) et du logiciel d'analyse d'images Quantity One.

11. Transformation de *S. thermophilus* et d'*E. coli*

A partir d'une préculture réalisée en lait, *S. thermophilus* a été ensemencé au 1/100^{éme} en milieu LM17 puis incubé 8 h à 42°C. Le milieu MCD a alors été ensemencé au 1/50^{éme} puis incubé 7 h à 42°C. Après détermination de la DO_{600nm}, un nouvel ensemencement a été réalisé en milieu MCD de façon à obtenir une DO de 0,05 et la culture incubée jusqu'à l'obtention d'une DO_{600nm} comprise entre 0,1 et 0,2. 100 µL de la culture ont ensuite été mis en contact avec de l'ADN (de 100 ng à 1 µg d'ADN linéaire ou circulaire) et incubées 1 h à 42°C. Les cellules ont alors été étalées sur milieu LM17 sélectif pour permettre la croissance des transformants. Le taux de transformation a été calculé en termes de colonies résistantes portant la construction/µg d'ADN ajouté. Les cellules non utilisées ont été centrifugées à température ambiante pendant 5 min à 8000 *g* et re-suspendues dans une solution composée de milieu MCD et de glycérol (concentration finale de 14%), puis aliquotées et congelées dans de l'azote liquide.

Les cellules chimio-compétentes d'*E. coli* TOP10 (Invitrogen) ont été transformées selon les instructions du constructeur.

12. Construction de la cassette chromosomique R-IVET

La stratégie utilisée pour construire la souche STUL5001 est présentée dans la Figure 8. Afin de construire le fragment mosaïque comportant le gène de résistance à la spectinomycine *specR* flanqué de 2 sites *loxP*, des réactions de PCR overlapping ont été réalisées en utilisant l'ADN polymérase Phusion.

Dans une première étape (Figure 8, 1A), les 3 fragments UP-loxP, specR, DN-loxP ont été amplifiés individuellement en utilisant respectivement les couples d'amorces #1/#24`, #2/#3 et #23/#4. Chaque réaction a été réalisée dans un volume final de 50 µL avec 0,02 U/µL d'ADN polymérase Phusion, 200 µM de chaque dNTP, 0,5 µM de chaque amorce et 100 ng d'ADN cible. L'ADN génomique de *S. thermophilus* LMD-9 a été utilisé pour l'amplification des fragments UP-loxP et DN-loxP et le plasmide pSET4S (Takamatsu *et al.*, 2001) a été utilisé pour l'amplification du gène *specR*. Les conditions de PCR utilisées ont été les suivantes : dénaturation initiale de 1 min à 98°C, 30 cycles de 3 étapes (dénaturation 20s à 98°C, hybridation 30s et extension 30s à 72°C) et une étape d'extension finale à 72°C pendant

10 min. Les températures d'hybridation utilisées ont été de 41,4°C, 60°C et 46,8°C pour l'amplification des fragments UP-loxP, specR, DN-loxP, respectivement.

Dans une deuxième étape (Figure 8, 1B), les 3 amplicons UP-loxP, specR, DN-loxP ont été mélangés (concentration équimolaire) et réunis en utilisant les amorces externes #1 et #4. Les conditions utilisées ont été les suivantes : 1 min de dénaturation initiale à 98°C ; 50 cycles de 3 étapes (20s de dénaturation à 98°C, 30s d'hybridation et 2 min d'extension à 72°C) ; une extension finale de 10 min à 72°C. Pour ce qui concerne la température d'hybridation, une PCR « touchdown » a été appliquée pour les 20 premiers cycles avec une diminution de 1°C par cycle (de 60°C à 40°C), suivie par une température d'hybridation constante à 42°C pour les 30 derniers cycles. Le fragment obtenu (environ 30 ng) a été utilisé directement pour la transformation de *S. thermophilus* LMD-9.

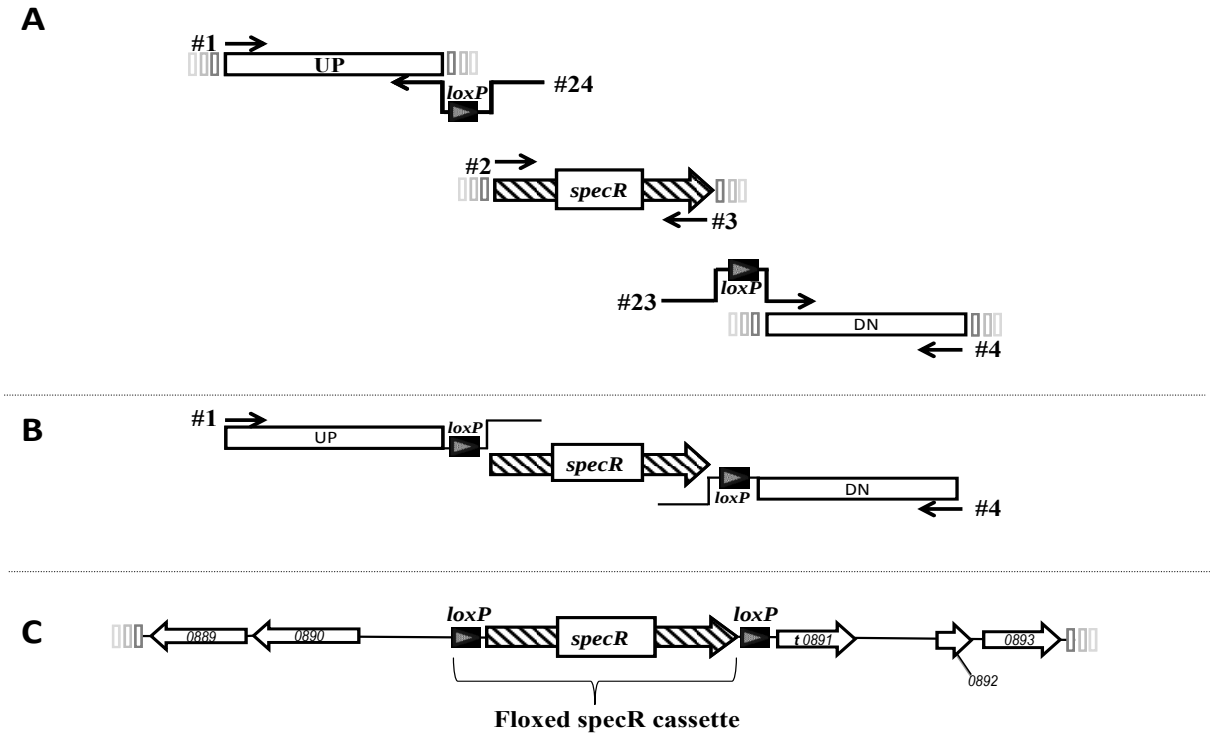

Figure 8. Représentation schématique des différentes étapes de construction du fragment d'ADN contenant la cassette chromosomique R-IVET par PCR "overlapping".

(A et B) et de la région chromosomique contenant la cassette R-IVET après transformation de *S. thermophilus* LMD-9 par le fragment final obtenu par PCR « overlapping » et intégration (C).

13. Construction du plasmide pULNcreB

Le plasmide pULNcreA (Figure 9) (et ses dérivés) a été construit à partir de la forme thermorésistante (pG^+host9^{TR}) de pG^+host9 (Maguin *et al.,* 1996), un plasmide qui peut se répliquer chez *E. coli* et *S. thermophilus* à 37°C. Le fragment portant le terminateur de transcription T*las*, un multi site de clonage comportant notamment les sites uniques de restriction *Sma*I et *Bgl*II et le gène *cre* dépourvu de son promoteur a été amplifié à partir du plasmide pNZ5520 (Bachman *et al.,* 2008) en utilisant les amorces #5 and #6 qui permettent d'introduire un site de restriction *Spe*I et *Sal*I aux extrémités 5' et 3' de l'amplicon. Le fragment de 1547-pb obtenu a été digéré par ces deux enzymes de restriction et ligaturé avec le réplicon pG^+host9^{TR} (Maguin *et al.,* 1996) préalablement digéré par les mêmes enzymes et déphosphorylé. Le plasmide pULNcreA a ainsi été obtenu et utilisé pour la construction du plasmide pULNcreB. Pour cela, le terminateur de transcription T*pepN* a été amplifié à partir du plasmide pNZ5520 en utilisant les amorces #7/#8 qui permettent d'incorporer à l'amplicon les sites de restriction *Kpn*I et *Sal*I côté 5' et 3', respectivement. Le fragment de 159-pb obtenu a été inséré en aval du gène *cre* dans le plasmide pULcreA après avoir digéré le plasmide et l'insert par les enzymes *Kpn*I et *Sal*I et déphosphorylé le vecteur. Le plasmide pULNcreB ainsi obtenu a été séquencé sur les deux brins et est présenté par la Figure 9.

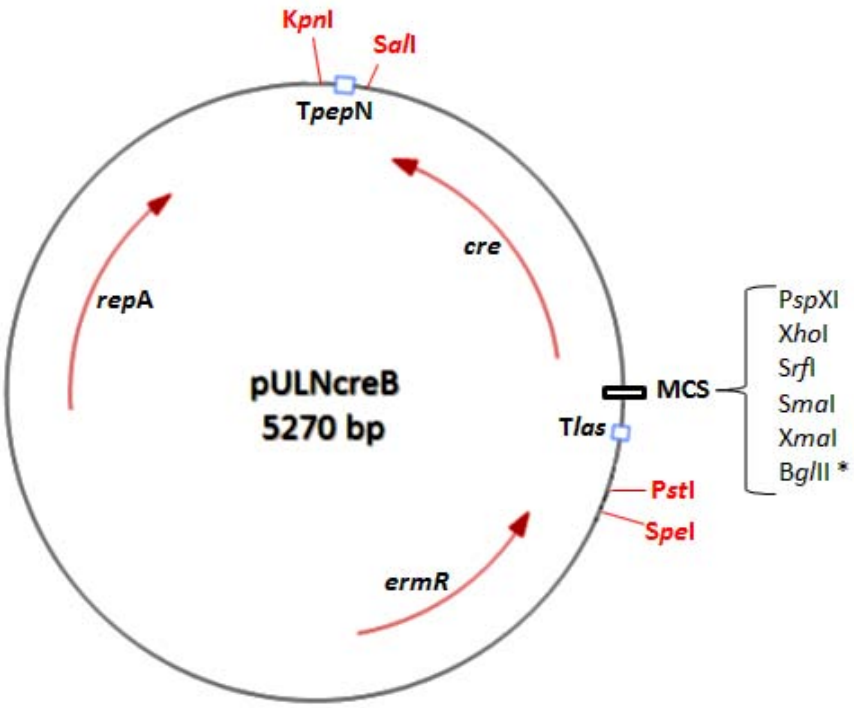

Figure 9. Carte du plasmide pULNcreB.

Le gène *cre* flanqué des terminateurs T*las* and T*pepN* (□) ainsi que le multi-site de clonage (MCS) situé en amont du gène *cre* sont indiqués. Le site utilisé pour le clonage des promoteurs est indiqué par une étoile et ceux utilisés pour la construction du plasmide sont indiqués en caractères rouges.

14. Etude de la stabilité du plasmide pULNcreB

La stabilité ségrégationnelle du plasmide pULNcreB dans la souche recombinante portant la cassette chromosomique R-IVET, *S. thermophilus* STUL5001, a été déterminée en maintenant cette souche en phase exponentielle de croissance en milieu LM17 sans érythromycine pendant plusieurs générations. Des échantillons ont été prélevés à différents temps, chaque temps correspondant à un nombre précis de générations, et étalés sur milieu LM17 gélosé. Après incubation 1 nuit à 37°C, 50 à 100 colonies ont été repiquées sur milieu LM17 supplémenté ou non en érythromycine. Le nombre de colonies Ery^R a été déterminé et la stabilité ségrégationnelle du plasmide pULNcreB déduite au temps considéré [(Nombre de colonies Ery^R/nombre de colonies repiquées sur milieu LM17) x 100]. Pour chaque temps, 10

colonies Ery^R ont été sélectionnées aléatoirement et le plasmide pULNcreB recherché par PCR sur colonie en utilisant les amorces #9/#10.

15. Clonage des promoteurs P*prtS*, P*shsp* et P*lac*

Les promoteurs P*prtS*, P*shsp* et le promoteur P*lac* de l'opéron lactose ont été amplifiés à partir de l'ADN génomique de *S. thermophilus* LMD-9, en utilisant les amorces #11/#12, #13/#14 et #15/#16. Les 6 amorces ont été dessinées de manière à introduire de chaque côté de l'amplicon obtenu un site *Bgl*II. Les amplicons de 211-pb (P*prtS*), 171-pb (P*shsp*) et de 324-pb (P*lac*) ont alors été digérés par l'enzyme de restriction *Bgl*II et clonés en amont du gène *cre* dans le plasmide PULNcreB, préalablement digéré par *Bgl*II et déphosphorylé. Les plasmides pULNcreB-P*prtS*, pULNcreB-p*shsp* et pULNcreB-p*lac* ont ainsi été obtenus et utilisés pour la validation *in vitro* et *in vivo* de l'outil R-IVET. Pour chacune des constructions, l'insert cloné a été séquencé sur les deux brins afin de s'assurer qu'aucune mutation n'était apparue au cours de l'amplification et/ou du clonage.

16. Extraction des protéines et quantification des protéines

Pour chaque souche de *S. thermophilus*, des cultures de 50 mL ont été réalisées en milieu LM17 à 42°C et stoppées en fin de phase exponentielle-début de phase stationnaire. Les cellules ont été récupérées par centrifugation à 4°C, pendant 15 min à 3900 *g* et le surnageant éliminé. Le culot a été lavé 3 fois avec du tampon phosphate de sodium (NaH_2PO_4/Na_2HPO_4, 50 mM à pH7) puis congelé pendant 3h à -20°C. Les cellules congelées ont ensuite été mises en suspension dans 5 mL de tampon phosphate de sodium et ont subi une sonication avec le sonicateur Vibra cell 600 W (Bioblock Scientific) pendant 3 min à une puissance de 100 W avec un cycle actif de 50%. Les débris cellulaires ont été séparés du contenu cellulaire soluble par centrifugation 10 min à 4°C à 12 000 *g*.

La quantification des protéines a été réalisée par la technique de Bradford en mesurant l'absorbance à 595 nm (Bradford, 1976). Pour cela, une gamme étalon de BSA (Bovine Serum Albumine) a été réalisée avec des concentrations allant de 0 à 100 µg/mL dans du tampon Tris-HCl (50 mM, pH8). Le dosage des protéines a été réalisé en mélangeant 100 µL d'échantillon avec 1 mL de réactif de Bradford (BioRad). La lecture de l'absorbance a été faite à 595 nm après 5 min de réaction.

17. Séparation des protéines par SDS-PAGE

Après avoir dosé les protéines dans chaque surnageant par la méthode de Bradford, 2 mg de protéines/mL de tampon d'échantillon (composé de 0,1 M de Tris-HCl pH 6,8 ; 5% de ß-mercaptoéthanol (v/v) ; 0,052 M de SDS ; 2% de glycérol (v/v) et 0,001% de bleu de bromophénol (m/v)) ont été chauffés pendant 5 min à 100°C et 10µL ont été déposés sur le gel d'acrylamide/bis-acrylamide. La migration des protéines a été effectuée dans un gel de concentration puis de séparation renfermant respectivement 5% et 15% d'acrylamide/bis-acrylamide (v/v) (Tableau 7). L'électrophorèse a été réalisée à 200 V, 500 mA et 250 W dans un tampon (Tris 25 mM, glycine 192 mM, SDS 0,1% (m/v), pH 8,3) de 45 min à 1 h à 4°C. La composition des gels et tampons utilisés est détaillée dans le Tableau 7.

Au terme de la migration, les protéines ont été fixées par incubation des gels dans une solution contenant 12% (m/v) d'acide trichloroacétique pendant 20 min, puis révélées par coloration au bleu de Coomassie R-250 en immergeant les gels pendant 4 h dans du colorant composé de 0,1% (m/v) de bleu de Coomassie R-250, 2% (m/v) d'acide trichloroacétique et 50% (v/v) d'éthanol. Les gels ont été ensuite décolorés grâce à une solution contenant 30% (v/v) d'éthanol et 7,5% (v/v) d'acide acétique.

Les gels ont été ensuite numérisés en utilisant un scanner GS-800 Calibrated Densitometer (Bio-Rad). Les masses des protéines ont été estimées grâce à un marqueur commercial (Precision Plus Protein™ All Blue Standards, Bio-Rad), qui est un mélange de 10 protéines ayant des masses moléculaires allant de 10 à 250 kDa. La taille des protéines a été estimée à l'aide du logiciel Quantity One (version 4.2.1 Bio-Rad).

Tableau 7. Composition des gels, tampons d'échantillon et de migration utilisés en électrophorèse SDS-PAGE.

A. Gel de concentration

Produits	Quantité (concentration)
Pourcentage de gel	5%
Acrylamide, bisacrylamide	1,3 mL (T=5%, C=2,6%)
Tris HCl pH6,8	2,5 mL (0,125 M)
eau	6,3 mL
Dégazer le mélange pendant 15 min	
SDS 10%	100 µL (2,6 mM)
TEMED	20 µL (3,3 mM)
Persulfate d'ammonium (100 mg/mL)	100 µL (2,2 mM)

Quantités pour 2 mini gels

B. Gel de séparation

Produits	Quantité
Pourcentage de gel	15%
Acrylamide, bisacrylamide	5,6 mL (T=15%, C=2,6%)
Tris HCl pH 8,8	3,75 mL (0,375 M)
eau	5,5 mL
Dégazer le mélange pendant 15 min	
SDS 10%	100 µL (2,6 mM)
TEMED	5 µL (3,3 mM)
Persulfate d'ammonium (100 mg/mL)	50 µL (2,2 mM)

Quantités pour 2 mini gels

C. Tampon échantillon

Produits	Quantité
Tris HCl pH 6,8	0,1M
β-mercaptoéthanol	5% (v/v)
Saccharose	4% (m/v)
Bleu de Bromophénol	0,001% (m/v)
SDS	0,052 M

D. Tampon d'électrodes pH 8,3

Produits	Quantité
Tampon tris	0,25 M
SDS	3,5 mM
Glycine	0,192 M

18. Détermination de la résistance au pH

A partir d'une culture de 20 h réalisée dans 1 mL de milieu LM17 à 37°C les souches de *S. thermophilus* ont été récupérées par une centrifugation 5 min à 13 000 *g* à température ambiante. Le culot bactérien a été re-suspendu dans 1 mL d'eau peptonée (EP ; 0,1% m/v) en conditions stériles. Puis la DO à 600 nm a été mesurée contre un blanc EP. Après un ajustement de la $DO_{600\ nm}$ à une valeur de 1, la suspension cellulaire a été divisée en deux échantillons de 500 µl chacun, le premier appelé «cellules stressées» (SC pour Stressed Cells) et le deuxième appelé «cellules non stressées» (NSC pour Non Stressed Cells). Les cellules du tube SC ont été récupérées par centrifugation 2 min à 3000 *g* à température ambiante et le culot re-suspendu dans 1 mL de milieu EP acidifié avec de l'acide chlorhydrique à pH 2 ou pH 4. Le volume dans le tube appelé NSC a été complété à 1 mL avec de l'EP. Les deux tubes ont été incubés à 37°C pendant 1 h puis centrifugés à 3000 *g* pendant 5 min à température ambiante et les culots cellulaires remis en suspension dans 1 mL d'EP. 100 µl de chaque échantillon ont été utilisés pour inoculer 900 µL de LM17. Une plaque de 384 puits a été remplie en conditions stériles avec 60 µl de LM17 dans chaque puits et 60 µl des suspensions bactériennes précédentes donnant une dilution finale de 1/40. A partir de cette dilution, deux autres dilutions en cascade, 1/80 et 1/160 ont été préparées pour les deux échantillons. La plaque a été ensuite incubée à 37°C. La croissance a été suivie par mesure de la densité optique à 600 nm toutes les 15 min pendant 19 h dans un lecteur ultra polyvalent de microplaques (Tecan). Le temps pris par les cellules de chaque échantillon pour atteindre le milieu de la phase exponentielle a été déterminé et la différence entre les deux temps (delta temps) a été calculée comme indice de résistance. Une faible valeur de delta temps indique un niveau de résistance élevé et inversement.

19. Détermination de la résistance aux sels biliaires

A partir d'une culture de 20 h réalisée dans 1 mL de milieu LM17 à 37°C les cellules bactériennes ont été récupérées par centrifugation 5 min à 13 000 *g* à température ambiante. Le culot bactérien a été re-suspendu dans 1 mL d'EP en conditions stériles. La DO à 600 nm a été mesurée contre un blanc EP et ajustée à une valeur de 1. La suspension cellulaire a été divisée en deux échantillons de 500 µl (SC et NSC) chacun et le volume de chaque tube a été complété à 1 mL avec de l'EP. Une solution composée de 50 mg/mL de chacun des deux principaux sels biliaires, le cholate de sodium (Sigma) et le déoxycholate de sodium (Sigma) a

été préparée. 10 µL de cette solution ont été ajoutés au milieu de culture de l'échantillon SC et 10 µl d'eau stérile ont été ajoutés dans l'échantillon NSC. Les deux échantillons (SC et NSC) ont été incubés à 37°C pendant une heure, puis les cellules récupérées par centrifugation 5 min à 13 000 *g* à température ambiante. Le culot bactérien a ensuite été re-suspendu dans 1 mL d'EP. A partir de chaque tube 100 µl ont été prélevés et mélangés à 900 µl de LM17. Les dilutions ont été faites comme expliqué précédemment dans la plaque 384 puits. La croissance a été suivie par mesure de la densité optique à 600 nm toutes les 15 min pendant 19 h dans un lecteur ultra polyvalent de microplaques (Tecan). Le temps pris par les cellules de chaque échantillon pou atteindre le milieu de la phase exponentielle a été déterminé et la différence entre les deux temps (delta temps) a été calculée comme indice de résistance. Une faible valeur de delta temps indique un niveau de résistance élevé et inversement.

20. Détermination de la résistance au stress oxydatif

A partir d'une culture de 20 h réalisée dans 1 mL de milieu LM17 à 37°C, les bactéries ont été récupérées par centrifugation 5 min à 13 000 *g* à température ambiante. Le culot bactérien a été re-suspendu dans 1 mL d'EP en conditions stériles, la $DO_{600\ nm}$ ajustée à une valeur de 1 et la suspension cellulaire divisée en deux échantillons de 500 µl chacun. Le volume de chaque échantillon (SC et NSC) a été complété à 1 mL avec de l'EP. 10,3 µl d'une solution de peroxyde d'hydrogène, H_2O_2 (Sigma), ont été ajoutés dans l'échantillon SC afin avoir une concentration finale de 10 mM et le même volume d'eau stérile a été ajouté à l'échantillon NSC. Les deux échantillons (SC et NSC) ont alors été incubés à 37°C pendant une heure, puis les cellules récupérées par une centrifugation 5 min à 13 000 *g* à température ambiante. Le culot a été ensuite re-suspendu dans 1 mL d'EP et 100 µl de chaque échantillon ont été ajoutés à 900 µl de LM17. 37,7 µl d'une solution de catalase (Sigma) ont été ajoutés afin d'avoir 2 unités de catalase/mL dans l'échantillon SC pour arrêter la réaction due à la présence de H_2O_2. Les dilutions ont été faites comme expliqué précédemment dans la plaque 384 puits. La croissance a été suivie par mesure de la densité optique à 600 nm toutes les 15 min pendant 19 h dans un lecteur ultra polyvalent de microplaques (Tecan). Le temps pris par les cellules de chaque échantillon pour atteindre le milieu de la phase exponentielle a été déterminé et la différence entre les deux temps (delta temps) a été calculée comme indice de résistance. Une faible valeur de delta temps indique un niveau de résistance élevé et inversement.

21. Test d'adhésion

Les cellules HT29-MTX ont été cultivées dans du DMEM (Lonza, Basel Suisse) supplémenté avec 2 mM de glutamine et 10% (v/v) de SVF (Lonza, Bâle, Suisse). Les cellules ont été maintenues à 37°C dans une atmosphère humide (10% de CO_2 et 90% d'air) et le milieu de culture changé tous les 2 jours. Les cellules HT29-MTX ont été ensemencées dans des plaques 24 puits (Costar) à une concentration de $0,1x10^6$ cellules/mL dans 500 µL de milieu et cultivées pendant 21 jours jusqu'à une densité de 50 000 cellules/cm^2.

Les bactéries provenant d'une culture de 20 h ont été récoltées par centrifugation 4000 *g* pendant 5 min à 4°C, puis lavées deux fois dans du PBS et re-suspendues pour obtenir une $DO_{600\ nm}$ de 1. Sachant qu'1 unité de $DO_{600\ nm}$ correspond à $3,0\ x10^8$ UFC / mL de *S. thermophilus* la taille de chaque inoculum a été ajustée pour avoir $6,0\ x10^8$ UFC/mL. Les plaques ont été incubées pendant 2 h à 37°C dans une atmosphère humide (10% de CO_2 et 90% d'air). Après deux heures d'incubation le surnageant a été récupéré à partir de chaque puits. Les bactéries présentes dans le surnageant ont été dénombrées par étalement sur le milieu LM17 après avoir fait les dilutions en cascade. Les plaques ont été délicatement lavées deux fois avec du PBS. Du triton a été ensuite ajouté aux puits à la concentration finale de 0,1%. Après incubation de 15 min à température ambiante, les puits ont été grattés afin de détacher les cellules adhérentes, le mélange récupéré et composé de cellules HT29-MTX et de cellules bactériennes étalé sur milieu LM17 gélosé et incubé à 37°C. La capacité d'adhésion des souches de *S. thermophilus* a été exprimée en pourcentage d'UFC déterminé à partir du mélange HT29-MTX-bactéries divisé par la somme de nombre d'UFC déterminé à partir du surnageant et le nombre d'UFC déterminé à partir du mélange HT29-MTX-bactérie.

22. Co-incubation avec les cellules HT29

Les cellules HT29 ont été ensemencées à la concentration de $0,1\ x\ 10^6$ cellules/mL dans 500 µL de milieu dans des plaques 24 puits et cultivées jusqu'à confluence pendant 7 jours à 37°C sous atmosphère humide (10% de CO_2 et 90% d'air). Le milieu de culture a été changé toutes les 24 h. Les cultures de *S. thermophilus* ont été réalisées à 37°C sur la nuit dans du milieu LM17 et les cellules bactériennes récupérées par centrifugation 5 min à 3600 *g* à température ambiante. Les culots bactériens ont été re-suspendus dans du DMEM frais complété avec 5% (v/v) de SVF et 2 mM de glutamine. La $DO_{600\ nm}$ a été mesurée et ajustée pour obtenir $1,46\ x\ 10^8$ UFC/mL. Les monocouches de cellules HT29 ont été lavées deux fois

avec du PBS (Lonza, Bâle, Suisse) et 450µL de DMEM frais supplémenté avec 5% (v/v) de SVF, 2 mM de glutamine et 0,1% (m/v) de pénicilline et de streptomycine ont été ajoutés à chaque puits. 5 ng/mL de TNFα (Sigma, Saint Louis, Etats-Unis) ont été ajoutés dans la moitié des puits. Pour chaque souche bactérienne, trois répétitions ont été effectuées dans les deux cas, c'est à dire avec TNFα et sans TNFα. Chaque puits a été inoculé avec des souches bactériennes, à raison d'une cellule HT29 pour 40 cellules bactériennes. Les plaques ont été incubées pendant 6 h à 37°C dans une atmosphère humide (10% de CO_2 et 90% d'air). A la fin de l'incubation, la viabilité cellulaire a été vérifiée en utilisant le bleu trypan (Sigma, Saint Louis, Etats-Unis), les plaques ont été centrifugées à 14 000 *g* pendant 5 min, les surnageants transférés sur une plaque de 96 puits (deep well) et de capacité de 2 mL (Corning bien VWR, Dublin, Irlande) et un cocktail antiprotéasique (Roche) a été ajouté à chaque échantillon. Ces surnageants ont été conservés à -80°C. Les plaques de 24 puits contenant les cellules HT29 ont été conservés à -20°C après deux lavages avec du SVF (Lonza, Bâle, Suisse) pour une détermination ultérieure de la concentration en protéines par la méthode de Bradford. L'interleukine IL-8 sécrétée a été quantifiée par ELISA (méthode immuno-enzymatique) comme décrit ci-dessous.

23. Co-incubation avec les cellules PBMC

Les cellules PBMC ont été décongelées selon les instructions du fournisseur et remises en suspension dans du RPMI-1640 supplémenté avec 2 mM de glutamine, 10% (v/v) de SVF et 1% (m/v) de PS. 1 mL de la suspension cellulaire a été distribué dans chaque puits d'une plaque 24 puits afin d'obtenir $1x10^6$ cellules/mL. Les cultures bactériennes ont été réalisées pendant 20 h dans du milieu LM17 à 37°C, et leur $DO_{600\ nm}$ ajustée à 1 avec du PBS. L'inoculation a été faite avec un rapport d'une cellule PBMC pour 10 cellules bactériennes et les co-cultures ont été incubées à 37°C sous une atmosphère de 10% de CO_2 pendant 4 h. Les surnageants ont ensuite été stockés à -80°C dans des plaques 96 puits (deep well) et de capacité de 2 mL (Corning bien VWR, Dublin, Irlande). Pour chaque expérience, un contrôle sans inoculation bactérienne a également été réalisé. Les interleukines IL-10 et IL-12p70 sécrétées ont été quantifiées par ELISA comme décrit ci-dessous.

24. ELISA

L'interleukine IL-8 sécrétée a été quantifiée dans les surnageants par un test ELISA classique en utilisant le test ELISA standard MAX ensemble kit (BioLegend, San Diego) constitué d'une plaque 96 puits, d'anticorps de capture et de détection et de protéines recombinantes type avidine-HRP requis pour la quantification précise de l'IL-8 humaine naturelle et recombinante. Les quantifications ont été réalisées conformément aux instructions du fabricant. La concentration en IL-8 a été exprimée en pg/mL et normalisée par la quantification dans chaque échantillon des protéines cellulaires.

Les interleukines IL-10 et IL-12p70 sécrétées ont été quantifiées dans les surnageants par un test ELISA classique, en utilisant la méthode ELISA pour l'interleukine-10 et 12 (p70) kits de Mabtech (Suède). Les quantifications ont été réalisées conformément aux instructions du fabricant. La concentration de l'IL-10 et IL-12p70 produites ont été déterminées en pg/mL.

25. Analyse des données

La tolérance des bactéries à pH 2, à pH 4, à l'H_2O_2 et aux sels biliaires ainsi que la capacité à l'adhésion bactérienne ont été soumises à une analyse en composantes principales (ACP). Les trois premiers axes ont été conservés pour réaliser une classification ascendante hiérarchique (CAH) en utilisant le logiciel SPAD 6.0. Pour la CAH, la méthode de Ward a été utilisée comme technique d'agglomération. Les six groupes obtenus optimisent les variabilités inter-groupes et intra-groupes. Les variables IL-8, IL-10 et IL-12 ont été ensuite utilisées comme variables illustratives (non utilisé dans les processus de calcul de l'ACP et de la CAH).

Résultats & Discussion

1. Un criblage *in vitro* de souches de *Streptococcus thermophilus* pour leur potentiel probiotique a révélé un fort *in vitro* potentiel anti-inflammatoire *in vitro* de trois souches

Un criblage *in vitro* de souches de *Streptococcus thermophilus* pour leur potentiel probiotique a révélé un fort in vitro potentiel anti-inflammatoire *in vitro* de trois souches

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Applied and Environmental Microbiology

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Résumé- *Streptococcus thermophilus* est une bactérie lactique thermophile très utilisée dans la fabrication des produits laitiers, ayant un statut GRAS et conférant des bénéfices santé aux produits alimentaires dans lesquels elle est présente (ex : yaourt). Après ingestion, les cellules de *S. thermophilus* peuvent être récupérées dans les fèces montrant que cette bactérie peut résister aux conditions du tractus gastro-intestinal (TGI). L'objectif principal de cette étude était de tester le potentiel probiotique de 30 souches de *S. thermophilus* de différentes origines en étudiant leurs capacités à résister aux différentes conditions de stress rencontrées pendant leur passage dans le TGI (pH faible, présence de sels biliaires, stress oxydatif), leur capacité à adhérer aux cellules épithéliales intestinales et leurs propriétés immunomodulatrices.

La résistance aux stress dans le tractus digestif a été évaluée en soumettant les souches à de faibles pH (pH 2 et pH 4), à des sels biliaires (cholate de sodium et déoxycholate de sodium) et du peroxyde d'hydrogène (H_2O_2). La croissance des bactéries stressées (SC pour stressed cells) et non stressées (NSC pour non stressed cells) a été déterminée et la différence entre le temps pris par les bactéries des échantillons SC et NSC pour atteindre le milieu de la phase exponentielle a été déterminée et constitue un indice de la résistance au stress pour chacune des souches testées. Les capacités des souches de *S. thermophilus* à adhérer aux entérocytes a été déterminée en utilisant la lignée cellulaire HT29-MTX qui est capable de produire du mucus. De plus, les propriétés immunomodulatrices de ces souches ont été étudiées en quantifiant les cytokines IL-8, IL-10 et IL-12 par ELISA, après co-incubation des bactéries avec des lignées de cellules HT29 ou PBMC. Il a ainsi été constaté que la résistance à des conditions acides, à l'H_2O_2 et aux sels biliaires ainsi que la capacité d'adhésion aux entérocytes était très variable d'une souche à l'autre. La majorité des souches réduit la production d'interleukine IL-8 (pro-inflammatoire) alors qu'elle induit la production d'interleukine IL-10 (anti-inflammatoire) et l'IL-12 (pro-inflammatoire). Sur la base du rapport IL-10/IL-12, qui indique le potentiel pro/anti-inflammatoire d'un probiotique, trois souches ont un fort potentiel anti-inflammatoire. L'Analyse en Composantes Principales (ACP) a permis de séparer les souches en 6 catégories différentes présentant des propriétés distinctes. A l'intérieur de chaque classe, une variabilité entre les souches a été observée et des caractéristiques intéressantes identifiées, mais aucune des classes ne peut-être considérée comme contenant le probiotique « parfait ». Cela n'est pas surprenant puisque les propriétés que doit posséder un probiotique dépendent fortement du type d'effet bénéfique attendu et de sa localisation. L'analyse par ACP pourrait permettre de prédire les potentialités d'une souche

probiotique comme sa capacité à survivre dans la première partie du tractus digestif ou d'adhérer à des entérocytes, comme le cas des membres de la classe 5.

Mots-clés : *S. thermophilus*, probiotique, stresses gastriques, tractus gastro-intestinal, adhésion, immunomodulations.

A High throughput *in vitro* screening of *Streptococcus thermophilus* strains revealed a high *in vitro* anti-inflammatory potential of three strains

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Abstract

Streptococcus thermophilus, a lactic acid bacterium widely used in dairy industry and having a GRAS status, contributes to the health benefits of dairy products. After consumption, it can be recovered from faeces showing its capacity to resist the harsh conditions of the gastro intestinal tract (GIT). The aim of this study was to determine the capacities of 30 *S. thermophilus* strains to resist to stress conditions encountered while GIT passage (i.e. pH, bile salt stress and oxidative stress), to adhere to intestinal enterocytes and their immunomodulatory properties. The strains were exposed to low pH (pH 2 and pH 4), bile salts (sodium cholate and sodium deoxycholate) and oxidative molecule (H_2O_2). Adhesion capacities of the strains to enterocytes were determined by using the mucous producing HT29-MTX cell line, and their immunomodulatory properties by quantifying the cytokines IL-8, IL-10 and IL-12 after co-incubation with the cell lines HT29 or PBMC. Resistance to the tested stresses as well as their adhesion capacity appeared to be highly variable between the strains. Majority of strains reduced the production of interleukin IL-8 (pro-inflammatory) while they induced production of interleukin IL-10 (anti-inflammatory) and of L-12 (pro-inflammatory). The Principal Component Analysis (PCA) method enabled us to cluster the

strains into 6 different classes displaying distinct properties. Finally, on the basis of the IL-10/IL-12 ratio, that indicates the pro/anti-inflammatory potential of a probiotic, three strains displayed a very strong *in vitro* anti-inflammatory potential.

Keywords**:** *S. thermophilus, probiotics, gastric stresses, gastro-intestinal tract, adhesion, immunoregulation.*

1.1. Introduction

The increasing costs of health as well as the desire of certain consumers such as the elderly to improve their quality of life stimulate the research and development of functional foods which are claimed to display health specific properties. Different approaches can be employed to produce functional foods: (i) adding of health promoting component(s), (ii) reducing/removing of harmful component(s) or (iii) modifying the nature or the bioavailability of specific component(s) (1). Among the health promoting component(s), probiotics which are defined by the Food Agricultural Organization/World Health Organization as live microorganisms conferring a health benefice on the host when administered in adequate amounts, are currently subjected to intense research to elucidate their health potential.

To exert their effect, probiotics have to be able to survive their passage through the GIT. Throughout whole extent of GIT, stress conditions are encountered starting from the harshness of saliva, followed by a very low pH in stomach, bile acid secretions, digestive enzymes, barrier effect of mucous and the presence of other microorganisms in the GIT. In these conditions, viability of the bacteria decrease along the passage through the GIT, the major causes of bacterial mortality being the acidic environment in the stomach and the bile secretions in the duodenum (2). Bacterial adhesion to the intestinal epithelium is considered to be important for probiotic action, since it may influence the residence time of the bacteria in the intestinal tract (3). The ability of some probiotic strains to inhibit pathogen colonization and to modulate the immune response(s) has also been related to their ability to adhere to intestinal mucus and/or epithelial cells (3). On the basis of this knowledge, several authors have proposed important criteria to rationalize the selection of the probiotic strains like (i) absence of pathogenicity or toxicity, (ii) large scale production perspectives, (iii) organoleptic properties and technological characters for use in food manufacturing, (iv) resistance to acids, (v) resistance to bile salts, (vi) adherence to the intestinal epithelium and/or to the mucous, (vii) production of bacteriocins, (viii) immunomodulatory properties etc (4, 5).

Lactic Acid Bacteria (LAB) are used for the manufacture of many food products such as yogurt, cheeses or fermented milks which can be considered as functional foods. Indeed, several of their components such as the peptides released during the hydrolysis of milk proteins as well as other substances released during fermentation process or the bacteria used for the fabrication of the dairy product can exert a healthy effect on the consumer, such as an immunomodulatory or an intestinal anti-inflammatory effect (6, 7). Among the LAB, *Streptococcus thermophilus* is the bacterium the most employed after *Lactococcus lactis* in the dairy industry. It is a thermophilic bacterium traditionally used in dairy products as a starter. In yogurt its metabolic properties in association with those of *Lactobacillus delbrueckii* ssp. *bulgaricus* contribute not only to the organoleptic properties but also to the health benefits of yogurt. Indeed, it has been shown that consumption of yogurt with live cultures improves digestion and absorption of lactose or may decreases stool frequency and duration of diarrheal episodes in children with acute watery diarrhea. It may also enhance the immune response particularly in certain populations such as the elderly (8). It has long been believed that *S. thermophilus* is unable to survive during its transit through the GIT, which explains that its probiotic potential has so far been little explored. However, this belief was nullified at the beginning of the 21th when the capacity of certain *S. thermophilus* strains to survive in the GIT was clearly established (9, 10, 11). Besides the positive health properties of the yogurt, several studies reported a positive effect of *S. thermophilus* also. For example, two *S. thermophilus* strains appeared to be capable of producing folate which is required for certain biological reactions (12) or it has been shown that *S. thermophilus* FB13 was able to produce an active β-galactosidase in the second half of the small intestine, explaining the role that could play this bacterium in alleviation of the lactose intolerance (13).

Thus, to improve and diversify the beneficial effects of yogurt consumption, it would be interesting to further investigate the probiotic properties of *S. thermophilus*. Given the complexity of the *in vivo* screening of large number of strains, *in vitro* experiments were performed on 30 *S. thermophilus* strains which have been isolated from yogurt or cheese. Resistance against acidic pH, bile salts and the oxidative molecule H_20_2 were determined. Capacities of the strains to adhere to the enterocytes were testing by using the mucous-producing HT29-MTX cells. Finally, the immunomodulatory profile of the strains was determined by monitoring the production of the interleukins like IL-8, IL-10 and IL-12 using two different cellular models: TNF-α activated HT29 and PBMC.

1.2. Material and Methods

1.2.1. Bacterial strains, media and growth conditions

S. thermophilus strains used in this work are listed in Table 1. They were isolated in our laboratory either from yogurt or cheese (P. Bracquart, personal communication), or came from the CNRZ (Centre National de Recherches Zootechniques, INRA, Jouy-en-Josas, France) or ATCC (American Type Culture Collection, Manassas, VA, USA) collections. Strains were stored at -80 °C in reconstituted skim milk (10%, w/v). They were inoculated at 1% in skim milk and incubated overnight at 42 °C before growth at 42°C in LM17, which corresponds to M17 medium (14) supplemented with lactose (20 g/l). *Lactobacillus acidophilus* NCFM is a human commercial strain which was grown under limited aeration at 37 °C in MRS medium (Difco).

Table 1. Origin and properties of resistance against various stresses prevailing in the GIT and of adhesion to the HT29-MTX cells of the *S. thermophilus* strains used in this work.

Strains	Origin	Gene shsp	Protein Hsp (16.4 KDa)	Resistance to gastric stresses				Adhesion Capacity % [b]
				pH 2	pH 4	Bile salts	Oxidative stress	
				Delta time between SC and NSC (h) [a]	Delta time between SC and NSC strain (h)[a]	Delta time between SC and NSC strain (h)[a]	Delta time between SC and NSC strain (h)[a]	
CNRZ455	Cheese	-	-	NG	0.49 ± 0.01	6.01 ± 0.27	8.97 ± 0.18	3.4 ± 0.26
WG19258M	PM	-	-	NG	0.46 ± 0.00	2.96 ± 0.25	6.32 ± 0.18	4.6 ± 0.08
ATE11PB6MJ	Yogurt	+	+	NG	0.59 ± 0.00	3.29 ± 0.11	7.25 ± 0.48	5.8 ± 0.13
EBL1066	Yogurt	-	-	NG	0.82 ± 0.02	6.14 ± 0.41	4.00 ± 0.21	2.5 ± 0.26
CNRZ160	Yogurt	-	-	NG	0.82 ± 0.01	3.31 ± 0.20	7.73 ± 0.41	4.6 ± 0.14
CNRZ21	Yogurt	-	-	NG	2.28 ± 0.00	8.09 ± 0.34	8.82 ± 0.35	0.0 ± 0.00
CNRZ302	Cheese	-	-	NG	0.37 ± 0.02	4.53 ± 0.14	3.46 ± 0.44	0.7 ± 0.17
EBL307	Cheese	-	-	NG	0.25 ± 0.00	7.49 ± 0.39	8.64 ± 0.42	0.7 ± 0.13
EBL308	Cheese	-	-	NG	1.26 ± 0.00	8.41 ± 0.27	9.22 ± 0.24	4.7 ± 0.53
EBL385	Cheese	-	-	NG	0.16 ± 0.00	4.16 ± 0.20	10.51 ± 0.20	1.8 ± 0.24
CNRZ391	Yogurt	+	+	NG	0.77 ± 0.00	8.44 ± 0.28	8.34 ± 0.35	0.0 ± 0.00
EBL404	Yogurt	+	+	NG	1.59 ± 0.00	6.75 ± 0.21	8.32 ± 0.48	1.8 ± 0.15
EBL407	Yogurt	-	-	NG	0.48 ± 0.01	4.14 ± 0.29	4.84 ± 0.98	3.7 ± 0.21
EBL445	Cheese	+	+	NG	0.66 ± 0.01	7.67 ± 0.27	12.50 ± 0.62	1.3 ± 0.24
4F44	Cheese	+	+	NG	0.42 ± 0.00	4.17 ± 0.36	4.57 ± 0.35	4.6 ± 0.17
EBLHAD17	Yogurt	-	-	NG	0.78 ± 0.01	7.14 ± 0.18	7.70 ± 0.44	4.5 ± 0.34
EBLHAD8α	Yogurt	-	-	NG	0.40 ± 0.01	7.36 ± 0.42	7.30 ± 0.18	1.6 ± 0.81
LMD9	Yogurt	+	+	4.75 ± 0.10	0.27 ± 0.01	5.88 ± 0.27	3.66 ± 0.31	11.8 ± 0.29
LMG18311	Yogurt	-	-	NG	0.78 ± 0.02	2.30 ± 0.40	7.58 ± 0.51	8.1 ± 0.33
PB18	Cheese	+	+	NG	0.61 ± 0.01	6.17 ± 0.34	8.78 ± 0.47	5.4 ± 0.22
PB18O	Cheese	+	+	4.51 ± 0.07	0.60 ± 0.01	3.98 ± 0.32	4.35 ± 0.40	6.6 ± 0.17
PB2(B17)	Unknown	-	-	NG	0.12 ± 0.00	4.28 ± 0.16	3.01 ± 0.18	0.9 ± 0.21
PB302	Yogurt	+	+	4.89 ± 0.12	0.64 ± 0.01	6.10 ± 0.27	3.27 ± 0.31	1.7 ± 0.14
PB385	Yogurt	+	+	NG	0.93 ± 0.01	3.95 ± 0.20	2.79 ± 0.22	0.6 ± 0.08
PB5MJ	Yogurt	-	-	NG	0.54 ± 0.01	4.21 ± 0.18	4.58 ± 0.32	0.0 ± 0.00
ST14	Yogurt	-	-	NG	1.72 ± 0.05	8.08 ± 0.34	9.15 ± 0.65	6.0 ± 0.17
EBLST19	Yogurt	-	-	NG	1.09 ± 0.01	3.63 ± 0.18	4.95 ± 0.28	4.6 ± 0.21
EBLST20	Yogurt	-	-	NG	0.43 ± 0.01	2.29 ± 0.34	5.79 ± 0.60	9.6 ± 0.24
ST88	Cheese	+	+	NG	0.31 ± 0.01	3.26 ± 0.10	5.96 ± 0.55	16.2 ± 0.34
EBLY4	Yogurt	+	+	NG	0.66 ± 0.01	5.49 ± 0.18	7.43 ± 0.29	4.0 ± 0.45

The presence of the *shsp* gene was determined by PCR while that of its corresponding protein by SDS-PAGE among soluble proteins of each strain. The results of resistance to the stresses tested in this study as well as those of adhesion correspond to the mean of at least three independent experiments. The delta time (expressed in hours) represents the difference between the time taken by the SC and NSC samples to reach the mid exponential growth phase. It was used as an index of stress resistance. [a] mean ± SD, [b] mean ± SEM, NG: No growth observed, SC Stressed Cells, NSC Non Stressed Cells, PM pasteurized Milk

1.2.2. Cell lines and culture configurations

The human colorectal adenocarcinoma cell lines HT29 were obtained from European Collection of Cell Cultures (ECACC) stored at -190°C or grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 1g/l Glucose, 10% of heat inactivated (56°C during 1h) Foetal Calf Serum (FCS, Lonza, Basel, Switzerland), 1% of non essential amino acids and 2 mM of glutamine (Lonza, Basel, Switzerland). Penicillin/Streptomycin (PS) (Lonza, Basel, Switzerland) was added at 0.1% (w/v) to the growth medium when necessary. Cells were grown at 37°C in a humidified atmosphere with 10% CO_2 and 90% air either in flasks (Becton Dickinson, Franklin Lake, USA) or in 24 well plates.

The mucous secreting HT29-MTX cell line (INSERM UMR S 938, Paris, France) was used for the adhesion tests. Cells were grown at 37°C during 21 days in DMEM supplemented with 10% FCS and 2 mM glutamine. The cell lines PBMC (Peripheral Blood Mononuclear Cells) were bought from Stemcells and cultivated in RPMI-1640 (Rosewell Park Memorial Institute) (Sigma-Aldrich) according to the supplier's instructions in 24 well plates and cultivated at 37°C under humidified atmosphere of 10% CO_2 and 90% air.

1.2.3. Protein extraction and separation

Whole soluble proteins were extracted in the conditions reported by Galia *et al*. (15). The SDS-PAGE and the staining of the gels were performed as already described (16).

1.2.4. Determination of resistance to pH, bile salts and H_2O_2

S. thermophilus cells were harvested from 1 ml of 20 h culture in LM17 and suspended in peptone water (0.1% w/v). After an adjustment of the O.D. $_{600\ nm}$ to the value of 1, the cell suspension was divided into two samples of 500 µl each. The first called "stressed cells" (SC), was subjected to the stress whereas the second, called "non stressed cells" (NSC) was used as the negative control. The SC and NSC samples were then centrifuged and cell pellets resuspended in 1ml of peptone water. 100µl of each sample was used to inoculate 900µl of LM17. Three dilutions i.e. 1/40, 1/80 and 1/160 were prepared for both samples and incubated in a 384 well plate. Growth was then monitored by measuring optical density at 600 nm every 15 min during 19 h in an Ultra multifunctional microplate reader (Tecan). The time necessary for the cells of each sample to attain the mid exponential growth phase was

determined and the difference between the two times (delta time) was calculated as the resistance index, a weak difference indicating a high resistance level. For each strain tested, at least three independent experiments were realized.

For the acidic tolerance assays, the SC sample was subjected during 1 h to low pH by adjusting the pH of the medium at 2 or 4 with hydrochloric acid. The control sample NSC was incubated in the same conditions without pH adjustment. To study the bile salt (BS) tolerance of *S. thermophilus*, two main bile salts, sodium cholate (Sigma) and sodium deoxycholate (Sigma) were added to the growth medium of the SC sample at the final equimolar concentration of 0.01% (w/v). Both samples (SC and NSC) were incubated at 37 °C for 1 h. Finally, the capacity of the different *S. thermophilus* strains to resist against oxidative stress was determined by adding hydrogen peroxide (Sigma) to the medium of SC sample at 10 mM final concentration. Both samples (SC and NSC) were incubated at 37 °C for one hour. Catalase (Sigma) was then added at 2 units/ml to the SC sample to stop the action of H_2O_2.

1.2.5. Bacterial adhesion to intestinal epithelium

HT29-MTX cells were grown in DMEM (Lonza, Basel Switzerland) supplemented with 2mM glutamine and 10% (v/v) FCS (Lonza, Basel Switzerland). The cells were maintained at 37°C in a humidified atmosphere (10% CO_2 and 90% air) and the culture medium was changed every 2 days. The HT29-MTX cells were seeded at a concentration of $0.1x10^6$ cells/ml in 500 µl of medium in 24-well plates (Costar) and grown for 21 days up to a density of 50,000 cells/cm^2. Bacteria from 20-h-old cultures were harvested by centrifugation (4,000 x g for 5 min at 4ºC), washed twice and suspended in PBS to obtain an $O.D._{600\ nm}$ of 1. Knowing that in the case of *S. thermophilus* 1 unit of $O.D._{600\ nm}$ corresponds to $3.0\ x10^8$ CFU/ml, the size of each inoculum was adjusted to have $6.0\ x10^8$ CFU/ml. The plates were incubated for 2 h at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. The supernatant was then recovered from each well for colony count and the plates were gently washed twice with PBS. Triton was then added to the wells to the final concentration of 0.1%. After 15 min incubation, the wells were scratched to remove the adhered cells and the mixture of HT29-MTX and bacterial cells were plated on LM17 agar plates and incubated at 37 °C. Adhesion capacity of the *S. thermophilus* strains was expressed as the percentage of CFU recovered from the HT29-MTX cells (adhered bacterial cells) relative to the CFU determined from the supernatants. Each assay was performed in triplicate, and conducted in three independent experiments.

1.2.6. Strain co-incubation with HT29 cell line

HT29 cells were seeded at the concentration of 0.1x 10^6 cells/ml in 500 µl medium in 24-well plates and grown to confluence for 7 days at 37 °C under humidified atmosphere of 10% CO_2 and 90% air. The culture medium was changed every 24 h. Overnight LM17 cultures of *S. thermophilus* were centrifuged (5 min at 3600 g) and bacterial cells suspended in fresh DMEM supplemented with 5% (v/v) FCS and 2 mM glutamine. O.D. $_{600\ nm}$ was measured and adjusted to obtain 1.46 x 10^8 CFU/ml. HT29 monolayers were washed twice with PBS (Lonza, Basel, Switzerland) and 450µl of fresh DMEM supplemented with 5% (v/v) FCS, 2 mM glutamine, and 0.1% (w/v) PS was added to each well. 5 ng/ml of TNFα (Sigma, Saint Louis, USA) was added to half of the wells. Three technical replicates for each bacterial strain were done in both cases i.e. with TNFα and without TNFα and then each well was inoculated with bacterial strains at the ratio of 1 HT29 cell for 40 bacterial cells. Plates were incubated for 6 h at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air. At the end of the incubation, cell viability was checked by trypan blue (Sigma, Saint Louis, USA), plates were centrifuged at 14000 g for 5 min, the supernatants were transferred to 96 deep well storage plates of 2 ml well capacity (Corning VWR, Dublin, Ireland) and antiprotease cocktail (Roche) was added to each sample. These supernatants were stored at−80 °C. The 24 well plates containing the HT29 cells were stored at -20 °C after washing twice with PBS (Lonza, Basel, Switzerland) for a later determination of the protein concentration using the Bradford method. A control without bacterial inoculation was also carried out for each experiment. Secreted IL-8 was quantified by ELISA as described below.

1.2.7. Strain co-incubation with PBMC

The PBMC cells were thawed according to the supplier's instructions and resuspended in RPMI-1640 supplemented with 2 mM glutamine, 10% (v/v) FCS and 1% (w/v) PS. 1ml of the cell suspension was distributed to each well of 24 well plates in order to obtain 1x10^6 cells/ml. Bacterial cultures were done for 20 h in LM17, and their O.D. $_{600\ nm}$ adjusted to 1 with PBS. Inoculation was done at the ratio of 1 PBMC cell for 10 bacterial cells and cultures were incubated at 37 °C under an atmosphere of 10% CO2 for 4 h. Three technical replicates for each strain were done as well as three independent experiments. The supernatants were then stored at −80 °C. For each experiment, a control without bacterial inoculation was also carried out. Secreted IL-10 and IL-12p70 were quantified by ELISA as described below.

1.2.8. ELISA

Secreted IL-8 was quantified in the supernatants by a classical ELISA test using the ELISA MAX Standard set kit (Biolegend, San Diego) consisting of a 96-well plate, capture and detection antibodies, recombinant protein standard and Avidin-HRP required for the accurate quantification of natural and recombinant human IL-8. Quantifications were performed according to the manufacturer's instructions. The concentration of IL-8 produced was calculated as pg/ml and normalized by the protein quantification of each cell sample. Three independent experiments were performed for each bacterial strain.

Secreted IL-10 and IL-12p70 were quantified in the supernatants by a classical ELISA test, using the ELISA for Human Interleukin-10 and 12 (p70) kits of Mabtech (Sweden). Quantifications were performed according to the manufacturer's instructions. The concentration of IL-10 and IL-12p70 produced were calculated as pg/ml.

1.2.9. Data Analysis

The low pH (pH 2 and pH 4), bile salt and H_2O_2 tolerance index values as well as bacterial adhesion were subjected to a Principal Component Analysis (PCA). The first three axes were submitted to hierarchical cluster analysis (HCA) using SPAD 6.0 data mining package. For HCA, squared Euclidean distance was used to measure similarity among clusters while Ward's method was used as an agglomeration technique, resulting in six clusters to a good partition and an optimal intergroup and intragroup variability. The PCA/HCA was elaborated to explore which bacteria followed the same profile and each cluster was illustrated by adding variables (not used into the calculation processes) and calculating on the average variables (IL-8, IL-10 and IL-12).

1.3. Results

1.3.1. Variability of the resistance to low pH, bile salts and oxidative stress in S. thermophilus

The 30 *S. thermophilus* were subjected to acidic conditions, bile salts and oxidative stress. For each strain, the time taken by the SC and the NSC samples to reach mid exponential phase was determined. The difference between these two times (delta time) gave the resistance index of the tested strain, a weak difference indicating a high resistance level.

Acidic resistance was tested at pH 2 and 4. The first pH corresponds to that prevailing in the stomach during interprandial phase while the second corresponds to that of the postprandial phase. At pH 2, only three strains PB18O, PB302 and LMD-9, resisted with an average delta time of 4.6 h (Table 1, Fig. 1A). All the other strains did not show any growth after being exposed to pH 2. Conversely, all of the tested strains appeared to be resistant to pH 4, with a delta time ranging from less than15 min for the strain PB2(B17) to more than 2 h for the strain CNRZ21(Table 1, Fig. 1B).

The *S. thermophilus* tolerance to bile salts was determined by incubating the 30 strains in presence of cholate and deoxycholate salt during 1 h. A high variability in the level of bile salt resistance was observed among the 30 strains, the strains EBLST20 and LMG18311 displaying the highest levels with a delta time of about 2.30 h, and the strains ST14, CNRZ21, EBL308 and CNRZ391 the lowest (delta time >8h) (Table 1, Fig. 2).

Finally, the 30 *S. thermophilus* strains were incubated for 1 h in presence of 10 mM H_2O_2 to determine their capacities to resist to such an oxidative stress. As observed for bile salt, a great variability of resistance level was displayed among the strains, PB385 being the most resistant strain (delta time of 2.8 h) and EBL385 and EBL445 being the most sensitive with a delta time longer than 10 h.

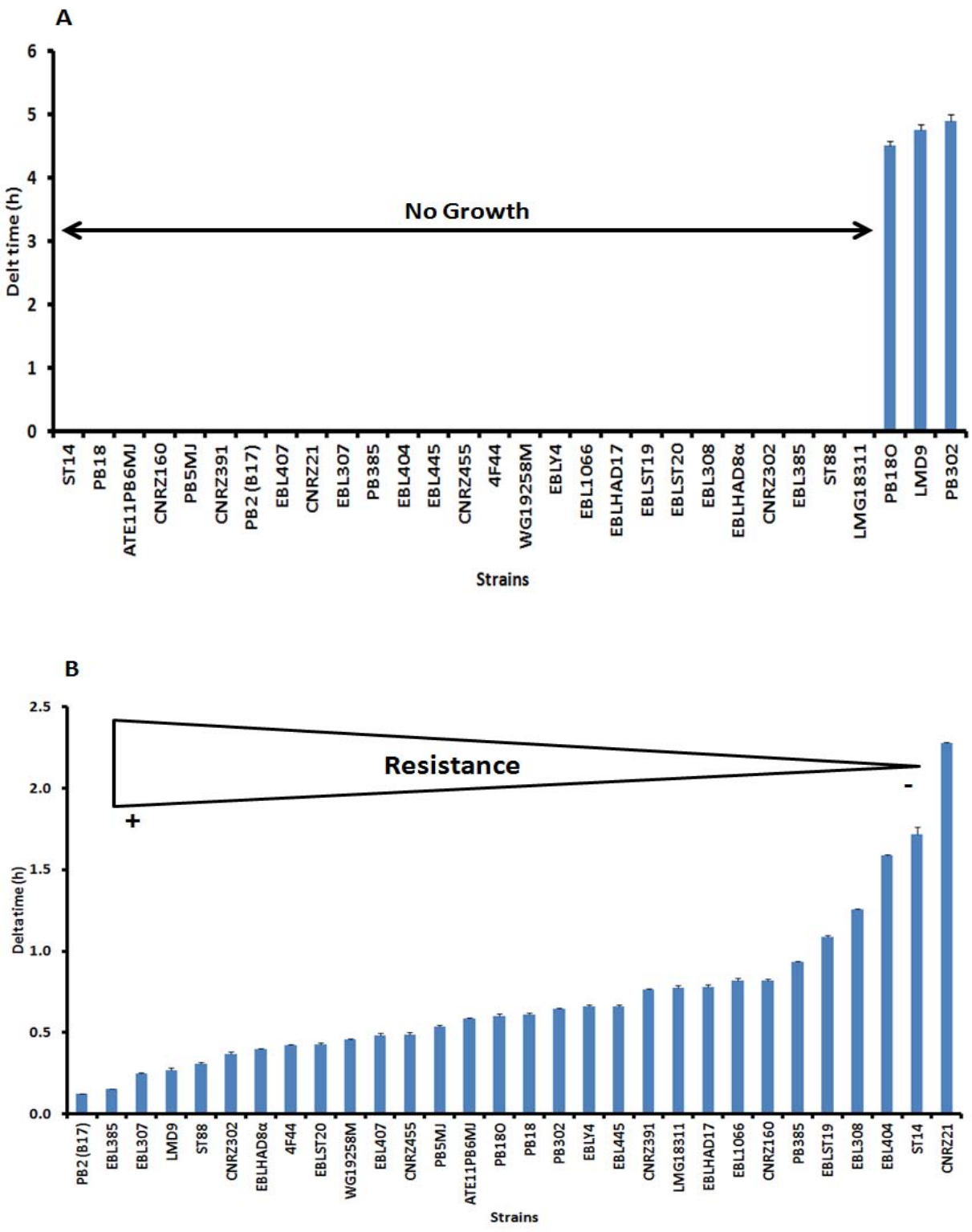

Fig. 1. Resistance of *S thermophilus* strains against acidic conditions. **(A)** resistance against pH= 2. **(B)** resistance against pH= 4. The delta time allows evaluating the level of resistance to acidic conditions. It corresponds to the difference between the time taken by the Stressed Cells (SC) and the Non Stressed Cells (NSC) to attain the mid exponential growth phase. For each strain, the mean delta time ± SEM deduced from at least three independent experiments is shown.

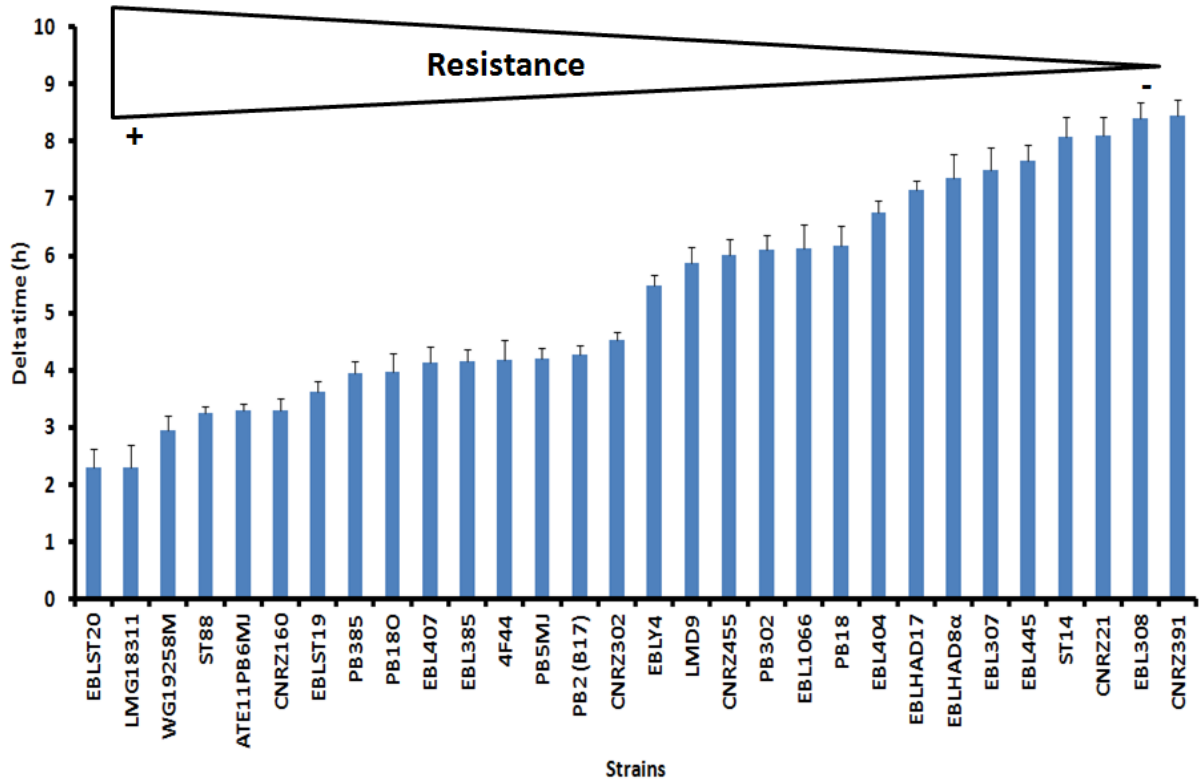

Fig. 2. Resistance of *S. thermophilus* strains against the bile salts (sodium cholate and sodium deoxycholate). The delta time allows evaluating the level of resistance to bile salts. It corresponds to the difference between the time taken by the Stressed Cells (SC) and the Non Stressed Cells (NSC) to attain the mid exponential growth phase. For each strain, the mean delta time ± SEM deduced from at least three independent experiments is shown.

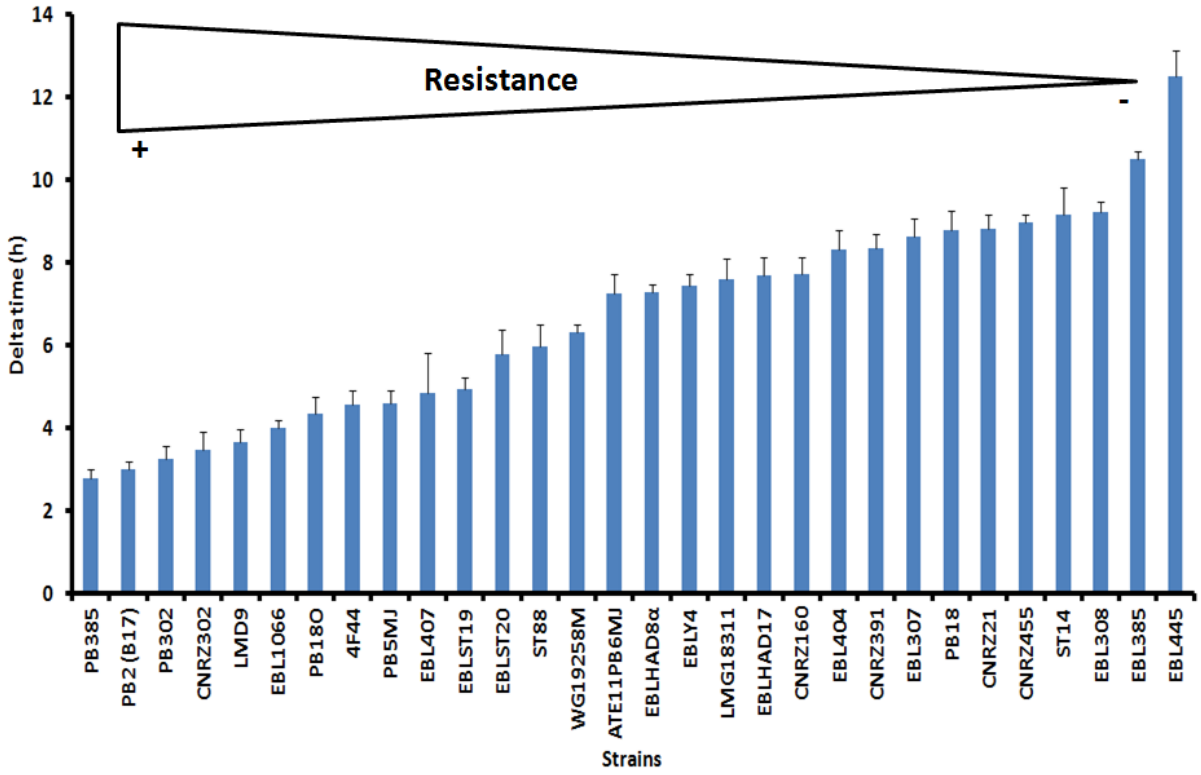

Fig. 3. Resistance of *S. thermophilus* strains against an oxidative stress (hydrogen peroxide). The delta time allows evaluating the level of resistance to the oxidative stress. It corresponds to the difference between the time taken by the Stressed Cells (SC) and the Non Stressed Cells (NSC) to attain the mid exponential growth phase. For each strain, the mean delta time ± SEM deduced from at least three independent experiments is shown.

1.3.2. Bacterial adhesion capacity

The adhesion capacity of the 30 *S. thermophilus* strains was determined using the mucin producing HT29-MTX cell line. *L. acidophilus* NCFM was used as a positive control. Except three strains (PB5MJ, CNRZ391 and CNRZ21) that were unable to adhere to the HT29-MTX cells, adhesion capacity was found to be highly variable between strains from less than 1 to about 16 % (Table 1, Fig. 4). Three groups could be clearly distinguished. The first contained 10 strains (strain PB385 to EBL1066) which displayed weak adhesion capacities ranging from less than 1% to about 3%. The second (from *L. acidophilus* NCFM to ST88) contained strains exhibiting the highest adhesion capacities ranging from 8 % to more than 16 %. Finally the third group (from strain CNRZ455 to PB18O) included the strains having intermediate adhesion capacities ranging between 3% and about 6%.

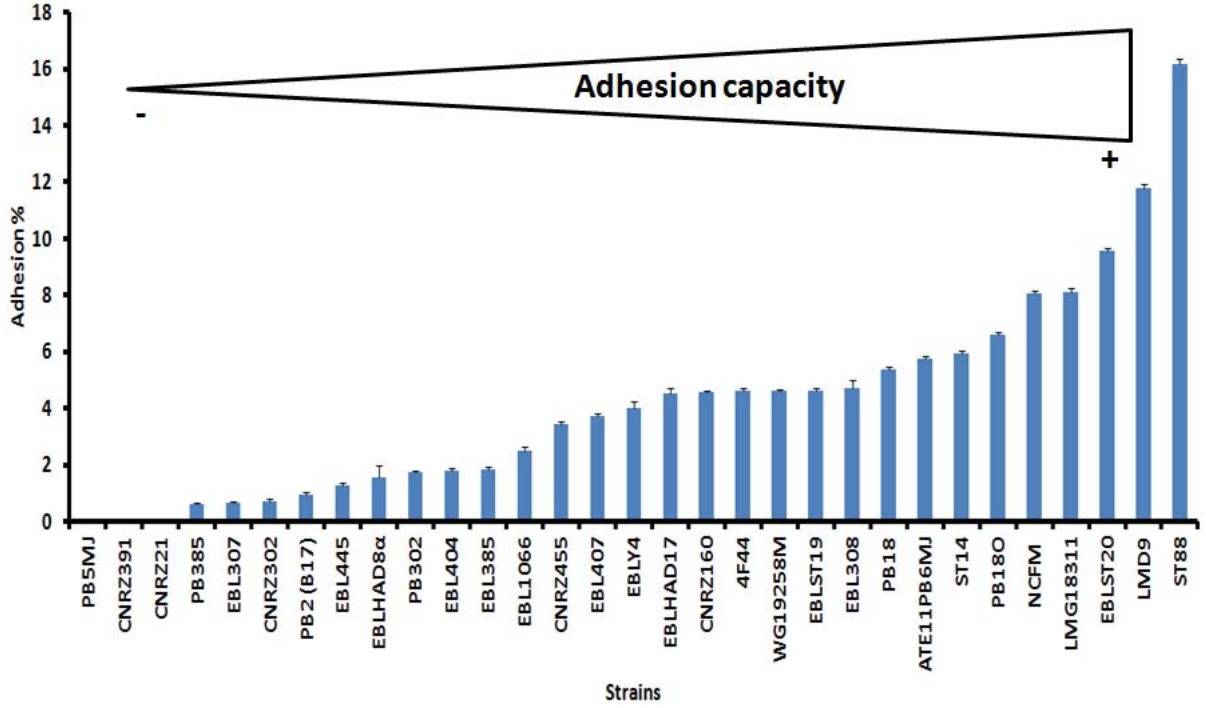

Fig. 4. Percentage of adhesion of the *S thermophilus* strains to HT29-MTX cells. For each strain, the mean ± SEM deduced from at least three independent experiments is shown.

1.3.3. Immunumodulatory properties of S. thermophilus

Immunomodulatory properties of the *S. thermophilus* strains were evaluated by quantifying cytokines produced after co-incubation of these bacterial cells with the cell line HT29 for IL-8 and PBMC for IL-10 and 12.

The effect of the 30 *S. thermophilus* strains on the secretion of the pro-inflammatory IL-8 was assayed with and without TNFα stimulation. Interleukin IL-8 was found to be secreted at concentration ranging from about 10 pg/ml to about 40 pg/ml, with 30 pg/ml obtained for the control consisting in co-incubate the HT29 cells with PBS (Fig. 5A). The majority of strains appeared to reduce IL-8 secretion. The most reducing strains EBLHAD17, EBLHAD8α, EBLST20 and ST14 were able to reduce IL-8 secretion induced by TNFα by 50%. In contrast, strains 4F44, ST88 and PB2(B17) were shown to increase IL-8 secretion, and the strain EBLY4 appeared to have no significant effect (Fig. 5A).

A distinct pattern for cytokines IL-10 and 12 was displayed by the 30 *S. thermophilus* strains (Fig. 5B and 5C). Concentrations of interleukins produced by PBMC cells varied between the strains from 900 to 2200 pg/ml for IL-10 (Fig. 5B), and from 5 pg/ml to 150

pg/ml for IL-12 (Fig. 5C). The determination of the IL-10/IL-12 ratio, commonly used to distinguish between strains exhibiting a pro-inflammatory (low ratio) or an anti-inflammatory (high ratio) profile (17), enabled us to distinguish three groups. The first, containing strains CNRZ302, EBL385 and PB18O, presented the lowest ratio whereas the second, containing strains CNRZ160, PB5MJ and CNRZ21 displayed the highest anti-inflammatory potential, with ratio values higher than 100. Finally, the third group containing the majority of the tested strains corresponded to strains with an intermediate ratio value of about 50 (Fig. 5D).

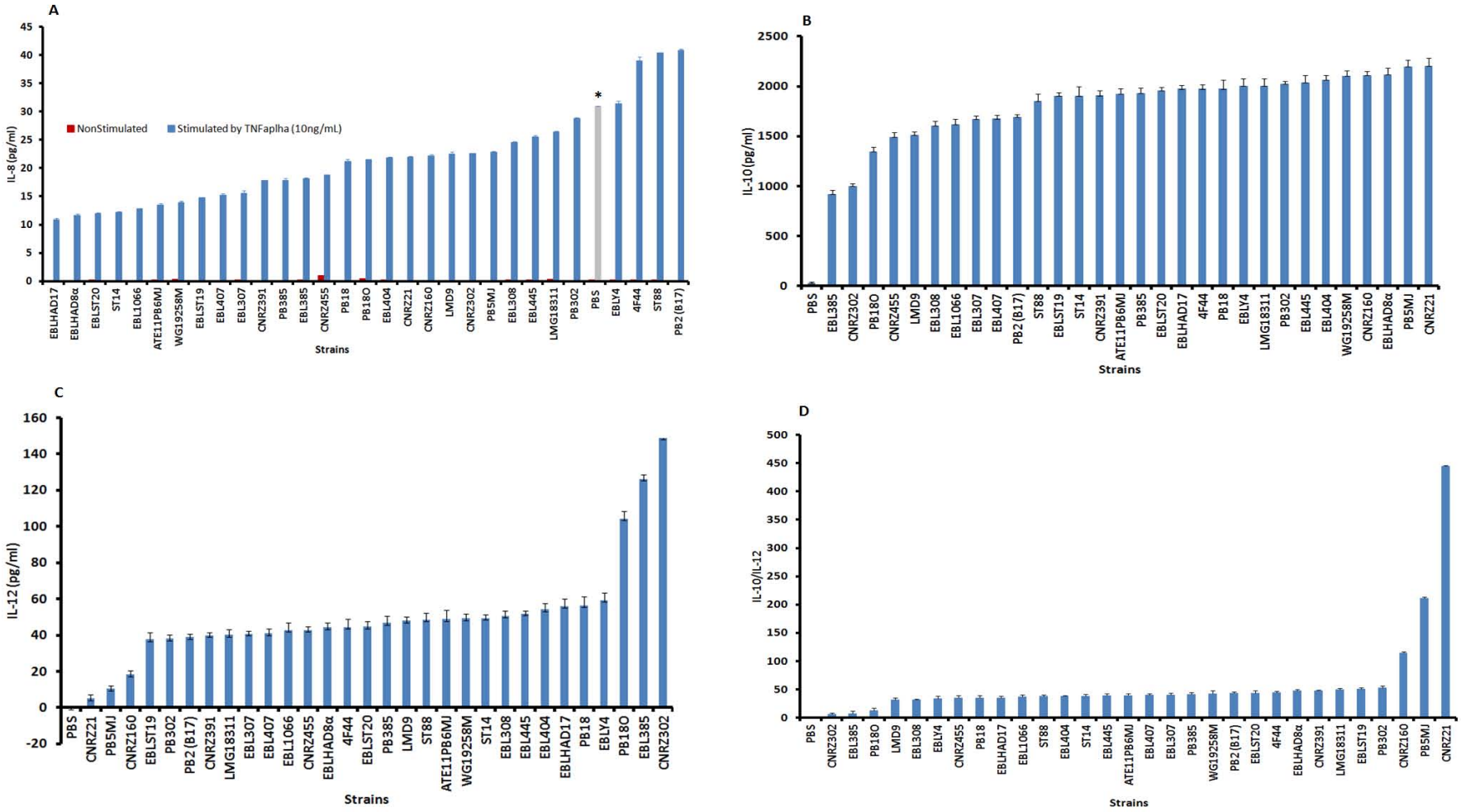

Fig. 5. Production of cytokines after co-incubation of the appropriate cells with different *S. thermophilus* strains. (A) production of IL-8 by the HT29 cells which were stimulated or not by TNFα (10ng/ml). (B) Production of IL-10 and (C) IL-12 by the human PBMC cells. (D) IL-10/IL-12 ratio. The values are expressed as means ± SEM.

1.3.4. Strain clustering by PCA analysis

Given the diversity of phenotypes observed in our strain collection, we determined whether they could be clustered into group displaying similar phenotypic properties by PCA analysis. Delta times obtained with stress pH 2, pH 4, H_2O_2, BS and the adhesion percentages were used as variables. For the statistical PCA analysis, the two first components contributed for about 59% of total variance and the third component for about 16%. With the hierarchical ascending classification, the 30 *S. thermophilus* strains could be grouped into 6 major classes showing a great inter-variability (Table 2, Fig. 6). Within each class, an additional intra-variability was also observed (Fig. 6B). Thus, the class 1 and 2 possessed low adhesion capacity and low resistance to bile salt and H_2O_2. Members of class 3 and 4 resisted well to bile salt and H_2O_2 and possessed a low capacity of adhesion, especially the members of the class 3 (capacity of adhesion of 1.12±0.84%). The three strains able to resist pH 2 constituted the class 6. They showed intermediate adhesion capacity (6-7%), a delta time of approximately 5 h when exposed to bile salts, as well as one of the higher level of resistance to H_2O_2 stress (delta time of about 3.7). Finally, the two strains of class 5, EBLST20 and ST88, resisted well to the gastric stresses and displayed the highest adhesion capacities (12.73±4.64%).

For the PCA analysis, interleukins (IL-8, IL-10 and IL-12) concentrations were used as illustrative variables for each class. Most of classes decreased the production of the pro-inflammatory interleukin IL-8 by HT29 cells stimulated by TNFα with respect to the control (stimulated cells incubated with PBS only) (Fig. 5A), while all of the classes significantly induced production of the anti-inflammatory cytokine IL-10 as compared to the control (Cells incubated with PBS only) (Fig. 5B), the members of the class 4 having the highest capacity to induce the production of IL-10 and the lowest capacity to induce the production of IL-12 (Table 2).

Table 2. Characteristics of the 6 deduced classes by PCA.

Classes (N= number of members)	Adhesion Capacity%	Stress Responses					IL-8 production by HT29 Stimulated by TNFα (pg/ml)	IL-10 production by PBMC (pg/ml)	IL-12 production by PBMC (pg/ml)
		Delta time between SC and NSC (h)				Time taken by NSC samples (h)			
		pH 2	pH 4	Bile Salt	Oxidative stress				
Class 1 (N=8)	2.17 ± 1.83	0	0.49 ± 0.21	6.59 ± 1.39	9.05 ± 1.71	5.30 ± 0.64	19.99 ± 6.11	1765.27 ± 398.26	57.68 ± 28.61
Class 2 (N=5)	3.38 ± 2.43	0	1.53 ± 0.56	7.69 ± 0.71	8.64 ± 0.63	7.31 ± 0.78	18.24 ± 6.27	1949.8 ± 223.28	43.00 ± 21.44
Class 3 (N=4)	1.12 ± 0.84	0	0.55 ± 0.37	4.72 ± 0.97	3.34 ± 0.57	5.27 ± 0.77	23.51 ± 12.2	1558.06 ± 396.54	69.31 ± 53.19
Class 4 (N=8)	4.46 ± 2.22	0	0.64 ± 0.23	3.50 ± 0.67	5.97 ± 1.40	7.04 ± 1.04	20.94 ± 8.7	1986.18 ± 160.22	36.20 ± 14.24
Class 5 (N=2)	12.73 ± 4.64	0	0.36 ± 0.08	2.77 ± 0.68	5.87 ± 0.11	5.78 ± 0.11	26.16 ± 20.06	1904.8 ± 74.64	46.65 ± 2.86
Class 6 (N=3)	6.64 ± 4.9	4.72 ± 0.19	0.51 ± 0.21	5.31 ± 1.16	3.7 ± 0.54	4.94 ± 0.07	24.22 ± 3.97	1627.78 ± 352.09	63.39 ± 35.70

All the values are expressed as mean ± SEM of all the member strains belonging to the same class. SC stressed cells, NSC non stressed cells. The interleukin secretion was quantified by using two different cell models i.e. IL-8 by HT29 cells and IL-10 and IL-12 by PBMC. For the tests of interleukin secretion incubation of cells with PBS was used as control.

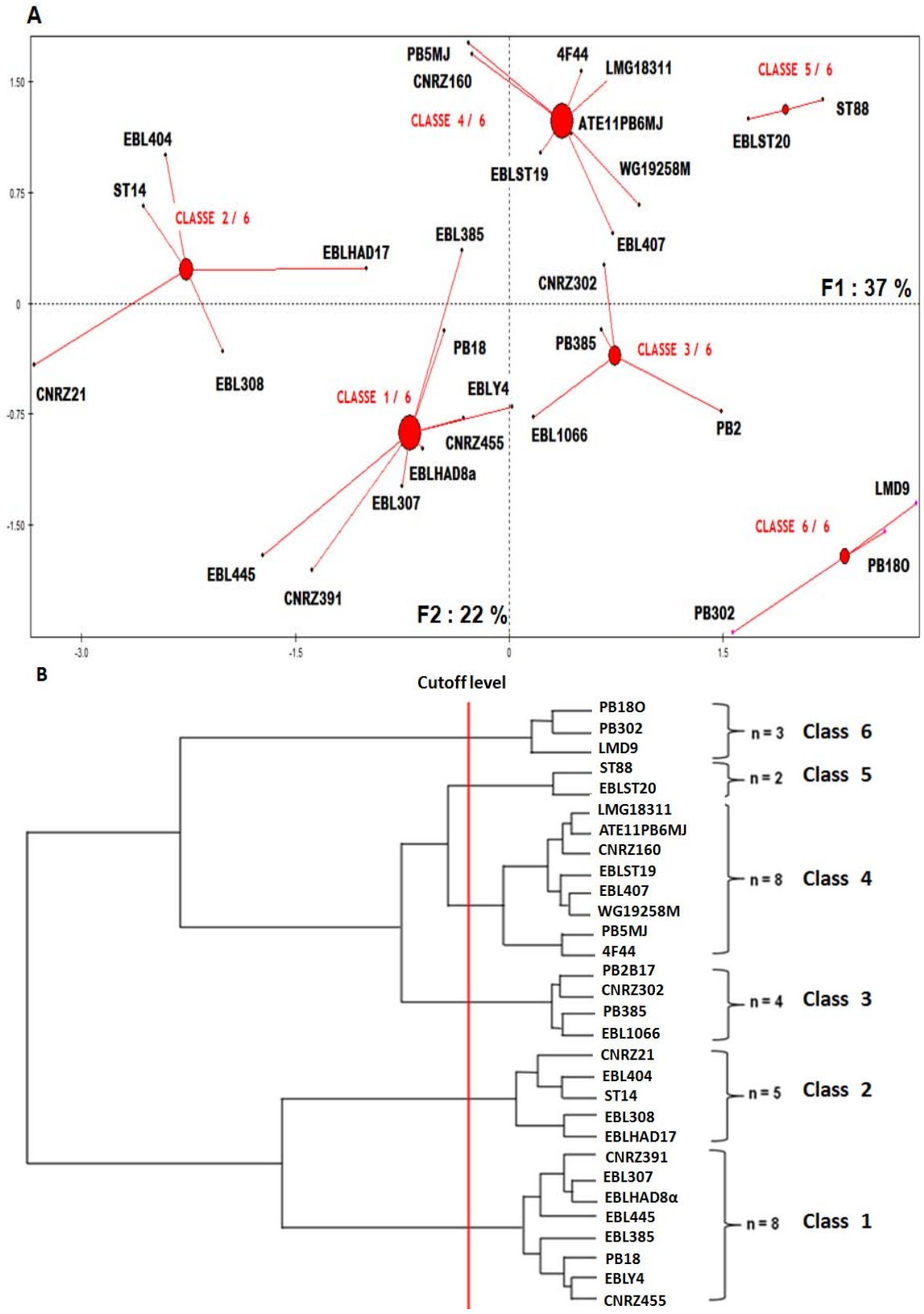

Fig. 6. Clustering of the thirty *S. thermophilus* strains used in this work into 6 classes on the basis of analysis of their probiotic properties. **(A)** Scattered plot generated through PCA (Principal Component Analysis). All the tested strains are divided into 6 classes on the basis of their resistance to pH=2, pH=4, H_20_2, bile salts and their adhesion capacities. **(B)** Dendrogram illustrating the similarities between the strains, in response to gastric stresses and adhesion to intestinal epithelial cells using the PCA.

1.4. Discussion

The needs of new probiotic candidates have drawn a lot of attention of scientists during the last few decades. The main criteria for selection of the probiotic strains include resistance to stresses encountered in the GIT and beneficial impacts on the health of the host. As several LAB used in food manufacture are also labeled as probiotics, we have investigated the potential probiotic properties of *S. thermophilus*, a LAB which is widely consumed as present in yogurt, considered as a functional food. Thirty *S. thermophilus* strains of various food origins were thus screened for their resistance to different stresses usually encountered in the GIT and for their adhesion and immunomodulatory properties.

The resistance of the 30 *S. thermophilus* strains to acidic environment was determined at pH 2 and 4. pH 2 corresponds to very stressful condition for bacteria but realistic since the pH in the stomach can be as low as 1.9 during interprandial phases (18). pH 4 corresponds to the pH the most frequently used in the *in vitro* experiments, where resistance of strains is usually checked against the pH 3 and 4 (19, 20). Indeed, when consumed the probiotics are generally included in a food matrix which buffers the pH and protects them against direct exposure to the extreme pH conditions of the stomach (21). In this study, we revealed that if all of the 30 strains were able to survive after a pH4 60 min incubation, only three (PB18O, PB302 and LMD-9) were able to resist at pH 2. Similar observations have been made for other LAB where no viable cells were obtained even after 30 min of pH stress (20, 22). Furthermore, our results are in harmony with the results already obtained (23) which showed that the survival rate of *S. thermophilus* decreased from 80% to 20% when pH decreased from 4 to 1.8. The fact that all of the strains tested in this work resist to pH 4 suggest that they may resist to the stomach conditions when included in a food matrix like yogurt, and be delivered alive in the small intestine.

Tolerance to the bile salts appears to be the most important factor for probiotic selection, since this capacity enables them to survive and exert their beneficial effect (2). Bile is a complex secretion consisting of water (85%), bile salts (10%), mucus and pigments (3%), fats (1%), inorganic salts (0.7%) and cholesterol (0.3%). Cholate and deoxycholate represent the most abundant bile salts justifying their use in this study at a final equimolar concentration of 0.01%. The mechanism of resistance to bile salts has been attributed in certain cases to the membrane proteins that can either take up or expulse the bile salts (24-28) or to enzymes transforming conjugated bile salts to disconjugated or primary to secondary bile salts (29).

Highly reactive free radicals and oxygen species of various origins are present in the biological systems and may oxidize nucleic acids, proteins or lipids and trigger degenerative diseases. Antioxidants scavenge free radicals such as peroxide, hydroperoxide and thus may inhibit the oxidative mechanisms that lead to the degenerative diseases. To test the antioxidant capacity of our 30 strains, we used hydrogen peroxide at the concentration of 10mM. Most of them resisted to this the stress with a delta time ranging from 2 to 9 h. *S. thermophilus* is an anaerobic aerotolerant microorganism, capable of growing in the presence of oxygen and surviving at low concentrations of superoxide and hydroxyl radicals (30). Among the various factors enabling it to withstand oxidative stress (31), *S. thermophilus* is known to accumulate a Mn-superoxide dismutase during the stationary phase rendering it more resistance to oxidative stresses (32, 33). A high variability in the level of resistance of the strains against bile salt and oxidative stress was display among the strains. The fact that most of them display a delta time of less than or equal to 5h when exposed to bile salts, suggests that they could resist in the small intestine.

Adhesion capacity of a probiotic to intestinal epithelium is regarded as the prerequisite, allowing possible colonization of the intestinal tract. It is very important to determine the resident time of a bacterium in the human gut in order to ensure its probiotic effects and its influence on immune system (3). Several studies had been conducted *in vitro* to evaluate the adhesion capacity of putative probiotic bacteria to the intestinal epithelial interface and their interactions with pathogens (34, 35). Different cell culture models like HT29 cells, Caco-2 cells and HT29-MTX have been used, the HT29-MTX cell line providing the most realistic human intestinal conditions (36, 37). The 30 *S. thermophilus* strains tested in this work displayed varying degrees of adhesion, which can be partially explained by the interaction of these bacteria with the mucin glycoprotein but also with other proteins located in the cell membrane of mature enterocytes (38) Different cell wall components have been identified as determinants of the bacterial adhesion capacity, such as mucopolysaccharides (39), carbohydrate capsule polymers (40), or the combinations of carbohydrates and proteinaceous factors (41). Recent studies have proved that the exopolysaccharide (EPS) production could be involved in the viability, adherence and competition of lactic acid bacteria, especially in Lactobacillus species (42), and *S. thermophilus* is known to produce different EPSs (43, 44). Finally, the aggregation capacities might also influence the bacterial adhesion capacity (45, 46) and their variation among the *S. thermophilus* strains tested here could also contribute to the adhesion variation observed.

Participation in the regulation of the immune system is also another important criterion to gain a probiotic acknowledgment. Cytokines are secreted by numerous cells and constitute signaling molecules which play a vital role in the intercellular communication. They play a vital role in the immuno-regulation. For the quantification of the central pro-inflammatory cytokine IL-8, the HT29 cells were co-incubated with *S. thermophilus* strains with or without TNFα which triggers the production of IL-8 (47). This cytokine is involved in systemic inflammation and stimulates the acute phase reaction. Most of the *S. thermophilus* strains (26/30) appeared to be able to reduce this IL-8 production, 6 out of the 30 strains (EBLHAD17, EBLHAD8α, EBLST20, ST14, EBL1066 and ATE11PB6MJ) dramatically decreasing this production (Fig. 5A). This property has been also observed in S. salivarius, a species phylogenetically closed from *S. thermophilus* (48), or in Lactobacillus and Bifidobacterium species (49). Further, if most of the strains tested here appeared to upregulate the production of the interleukin IL-10 (anti-inflammatory) and of IL-12 (pro-inflammatory), the ratio IL-10/IL-12 which can be used to evaluate the anti-inflammatory potential of a probiotic showed that 3 of them (CNRZ160, PB5MJ and CNRZ21) had the highest potential. Indeed, these three strains induced the highest levels of IL-10 as well as the lowest levels of IL-12 production (Fig. 5B and 5C). Although it should be very careful since this bacterium has not been included in our tests, the ratio IL10/IL12 observed for the strains CNRZ160, PB5MJ and CNRZ21 appeared to be similar or higher than that reported for *Faecalibacterium prausnitzii,* a commensal bacterium having one of the best anti-inflammatory properties (17). Knowing that there is a good correlation between the cytokine profile released by PBMCs (50) and *in vivo* immunomodulatory potential of bacteria, the results obtained with these three strains are promising.

Although the *S. thermophilus* species displays a weak genetic variability (51), a high phenotypic variability has been observed in our study. The data obtained were thus subjected to a PCA which allowed to cluster the *S. thermophilus* 30 strains into 6 classes which mainly depended from the resistance to gastric stresses and their adhesion capacity to the intestinal cells. Within each class, interesting characteristics could be identified. For example, members of the class 5 exhibit a high resistance profile to gastric stresses as well as the highest adhesion capacities, while members of the class 6 were able to resist to pH 2. The strains CNRZ160, PB5MJ and CNRZ21 which displayed a high anti-inflammatory profile *in vitro* belonged to the class 4 (for the strains CNRZ160 and PB5MJ) and 2 (strain CNRZ21). Thus, strain CNRZ21 belonged to the class which exhibited the lowest capacities to resist gastric

stresses and to adhere to enterocytes. Consequently, its use, if its probiotic potential was confirmed *in vivo*, implied to increase its resistance index. As a more exhaustive work, it would be now interesting to analyze the gene pool or the allele combination of each class to identify genes which could be responsible of the distinct properties observed.

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2. Développement de la Recombinase-based *In vivo* Expression Technology (R-IVET) chez *Streptococcus thermophilus* et validation dans le tractus gastro-intestinal des souris en utilisant le promoteur de l'opéron lactose

Développement de la Recombinase-based *In vivo E*xpression Technology (R-IVET) chez *Streptococcus thermophilus* et validation dans le tractus gastro-intestinal des souris en utilisant le promoteur de l'opéron lactose

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Résumé

Objectifs: Le but de ce travail était de construire un outil basé sur la technologie R-IVET (Recombinase-based *In vivo* Expression Technology) chez *Streptococcus thermophilus* et de le valider en évaluant l'activité du promoteur de l'opéron lactose des bactéries après un transit dans le tractus gastro-intestinal (TGI) de la souris.

Méthodes et Résultats: La technologie R-IVET mise au point chez *S. thermophilus* se compose de deux éléments : un vecteur plasmidique portant le gène codant une recombinase site-spécifique sans son promoteur (*cre*) et une cassette chromosomique comportant un gène de résistance à la spectinomycine flanqué par les sites *loxP,* qui sont reconnus par la recombinase Cre. L'induction d'une séquence promotrice clonée en amont du gène *cre* conduit à l'expression et l'excision de la cassette chromosomique qui rend la cellule bactérienne sensible à la spectinomycine. Dans cette étude, nous avons construit le plasmide pULNcreB portant *cre*. Ce plasmide a été introduit par transformation naturelle dans la souche recombinante de *S. thermophilus* STUL5001 qui correspond à la souche LMD-9 portant la cassette chromosomique *loxP-SpecR-loxP*. La fonctionnalité de l'outil R-IVET a ensuite été testée en clonant trois promoteurs différents de *S. thermophilus* (P*prtS*, P*shsp* and P*lac*) en amont de *cre* et en déterminant si la cassette chromosomique était excisée ou non selon différentes conditions expérimentales.

Conclusions: L'outil R-IVET que nous avons développé chez *S. thermophilus* est stable et pleinement fonctionnel. Cela a été validé *in vitro* avec trois promoteurs différents lorsque les cellules étaient cultivées en milieu M17 et avec le promoteur de l'opéron lac (P*lac*) lorsque les cellules bactériennes avaient transité par le TGI d'une souris.

Importance et impact de l'étude: Ce travail est la première adaptation du R-IVET chez *S. thermophilus*. Il peut maintenant être utilisé pour identifier les gènes qui sont activés dans de nombreux environnements complexes. Cela fournit un outil très précieux de transcriptomique permettant une exploration de l'état physiologique de *S. thermophilus* lorsque les cellules sont dans des niches complexes comme le TGI de mammifères, les procédés de fermentation ou dans les produits laitiers.

Mots-clés: *Streptococcus thermophilus*, Recombinase-based *In vivo* Expression Technology, site-spécifique recombinase, promoteur, lactose opéron

Development of the Recombinase-based *In vivo* Expression Technology (R-IVET) in *Streptococcus thermophilus* and validation in the gastrointestinal tract of mice using the lactose operon promoter

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Abstract

Aims: The aim of this work was to construct and validate the Recombinase-based *in vivo* Expression Technology (R-IVET) tool in *Streptococcus thermophilus* (ST).

Methods and Results: The transcriptional fusion plasmid vector pULNcreB was constructed with the promoterless *cre* gene (‘*cre*), encoding a site-specific recombinase (Cre). The R-IVET cassette containing a spectinomycin resistance marker gene flanked by the *loxP* sites was generated by overlapping PCR and introduced into the chromosome of the LMD-9 strain by natural transformation. To test its functionality, the R-IVET tool was assayed with the three different streptococcal promoters P*prtS,* P*hsp* and P*lac* by monitoring the spectinomycin resistance phenotype.

Conclusions: The R-IVET tool developed in ST is stable and fully functional. We showed that the promoter of the lactose operon was induced during the transit in the mouse Gastro-Intestinal Tract (GIT).

Significance and impact of the study: This first adaptation of R-IVET to ST provides a highly valuable tool allowing an exploration of the physiological state of ST when the cells are present in the GIT of mammals, fermentation processes or dairy products.

Keywords: *Streptococcus thermophilus* (ST), Recombinase-based *In vivo* Expression Technology, promoter, lactose operon

2.1. Introduction

Streptococcus thermophilus is a lactic acid bacterium widely used as a starter in the dairy industry. It is traditionally used in combination with other lactic acid bacteria like *Lactobacillus delbrueckii* subsp. *bulgaricus* in yogurt or *Lactobacillus helveticus* in hard cheeses (Fox 1993; Tamime and Deeth 1980). It is phylogenetically related to pathogenic streptococcal species like *S. pneumonia, S. pyogenes* or *S. mutans* causing pneumonia, meningitis or dental cavities, respectively, and is most closely related to the commensal species *S. salivarius* (Mitchell 2003; Tettelin 2004). However, ST has never been associated with pathogenic incidences, and combined with the very long history of use in food industry, led to the GRAS (Generally Recognized As Safe) status assignment by the American Food and Drug Administration and the QPS (Qualified Presumption of Safety) status by the European EFSA (European Food Safety Authority) to the species ST. Comparative genomic analysis of the first two sequenced ST strains (http://www.ncbi.nlm.nih.gov/genome) with their pathogenic relatives highlighted that the genes related to virulence were either absent or present as pseudogenes (Bolotin *et al.* 2004). This comparative genomic analysis also revealed that horizontal gene transfer has played an important role in the evolution of ST rendering it more adapted to milk.

In contrast to other lactic acid bacteria like some lactobacilli, the probiotic status of ST is still questioned (Guarner *et al.* 2005). Indeed, about two decades ago this bacterium was still documented to be unable to survive in the gastro-intestinal tract (GIT). However, studies using more specific and sensitive detection methods revealed the presence of ST in feces of consumers (Brigidi *et al.* 2003, Mater *et al.* 2005, Elli *et al.* 2006). Moreover, metagenomic sequencing performed on fecal specimen of 124 Europeans had shown the presence of ST DNA in the feces of 90 % of the individuals (Qin *et al.* 2010). Several studies have proposed ST to have some probiotic potential (for review see Guarner *et al.* 2005). But, the experimental evidence for probiotic properties of this species are currently restricted to animal

models or *in vitro* cultures ie, intestinal β-galactosidase activity in mouse models (Droualt *et al*. 2002, Mater *et al*. 2006), capacity to synthesize antimicrobial compounds (Kabuki *et al.* 2011), adhesion to epithelial cells (Fernández de Palencia *et al.* 2008), action as an antioxidant (Ito *et al.* 2003), stimulation of immunemodulatory responses (Kekkonen 2008). Even if human studies with ST as a probiotic are still quite limited to date, ST represents an interesting species for the development of probiotic products with specific health benefits. Before ST strains can be credited with potential probiotic allegations, further studies must be performed to better decipher their physiology in the GIT of humans and their aptitude to exert probiotic functions *in situ*.

The complete genome sequence of 6 strains of ST provides a rich source of information from which genes expressed in the GIT can be discovered. The use of post-genomics technologies combined with appropriate animal models and *in vitro* human systems will ultimately allow the understanding of the complex interactions of the bacterial cells with the human host cells and with the rest of the microbiota (de Vos *et al.* 2004). A comprehensive investigation of the physiological state of ST when passing through the GIT of gnotobiotic rats has already been performed by proteomic analysis (Rul *et al.* 2011; Thomas *et al.* 2011), revealing a massive use of the glycolysis pathway. In order to identify genes which are activated when ST transits through the GIT, we aimed to evaluate transcriptional responses in situ by performing the elegant genetic screening provided by Recombinase-based *In vivo* Expression Technology (R-IVET).

R-IVET is a promoter-trapping technology devised to identify genes which are activated in particular complex environments like infected tissues, GIT, fermented food or soils (Rediers *et al.* 2005). It is based on a transcriptional fusion between a promoterless recombinase-gene and random genomic DNA fragments which are tested for their promoter functionality (Camilli *et al.* 1994). R-IVET systems are generally composed of two different elements: the recombinase transcriptional fusion located on a plasmid, and the chromosomal target module that contains one or several reporter genes which are deleted by the action of the recombinase produced upon the transcription of the trapped promoter. As a result of a promoter induction, the bacterial cell is irreversibly labeled and can be analyzed at any later collection time. R-IVET systems, initially developed to reveal virulence functions induced when pathogenic bacteria infected host tissues (Angelichio and Camilli 2002), have been applied to lactic acid bacteria in order to detect genes which are active in their natural niches, in the GIT of mice (Bron *et al.* 2004) or in fermented dairy environment (Bachmann *et al.*

2010). This system applied to *Lactobacillus plantarum* led to the identification of 72 genes specifically induced in the mouse GIT (Bron *et al.* 2004). An alternative valuable utilization of the R-IVET system is the transcription monitoring of a selected gene as a function of time in a specific environment (Angelichio and Camilli 2002).

In the present study, we describe the construction of the two genetic components of the R-IVET system in the *S. thermophilus* strain LMD-9. After testing the stability of the genetic tool, we investigated its functionality by using three different *S. thermophilus* promoters (P*prtS,* P*hsp* and P*lac*) and analyzed whether the chromosomal antibiotic cassette could be deleted under different growth conditions in M17 medium. As a proof of concept for a subsequent use in complex environments, the R-IVET system containing the promoter of the *lac* operon (P*lac*) was tested in the GIT of mice.

2.2. Material and Methods

2.2.1. Bacterial strains, media, growth and transformation conditions

The strains used in this work are listed in Table 1. The *S. thermophilus* strain used to implement the R-IVET system is LMD-9 from the American Type Culture Collection (Manassas, VA, USA). *S. thermophilus* strains were grown in anaerobic conditions (AnaeroGen, oxoid, Basingstoke, UK) at 42°C in M17 medium (Terzaghi and Sandine 1975) supplemented with 2% (w/v) lactose (LM17) or 0.5% (w/v) glucose (GM17). When needed, antibiotics purchased from Sigma (Saint-Quentin Fallavier, France) were added to the media at the concentration of 300 µg ml^{-1} for spectinomycin or 5 µg ml^{-1} for erythromycin. Growth kinetics of *S. thermophilus* strains were monitored using the BIOSCREEN C Microbiology Reader (Thermo Fisher Scientific, France). Wells were loaded with 350 µl of culture adjusted to an $O.D._{600nm}$ value of 0.05 and covered with 50 µl of oil to reduce the oxygen tension. Values of $O.D._{600nm}$ were recorded every 15 min for 6 h until reaching the stationary phase. Generation times were calculated as log(2)/slope. Growth kinetics of three independent cultures was assayed for each strain. Data points represent average values and standard deviations of measurements from three independent cultures.

Table 1. Bacterial strains and plasmids used in this study

Strains	Relevant markers and characteristics	Reference or source
Streptococcus thermophilus		
LMD-9	Wild type strain for which genome sequence is available	ATCC BAA-491
STUL5001	$Spec^R$; LMD-9 derivative containing the *loxP-specR-loxP* cassette in the STER_0891 locus	This study
Escherichia coli		
TOP10	One Shot® TOP10 Chemically Competent *E. coli* cells	Invitrogen
Plasmids		
pSET4s	$Spec^R$; replication function of pG^+host3 and pUC19, *lacZ' spec*^{R}	Takamatsu *et al.,* 2001
pNZ5520	Cm^R; pNZ18 derivative containing a linker with an *Bgl*II restriction site upstream of '*cre*	Bachmann *et al.,* 2008
pG^+host9	Em^R; pWV01 derivative with thermosensitive replication function	Maguin *et al.,* 1996
pULNcreA	Em^r; pG^+host9^{TR} derivative containing the promoterless *cre* gene with the *las* transcriptional terminator (T*las*) upstream of *cre*, with thermoresistant replication function	This study
pULNcreB	pULNcreA with the *pepN* transcriptional terminator downstream of '*cre*	This study
pULNcreB-Ps*hsp*	pULNcreB derivative containing Ps*hsp* cloned in the *Bgl*II site upstream of '*cre*	This study
pULNcreB-P*lac*	pULNcreB derivative containing P*lac* cloned in the *Bgl*II site upstream of '*cre*	This study
pULNcreB-P*prtS*	pULNcreB derivative containing P*prtS* cloned in the *Bgl*II site upstream of *cre*	This study

Competent cells of *S. thermophilus* LMD-9 were prepared by growing them in a chemically defined medium (CDM) described by Letort and Juillard (2001) and containing only amino acids as nitrogen source. Cells were grown overnight at 42°C in CDM after which the culture was diluted in fresh CDM and further grown to a final $O.D._{600nm}$ of 0.2. The competent cells were either transformed immediately or stored at -80°C for a subsequent use (Blomqvist *et al.* 2006; Fontaine *et al.* 2010; Gardan *et al.* 2009). When used immediately, 250-500 ng of plasmidic DNA or 20-50 ng of linear DNA were added to one ml of the culture

and incubated at 42°C for 1 h. Transformed cells were then recovered by centrifugation at 8000 g at room temperature for 5 min resuspended in 200 µl of MCD and plated onto the solid M17 medium containing the appropriate antibiotic. When competent cells were used later, the culture grown at $O.D._{600nm}$ of 0.2 was centrifuged at 8000 g at room temperature during 5 minutes and resuspended in a solution (1/10 volume of the initial culture) of MCD and 14% glycerol. Competent cells were then aliquoted and frozen in liquid nitrogen.

Escherichia coli strain TOP10 was used as an intermediate cloning host for the construction of recombinant plasmids. Chemically competent TOP10 cells purchased from Invitrogen (Breda, the Netherlands) were transformed according to the manufacturer's instructions. *E. coli* strains were grown at 37°C aerobically in LB medium (Sambrook and Russell 2001). When needed, erythromycin was added to the medium at the concentration of 400 μg ml^{-1}.

2.2.2. Animal experiments

Mouse experiments were performed according to the guidelines N°86/609/CEE of the French Government. Six-week-old C57BL/6J and 18-week-old Swiss mice were used for the assessment of the R-IVET tool *in vivo* stability and for the lactose operon promoter (Plac) experiment, respectively. Mice had free access to standard mouse chow and water. Mice were checked for their erythromycin sensitive fecal flora before the intragastric administration of the *S. thermophilus* R-IVET cells. *S. thermophilus* cells used for mouse gavage were prepared as follows: after an overnight culture in M17, cells were pelleted by centrifugation and suspended at the concentration of 5.109 $CFU.ml^{-1}$ with M17 medium. Each mouse was inoculated by intragastric gavage with a 200 µl dose (ca. 109 CFU) of the freshly prepared bacterial suspension. For the Plac experiment, mice were given water supplemented with lactose at the concentration of 5% (w/v) during 20 h before the *S. thermophilus* administration and until the end of feces collection. This was performed in order to induce the activity of the lactose operon and to increase *S. thermophilus* survival in the mouse GIT (Mater et al. 2006). Fresh fecal samples were collected, immediately homogenized in phosphate buffer and then, appropriate serial dilutions were spread on LM17 or GM17 agar supplemented either with erythromycin or spectinomycin.

2.2.3. DNA extraction, cloning and sequence analysis

Plasmid DNA was isolated from *E. coli* cells with a Miniprep Kit (Fermentas, Villebon sur Yvette, France) according to the manufacturer's instructions. *S. thermophilus* genomic DNA was extracted from 50 ml overnight LM17 culture according to Fischer and coworkers (Fischer *et al.* 1997) and was used as target DNA for PCR.

DNA fragments were separated by electrophoresis in agarose gels using 0.5x TBE buffer at 100V. 1 kb and 100 bp DNA ladders (Fermentas, Villebon sur Yvette, France) were used as molecular weight markers. Gels were stained with ethidium bromide and imaged using a GelDoc-It Imaging System (Biorad, Marne-la-Coquette, France).

Restriction endonucleases (Fermentas, Villebon sur Yvette, France), the T4 DNA ligase and the calf intestinal alkaline phosphatase (CIAP) (New England Biolabs, Leusden, The Netherlands) were used according to the manufacturer's recommendations.

DNA cloning was carried out using standard procedures as described in Sambrook and Russel, 2001. DNA fragments used for the ligation reactions were purified with the High Pure DNA purification Kit purchased from Roche (Roche Molecular Biochemicals, Mannheim, Germany) before and after endonuclease restriction. 10 µl final volume ligation reactions were performed at 16°C overnight with 50 ng of digested and CIAP dephosphorylated vector and 15 ng of inserts (ratio of 5 copies of insert for 1 copy of vector).

DNA sequences were determined from PCR products on both strands by the company Beckman Coulter Cogenics (Takeley, United Kingdom). Sequence comparisons and alignments were performed with the Bioedit software (free access, http://www.mbio.ncsu.edu/BioEdit/page2.html) using the ClustalW algorithm.

2.2.4. Standard PCR amplifications

PCR were carried out in a Mastercycler pro thermocycler (Eppendorf, Hambourg, Germany). Oligonucleotides used as primers were purchased from Eurogentec (Serain, Belgium) and their sequences are described in Table 2.

Analytical PCRs were done in 20 μl containing 0.5 units of *Taq* DNA polymerase (Fermentas, Villebon sur Yvette, France), 200 mM of each dNTP, 25 mM of each primer, 2 μl of the 10x concentrated Fermentas buffer, 1.5 mM $MgCl_2$ and 2 μl of colony lysate obtained

by heating colony cells during 3 min in a microwave oven at maximum power or 50 ng of purified DNA. Temperature cycle conditions were as follows: 3 min of denaturation at 95°C; 30 cycles of three steps of 30 s each (denaturation step at 95°C, annealing step at 54°C and extension step at 72°C); and a final extension at 72°C for 10 min.

Preparative PCR were performed to produce DNA fragments intended for subsequent cloning or sequencing reactions. PCR were performed in a 50 μl final volume with 2.5 units of *AccuTaq* polymerase (Sigma-Aldrich, California, USA) using the analytical PCR cycle conditions but with extension steps carried out at 68°C.

Table 2. Oligonucleotides used in this study with their relevant characteristics

#	Sequence 5' to 3'	Characteristics
1	ATCCGTAAAACCATCAAATCTTAATT	Construction of the floxed specR cassette, 3616-bp amplicon with #4 for the undeleted cassette or 2382 bp for the deleted cassette
2	TGACTCCCCGTCGTGTAGATAACTAG	Amplification of the spectinomycin resistance gene from pSET4S with #3 (1201 bp)
3	CGCTACGATAACGCCTGTTT	Used with #2
4	TTAAACCAATCTGTCCTCGGG	Used with #1
5	ATAATAACTAGTGAGAGGCCGCCACGGCGATGTGC	*Spe*I, construction of pULNcreA, 1457-bp amplicon with #6
6	ATAATAGTCGACGCTTCTGGAGCTCAATTGAAAG	*Sal*I, used with #5
7	ATATATGGTACCTCGCTTTGATTGTTCTATCG	*Kpn*I, construction of pULNcreB, 159-bp amplicon with #8
8	ATAATAGTCGACAAGCAGCAGTTGATAAAGC	*Sal*I, used with #7
9	CCATTTTGAACGATGACCTC	Confirmation of *creB* presence, 2041-bp amplicon with #10
10	GACAGCTTCCAAGGAGCTAA	Used with #9
11	ATAATAAGATCTTCATGAATGTCTTGCTCACTCC	*Bgl*II, construction of pULNcreB-P*prtS*, 211-bp amplicon with #12
12	ATAATAAGATCTTCCTCCTAGTTTTTCTCCTT	*Bgl*II, used with #11
13	ATAATAAGATCTGGACGAACTAAAATAGTGACG	*Bgl*II, construction of pULNcreB-P*shsp*, 171-bp amplicon with #14
14	ATAATAAGATCTCATAATAATCACTCCTTTC	*Bgl*II, used with #13
15	ATAATAAGATCTACGATGGATGATATCGAAG	*Bgl*II, construction of pULNcreB-P*lac*, 324-bp amplicon with #16
16	ATAATAAGATCTTTCGGAAACCTCCTATTATTTG	*Bgl*II, used with #15
17	ACACAAAGTCGCTGTTCTCG	Sequencing of the R-IVET cassette
18	TGCAAGTAAAATTGCACCTGTT	Sequencing of the R-IVET cassette
19	ATCTTCCGCTTTAGTTTTTGGC	Sequencing of the R-IVET cassette
20	CCATGGCAATTATTTGGTTACG	Sequencing of the R-IVET cassette
21	TGGTACCGTGGAATCATCCT	Confirmation of *specR* presence, 361-bp amplicon with #22
22	GGAGAAGATTCAGCCACTGC	Used with #21
23	AAACAGGCGTTATCGTAGCG***ATAACTTCGTATAGCATACATTATACGAAGTTAT***TAATGTTTGGTGTAGGTAATA	
24	TAGTTATCTACACGACGGGGAGTCA***ATAACTTCGTATAATGTATGCTATACGAAGTTAT***TCCCCAAGCAAAAATTGGA	

[a] Underlined sequences indicate restriction sites used in cloning procedures. Sequence in bold and italics is the sequence of *loxP* sites

2.2.5. Construction of the floxed spectinomycin resistance fragment for chromosomal integration

The strategy used to construct the reporter *S. thermophilus* STUL5001 containing the floxed specR gene in the chromosome of the LMD-9 strain is presented in Fig.1. In the first series of PCR (Fig. 1.A), the three fragments UP-loxP, specR, and loxP-DN fragments were amplified individually with primer couples #1#24, #2#3 and #23#4, respectively. Each reaction was performed in a final volume of 50 µl with 1 U of Phusion High Fidelity DNA polymerase, 10 µl of Phusion 5x buffer (Finnzymes, Thermo Scientific, Finland), 200 µM of each dNTP, 0.5 µM of each primer and 100 ng of template DNA. Genomic DNA of the strain LMD-9 was used as template to amplify the UP-loxP and the loxP-DN fragments. The plasmid pSET4S (Table 1, Takamatsu et al. 2001) was used to amplify the specR gene encoding a spectinomycin adenyltransferase. For the three reactions, c12ycling conditions were 1 min of initial denaturation at 98°C; 30 cycles of three steps (20 s denaturation at 98°C, 30 s annealing and 30 s extension at 72°C) and a final extension at 72°C for 10 min. Specific annealing temperatures were set at 41.4°C, 60°C and 46.8°C for the amplifications of the three fragments UP-loxP, specR, and loxP-DN, respectively.

In the second amplification phase (Fig 1.B), an overlapping PCR was carried out with primers #1#4 using the three fragments UP-loxP, specR and loxP-DN mixed together in equimolar concentration. Cycling conditions were 1 min of initial denaturation at 98°C; 50 cycles of three steps (20 s of denaturation at 98°C, 30 s of annealing and 2 min of extension at 72°C); and a final extension at 72°C for 10 min. A touchdown PCR was applied for the first 20 cycles with a 1°C decrease per cycle from 60°C to 40°C followed by a constant 42°C annealing temperature for the last 30 cycles. Thirty ng of the resulting amplified fragments were used for the transformation of LMD-9 competent cells. A spectinomycin resistant clone confirmed to have the cassette at the STER_0891 locus by colony PCR with primers #1#4 was chosen (STUL5001). Its floxed specR cassette and junction regions with the chromosome were sequenced to check that no mutation had occurred during the construction of the mutant.

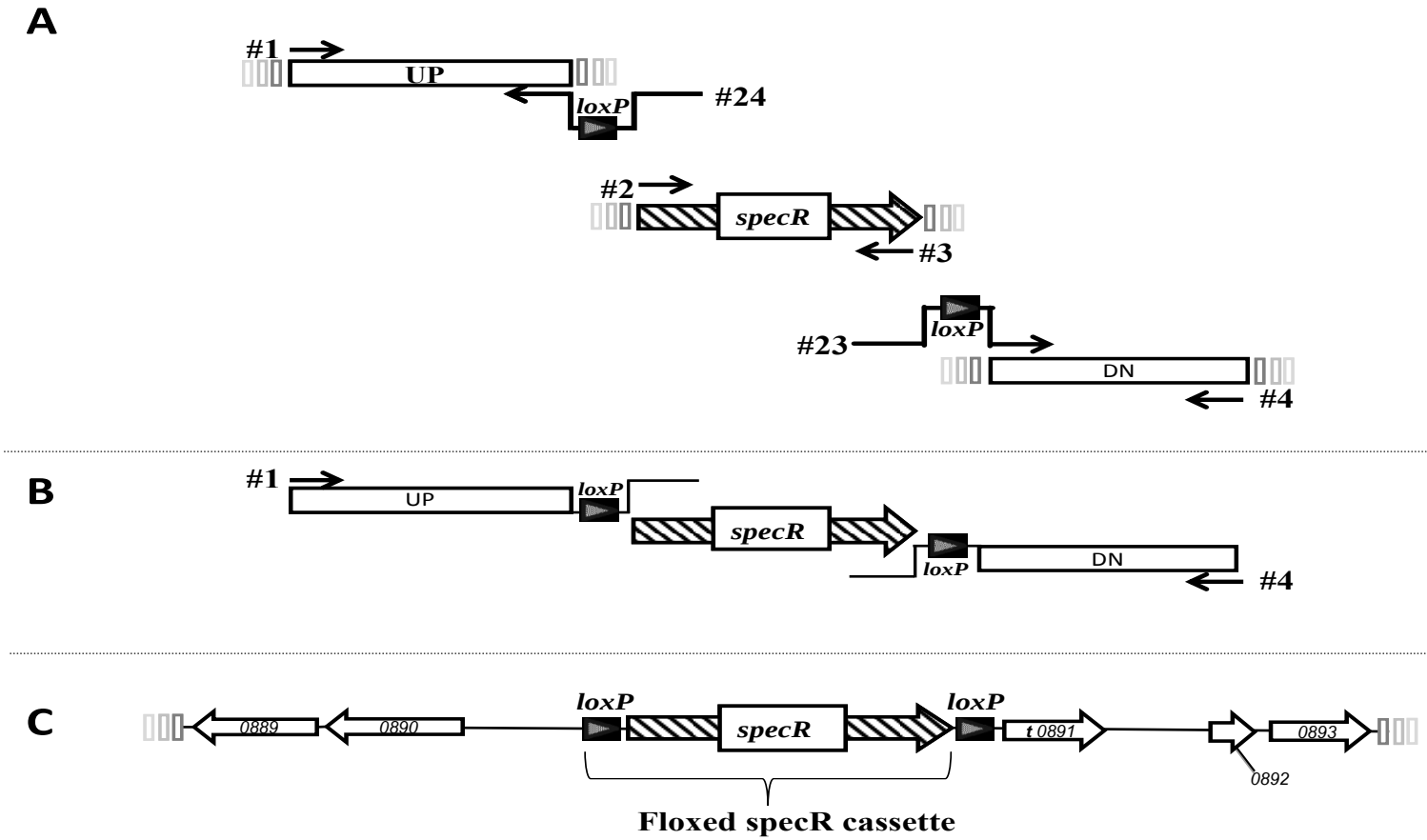

Figure 1. Construction of the *S. thermophilus* mutant STUL5001 containing the floxed specR cassette. The first sets of PCR reaction are presented in (A) and the final overlapping PCR is shown in (B). The resulting chromosomal region containing the floxed specR cassette obtained after transformation with the final overlapping PCR fragment is shown in C. Oligonucleotides used as amplification primers are indicated as black arrows and labelled with their numbers as detailed in Table 2. UP and DN regions correspond to upstream and downstream sequences flanking the floxed specR cassette. *specR* corresponds to the gene which encodes for a spectinomycin adenyltransferase conferring resistance to the antibiotic spectinomycin. The black square containing the grey triangle correspond to the *loxP* sequences.

2.2.6. Construction of the transcriptional fusion plasmid pULNcreB

To construct the cre transcriptional fusion vector suitable for *S. thermophilus*, the promoterless gene of the site-specific recombinase Cre was cloned into pG+host9TR, a thermoresistant replication derivative of the pG+host9TR plasmid (Maguin et al. 1996, Table 1) carrying an erythromycin resistance gene as the selection marker. The fragment bearing, in this order, the las operon transcriptional terminator Tlas, a multiple cloning site and the promoterless cre gene was amplified from the plasmid pNZ5520 (Bachmann et al. 2008, Table 1) using primers #5#6 (Table 2), incorporating a SpeI restriction site at the 5' end of the amplicon and a SalI site at its 3' end. The PCR fragment was SpeI-SalI double-digested and cloned into the dephosphorylated and similarly digested pG+host9TR giving pULNcreA (Table 1). In order to improve the segregational stability of this vector, we inserted the transcriptional terminator sequence of the pepN gene (TpepN) downstream of the gene cre. This was intended to prevent any read-through transcription that might be responsible for the synthesis of an antisens-RNA interfering with the expression of the replication genes. The terminator TpepN amplified from pNZ5520 (Bachmann et al. 2008, Table 1) using primers #7#8 was digested with both KpnI and SalI enzymes and cloned into the dephosphorylated and similarly digested pULNcreA leading to pULNcreB. The plasmid was completely sequenced on both strands and a restriction map is given in Fig.2.

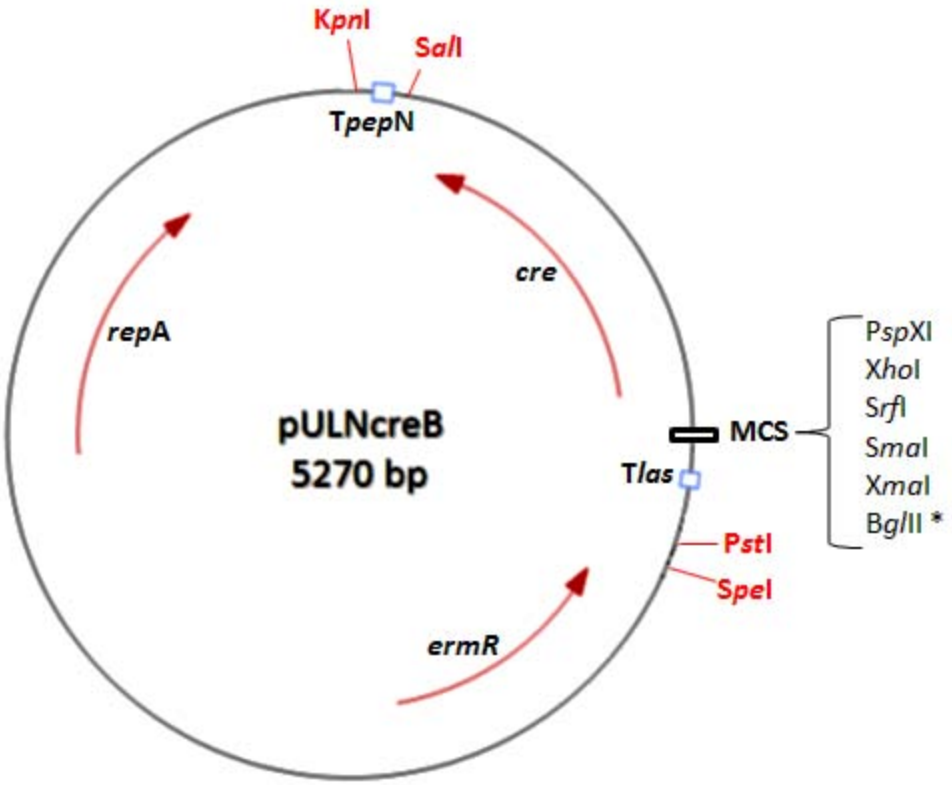

Figure 2. Restriction and genetic map of pULNcreB.indicating the promoterless gene '*cre* flanked by terminators T*las* and T*pepN*. The multiple cloning site is located upstream of the gene '*cre*. (□) represents the terminator sequences, (▭) represents the multiple cloning site, (*) represents the site used for cloning of promoter sequences and the restriction sites indicated in red characters are used for the construction of the plasmid.

2.2.7. Assessment of the segregational stability of pULNcreB and the floxed specR cassette

The segregational stability of pULNcreB in the recombinant strain STUL5001 was determined in a maintained exponential-growing culture performed in LM17 broth without erythromycin. Samples were taken at different culture times corresponding to a defined number of generations and plated on LM17 agar without erythromycin. For each time, 50 to 100 colonies were repicked on LM17 agar containing erythromycin. The segregational stability of pULNcreB was determined as the percentage of colonies resistant to erythromycin compared to the total number of colonies. For confirmation, 10 EryR colonies randomly chosen for each sampling time were analyzed by PCR with primers #9#10 (Table 2) to ensure that the EryR phenotype resulted from the presence of the plasmidic sequence and not from a spontaneous chromosomal EryR mutation.

2.2.8. Cloning of promoter sequences into the R-IVET vector pULNcreB

The promoter region of the *S. thermophilus* genes prtS, shsp and that of the lac operon were amplified from the genomic DNA of LMD-9 using the primer sets #11#12, #13#14 and #15#16, respectively (Table 2). All six primers were designed to contain a BglII restriction site at their 5' extremity in order to clone the PCR fragments into the BglII cloning site of pULNcreB located upstream of the promoterless cre gene. PCR products of 211-bp, 171-bp and 324-bp were digested with BglII and cloned into in the dephosphorylated and BglII-digested pULNcreB giving rise to plasmids named pULNcreB-PprtS, pULNcreB-Pshsp, and pULNcreB-Plac, respectively (Table 1).

2.3. Results

2.3.1. Construction of the loxP-specR-loxP reporter strain STUL5001 and assessment of its growth capacities and stability

The *S. thermophilus* strain LMD-9 was selected to implement the R-IVET system because its genome sequence is available (Makarova *et al.* 2006) and it exhibits natural transformation capacities (Blomqvist *et al.* 2006; Fontaine *et al.* 2010; Gardan *et al.* 2009). The locus STER_0891 encoding a putative glucose permease, located between positions 823822 and 824709 of the LMD-9 chromosome, was used to integrate the cassette *loxP-specR-loxP* into the chromosome.

Overlapping PCR was used to generate a mosaic PCR fragment consisting of an internal *loxP-spec*R-*loxP* region flanked by sequences corresponding to the upstream (UP) and downstream (DN) of the STER_0891 target locus (Fig. 1). One spectinomycin resistant clone obtained after transformation with the mosaic PCR fragment and confirmed by PCR and sequencing to have the cassette integrated was named STUL5001 (*STER_0891::loxP-specR-loxP*).

In order to assess the impact of the partial replacement of the putative glucose permease locus by the *loxP-specR-loxP* cassette, we monitored growth kinetics of both the wild type LMD-9 strain and the STUL5001 transformant in M17 medium containing either lactose (LM17) or glucose (GM17) as the source of carbon (Fig. 3). The growth rate of STUL5001 was not found to be different to the wild type strain LMD-9 when cultured in LM17 with generation times of 33.0 and 31.9 min respectively. When both strains were cultivated in GM17, growth was slower than in M17 medium containing lactose, but no significant difference was observed

between STUL5001 and LMD-9 (43.6 and 41.2 min, respectively). The faster growth of *S. thermophilus* in lactose compared to glucose is well known (Geertsma *et al.* 2005). In *S. thermophilus*, lactose does not elicit carbon catabolite regulation (van den Bogaard *et al.*, 2000) supporting preference for lactose over glucose as the main source of carbon and energy. More importantly and as observed before (Fontaine *et al.* 2010), comparable growth rates in glucose medium for LMD-9 and a strain mutated in the putative glucose permease locus indicated that the R-IVET cassette does not seem to affect growth in a complex environment such as the M17 medium.

The chromosome stability of the *loxP-specR-loxP* cassette in STUL5001 was assessed after 30 generations of growth in non-selective medium. Replica plating of 100 individual colonies, amplification of the *specR* gene in 20 randomly selected colonies and resequencing over the integration locus of one clone confirmed the stability of the mutated strain phenotype and genotype.

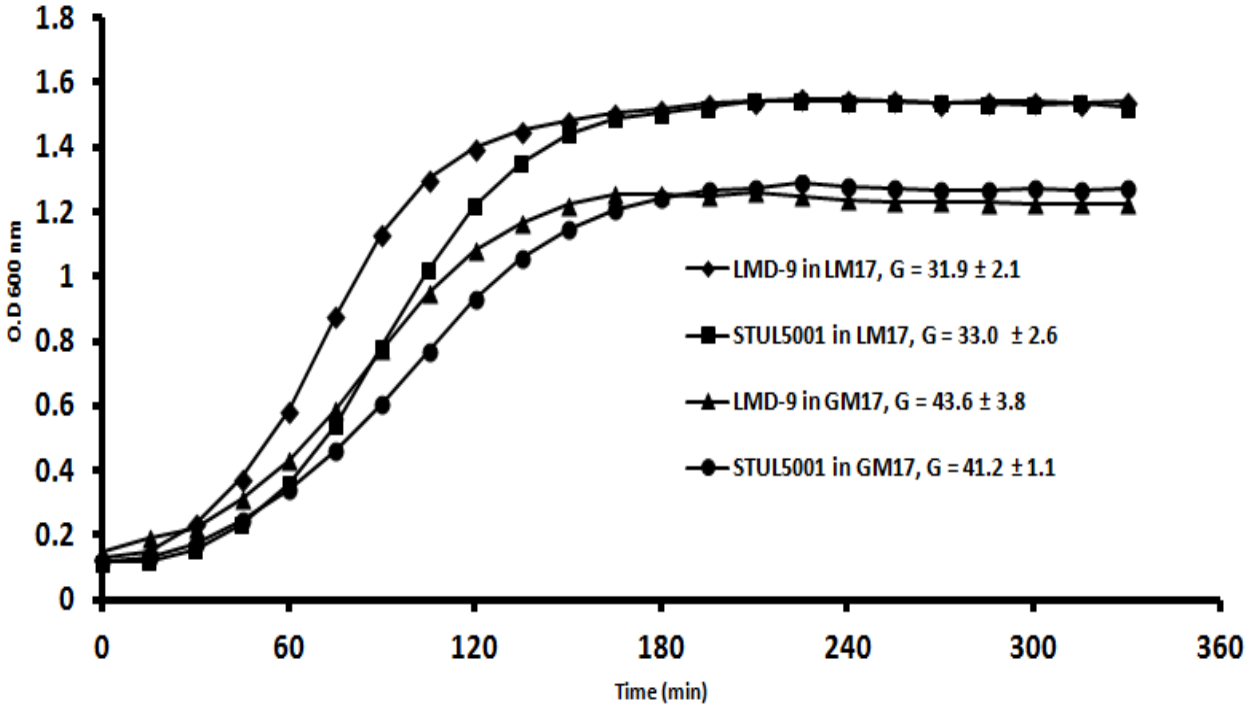

Figure 3. Growth curves of the LMD-9 strain and its derivative STUL5001 strain in LM17 and in GM17 media. Growth expressed as the optical density at 600 nm was monitored at 15 min intervals with the Bioscreen CTM. Each point represents the mean value of triplicates for both strains. Generation times are given in min (mean ± standard deviation).

2.3.2. Construction of the cre expression vector pULNcreB and stability assessment of the R-IVET tool in S. therlophilus

The second component of the R-IVET system is the plasmid carrying the promoterless cre gene which constitutes the basis to construct a random genomic DNA library or a clone containing a specific promoter to be tested. The choice of a replicon able to be maintained in *S. thermophilus* was a critical point as only a few segregationally stable exogenous plasmids exist for *S. thermophilus* (Girard and Moineau 2007). Moreover, the LMD-9 strain used to construct the R-IVET system is known to contain two indigenous plasmids which may represent a supplementary constraint with respect to the feasibility to introduce an additional plasmid. The vector pG+host9^{TR}, a thermoresistant replication derivative of pG+host9^{TR} (Maguin et al. 1996) was used as a replicon base as it was shown to generate high transformation rates in *S. thermophilus* (Fontaine et al. 2010). The R-IVET vector pULNcreA was constructed by cloning into pG+host9TR the functional region from pNZ5520 (Bachmann et al. 2008) which contains in the order (1) the las operon transcriptional terminator, (2) a multiple cloning site including *Sma*I and *Bgl*II restriction sites and (3) the promoterless cre gene. The introduction of TpepN (Bachman et al. 2008) downstream of the cre gene in pULNcreA was designed in order to prevent any read-through transcription initiated from promoters cloned into the cloning site (Bron et al. 2004).

The segregational stability of the vector pULNcreB in the recombinant strain was assessed *in vitro* by growing the recombinant strain in LM17 medium without erythromycin and was measured by comparing the number of CFU recovered on medium with and without erythromycin at each sampling point. Erythromycin resistance was found in 90% (72/80) of the clones after 1 generation, 73% (42/57) after 34 generations and a maintained rate of 38% (62/165) for the period extending from 44 to 52 generations. The presence of pULNcreB in the EryR clones was confirmed by colony PCR performed on 10 EryR colonies for each sampling time.

Keeping in mind that the R-IVET system developed in this study was intended to be used to detect genes which are induced in the gastrointestinal tract of mammals, the stability of both chromosomal and plasmid constructions of the recombinant strain STUL5001 harboring a promoter-less pULNcreB was assessed in feces after the passage in the GIT of a mouse. One hundred random picked colonies showed both EryR and SpecR phenotype indicating that they carried both components of the R-IVET system. This was confirmed by a multiplex-PCR analysis performed on genomic DNA extracted from 5 clones with the two sets of primers specific for the floxed specR cassette and for pULNcreB (Fig. 4). These results justified the suitability of pULNcreB as a vector for the implementation of the R-IVET technology in *S. thermophilus*.

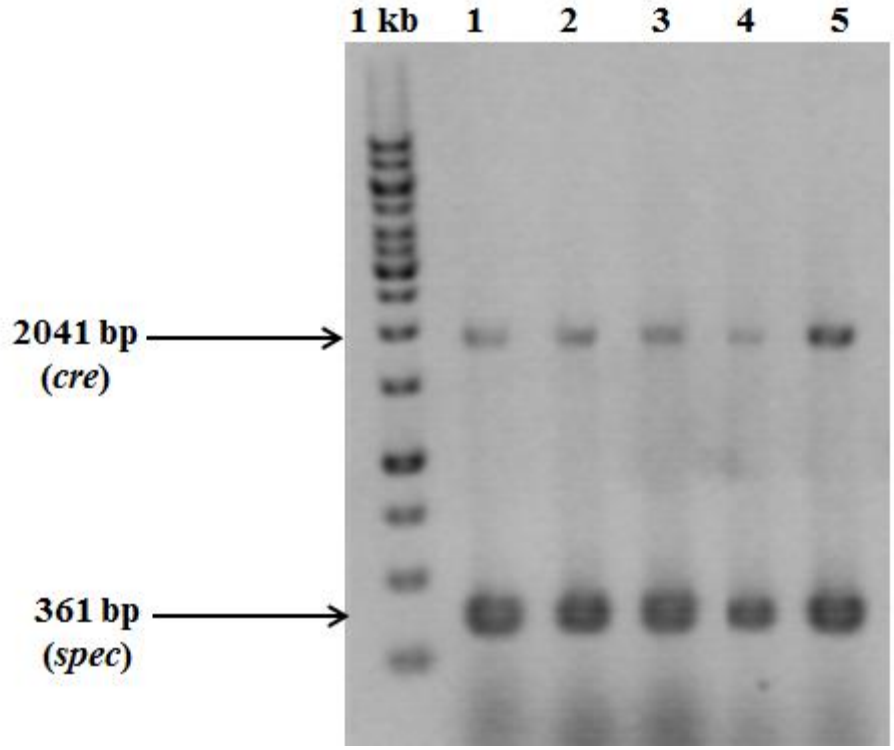

Figure 4. Multiplex amplification of the two R-IVET components in *S. thermophilus* recovered from mouse feces after GIT transit. Cell lysates of 5 colonies (lanes 1 to 5) recovered from the feces were used as template for amplification with primers #9#10 (gene cre, upper band) and with the cassette primers #21#22 (spec, lower band). Fragments were separated on 1% (w/v) agarose gel in 0.5x TBE at 100 V for 1 h. The 1kb DNA ladder (lane 1kb) was used as molecular weight marker.

2.3.3. Assessment of prtS, shsp and lac operon promoter induction in in vitro conditions

To evaluate the functionality of the developed R-IVET tool *in S. thermophilus,* we choose to test promoters of three *S. thermophilus* genes (i.e. *prtS, shsp* and *lac* operon) for which activity can be induced *in vitro*. The *prtS* gene is chromosomal and encodes the single cell-wall protease of *S. thermophilus* which has been shown to facilitate its growth in milk (Courtin *et al.* 2002). The *shsp* gene is plasmid borne and encodes a low-molecular-weight heat stress protein which displays a basal expression level and is strongly induced in response to the stress environment such as heat and acid (González-Márquez *et al.* 1997; Petrova and Gouliamova 2006). The promoter of the *lac* operon, involved in the transport and metabolism of lactose, was used because of its inducibility *in vitro* and *in vivo* (Drouault *et al.* 2002; Mater *et al.* 2006).

The promoter region of the three genes were generated by PCR and cloned into pULNcreB upstream of the promoter-less *cre* gene. Transformants of STUL5001 obtained with each of the three recombinant plasmids were selected on LM17 medium containing erythromycin. All randomly selected colonies (100/100) were found to be spectinomycine sensitive. PCR performed on 10 colonies of each recombinant strain confirmed that the *loxP-specR-loxP* cassette had been deleted by site-specific recombination leaving a single chromosomal *loxP* site as demonstrated by sequencing. These results established that the three promoters P*prtS*, P*shsp* and P*lac* had been activated in all *S. thermophilus* cells during their overnight growth on the agar LM17 medium. When transformants obtained with pULNcreB-P*lac* were grown on glucose instead of lactose, spectinomycin sensitive transformants appeared at the rate of 39% (182/300) and 72% (129/180) after culture for 24h and 36h, respectively.

The recombinant plasmids pULNcreB-P*prtS* and pULNcreB-P*shsp* were introduced into STUL5001 by natural transformation. Selection on LM17 containing erythromycin generated 162 colonies for pULNcreB-P*prtS* and 183 colonies for pULNcreB-P*shsp*. When the native plasmid pULNcreB was used, 201 transformants were obtained. For each of the three constructs, replica plating of 100 colonies was carried out on LM17 with spectinomycin. All transformants obtained with pULNcreB-P*prtS* and pULNcreB-P*shsp* were phenotypically spectinomycin sensitive whereas all pULNcreB transformants displayed resistance to spectinomycin. PCR performed on 10 colonies of each pULNcreB-P*prtS* and pULNcreB-P*shsp* transformants revealed a 2382-bp amplicon with primers stu0855-UPF/stu0855-DNR. This indicated that the *loxP-specR-loxP* cassette had been deleted by site-specific recombination. The sequencing of the 2382-bp amplicon obtained with both plasmids with the two couples of primers stu0855-

UPF/stu0855-DNR and UPin-F/DNin-R confirmed that the chromosomal cassette had been deleted by site-specific recombination leaving a single *loxP* site as expected. These results indicate that both the promoters, *prtS* and *shsp* had been activated in all *S. thermophilus* cells during their overnight growth on the agar LM17 medium.

The recombinant plasmid pULNcreB-P*lac* was introduced into STUL5001 and 180 transformants were obtained on GM17 containing erythromycin. In this experiment, glucose had been used instead of lactose in order to limit the activity of the lactose operon promoter. Replica plating onto GM17 containing spectinomycin showed that 72% (129/180) of the clones were sensitive to spectinomycin indicating a partial activation of the lactose operon during growth on glucose. When the spectinomycin resistant colonies were replica plated in a second step onto M17 containing lactose and spectinomycin, all resulting clones were found to be sensitive to spectinomycin. The deletion of the chromosomal cassette confirmed by PCR in 10 clones indicated that growth on lactose induced the full activity of the lactose operon promoter.

2.3.4. Assessment of the lac operon promoter induction in the GIT

Because of the partly inducible activity of P*lac* observed *in vitro*, we choose to evaluate inducibility of the lactose promoter *in vivo* with *S. thermophilus* recombinant cells passing through the GIT of mouse. Cells of STUL5001 pULNcreB-P*lac* were prepared in GM17 medium containing spectinomycin to select clones with an undeleted chromosomal cassette before the mouse feeding. Fecal samples were collected 3h, 6h and 24h following the intragastric feeding and suitable dilutions were plated on GM17 containing erythromycin to specifically select R-IVET *S. thermophilus* strains. For the first two collection points, 100 colonies were collected, pooled by lot of 10 and analyzed by colony PCR to test if the floxed *specR* cassette was intact or deleted (Fig. 5). At 3h, clones displayed the amplicon indicative of the intact cassette whereas clones collected at 6h and 24h gave the fragment indicative of the deleted cassette. The disappearance of the 3616-bp and the appearance of the 2382-bp in the samples collected at 6h and 24h confirmed that the promoter P*lac* had been induced in the digestive tract as was already previously established (Drouault *et al*. 2002; Mater *et al*. 2006).

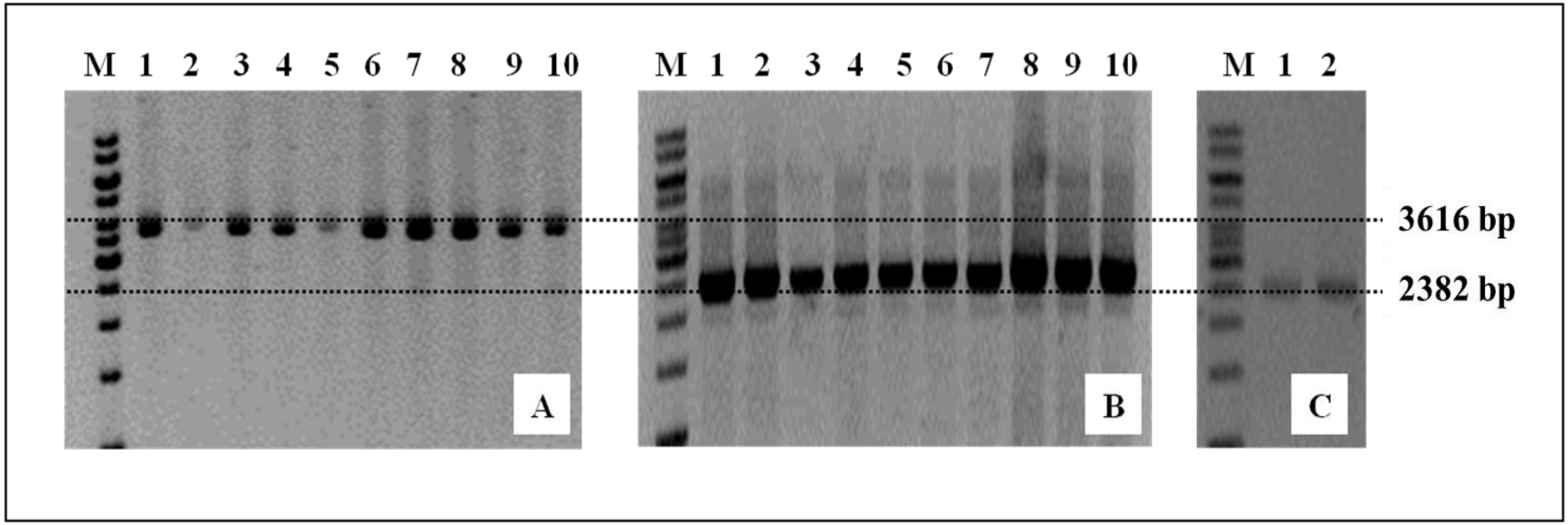

Figure 5. Monitoring of the cassette resolution in *S. thermophilus* after GIT transit. PCR products obtained from cell lysates of colonies obtained from mouse feces at 3h (A), 6h (B) and 24 h (C) following the intragastric administration. Each cell lysate corresponding to a lane was prepared from 10 colonies. PCR was carried out with the primers #1 #4 to detect the presence (3616 bp) or the deletion (2382 bp) of the floxed specR cassette. PCR products were separated on 0.8% (w/v) agarose gel in 0.5x TBE at 100 V during 1 h. The 1kb DNA ladder (lane M) was used as molecular weight marker.

2.4. Discussion

S. thermophilus is a bacterium found in the feces of humans after consumption of yogurts (Brigidi *et al*. 2003; Mater *et al.* 2005; Elli *et al.* 2006). Although health claims were attributed to live yogurt cultures to improve lactose digestion in 2010 by the European Food Safety Authority, the status of *S. thermophilus* as a probiotic is still questioned. Health benefits associated with *S. thermophilus* consumption remain to be established. A better knowledge on the physiology of the bacterium when present in the GIT will help understand potential influence on the consumer.

In order to get an exhaustive inventory of bacterial functions triggered when residing in the GIT, sophisticated genetic screening technologies have been developed (An and Grewal 2012). Hybridization-based approaches, including techniques like microarrays and SCOTS, monitor gene expression changes by quantifying transcripts. The whole genome transcriptional profiling using DNA microarrays performed on intestine-colonizing *Lactobacillus plantarum* strains revealed a convergence of gut-adaptive responses in human and mice (Marco *et al.* 2010). However, hybridization-based approaches require bacterial mRNA of high quality and in high quantity and can therefore not be applied to microorganisms underrepresented compared to the rest of the microbiota and host cells. Alternatively, promoter-trapping techniques including IVET, R-IVET and DFI can be used to detect activated promoters of the microorganism in any complex environment. We decided to explore the physiology of *S. thermophilus* when transiting the GIT by the R-IVET technology. As a transcriptomics technique based on a transcriptional fusion to a reporter gene, it is suitable to capture any active promoters in the complex environment of the gut. Moreover, the R-IVET technology is suited to monitor the spatial and temporal activation of a particular promoter in a specific environment, by simply measuring the proportion of clones which have lost the reporter function present in the floxed cassette.

In the R-IVET tool we developed for *S. thermophilus*, we used the recombinase *cre* gene which has already been successfully used in other lactic acid bacteria R-IVET systems (Bron *et al.* 2004; Bachman *et al.* 2008). We showed that the two components of this system were stable in recombinant bacteria during their transit through the GIT of mice which is a prerequisite to use this tool for identifying genes induced in the GIT. Subsequently monitoring the promoter activity of both *prtS* and *shsp* genes with our R-IVET system was therefore interesting to test its functionality and gauge its sensitivity. Deletion of the R-IVET

cassette observed for clones obtained with P*prtS* and P*shsp* indicated that both promoters had been activated in all *S. thermophilus* cells during their overnight growth on the agar LM17 medium. This was expected for both genes *prtS* and *shsp* as they are expressed throughout the growth of *S. thermophilus* in M17 medium (Galia *et al.* unpublished results; Gonzalez-Marquez *et al.* 1997).

To test further our R-IVET tool in *in vitro* and *in vivo* conditions, the promoter of the *lac* operon, involved in the transport and metabolism of lactose, was used as an "inducible" promoter. Using our R-IVET system, we observed that 39% of the clones displayed a lactose promoter activity after 24 h growth on glucose agar. This was expected as transcription of the lactose operon is induced during growth in the presence of lactose but a basal level of transcription is persistent during growth in the presence of glucose (van den Bogaard *et al.* 2000). However, this value is difficult to compare with values published elsewhere as it depends on the method of promoter induction monitoring, the medium used and the time of sampling. For instance, in a study using Northern blot, transcription of the lactose operon was observed to be 2-fold higher in LM17 than in GM17 (van den Bogaard *et al.* 2000) and 9-fold higher with a transcriptional fusion to the luciferase genes (Drouault *et al.* 2002). Furthermore, the gene expression percentage obtained in the R-IVET experiment represents the cumulative rate of clones which have had their promoter activated during the course of the experiment in contrast to other experimental procedures where values indicate the mean percentage of clones showing a promoter activity at the moment of the assay. When tested in the murine GIT, we observed that the lactose promoter had not been induced in *S. thermophilus* cells recovered from feces collected 3 hours after the intragastric administration but had been induced in cells collected at 6 and 24 h. The counter-selection performed on the R-IVET library cells with spectinomycin during the preparation of the cells guarantees the presence of 100% of cells containing the floxed specR cassette when inoculated to the mouse. It is however surprising to observe that cells recovered from feces at 3h did not show any P*lac* induction. This can not be explained by the absence of the lactose inducer in the GIT since lactose is continuously provided to the mouse. In a recent study performed in gnotobiotic rats, Thomas and coworkers established that lactose enhanced the level of colonization of *S. thermophilus* in the GIT (Thomas *et al.* 2011). This can be directly correlated with our findings where the first *S. thermophilus* cells found in the feces did not show any lactose operon activity whereas all cells excreted later at 6h and 24h showed an activity. *S. thermophilus* cells which did not have lactose catabolism were not able to thrive in the GIT of

mice and were expulsed rapidly in the feces (3h), while those which expressed it could be maintained in the GIT for 6h or 24h.

Our R-IVET tool is now available to identify promoters of *S. thermophilus* specifically induced in complex environments like the GIT tract of animals but also in fermented products. It involves the construction of a plasmid library made of random genomic DNA of *S. thermophilus* cloned into the plasmid pULNcreB and places the R-IVET recombinant *S. thermophilus* library in the complex biological niche. After excision of the R-IVET cassette, the analysis of appearing spectinomycin-sensitive clones will enable the identification of genes which are transiently expressed under the tested conditions. The system described in this report can be improved by adding a positive-selection system where the recombination mediated by Cre can activate the transcription of a second antibiotic resistance marker (Hanin *et al.* 2010) or of a fluorescent / color marker (Bachman *et al.* 2008; Lozano *et al.* 2011) or eliminate a lethal gene like *sacB* (Osorio *et al.* 2005).

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Conclusion générale & Perspectives

Une partie de l'activité de recherche de l'équipe PB2P de l'UR AFPA, dans laquelle ce travail a été réalisé, concerne la bactérie lactique *S. thermophilus*. Le but poursuivi à long terme par l'équipe est de développer dans un but de prévention, des aliments fonctionnels et plus particulièrement des laits fermentés ayant des fonctionnalités variées et répondant à des besoins de prévention de pathologies complexes, telles que celles liées au stress, aux maladies cardiovasculaires ou inflammatoires. La fonctionnalité de tels aliments pourrait provenir d'une part d'ingrédients, tels que des peptides bioactifs, libérés lors de la fabrication de l'aliment et/ou de propriétés probiotiques de la bactérie utilisée pour leur fabrication. C'est ainsi que l'équipe PB2P étudie le système protéolytique de *S. thermophilus* afin de l'utiliser pour produire des peptides bioactifs à partir des protéines laitières et s'intéresse aux propriétés probiotiques de cette bactérie. Mon travail de recherche a concerné ce dernier point, les objectifs étant :

- d'explorer les capacités de survie dans le tractus digestif de différentes souches de *S. thermophilus* et des propriétés probiotiques ;

- de développer un outil basé sur la technologie R-IVET afin d'identifier les gènes qui sont exprimés par *S. thermophilus* dans le tractus digestif, et de là, contribuer à la caractérisation de son état physiologique *in vivo*.

1. Exploration des propriétés de survie et des propriétés probiotiques de *S. thermophilus*

Différentes propriétés permettant à *S. thermophilus* de survivre dans le tractus intestinal ont été explorées *in vitro*. Il s'agit de la résistance à des pH acides qui sont ceux des phases inter-prandiales (pH 2) et post-prandiales de l'estomac (pH4) (McLauchlan *et al.*, 1987) ; (Both *et al.*, 2010), de la résistance aux sels biliaires et à un stress oxydatif causé par du peroxyde d'hydrogène. La résistance aux bas pH permet de prédire la résistance de la souche au passage de l'estomac et celle aux sels biliaires permet de prédire la résistance des souches dans l'intestin grêle.

La capacité d'adhésion d'un probiotique aux entérocytes est importante car elle influera à la fois sur son action bénéfique sur l'hôte et sur le temps de séjour du probiotique dans le tractus digestif, deuxième paramètre qui influence bien évidemment le premier (Servin et Coconnier, 2003). Pour ce qui nous concerne, cette capacité d'adhésion a été déterminée en

utilisant les cellules entérocytaires productrices de mucus de la lignée HT29-MTX. Enfin, les propriétés immunomodulatrices des souches de *S. thermophilus* de notre collection ont été étudiées par évaluation de la production des cytokines IL-8, IL-10 et IL-12.

Si le niveau de variabilité génétique au sein de l'espèce *S. thermophilus* apparait relativement faible par rapport à d'autres espèces, une variabilité phénotypique pour chacun des caractères testés a été observée, que ce soit en termes de degré de résistance aux différents stress, de capacité d'adhésion ou de propriétés immomodulantes. L'analyse de ces résultats en composante principale (ACP) a amené à la constitution de 6 classes de souches, chaque classe affichant divers degrés de résistance aux stress gastriques, d'adhésion aux cellules épithéliales et de potentiel immunomodulant. Ces résultats ouvrent des perspectives à court et moyens termes.

A court terme et *in vitro*, il serait intéressant de :

- d'étudier la résistance des souches à des pH bas mais au sein d'une matrice alimentaire ;
- de déterminer leur capacité de résistance à un mélange de sels biliaires plus complexe tel qu'un jus pancréatique et de rechercher de déterminants permettant cette résistance ;
- de comparer les capacités d'adhésion des différentes souches de *S. thermophilus* à celles d'autres probiotiques déjà utilisés dont des lactobacilles et de comparer également leurs propriétés immunomodulantes.

Pour ce qui concerne la résistance aux pH acides, seuls 3 souches résistent au stress « pH 2 » mais toutes se sont révélées capables de résister au stress « pH 4 ». La sensibilité des souches à pH 2 n'apparait pas rédhibitoire *a priori* dans la mesure où les bactéries seront administrées au sein d'une matrice alimentaire, et devraient donc être protégées, et ce d'autant que, certains éléments de la matrice comme les caséines ont un pouvoir tampon.

La résistance aux sels biliaires est par contre absolument indispensable pour que la bactérie reste vivante dans le tractus digestif (Blanquet-Diot *et al.*, 2012). L'étude des capacités de résistance aux deux sels biliaires utilisés devra être complétée dans un premier temps par des études *in vitro* utilisant un jus plus complexe de sels biliaires. Par la suite, les déterminants de cette résistance devront être identifiés afin de pouvoir les améliorer chez une

souche qui serait faiblement résistante mais qui aurait des propriétés probiotiques intéressantes.

Dans l'étude que nous avons menée, plusieurs souches de *S. thermophilus* se sont révélées plus adhérentes sur le modèle cellulaire utilisé que *Lactobacillus acidophilus* NCFM, souche probiotique déjà commercialisée. Il s'agit d'un résultat encourageant et il serait intéressant de comparer la capacité d'adhésion de nos souches à celles d'autres probiotiques utilisés et notamment d'autres lactobacilles.

Trois cytokines (IL-8, IL-10 et IL-12) ont été quantifiées lors de l'étude des propriétés immunomodulantes des souches de *S. thermophilus*. La quantification de la cytokine IL-8 (pro-inflammatoire) a été effectuées en co-incubant les différentes souches avec les cellules HT29 avec ou sans TNFα, utilisé comme immunostimulant pour déclencher la production d'IL-8. Cette cytokine est notamment impliquée dans l'inflammation systémique. 26/30 souches de *S. thermophilus* se sont avérées capables de diminuer la production d'IL-8. En même temps, la quantification des cytokines IL10 (anti-inflammatoire) et IL-12 (pro-inflammatoire) a été réalisée en utilisant les cellules PBMC. Le rapport d'IL-10/IL-12 a été utilisé pour évaluer le potentiel anti-inflammatoire de ces souches. Sur la base de la valeur de ce rapport, 3 souches semblent avoir le meilleur potentiel anti-inflammatoire. La critique majeure que nous pouvons faire à ces résultats est le manque d'élément de comparaison par rapport à d'autres probiotiques déjà considéré comme « anti-inflammatoire », qui pourrait nous permettre de déterminer si *S. thermophilus* a un potentiel dans ce domaine.

A plus long terme, il serait intéressant de :

- tester la capacité de survie des souches de *S. thermophilus* en modèle rongeur mais aussi en conditions digestives humaines simulées ;
- d'étudier les propriétés anti-inflammatoires *in vivo*, dans des modèles souris d'inflammation de l'intestin ;
- de comparer les pools de gènes entre les 6 classes de souches afin de voir si certaines propriétés spécifiques à une classe peuvent être reliées à un profil génétique particulier.

Même s'il peut être intéressant de déterminer la survie de nos souches chez la souris, l'utilisation de conditions digestives humaines simulées (utilisation du « TNO gastro-

intestinal tract model ») est certainement une voie plus prometteuse. Ces systèmes ne sont pas exactement les mêmes que les systèmes naturels, mais ils sont les meilleurs imitateurs de celui d'origine. Presque tous les facteurs peuvent être contrôlés et modifiés de sorte que le comportement des souches peut être observé dans certaines conditions particulières. En même temps, l'influence de la matrice alimentaire (lait ou lait fermenté, repas etc.) sur le taux de survie des bactéries pourra être étudiée.

Les maladies inflammatoires chroniques de l'intestin affectent de nombreux individus et pour étudier ces maladies complexes, des modèles d'inflammation ont été mis au point chez la souris. Les flores en transit constituent une voie attractive pour tenter de diminuer *in situ* le degré d'inflammation. Il serait donc intéressant de passer dans de tels modèles celles de nos souches qui paraissent avoir un fort potentiel anti-inflammatoire, s'il s'avère aussi important que celui de probiotiques déjà utilisés. En tant qu'élément d'adhésion mais aussi d'immunostimulation, il serait intéressant enfin d'étudier la production d'exopolysaccharides au sein de notre collection des souches. En effet, la production d'exopolysaccharides pourrait être impliquée dans la viabilité et l'adhésion des bactéries lactiques, en particulier chez les espèces du *Lactobacillus* (Darilmaz *et al*., 2011).

Enfin, et pour conclure sur cette partie, il serait d'un grand intérêt aussi d'explorer d'autres propriétés probiotiques chez ces souches.

2. Construction de l'outil R-IVET

Dans la deuxième partie de ce travail, un outil permettant d'implanter la technologie R-IVET chez *S. thermophilus* a été construit. Il comporte deux composants : un composant plasmidique qui porte le gène *cre* dépourvu de son promoteur et le composant chromosomique, consistant en une cassette comprenant le gène de résistance à la spectinomycine flanqué des sites *loxP* qui sont reconnus par la recombinase Cre et qui sont tous deux orientés dans la même direction. L'expression du gène *cre,* suite à l'introduction d'une séquence promotrice en amont de ce gène conduit à l'excision de la cassette et à la perte de la résistance à la spectinomycine. Cet outil a été validé *in vitro* mais aussi *in vivo*. Il constitue la première étape indispensable à l'utilisation de la technologie R-IVET pour identifier des gènes de *S. thermophilus* spécifiquement exprimés dans le tractus digestif et essayer de comprendre son état physiologique dans ces conditions. L'utilisation de cette

technologie dans le même but a d'ailleurs été couronnée de succès chez d'autres LAB (Bachmann *et al*., 2008; Bron *et al*., 2004)

La perspective immédiate de ce travail consiste en la construction d'une banque d'ADN génomique de *S. thermophilus* en amont du gène *cre,* puis à l'introduction de cette banque chez la souris ou dans le TNO afin d'identifier des promoteurs, et par voie de conséquence des gènes, qui sont exprimés *in vivo*, c'est-à-dire au cours du transit de la bactérie. Cependant, certains points restent à améliorer si nous voulons utiliser facilement cette technologie pour identifier des gènes exprimés spécifiquement dans différents contextes. Le premier est le mode de sélection. En effet, l'utilisation d'un crible négatif est difficile car implique le repiquage d'un grand nombre de clones en sortie du tractus digestif. Aussi l'élaboration d'un crible de sélection positive dans la cassette chromosomique aiderait à réduire le temps expérimental et permettrait d'éviter des doubles étapes de sélection. Le deuxième paramètre à améliorer concerne la sensibilité du système. En effet, compte tenu du caractère multi-copie du réplicon utilisé pour la construction du composant plasmidique, une faible expression, même transitoire, du promoteur *in vitro* pourrait aboutir à la contre-sélection du clone avant son introduction dans la condition testée. La modification des sites *loxP* pourrait être une voie possible de diminution de la sensibilité du système.

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Valorisation des travaux de recherche

Le travail qui a été réalisé au cours de cette thèse a donné lieu aux publications et communications listées ci-dessous.

I. Publications

1. **Junjua, M.,** Roussel, Y., Dary-Mourot, A. Lactic acid bacteria and study of their physiological state in the host. Séminaire Ecole Doctorale RP2E, 20 janvier 2011 Nancy, France. ISBN 2-9518564-9-0

2. **Junjua, M.,** Kechaou, N., Chain, F., Roussel, Y., Perrin, C., Langella, P., Bermudez-Humaran, L.G., Le Roux, Y., Chatel, J.M., Dary-Mourot, A. High throughput *in vitro* screening of *Streptococcus thermophilus* strains for their probiotic potential. Article soumis dans International Journal of Food Microbiology.

3. **Junjua, M.,** Galia, W., Gaci, N., Genay, M., Bachmann, H., Kleerebezem, M., Dary, A., Roussel, Y. Development of the Recombinase-based *In vivo* Expression Technology (R-IVET) in *Streptococcus thermophilus* and validation in the gastrointestinal tract of mice using the lactose operon promoter. Article préparé pour une soumission dans le Journal of Applied Microbiology.

II. Communications avec actes

1. Roussel, Y., Genay, M., **Junjua, M.,** Dary, A. Cloning of resolvase gene from *Lactococcus lactis* and potential use in molecular genetics. 16ème Colloque du Club des Bactéries Lactiques, 27-29 mai 2009, Toulouse, France.

2. **Junjua, M.,** Genay, M., Roussel, Y., Dary, A. Construction d'un outil R-IVET pour identifier des gènes exprimés *in vivo* chez *Streptococcus thermophilus.* 16ème Colloque National de la Recherche dans les IUT, 9-11 juin 2010, Angers, France.

3. **Junjua, M.,** Roussel, Y., Dary, A. Application de la technologie R-IVET à *Streptococcus thermophilus* pour identifier les gènes actifs dans le tractus gastro-intestinal. 17ème Colloque du Club des Bactéries Lactiques, 27 -29 Octobre 2010, Nancy, France.

4. **Junjua, M.**, Chain, F., Kechaou, N., Roussel, Y., Le Roux, Y., Langella, P., Bermudez-Humaran, L.G., Chatel, J.M., Dary-Mourot, A. *In vitro* screening of

Streptococcus thermophilus strains for their resistance against different stresses encountered in the gastro-intestinal tract and for their immunomodulatory properties. 18ème Colloque du Club des Bactéries Lactique, 22-24 mai, 2012, Clermont Ferrand, France.

5. **Junjua, M.**, Roussel, Y., Dary, A. Use of Recombination Based *in vivo* Expression Technology in *Streptococcus thermophilus*. Séminaire de l'Ecole Doctorale RP2E, 20 janvier, 2011, Nancy, France.

UNIVERSITÉ DE LORRAINE

A Nancy, le 21 février 2013

No étudiant : 30711875

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Madame,

Par décision en date du 21 février 2013, vous avez été autorisée à présenter en soutenance vos travaux en vue de l'obtention du diplôme :

DOCTORAT SCIENCES AGRONOMIQUES

La soutenance aura lieu le 19 mars 2013 à 14h30 à l'adresse suivante :

Université de lorraine - site Brabois - Salle Gallé - 2 avenue de la Forêt de Haye - 54500 Vandoeuvre-lès-Nancy

La soutenance sera publique.

Je vous prie d'agréer, Madame, l'expression de mes salutations distinguées.

Le Président

Pierre MUTZENHARDT

Pour le Président et par délégation,
La Vice-Présidente du CS

Clotilde BOULANGER

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Résumé :
Streptococcus thermophilus est la bactérie lactique la plus utilisée après *Lactococcus lactis* dans l'industrie laitière pour la fabrication de yaourts et de fromages à pâte cuite (Emmental, gruyère), filée (Mozarella) ou pressée (Cheddar). Il s'agit du seul streptocoque à avoir le statut de bactérie GRAS (Generally Recognized As Safe). Dans un premier temps le potentiel probiotique de 30 souches de *S. thermophilus* de différentes origines a été testé par l'étude de leurs capacités à résister aux différentes conditions de stress rencontrées pendant leur passage dans le tractus gestro-intestinal (bas pH, sels biliaires et stress oxydant), de leur capacité à adhérer aux cellules épithéliales intestinales et de leurs propriétés immunomodulatrices. La majorité des souches réduit la production d'interleukine IL-8 (pro-inflammatoire) alors qu'elles induisent la production d'interleukine IL-10 (anti-inflammatoire) et l'IL-12 (pro-inflammatoire). Sur la base du rapport IL-10/IL-12, qui permet d'apprécier le potentiel anti-inflammatoire d'une souche, nous avons observé que certaines d'entre-elles pourraient avoir un fort potentiel anti-inflammatoire. L'Analyse en Composantes Principales (ACP) nous a permis de séparer les souches en 6 catégories différentes présentant des propriétés distinctes. A l'intérieur de chaque classe, une variabilité entre les souches a été observée et des caractéristiques intéressantes identifiées. Cependant, aucune des classes ne peut être considérée comme contenant le probiotique « parfait ». Suite à cette étude et sur la base de sa résistance aux stress gastriques et de ses capacités d'adhésion, et sachant que la séquence de son génome est disponible, la souche LMD-9 a été sélectionnée pour la deuxième partie de ce travail, à savoir la construction d'un outil basé sur la technologie R-IVET pour étudier l'état physiologique de *S. thermophilus* dans le tractus digestif. L'otil R-IVET mis au point se compose de deux éléments: un vecteur plasmidique portant le gène *cre* codant une recombinase spécifique dépourvu de son promoteur et une cassette chromosomique composée d'un gène de résistance à la spectinomycine flanqué par des sites *loxP*, reconnus par la recombinase Cre. La fonctionnalité de l'outil R-IVET a ensuite été testée par le clonage de trois promoteurs différents de *S. thermophilus* (P*prtS*, P*shsp* and P*lac*) en amont de *cre*. Le système a été valide *in vitro* avec les trois promoteurs et *in vivo* avec le promoteur P*lac*.

Mots clés : *S. thermophilus*, probiotique, stresses gastriques, tractus gastro-intestinal, adhésion, immunomodulation, Recombinase-based *In vivo* Expression Technology, site-spécifique recombinase

Abstract:
Streptococcus thermophilus is a lactic acid bacterium used after *Lactococcus lactis* in the dairy industry for the production of yogurt and cheeses like Emmental, Gruyere, Mozarella and Cheddar. It is the only streptococcus to have the GRAS (Generally Recognized As Safe) status. In this work, the probiotic potential of 30 *S. thermophilus* strains from different origins was tested by studying their ability to resist different stress conditions encountered during their passage through the GIT (low pH, bile salts and oxidative stress), their ability to adhere to intestinal epithelial cells and their immunomodulatory properties. Majority of the strains reduced the production of interleukin IL-8 (pro-inflammatory) and induced the production of interleukin IL-10 (anti-inflammatory) and IL-12 (pro-inflammatory). On the basis of the ratio IL-10/IL-12 which allows to evaluate the anti-inflammatory potential of a probiotic, several strains appeared to have a high the anti-inflammatory potential. Principal Component Analysis (PCA) allowed us to classify strains in 6 different categories with different properties. Within each class, variability and interesting features were observed, but none of the classes could be considered as containing the perfect probiotic. Following this study and on the basis of its resistance to gastric stress and its adhesion capacity and knowing that its genome sequence is available, the strain LMD-9 was selected for the second part of this work, namely the construction of a tool based on R-IVET to study the physiological state of *S. thermophilus* in the digestive tract. This tool is composed of two elements: a plasmid vector (pULNcreB), carrying the gene *cre* encoding a site-specific recombinase without its promoter and a chromosomal cassette composed of a gene of resistance to spectinomycin flanked by *loxP* sites which are recognized by the recombinase Cre. The functionality of the tool R-IVET was then tested by cloning three different promoters of *S. thermophilus* (P*prtS*, P*shsp* and P*lac*) upstream of *cre*. System was valid *in vitro* with all the three promoters and *in vivo* by using the lactose operon promoter P*lac*.

Keywords: *S. thermophilus*, probiotics, gastric stress, gastro-intestinal tract, adhesion, immunomodulations, Recombinase-based *In vivo* Expression Technology, site specific recombinase.