Les adaptations bactériennes améliorent la survie de Propionibacterium freudenreichii durant le séchage et le stockage

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THESE DE DOCTORAT DE

AGROCAMPUS OUEST
COMUE UNIVERSITE BRETAGNE LOIRE

ECOLE DOCTORALE N° 600
Ecole doctorale Ecologie, Géosciences, Agronomie et Alimentation
Spécialité : « *Biochimie, biologie moléculaire et cellulaire* »

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« Improving the survival of beneficial bacteria during drying and storage by the exploitation of bacteria adaptations»

Thèse présentée et soutenue à « Rennes », le « 12 décembre 2019 »
Unité de recherche : INRA Agrocampus Ouest - Science et Technologie du Lait et de l'Œuf.

Thèse N° : 2019-24_B-329

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DOCTEUR DE L'INSTITUT SUPERIEUR DES SCIENCES AGRONOMIQUES, AGROALIMENTAIRES,

HORTICOLES ET DU PAYSAGE

Spécialité : «Biochimie, biologie moléculaire et cellulaire»

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Nous portons en nous des merveilles que
nous cherchons en dehors de nous.

Thomas Browne (1605-1682)

Acknowledgments

Je suis arrivée au STLO en 2016 pour un stage, puis j'ai eu l'opportunité de continuer pour trois ans de thèse. Le travail de thèse n'aurait pas pu être réalisé sans l'aide de nombreuses personnes : un vrai travail collectif !

Je souhaiterais remercier dans un premier temps, Joëlle Léonil et Yves Le Loir (ex-directrice et directeur du STLO) pour m'avoir accueillie dans le laboratoire.

Je voudrais remercier les membres du jury, Vianney Pichereau, Fernanda Fonseca, Nathalie Desmasure, Muriel Thomas et Michel Gautier pour avoir accepté d'évaluer ce travail.

Je tiens à remercier particulièrement, mes encadrants de thèse pour leur aide, leur investissement, et leur soutien. Merci pour ces trois années. Merci à Gwénaël pour ta motivation, pour tes inspirations notamment pour les remerciements (Sarah Klet reste le meilleur) et JAN passe. Romain, merci beaucoup pour ton avis éclairé, et ton humour inimitable. Enfin, merci Sylvie pour ta gentillesse, tes encouragements et ta patience.

Un grand merci à Bioprox et l'ANRT (Association National de la Recherche et de la Technologie), financeurs de la thèse et particulièrement Philippe Blanc et Pierre Marchand pour leur implication, leurs encouragements et leur aide pour les expérimentations. Merci également à Carmen Lapadatescu pour avoir monté le projet.

Merci au Professeur Vasco Azevedo pour m'avoir accueillie au sein de son laboratoire à l'UFMG pour des expérimentations. Merci particulièrement à Fillipe pour le temps passé pour l'organisation et pour toute l'aide durant le séjour. Tu as également un avenir en tant que guide touristique ;-). La visite du marché de Belo Horizonte restera un grand moment

J'adresse un grand merci à Renand Goude et Achim Quaisier pour avoir participé aux comités de thèse.

Merci à Koffigan pour le travail important effectué pendant son stage de master.

Je remercie Julien Jardin et Valérie Briard-Bion pour l'aide et l'implication pour les manips de protéomiques et Anne Dolivet pour les dosages NPN. Merci également à Sandrine Pottier et Arnaud Bondon pour leur aide en RMN et leur réactivité. Je remercie également Marie-Bernadette Maillard pour les analyses express des sucres et des acides gras volatiles ainsi que Jordane Ossemond pour les analyses de lipides, ta patience et ta pédagogie.

Je remercie également Benoit Robert et Serge Méjean pour toute l'aide apportée durant les expérimentations de séchage. Un grand merci aux filles du CIRM (Marie-No, Victoria, Anne-Sophie, Sophie, Florence) pour l'aide et leurs bons conseils.

Je remercie chaleureusement Paulette pour la création et l'organisation des légendaire pauses goûter. Un très très grand et même énorme merci à Jessica pour toute l'aide et les sauvetages de manips.

Merci à l'ensemble de l'équipe microbio et autres locataires (Aurélie, Nathalie, Christophe et Adélaïde) des labo MB1 et MB7 pour la bonne ambiance et les discussions durant les manips. Ça aura animé les numérations et les comptages de boîtes !! Un grand merci également à Nadia pour tous ses conseils.

Merci à mes colocs de bureau. Un grand merci Alexia pour ton enthousiasme à Stefano pour les encouragements mutuels durant la rédaction, à Jun pour ton sourire, à Elham pour toute ta motivation, et à Fanny et Julien pour vos blagues toujours très pertinentes. Merci aussi à François tu fais à moitié partie du bureau ! Merci aux étudiants des autres bureaux, vous étiez sympas aussi ;-) Je remercie Domitille pour sa bonne humeur et d'avoir laissé utiliser son bureau comme salle d'attente lorsque je cherchais Romain. Merci également à Jordane, Carlos, Simon et à Lucas pour votre gentillesse.

Un grand merci à Vinícius. Je me souviendrai toujours de ta motivation (surtout pour sortir), tes vidéos chelous, et les débats politiques/philosophiques/scientifiques. Un vrai poète même en français !!

Ha Song... très heureuse de t'avoir rencontré. Un grand merci pour tous les bons moments passés, à Rennes, en Chine et chez E. Leclerc. Je reviendrais voir ta petite famille en Chine ! Mais reviens travailler en France quand même ça sera plus simple !

Un énorme merci à Houyem, pour tes conseils, ton soutien, ta motivation et toutes les heures passer à discuter. On aura eu l'occasion de faire une grande partie de notre thèse ensemble et de partager nos connaissances, nos manips qui ont presque toutes marché ^^, mais surtout les voyages et les pizzas !

Je remercie également les footeuses de Bréquigny, de Mordelles et les coachs pour ces moments de détente indispensables.

Un très grand merci à Adeline et Caro pour les skypes du dimanche, d'avoir été présentes toute au long de cette thèse, et merci à Florian pour ta gentillesse.

Un immense merci à Anas, Didine, Fanny, Ju, Romain, Vir, Lora et à la GROSSE MOCHE pour tout !!!

Mes derniers remerciements iront à ma famille et particulièrement à mes parents, et à mes frères et sœurs, Pierre-Alexandre, Marie, Guillaume et Arnaud. Merci pour votre soutien, je ne vous en voudrais pas si vous arrêtez de lire à partir d'ici ;-)

Thesis outputs

Review article

F. Gaucher, S. Bonnassie, H. Rabah, P. Marchand, P. Blanc, R. Jeantet, R, G. Jan. Review: Adaptation of Beneficial Propionibacteria, Lactobacilli, and Bifidobacteria Improves Tolerance Toward Technological and Digestive Stresses. **Frontiers in Microbiology** (2019) 10, 841. doi:10.3389/fmicb.2019.00841.

Published or submitted research articles

F. Gaucher, S. Bonnassie, H. Rabah, P. Leverrier, S. Pottier, J. Jardin, V. Briard-Bion, P. Marchand, R. Jeantet, P. Blanc, G. Jan. Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*. **Journal of Proteomics**. (2019) 103400. doi:10.1016/j.jprot.2019.103400

F. Gaucher, K. Kponouglo, H. Rabah, S. Bonnassie, J. Ossemond, S. Pottier, J. *Jardin, V. Briard-Bion, P. Marchand, P. Blanc, R. Jeantet, G. Jan. Propionibacterium freudenreichii* osmoadaptation coupled to acid-adaptation, increases its viability during freeze-drying. Frontiers in Microbiology, **Frontiers in Microbiology** (2019) 10, 841. doi: 10.3389/fmicb.2019.02324

F. Gaucher, V. Gagnaire, H. Rabah, M-B, Maillard, S. Bonnassie, S. Pottier, P. Marchand, G. Jan, P. Blanc, R. Jeantet. Exploiting bacterial adaptation in order to optimize industrial production of dry *Propionibacterium freudenreichii*. **Microorganisms** (2019) 7, 477. doi: 10.3390/microorganisms7100477.

F. Gaucher, H. Rabah, K. Kponouglo, S. Bonnassie, S. Pottier, A. Dolivet, P. Marchand, R. Jeantet, P. Blanc, G. Jan. Fine-tuning of intracellular compatible solute concentrations to optimize *Propionibacterium freudenreichii* during drying. **Applied Microbiology and Biotechnology**, **in revision.**

F. Gaucher, S. Bonnassie, H. Rabah, P. Leverrier, S. Pottier, J. Jardin, V. Briard-Bion, P. Marchand, R. Jeantet, P. Blanc, G. Jan. Data from a proteomic analysis highlight different osmoadaptations in two strain of *Propionibacterium freudenreichii.* Data in Brief, **in revision.**

S. Huang, F. Gaucher, C. Cauty, J. Jardin, Y. Le Loir, R. Jeantet, X.D. Chen, G. Jan, Growth in Hyper-Concentrated Sweet Whey Triggers Multi Stress Tolerance and Spray Drying Survival in *Lactobacillus casei* BL23: From the Molecular Basis to New Perspectives for Sustainable Probiotic Production, **Frontiers in Microbiology**. 9 (2018). doi:10.3389/fmicb.2018.02548.

H. Rabah, O. Ménard, F. Gaucher, F.L.R. do Carmo, D. Dupont, G. Jan, Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during in vitro digestion, **Food Research International**. 106 (2018) 712–721. doi:10.1016/j.foodres.2018.01.035.

F.L.R. do Carmo, H. Rabah, S. Huang, F. Gaucher, M. Deplanche, S. Dutertre, J. Jardin, Y. Le Loir, V. Azevedo, G. Jan, *Propionibacterium freudenreichii* Surface Protein SlpB Is Involved in Adhesion to Intestinal HT-29 Cells, **Frontiers in Microbiology**. 8 (2017). doi:10.3389/fmicb.2017.01033.

F.L.R. do Carmo, H. Rabah, R.D. De Oliveira Carvalho, F. Gaucher, B.F. Cordeiro, S.H. da Silva, Y. Le Loir, V. Azevedo, G. Jan, Extractable Bacterial Surface Proteins in Probiotic–Host Interaction, **Frontiers in Microbiology**. 9 (2018). doi:10.3389/fmicb.2018.00645.

H. Rabah, S. Ferret-Bernard, S. Huang, L. Le Normand, F.J. Cousin, F. Gaucher, R. Jeantet, G. Boudry, G. Jan, The Cheese Matrix Modulates the Immunomodulatory Properties of *Propionibacterium freudenreichii* CIRM-BIA 129 in Healthy Piglets, **Frontiers in Microbiology**. 9 (2018). doi:10.3389/fmicb.2018.02584.

S. Huang, H. Rabah, S. Ferret- Bernard, L. Le Normand; F. Gaucher, S. Guérin, I. Nogret; Y. Le Loir, XD. Chen, G. Jan, G. Boudry, R. Jeantet, Propionic fermentation by the probiotic *Propionibacterium freudenreichii* to functionalize whey. **Functional Foods** (2018)

Oral presentation

F. Gaucher, Houem Rabah, S. Bonnassie, P. Marchand, P. Blanc, R. Jeantet, G. Jan. L'adaptation de *Propionibacterium freudenreichii* permet d'augmenter sa survie au séchage, Presented at 14th national congress of the Société Française de Microbiologie : SFM 2018

F. Gaucher, S. Bonnassie, Houem Rabah, P. Marchand, P. Blanc, R. Jeantet, G. Jan. Osmotic adjustments may exert benefits or drawbacks on *Propionibacterium freudenreichii* viability during freeze-drying. Presented at 13th international scientific conference on probiotics prebiotics, gut microbiota and health: IPC 2019, Prague, Czech Republic.

Poster presentation

F. Gaucher, H. Rabah, K. Kponouglo, S. Bonnassie, S. Pottier, A. Dolivet, P. Marchand, R. Jeantet, P. Blanc, G. Jan. Driving the nitrogen/carbon ratio of the culture medium determines *Propionibacterium freudenreichii* survival during spray drying. Poster presented at IDF Dairy for the next generation, 2018, Daejeonn, South Korea.

F. Gaucher, H. Rabah, K. Kponouglo, S. Bonnassie, S. Pottier, A. Dolivet, P. Marchand, R. Jeantet, P. Blanc, G. Jan. Driving the nitrogen/carbon ratio of the culture medium determines *Propionibacterium freudenreichii* survival during spray drying. Poster presented at Journées Scientifiques de l'ED EGAAL, 2019, Rennes, France.

Supervision

Lab-supervision of a master student (Koffigan Kponouglo, Unniverstié de Rennes 1) from 01/01/2018 to 15/07/2018.

Table of contents

List of Abbreviations

ABC: ATP-binding cassette transporters
aw: water activity
BAME: bacterial acid methyl ester
BFA; branched chain fatty acid
BHI: brain heart infusion medium
bsh: bile salts hydrolase
CAGR: Compound annual growth rate
CFU: colony-forming unit
CH : carbohydrates
CIRM-BIA: centre international de ressources microbiennes et de bactéries d'intérêt alimentaire
Clp: caseinolytic protease
COG: Clusters of Orthologous Group
Csp: Cold Shock Protein
DAPI: 4',6-diamidino-2-phénylindole
DC-SIGN : Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DE : Dextrose Equivalent
DNA : Deoxyribonucleic Acid
EF-Tu: elongation factor thermo unstable
EFSA: European Food Safety Authority
FAO: food and agriculture organization
Foxp3: forkhead box protein P3
GB: Glycine betaine
GRAS: generally recognized as safe
HSPs: Heat shock proteins
IDF: International Dairy Federation
IL-: interleukine
ITG-P: Institut Technique du Gruyère
KEGG : Kyoto Encyclopedia of Genes and Genomes
LAB : Lactic Acid Bacteria
MMO: Medium Minus Osmoprotectant
MRS: Man Rogosa and Sharp
MS: Mass Spectomerty
MU: Milk Ultrafiltrate
NAD: Nicotinamide adenine dinucleotide
NADP: Nicotinamide adenine dinucleotide phophate
NMR: Nuclear magnetic resonance
NPN: Non-protein nitrogen
OD: Optical density
PCA: Principal component analysis
PLP: pyridoxal 5'-phosphate
PVDF: PolyVinyliDene Fluoride
RNA: ribonucleic acid
ROS: Reactive Oxygen Specices
rRNA: Ribosomal ribonucleic acid
RU: Relative Unit
SD: standard deviation
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SFA: saturated fatty acid
Slp: Surface layer protein
SW: Sweet Whey
TMSP: Trimethylsilylpropanoic acid
pHi: Intracellular pH
Th17: T helper 17 cells
UFA: Unsaturated fatty acids
w/w: weight/ weight
WHO: World Health Organization
XIC: extracted ion chromatogram
YEL: Yeast Extract Lactate
YEL+L: YEL+Lactose

General introduction

0.1 Economic context of beneficial bacteria

Microorganisms, including bacteria, are present in all the ecological niches. Complex communities of microorganisms colonize host animals, plants and soil (Fig. 1, Ikeda-Ohtsubo et al., 2018). Multicellular organisms include plants and animals such as fishes, mollusks, and as well as humans. As shown in Figure 1, numerous ecological niches can be found in multicellular organisms: the skins and the intestines of livestock animals, humans and fish, the gill of fish, the phyllosphere and the rhizosphere of plants. Temperature, pH, oxygen level and nutrition availability impact the microbiota. Microbiota is defined as "the microorganisms that typically inhabit a particular environment, such as the soil, a body of water, or a site on or in an organism, considered as a group" (Miller-Kane and O'Toole, 2003). Livestock animal, fish and plant microbiota are highly diverse and can be composed by more 20 bacteria phyla. The most commonly phyla found in global microbiota throughout the body are Proteobacteria, Firmicutes and Bacteroidetes, but Actinobacteria is also found in lower amounts (Fig. 1, (Ikeda-Ohtsubo et al., 2018). Figure 1 highlights the difference between bacteria present in rhizosphere and in animals gut. Animal gut microbiota is influenced by the alimentation. Indeed, carnivorous and omnivorous animal microbiota contain a high proportion of Proteobacteria while herbivorous animal microbiota mostly contains Firmicutes.

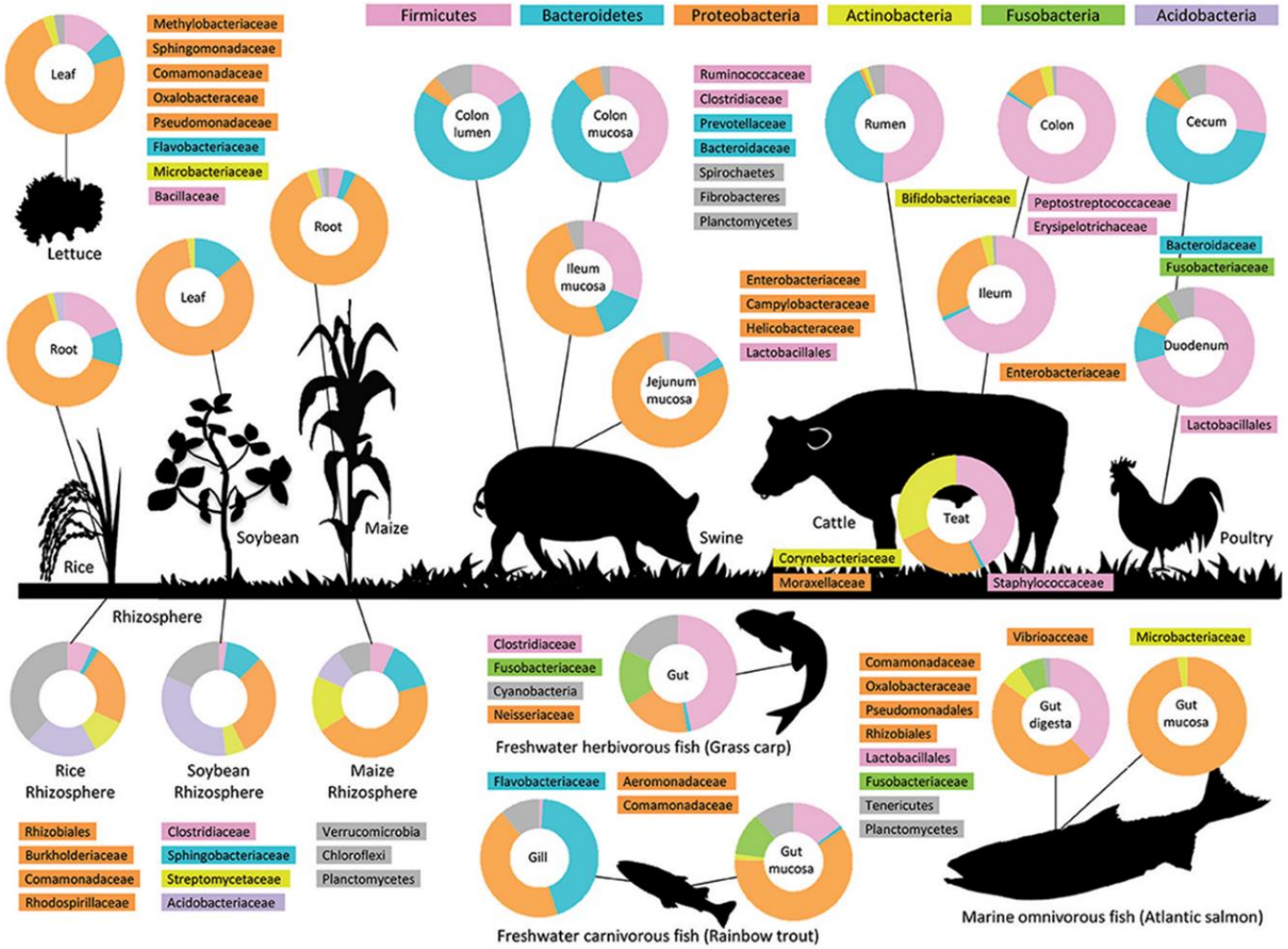


Figure 1: Microbiota in agriculture. The figure provides an overview of the bacterial composition of the microbiota of different parts of livestock animals, gill and intestines of fish, and phyllosphere and rhizosphere of plants at the phylum-level (pie-charts) and lower taxonomic levels. The data sources are 16S rRNA or metagenomic analyses of intestinal samples from pigs, cattle, chicken, Atlantic salmon, grass carp, gill and mucosal samples from rainbow trout, leaf samples from lettuce, leaf and rhizosphere samples from soybean, root and rhizosphere samples from maize, rice (Ikeda-Ohtsubo et al., 2018)

Selected strains of microorganisms present in the different environments, including plants and raw milk, are commonly used as starters. As an example, *Propionibacterium freudenreichii* can be found in the soil, silage, rumen and waste water (Falentin et al., 2010); as a starter, it is used during the Swiss-type cheese making where it plays a key role in aroma formation and curd eyes opening. Generally speaking, starters are beneficial bacteria used for food fermentation and conservation, as well as to improve organoleptic qualities of products (Fig. 2).

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001), this latter being livestock animals or human. In agriculture, some probiotics can improve livestock animal, fish and plant growth and improve the organism health (Fig. 2, Mehmood et al.; Pandiyan et al., 2013; García-Fraile et al., 2015; Hoseinifar et al., 2016; Vejan et al., 2016; Jahangiri and Esteban, 2018). In particular, probiotics can increase the body weight and the gain of weight for livestock animals (Hou et al., 2015; Mahfuz et al., 2017). Impact of biotic or abiotic stresses can be decreased by probiotics (Vejan et al., 2016; Zorriehzahra et al., 2016). In agriculture or in human, probiotics are reported to decrease pathogens presences (Yadav et al., 2013; Hynönen et al., 2014; Hou et al., 2015; Mahfuz et al., 2017). Due to the spread of antimicrobial resistance from agriculture to human society, taking into account and better understanding the microbiota related topics in agriculture is of prime importance, and probiotics constitute a potential alternative to feed-antibiotics.
In humans, probiotics can exert immunomodulation, can have anti-inflammatory properties and can facilitate the digestion thanks to enzymatic contribution. Promising results were also obtained in the context of cancers and related cure (Ma et al., 2019). Some probiotics need to be alive in order to exert beneficial effect. Probiotics benefits depend also on the adequate dose administered (Mimura, 2004; Johansson et al., 2015) and the International Dairy Federation (IDF) recommends a minimum of 10^7 live probiotic bacterial cells per gram or milliliter of product at the time of consumption (Corona-Hernandez et al., 2013).

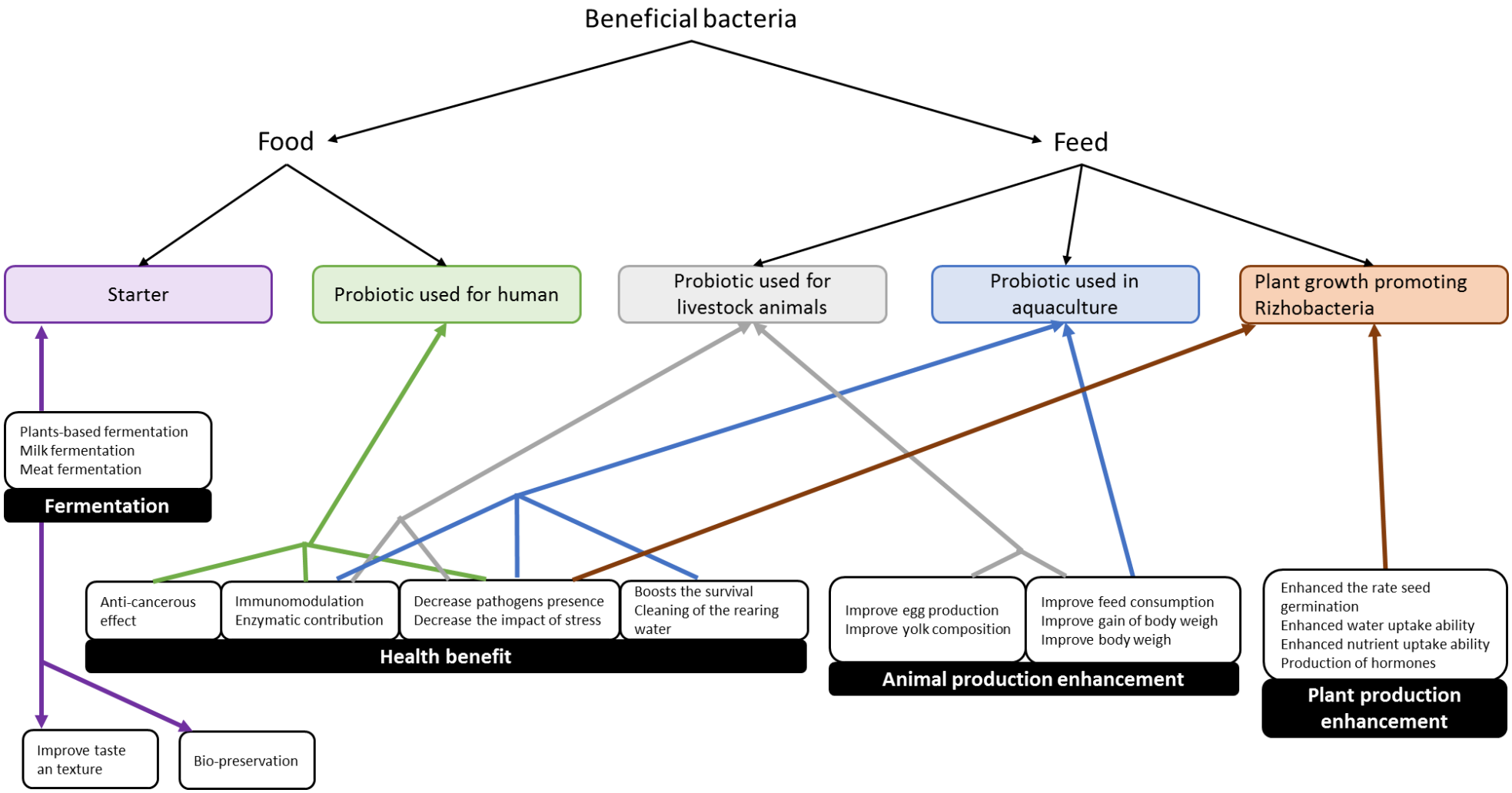


Figure 2: Beneficial bacteria market. The figure provides an overview of the possible utilization of beneficial bacteria as starters or probiotics.

These benefits and perspectives drive a rapid growth of starter and probiotics production and trade. The starter market is expected to grow regularly between 2019 and 2025 (Marketwatch.com, 2019). The global demand for probiotics is also increasing, and the global probiotics market was recently expected to grow from US$ 49.4 billion in 2018 up to US$ 69.3 billion by 2023 (marketsandmarkets.com, 2019), coming with a mean annual growth of 8%. Food and beverages containing probiotics currently dominate the probiotic market, but livestock animal probiotics is projected to have the fastest growth during the forecast period. In the probiotic market, bacteria are projected to dominate the market and Lactobacilli, Bifidobacteria, *Streptococcus thermophilus*, and to a lesser extent Enterococci, Bacilli and Pediococci are the most widely consumed. At present, Danone (France), Yakult Honsha (Japan), Nestlé (Switzerland), DowDuPont (US) and Chr. Hansen (Denmark) are sharing more than half of the probiotic market.

Probiotics are commonly produced under a powder form to extend the shelf life, facilitate the transport, storage and dosage and more generally to generate stable easy-to-use preparation (Huang et al., 2017b). Two drying processes can be used: the freeze-drying, that constitutes the benchmark, and the spray drying. The freeze-drying process has the advantage to be well adapted for bacteria production, as it gives rise to high bacterial viability. However, this process is expensive and high energy consuming (in the range of 5-10 kWh/kg of water evaporated). The spray drying process is continuous, has higher productivity, consumes less energy (in the range of 1-2 kWh/kg of water evaporated), but does not at the moment permit to obtain satisfying bacterial viability during drying. Both drying processes are stressful and an important part of bacteria may die after drying (described in chapter 1), which is detrimental as starters and some probiotics need to be alive to provide their beneficial effects. Indeed, spray drying imposes heat, osmotic and oxidative stresses while freeze-drying imposes cold, osmotic and oxidative (to a lesser extent than spray drying, as it only concerns final desorption stage) stresses (Santivarangkna et al., 2007; Huang et al., 2017b). During storage, bacteria suffer also of oxidative stress, which can be responsible for the viability decrease (Santivarangkna et al., 2007; Huang et al., 2017b). Bacteria adaptation processes, such as osmoadaptation, can be used to increase bacteria survival during drying and storage.

0.2 Context of the PhD project

During a first part of this PhD project, osmoadaptation was used to increase bacteria survival during spray drying. This topic was initially considered in another PhD project (Dr Song Huang) jointly supervised between Soochow University (School of Chemical and Environmental Engineering; Suzhou, China) and Agrocampus Ouest (Science et Technologie de Lait et de l'Oeuf - STLO, Agrocampus Ouest, INRA; Rennes, France). STLO investigates on technological and probiotics abilities of dairy starter, especially on starters having probiotics properties. More precisely, Song Huang worked with *P. freudenreichii* and *Lactobacillus casei*. As a first part of my PhD thesis work, I took part in the research work leading to one of his published papers, which deals with the use of hyper-concentrated sweet whey in order to improve *L. casei* survival during spray drying.

Growth in the hyper-concentrated sweet whey increased *L. casei* viability during spray drying. Sweet whey, a dairy industry by-product, can be used as a food-grade growth medium by industry for the production of probiotic and starters. A patent corresponding to this innovation, Biospraydry, was deposited and complementary experiments revealed that the viability of both *P. freudenreichii* CIRM-BIA 129 and *L. casei* BL23, during spray drying, was increased upon growth in hyper-concentrated sweet whey. This medium provokes the osmoadaptation of both strains, leading to enhanced tolerance towards heat, acid, bile salts and spray drying challenges. However, the mechanisms leading to enhanced resistance to spray drying remain within a "black box". Indeed, this patent highlights various questions:

- Does the increase of osmotic pressure alone permit higher bacteria tolerance to spray drying?
- Does the increase of osmotic pressure by addition of salts also permit higher bacteria tolerance to spray drying?
- Does the very high concentration of lactose in the hyper-concentrated sweet whey have another role than increasing osmotic pressure in bacteria adaptation?
- Does the high mineral salts concentration, such as calcium and phosphate, trigger increased bacterial resistance to spray drying?

Osmoadaptation permits higher bacterial tolerance to heat, acid, bile salts, freeze-drying and spray drying, this is called cross-protections (Jan et al., 2000; Desmond et al., 2001; Carvalho et al., 2003; Huang et al., 2016b). Other bacterial adaptation such as acid or heat-adaptation can also provide cross-protections (Jan et al., 2000; Li et al., 2009; Broadbent et al., 2010; Paéz et

al., 2012). These bacterial adaptations, alone or combined to osmoadaptation, can be promising in order to increase bacteria survival during drying.

The hyper-concentrated sweet whey is very interesting and can be used for probiotic or starter bacteria production for the dairy market, including infant formula, fermented milks, or cheese. For other markets, lactose presence can be a problem for consumer demand. Another issue with the use of hyper-concentrated sweet whey is the low concentration of bacteria in the powder. Indeed, the major constituent of the obtained powder is whey *per se*.

The production of probiotic or starter bacteria using hyper-concentrated growth media opens interesting avenues, but can be improved. For this, we need to better understand bacterial adaptations and cross-protections that result from such growth conditions. In addition, industrial constraint must be taken into account.

In this context, an industrial producer of starters and probiotics, Bioprox, and the STLO laboratory wanted to go further with this project. Bioprox financed my thesis project entitled VABENE (Viabilité Améliorée de Bactéries BENEfiques) under the form of a CIFRE contract.

Gwenaël Jan, Romain Jeantet and Sylvie Bonnassie, from UMR STLO and Philippe Blanc and Pierre Marchand, from Bioprox, for the industrial part, supervised my work during the thesis. One of the principal objectives of INRA is to ensure a good, healthy and sustainable diet. My thesis is part of the MICA (MIcrobiologie et Chaîne Alimentaire) department and more precisely in the part “Microbial” food (pre, pro and sym-biotic fermented foods) as levers of well-being and health. Bioprox is specialized in the production of probiotics, starters for dairy product, bread, and biofertilizants. The efficient production of probiotics and starters, more stable during storage, is one of their principal objectives.

During the thesis we tried to increase bacteria survival during spray drying but also during freeze-drying and during storage. In fact, freeze-drying is still highly used by Bioprox and it is possible that drying some weak and fragile bacteria by spray drying can be very difficult. We focused on *P. freudenreichii*, which is well known in the STLO laboratory and has the advantage to be robust. A second bacterium: *L. rhamnosus*, which is commercially important for Bioprox for different applications (dairy/probiotics), is also studied during the project. This bacterium has lower stress tolerance (tests internes), compared to *P. freudenreichii*. The aim of the first part of the thesis is a literature review on bacterial adaptation commonly used to increase beneficial bacteria tolerance to technological and digestive stress (Chapter 1). This is followed by the description of the objectives and strategy of the thesis (Chapter 2). In a first

part of the thesis the work was focused on the osmoadaptation, in a chemically defined medium, of *P. freudenreichii*, in order to better understand the underlying mechanisms (Chapter 3). Then, more complex media were used and their compositions were modulated to fine-tune the osmoadaptation (Chapter 4). Cross-protection provided by acid and heat-adaptation were then studied to increase *P. freudenreichii* tolerance (Chapter 5). Experimental design was then used to optimize *P. freudenreichii* and *L. rhamnosus* viability during drying and storage (Chapter 6).

Chapter 1: Adaptation of beneficial propionibacteria, lactobacilli and bifidobacteria improves tolerance towards technological and digestive stresses: a review

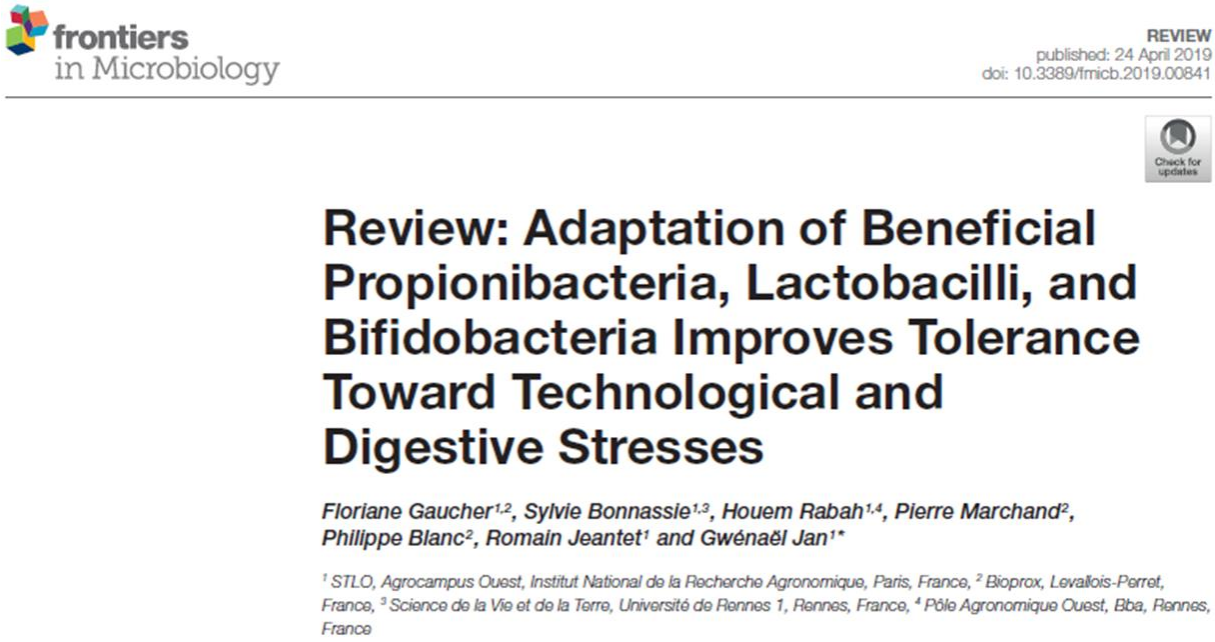
This chapter aims at providing a literature review of bacterial adaptations that may be used to improve probiotic and starter viability during drying, storage and digestion processes. The use of propionibacteria, Lactobacilli and bifidobacteria as probiotic and/or starter is first introduced. Then the stresses encountered by these bacteria during industrial production and digestion process are exposed. Bacteria possess many adaptation mechanisms, which may modulate their viability during stresses, these mechanisms are then described. Adaptive mechanisms include accumulation od compatible solutes and of energy storage compounds, which can be largely modulated by the culture conditions. They also include the regulation of energy production pathways, as well as the modulation of the cell envelope, i.e., membrane, cell wall, surface layer, and exopolysacharides. They finaly lead to the overexpression of molecular chaperones and of stress-responsive protease. Reported adaptations, which can trigger adaptive mechanism, and can increase bacteria survival during technological and digestive stresses, are then reviewed. The final section of this chapter, displays the aims, the originality and the strategy of this PhD project. Indeed, well understanding bacteria adaptation and the effect on bacteria stress tolerance is essential to propose alternative solution to the preservation of their properties upon long-term storage.

The aims of this chapter are to:

- Provide insight on bacteria adaptive mechanisms
- Highlight the benefits of using bacterial adaptation to improve bacteria survival during technological and digestives processes.
- Define the PhD research project and display the underlying research question

The main content in this chapter has been published as:

F. Gaucher, S. Bonnassie, H. Rabah, P. Marchand, P. Blanc, R. Jeantet, R, G. Jan, Review: Adaptation of Beneficial Propionibacteria, Lactobacilli, and Bifidobacteria Improves Tolerance Toward Technological and Digestive Stresses. Frontiers in Microbiology (2019) 10, 841. doi:10.3389/fmicb.2019.00841.



frontiers in Microbiology

REVIEW
published: 24 April 2019
doi: 10.3389/fmicb.2019.00841

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Review: Adaptation of Beneficial Propionibacteria, Lactobacilli, and Bifidobacteria Improves Tolerance Toward Technological and Digestive Stresses

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1.1 Introduction

Bacteria may constitute useful fermentation starters, healing probiotics, or both for the so-called "2-in-1" bacteria. These beneficial bacteria, within fermented foods as starter or within functional foods supplements as probiotic, are ingested in high amount and this constitutes a means to modulate the activity of the human gut microbiota (Collins and Gibson, 1999; Parvez et al., 2006; Moens et al., 2017). The ingested bacteria are essential for normal development of the immune system (Marco et al., 2017). Indeed, gut microbiota dysbiosis is increasingly correlated to various diseases, such as inflammatory bowel diseases and obesity, which are contisnously growing and constitute a public health problem linked to the Western diet (David et al., 2014; Plé et al., 2016). In order to decrease the consequences of such health problems, probiotics and fermented foods could be part of the solution. Indeed, the size of the probiotic market exceeded US$42 billion in 2016 and is expected to exceed US$64 billion in 2022 (marketsandmarkets.com, 2014). Probiotics are also increasingly incorporated into non-fermented functional foods such as infant formula, ice creams and cereal bars (Homayouni et al., 2008; Braegger et al., 2011; Bampi et al., 2016). These products constitute a growing market as well. The starter culture market, worth US$1.0 billion by 2018, is projected to grow at a CAGR (Compound Annual Growth Rate) of 5.6% (marketsandmarkets.com, 2014).

Starters and probiotics are usually dried to produce easy-to-use ingredients that are stable and flexible for different applications like food, feed and pharmaceutical products. At the industrial scale, two types of drying processes are implemented: freeze-drying and spray drying. In addition, drying is a way to keep bacteria alive on the long term at ambient temperature and to facilitate their storage and transport. At the industrial level, the use of frozen starter culture has the disadvantage of high energy costs during transportation and storage. Therefore, frozen starters go hand-in-hand with high operating costs (Santivarangkna et al., 2007). During powder production, storage and digestion, bacteria of interest may encounter multiple stresses, which may affect their survival and their beneficial effects. Indeed, they must survive during powder production and storage in a first time, and in a second time, during fermentation (starter) and digestion (probiotics) (Picot and Lacroix, 2004). A stress is defined as "any change in the genome, proteome or environment that imposes either reduced growth or survival potential. Such changes lead to attempts by a cell to restore a pattern of metabolism that either fits it for survival or faster growth" (Booth, 2002). Different pretreatments can induce an enhanced tolerance to various stresses, which may occurs during bacterial powders production and consumption. In this paper, we reviewed stresses encountered during drying processes, storage

and digestion. Then, we summarized the main molecular adaptation mechanisms induced by different pretreatments described in propionibacteria, lactobacilli and bifidobacteria, recognized as beneficial bacteria. We then describe the impact of the adaptation mechanisms induced by pretreatments on tolerance towards technological and digestive stresses. These data should contribute to making an informed choice of the best treatments for reinforcing bacteria during drying, storage and digestion

1.2 Beneficial effects of propionibacteria, bifidobacteria and lactobacilli

1.2.1 General features of propionibacteria, bifidobacteria and lactobacilli

This review is focused on propionibacteria, lactobacilli and bifidobacteria, which are Generally Recognized As Safe (GRAS) (Avalljaaskelainen and Palva, 2005; Picard et al., 2005; Cousin et al., 2010b). They constitute, to the best of our knowledge, the main bacterial genera considered as starter or/ and as probiotics.

Propionibacteria and bifidobacteria both belong to the Actinobacteria class, which comprises non-sporulating Gram-positive bacteria with a high G+C content (Cousin et al., 2010b). Propionibacteria are currently described as non-motile pleomorphic rods. Their optimal growth temperature is 30°C with a pH of 7.0. They are anaerobic aerotolerant, and many of them are catalase-positive. Propionibacteria can use a wide range of carbon sources such as organic acids (lactate), carbohydrates (lactose, glucose, galactose, and fructose) and alcohol (glycerol) (Cousin et al., 2010b). They are hetero-fermentative bacteria and their favorite substrate is lactate. For a consumption of 3 moles of lactate, propionibacteria produce 1 mole of acetate, 2 moles of propionate and 1 mole of carbon dioxide, according to the Fitz equation (Fitz, 1878). Bifidobacteria require cysteine in their growth medium, consume carbohydrates and produce lactate and acetate at molar ratio of 2:3 respectively, thus decreasing the pH of the culture medium. Their optimal growth temperature is 37°C (Hsu et al., 2007). Bifidobacteria are bifid or multiple-branched rods, and are strictly anaerobic and catalase-negative. They are naturally present in the gastrointestinal tract and vagina of animals (Schell et al., 2002; Savijoki et al., 2005; De Dea Lindner et al., 2007).

Lactobacilli are Gram-positive, firmicute bacteria with a low G+C content. Their natural habitat includes the digestive or reproductive tract of animals, raw milk, decomposing plants and fermented products. Most of them are anaerobic and non-sporuling. Lactobacilli require rich growth media that contains carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic

acid derivatives and vitamins (Chervaux et al., 2000; Elli et al., 2000). Lactobacilli optimal growth temperature is generally between 30°C and 40°C (Hammes and Hertel, 2015). Like bifidobacteria, lactobacilli use carbohydrates (as carbon and energy source) and produce lactic acid, therefore inducing a significant decrease in the pH of the medium. Among lactic acid bacteria, members of the genera *Streptococcus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* are also widely used as starters. The majority of available literature on probiotic effects and/or studies deals with lactobacilli, the largest genus within the group of lactic acid bacteria. In addition to lactobacilli, this review also deals with propionibacteria and bifidobacteria, used as probiotics and/or as starters.

1.2.2 Potential of propionibacteria, bifidobacteria and lactobacilli as probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Potential probiotics can be isolated from many sources (Mills et al., 2011). A minimum of 10^7 live probiotic bacterial cells per gram or milliliter of product at the time of consumption is recommended by the International Dairy Federation (IDF) (Huang et al., 2017b). Since these bacteria are included in probiotic preparations, the amount of live bacteria in these latter (tablets or capsules) have to be optimized (Gagnaire et al., 2015; Huang et al., 2017b), as does their viability during digestion (Kailasapathy, 2006; Rabah et al., 2018). Probiotic bacteria can enhance or preserve health, in strain-dependent manner among probiotic species (Lebeer et al., 2008; Le Maréchal et al., 2015; Plé et al., 2016).

As mentioned previously, the consumption of probiotics modulates the gut microbiota, which is a pivotal effect. Indeed, the presence of bifidobacteria in the intestine is very important, especially during the first years of life (De Dea Lindner et al., 2007), when they represent a majority of the intestinal microbiota. This part decreases with time over a lifetime (Sánchez et al., 2013).

The probiotic effects include also immunomodulation, described in propionibacteria, bifidobacteria and lactobacilli, which prevent and help to treat various immunes diseases as inflammatory bowel diseases or allergy (Picard et al., 2005; Steed et al., 2010; DuPont et al., 2014; Rong et al., 2015, 2015; Saez-Lara et al., 2015). Indeed, this early colonization of the gut by bifidobacteria and propionibacteria seems to prevent necrotizing enterocolitis (Colliou et al., 2017; Young et al., 2017). In addition, the manipulation of the gut microbiota with probiotics has been considered as a possible manner to prevent and treat obesity and cancers (Saez-Lara

et al., 2015; Dasari et al., 2017; Rabah et al., 2017; Brusaferro et al., 2018). Some *Propionibacterium freudenreichii* inhibits pathogens such as *Salmonella* Heidelberg (Nair FIM 2018) and meticillin-resistant *Staphylococcus aureus* (Sikorska and Smoragiewicz, 2013). Some *Lactobacillus amylovorus* inhibits *Escherichia coli* adhesion to intestinal cells (Hynönen et al., 2014) while some *Lactobacillus plantarum* inhibits *E. coli* 0157:H7 adhesion to collagen (Yadav et al., 2013). Selected strains of bifidobacteria were reported to inhibit growth and toxicity of *Clostridium difficile* (Valdés-Varela et al., 2016), or to affect the virulence of *Listeria monocytogenes in vitro* (Rios-Covian et al., 2018). They may protect against gastrointestinal disorders (Sanchez et al., 2007) and prevent diarrhea through their effects on the immune system and/or through enhanced resistance to colonization by pathogens, as *Clostridium difficile* (Cantero et al., 2018). Alleviation of lactose intolerance symptoms is demonstrated by lactobacilli, which provide the missing enzyme for lactose-intolerant people and, therefore, complementary to a host that is deficient in β-galactosidase (Levri et al., 2005; Pakdaman et al., 2015). The preclinical studies are promising and data highlight the strain-dependent aspect of these effects among propionibacteria, bifidobacteria and lactobacilli species. However, more investigations are needed to assess the probiotic effectiveness at clinical level and to determine the precise molecular mechanisms involved (Khalesi et al., 2019).

The molecular mechanisms identified in probiotics effects seem to be similar among propionibacteria, lactobacilli and bifidobacteria species. They include the production of secreted metabolites as short fatty acids (Lan et al., 2008; LeBlanc et al., 2017; Rabah et al., 2017) , and the presence of key surface components (Konstantinov et al., 2008; Foligné et al., 2013; Sengupta et al., 2013; Taverniti et al., 2013; Le Maréchal et al., 2015; Lightfoot et al., 2015; Sarkar and Mandal, 2016; Louis and Flint, 2017; do Carmo et al., 2018). In the dairy propionibacterium *P. freudenreichii*, the immunomodulatory properties are linked to the ability of selected strains to induce the release of the regulatory IL-10 by immune cells (Foligne et al., 2010). This property is mediated by surface proteins of the S-layer family (Le Maréchal et al., 2015; Deutsch et al., 2017). Accordingly, propionibacteria belonging to the human gut microbiota protect new-borns from necrotizing enterocolitis via Th17 cell regulation (Colliou et al., 2017). This property is also dependent on proteins of the surface layer and induce the generation of bacteria-specific Th17 cells, while maintaining IL-10^{+} regulatory T cells (Ge et al., 2019). In *Lactobacillus acidophilus*, immunomodulatory properties are dependent on the surface-layer protein SlpA, it occurs via binding to DC-SIGN receptors on dendritic cells and inducing a concentration-dependent production of IL-10 (Konstantinov et al., 2008). This

binding plays a pivotal role in *L. acidophilus* ability to mitigate induced colitis (Lightfoot et al., 2015). By contrast, in the probiotic *Bifidobacterium bifidum*, such a protective ability, as well as the immunomodulatory properties, are linked to the presence of cell surface polysaccharides. These last also act via regulatory dendritic cells, but through a partially Toll-like receptor 2-mediated mechanism, inducing the generation of Foxp3+ regulatory T cells (Verma et al., 2018). The strain-dependent nature of the probiotics beneficial effects is closely correlated to the strain-dependent nature of the ability of probiotics to express or produce these probiotics effectors.

1.2.3 Potential of propionibacteria, bifidobacteria and lactobacilli as starters

Fermented foods have been produced and consumed worldwide for centuries (Ebner et al., 2014; Marco et al., 2017). Fermented products can be produced from dairy, meat or plant matrices and then used to produce a large diversity of fermented foods. As a result, there is a multitude of food matrix-starter combinations. Some fermentations can be spontaneous, but many products require inoculation by starters (Rong et al., 2015; Walsh et al., 2016; Marco et al., 2017). To produce fermented foods, a large number of bacteria may be used. This number was shown to be stable over time and between countries (Fortina et al., 1998; De Dea Lindner et al., 2007), although the diversity of fermented foods tends to decrease because of industrialization (Marco et al., 2017).

Lactobacilli are extensively used as starters in the fermentation of dairy products, e.g., *L. acidophilus* in cheese, *Lactobacillus delbrueckii* in yogurt and *Lactobacillus kefiranofaciens* in kefir. *L. acidophilus* is also used in fermented plant products such as kimchi (Meira et al., 2015; Marco et al., 2017; Wang et al., 2018), that also provides other lactobacilli such as *Lactobacillus sakei* (Kwon et al., 2018), *L. plantarum* (Lim et al., 2018) and *Lactobacillus fermentum* (Yoo et al., 2017). Propionibacteria are widely used as ripening starters in the manufacture of Swiss-type cheeses (Thierry et al., 2011) and also contribute to the fermentation of vegetable products (Yu et al., 2015).Although not really considered as fermentation starters, bifidobacteria can be added before the fermentation process, with an impact on the final organoleptic properties of the product (Kailasapathy, 2006).

During fermentation, bacteria modify the matrices and contribute to the final flavor, texture , nutrition and organoleptic qualities (Marco et al., 2017). They offer an increased availability of bioactive molecules, vitamins, and other constituents due to the process of fermentation. Thus,

fermentation leads to increased digestibility of dairy products and plant matrices (Marco et al., 2017; Moens et al., 2017). Fermented dairy products have a low lactose content. Indeed, this carbohydrate is digested by the starter (Guarner et al., 2005), like other oligosaccharides (Sánchez et al., 2013; Moens et al., 2017). Furthermore, starters improve food storage and preservation, and may thus make it possible to decrease the use of additives (Mende et al., 2016).

Traditional fermented foods products can have a probiotic effect *per se* (Levri et al., 2005). Combining selected strains of probiotic bacteria with two-in-one abilities (both efficient starters and probiotics) leads to new functional fermented foods (Dimitrellou et al., 2016). Probiotics can be added to foods like in the case of bifidobacteria in fermented milk (Picot and Lacroix, 2004), but these type of processes require adequate technologies to keep bacteria alive (Racioppo et al., 2017). The use of probiotics in food is growing, but mechanisms used by bacteria to exert health benefits are not fully elucidated (Savijoki et al., 2005). The main troubles targeted are antibiotic-associated diarrhea, traveler's diarrhea, pediatric diarrhea, inflammatory bowel disease and irritable bowel disease. Although there is a limited number of clinical studies with fermented foods (Marco et al., 2017), preclinical studies show promising positive effects (Chen et al., 2014; Gao et al., 2015; Kato-Kataoka et al., 2016; Plé et al., 2016). All the above-described probiotic effects should be considered as potential effects. Indeed, the European Food Safety Authority (EFSA) requires substantial clinical proof before allowing a functional claim.

For all probiotics and starters, survival during industrial production processes, storage step and supply chain is a prerequisite. For *in situ* probiotic efficacy, effects that rely on the local production of beneficial metabolites such as short-chain fatty acids or vitamins require live bacteria capable of surviving digestive tract constraints. In contrast, for effects that rely on cellular fractions such as cell wall immunomodulating compounds, viability may be less crucial. Anyway, the probiotic bacterium should adapt industrial constraints to keep alive and then used.

1.3 Stress encountered during industrial production and digestion processes

1.3.1 Industrial drying process and storage

1.3.1.1 Freeze-drying process

Frozen starter culture is benchmark, as a high cooling rate and very low temperature (- 80°C) permit to increase bacteria viability. However, the use of frozen starter culture at the industrial level has the disadvantage of requiring negative temperatures during transportation and storage. Therefore, frozen starters go hand-in-hand with high operating costs (Santivarangkna et al., 2007). Freeze-drying is the most conventional process, i.e., the most frequently used with regard to its efficiency. The drying process of bacteria is conducted by sublimation, with the advantage of providing high bacterial viability (Santivarangkna et al., 2007). However, this process is discontinuous and expensive. Moreover, the ice crystals produced during freezing in the intracellular and extracellular compartments may be responsible for cell damage: compromised cellular integrity, broken DNA strands, altered transcription and replication, and reduced membrane fluidity. Freeze-drying favors also the appearance of holes in the cell membrane, which may cause cell death if another stress occurs (Carvalho et al., 2004; Giulio et al., 2005). During the freezing step, the cooling rate is an important factor for maintaining bacterial viability. In addition, bacteria suffer from osmotic stress (decrease in surrounding water activity) during freeze-drying, that represents a common constraint encountered during the drying of bacteria. The composition of drying media is highly studied in the aim of improving bacterial viability during the process. As an example, skim milk can be used as a drying matrix since it has the advantage of stabilizing the cell membrane constituents and contains proteins that build a protective coating for the bacteria (Carvalho et al., 2004). Protective agents can be added to the drying matrix in order to improve bacterial viability during storage. These molecules need to have an amino group, a secondary alcohol group, or both (Carvalho et al., 2004). Suitable protective agents should provide cryoprotection for the bacteria during freezing, be easily dried, and improve drying matrix stability and rehydration (Zhao and Zhang, 2005). Several sugars are used to protect bacteria, e.g., glucose, fructose, lactose and trehalose. Sugar alcohols like sorbitol and inositol can also be used. Monosodium glutamate can stabilize protein structure. Antioxidants such as ascorbate can be also added in order to protect membrane lipids against damage caused by the freeze-drying process (Carvalho et al., 2004; Kurtmann et al., 2009). The efficiency of these different agents to limit freeze-drying stresses are bacteria dependent (Zhao and Zhang, 2005).

1.3.1.2 Spray drying process

Spray drying decreases the cost of water removal by a factor of six, in comparison to freeze-drying (Paéz et al., 2012), while producing powder with a yield four to seven times higher than that of freeze-drying (Golowczyc et al., 2011). Spray drying has the advantage of being a continuous process, drying and encapsulating bacteria occurre in the same time (Peighambardoust et al., 2011). This process constitutes thus an emerging alternative to freeze-drying (Huang et al., 2016b).

Spray drying can be divided into two steps: an initial constant rate evaporation stage, which is at the wet-bulb temperature, and the falling rate evaporation stage, which leads to product temperature increases towards the outlet air temperature at the end of drying (Huang et al., 2017b). The time-temperature combination is an important factor that determines the extent of cell death, in particular depending on the outlet temperature value (Santivarangkna et al., 2007). The spray drying process usually leads to high temperatures over a short time. Heat stress is a commonly encountered stress, affecting bacterial viability during spray drying. Cell death is due to the destruction of more than one critical component (Peighambardoust et al., 2011), including major damage to membrane lipids and/or aggregation of membrane protein (Mills et al., 2011). Indeed, the membrane bilayer structure is thermodynamically unstable (Peighambardoust et al., 2011) so that damage caused to the membrane is the first cause of viability losses (Simpson et al., 2005). Other damage may occur inside the cell during drying. It affects DNA (Simpson et al., 2005), intracellular proteins and ribosomes (Mills et al., 2011). RNAs may also be impaired as a result of the escape of Mg^{2+} (Huang et al., 2017b). Indeed, the use of lower temperatures during spray drying is possible to enhance viability, but the high water activity (a_w) obtained in the powder limits its stability during storage. Moreover, bacteria suffer from oxidative and osmotic stresses that are coupled to heat stress during spray drying (Huang et al., 2017b), first, from the drying air and, second, because of the concomitant increase in osmotic pressure with the loss of water (Desmond et al., 2001).

Survival rate during spray drying is species and strain-dependent, which is related to species- and strain-dependent heat tolerance ability (Peighambardoust et al., 2011a). *Propionibacterium freudenreichii* has a better chance to survive than *Lactobacillus casei* (Huang et al., 2016a), and thermotolerant *S. thermophilus* survives better than lactobacilli (*L. delbrueckii ssp bulgaricus* and *L. acidophilus*) (Huang et al., 2017b). One of the most important factors for spray drying

survival is the intrinsic resistance to heat that is species- and strain-dependent and determine the survival of the bacteria during the process. The difference in heat resistance is more important when the heat challenge increases (Paéz et al., 2012). The thermic intrinsic sensitivity of a strain is essential to determine survival (Simpson et al., 2005) and can be optimized to enhance viability (Santivarangkna et al., 2007). In contrast, the oxygen tolerance of bifidobacteria is not correlated with a good viability during drying (Simpson et al., 2005).

Statistical models were developed to predict survival during spray drying based on the hypothesis that bacterial death during spray drying followed a probability distribution (Perdana et al., 2014). However, this prediction was not very precise because bacteria can be pre-adapted to technological stress, and because the drying medium may play a protective role. Viability can be improved by adding either trehalose, which stabilizes membranes and proteins and decreases membrane phase transition temperatures, or gelatin, gum Arabic or fruit juice (Huang et al., 2017b). It is thus possible to modulate the drying media composition to enhance bacterial survival (Sollohub and Cal, 2010). However, adding protective agents to the drying medium will not necessarily lead to optimal bacteria survival during storage (Santivarangkna et al., 2007).

1.3.1.3 Storage

During storage, bacterial viability generally remains higher for freeze-dried powders compared to spray-dried powders (Santivarangkna et al., 2007), which may be due to heat stress (Huang et al., 2017b). Viability indeed decreases during storage, particularly at ambient temperature (Simpson et al., 2005). During storage, the water activity of the dry product, the glass transition temperature, the environmental relative humidity, temperature and light are important factors for maintaining cell viability (Santivarangkna et al., 2007; Huang et al., 2017b). For freeze-dried powders, stability is greater at low temperatures and in oxygen-free environments (Santivarangkna et al., 2007). Viability is inversely correlated to storage temperature (Paéz et al., 2012), but industry generally looks for stable dry cultures at ambient temperature for marketing purposes. The presence of oxygen decreases viability because of lipid oxidation. Some authors therefore attempted to add antioxidants to decrease the impact of oxidative stress, but the results were not convincing for long-term storage (Santivarangkna et al., 2007).

1.3.2 Digestion

Some probiotic effects require live bacteria in sufficient amounts in the large intestine. Probiotic bacteria have to pass the stomach, which seems to be the most difficult part of the gastrointestinal tract to cross during digestion. Bacteria have to survive the acid conditions and the presence of bile salts (Mainville et al., 2005). The harsh conditions of the gastrointestinal tract also include variations in the redox potential, and the presence of hydrolytic enzymes such as lysozyme, of various proteases and lytic compounds found in pancreatin, and of bile salts. Acid stress is a major limit to fermentation by beneficial bacteria. A decrease in the pH is generally the reason why fermentation stops. Probiotics such as bifidobacteria are also added to acidic yogurt-type products and then stored at 4°C before consumption. Finally, all the beneficial bacteria face severe acid stress during the first step of digestion (ie. stomach), which limits the *in situ* activity. Viability in the intestinal compartment is species and strain-dependent (Picot and Lacroix, 2004; Alcantara and Zuniga, 2012). For example, *L. casei* and other lactobacilli have low tolerance to low pH (Broadbent et al., 2010), although it is still higher than that of some bifidobacteria (except for *Bifidobacterium infantis*). The capacity to resist bile salts is strain-dependent, and probiotic strains can thus be selected for their tolerance to bile salts. Bile salts act as detergents (Alcantara and Zuniga, 2012; Papadimitriou et al., 2016) and cause cell damage and cytotoxicity (Arnold et al., 2018). Molecular mechanisms leading to bile adaptation were evidenced in dairy propionibacteria (Leverrier et al., 2003, 2004), in lactobacilli (Ruiz et al., 2013; Goh and Klaenhammer, 2014) and in probiotic bifidobacteria (Sanchez et al., 2007; Ruiz et al., 2013).

Lactobacilli and bifidobacteria have bile salts hydrolase *(bsh)* genes, which can have a positive or negative impact on bile salts tolerance, so the selection requires a careful examination of microbial physiology (Arnold et al., 2018). Since osmolarity is constantly changing in the gastrointestinal tract, bacteria survival may be affected by osmotic stress (De Dea Lindner et al., 2007).

1.4 Mechanisms of stress adaptation triggered by pretreatements

Beneficial bacteria used as technological starters will have to face technological stresses such as heat, cold, oxidative and osmotic stresses. Probiotic bacteria should also survive digestive stress, in addition to the above-mentioned stresses. Digestive stresses include acid, bile salts and osmotic constraints. The main adaptive mechanisms triggered by bacteria to deal with such

damage are DNA repair, metabolic pathways of lipid modification, chaperones and proteases, accumulation of compatible solutes and Reactive Oxygen Specices (ROS) detoxification (Desmond et al., 2001; Sheehan et al., 2006; Li et al., 2009). The medium culture choice is crucial since its composition may induce different adaptation mechanisms, as detailed below. General adaptive mechanisms induced by different pretreatments of propionibacteria, bifidobacteria and lactobacilli are presented in the Figure 3. Adaptive mechanisms are induced by specific pretreatments are reported in Table 1.

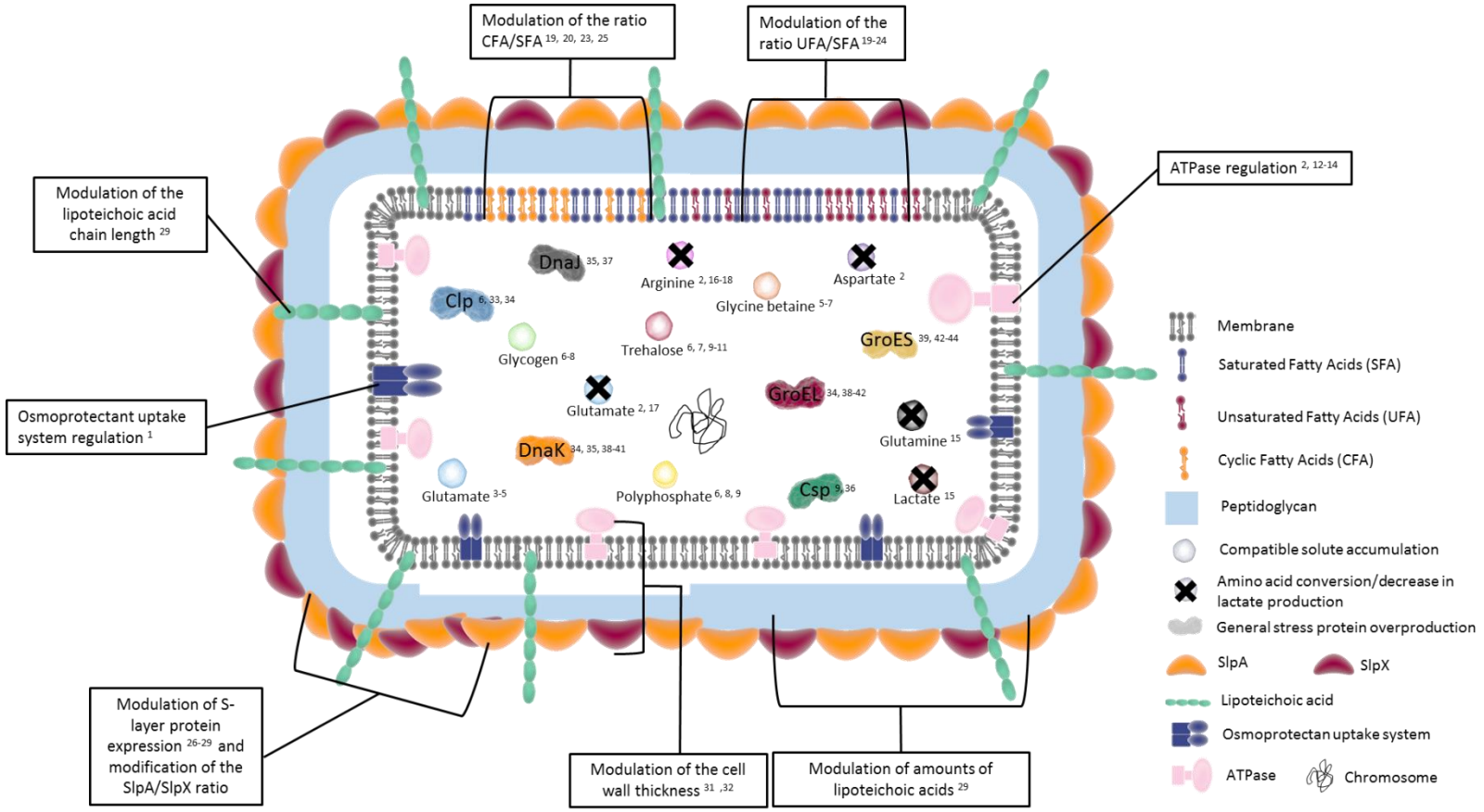


Figure 3: Key actors of adaptive mechanisms in bacteria during osmotic, acid, oxidative, heat, cold and bile salts adaptation. General adaptive bacterial mechanisms during osmotic, acid, oxidative, heat, cold and bile salts treatment are represented. Peptidoglycan is represented in blue. Membrane lipids under normal growth are represented in grey. Amounts of saturated (blue), unsaturated (red) and cyclic (yellow) fatty acids are modulated by treatments. S-layer proteins, which may be involved in adaptation, are represented in yellow and red outside the peptidoglycan. Liptechoic acids, whose length is modulated, are presented in green. Inducible transmembrane ATPase and osmoprotectant uptake systems are represented in pink and blue, respectively. In the cytoplasm, general stress proteins are represented by different colors. Colored circles represent different compatible solutes and energy storage compounds. Crosses on circles mean the conversion of the molecule. The chromosome is represented in black. The numbers indicate corresponding references in the tables.

Table 1: Adaptive mechanisms induced by stressing conditions or by modifications of the growth medium in bifidobacteria, propionibacteria and lactobacilli.

+ : indicates an improvement of survival; - : indicates a decrease of survival; 0 : indicates no effect on survival; *: indicates a cited reference; a: *Lactobacillus*; b: *Propionibacterium*; c: *Lactococcus*; d: *Bifidobacterium*

Adaptive mechanism	Stress	Bacteria	References	Corresponding number in the figures
ABC transporter	Heat	*L.*[a] *rhamnosus*	(Prasad et al., 2003)	1
Arginine accumulation	Addition of arginine	*P.*[b] *acidopropionici*	(Guan et al., 2013)	2
Glutamate accumulation	Addition of glutamate	*L. sakei*	(Ferreira et al., 2005)	3
Glutamate accumulation	Osmotic	*L. plantarum*	(E Kets, P Teunissen and J de Bont, 1996; Glaasker et al., 1996)	4, 5
Glycine betaine accumulation	Osmotic	*P. freudenreichii*	(Huang et al., 2016)	6
Glycine betaine accumulation	Osmotic	*Lc.*[c] *Lactis*	(Romeo et al., 2003)	7
Glycine betaine accumulation	Osmotic	*L. plantarum*	(Glaasker et al., 1996)	5
Glycogen accumulation	Addition of carbon (raffinose and trehalose)	*L. acidophilus*	(Goh and Klaenhammer, 2014)	8
Glycogen accumulation	Cold	*P. freudenreichii*	(Dalmasso et al., 2012a)	7
Glycogen accumulation	Osmotic	*P. freudenreichii*	(Huang et al., 2016)	6
PolyP accumulation	Addition of polyphosphate	*Lactobacillus*	(Alcantara et al., 2014)	8
PolyP accumulation	Cold	*P. freudenreichii*	(Dalmasso et al., 2012b)	9
PolyP accumulation	Osmotic	*P. freudenreichii*	(Huang et al., 2016)	6
Trehalose accumulation	Acid	*P. freudenreichii*	(Cardoso et al., 2007)	10
Trehalose accumulation	Cold	*P. freudenreichii*	(Dalmasso et al., 2012b, 2012a)	9, 7
Trehalose accumulation	Osmotic	*P. freudenreichii*	(Cardoso et al., 2004; Huang et al., 2016)	11,6

Trehalose accumulation	Oxydative	*P. freudenreichii*	(Cardoso et al., 2007)	10
F_0F_1-ATPase upregulated	Acid	*B.*[d] *longum*	(Sanchez et al., 2007)	12
F_0F_1-ATPase upregulated	Acid	*L. rhamnosus*	(Corcoran et al., 2005)	13
F_0F_1-ATPase upregulated	Acid	*P. acidopropionici*	(Guan et al., 2013)	2
F_0F_1-ATPase upregulated	Addition of glucose	*L. rhamnosus*	(Corcoran et al., 2005)	13
F_0F_1-ATPase upregulated	Bile salt	*B. animalis*	(Sanchez et al., 2006)	14
F_0F_1-ATPase upregulated	Increase of NAD/NADH	*P. acidopropionici*	(Guan et al., 2013)	2
L-lactate deshydrogenase downregulated	Acid	*L. rhamnosus*	(Koponen et al., 2012)	15
Arginine conversion	Acid	*L. reuteri*	(Rollan et al., 2003; Teixeira et al., 2014)	16, 17
Arginine conversion	Acid	*P. acidopropionici*	(Guan et al., 2013)	2
Arginine conversion	Heat	*L. fermentum*	(Vrancken et al., 2009)	18
Arginine conversion	Osmotic	*L. fermentum*	(Vrancken et al., 2009)	18
Aspartate conversion	Acid	*P. acidopropionici*	(Guan et al., 2013)	2
Glutamate conversion	Acid	*L. reuteri*	(Teixeira et al., 2014)	17
Glutamate conversion	Acid	*P. acidopropionici*	(Guan et al., 2013)	2
Glutamine conversion	Acid	*L. reuteri*	(Koponen et al., 2012)	15
Decrease in unsaturated/saturated fatty acid ratio	Acid	*L. casei*	(Broadbent et al., 2010)	19
Decrease in unsaturated/saturated fatty acid ratio	Acid	*L. delbrueckii*	(Streit et al., 2008)	20
Decrease in unsaturated/saturated fatty acid ratio	Osmotic	*L. casei*	(Machado et al., 2004)	21
Increase in unsaturated/saturated fatty acid ratio	Bile salts	*L. reuteri*	(Taranto et al., 2003)	22
Increase in unsaturated/saturated fatty acid ratio	Cold	*L. acidophilus*	(Wang et al., 2005)	23
Increase in unsaturated/saturated fatty acid ratio	Heat	*L. helveticus*	(Lanciotti et al., 2001)	24
Increase in unsaturated/saturated fatty acid ratio	Oxidatif	*L. helveticus*	(Lanciotti et al., 2001)	24
Decrease in the number of cycloporpane fatty acids	Acid	*L. delbrueckii*	(Streit et al., 2008)	20
Decrease in the number of cycloporpane fatty acids	Acid	*L. bulgaricus*	(Li et al., 2009)	23

Increase in the number of cycloporpane fatty acids	Acid	*L. casei*	(Broadbent et al., 2010)	19
Increase in the number of cycloporpane fatty acid	Acid	*L. acidophilus*	(Wang et al., 2005)	23
Increase in the number of cycloporpane fatty acid	Heat	*L. bulgaricus*	(Li et al., 2009)	25
Increase in S-layer production	Acid	*L. acidophilus*	(Khaleghi and Kasra, 2012)	26
Increase in S-layer production	Bile salts	*L. acidophilus*	(Grosu-Tudor et al., 2016; Khaleghi et al., 2010)	27, 28
Increase in S-layer production	Heat	*L. acidophilus*	(Grosu-Tudor et al., 2016; Khaleghi and Kasra, 2012)	27, 26
Increase in S-layer production	Osmotic	*L. acidophilus*	(Grosu-Tudor et al., 2016; Palomino et al., 2013, 2016)	27, 29, 31
Thinning of the cell wall	Osmotic	*L. casei*	(Piuri et al., 2005)	30
Increase in the surface hydrophobicity	Osmotic	*L. casei*	(Machado et al., 2004)	21
Reduction of lipotecoïc acid	Osmotic	*L. casei*	(Palomino et al., 2013)	29
Increase in the negative charge of the cell wall	Osmotic	*L. casei*	(Palomino et al., 2013)	29
Reduction of the lipotecoïc chain	Osmotic	*L. casei*	(Palomino et al., 2013)	29
Increase in SlpA/SlpX ratio	Osmotic	*L. acidophilus*	(Palomino et al., 2016)	31
Increase in the density and the thickness of the cell wall	Addition of transglutaminase	*Lc. Lactis*	(Li et al., 2015)	32
ClpB overproduction	Acid	*L. plantarum*	(Bove et al., 2013)	33
ClpB overproduction	Bile salt	*P. freudenreichii*	(Leverrier et al., 2004)	34
ClpB overporudction	Heat	*B. breve*	(De Dea Lindner et al., 2007)	35
ClpB overproduction	Heat	*P. freudenreichii*	(Leverrier et al., 2004)	34
ClpB overporudction	Osmotic	*B. breve*	(De Dea Lindner et al., 2007)	35
ClpB overproduction	Osmotic	*P. freudenreichii*	(Huang et al., 2016; Leverrier et al., 2004)	6, 34
ClpC overproduction	Acid	*P. freudenreichii*	(Leverrier et al., 2004)	34
ClpE overproduction	Acid	*L. plantarum*	(Bove et al., 2013)	33
ClpP overproduction	Acid	*L. plantarum*	(Bove et al., 2013)	33
CspA overproduction	Cold	*P. freudenreichii*	(Dalmasso et al., 2012b)	9

CspB overproduction	Cold	*P. freudenreichii*	(Dalmasso et al., 2012b)	9
CspC overproduction	Cold	*L. plantarum*	(Derzelle et al., 2000)	36
CspL overproduction	Cold	*L. plantarum*	(Derzelle et al., 2000)	36
CspP overproduction	Cold	*L. plantarum*	(Derzelle et al., 2000)	36
DnaJ1 overproduction	Acid	*B. longum*	(Jin et al., 2015)	37
DnaJ1 overproduction	Heat	*B. breve*	(De Dea Lindner et al., 2007)	35
DnaJ1 overproduction	Osmotic	*B. breve*	(De Dea Lindner et al., 2007)	35
DnaK overproduction	Acid	*L. delbrueckii*	(Gouesbet et al., 2002; Lim et al., 2001)	38, 39
DnaK overproduction	Bile salt	*P. freudenreichii*	(Leverrier et al., 2004)	34
DnaK overproduction	Bile salt	*P. freudenreichii*	(Savijoki et al., 2005)	40
Dnak overproduction	Heat	*B. breve*	(De Dea Lindner et al., 2007)	35
DnaK overproduction	Heat	*P. freudenreichii*	(Savijoki et al., 2005)	40
DnaK overproduction	Heat	*L. rhamnosus*	(Prasad et al., 2003)	41
Dnak overproduction	Osmotic	*B. breve*	(De Dea Lindner et al., 2007)	35
DnaK overproduction	Osmotic	*P. freudenreichii*	(Leverrier et al., 2004)	34
DnaK overproduction	Heat	*P. freudenreichii*	(Leverrier et al., 2004)	34
GroEL overproduction	Acid	*L. delbrueckii*	(Gouesbet et al., 2002; Lim et al., 2001)	38, 39
GroEL overproduction	Acid	*P. freudenreichii*	(Jan et al., 2001; Leverrier et al., 2004)	42, 34
GroEL overproduction	Bile salt	*P. freudenreichii*	(Savijoki et al., 2005)	40
GroEL overproduction	Heat	*B.breve*	(Ventura et al., 2004)	43
GroEL overproduction	Heat	*L. rhamnosus*	(Prasad et al., 2003)	41
GroEL overproduction	Heat	*P. freudenreichii*	(Savijoki et al., 2005)	40
GroES overproduction	Acid	*L. delbrueckii*	(Lim et al., 2001; Silva et al., 2005)	39, 44
GroES overproduction	Acid	*P. freudenreichii*	(Jan et al., 2001)	42
GroES overproduction	Heat	*B. breve*	(Ventura et al., 2004)	43
GroESL overproduction	Heat	*L. johnsonii*	(Walker et al., 1999)	45
grpE overproduction	Heat	*B. breve*	(De Dea Lindner et al., 2007)	35
grpE overproduction	Osmotic	*B. breve*	(De Dea Lindner et al., 2007)	35
HtrA overproduction	Bile salt	*P. freudenreichii*	(Savijoki et al., 2005)	40

HtrA overproduction	Heat	*P. freudenreichii*	(Savijoki et al., 2005)	40
SodA overproduction	Bile salt	*P. freudenreichii*	(Leverrier et al., 2004)	34
SodA overproduction	Heat	*P. freudenreichii*	(Leverrier et al., 2004)	34
SodA overproduction	Osmotic	*P. freudenreichii*	(Leverrier et al., 2004)	34

1.4.1 Accumulation of compatible solute and energy storage

A compatible solute is a small organic molecule that is polar, highly soluble in water, and that has a neutral isoelectric point. It behaves like an osmolyte, allowing a live cell to adapt to an osmotic stress (Csonka, 1989). During osmotic stress, bacteria accumulate compatible solutes (Csonka, 1989), either transported from the external medium, or synthesized *de novo*, to restore turgescent pressure and enable cell growth and division (Csonka and Hanson, 1991). Compatible solutes are unable to rapidly cross bacterial membranes without the involvement of a transport system and, for the most part, do not carry an electrical charge at a neutral pH. Uncharged molecules can be accumulated at a high concentration without disturbing the metabolism (Csonka, 1989). There is a limited number of molecules considered to be compatible solutes, and they can be divided into two categories: the first one corresponds to sugars and polyols, and the second is composed of alpha and beta amino acids and their derivatives (Roesser and Müller, 2001). Compatible solutes preserve the conformation of proteins submitted to osmotic constraint. Generally, compatible solutes are excluded from the immediate vicinity of the proteins by an unfavorable interaction between the protein surface and the compatible solutes. This mechanism is known as "preferential excluding" (Roesser and Müller, 2001).

1.4.1.1 Accumulation of sugar and polyol

1.4.1.1.1 Accumulation of trehalose

The versatile role of trehalose accumulation in stresses adaptation was schematized in the Figure 4. Indeed, trehalose is a stable molecule (Giulio et al., 2005) which prevents protein aggregation, facilitates refolding, and protects cells and cellular proteins from damages caused by oxygen (Cardoso et al., 2004). It is also suggested that trehalose plays a role in the stabilization of cell membranes (Cardoso et al., 2007). Trehalose can act as an intracellular carbon stock and is consumed after exhaustion of the external carbon source (Cardoso et al., 2004). High sugar concentration in the culture medium promotes trehalose accumulation. Osmotic stress triggers accumulation of trehalose in *P. freudenreichii* and *L. casei* (Cardoso et al., 2004; Huang et al., 2016b). The accumulation of compatible solutes is facilitated in a rich medium as opposed to a chemically-defined media. Trehalose accumulation by *P. freudenreichii* may also be induced by other stresses such as cold (Dalmasso et al., 2012b), oxidative and acid stresses (Cardoso et al., 2004, 2007). Moreover, trehalose can decrease the loss of viability during storage after freeze-drying (Giulio et al., 2005).

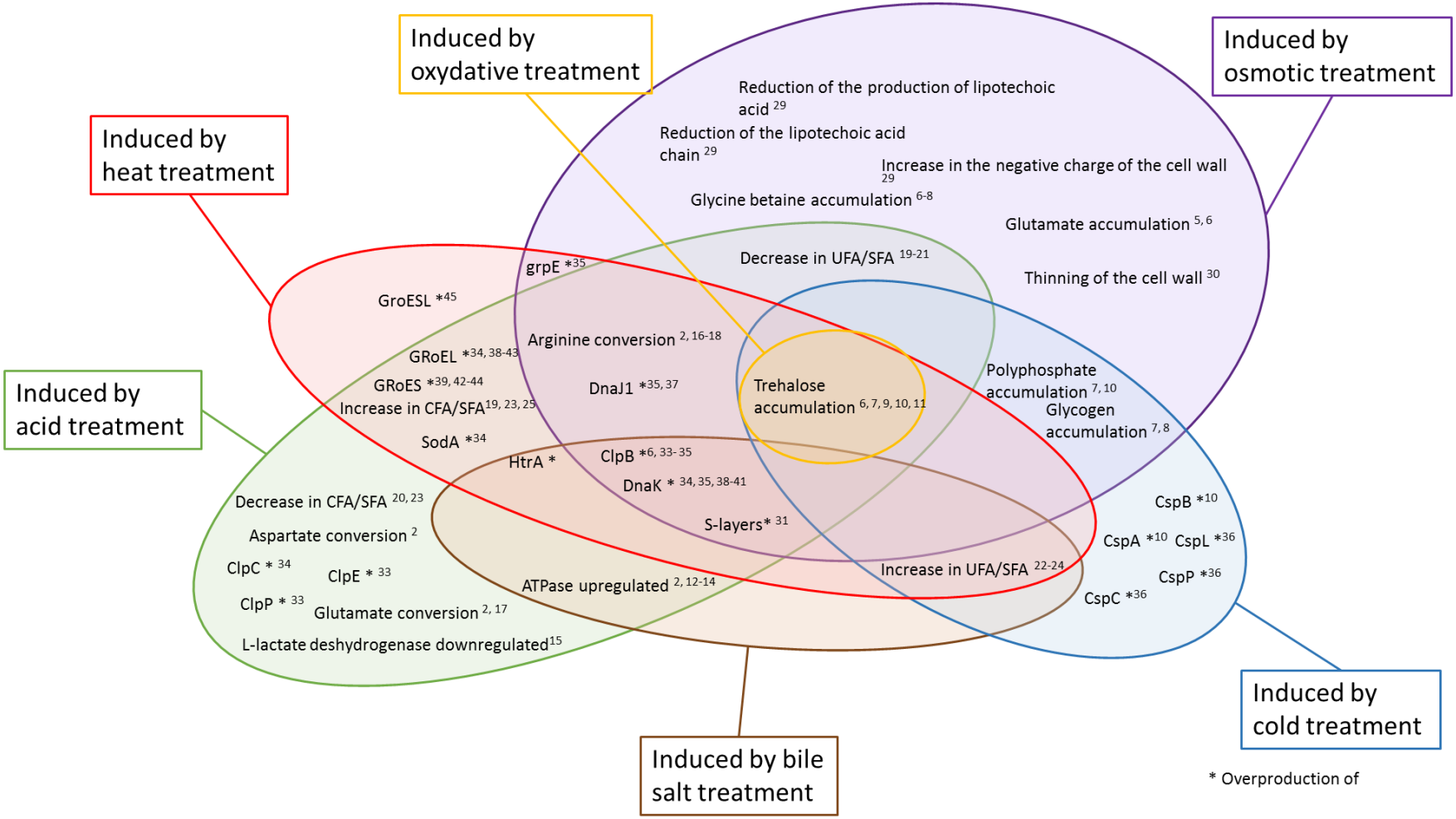


Figure 4: Different treatments modulate the key actors of adaptive mechanisms. Colored areas represent the different treatments studied. In yellow: oxidative; in red: heat; in green: acid; in brown: bile salts; in blue: cold; and in purple: osmotic treatment. The key actors of adaptive mechanisms indicated inside a bubble are modulated by the corresponding treatment. The numbers indicate corresponding references in the tables.

1.4.1.1.2 Accumulation of glycerol

Huang et al. observed that Glycerol 3–phosphate dehydrogenase was over-expressed by *P. freudenreichii* in hyperconcentrated sweet whey medium (Huang et al., 2016b). During osmotic stress, glycerol modification on oligosaccharides is increased. This phenomenon induce a possible increase of hydroxyl groups available number in saccharide molecules and replace water molecules for cellular interaction, during osmotic stress, for example (Prasad et al., 2003).

1.4.1.2 Accumulation of amino acids

In osmotic adaptation, lactobacilli regulate the intracellular concentration of amino acids like proline and glutamate (Papadimitriou et al., 2016). Glutamate is a key metabolite which plays a role in various cell stress responses (Feehily and Karatzas, 2013). This amino acid is crucial for the primary response to hyper-osmotic shock (Glaasker et al., 1996). The first response to a high osmolarity is the accumulation of K^+ and its counterion, glutamate. Glutamate has a minor influence on the acid tolerance capacity of *Acidipropionibacterium acidipropionici* (Guan et al., 2013). The addition of glutamate in the growth medium enhanced glutamate accumulation by *L. sakei,* even in the absence of stressing conditions (Ferreira et al., 2005).

An increase in lysine production during stress has been observed in lactobacilli (Papadimitriou et al., 2016). In *A. acidipropionici*, a similar phenomenon was observed for other amino acids such as arginine and aspartate, which were accumulated during acid-adaptation (Guan et al., 2013). GABA (decarboxylation of glutamate to γ-aminobutyrate) is involved in acid tolerance (Bron et al., 2006; Guan et al., 2013). The lysine degradation pathway is activated under acidic conditions in *L. plantarum* (Heunis et al., 2014).

Glycine betaine is known to protect different bacterial species against high osmolarity and is considered to be the most effective compatible solute. (Roesser and Müller, 2001). During osmotic stress, *P. freudenreichii* accumulation of glycine betaine occurs via the OpuABC (or Bus ABC) transporter, which is osmotically induced (Fig. 3, (Huang et al., 2016b). The glycine betaine transporter OpuABC of *Lactococcus lactis* was also well described by Romeo et al. (2003), as well the QacT transporter in *L. plantarum* by (Glaasker et al., 1998).

1.4.1.3 Accumulation of energy storage compounds

1.4.1.3.1 Accumulation of phosphates

Polyphosphates are not only used for energy storage but they are also accumulated by *P. freudenreichii* during cold (Dalmasso et al., 2012a) and osmotic stresses (Huang et al., 2016b). For lactobacilli, the accumulation of polyphosphates is dependent on high phosphate concentration in the growth medium (Alcántara et al., 2014). Polyphosphates act as chaperones in other bacteria and interact with misfolded proteins in oxidative stress conditions (Gray and Jakob, 2015). Polyphosphate accumulation is recognized as a key factor of stress tolerance in *L. casei* (Huang et al., 2018).

The Polyphosphate kinase, responsible for polyphosphate (Ppk) synthesis, catalyzes the ATP-dependent formation of a phosphoanhydride bond between a polyphosphate chain and orthophosphate. Some lactobacilli have more than one *ppk* gene involved in polyphosphate synthesis. The number of *ppk* genes was shown to be correlated with the accumulation of elevated phosphate concentrations (Alcántara et al., 2014).

1.4.1.3.2 Accumulation of glycogen

Low temperatures induce glycogen accumulation in *P. freudenreichii* (Dalmasso et al., 2012b). This bacterium also accumulates glycogen in hyper-concentrated sweet whey medium, which has a high osmotic pressure and provides an abundance of carbon substrate (Huang et al., 2016b). Glycogen accumulation depends on the type of sugar substrate present in the culture medium. Raffinose and trehalose activate the accumulation of glycogen, whereas glucose represses it in lactobacilli (Goh and Klaenhammer, 2014).

1.4.2 Regulation of energy production

Intracellular pH (pHi) homeostasis is a prerequisite to normal growth or to survival during stress (Guan et al., 2013). Bacteria triggered different mechanisms to regulate their pHi, such as the up-regulation of ATPase activity and the conversion of different substrates.

1.4.2.1 Regulation of ATPase activity

Lactobacilli acid tolerance is attributed to the presence of a constant gradient between extracellular and intracellular pH. The ATPase protein is a known mechanism used for protection against acid stress (Fig. 3). This protein generates a proton driving force via proton expulsion (Corcoran et al., 2005; Guan et al., 2013). The mechanism is similar for bifidobacteria during acid (Sanchez et al., 2007) and bile salts stresses (Sanchez et al., 2006). The regulation of ATPase activity occurs at the transcriptional level (Broadbent et al., 2010). There is a correlation between APTase activity and acid tolerance (Guan et al., 2013): the higher the ATPase activity is, the higher the acid tolerance will be. However, some lactobacilli species (like *L. casei*) do not use ATPase as a tolerance response to acid stress, instead, they keep the intracellular pH low and reduce the energy demand for proton translocation, in addition to preventing intracellular accumulation of organic acid (Broadbent et al., 2010).

1.4.2.2 Regulation of substrate conversion

During an acid stress, lactobacilli decrease the production of lactate (Papadimitriou et al., 2016). In cold stress conditions, propionibacteria decrease the production of propionate and acetate from lactate substrate via a redirection of pyruvate from the Wood-Werkman pathway to other metabolic pathways (Dalmasso et al., 2012a). To limit the decrease of the intracellular pH, *A. acidopropionici* and lactobacilli increase the activity of the arginine deaminase (ADI) system by a factor of three to five. This system allows the degradation of arginine (pKa ~ 2, producing ATP, NH_4^+ and CO_2 (Rollan et al., 2003; Guan et al., 2013; Teixeira et al., 2014). The production of NH_4^+ and CO_2 allows pH homeostasis (Guan et al., 2013), and the ATP produced leads to an exclusion of protons by the APTase. Guan and al. (2013) showed that the conversion of aspartic acid into alanine makes it possible for the latter to contribute to the ADI system. In addition, arginine deaminase induction can also occur during osmotic and heat stress in *L. fermentum* (Vrancken et al., 2009). During acid treatment, *Lactobacillus reuteri* over-expresses glutamine deaminase and glutamate decarboxylase (Fig. 3, (Su et al., 2011; Teixeira et al., 2014). These enzymes also generate NH_4^+ and CO_2 and are involved in pH_i homeostasis.

At a low pH, enzymes involved in catabolism and energy production are overproduced (Sanchez et al., 2007). This includes enzymes involved in the consumption of complex carbohydrates that contribute to the bifid shunt (Sanchez et al., 2007). Carbon utilization is more efficient and produces more ATP, which could contribute to the proton exclusion via

ATPase. (Sanchez et al., 2007). The addition of glucose in the growth medium can improve acid tolerance by providing the ATP pool required for proton extrusion by ATPase (Corcoran et al., 2005). NAD is used in glycolysis so that the NAD/NADH ratio has to be optimized for high ATP production (Guan et al., 2013).

1.4.3 Impact on the bacterial envelope

Lactobacilli, propionibacteria and bifidobacteria encountere damages during various stresses (Papadimitriou et al., 2016). Indeed, the cytoplasmic membrane acts as a barrier for most solutes. The cell membrane also plays a role in other stresses like acid, cold, heat and bile salts (Table 1). The cell envelope plays also a key role in the regulation of osmotic stress; and maintains cell shape and counteracts the high intracellular osmotic pressure (Papadimitriou et al., 2016). To restore membrane and cell wall integrity, different adaptation mechanisms are adopted by bacteria.

1.4.3.1 Regulation of membrane fluidity

The membrane has such an important role that the relationship between membrane fluidity and stress tolerance has been used to predict the outcome of cell tolerance to stress (Muller et al., 2011). Indeed, stability and permeability of membranes are both key parameters of adaptation and tolerance towards various stresses (Machado et al., 2004). Modulation of membranes composition as adaptation mechanism, which can occur under stressing conditions, tends to counteract variations of fluidity in order to maintain the structure of the bilayer (Fig. 3).

Changes in membrane lipids composition are strain-dependent. As presented in the Figure 4, the cyclic/saturated fatty acid ratio can be increased or decreased during osmotic stress. During osmotic adaptation, an increase in the amount of cyclopropane fatty acids is observed in *Lc. lactis*, while the unsaturated/saturated ratio in membrane fatty acids remains unchanged (Guillot et al., 2000). However, for other bacteria, a decrease in the unsaturated/saturated lipid ratio in the membrane composition can be observed during osmotic adaptation (Machado et al., 2004). *L. casei* can do both during acid stress, increasing the number of cyclopropane fatty acids and decreasing the unsaturated/saturated ratio. In acidic conditions, bacteria have to counteract proton influx by increasing the rigidity and compactness of the cytoplasmic membrane (Broadbent et al., 2010). The unsaturated/saturated ratio and the cyclic/saturated ratio decrease in *L. delbreuckii* subsp *bulgaricus* in membrane under acidic conditions, thus leading to a

decrease in membrane fluidity (Streit et al., 2008). During heat stress, *L. helveticus* decreases its membrane fluidity by increasing the number of unsaturated fatty acids (Lanciotti et al., 2001). *P. freudenreichii*, which contains a majority of odd-numbered membrane unsaturated fatty acids, changes its fatty acid composition under cold stress conditions were observed. It reduces the amount of iso-fatty acids in favor of anteiso-fatty acids (Dalmasso et al., 2012a). As an example, in propionibacteria, branched-chain fatty acids are synthesized due to the activity of branched alpha-keto acid dehydrogenase under cold stress during cheese storage (Dalmasso et al., 2012a). *P. freudenreichii* uses different enzymes to synthesize branched-chain fatty acids, which result from the catabolism of branched amino acids (Gagnaire et al., 2015), maintaining the fluidity of the membrane in order to counteract the cold stress.

The role of cyclic fatty acids (poorly understood) include the modulation of the fluidity in order to increase the tolerance to different stresses (Machado et al., 2004, Velly et al., 2015). Cyclic fatty acid concentration increases during the stationary phase (Muller et al., 2011). The number of double bonds in unsaturated fatty acids is important: linoleic and linolenic acids have two and three double bonds, respectively, causing more steric hindrances than oleic acid (one double bond). Several double bonds could result in the loss of membrane integrity and cell death (Muller et al., 2011). Indeed, *Lactobacillus johnsonii* NCC533 supplemented with unsaturated fatty acid shows a higher sensitivity to heat and acid stress.

Fatty acid biosynthesis, or neosynthesis, requires a great deal of energy, but bacteria have the possibility of modifying existing fatty acids. For example *L. casei* ATCC 334 possesses an enzyme that can add a methylene residue across the cis double bond of C16:1n(9), C18:1n(9) or C18:1n(11) unsaturated fatty acids to form a cyclopropane derivative, thus allowing bacterial adaptation with a minimal energy requirement (Broadbent et al., 2010).

Modulation of the membrane bilayer fatty acid composition seems to be stress-dependent as well as strain-dependent (Fig. 4). Different bacteria reach the same goal, environmental adaptation, in different ways.

1.4.3.2 Regulation of the cell wall

Peptidoglycan is a key element for the stability of bacteria. It is composed of glycan chains of repeating N-acetyl-glucosamine and N-acetyl-muramic acid residues, and cross-linked by peptide side chains (Piuri et al., 2005). During osmotic treatment, bacteria are affected by an enlargement of the cell (Piuri et al., 2005). During enlargement, the cell wall loses a layer. Two

layers can be observed in high salt conditions, whereas three layers are observed under normal conditions. The cell wall is thus thinned (Fig. 3). Under salt stress adaptation, the structure is irregular and seems to be detached from the cytoplasmic membrane. This phenomenon can be attributed to plasmolysis. The presence of protein and teichoic acid in controlled conditions may be responsible for the presence of a third layer (Piuri et al., 2005).

Cells grown in high osmolarity increase the hydrophobicity of their surface, revealed by a higher adherence to the organic solvent. In Gram-positive bacteria, lipoteichoic acids and proteins are the most important cell wall components responsible for surface hydrophobicity. This high hydrophobicity helps bacteria to tolerate the osmotic stress (Machado et al., 2004). *L. casei* grown in high salt conditions limits its production of lipoteichoic acids. In addition, lipoteichoic acids exhibit a lower mean chain length and a lower D-alanine substitution during osmotic treatment. D-alanine substitution increases negative charges in the cell wall and contributes to bacteria tolerance by helping to evacuate toxic Na^+ from the cell wall (Palomino et al., 2013).

1.4.3.3 Regulation of the different S-layers

S-layer proteins are the outer layer component and have several different functions; they maintain cell shape, provide a protective coating, and adhere to the host cell. Lactobacilli overexpress S-layer proteins under stress conditions like bile salts, acid, heat and salt stresses (Khaleghi et al., 2010; Khaleghi and Kasra, 2012; Grosu-Tudor et al., 2016). Moreover, they are involved in osmoadaptation (Palomino et al., 2016). During osmotic treatment, there is an overproduction of the surface layer proteins A (SlpA) and X (SlpX), and the SlpA/SlpX ratio is modified in *L. acidophilus* (Palomino et al., 2016). S-layer proteins may have a role as a protective sheath and protect cells against mechanical and osmotic insults. Bacteria may increase the S-layer gene expression in order to maintain the integrity of the cell envelope structure (Fig. 3), mainly because of the decrease of the cell wall thickness (Palomino et al., 2016).

1.4.3.4 Regulation of exopolysaccharide

Exopolysaccharides include various forms of polysaccharides and are located outside the microbial cell wall. They consist of repeating units of homo- or heteropolysaccharides (Caggianiello et al., 2016). The exopolysaccharides can be strongly or weakly bound to the cell

surface and protect bacteria against high temperature, acid, bile salts and osmotic stress (Alp and Aslim, 2010; Stack et al., 2010; Caggianiello et al., 2016). Exopolysaccharide production is improved under acidic conditions (Torino et al., 2001) and enhances thus the bacterial tolerance response.

1.4.4 Overexpression of molecular chaperones and stress-responsive proteases.

The synthesis of chaperones and proteases is quickly induced under various stressing conditions (Fig. 4) to decrease the deleterious impact of the aggregation of denatured proteins and to refold misfolded ones. Proteases act like the last line of defense when damage is irreversible and lead to amino acids recycling. Proteolysis of cellular proteins, which is a regulated process, can greatly contribute to homeostasis by degrading proteins whose functions are no longer required after modification of environmental parameters (Papadimitriou et al., 2016). DnaK (Hsp70) is one of the well-conserved bacterial chaperones that can refold misfolded proteins (Papadimitriou et al., 2016).

One of the ways to counteract stress is the regulation of membrane fluidity. This rather complex regulation involves GroEL and HSPs universal protein chaperones (Papadimitriou et al., 2016). Indeed, small heat shock proteins regulate membrane lipid polymorphism (Tsvetkova et al., 2002). Accordingly, inactivation of small heat shock proteins affects membrane fluidity in *L. plantarum* (Capozzi et al., 2011). GroEL and GroES (Hsp60) proteins are overproduced under various stress conditions (Fig. 4, (Papadimitriou et al., 2016). It has been shown that *L. paracasei* overproduces GroESL during heat stress (Santivarangkna et al., 2007). GroEL and GroES work in tandem to ensure the correct folding of proteins in an ATP-regulated manner (Fig. 4, (Mills et al., 2011).

De Dea Lindner et al., 2007 showed that there are two types of stress protein regulation. In the first type, GroEL, GroES and ClpC are rapidly induced to a high level after moderate heat stress (+5°C) but are not induced by severe heat treatment (+13°C). In the second type, DnaK, GrpE, DnaJ1 and ClpB are strongly induced in a high heat treatment but not in moderate one (De Dea Lindner et al., 2007). Heat shock proteins are highly conserved but it is not the case for their expression regulation mechanisms. The heat shock response is controlled by a combination system like transcriptional repression and activation (De Dea Lindner et al., 2007).

Bifidobacterium breve overexpresses DnaK, DnaJ and ClpB in high salt conditions and during high heat treatment, which probably means that there is an overlapping regulatory network that

controls both osmotic and severe heat stress (De Dea Lindner et al., 2007). This is consistent with observed cross-protections between heat and salt treatments. These proteins are also overproduced during bile and acid stresses (Leverrier et al., 2004; Bove et al., 2013; Jin et al., 2015).

In addition, *P. freudenreichii* overproduces HtrA, DnaK and GroEL during bile salts stress, and HtrA is known to have protease and chaperone activities (Leverrier et al., 2003, 2004). This suggests that bile and salt adaptation are closely related (Savijoki et al., 2005).

The Clp protease family constitutes a major system for general protein turnover in lactobacilli. These proteins work with ATPase to degrade proteins (Papadimitriou et al., 2016). Chaperones and Clp proteins are stress-induced, with induction occurring at the transcription level, and can be regulated by the transcriptional repressors CtsR and HrcA (Papadimitriou et al., 2016).

Cold shock proteins (Csp) are produced in cold conditions (Fig. 4) and are involved in mRNA stabilization (Mills et al., 2011). These proteins are essential for growth in cold temperatures (Dalmasso et al., 2012a). The production of three Csps, CspL, CspP and CspC, in cold conditions resulted in an increased of bacterial tolerance towards temperature downshift (Mills et al., 2011). In cold and other stress conditions, *P. feudenreichii* up-regulates genes coding for two Csp (Dalmasso et al., 2012a). Bifidobacteria have less different chaperones and proteases than other bacteria (De Dea Lindner et al., 2007), in accordance with their great sensitivity to stress.

Chaperones can also behave as moonlight proteins. Moonlighting DnaK and GroEL, for example, have adhesive functions. The overexpression of these proteins during bile or acid stresses can help the bacteria to adhere to intestinal cells and to persist within the digestive tract (Bergonzelli et al., 2006; Candela et al., 2010).

The whole studies reveal that some adaptive mechanisms are common to different treatments. They may be induced by several treatments and explain the observed cross-protections (Fig. 4). Osmotic and acid treatments induce large changes in bacterial cells, and only a few adaptation mechanisms are common to both treatments. Oxidative treatment has been less studied than the other treatments.

1.5 Stress adaptation improves bacterial tolerance to technological and digestive stress

Understanding and mastering bacterial adaptation is crucial for strains selection and of pretreatments to improve viability during technological stresses, storage and digestion (Fig. 5). Bacterial survival during technological and digestive stresses is strain-dependent. Some bacteria are naturally more resistant and have the ability to adapt to various stresses. Some growth medium modifications and some physicochemical treatments can improve bacterial survival during technological stresses, thanks to adaptation (Tables 2 and 3; Fig. 5), but only a few studies have focused on which adaptive mechanisms are responsible for enhanced survival.

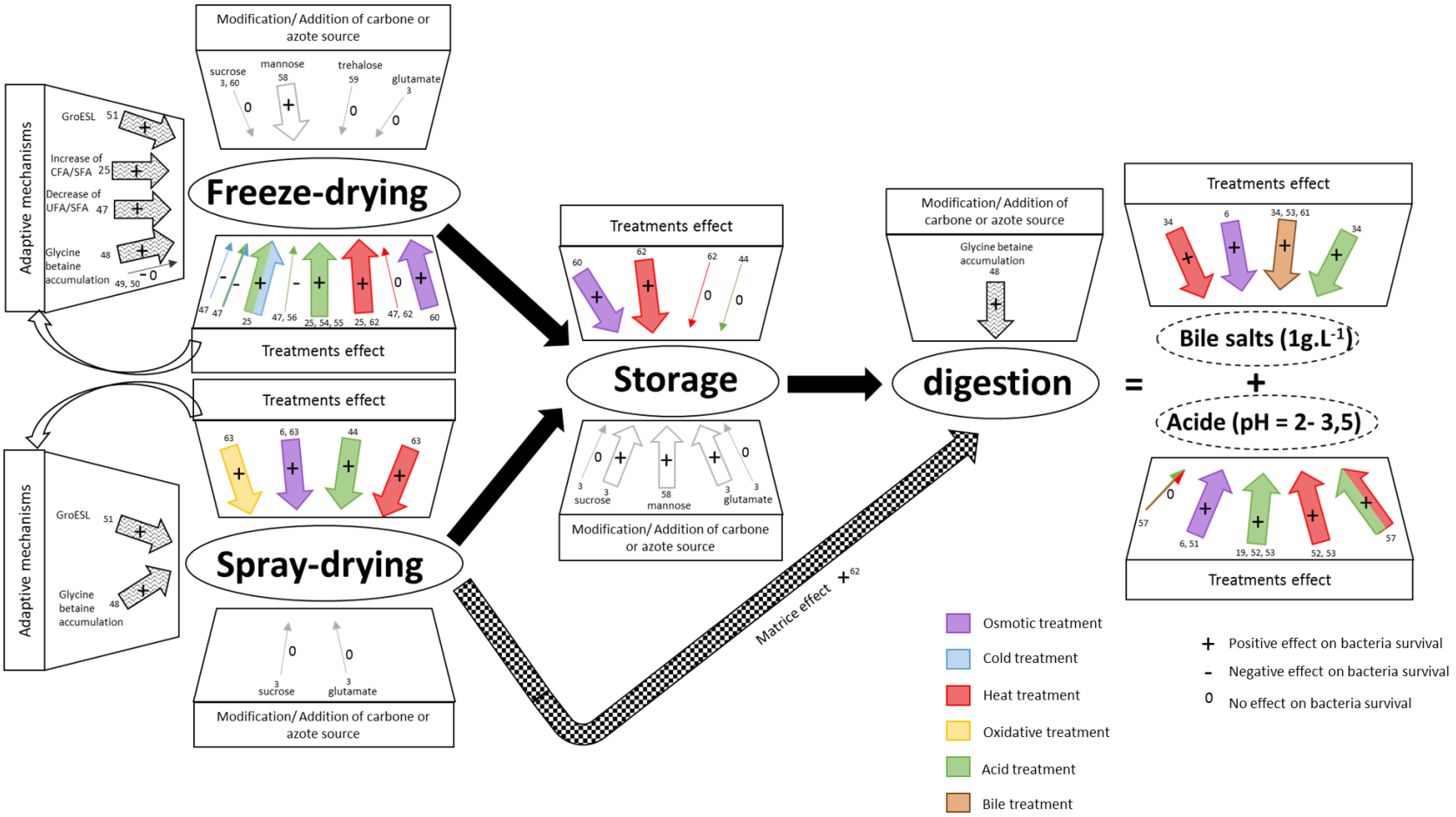


Figure 5: Stressing pre-treatments and modifications of the growth medium modulate survival during technological and digestive stresses. Technological and digestive stresses are represented in the figure. Digestion triggers two main stresses: bile salts and acid stress. For each stress, the impact of stressing pre-treatments and of modifications of the growth medium on bacteria survival is indicated (+: positive; -: negative; and 0: no effect). Strain-dependent modulations are represented by arrows (purple: osmotic; blue: cold; red: heat; yellow: oxidative; green: acid; brown: bile salts treatment). Large arrows indicate a positive effect and thin arrows indicate either no effect or a negative effect. The numbers indicate corresponding references in the tables

Table 2: Adaptive mechanisms reportedto modulate the survival of propionibacteria, bifidobacteria and lactobacilli under technological and digestive stresses.

+ : indicates an improvement of survival; - : indicates a decrease of survival; 0 : indicates no effect on survival; *: indicates a cited reference; a: *Lactobacillus*; b: *Bifidobacterium*

Cell modification	Effect	Technological stress or digestion	Bacteria	References	Corresponding number in the figures
Conversion of glutamate to GABA	+	Acid stress (pH=2.5)	*L.*[a] *reuteri*	(Su et al., 2011)	46*
Decrease in unsaturated fatty acid/saturated fatty acid ratio	+	Freeze-drying	*L. coryniformis*	(Schoug et al., 2008)	47
Glycine betaine accumulation	+	Digestion	*B.*[b] *breve*	(Sheehan et al., 2006)	48
Glycine betaine accumulation	0	Freeze-drying	*B. animalis*	(Saarela et al., 2005)	49
Glycine betaine accumulation	-	Freeze-drying	*L. coryniformis*	(Bergenholtz et al., 2012)	50
Glycine betaine accumulation	+	Freeze-drying	*L. salivarius*	(Sheehan et al., 2006)	48
Glycine betaine accumulation	+	Spray-drying	*L. salivarius*	(Sheehan et al., 2006)	48
GroESL overproduction	+	Freeze-drying	*L. paracasei*	(Corcoran et al., 2006)	51
GroESL overproduction	+	Spray-drying	*L. paracasei*	(Corcoran et al., 2006)	51
Increase in cyclopropane fatty acid number	+	Freeze-drying	*L. bulgaricus*	(Li et al., 2009)	25

Table 3: Treatments and modifications of the growth medium that modulate the survival of propionibacteria, bifidobacteria and lactobacilli under technological and digestive stresses.

+ : indicates an improvement of survival; - : indicates a decrease of survival; 0 : indicates no effect on survival; *: indicates a cited reference; a: *Lactobacillus;* b: *Propionibacterium;* c: *Bifidobacterium*

Adaptation	Effect	Technological stress or digestion	Bacteria	References	Corresponding number in the figures
Acid adaptation	+	Acid stress (pH=2)	*L.*[a] *casei*	(Broadbent et al., 2010)	19*
Acid adaptation	+	Acid stress (pH=2)	*P.*[b] *freudenreichii*	(Jan et al., 2000, 2002)	52, 53
Acid adaptation	-	Bile salt stress (1g.L-1)	*P. freudenreichii*	(Leverrier et al., 2004)	34
Acid adaptation	+	Freeze-drying	*L. bulgaricus*	(Li et al., 2009)	25
Acid adaptation	-	Freeze-drying	*L. coryniformis*	(Schoug et al., 2008)	47
Acid adaptation	+	Freeze-drying	*L. reuteri*	(Koch et al., 2008; Palmfeldt and Hahn-Hägerdal, 2000)	54, 55
Acid adaptation	-	Freeze-drying	*L. rhamnosus*	(Ampatzoglou et al., 2010)	56
Acid adaptation	+	Spray-drying	*L. delbrueckii*	(Silva et al., 2005)	44
Acid adaptation	0	Storage (spray dryingD)	*L. delbrueckii*	(Silva et al., 2005)	44
Cold + acid adaptation	-	Freeze-drying	*L. coryniformis*	(Schoug et al., 2008)	47
Cold + acid adaptation	+	Freeze-drying	*L. bulgaricus*	(Li et al., 2009)	25
Acid + heat adaptation	+	Acid stress (pH=3,5 in Synthetic Gastric Fluid)	*B.*[c] *lactis*	(Maus and Ingham, 2003)	57
Acid + heat adaptation	0	Acid stress (pH=3,5 in Synthetic Gastric Fluid)	*B. longum*	(Maus and Ingham, 2003)	57
Addition of mannose	+	Freeze-drying	*L. delbrueckii*	(Carvalho et al., 2008)	58
Addition of mannose	+	Storage (freeze-drying)	*L. delbrueckii*	(Carvalho et al., 2008)	58
Addition of trehalose	0	Freeze-drying	*L. salivarius*	(Zayed and Roos, 2004)	59

Addition of glutamate	0	Freeze-drying	*L. sakei*	(Ferreira et al., 2005)	3
Addition of glutamate	0	Spray-drying	*L. sakei*	(Ferreira et al., 2005)	3
Addition of glutamate	0	Storage (freeze-drying)	*L. sakei*	(Ferreira et al., 2005)	3
Addition of glutamate	+	Storage (spray drying)	*L. sakei*	(Ferreira et al., 2005)	3
Addition of sucrose	0	Freeze-drying	*L. sakei*	(Ferreira et al., 2005)	3
Addition of sucrose	0	Freeze-drying	*L. bulgaricus*	(Carvalho et al., 2003)	60
Addition of sucrose	0	Spray-drying	*L. sakei*	(Ferreira et al., 2005)	3
Addition of sucrose	+	Storage (spray drying)	*L. sakei*	(Ferreira et al., 2005)	3
Addition of sucrose	0	Storage (freeze-drying)	*L. sakei*	(Ferreira et al., 2005)	3
Bile salt adaptation	+	Bile salt stress (1g.L-1)	*P. freudenreichii*	(Jan et al., 2002; Leverrier et al., 2003, 2004)	53, 61, 34
Cold adaptation	-	Freeze-drying	*L. coryniformis*	(Schoug et al., 2008)	47
Heat adaptation	+	Acid stress (pH=2)	*P. freudenreichii*	(Jan et al., 2000, 2002)	52, 53
Heat adaptation	+	Bile salt stress (1g.L-1)	*P. freudenreichii*	(Leverrier et al., 2004)	34
Heat adaptation	0	Freeze-drying	*L. acidophilus*	(Paéz et al., 2012)	62
Heat adaptation	+	Freeze-drying	*L. bulgaricus*	(Li et al., 2009)	25
Heat adaptation	+	Freeze-drying	*L. casei*	(Paéz et al., 2012)	62
Heat adaptation	0	Freeze-drying	*L. coryniformis*	(Schoug et al., 2008)	47
Heat adaptation	+	Freeze-drying	*L. plantarum*	(Paéz et al., 2012)	62
Heat adaptation	+	Spray-drying	*L. paracasei*	(Desmond et al., 2001)	63
Heat adaptation	0	Storage (freeze-drying)	*L. acidophilus*	(Paéz et al., 2012)	61
Heat adaptation	0	Storage (freeze-drying)	*L. casei*	(Paéz et al., 2012)	61
Heat adaptation	+	Storage (freeze-drying)	*L. plantarum*	(Paéz et al., 2012)	61
Osmoadaptation	+	Acid stress (pH=2)	*P. freudenreichii*	(Huang et al., 2016; Jan et al., 2000)	6, 51
Osmoadaptation	+	Bile salt stress (1g.L-1)	*P. freudenreichii*	(Huang et al., 2016)	6
Osmoadaptation	+	Freeze-drying	*L. bulgaricus*	(Carvalho et al., 2003)	60
Osmoadaptation	+	Spray-drying	*L. paracasei*	(Desmond et al., 2001)	63
Osmoadaptation	+	Spray-drying	*P. freudenreichii*	(Huang et al., 2016)	6

Osmoadaptation	+	Storage (freeze-drying)	*L. bulgaricus*	(Carvalho et al., 2003)	60
Oxidative adaptation	+	Spray-drying	*L. paracasei*	(Desmond et al., 2001)	63
Spray-dried	+	Digestion	*L. acidophilus*	(Paéz et al., 2012)	61
Spray-dried	+	Digestion	*L. casei*	(Paéz et al., 2012)	61

1.5.1 Enhanced tolerance toward drying process and storage

1.5.1.1 Freeze-drying process

Bacteria survival during freeze-drying has been well studied. Many studies presenting the best way to increase survival are available. However, only few studies have focused on the role of bacterial adaptive mechanisms on bacterial survival during this process.

The accumulation of glycine betaine leads to an enhanced survival during freeze-drying for *Lactobacillus salivarus* (Sheehan et al., 2006). Unfortunately, this mechanism is strain-dependent, as shown in the Figure 5. Glycine betaine accumulation can be triggered by osmotic stress (Table 1); (Glaasker et al., 1996; Romeo et al., 2003; Huang et al., 2016b). Indeed, as listed in Table 3, the osmoadaptation enhanced *Lactobacillus bulgaricus* survival during freeze-drying (Carvalho et al., 2003). Osmotic constraint induces bacterial adaptation, and glycine betaine accumulation can be responsible for the better survival of *L. bulgaricus* and of other species. Figure 5 shows that acid, heat and osmotic treatments can improve bacterial survival during freeze-drying in strain-dependent way. Osmotic stress can be also induced by the addition of either salt or sugar. The advantage of a sugar like mannose is that it can increase bacterial survival during freeze-drying (Carvalho et al., 2008). In fact, a growth medium with a high sugar concentration leads to the accumulation of trehalose (Cardoso et al., 2004). In addition, sugars have a positive impact on bacterial survival when they are added at a high concentration to the drying medium (Giulio et al., 2005).

A strain of *Lactobacillus paracasei* which overexpresses GroESL, presents a better survival rate after extreme stresses challenges in comparaison than other *L. paracasei* strains (Mills et al., 2011). GroESL is a chaperone protein which can be induced during heat treatment (Walker et al., 1999). The overproduction of GroESL or of other general stress proteins is induced by various treatments (Table 1, Schoug et al., 2008), and their synthesis probably has a considerable impact on bacterial survival during freeze-drying.

Modulation of membrane lipids composition may contribute to enhancing bacterial survival during freeze-drying. In fact, lipid membrane modifications depend on the strain-treatment couple, like other adaptive mechanisms. In the literature, two lipid modifications have been reported to increase bacterial survival during freeze-drying. It includes the increase of the cyclic fatty/saturated fatty acid ratio and the decrease of the unsaturated/saturated membrane fatty acid ratio, these ratios being key features of stress adaptation (see Section 2.3.1, Table 2 and Fig. 5).

Combinations of stress seem to be a promising way to induce multiple adaptation mechanisms, which unfortunately are strain-dependent. The combination of cold and acid treatment was tested, but this association decreased *Lactobacillus coryniformis* survival during freeze-drying (Schoug et al., 2008). However, Li et al. demonstrated that *L. bulgaricus* viability is enhanced during freeze-drying , when it is grown in cold and acid conditions (30°C; pH=5) or when it is submitted to a mild cold treatment (30°C) (Li et al., 2009).

1.5.1.2 Spray drying process

To enhance the latter, it is possible firstly to select strains with high intrinsic stress tolerance and the ability to adapt upon pretreatment, which is strain-dependent. Indeed, bifidobacteria species with high heat and moderate oxygen tolerance have a better survival rate after spray drying (68-102%) compared to species with no intrinsic tolerance to oxygen and to heat stress (Santivarangkna et al., 2007). In addition, it is important to remember that growth phase harvesting may influence the bacterium viability during spray drying. The stationary phase seems to be a favorable phase to harvest bacteria for drying (Peighambardoust et al., 2011a).

The three treatments - osmotic, heat and acid - can improve bacterial survival during spray drying or freeze-drying (Fig. 5). Although these two drying processes impose opposite stresses - cold and heat stress - the treatments that improve bacterial survival are indeed the same. It is possible to rank treatments according to their efficiency toward protection during spray drying for *L. paracasei* NFBC 338. Decreasing efficiency is observed with heat > salt > hydrogen peroxide > and bile treatment (Desmond et al., 2001). Obviously, this ranking is strain-dependent and the addition of another treatment may make more effective bacterial adaptation possible.

The heat tolerance of bacteria is defined by the decimal reduction value (D_θ value) that represents the time needed to kill 90% of the bacteria at a given temperature θ. Upon heat-adaptation, the D_{60} value of *L. paracasei* can increase from 1.7 to 3.1 min (Desmond et al., 2001), in accordance with enhanced tolerance towards spray drying. Survival of *L. salivarius* and *L. paracasei* is also enhanced by heat or oxidative treatments before spray drying (Desmond et al., 2001). Moreover, during heat stress, GroESL can be overproduced and enhance bacterial survival during spray drying (Walker et al., 1999; Corcoran et al., 2006). Silva et al., 2005 showed that acid-adapted *L. delbrueckii* achieves a better tolerance to heat and spray drying due to the production of heat shock proteins.

In addition, the accumulation of glycine betaine improves lactic acid bacteria viability during spray drying (Sheehan et al., 2006; Peighambardoust et al., 2011a). High fermentable sugar concentrations in the growth medium permit the production of metabolites like mannitol. Non-fermentable sugars increase the osmotic pressure of the medium and induce osmoadaptation of bacteria, so that both types of sugars improve cell survival during spray drying (Peighambardoust et al., 2011a). Osmoadaptation increases the survival of several species during spray drying (Desmond et al., 2001; Huang et al., 2016a, 2016b).

Spray drying is a stressful process that affects the bacterial membrane. Acid, osmotic, heat and oxidative treatments lead to the modification of the membrane composition (Fig. 4). Membrane fluidity should be optimized to increase bacteria survival. If the fluidity is too high or too low, bacterial survival may decrease.

Pretreatments prior to drying are thus promising tools to improve bacterial viability during technological processes, but must be adapted for each strain. It can be observed that bacterial adaptation has its own limits. As an example, the heat-adaptation of *L. paracasei* would be useful in order to deal with high drying outlet temperatures (higher than 95°C) and, conversely, less useful for lower outlet temperatures (85-90°C) because the temperature would cause less damage in this case (Desmond et al., 2001). Moreover, the outlet temperature can be decreased while optimizing the drying parameters. It seems that the best pretreatment would be acid and osmotic stresses for spray drying.

1.5.1.3 Storage

The challenge is not only to maintain the viability of bacteria during the drying process, but also during storage. Relative humidity and temperature are two key factors controlling the loss of viability upon storage. Since the oxidation of lipids increases with relative humidity (Golowczyc et al., 2011) and temperature, the stability is negatively correlated with these two factors. Cells have the best stability during storage with a relative humidity of 0%, even if the temperature is 30°C (Golowczyc et al., 2011).

To increase the stability of cells during storage, a protective agent can be added during growth. When monosodium glutamate or fructo-oligosaccharides (FOS) are added during the growth of *Lactobacillus kefir*, the cells have a higher stability after drying and during storage at 0-11% relative humidity (Golowczyc et al., 2011). Sugars like glucose, fructose, mannose and sorbitol can provide protection (Peighambardoust et al., 2011a). Protection through the addition of

sucrose is strain-dependent (Golowczyc et al., 2011). Storage at relative humidity higher than 23% resulted in low stability, regardless of the protective agent, with a correspondingly low D_{20} value (Golowczyc et al., 2011). This highlights the importance of decreasing the relative humidity of the storage room.

Another way to prepare bacteria to storage conditions is cross-protection. Cells cultivated with a moderate stress can have better stability during storage. Only heat and osmotic treatments are reported to increase bacterial survival upon storage (Fig. 5). Indeed, mild heat treatment enhances the stability of *L. paracasei* (Paéz et al., 2012) and of *Lactobacillus rhamnosus* (Prasad et al., 2003) during storage, these results being strain-dependent (Paéz et al., 2012).

Osmotic adaptation can also improve stability during storage for *L. rhamnosus* (Prasad et al., 2003). In powders, the absence of water may have deleterious effects on bacteria. Osmotic treatment during growth induces the accumulation of compatible solutes like glycine betaine, glycerol and trehalose, and leads to changes in carbohydrate metabolism, resulting in the accumulation of glycerol linked to polysaccharides. These molecules interact with macromolecules instead of water. This protection increases the stability of *L. rhamnosus* during storage (Prasad et al., 2003).The modification of membrane composition during heat and osmotic treatments may reduce membrane damage, which occurs during storage.

L. delbrueckii spp. *bulgaricus* grown under uncontrolled pH, thus experiencing acid stress, do not have a higher stability during storage (Silva et al., 2005).

1.5.2 Enhanced tolerance toward digestive stresses

During digestion, bile salts and acid stresses are two major stresses which affect probiotics survival and thus their beneficial effects. The effect of adaptation on bacterial survival during digestion is not well known (Fig. 5).

Pre-adaptation of bifidobacteria to bile salts induces many metabolic changes. The first is the more efficient use of maltose by *Bifidobacterium animalis*. In addition, *Bifidobacterium longum* over-produces mucin-binding protein in acid pH conditions. This should facilitate its efficient targeting to the colon, which is its natural habitat. Moreover, *B. animalis* overproduces the membrane-bound ATPase that controls the intracellular pH (Sánchez et al., 2013). Finally, adapted bifidobacteria are then able to consume raffinose and maltose, in addition to glucose

(Sanchez et al., 2007). All these elements lead to a better survival within the gut, particularly because acid and bile salts adaptation prepare cells to use carbon sources, which cannot be used by the indigenous microbiota (Collado and Sanz, 2007; Sánchez et al., 2013).

Acid response is well documented for bifidobacteria. More than 20 bifidobacteria showed an increase of their tolerance to a simulated GIT after acid-adaptation (Sánchez et al., 2013). Acid and heat-adaptation can be used together at the same time to improve *Bifidobacterium lactis* survival to acid stress (Maus and Ingham, 2003).

Heat and osmotic treatments can be used to adapt bacteria to acid stress. In addition, *L. casei* can be acid-adapted with a treatment at pH 4.5 for 10 or 20 minutes, with an enhanced viability to acid challenge (Broadbent et al., 2010) and, consequently, to digestion. During bile salts adaptation, some probiotics express a range of bile salts hydrolases which lead a better bacterial viability (Papadimitriou et al., 2016). The spray drying process can improve the tolerance to simulated gastrointestinal digestion as a result of encapsulation (Fig. 5, Paéz et al., 2012).

The probiotic delivery vehicle, used as drying matrix, has a high impact on viability during digestion. Several studies reported that spray drying is the best method to maintain and improve the effect of probiotics. The protection of bacteria during digestion, both by encapsulation or by a food matrix, seems to be an important factor and has to be further studied in order to improve the effect of probiotics.

1.6 Conclusion

Treatments used to adapt bacteria before technological and digestive challenges offer promising opportunities for improvement of the efficacy of probiotics and starters. Osmotic and heat treatments reportedly enhance bacteria survival during stressing challenges, via accumulation of compatible solutes and overexpression of key stress proteins, respectively. In the literature, these treatments are reported to exert positive impacts on bacterial survival during freeze-drying, spray drying, storage and, to a lesser extent, during digestion. Acid treatment also seems promising because it can exert a positive impact during drying, bile salts and acid stress challenges. Treatments including bile salts have not been well studied, particularly in terms of their impact on bacterial survival during technological stresses. The combination of two or three of these stresses could be very interesting, especially the osmotic-acid combination. Indeed, it can be observed that these two treatments can induce a high number of adaptive mechanisms.

Starter bacteria apart from two-in-one bacteria do not require an adaptation to digestive stresses because they will grow in a food matrix. For starters, osmotic or heat treatment, or the combination thereof, seem to afford protection towards technological constraints. To trigger the adaptation of beneficial bacteria, the growth medium should be adapted, as carbon (saccharides) and nitrogen (amino acids) sources can be modulated. Growth conditions have to be chosen according to the subsequent probiotic/starter use and to the strain, since this review shows that most of the treatments' effects are strain-dependent.

Chapter 2: Objectives and strategy

This chapter aims at introducing the objectives, the originality and strategy of this PhD research project. Better understanding bacterial adaptation mechanisms is of prime importance; bacterial adaptation coupling is even poorly known and can lead to high beneficial bacteria survival during technological and digestive stresses. This will help the manufacturers to improve their competitiveness on a challenging market.

The aims of this chapter were to:

- Define the PhD research area and the research question
- Provide the PhD strategy

2.1 Research question

As described in chapter 1, different adaptation mechanisms exist for bacteria, including modulation of the membrane fatty acids composition, accumulation of compatibles solutes and overexpression of general stress proteins. It is essential to keep in mind that adaptation mechanisms can be induced by pretreatments. It has been previously reported that osmoadaptation increased bacteria survival during technological and digestive stresses. A better understanding of adaptation mechanisms, which have a key role in the higher bacteria resistance to further processing or digestive stresses, therefore constitutes a key issue. The main and central research question is:

How to adapt beneficial bacteria in order to increase their survival towards technological and digestives stresses?

2.2 Objective and strategy

The aim of the PhD project was to provoke adaptation to improve *P. freudenreihcii* survival during different lethal challenges and drying processes (Figure 6). We provoked three different adaptations: osmoadaptation, acid-adaptation and heat-adaptation on *P. freudenreichii* during or after the growth. We tried to understand the adapatation mechanisms used by *P. freudenreichii* thanks to omics analyses. We thus analysed the accumulation of compatibles solutes, the membrane fatty acids composition and the proteomes. This adaptives mechanisms are known to have an important impact on bacteria survival during drying processes (Fonseca et al., 2019, Huang et al., 2016b). We then tried to correlate the adapatation mechanisms used by *P. freudenreichii* to it survival during different lethal challenge and during drying processes (Figure 6). The heat and the oxidative challenges were chosen to represent the spray drying processes, and the acid and bile salts challenges were chosen to represent the digestion process.

We decided to focus on a robust and a fragile strain. In this view, *Propionibacterium freudenreichii* CIRM-BIA 129 (also called ITG-P20) was first considered as the robust strain, and the study was then widened to a more fragile strain used by Bioprox: *Lactobacillus rhamnosus* CIRM-BIA 1113.

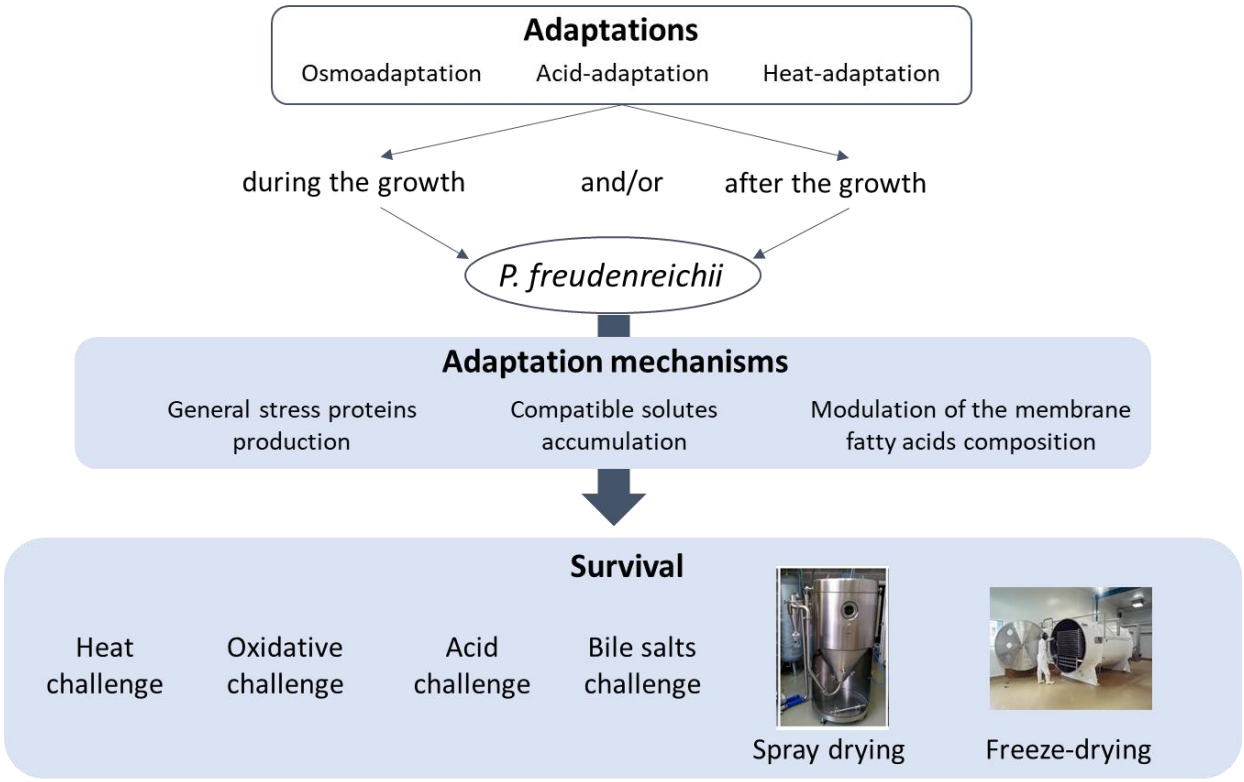


Figure 6: General strategy of the thesis. Different adaptations were imposed to *P. freudenreichii* during or after the growth. Three adaptation mechanisms were studied to understand the effect of the adaptations on P. freudenreichii survival during challenges and drying process.

The above-mentioned research question has been by divided into the following sub-questions, overall forming a general strategy scheme (Fig. 7).

- **In chapter 3**, the main question was: **how do the cross-protections provided by osmoadaptation work?** In order to answer this question, the osmoadaptation of the strain *P. freudenreichii* CIRM-BIA 129 (osmotolerant) was compared to the osmoadaptation of the strain *P. freudenreichii* CIM-BIA 1025 (osmosensitive). Cross-protections provided by the osmoadaptation were compared for each strain, and correlated with the accumulation of compatible solutes and to strains' proteome.

- **In chapter 4**, we aimed at answering the question: **can we fine-tune *P. freudenreichii* osmoadaptation so to optimize cross-protections?** For this purpose, different growth media, with different carbon and nitrogen composition, were used for *P. freudenreichii* growth and osmoadaptation. The impact of growth medium composition, and particularly of the lactose/NPN ratio, on compatibles solutes accumulated by *P. freudenreichii* during

osmoadaptation, was studied. We then analyzed the role of the compatible solutes accumulation on *P. freudenreichii* viability during freeze-drying and spray drying.

-In chapter 5, we aimed at improving *P. freudenreichii* viability during freeze-drying. Other adaptation can be performed, or combined to the osmoadaptation. Combination of different bacteria adaptation stimuli has been to date very poorly studied. The question was: **can the coupling of adaptations increase *P. freudenreichii* survival during freeze-drying?** We studied therefore *P. freudenreichii* resistance after acid or heat-adaptations, coupled or not to osmoadaptation. Adaptive mechanisms of *P. freudenreichii* during such adaptations were then highlighted to determine the best adaptative way in order to increase viability during freeze-drying.

-In chapter 6, the initial question was: **how can we optimize *P. freudenreichii* osmoadaptation to increase its survival during spray drying?** We thus used an experimental design to improve *P. freudenreichii* resistance to heat and oxidative challenges. Cultures with best bacterial adaptations were then spray dried to confirm the methods. Compatible solutes accumulated by *P. freudenreichii* in the best conditions were analyzed to explain its higher resistance during spray drying. *P. freudenreichii* dried by spray drying was then used as starter to test its revivification. In this chapter, we consiedred a second question: **Is it possible to transpose the optimization strategy of stress adaptation to other bacteria?** In the same way, *L. rhamnosus* adaptation was optimized by an experimental design. Optimized cultures were then dried by freeze-drying, and two different spray drying methods.

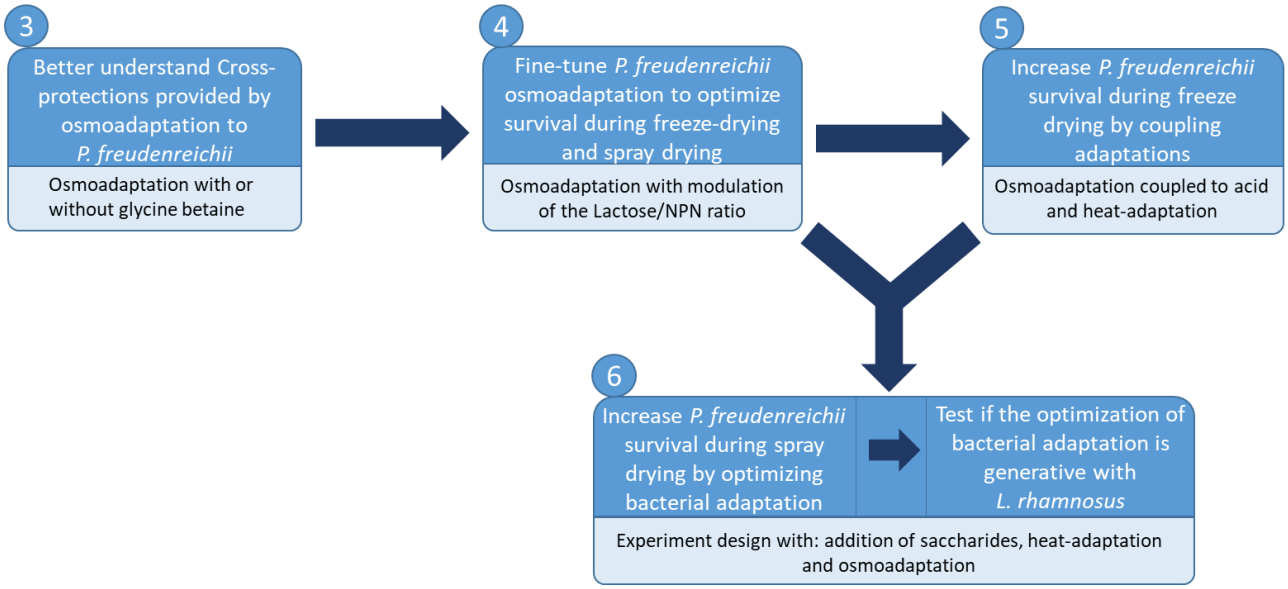


Figure 7: Schema of the thesis general plan to answer the research question: "How to adapt beneficial bacteria in order to increase their survival towards technological and digestives stresses?" The research question is divided into five different questions dealing with bacteria adaptation. Chapter number are indicated in circles. White writing indicates objective and black writing indicates methods.

Chapter 3: Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*

In this chapter, we try to better understand *Propionibacterium freudenreichii* osmoadaptation, as well as its impact on *P. freudenreichii* tolerance towards relevant stress challenges. This was performed thanks to a chemically defined medium. Osmotic adaptation within a rich medium was already shown to enhance *P. freudenreichii* CIRM-BIA 129 tolerance towards stresses, which are encountered during freeze-drying and during digestion.

In this report, we compared the osmoadaptation molecular mechanisms of two *P. freudenreichii* strains in a chemically defined medium, in the presence or absence of salt, with or without added glycine betaine. Compatible solutes accumulated were identified and quantified, and then proteomes of the two strains in the thesis's conditions were compared.

It was revealed that both osmotolerance and osmoadaptation were strain-dependent and had different effects on multiple stress tolerance, depending on the presence of compatible solutes. Availability of glycine betaine (GB) restored the growth of one of the two strains. In this strain, osmotic preadaptation enhanced heat, oxidative and acid stress tolerances, as well as survival upon freeze-drying. However, addition of GB in the medium had deleterious effects on stress tolerance, while restoring optimal growth under hyperosmotic constraint. In the other strain, neither salt nor GB enhanced stress tolerance, which was constitutively low. Accordingly, whole cell proteomics revealed that mechanisms triggered by salt in the presence and in the absence of GB are different between strains.

P. freudenreichii is used as starter and as procbiotic. During the production, different stresses are imposed to bacteria, in particularly during drying. Osmotic adjustments may modulated tolerance towards drying. However, they are strain-dependant, medium-dependant and may either reduce or increase stress tolerance toward drying. A case-by-case study, for each strain-medium thus seems necessary. In this chapter we identify key proteins involved in osmoadaptation and give new inshights into adapatation mechanisms in *P. freudenreichii*. This opens new perspectives for the selections of strains and for the choice of the growth medium composition.

The aims of this chapter were to:

- Investigate the impact of *P. freudenreichii* osmoadaptation on its tolerance
- Better understand the molecular mechanism involved in *P. freudenreichii* osmoadaptation
- Evaluate the impact of the growth medium composition on *P. freudenreichii* osmoadaptation
- Evaluate the importance of strain selection

The main contents in this chapter have been published as:

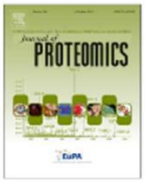
F. Gaucher, S. Bonnassie, H. Rabah, P. Leverrier, S. Pottier, J. Jardin, V. Briard-Bion, P. Marchand, R. Jeantet, P. Blanc, G. Jan, Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*, Journal of Proteomics. (2019) 103400. doi:10.1016/j.jprot.2019.103400

Journal of Proteomics 204 (2019) 103400

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*

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3.1 Introduction

As reviewed in Chapter 1, *P. freudenreichii* is a promising probiotic bacterium consumed in high amounts, mainly in Swiss-type cheeses, but also in functional probiotic food supplements. Efficacy of probiotics depends on the amount of live probiotic bacteria within a preparation, which often remains to be optimized (Gagnaire et al., 2015). It also depends on probiotic bacteria survival during the digestion (Rabah et al., 2018). The international Dairy Federation (IDF) recommends a minimum of 10^7 live probiotic bacterial cells per gram or milliliter of product at the time of consumption (Huang et al., 2017b). To produce tablets or capsules, the drying process and the probiotic product formulation require optimization, in order to keep alive the maximum of probiotic bacteria per gram during drying and storage. The freeze-drying process is a well-known process, the most used, and it allows drying of bacteria with a high viability (Santivarangkna et al., 2007). However, freeze-drying imposes osmotic and cold stresses, whereas spray drying imposes thermal, osmotic and oxidative stresses (Gaucher et al., 2019a). Ingested probiotic bacteria are submitted to the digestion process and should reach the colon alive, thus surviving acidic conditions of the stomach and presence of enzymes and bile salts in the intestine (Mainville et al., 2005). Furthermore, osmolality within the gastrointestinal tract is constantly changing as a result of water absorption (De Dea Lindner et al., 2007).

Depending on bacterial species and strains, stress adaptation may lead to increased viability during drying (Desmond et al., 2001; Carvalho et al., 2004; Silva et al., 2005; Li et al., 2009; Paéz et al., 2012), acid stress (Jan et al., 2002; Broadbent et al., 2010), and bile salts stress (Leverrier et al., 2003, 2004), thanks to cross protection. During osmotic stress, general stress proteins are overproduced (Leverrier et al., 2004; De Dea Lindner et al., 2007; Huang et al., 2016b). Chaperones and proteases limit the impact of deleterious aggregation of denatured proteins (Papadimitriou et al., 2016). During osmoadaptation, bacteria accumulate compatibles solutes (Csonka, 1989), which can be imported inside the cell, or synthesized *de novo*. Compatibles solutes may preserve turgescent pressure and enable cell growth and division (Csonka and Hanson, 1991). Bacteria like *L. plantarum* can accumulate glutamate during osmoadaptation (Glaasker et al., 1996; Kets et al., 1996). Some authors reported that *P. freudenreichii* is able to accumulate trehalose and glycine betaine (GB) under osmotic constraint (Cardoso et al., 2007; Huang et al., 2016a). During osmoadaptation, *P. freudenreichii* can accumulate energy storage molecules like glycogen and polyphosphate, in a very rich growth medium (Huang et al., 2016b). This specific growth conditions seemed promising in the aim to produce more resistant probiotic, as they enhanced *P. freudenreichii* resistance to

acid, to bile salts stress and to spray drying (Jan et al., 2000; Huang et al., 2016b). Osmoadaptation depending on both the strain and the culture medium, a screening is required to optimize probiotic products and to understand the effects of growth conditions. In this report, we investigated benefits and drawbacks of osmotic adjustment in two different strains of *P. freudenreichii*.

3.2 Materials and methods

3.2.1 Strains and pre-culture

Propionibacterium freudenreichii CIRM-BIA 129 (equivalent ITG P20) and CIRM-BIA 1025 (equivalent ITG P1) were provided, stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France). The strains are routinely cultivated in yeast extract lactate (YEL) medium (Malik et al., 1968) in this study, they are also cultivated in MMO (Medium Minus Osmoprotectants). MMO is a synthetic medium which was derivated from CdM described previously (Gagnaire et al., 2015), MMO medium has the same composition as CdM but glutamate, glutamine and proline, which are considered as potent sources of osmoprotectants, were removed. *P. freudenreichii* was grown at 30°C without agitation under microphilic condition. Composition of MMO is detailed in Table 4.

Table 4: Composition of the MMO (Medium Minus Osmoprotectants) medium.

Constituent	Final concentration
Sodium lactate	12.8 g/L
KH2PO4	0.6 g/L
Potassium acetate	0.4 g/L
MgSO4.7H2O	50 mg /L
MnSO4.4H2O	5 mg/L
FeSO4.7H2O	2.5 mg/L
CuSO4	2.5 mg/L
Cobalt acetate	0.25 mg/L
ZnSO4	15 µg/L
H3BO3	1 µg/L
Na2MoO4	1 µg/L
Thiamine	50 µg/L
Pyridoxal	100 µg/L
Calcium pantothenate	50 µg/L
Riboflavine	50 µg/L
Nicotinamide	100 µg/L

p-Aminobenzoic acid	10 µg/L
Biotine	4 µg/L
Folic acid	20 µg/L
Cyanocobalamine	2 µg/L
L-Ala	50 mg/L
L-Arg	160 mg/L
L-Asn	150 mg/L
L-Asp	250 mg/L
L-Cys	140 mg/L
Gly	80 mg/L
L-His	100 mg/L
L-Ile	180 mg/L
L-Leu	300 mg/L
L-Lys	220 mg/L
DL-Met	60 mg/L
L-Phe	170 mg/L
L-Ser	180 mg/L
L-Thr	150 mg/L
L-Trp	50 mg/L
L-Tyr	60 mg/L
DL-Val	480 mg/L
Adenine	5 mg/L
Guanine	5 mg/L
Uracile	5 mg/L
Xanthine	5 mg/L

3.2.2 Bacterial growth under osmotic stress

P. freudenreichii CIRM-BIA 129 and CIRM-BIA 1025 were grown under different condition: in YEL medium (0.429 osmol), in YEL medium with 0.9M NaCl (YEL+NaCl, 1.958 osmol) and in MMO, MMO with salt 0.4M (MMO+NaCl, 0.958 osmol) and MMO with 0.9 M NaCl and 1 mM of GB (MMO+NaCl+GB 0.960 osmol). 0.4 M NaCl is the highest salt concentration allowing *P. freudenreichii* growth in MMO medium. 0.4 M NaCl is the highest salt concentration allowing *P. freudenreichii* growth in YEL medium. The GB concentration used in the chemically defined MMO medium was 1 mM, reported as the optimal concentration in previous reports including the actinobacteria *Brevibacterium ammoniagenes* (Gouesbet et al., 2002) and *Propionibacterium freudenreichii* (Leverrier et al., 2003). Bacterial populations were followed by Optical Density (OD) at 650nm.

3.2.3 Stress challenges

Heat, oxidative, bile salts and acid challenge were applied to cultures at the beginning of stationary-phase (when maximal OD was reached). Heat challenge was performed by placing 2mL (in a 15 mL Falcon tube) of *P. freudenreichii* culture in a water bath at 60°C for 10 min (Leverrier et al., 2004). Oxidative challenge was applied by adding 1.25 mM of hydrogen peroxide (Labogros, France) to 2mL of *P. freudenreichii* culture during 1 hour at 30°C (Serata et al., 2016). Acid challenge was applied by re-suspending *P. freudenreichii* in MMO medium adjusted to pH 2.0 by using HCl at 30°C followed by a 1 hour incubation (Jan et al., 2000). Bile salts challenge was performed by adding $1g.L^{-1}$ of a bile salts mixture (an equimolar mixture of cholate and deoxycholate; Sigma Chemical, St. Louis, MO, USA) in the culture during 1 hour at 37°C (Leverrier et al., 2003). CFU counting was performed before and after challenge. In order to calculated survival percentage, a CFU counting was made, with untreated culture left for the same time at 30°C as a control.

3.2.4 Identification and quantification of compatible solutes accumulated by *P. freudenreichii* CIRM-BIA 129

3.2.4.1 Extraction of accumulated compatibles solutes

P. freudenreichii CIRM-BIA 129 was grown in MMO, MMO+NaCl and MMO+NaCl+GB. During exponential phase (OD= 0.8), cells were harvested by centrifugation (8000g 10 minutes). Cells were washed twice in a NaCl solution with the same osmolality than the culture medium. Cells were then re-suspended in 2mL of distilled water, then 8mL of absolute ethanol were added. The suspension was homogenized and centrifuged (8000g, 10 minutes) in order to remove cell fragments. The supernatant extract was evaporated during 7 hours with a rotary evaporator. Dried extracts were then solubilized in deuterium oxide (Sigma-Aldrich, USA).

3.2.4.2 NMR (Nuclear Magnetic Resonance) analyses.

All ^{1}H and ^{13}C NMR spectra were recorded at 298 K on a Bruker Avance 500 spectrometer equipped with a 5 mm TCI triple-resonance cryoprobe (PRISM core facility, Rennes). ^{1}H spectra were acquired with a 6 kHz spectral width, 32 K data points and a total repetition time of 6.73s. ^{13}C spectra were acquired using a proton power-gated decoupling sequence with a 30° flip angle, a 30 kHz spectral width, 64 K data points and a total repetition time of 3.08s. The data were processed with Topspin software (Bruker Biospin). Before applying the Fourier

transform, free induction decays of ^{1}H spectra were treated with an exponential broadening of 0.3 Hz.

Samples were solubilized in D_2O. 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP-d4) (Sigma-Aldrich, USA) served as an internal reference for ^{1}H and ^{13}C chemical shifts. Relative concentration of trehalose, glutamate and glycine betaine in the samples was determined by integration of their ^{1}H signals. Results are expressed as NMR relative units (RU).

3.2.5 Label free proteomics

3.2.5.1 Whole-cell protein extraction and protein tryptic digestion

The label free proteomics was have been conducted as Huang et al. 2018 (Huang et al., 2018). At the beginning of stationary phase, *P. freudenreichii* cells were harvested by centrifugation and washed twice with PBS buffer (NaCl 8g.L^{-1}, KCl 2g.L^{-1} KH_2PO_4 2g.L^{-1}, Na_2HPO_4 $12H_2O$ 35,8g.L^{-1}). Cell pellets were then re-suspended in lysis solution (lysis solution: 0.5 mL pH 7.5, 157g Tris-HCl, 0.03 g SDS, 0.3 g DTT and 9.5 mL H_2O), with 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, USA). The solution was frozen for 1 hour, then sonicated (2 min 30 HZ), and cells were broken using zirconium beads in the homogenizer (Homogénéisateur Precellys Evolution - Bertin Instruments, France). The resulting SDS extracts were recovered by centrifugation (21,000 × *g*; 20 °C; 20 min) and then cleaned and quantified using the two-dimensional (2-D) clean-up kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the 2-D quant kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), respectively. Tryptic digestion was performed on 100 µg of whole-cell proteins from each sample during 15 hours at 37 °C using Sequencing Grade Modified Trypsin (Promega, Madison, USA) according to the manufacturer's instructions and as described previously (Huang et al., 2018). Spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma-Aldrich, USA) was added in order to stop the digestion.

3.2.5.2 Nano-LC-MS/MS

Experiments were performed as previously described (Huang et al., 2018). Experiments were performed using a nano RSLC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nano-electrospray ion source. A preliminary sample concentration step was performed on a C18 pepMap100 reverse phase column (C18 column, 300-µm inner diameter (i.d.), 5 mm length, 5 µm particle size, 100 Å

pore size; Dionex, Amsterdam, The Netherlands). Peptides separation was performed on a reversed-phase column (PepMap 100 C18, 75 µm i.d., 250 mm length, 3 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) with a column temperature of 35°C, using solvent A (2% (v/v) acetonitrile (Honeywell, USA), 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water). Peptides were separated using a gradient of 5 to 35% solvent B over 80 min followed by 35 to 85% solvent B over 5 min at a flow rate of 0.3 µL/min. Eluted peptides were directly electro sprayed into the mass spectrometer operating in positive ion mode with a voltage of 2kV. Spectra were recorded in full MS mode and selected in a mass range 250-2000 m/z for MS spectra with a resolution of 70,000 at m/z 200. For each scan, the ten most intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at m/z 200 and the parent ion was subsequently excluded from MS/MS fragmentation for 20 s. The instrument was externally calibrated according to the supplier's instructions.

3.2.5.3 Protein identification

Proteins identification was performed as previously described (Huang et al., 2018). Peptides were identified from the MS/MS spectra using X!Tandem pipeline software (Langella et al., 2017). The search was performed against a database composed of proteomes of strains *P. freudenreichii* CIRM-BIA 129 and CIRM-BIA 1025 (downloaded from NCBI.nlm.nih.gov on the 23rd of August 2018). Database search parameters were specified as follow: trypsin cleavage was used and the peptide mass tolerance was set to 10 ppm for MS and 0.05 Da for MS/MS. Oxidation of methionine and phosphorylation of threonine, serine and tryptophan were selected as a variable modification. For each peptide identified, a minimum score corresponding to an e-value below 0.05 was considered as a prerequisite for peptide validation.

3.2.5.4 Protein quantification

Protein quantification was performed as previously described (Huang et al., 2018). Every peptide identified by tandem mass spectrometry was quantified using the free MassChroQ software (Valot et al., 2011) before data treatment and statistical analysis within the R software (R 3.2.2, Project for statistical computing). A specific R package called 'MassChroqR' was used to automatically filter dubious peptides for which standard deviation of retention time was superior to 40 s and to regroup peptide quantification data into proteins. Two different and

complementary methods of analysis were used, based on peak counting or XIC (eXtracted Ion Current). For peak counting analysis, variance analysis was performed on proteins with a minimum peak ratio of 1.5 between both culture conditions. Proteins with an adjusted p-value <0.05 were considered significantly different. For XIC based quantification, normalization was performed to take into account possible global quantitative variations between LC-MS runs. Peptides shared between different proteins were automatically excluded from the data set as well as peptides present in less than 80% of samples. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein (Blein-Nicolas et al., 2015). Analysis of variance was used to determine proteins with significantly different abundance between our two culture conditions.

3.2.6 Freeze-drying

P. freudenreichii strains were grown in the three different growth media (MMO, MMO+NaCl and MMO+NaCl+GB). At the beginning of the stationary phase, cultures were harvested (8000g, 10 minutes, 30°C). Pellets were then homogenized in a maltodextrin solution (100g.L^{-1}) (Roquette, France). The bacterial solutions were then freeze-dried (2253-04, Serail, France).

3.2.7 Statistical analysis

The data were from triplicate samples. All the results are presented as mean value with standard deviation. Statistical significance was set at $p<0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows).

3.3 Results

3.3.1 *P. freudenreichii* growth in hypertonic media is strain-dependent

The growth of *P. freudenreichii* CIRM-BIA 129 and 1025 was analyzed in YEL medium containing increasing NaCl concentrations, or in MMO supplemented or not with 0.4M NaCl and 1 mM GB (Fig. 8). The CIRM-BIA 129 strain was able to grow in the rich YEL medium supplemented by NaCl, up to 0.9 M, with a final OD close to the control without NaCl. High salt concentrations reduced significantly the growth rate (Fig. 8A). In the same conditions, the CIRM-BIA 1025 growth in YEL 0.4 M NaCl was similar to that of CIRM-BIA 129 but was completely inhibited in the presence of 0.9 M of NaCl, indicating that this strain is more susceptible to osmotic stress (Fig. 8B). In the chemically defined MMO medium, the two strains

displayed a similar growth profile. With the addition of 0.4 M NaCl, both strains had a limited growth with a reduced finale OD and growth rate (Fig. 8C and D), while no growth occurred for both strains in the presence of 0.9 M NaCl (data not shown). Addition of GB partially restored growth of *P. freudenreichii* CIRM-BIA 129 in salted MMO. By contrast, growth of CIRM-BIA 1025 was similar despite the presence or not of GB (Fig. 8D).

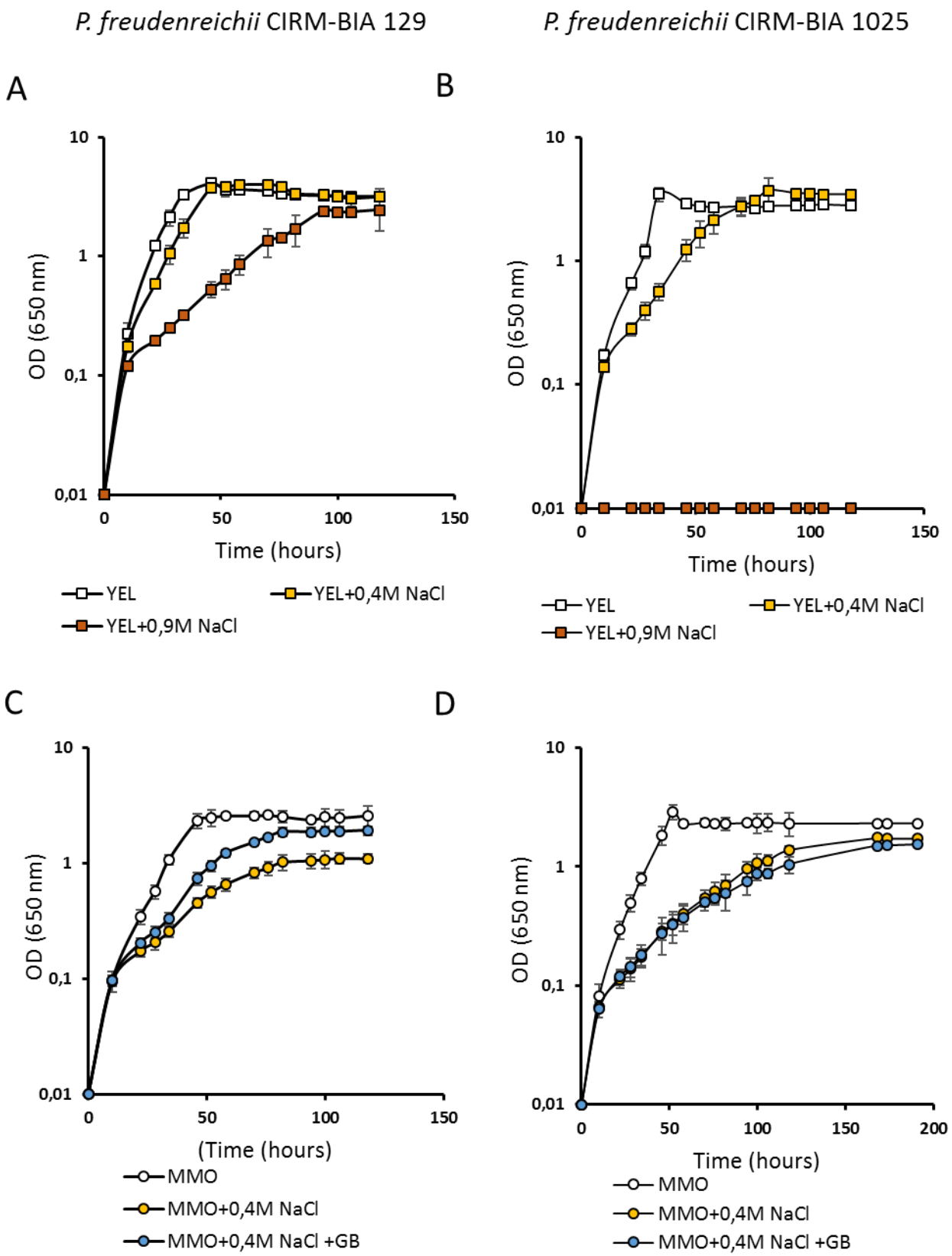

Figure 8: *P. freudenreichii* growth in the presence of salt is strain-dependent. *P. freudenreichii* CIRM-BIA 129 (A & C) and CIRM-BIA 1025 (B & D) were cultivated in YEL medium (A & B) with different salt concentrations (0, 0.4, and 0.9 M of NaCl) or in chemically-defined MMO medium, with or without salt (0.4 M NaCl), and with or without 1mM glycine betaine (C & D).

3.3.2 Compatible solutes accumulation in *P. freudenreichii* is strain-dependent

Intracellular accumulation of compatible solutes was quantified by NMR analysis. *P. freudenreichii* CIRM-BIA 129 grown in MMO+ 0.4 M NaCl accumulated trehalose (62.3 RU) and glutamate (37.7 RU) (Fig. 9). The strain CIRM-BIA 1025 accumulated the same compounds with nearly the same proportion, 60.9% and 39.1%, respectively. In the presence of glycine betaine, the CIRM-BIA 129 strain accumulated GB as the bulk majority of intracellular compatible solutes (89.5 RU), with only limited amounts of glutamate (8 RU) and nearly no trehalose (2.5 RU). In the same conditions, the CIRM-BIA 1025 strain accumulated more diverse compatible solutes, with much less GB (29.4 RU) and a majority of trehalose (46 RU), while glutamate (24.5 RU) was also accumulated. These results suggest that both strains possess GB uptake system. However, the replacement of endogenous osmolytes by GB, classically observed in many bacteria, is only effective for CIRM-BIA 129.

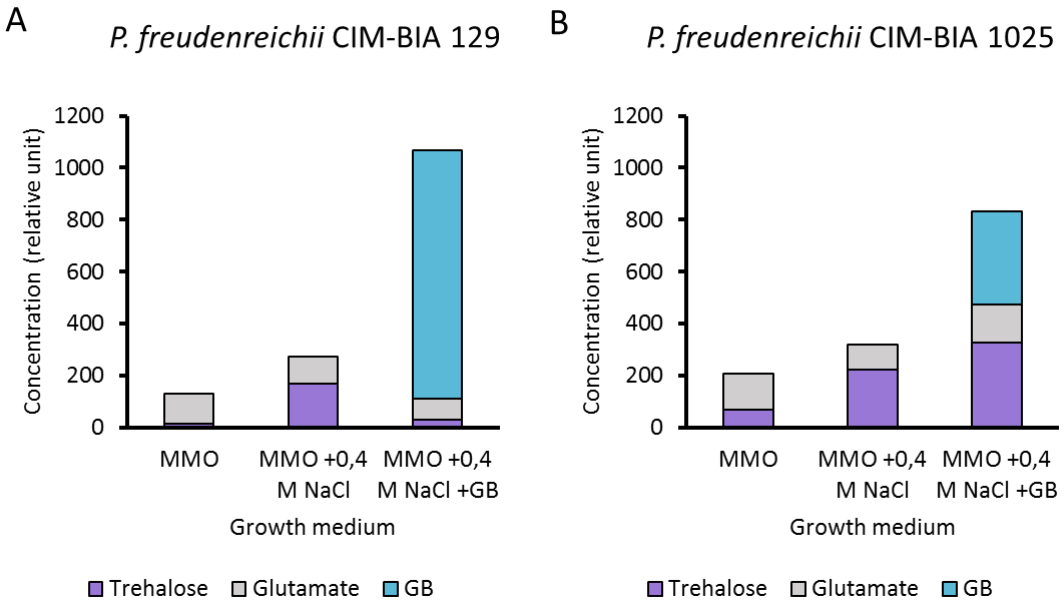

Figure 9: *P. freudenreichii* compatible solutes accumulation is strain and medium-dependent. *P. freudenreichii* CIRM-BIA 129 (A) and CIRM-BIA 1025 (B) were cultivated in the different growth media described in section 3.2.2. Cytoplasmic extract were made. Compatible solutes were identified and quantified by NMR analysis in these extracts. Compatible solutes accumulation is expressed as relative concentrations.

3.3.3 Multiple stress tolerance of *P. freudenreichii* is strain- and medium-dependent

P. freudenreichii viability was monitored by numeration, before and after stress challenges, in order to determine viability. We selected a series of stresses relevant to both technological and

digestive processes. CIRM-BIA 129 tolerance towards heat, acid and oxidative lethal challenges was higher, when grown in salted MMO medium, compared to control MMO (Fig. 10A, B, and C). This evidences osmotically induced multitolerance. It depends on the stress applied since the addition of NaCl, in the MMO medium, decreased CIRM-BIA 129 tolerance towards a bile salts lethal challenge, compared to control MMO (Fig. 10 D). With the addition of GB, the viability of the CIRM-BIA 129 strain decreased for all lethal challenges, compared to MMO+NaCl medium. This indicates abrogation of osmotically induced multitolerance, as a result of GB accumulation. By contrast, neither the addition of NaCl, nor that of GB, had any impact on *P. freudenreichii* CIRM-BIA 1025 viability after heat challenge (Fig. 10 E). These additions even decreased the viability after oxidative and acid challenges in this strain (Fig. 10F, G and H). Then, the response of 1025 and 129 are clearly different despite the accumulation of compatibles solutes, when submitted to osmotic stress.

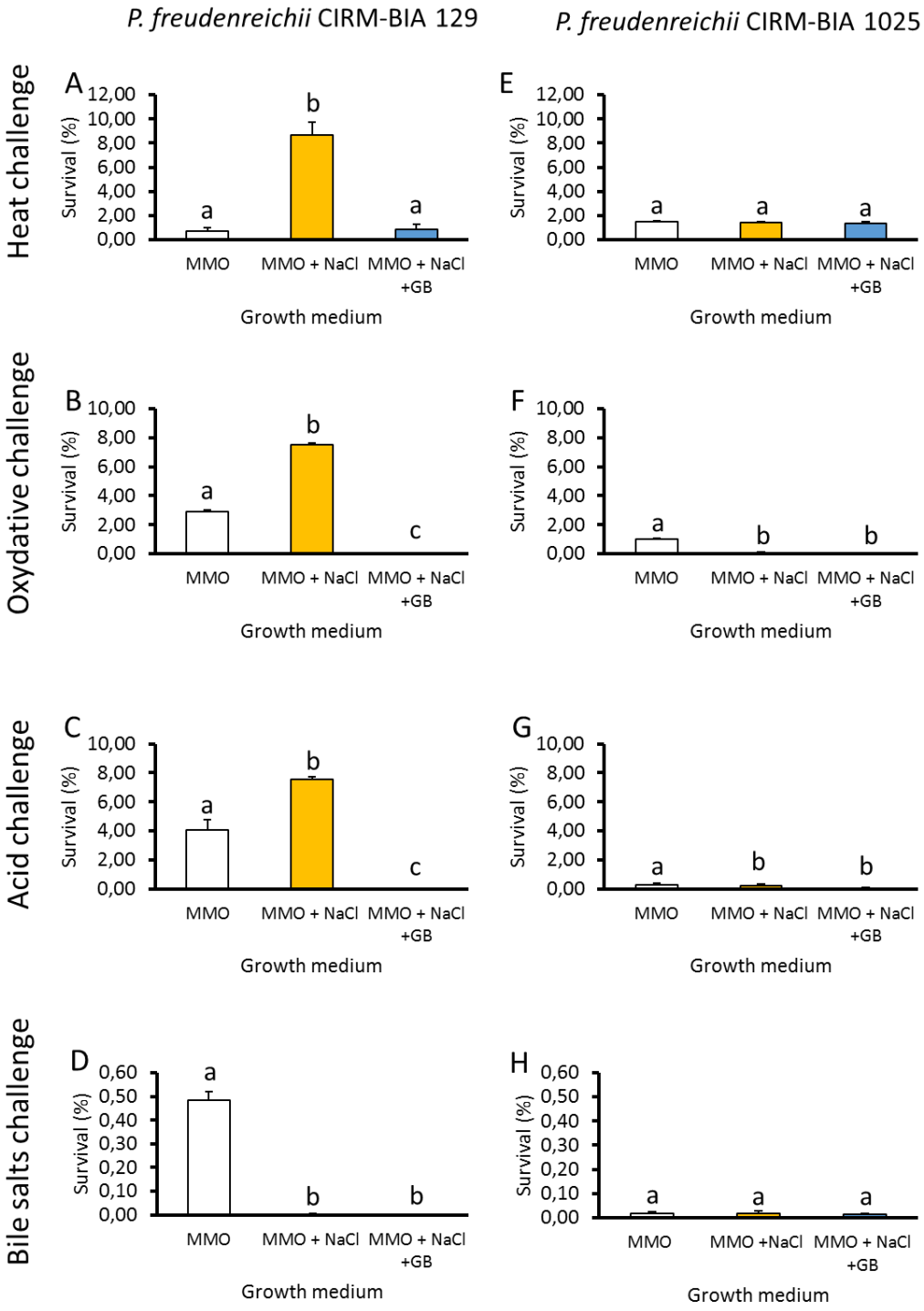

Figure 10: Cross-protections conferred to *P. freudenreichii* are strain-dependent. *P. freudenreichii* CIRM-BIA 129 (A, B, C, & D) and CIRM-BIA 1025 (E, F, G, & H) were cultivated until the beginning of stationary phase in three different growth media: MMO, MMO+NaCl and MMO+NaCl+GB. They were then subjected to heat (A & E, 60°C for 10 min), oxidative (B & F, 1.15 M H_2O_2 for 1 h), bile salts (C & G, 1 $g.L^{-1}$ for 1 h) or acid challenges (D & H, pH 2.0 for 1 h) as described in materials and methods. Surviving propionibacteria were enumerated by CFU counting in treated and control samples. Results are expressed as percent of survival. Error bars represent the standard deviation for triplicate experiments. Significant difference are reported with different letters above the columns ($p>0.05$).

3.3.4 Modulation of protein expression during osmoadaptation

To understand the difference observed between these two strains, we realized a proteomic differential analysis. We focused on proteins that were differentially expressed for at least one condition between MMO, MMO+NaCl and MMO+NaCl+GB, for *P. freudenreichii* CIRM-BIA 129 or for *P. freudenreichii* CIRM-BIA 1025, with a minimum ratio of 1.5 or less than 0.66. A total of 387 proteins were significantly modulated (See Supplemental Table 1). Among these, 58 proteins were involved in energy production, conversion of carbohydrate and transport (Table 5), 53 were involved in amino acid metabolism and transport (Table 6) and 35 in post-translational modification, protein turnover, and chaperone function (Table 7).

Table 5: Salt and glycine betaine-mediated modulation of proteins belonging to the COG functional category C "energy production and conversion" or G "carbohydrate metabolism and transport".

Ratio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.

a: Determined by using UniProtKB database corresponding to …

b: Ratio of the protein level in the MMO+NaCl medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl.

c: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

d: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO+NaCl medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

e: Non significant

accession	Description [a]	CIRM-BIA 129			CIRM-BIA 1025		
		MMO+ NaCl/ MMO[b]	MMO+ NaCl+ GB/ MMO[c]	MMO+ NaCl+ GB/ MMO+ NaCl[d]	MMO+ NaCl/ MMO[b]	MMO+ NaCl+ GB/ MMO[c]	MMO+ NaCl+ GB/ MMO+ NaCl[d]
emb|CDP49220.1|	Phosphocarrier, HPr family	0,38	0,53	NS[e]	0,49	0,56	NS
emb|CDP48824.1|	Dihydroxyacetone kinase	0,38	NS	2,44	0,39	0,44	NS
emb|CDP48902.1|	Fructose-bisphosphate aldolase class I	0,41	NS	2,72	NS	NS	NS
emb|CDP49440.1|	Ferredoxin	0,43	0,45	NS	NS	NS	NS
emb|CDP49591.1|	Cytochrome d ubiquinol oxidase, subunit II	0,53	NS	2,10	NS	NS	NS
emb|CDP49430.1|	Glucose-1-phosphate adenylyltransferase	0,53	NS	2,12	0,51	0,57	NS
emb|CDP49666.1|	Coenzyme A transferase	0,54	NS	NS	NS	NS	NS
emb|CDP48965.1|	nucleoside-diphosphate-sugar epimerases	0,55	NS	NS	NS	NS	NS
emb|CDP47837.1|	Malate dehydrogenase	0,58	NS	NS	0,63	NS	NS
emb|CDP49639.1|	Gluconate kinase	0,59	NS	1,57*	0,63	NS	NS
emb|CDP49605.1|	3-carboxymuconate cyclase	0,60	0,63	NS	NS	NS	NS
emb|CDP48837.1|	Aldose 1-epimerase	0,60	NS	NS	NS	NS	NS

emb|CDP48900.1|	Citrate synthase	0,60	NS	1,50	0,53	0,55	NS	
emb|CDP47731.1|	DhaK PTS-dependent dihydroxyacetone kinase,dihydroxyacetone-binding subunit	0,64	NS	1,65	NS	NS	NS	
emb|CDP48996.1|	Galactokinase	0,64	NS	1,80	NS	NS	NS	
emb|CDP48782.1|	Glycosyl hydrolase, family 13	0,65	NS	NS	NS	NS	NS	
emb|CDP49592.1|	Cytochrome d ubiquinol oxidase subunit I	0,65	NS	NS	NS	NS	NS	
emb|CDP47594.1|	Succinate dehydrogenase cytochrome B-558 subunit	0,66	NS	NS	NS	NS	NS	
emb|CDP48732.1|	L-lactate dehydrogenase	0,66	NS	NS	NS	NS	NS	
emb|CDP47815.1|	Pyruvate:ferredoxin oxidoreductase	0,66	NS	NS	NS	NS	NS	
emb|CDP47853.1|	NADPH:quinone reductase related Zn-dependent oxidoreductase	NS	NS	NS	0,60*	NS	NS	
emb|CDP47989.1|	Thiamine pyrophosphate (TPP family)	NS	NS	NS	1,56	1,54	NS	
emb|CDP48002.1|	NADH-quinone oxidoreductase chain F	NS	NS	1,65	NS	NS	NS	
emb|CDP48006.1|	NADH-quinone oxidoreductase chain B	NS	1,55	NS	NS	NS	NS	
emb|CDP48009.1|	electron transfer oxidoreductase	NS	NS	1,61	NS	NS	NS	
emb|CDP48026.1|	Zinc-containing alcohol dehydrogenase superfamily	NS	NS	NS	0,62	0,59	NS	
emb|CDP48084.1|	FAD linked oxidase domain protein	NS	NS	1,67	NS	NS	NS	
emb|CDP48129.1|	Xylulokinase protein, Carbohydrate kinase	NS	NS	1,64	0,61	0,66	NS	
emb|CDP48130.1|	Glycerol-3-phosphate dehydrogenase	NS	1,93	2,07	NS	NS	NS	
emb|CDP48449.1|	Succinate dehydrogenase, subunit B	NS	1,52	1,69	NS	NS	NS	
emb|CDP48511.1|	Nitroreductase	NS	NS	NS	1,51	1,80	NS	
emb|CDP48893.1|	Oxidoreductase	NS	1,58	NS	NS	NS	NS	
emb|CDP49268.1|	Glycerol kinase	NS	NS	1,77	NS	NS	NS	
emb|CDP49349.1|	Aldehyde dehydrogenase	NS	NS	1,73	NS	NS	NS	
emb|CDP49526.1|	Beta-galactosidase	NS	NS	1,55	NS	NS	NS	
emb|CDP49788.1|	Galactokinase	NS	NS	NS	1,74	1,82	NS	
emb|CDP49834.1|	Ribose-5-phosphate isomerase 3	NS	NS	NS	2,14	1,99	NS	
emb|CEG97971.1|	Inositol-1-monophosphatase	NS	NS	NS	0,58	0,62	NS	

emb|CEG98002.1|	Putative aldo/keto reductase	NS	NS	NS	1,63	1,52	NS
emb|CEG98016.1|	Pyruvate phosphate dikinase	NS	NS	NS	0,52	0,60	NS
emb|CEG99056.1|	Phosphate acetyltransferase	NS	NS	NS	0,49	0,50	NS
emb|CEG99057.1|	Acetate kinase	NS	NS	NS	0,64	NS	NS
emb|CEG98960.1|	Methylmalonyl-CoA carboxytransferase 5S subunit.	NS	NS	NS	0,63	0,65	NS
emb|CDP48804.1|	ATP synthase B chain	1,61	1,75	NS	2,17	2,30	NS
emb|CDP49578.1|	Electron transfer flavoprotein-quinone oxidoreductase (FixC protein)	1,61	1,55*	NS	NS	NS	NS
emb|CDP48974.1|	Enolase 1	1,63	NS	0,60	NS	NS	NS
emb|CDP49579.1|	Electron transfer flavoprotein, carnitine metabolism (FixB protein)	1,63	NS	0,60	NS	NS	NS
emb|CEG97844.1|	ATP synthase F1 sector subunit beta	1,63	1,67	NS	NS	NS	NS
emb|CDP48428.1|	pyruvate dehydrogenase E1 component	1,83	NS	NS	NS	NS	NS
emb|CDP48799.1|	ATP synthase F1 sector epsilon subunit	1,85	1,70	NS	1,99	1,94	NS
emb|CDP49189.1|	Fructose-bisphosphate aldolase class II	1,88	NS	0,63	NS	NS	NS
emb|CDP48899.1|	Oxidoreductase	1,91	1,51	NS	NS	NS	NS
emb|CDP48803.1|	ATP synthase delta chain	1,99	1,93	NS	NS	NS	NS
emb|CDP49838.1|	Sulfite reductase	2,06	1,94	NS	NS	NS	NS
emb|CDP48122.1|	Betaine-aldehyde dehydrogenase	0,59*	NS	NS	NS	NS	NS
emb|CDP49431.1|	Glycosyltransferase	0,64*	NS	NS	NS	NS	NS
emb|CDP49577.1|	Ferredoxin-like protein fixX	2,06*	NS	NS	NS	NS	NS
emb|CEG98306.1|	sugar transporter, major facilitator superfamily	2,59*	2,28*	NS	NS	NS	NS

Table 6: Salt and glycine glycine betaine-mediated modulation of proteins belonging to the COG functional category E "amino acid metabolism and transport".

Ratio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.

a: Determined by using UniProtKB database corresponding to …

b: Ratio of the protein level in the MMO+NaCl medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl.

c: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

d: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO+NaCl medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

e: Non significant

accession	Description [a]	CIRM-BIA 129			CIRM-BIA 1025		
		MMO+ NaCl/ MMO[b]	MMO+ NaCl+ GB/ MMO[c]	MMO+ NaCl+ GB/ MMO+ NaCl[d]	MMO+ NaCl/ MMO[b]	MMO+ NaCl+ GB/ MMO[c]	MMO+ NaCl+ GB/ MMO+ NaCl[d]
emb|CDP48506.1|	Alanine dehydrogenase	0,07	NS[e]	11,62	0,30*	0,32*	NS
emb|CDP48687.1|	Glycine cleavage H-protein (lipoate-binding)	0,38	0,46	NS	0,51	0,57	NS
emb|CDP49124.1|	Glutamine amidotransferase of anthranilate synthase or para-aminobenzoate synthase	0,45	NS	1,83	NS	NS	NS
emb|CEH00437.1|	Xaa-Pro aminopeptidase I	0,48	NS	1,78	NS	NS	NS
emb|CDP48503.1|	Amidohydrolase	0,53	NS	2,00	NS	NS	NS
emb|CDP48646.1|	anthranilate synthase component I	0,57	NS	NS	NS	NS	NS
emb|CDP49409.1|	Glutamine synthetase	0,59	1,51	2,56	NS	NS	NS
emb|CDP47865.1|	Cysteine synthase 1	0,60	NS	NS	NS	NS	NS
emb|CDP48633.1|	Histidinol-phosphate aminotransferase	0,60	NS	NS	NS	NS	NS
emb|CDP48419.1|	4-aminobutyrate aminotransferase	0,61	NS	NS	NS	NS	NS
emb|CDP49173.1|	Dihydroxy-acid dehydratase	0,62	NS	NS	0,62	0,62	NS

emb|CDP49134.1|	L-serine dehydratase	0,63	NS	NS	0,62	0,62	NS
emb|CDP48561.1|	polar amino acid ABC transporter, binding protein component	0,65	NS	NS	NS	NS	NS
emb|CDP49308.1|	Cysteine desulphurases, SufS	1,66	NS	0,61	NS	NS	NS
emb|CDP48772.1|	3-isopropylmalate dehydratase small subunit	1,66	NS	0,62	NS	NS	NS
emb|CDP49711.1|	Nitrogen regulatory protein P-II	0,18*	2,16*	12,1*	4,41*	4,41*	NS
emb|CEG99164.1|	Alanine dehydrogenase	0,36*	NS	2,57*	0,25	0,30	NS
emb|CEG98734.1|	Glutamate synthase large subunit (Ferredoxin)	0,65*	NS	NS	NS	NS	NS
emb|CDP49001.1|	glutamate dehydrogenase	0,65*	NS	NS	NS	NS	NS
emb|CEG99336.1|	Aminopeptidase N, Lysyl aminopeptidase	NS	1,51	NS	NS	NS	NS
emb|CDP49614.1|	Argininosuccinate synthase	NS	1,70	1,65	NS	NS	NS
emb|CEG97416.1|	Protein of unknown function	NS	NS	NS	NS	0,50*	NS
emb|CDP48480.1|	Imidazole glycerol phosphate synthase subunit HisF	NS	NS	NS	1,50	NS	NS
emb|CDP47871.1|	Chorismate mutase	NS	1,60	NS	2,36	3,08	NS
emb|CDP48779.1|	3-isopropylmalate dehydrogenase	NS	0,64	NS	NS	NS	NS
emb|CDP47561.1|	N-acetyl-gamma-glutamyl-phosphate reductase	NS	NS	1,81	NS	NS	NS
emb|CDP49606.1|	Shikimate 5-dehydrogenase	NS	NS	NS	0,66	0,67	NS
emb|CDP48345.1|	Phosphoserine phosphatase/homoserine phosphotransferase bifunctional protein	NS	NS	NS	1,80	2,02	NS
emb|CEG98743.1|	Phosphoribosyl-AMP cyclohydrolase	NS	NS	NS	0,46	0,44	NS
emb|CDP48360.1|	3-phosphoshikimate 1-carboxyvinyltransferase	NS	NS	NS	1,52	1,60	NS
emb|CDP48589.1|	methionine synthase	NS	2,11	NS	NS	NS	NS
emb|CDP47558.1|	Acetylornithine and succinylornithine aminotransferase	NS	NS	1,75	NS	NS	NS
emb|CEG99593.1|	Aspartate ammonia-lyase (Aspartase)	NS	NS	0,63	NS	NS	NS
emb|CDP47560.1|	Arginine biosynthesis bifunctional protein ArgJ	NS	1,59	NS	NS	1,70*	NS
emb|CDP48567.1|	Bifunctional PLP-dependent enzyme with beta-cystathionase and maltose regulon repressor activities	NS	NS	NS	1,94	2,11	NS
emb|CDP49050.1|	Phosphoserine aminotransferase	NS	0,54	0,44	NS	NS	NS
emb|CDP47562.1|	Aspartate aminotransferase	NS	NS	NS	1,57	1,64	NS

emb|CEG98065.1|	Amino acid permease. membrane protein	NS	NS	2,81*	NS	NS	NS
emb|CEG99028.1|	Amino acid permease-associated region. Membrane protein	NS	NS	NS	1,60	NS	NS
emb|CDP49113.1|	Propanediol utilization protein PduU	NS	NS	NS	NS	12,00*	NS
emb|CEH00744.1|	Thiamine pyrophosphate enzyme	NS	NS	NS	0,55	0,54	NS
emb|CDP47931.1|	solute binding protein of the ABC transport system	NS	0,63	NS	NS	NS	NS
emb|CEG97415.1|	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	1,65*	NS	NS	NS	NS	NS
emb|CDP47878.1|	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	1,68*	NS	NS	NS	NS	NS
emb|CEG97413.1|	binding protein of choline ABC transporter	2,15*	1,63*	NS	NS	NS	NS
emb|CDP47876.1|	binding protein of choline ABC transporter	2,34*	1,71*	NS	NS	NS	NS

Table 7: Salt and glycine betaine-mediated modulation of proteins belonging to the COG functional category O "post translational modification, proteins turnover or chaperone functions".

Ratio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.

a: Determined by using a database composed of proteomes of strains *P. freudenreichii* CIRM-BIA 129 and CIRM-BIA 1025 (downloaded from NCBI.nlm.nih.gov on the 23rd of August 2018).

b: Ratio of the protein level in the MMO+NaCl medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl.

c: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

d: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO+NaCl medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

e: Non significant.

accession	Description [a]	CIRM-BIA 129			CIRM-BIA 1025		
		MMO+ NaCl/ MMO[b]	MMO+ NaCl+ GB/ MMO[c]	MMO+ NaCl+ GB/ MMO+ NaCl[d]	MMO+ NaCl/ MMO[b]	MMO+ NaCl+ GB/ MMO[c]	MMO+ NaCl+ GB/ MMO+ NaCl[d]
emb|CDP48273.1|	Surface layer protein B	0,14	0,26	1,83	NS[e]	NS	NS
emb|CDP48952.1|	peptide-methionine (S)-S-oxide reductase	0,41	0,57	NS	0,47*	0,54*	NS
emb|CDP48879.1|	Thioredoxin	0,49	0,58	NS	NS	NS	NS
emb|CDP48588.1|	SppA, Periplasmic serine proteases	0,61	NS	NS	NS	NS	NS
emb|CDP49020.1|	Chaperone protein dnaJ 1	0,66	NS	NS	0,55	0,57	NS
emb|CDP49048.1|	Thioredoxine	0,67	NS	NS	NS	NS	NS
emb|CDP47745.1|	groES protein 2	1,51	NS	NS	NS	NS	NS
emb|CDP49311.1|	FeS assembly protein SufD	1,51	NS	NS	NS	NS	NS
emb|CDP48411.1|	SmpB SsrA-binding protein	1,71	NS	NS	NS	NS	NS
emb|CDP47705.1|	ATP-dependent Clp protease proteolytic subunit 2	1,78	NS	NS	1,54	1,61	NS
emb|CDP49309.1|	ABC-type transport system involved in Fe-S cluster assembly, ATPase component, SufC	1,85	NS	0,58	NS	NS	NS
emb|CDP49617.1|	thiol peroxidase	1,88	NS	0,59	NS	NS	NS

emb|CDP49261.1|	Secreted protein	0,57*	NS	NS	0,35*	0,41*	NS
emb|CDP48858.1|	Surface protein with SLH domain	0,61*	NS	NS	NS	NS	NS
emb|CEH00247.1|	Surface layer protein B	0,65*	NS	NS	NS	NS	NS
emb|CDP47874.1|	groEL protein 1	NS	NS	0,63	NS	NS	NS
emb|CDP48789.1|	Thioredoxin	NS	NS	0,65	NS	NS	NS
emb|CEG99654.1|	Heat shock protein 20 kDa 2	NS	NS	NS	1,56	NS	NS
emb|CEG97258.1|	Heat shock protein 20 kDa 1	NS	NS	NS	1,85	1,82	NS
emb|CDP47875.1|	groES protein 1	NS	NS	NS	1,96	1,94	NS
emb|CDP47709.1|	Trigger factor	NS	NS	0,66	NS	NS	NS
emb|CDP47983.1|	Alkyl hydroperoxide reductase subunit C	NS	NS	NS	1,78	1,79	NS
emb|CDP49391.1|	Probable peptidyl-prolyl cis-trans isomerase A	NS	0,65	NS	NS	NS	NS
emb|CEH00703.1|	Co-chaperone protein GrpE2	NS	NS	NS	1,91	2,08	NS
emb|CDP49595.1|	peptidyl-prolyl cis-trans isomerase	NS	0,51	0,64	NS	NS	NS
emb|CEH01092.1|	thiol peroxidase	NS	NS	NS	2,58	2,46	NS
emb|CDP49795.1|	Thioredoxin	NS	0,44	0,41	0,58	0,56	NS
emb|CDP47990.1|	Metalloprotease (Peptidase family M13)	NS	NS	NS	1,62	1,70	NS
emb|CDP49400.1|	HesB protein	NS	NS	NS	0,66	0,63	NS
emb|CDP47657.1|	Chaperone protein dnaJ 3	NS	NS	0,65	0,62	0,62	NS
emb|CDP48201.1|	peptidyl-prolyl cis-trans isomerase	NS	0,64	0,59	NS	NS	NS
emb|CDP47586.1|	Magnesium chelatase, subunit ChlI	NS	NS	NS	3,59*	3,59*	NS
emb|CDP49125.1|	groEL protein 2	NS	NS	0,62	NS	NS	NS
emb|CEG97257.1|	Heat shock protein 20b 3 (20 kDa chaperone 3)	1,90*	1,90*	NS	NS	1,63	NS
emb|CDP48339.1|	Heat shock protein 20 kDa 3	1,97*	1,90*	NS	NS	NS	NS

A PCA analysis was performed on the abundances of the 1362 propionibacterial proteins that were detected in this proteomic investigation (Fig. 11A). It revealed that *P. freudenreichii* CIRM-BIA 129 displayed three different profiles, according to the culture conditions. By contrast, CIRM-BIA 1025 only displayed two different profiles: one for the MMO medium, while the second profile was the same for the MMO+NaCl and for the MMO+NaCl+GB culture media (Fig. 11A). Salt addition strongly affected cellular proteome in both strains. Indeed, Figure 11A clearly shows a shift of the global proteome towards increased values of the Axis 1, yet reduced values of the Axis 2, whatever the strain. However, glycine betaine addition further changed *P. freudenreichii* CIRM-BIA 129 global proteome by partially restoring the initial value of Axis 1. By contrast, glycine betaine addition adds little or no effect on CIRM-BIA 1025 global proteome. This is consistent with the absence of effect of GB on CIRM-BIA 1025 growth. The corresponding heatmap (Fig. 11B) further evidences three very different proteomes in the CIRM-BIA 129 strain, while only two proteomic patterns (MMO+NaCl and MMO+NaCl+GB) are very close concerning the CIRM-BIA 1025 strain. We then selected 321 differential proteins exhibiting ratios below 0.66 or above 1.5. The corresponding Venn diagram, shown in Figure 11C, further indicates strain-dependent proteomic readjustments. Among the 321 regulated proteins, only 60 were regulated in both strains. By contrast, 134 proteins were exclusively regulated in CIRM-BIA 129 and 127 exclusively in CIRM-BIA 1025. In CIRM-BIA 129, a high number of stress proteins was detected in the presence of salt without GB. By contrast, in CIRM-BIA 1025, a high number of stress proteins were observed, whatever the presence or absence of GB.

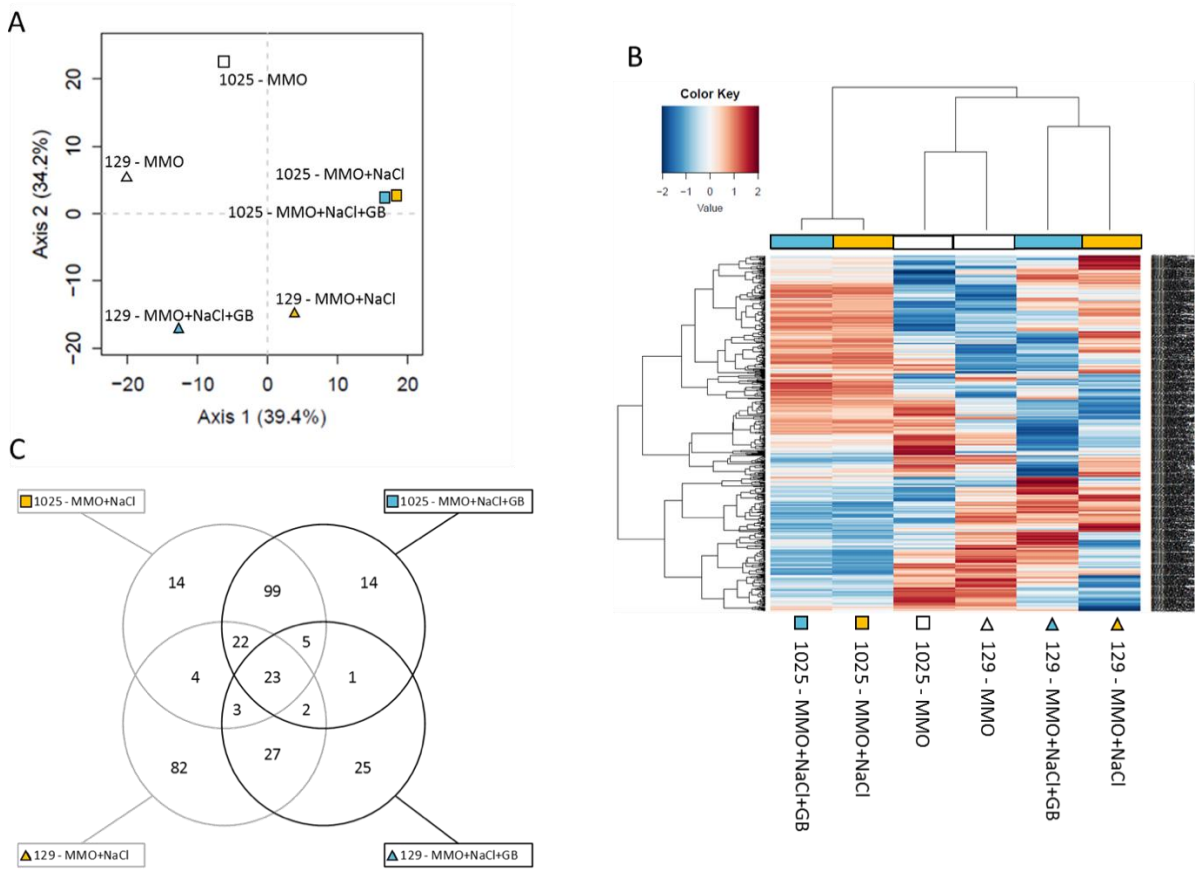

Figure 11: Glycine betaine effect on the stress proteome is strain-dependent. The PCA represents the distribution of the couple strain-growth medium, according to the protein abundances (A). Analysed proteomes were extracted from *P. freudenreichii* CIRM-BIA 129 (square) or CIRM-BIA 1025 (triangle), cultivated in different growth medium: MMO (white), MMO+NaCl (grey), MMO+NaCl+GB (black). Proteins were extracted, identified and quantified as described in materials and methods. The Venn diagram (C) represents the numbers of stress proteins exhibiting a modified expression in CIRM-BIA 129 or CIRM-BIA 1025, in presence of NaCl, with or without GB, compared to the control MMO medium

When looking deeper into the expression of specific proteins, crucial differences, between strains, were evidenced, such as the induction of compatible solutes uptake OpuA in CIRM-BIA 129, yet not in CIRM-BIA 1025, as a result of salt addition. This confirmed different behaviors of these strain. Furthermore, looking at proteins involved in energy production and conversion or carbohydrate and transport (Table 5), many adjustments were observed in *P. freudenreichii* CIRM-BIA 129, yet not in the strain CIRM-BIA 1025. As an example, proteins involved in ATP synthesis (ATP synthase F1 sector, ATP synthase delta and B chain), in electron transfer chain (electron transfer flavoprotein, cytochrome d'ubiquinol oxidase, oxidoreductase), and in carbohydrate utilization (enolase, galactokinase, beta-galoctosidase),

were modulated by NaCl addition, and restored by GB addition in the CIRM-BIA 129 strain, yet not in CIRM-BIA 1025 strain. By contrast, some modulations were specific of the strain CIRM-BIA 1025, such as the repression of propionic fermentation enzyme (methylmalonyl-CoA carboxytransferase 5S subutnit) or induction of proteins involved in oxidative stress remediation (nitroreductase, aldo-keto reductase). About proteins involved in amino acid metabolism and transport (Table 6), transporters of compatible solutes and/or of aminoacids were induced in the presence of NaCl, yet not in the presence of NaCl+GB, including betaine/carnitine/choline ABC transporter ATP-binding protein opuCA of glycine and betaine/carnitine/choline ABC transporter, while one involved in polar aminoacids (polar amino acid ABC transporter, binding protein component) was repressed, in CIRM-BIA 129. These modulations did not occur in *P. freudenreichii* CIRM-BIA 1025. Proteins involved in compatible solutes neosynthesis were also regulated in *P. freudenreichii* CIRM-BIA 129 (glutamine synthetase, glutamate deshydrogenase, glutamate synthase large subunit), yet not in 1025. In 1025, by contrast, proteins involved in signal transduction and regulation (nitrogen regulatory protein P II and bifunctional PLP-dependent enzyme with beta cystathionase and maltose regulon repressor activities) were specifically induced.

Concerning proteins involved in post translational modification, protein turnover, chaperone function (Table 7), general stress adaptation proteins were specifically modulated in CIRM-BIA 129, i.e. induced in the presence of NaCl, yet not in the presence of NaCl+GB, including groES protein 2 and thiol peroxidase. Addition of GB further repressed groEL protein 1, groEL protein 2, which also participate in general stress response. A different set of stress proteins were specifically induced in CIRM-BIA 1025, including heat shock protein, groES protein 1 and chaperone protein Hsp20. Moreover, ATP-dependent protease Clp2, which is involved in misfolded proteins turnover, was induced by salt in both strains, but repressed by the addition of GB only in *P. freudenreichii* CIRM-BIA 129.

3.3.5 *P. freudenreichii* viability after freeze-drying.

P. freudenreichii CIRM-BIA 129, when cultivated in MMO medium, displayed a low viability upon freeze-drying (3.3 %) (Fig. 12). As previously shown for individual stress challenge (Fig. 10), viability was enhanced as a result of salt addition to this medium (9.4%). However, such improvement was reduced by the addition of GB, and the viability of *P. freudenreichii* CIRM-BIA 129 decreased to 6.8%. By contrast, the CIRM-BIA 1025 strain exhibited its best viability

when cultivated in MMO medium (6.5%). With the addition of salt with or without GB, the *P. freudenreichii* CIRM-BIA 1025 viability decreased to 4%.

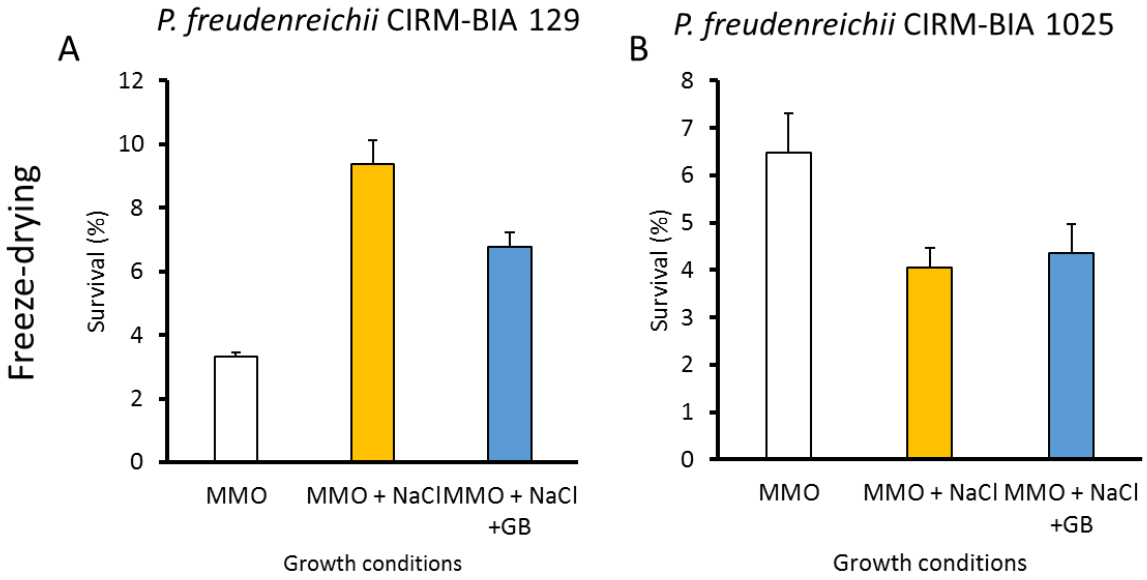

Figure 12: Osmoadaptation modulates the viability of *P. freudenreichii* submitted to freeze-drying. *P. freudenreichii* CIRM-BIA 129 and CIRM-BIA 1025 were cultivated until the beginning of stationary phase in three different growth medium: MMO, MMO+NaCl and MMO+NaCl+GB (A and B respectively). Propionibacteria were then freeze-dried as described in materials and methods. Results are expressed as percent survival. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p>0.05$).

3.4 Discussion

3.4.1 Osmotic tolerance and adaptation is strain-dependent in *P. freudenreichii*

P. freudenreichii CIRM-BIA 129 and CIRM-BIA 1025 strains are both able to grow in the rich YEL medium with a low concentration of salt (0.4 M NaCl). The addition of a high concentration of salt (0.9 M NaCl) is more selective and allows growth of CIRM-BIA 129, yet not of CIRM-BIA 1025. This suggests that the rich YEL medium contains compatible solutes, which CIRM-BIA 129 would better use than CIRM-BIA 1025 does. The restoration of *P. freudenreichii* CIRM-BIA 129 growth in MMO+NaCl, as a result of GB addition, indicates a key role of GB in its osmoadaptation. By contrast, GB did not restore CIRM-BIA 1025 growth in MMO+NaCl. This suggested that GB is imported and accumulated by CIRM-BIA 129, yet not by CIRM-BIA 1025. This hypothesis was then shown to be false, as NMR evidenced accumulation of GB in both strains. However, GB levels were much lower in CIRM-BIA 1025

than in CIRM-BIA 129. This small accumulation had no positive impact on CIRM-BIA 1025 growth, in contrast with CIRM-BIA 129. Both strains accumulated glutamate, trehalose and glycine betaine. Glutamate accumulation in hyperosmotic conditions was already reported for *Lactobacillus plantarum* (Glaasker et al., 1996; Kets et al., 1996). Trehalose is a key protectant molecule in *P. freudenreichii*, it can be accumulated during acid, cold, osmotic and oxidative treatments (Cardoso et al., 2004, 2007; Dalmasso et al., 2012b; Huang et al., 2016b). GB accumulation has already been observed during *P. freudenreichii* growth in hyper-concentrated rich media (Huang et al., 2016b), but its beneficial effect is shown here to be strain-dependent.

3.4.2 Benefits and drawbacks of osmoadaptation on cross-protection toward other stress are strain-dependent

NaCl adaptation enhanced *P. freudenreichii* CIRM-BIA 129 tolerance towards heat, oxidative and acid challenges, as well as towards freeze-drying. Restoration by GB in these hyperosmotic conditions however suppressed this cross-protection and even triggered hypersensitivity towards oxidative, bile salts, acid and freeze-drying challenges. Since GB replaces trehalose and glutamate accumulation inside the cells, we can suggest that these molecules could play a major role in cross protection (Santivarangkna et al., 2008). By contrast, addition of salt failed to provide cross protection in *P. freudenreichii* CIRM-BIA 1025, and so did the addition of GB. Finally, osmoadaptation had a negative impact on tolerance of both strains towards freeze-drying (Fig.12).

3.4.3 Proteomic patterns may explain differential adaptation and stress tolerance

Osmoadaptation triggered major modulations of cellular proteins' expression. Indeed, growth under hyperosmotic constraint and in the absence of compatible solute induced expression of many proteins involved in stress adaptation (ie compatible solutes uptake systems) or reflecting metabolic readjustments. The heatmap in Figure 11B and Venn diagram in Figure 11C clearly evidence differences in terms of proteomics variations, among strains. Among the 321 stress proteins identified, only 23 were induced whatever the strain and medium. Such a discrepancy highlights the strain-dependence of osmoadaptation. Major proteome changes induced by stress in CIRM-BIA 129 may explain the efficiency of osmoadaptation in this strain. This strain, which was shown to adapt hyperosmotic constraint and to transport GB, overexpressed proteins involved in carbohydrates transport and utilization, electron transfer chain and ATP synthesis (Table 5). Enolase, as an example, was previously shown to participate in multiple stress

adaptation in *P. freudenreichii* (Leverrier et al., 2004) and in other food-grade bacteria (Chen et al., 2017). This glycolytic enzyme is also a component of RNA degradosome, which is involved in RNA processing and gene regulation, in accordance with its role in stress tolerance acquisition in many bacteria (Weng et al., 2016). Overexpression of ATPase subunits, allowing enhanced energy production, is a key parameter of adaptation, of intracellular homeostasis and of metabolic readjustments. It was already reported to be involved in acid-adaptation in *P. acidipropionici* (Guan et al., 2013). As another key difference between the two strains, CIRM-BIA 129 was able to import GB thanks to the ATP binding protein opuCA of the glycine betaine/carnitine /choline ABC transporter, which were induced here by salt (Table 6). This strain was already reported to overexpress opuCA in hyper concentrated sweet whey (Huang et al., 2016b). This induction explains the high GB accumulation in MMO+NaCl+GB (Fig. 9) and the restoration of growth (Fig. 8). Furthermore, CIRM-BIA 129 also overexpressed the binding protein of an ABC choline transporter, suggesting the ability to accumulate other compatible solutes, when provided in the growth medium (Table 6). Salt further triggered over-expression of the GroES chaperone and of proteins involved in oxidative stress remediation (Table 7). By contrast, enzymes such as glutamine synthetase and glutamate dehydrogenase, which are susceptible to reduce intracellular compatible solute concentration, were repressed (Table 6). Finally, the overexpression of several proteins involved in stress adaptation is consistent with the enhanced tolerance towards stresses (Fig. 10) and towards freeze-drying (Fig. 12).

3.4.4 Glycine betaine modulates both stress proteome and resistance

P. freudenreichii CIRM-BIA 129 over-produced compatible solutes transporters in MMO+NaCl. This over-production was however no more significant when GB was added, while GB restored growth (Table 6). Indeed, the absence of potent compatible solute in MMO+NaCl triggers the need for increased activity of such transporters. In many bacteria, such transporters are induced by the hyperosmotic constraint (Wood, 1999; Poolman et al., 2004; Hoffmann et al., 2013). By contrast, once turgor pressure and growth are restored by GB, opuA expression is downregulated in response to the increase in the intracellular GB pool (Hoffmann et al., 2013). The “normalization” of the expression of many stress proteins, as a result of growth restoration by the availability of GB, is consistent with the loss of cross-protection towards stresses (Fig. 10) and towards freeze-drying (Fig. 12). Similarly, GB import limits Salmonella stress resistance, while salt adaptation without GB import leads to trehalose accumulation and to enhanced resistance in this bacterium (Pilonieta et al., 2012). Interestingly, the regulation of

expression of the ATP-dependent stress protease Clp indicates that hyperosmolarity causes accumulation of misfolded proteins in both strains, and that GB-mediated restoration suppresses this intracellular stress in CIRM-BIA 129, yet not in CIRM-BIA 1025.

3.4.5 Salt stress proteome is strain-dependent

Our study clearly indicated that osmoadaptation is more efficiently induced by NaCl in CIRM-BIA 129 than in CIRM-BIA 1025. In hyperosmotic conditions, *P. freudenreichii* CIRM-BIA 1025, which failed to adapt to strong hyperosmotic constraint and to be protected by GB, overexpressed a different set of proteins. This included heat shock proteins Hsp20, which reflect intracellular macromolecules damages (Table 7). This also included proteins involved in intracellular detoxification such as aldo/keto reductase, as well as ribose-5-phosphate isomerase, which is involved in the pentose phosphate pathway, producing NADPH and remediating oxidative stress. Moreover, PLP-dependent cystathionase, with a cystathionine beta-lyase activity, which is involved in remediation of intracellular reactive metabolites spontaneously damaging macromolecules in *Salmonella* (Ernst et al., 2018), was also induced by salt in CIRM-BIA 1025. Accordingly, PII nitrogen regulatory protein was strongly induced and plays a pivotal role in nitrogen stress remediation and stringent response (Brown et al., 2014; Merrick, 2015). Moreover, overexpression of chorismate mutase and of 3-phosphoshikimate 1-carboxyvinyltransferase indicate induction of the shikimate pathway, which is used to protect against reactive oxygen species (ROS) generated by salt stress in plants (Lee et al., 2016). Such over-expressions were not correlated with cross-protection of the strain CIRM-BIA 1025 towards challenge tests, in contrast with CIRM-BIA 129.

3.5 Conclusion

As a conclusion, this chapter highlights the strain-dependence of osmoadaptation, and of the resulting cross-protections, in *P. freudenreichii*. Indeed, *P. freudenreichii* CIRM-BIA 129 acquired a better tolerance, thanks to osmoadaptation without glycine betaine, to heat, oxidative and acid challenged and also to freeze-drying. Wherease osmoadaptation failed to exert positive impact on *P. freudenreichii* CIRM-BIA 1025 tolerance towards these challenges and towards freeze-drying. This work confirms that strains selection is an important prerequisite to industrial production of bacteria. *P. freudenreichii* CIRM-BIA 129, which previously revealed a high probiotic potential, is able to adapt hyperosmotic conditions. We thus intended to improve survival of this strain under technological stresses. Identification of relevant differential

proteins provided new insights into strain-dependent osmotic adaptation. However, accumulation of GB, considered as universal compatible solute, seemed to have deleterious effects on stress tolerance in this strain. Finally, growth conditions constitute key parameters for *P. freudenreichii* stress tolerance. Osmoadaptation was studied here in a chemically defined medium and should then be tested in rich media in order to improve *P. freudenreichii* resistance.

Important points of the chapter 3:

- *P. freudenreichii* cross-protections upon osmoadaptation is strain-dependent.
- Glycine betaine is not necessarily beneficial for *P. freudenreichii* stress tolerance.
- Osmoadaptation increases *P. freudenreichii* CIRM-BIA 129 viability during freeze-drying.
- *P. freudenreichii* CIRM-BIA 129 freeze-drying tolerance is higher following osmoadaptation in the absence of glycine betaine.

Chapter 4: Driving intracellular compatible solute concentrations to optimize *Propionibacterium freudenreichii* survival during drying.

In a previous study, osmoadaptation in hyper-concentrated sweet whey increased *Lactobacillus casei* and *Propionibacterium freudenreichii* survival upon spray drying (Huang et al., 2016b). During growth in hyper-concentrated sweet whey, these bacteria over-expressed stress proteins, and accumulated polyphosphates and high amounts of trehalose. Nevertheless, the mechanisms underlying the increased resistance towards spray drying remain a black box. In the previous chapter, osmoadaptation seemed to provide cross-protections to *P. freudenreichii* CIRM-BIA 129. However, we saw that addition of glycine betaine in the growth medium suppressed the protections provided by the osmoadaptation. Growth medium composition, and in particular the presence of compatible solutes and/or their precursors, can be promising to improve *P. freudenreichii* resistance.

In this chapter, we used rich growth medium to increase the biomass and to be closer to industrial conditions. We selected two dairy media, the sweet whey and the milk ultrafiltrate, and we modulated the composition of the YEL (Yeast Extract Lactate) medium with lactose addition. The different media contain different amounts of carbon and nitrogen sources and permit to explore the impact of the growth medium composition on accumulated compatible solutes. *P. freudenreichii* resistance was shown to be linked to the amount of available non-protein nitrogen and to the lactose concentration. Fine-tuning the ratio between the carbohydrates provided and non-protein nitrogen during growth under osmotic constraint modulated compatible solutes accumulation. This, in turn, was correlated with P. freudenreichii tolerance towards different stresses, on the one hand, and towards freeze-drying and spray drying, on the other. Surprisingly, trehalose accumulation correlated with spray-drying survival and glycine betaine accumulation with freeze-drying. This first report showing the ability to fine-tune the trehalose/GB ratio in osmoprotectants accumulated by a probiotic bacterium opens new perspectives for the optimization of probiotics production.

The aims of this chapter were to:

- Highlight the impact of the growth medium composition on the compatible solutes accumulated by *P. freudenreichii*
- Investigate the effect of the amount of compatible solutes accumulated by *P. freudenreichii* on its resistance to freeze-drying and to spray drying

The main contents in this chapter have been submitted as:

F. Gaucher, H. Rabah, K. Kponouglo, S. Bonnassie, S. Pottier, A. Dolivet, P. Marchand, R. Jeantet, P. Blanc, and G. Jan, Fine-tuning of intracellular compatible solute concentrations to optimize *Propionibacterium freudenreichii* survival during drying, Applied Microbiology and Biotechnology, submitted

4.1 Introduction

Probiotic microorganisms, including yeasts and bacteria, are widely dried to generate stable and easy-to-use preparations that are used in the food, feed and pharmaceutical industries (Huang et al., 2017b).The drying technology used should thus provide a stable product with extended shelf life, maintain a large quantity of live cells, and adapt probiotic microorganisms conducive to technological and digestive processes (Huang et al., 2017b). Freeze-drying is very widely used for this purpose and remains the preferred and reference technology. It allows efficient drying of live probiotic cells, but remains time-consuming, expensive and is a discontinuous process. In contrast, spray drying is a technology predominantly used in the dairy industry (Schuck et al., 2016). It constitutes a continuous process with a lower specific energy cost than freeze-drying and a higher productivity (Huang et al., 2017b). During freeze-drying, bacteria suffer from cold, oxidative and osmotic stresses (Gaucher et al., 2019b). Spraying increases the air/product contact surface by generating fine droplets (10 to 150 µm) into a stream of hot and dry air (150 to 200°C), maximizing the heat and mass transfer and drying within a few seconds. However, it can lead to a dramatic loss in bacterial viability because of the oxidative and thermal stresses imposed. Spray drying was recently applied to cultures of the probiotic bacterium *Propionibacterium freudenreichii* with diverse degrees of effectiveness (Huang et al., 2016a, 2016b, 2017a). Probiotic bacteria are further exposed to harsh conditions during the storage and the digestion process. However, their mode of action often requires them to reach the colon alive for optimal efficacy. Tolerance towards acidity and bile salts thus constitutes a limit to their efficacy. Probiotic growth conditions can determine such tolerance.

We have recently shown that although standard laboratory cultures of *P. freudenreichii* experience massive cell death upon spray drying, growth in hyperconcentrated sweet whey dramatically enhances its survival during this stressful process (Huang et al., 2016a, 2016b, 2017a). Sweet whey constitutes a very rich and complex growth medium that was used in a four- to five-time concentrated hypertonic form in this study. Growth of *P. freudenreichii* in such medium triggered complex and diverse modifications within propionibacteria including changes in morphology, overexpression of stress proteins including ClpB, SodM, EF-Tu and Hsp20, and accumulation of storage compounds including glycogen and polyphosphate (Huang et al., 2016b). During osmoadaptation, *P. freudenreichii* is able to accumulate trehalose, glycine betaine and glutamate (Gaucher et al., 2019a). Accumulated intracellular trehalose has already been reported to protect bacteria during freeze-drying (Termont et al., 2006) and glycine betaine accumulation can have a positive or negative impact on drying depending on the bacterial

species (Saarela et al., 2005; Sheehan et al., 2006; Bergenholtz et al., 2012). However, tolerances conferred by each compatible solute are not well known. Owing to the great complexity of the hyperconcentrated sweet whey growth and drying medium, the mechanisms triggered that lead to general stress tolerance remained a black box. In particular, the nature and the amount of the different compatible solutes accumulated were poorly addressed. Indeed, different compatible solutes may be accumulated, with different impacts on tolerance acquisition.

In this study, we modulated *P. freudenreichii* growth conditions by changing concentrations of available carbohydrates (CH) and non-protein nitrogen (NPN). We monitoredcompatible solute accumulation and stress tolerance acquisition. Driving the ratio between CH and NPN during growth under osmotic constraint modulated the ratio between glycine betaine (GB) and trehalose. This, in turn, affected *P. freudenreichii* tolerance towards different stresses, towards freeze-drying and towards spray drying, thus opening new perspectives for the optimization of freeze-drying and spray drying processes.

4.2 Materials and methods

4.2.1 Growth media and *P. freudenreichii* growth

Propionibacterium freudenreichii CIRM-BIA 129 (equivalent ITG P20) was provided, stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France). *P. freudenreichii* is routinely cultivated in yeast extract lactate (YEL) medium (Malik et al., 1968). *P. freudenreichii* CIRM-BIA 129 was grown in different media: laboratory medium: YEL medium, YEL medium with 0.9 M NaCl (YEL+NaCl), YEL medium with 34 $g.L^{-1}$ of lactose (YEL+Lactose) and YEL medium with 34 $g.L^{-1}$ of lactose and 0.9 M NaCl (YEL+Lactose+NaCl); and in dairy-type medium: sweet whey (SW), sweet whey with 0.7 M NaCl (SW+NaCl), Milk Ultrafiltrate (MU), and Milk Ultrafiltrate with 0.7 M NaCl (MU+NaCl). Non-Protein Nitrogen (NPN) was determined as previously described (Desmazeaud et al., 1976) and Total Nitrogen (TN) was determined using the Kjeldahl method (FIL/IDF, 1993). Osmolarity and growth medium composition are reported in Table 8. Bacterial populations were monitored by Optical Density (OD) at 650 nm. Salt concentrations are the highest concentrations that enable *P. freudenreichii* "normal" growth in the different media.

Table 8: Composition and osmotic pressures of the different culture medium.
YEL: Yeast Extract Lactate. L: Lactose. MU: Milk Ultrafiltrate. SW: Sweet Whey.

	YEL	YEL+NaCl (0.9 M)	YEL+L	YEL+L+NaCl (0.9 M)	MU	MU+NaCl (0.7 M)	SW	SW+NaCl (0.7 M)
Saccharides (Lactose) ($g.L^{-1}$)	0	0	34	34	48	48	33.4	33.4
Nitrogen total (g.L-1)	14.6	14.6	14.6	14.6	2.3	2.3	4.2	4.2
Non-protein Nitrogen (NPN) (g.L-1)	14.2	14.2	14.2	14.2	1.5	1.5	0.2	1.6
Lactose/NPN	0	0	2.4	2.4	32	32	167	20.9
Osmotic pressure (osmol)	0.308	2.028	0.406	2.175	0.259	1.996	0.192	1.903

4.2.2 Identification and quantification of compatible solutes accumulated by *P. freudenreichii* CIRM-BIA 129

4.2.2.1 Extraction of accumulated compatible solutes

P. freudenreichii CIRM-BIA 129 was grown in the different media. During the exponential growth phase (OD=0.8), cells were harvested by centrifugation (8000 g, 10 min, 30°C). Compatible solutes were then extracted as previously described in chapter 3 (Gaucher et al., 2019a).

4.2.2.2 NMR (Nuclear Magnetic Resonance) analyses.

Dried extracts were then analyzed by NMR as previously described in chapter 3 (Gaucher et al., 2019a). Results are expressed as NMR relative units (RU).

4.2.3 Stress challenges

Heat, oxidative, bile salt and acid challenges were applied to cultures at the beginning of the stationary phase (when maximal OD was reached). The heat challenge was performed by placing 2 mL (in a 15-mL Falcon tube) of *P. freudenreichii* culture in a water bath for 10 min at 60°C, corresponding to the Spray drying temperature (Leverrier et al., 2004). The oxidative challenge was applied by adding 1.25 mM of hydrogen peroxide (Labogros, France) to 2 mL of *P. freudenreichii* culture for 1 h at 30°C (Serata et al., 2016). The acid challenge was applied by re-suspending *P. freudenreichii* in culture medium adjusted to pH 2.0 by using HCl at 30°C followed by a 1-h incubation (Jan et al., 2000). The bile salt challenge was performed by adding 1 $g.L^{-1}$ of a bile salt mixture (an equimolar mixture of cholate and desoxycholate; Sigma Chemical, St. Louis, MO, USA) to the culture for 1 h at 37°C (Leverrier et al., 2003). CFU counting was performed after the challenges. In order to calculate the survival percentage, a

CFU counting was made on untreated culture left for the same length of time at 30°C (for the heat, oxidative and acid challenges) or 37°C (for the bile salt challenge) as a control.

4.2.4 Spray drying and powder storage

Spray drying was performed on a laboratory-scale spray dryer (Mobile Minor^{TM}, GEA Niro, Denmark). *P. freudenreichii* was cultivated in the different YEL media. At the beginning of the stationary phase, cells were harvested by centrifugation (8000 g, 20 min, 30°C) and resuspended in a maltodextrin solution (DE= 6-8) (Roquette, France). In the bacterial solution obtained, maltodextrin concentration was 20% of the dry matter, whereas the bacterial concentration was 4% of the dry matter. These different suspensions (~2 L) of *P. freudenreichii* were agitated for 10 min prior to delivery to the dryer by a peristaltic pump (520S, Watson-Marlow, France). A two-fluid nozzle with a diameter of 0.8 mm was used for atomization. The inlet air temperature was fixed at 160°C. The temperature outlet air was controlled at 60 ± 2°C, by adjusting the feed rate. The bacterial viabilities were estimated by numeration on YEL agar plates incubated at 30°C before and after spray drying, as previously described (Huang et al., 2016a). The powders were collected and sealed in sterilized polystyrene bottles (Gosselin, France), stored at a controlled temperature of 4, 20 or 37 °C, and kept away from light. The samples were stored for analyses for 112 days (approximately 4 months). One gram of powder was solubilized in 9 g of sterile water and bacterial viability was tested by numeration on YEL agar plates incubated at 30°C.

4.2.5 Freeze-drying

P. freudenreichii strains were grown in the YEL, YEL+NaCl, YEL+Lactose and YEL+Lactose+NaCl media. At the beginning of the stationary phase, cultures were harvested (8000 g, 10 min, 30°C). Pellets were then homogenized in a maltodextrin solution (DE= 6-8) (Roquette, France) with a final concentration of 10% (w/w). The bacterial solutions were then freeze-dried (2253-04, Serail, France). The bacterial viabilities were estimated by numeration on YEL agar plates incubated at 30°C before and after freeze-drying.

4.2.6 Statistical analysis

The data were from triplicate samples. All the results are presented as a mean value with standard deviation. Statistical significance was set at $p<0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows).

4.3 Results

4.3.1 *P. freudenreichii* growth parameters depend on carbon sources and on salt concentration

The addition of lactose to the rich YEL medium increased the final population of *P. freudenreichii* in Fig. 13 A. The presence of NaCl inhibited *P. freudenreichii* growth in all media. However, only the rich YEL medium sustained growth of *P. freudenreichii* up to 0.9 M NaCl, in agreement with the presence of compatible solutes. The dairy culture media, MU (milk ultrafiltrate) and SW (sweet whey), failed to allow growth of *P. freudenreichii* in the presence of such high salt concentrations (Fig. 13A). The addition of salt to YEL+Lactose, to MU and to SW+NaCl decreased both parameters, i.e., growth rates and final populations, compared to a non-salty medium (Fig. 13B and C). In contrast, the addition of salt to YEL without lactose decreased the growth rate but the final population was maintained, compared to the YEL control. The presence of lactose in YEL+Lactose or in YEL+Lactose+NaCl had no impact on the *P. freudenreichii* growth rate but increased final populations, in agreement with its role as a fermentation substrate. Accordingly, the pH decreased down to values close to 5 in all media containing lactose, while it remained close to 6.5 in YEL without lactose.

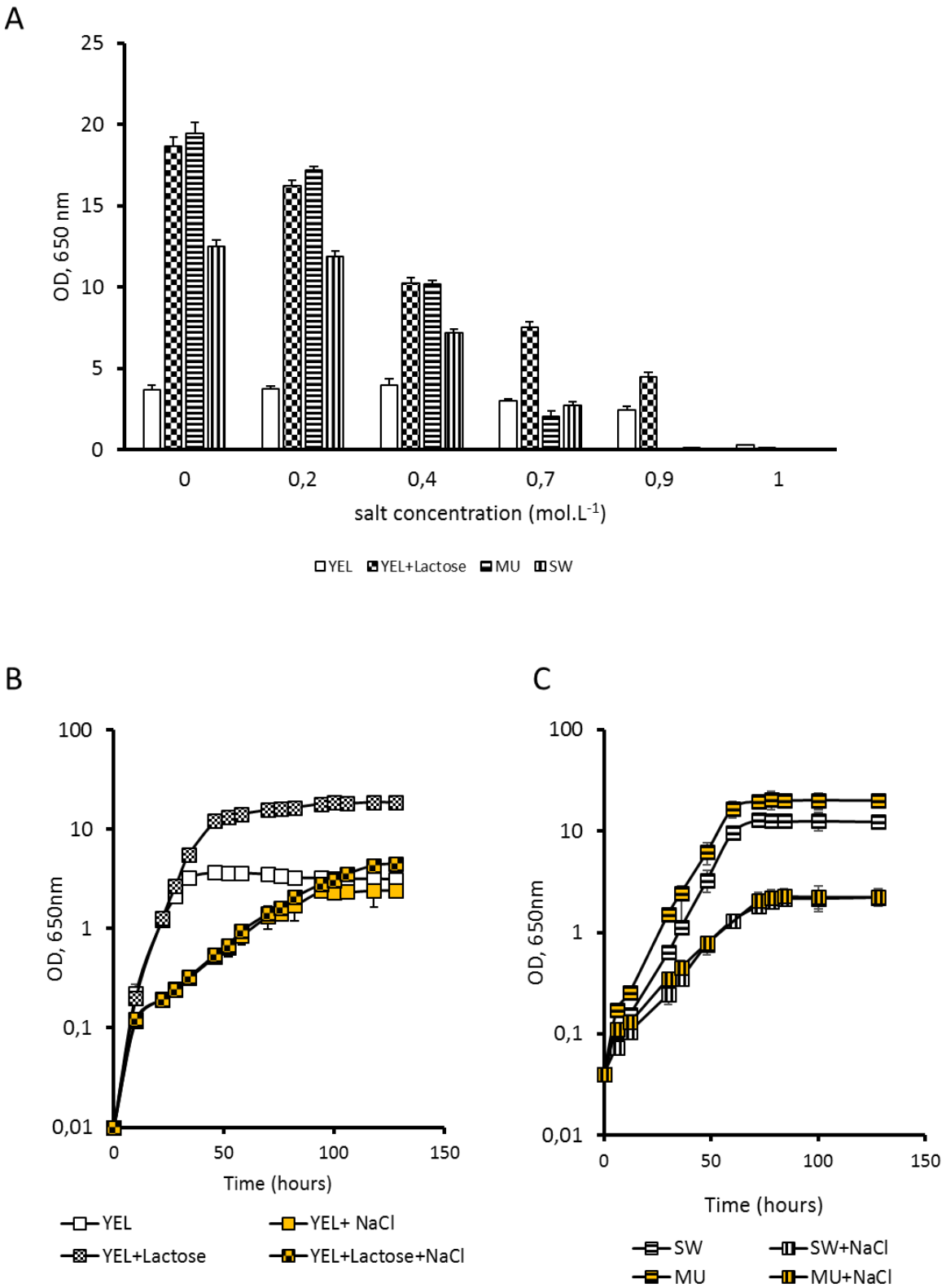

Figure 13: *P. freudenreichii* growth in media supplemented or not with NaCl. (A) Final optical densities were measured after 130 hours of growth in YEL-type media (YEL, YEL+NaCl YEL+Lactose and YEL+Lactose+NaCl) and in dairy-type media (SW, SW+NaCl, MU and MU+NaCl) in different salt concentration. B & C. *P. freudenreichii* CIRM-BIA 129 growth was then monitored in these media, in the presence of the highest NaCl concentration allowing growth. YEL: yeast extract lactate. MU: Milk Ultrafiltrate. SW: Sweet Whey

4.3.2 Compatible solute accumulation in *P. freudenreichii* is medium-dependent

Salt addition improved the total amount of compatible solutes accumulated by *P. freudenreichii*, regardless of the culture medium (Fig. 14 A and B). The presence of lactose had a similar impact: in YEL+Lactose and YEL+Lactose+NaCl, the total amount of compatible solutes accumulated are higher than in YEL and in YEL+NaCl, respectively. This is in agreement with the additional 0.1 osmol of osmotic pressure due to 34 $g.L^{-1}$ of lactose and with the acidification of the growth medium. As a negative control, the YEL medium, with an osmolality of 0.308 osmol, causes no osmotic stress and, consequently, no compatible solute accumulation inside bacteria. In the presence of salt (YEL+NaCl), *P. freudenreichii* mainly accumulated glycine betaine (1370 relative units (RU)) and low amounts of glutamate (132 RU) and trehalose (74 RU). In the presence of lactose (YEL+Lactose+NaCl), *P. freudenreichii* accumulated higher proportions of trehalose (328 RU) and lower proportions of glycine betaine (739 RU). In MU and in SW, which are both rich in lactose, the addition of salt led *P. freudenreichii* to mainly accumulate trehalose (244 RU and 467 RU, respectively), as well as small amounts of glycine betaine and of glutamate (14 RU and 26 RU, respectively, for MU+NaCl and 228 RU, and 114 RU, respectively, for SW+NaCl).

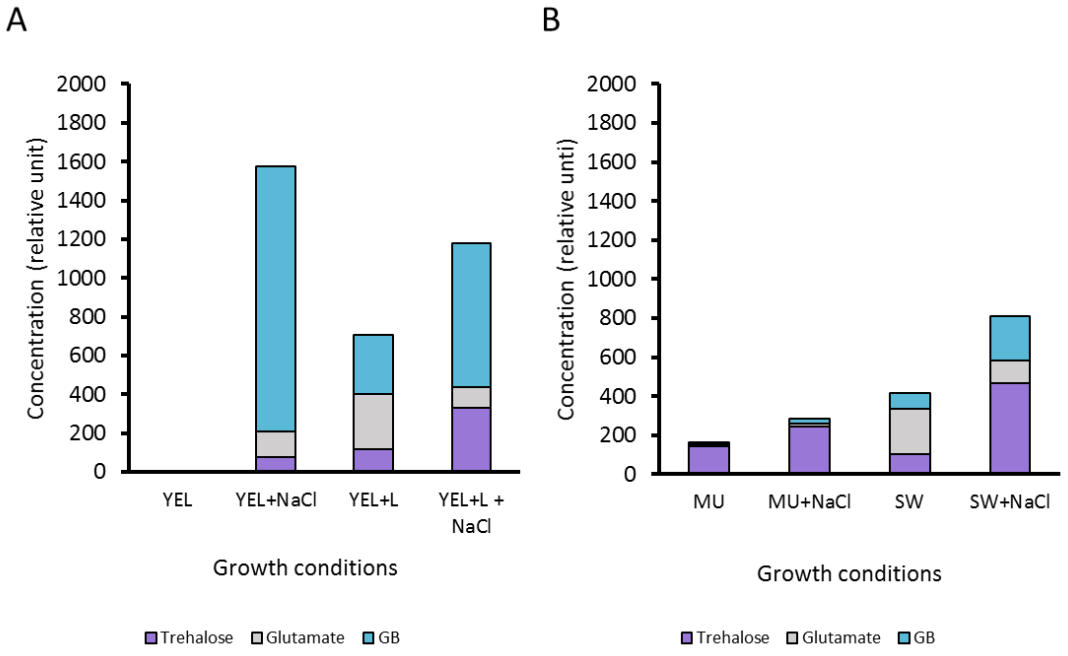

Figure 14: *P. freudenreichii* compatibles solutes accumulation is medium-dependent. *P. freudenreichii* CIRM-BIA 129 was cultivated in YEL type-media (A) and dairy type-media (B), containing or not NaCl. Cytoplasmic extracts were made as described in materials and methods. Intracellular compatible solutes were then identified and quantified by NMR analysis. Compatible solutes accumulations are expressed as relative units. YEL: Yeast Extract Lactate. L: Lactose. MU: Milk Ultrafiltrate. SW: Sweet Whey.

In conclusion, the three growth media that contained lactose (YEL+Lactose, MU and SW) triggered compatible solute accumulation even in the absence of salt. Salt triggered GB accumulation in YEL, and this accumulation was reverted by lactose addition, which leads to trehalose accumulation. In YEL+NaCl, GB is the main compound accumulated, whereas in MU+NaCl and SW+NaCl, trehalose is the main compound accumulated.

4.3.3 Multiple stress tolerance of *P. freudenreichii* is medium-dependent

P. freudenreichii stress tolerance was monitored by subjecting bacteria to heat, oxidative, acid and bile salt challenges. *P. freudenreichii* viability was determined by numeration before and after stress challenges. We selected a series of challenges relevant for both technological and digestive constraints. The presence of NaCl in YEL+NaCl medium had no impact on *P. freudenreichii* heat resistance at 60°C (Fig. 15 A), but had a positive effect in the YEL+Lactose+NaCl, SW+NaCl and MU+NaCl media (97.9%, 82.7% and 56.6%, respectively), compared to the non-salty media (60.0%, 31.0% and 44.8%, respectively) (Fig. 15 A and B). However, the presence of salt in the four media (YEL+NaCl, YEL+Lactose+NaCl, SW+NaCl and MU+NaCl) had a negative effect or no impact on *P. freudenreichii* survival to oxidative, acid and bile salt (Fig. 15 C-H). These results reveal a cross-protection between osmoadaptation and heat stress, but not for the other stresses. The presence of lactose (YEL+Lactose and YEL+Lactose+NaCl) had a positive impact for the four different stress challenges.

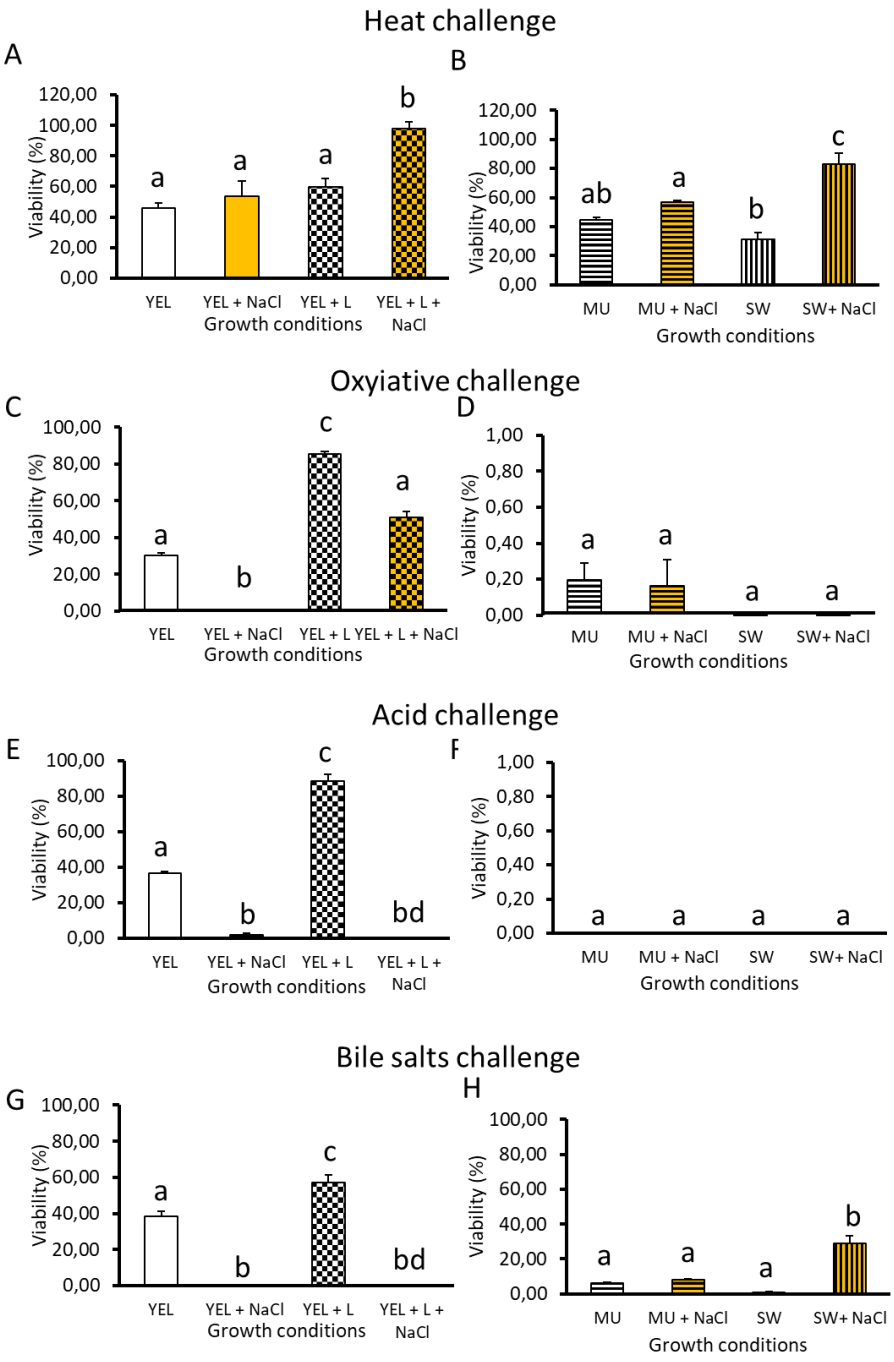

Figure 15: Cross-protections conferred to *P. freudenreichii* are medium-dependent. *P. freudenreichii* CIRM-BIA 129 was cultivated until the beginning of stationary phase in YEL type-media (A, C, E, G) and dairy-type media (B, D, F, H), with and without NaCl. Cultures were then subjected to heat (A and B, 60°C for 10 min), oxidative (C and D, 1.15 mM H_2O_2 for 1 h), acid challenges (E and F, pH=2 for 1 h) or bile salts (G and H, 1 $g.L^{-1}$ for 1 h) challenges as described in materials and methods. *P. freudenreichii* viability was determined by CFU counting in treated and control samples. Results are expressed as percent of survival. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p>0.05$). YEL: Yeast Extract Lactate. L: Lactose. MU: Milk Ultrafiltrate. SW: Sweet Whey.

4.3.4 *P. freudenreichii* viability after drying.

Different *P. freudenreichii* cultures, with or without the addition of salt, were subjected to freeze-drying or spray drying in order to evaluate tolerance towards industrial drying processes. The presence of salt in YEL+NaCl and YEL+Lactose+NaCl medium had a positive impact on *P. freudenreichii* viability during freeze-drying (98.0% and 53.7%, respectively) compared to YEL and YEL+Lactose medium (85.2% and 32.8%, respectively) (Fig. 16 A). The addition of lactose had a negative impact on *P. freudenreichii* viability during freeze-drying.

The mesures of the water activity of the powders were 0.17 ± 0.04. A similar positive effect of NaCl was observed for bacteria grown in YEL+Lactose+NaCl compared to YEL+Lactose (102% and 73%, respectively, Fig. 16 B) during spray drying process. In contrast, bacteria grown in YEL+NaCl exhibited a lower viability compared to bacteria grown in YEL (13% and 28%, respectively, Fig. 16 B).

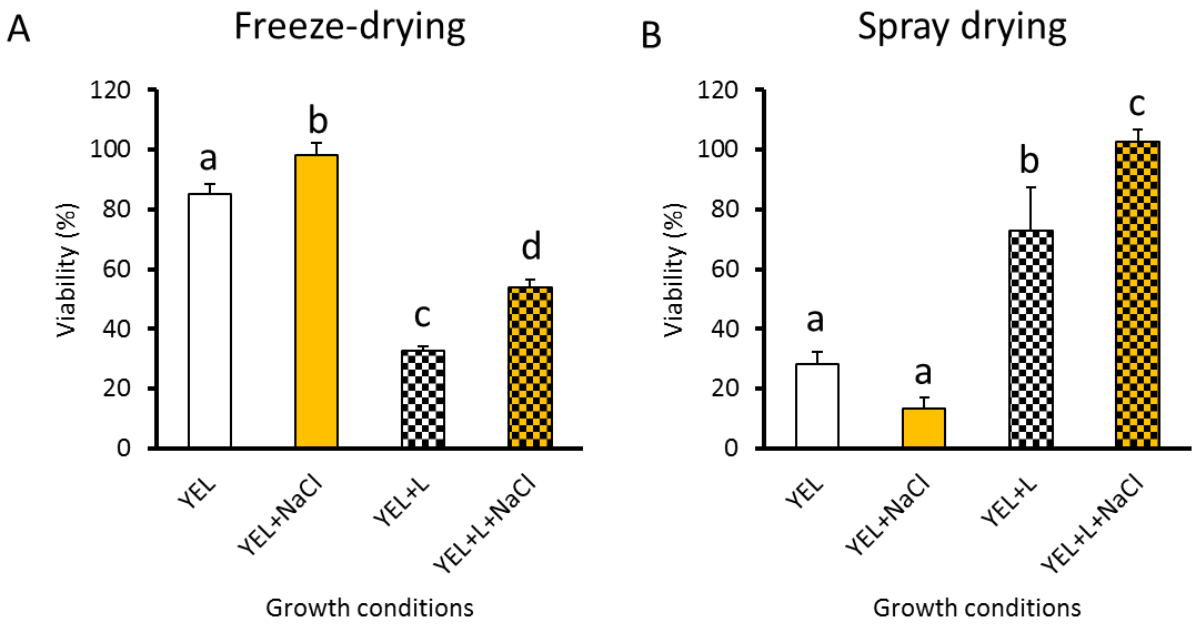

Figure 16: *P. freudenreichii* adaptations modulates its viability during freeze-drying and spray drying. *P. freudenreichii* CIRM-BIA 129 was cultivated until the beginning of stationary phase in YEL medium, in the presence or absence of NaCl and of lactose. *P. freudenreichii* were harvested, and then suspended in a maltodextrine solution (20%). The suspension was freeze-dried (A) and spray-dried (B) as described in materials and methods. Results are expressed as percent survival. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p>0.05$). YEL: Yeast Extract Lactate. L: Lactose.

4.3.5 *P. freudenreichii* viability after spray drying and powder storage.

The viability *P. freudenreichii* within spray dried powders depends on growth conditions and on storage temperature. During storage at 4°C, bacterial viability was stable over time (Fig. 17A). At 20°C, the viability decreased. During storage at 37°C, *P. freudenreichii* viability decreased faster than during storage at 20°C (Fig. 17 B and C). At 37°C, powders resulting from cultures in YEL medium containing lactose and NaCl exhibited a lower stability over time than the other cultures.

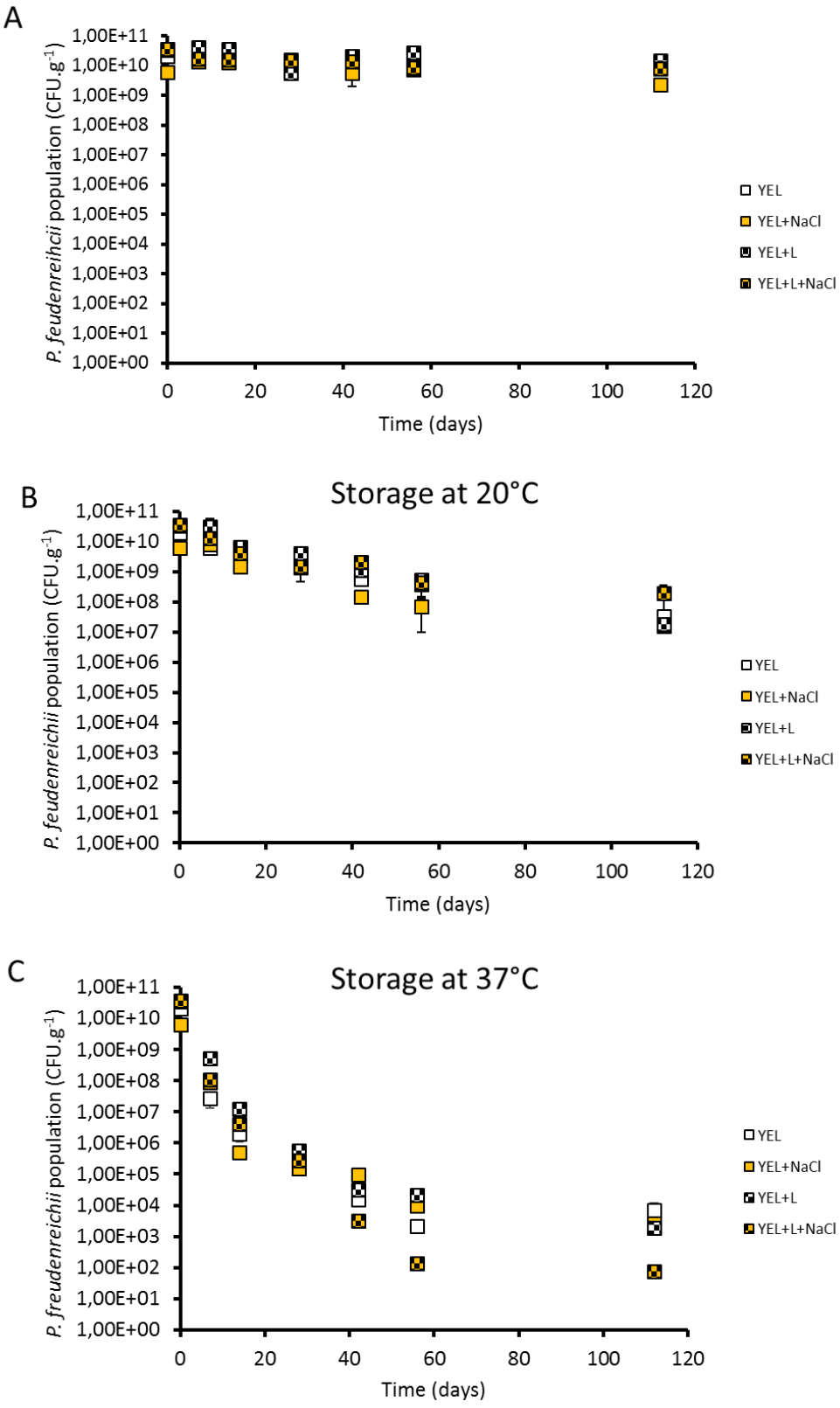

Figure 17: Storage temperature and *P. freudenreichii* adaptation modulates its viability during storage of powders. The different powders were stored at 4°C (A), 20°C (B) and 37°C (C) during 115 days. *P. freudenreichii* population in spray-dried powders were quantified by CFU counting (as described in material and methods) during storage. YEL: Yeast Extract Lactate. L: Lactose.

4.4 Discussion

4.4.1 The growth medium composition drives *P. freudenreichii* adaptation and compatible solute accumulation.

This study revealed that *P. freudenreichii* is able to adapt to various concentrations of salt, and that different culture media support this adaptation. *P. freudenreichii* grew in the presence of 0.4 M NaCl, regardless of the medium. Growth was further observed up to 0.9 M NaCl in YEL medium and final populations were further enhanced by the addition of lactose. This indicates that, compared to milk ultrafiltrate (MU), YEL provides more potent compatible solutes. It contains yeast extract and tryptone, which are sources of non-protein nitrogen (NPN) such as amino acids, peptides, glycine betaine and soluble vitamins (Frings et al., 1993; Maria-Rosario et al., 1995; Robert et al., 2000). This large amount of NPN that provided potent compatible solutes led to the efficient osmoadaptation of *P. freudenreichii* and a better tolerance to salt than MU and SW media, which contain less NPN. For all culture media, the salt concentration chosen was the highest that would allow *P. freudenreichii* to grow. However, in all salty growth media, *P. freudenreichii* exhibited slower growth compared to non-salty medium.

During osmoadaptation, *P. freudenreichii* is able to accumulate glycine betaine, trehalose and glutamate, as already reported (Huang et al., 2016b; Gaucher et al., 2019a). Accumulation of trehalose is a key constituent of stress response in *P. freudenreichii* (Cardoso et al., 2007; Dalmasso et al., 2012b). More generally, this disaccharide is accumulated by different species in many stressful conditions such as acid, cold, osmotic and oxidative stress (Cardoso et al., 2004, 2007; Thierry et al., 2011; Huang et al., 2016b). Glutamate accumulation in hyperosmotic conditions is well known and has been reported for *Lactobacillus plantarum* (Kets et al., 1996; Glaasker et al., 1998). It constitutes the primary response to osmotic upshift and its transient accumulation is followed by its replacement with compatible solutes such as trehalose in many types of cells (Csonka, 1989).

The YEL+NaCl medium, which contains only lactate as a carbon source as well as large amounts of NPN, allowed the accumulation of high intracellular concentrations of GB. Other bacteria, including *Brevibacterium*, *Corynebacterium* and *Enterococcus faecalis*, accumulate glycine betaine under hyperosmotic constraint in the presence of yeast extract (Frings et al., 1993; Pichereau et al., 1999). The presence of lactose in YEL+Lactose+NaCl improved trehalose accumulation at the expense of GB accumulation. The same trend was observed in the dairy media. In MU, GB concentration is too low, whereas lactose is abundant (Table 8),

leading to the accumulation of trehalose in hyperosmotic conditions. SW has a lower lactose/NPN ratio than MU (18.9 and 32.6, respectively), and *P. freudenreichii* accordingly accumulated compatible solutes with a lower trehalose/glycine betaine ratio in SW+NaCl than in MU+NaCl (18.9 and 32.6, respectively). These data suggested a correlation between compatible solute accumulation and growth medium composition. We thus plotted the trehalose/GB ratio as a function of the lactose/NPN ratio (Fig. 18 A), using values calculated from table 8 and Figure 14. This confirms such a correlation. In contrast, Fig. 18 B and C do not suggest any correlation between the lactose and intracellular trehalose provided *per se* or the NPN and intercellular GB provided *per se*. There is a correlation between the intracellular trehalose/GB ratio accumulated by *P. freudenreichii* and the lactose/NPN ratio of the growth medium composition (Fig. 18 A). The higher the lactose/NPN concentration ratio is, the higher the trehalose/NPN ratio will be. Modulating the nitrogen and carbon composition of the growth medium thus driving the amount and composition of intracellular compatible solutes in *P. freudenreichii.*

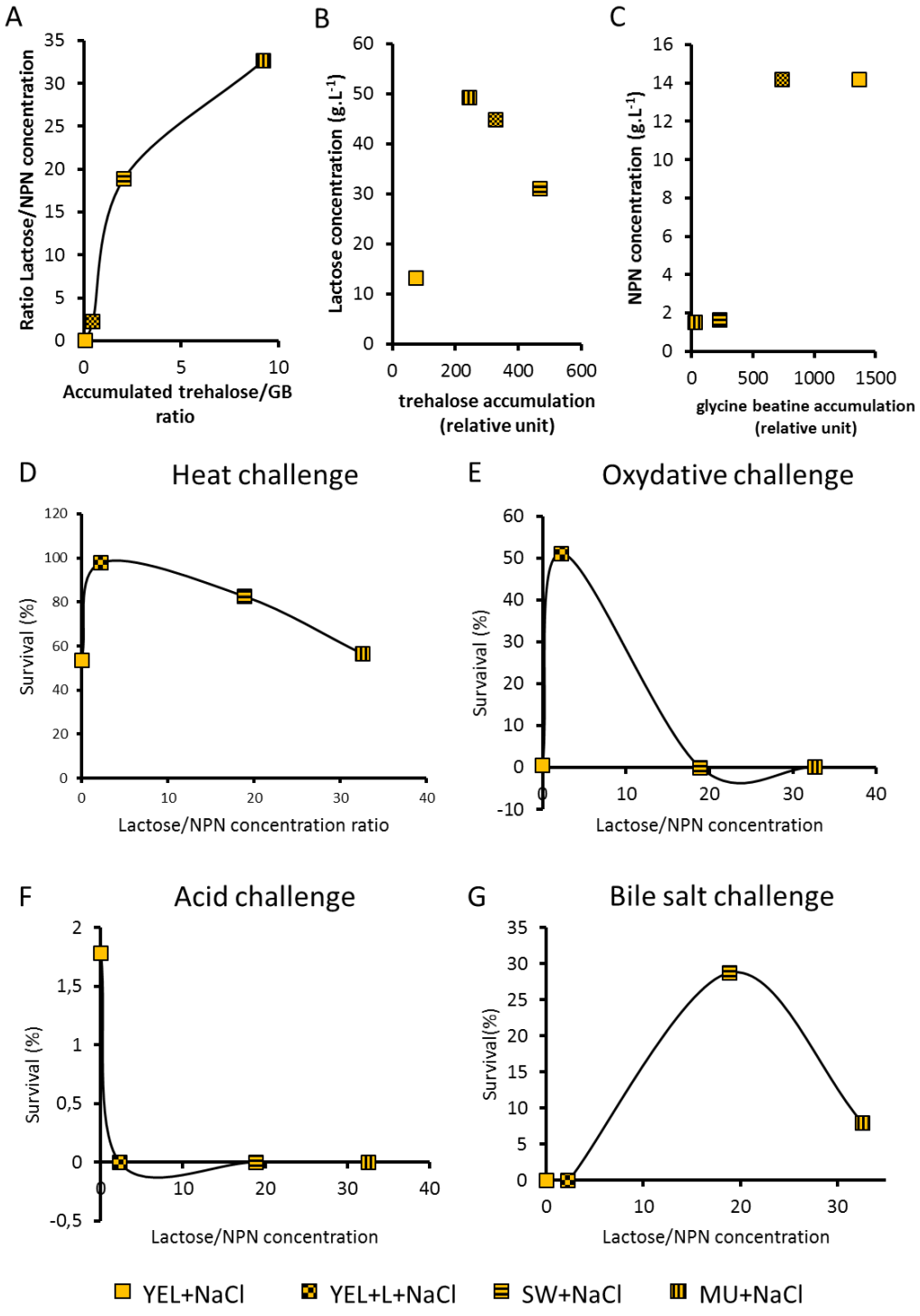

Figure 18: Driving the lactose/NPN ratio modulates physiological parameters including compatible solutes and stress tolerance. Data from the results section were used to seek correlation between lactose/NPN ratio and physiological parameters. The ratio lactose/NPN of growth media was plotted as a function of trehalose/NPN (A), the trehalose accumulation as a function of lactose concentration in growth media (B), the GB accumulation as a function of NPN concentration in growth media. The ratio lactose/NPN was also plotted as a function of heat tolerance (D), of oxidative tolerance (E), of acid tolerance (F), of bile salts tolerance (G). This suggests that ratio lactose/NPN is correlated with ratio trehalose/GB. This suggests that an optimal lactose/NPN ratio exists for each stress challenge.

4.4.2 Growth medium composition determines viability during stress challenges.

P. freudenreichii-osmoadapted cultures exhibited enhanced viability upon heat challenges, as already described (Huang et al., 2016b), regardless of the growth medium. This confirms that a cross-protection exists towards heat stress. *Escherichia coli* and *Pantoea agglomerans* also exhibited higher thermotolerance following GB accumulation driven by osmoadaptation (Teixido et al., 2005; Pleitner et al., 2012). In contrast, in our study, osmoadaptation decreased *P. freudenreichii* tolerance towards oxidative, acid and bile salt challenges. Pichereau et al. (1999) reported a decreased bile salt tolerance in *Enterococcus faecalis* as a result of GB accumulation. In our study, the addition of lactose to YEL type-medium triggered high trehalose accumulation and increased *P. freudenreichii* tolerance towards oxidative, acid and bile salt challenges. Trehalose accumulation accordingly leads to enhanced tolerance of *Lactococcus lactis* towards acid and bile salt challenges (Termont et al., 2006; Carvalho et al., 2011).

Huang et al. reported that *P. freudenreichii* osmoadaptation in hyperconcentrated SW, which contains high amounts of lactose, triggered increased tolerance towards heat, acid and bile salt challenges. Hyperconcentrated SW imposed osmoadaptation to *P. freudenreichii*, without variation of the trehalose/NPN ratio (Huang et al., 2018). This enhanced tolerance towards different challenges could thus be explained by trehalose accumulation during osmoadaptation and by the matrix protection of the hyperconcentrated SW.

We then plotted the survival rates upon the different stress challenges (from Fig. 15) as a function of the lactose/NPN ratio (from Table 8). For each challenge, the resulting curve (Fig. 18 D-G) suggests that an optimal lactose/NPN ratio corresponds to an optimal survival rate. In these experiments, a ratio close to 2.3 correlated with the highest tolerance to heat and oxidative challenges (Fig. 18 B and C), while a ratio close to 20 correlated with the highest tolerance to bile salt challenges (Fig. 18 D). Acid tolerance of *P. freudenreichii* occurred when the accumulation of GB was the highest (Fig. 18 F).

4.4.3 Driving *P. freudenreichii* osmoadaptation makes it possible to increase its survival during drying and storage

Different *P. freudenreichii* YEL cultures, with or without NaCl and with or without lactose, exhibited various survival rates upon drying. Concerning freeze-drying, osmoadaptation in YEL+NaCl led to the best viability after freeze-drying. Such a protection seems to be correlated with the high accumulation of glycine betaine. Accumulation of GB can either increase

Lactobacillus salivarius tolerance or decrease *Lactobacillus coryniformis* tolerance towards freeze-drying (Sheehan et al., 2006; Bergenholtz et al., 2012). Concerning spray drying, the best tolerance was observed for *P. freudenreichii* grown in YEL+Lactose+NaCl. Its osmoadaptation in medium containing lactose, leading to more trehalose accumulation, allowed an enhanced adaptation towards spray drying. This is in accordance with the enhanced tolerance towards heat and oxidative challenges (stresses encountered during spray drying), which was observed in the present work. Different osmoadaptations thus have different impacts on *P. freudenreichii* tolerance towards both drying processes. In contrast, GB accumulation in *L. salivarius* improved its resistance to freeze-drying and to spray drying (Sheehan et al., 2006). *P. freudenreichii* grown in YEL+Lactose+NaCl experienced a high viability loss during storage at high temperatures (Fig. 17 C). *P. freudenreichii* adaptation can be driven to obtain better viability during a particular process, but it was not possible in this work to provide protection to all technological stresses tested with the same culture ccondition.

4.5 Conclusion

In this chapter, we proposed a relationship between *P. freudenreichii* resistance to freeze-drying and to spray drying with the compatible solutes accumulated by *P. freudenreichii*.

P. freudenreichii adaptation can be driven to optimize viability during specific industrial processes. The ratio trehalose/glycine betaine accumulated is crucial for *P. freudenreichii* survival to spray drying, whereas an important accumulation of glycine betaine permits higher survival during freeze-drying. Growth medium composition, like carbon sources, nitrogen sources and the ratio thereof, can be controlled in order to optimize bacterial adaptation.

In the chapter 3, we saw that the accumulation of glycine betaine during the adaptation of *P. freudenreichii* CIRM-BIA 129 in a chemically defined medium without compatible solutes (MMO) had a negative impact on its survival during freeze-drying. This is a food for thought, accumulation of glycine betaine is not sufficient to improve *P. freudenreichii* survival upon freeze-drying. The MMO medium contains only amino acids as nitrogen sources, this medium is poorer than the YEL medium. Other adaptation mechanisms, such as over-expression of general stress proteins and modification of membrane fatty acids, have also an important impact on *P. freudenreichii* resistance. Bacterial adaptations, such as heat, acid, oxidative and osmotic adaptations, can be used alone or can be coupled to optimize bacterial adaptation mechanisms and obtain higher *P. freudenreichii* survival during stressing processes. In addition, an

adaptation can improve bacterial viability during a technological stress but not during all the production and digestion processes.

Important points of the chapter 4:

- *P. freudenreichii* osmoadaptation can be driven to optimize its survival during drying.
- The lactose/NPN ratio modulates the trehalose/glycine betaine ratio accumulated.
- Modulation of the lactose/NPN ratio increases *P. freudenreichii* viability during spray drying.

Chapter 5: *Propionibacterium freudenreichii* CIRM-BIA 129 osmoadaptation coupled to acid-adaptation increases its viability during freeze-drying

In the previous chapters, glycine betaine accumulation in a chemically defined medium decreased *Propionibacterium freudenreichii* survival during freeze-drying. By contrast, *P. freudenreichii* osmoadapted in the rich YEL medium, accumulated high amounts of glycine betaine, and exhibited higher survival than non-osmoadapted *P. freudenreichii*. Several adaptation mechanisms affect *P. freudenreichii* viability during freeze-drying, including the modulation of the membrane fatty acids composition and the over-expression of general stress proteins. As a reminder, in the chapter 3, the proteomes and resistance of *P. freudenreichii* CIRM-BIA 129 after osmoadaptation with or without glycine betaine in the culture medium were different. A high number of general stress proteins, which were up regulated by the addition of salt in the MMO+NaCl, where down regulated by the addition of glycine betaine.

The modulation of membrane fatty acids composition must be analyzed, as the proteome of *P. freudenreichii* osmoadapted in the riche YEL medium, with a particular focus on general stress proteins. Indeed, membrane fatty acids composition determines membrane fluidity and affects bacterial resistance to drying and storage (Fonseca et al., 2019).

In this chapter, *P. freudenreichii* CIRM-BIA 129 was cultured under hyperosmotic constraint in order to trigger osmoadaptation. This adaptation was then combined with acid or thermal pre-treatment. Such combination led to accumulation of key stress proteins, of intracellular compatible solute glycine betaine, to modulation of the propionibacterial membrane composition, and to enhanced survival upon freeze-drying.

The aims of this chapter were to:

- Investigate the effect of combining different adaptations on *P. freudenreichii* stress tolerance
- Evaluate the impact of adaptation conditions on the compatible solutes accumulation, on the composition of membrane fatty acids and on the proteins expression.
- Explain the best survival obtained via optimized bacterial adaptations with bacterial adaptation mechanisms

The main contents in this chapter have been published as:

F. Gaucher, K. Kponouglo, H. Rabah, S. Bonnassie, J. Ossemond, S. Pottier, J. Jardin, V. Briard-Bion, P. Marchand, P. Blanc, R. Jeantet, G., Jan, *Propionibacterium freudenreichii* CIRM-BIA 129 osmoadaptation coupled to acid-adaptation increases its viability during freeze-drying. Frontiers in Microbiology (2019) DOI: 10.3389/fmicb.2019.02324.

ORIGINAL RESEARCH
published: 09 October 2019
doi: 10.3389/fmicb.2019.02324

Propionibacterium freudenreichii CIRM-BIA 129 Osmoadaptation Coupled to Acid-Adaptation Increases Its Viability During Freeze-Drying

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5.1 Introduction

Most of the time, probiotic bacteria are stored, transported and consumed under a powder form. Probiotics microorganisms like bacteria and yeasts are dried to produce easy-to-use preparations, which can be implemented in food, feed or pharmaceutical industry (Huang et al., 2017b). Freeze-drying process imposes cold and osmotic stresses (Gaucher et al., 2019b), and causes the appearance of holes in the membrane, which can lead to cell death (Carvalho et al., 2004; Giulio et al., 2005). After drying, bacteria used as probiotic will further be exposed to harsh conditions during the digestion process. Some of the probiotic actions however need live bacteria in the colon. Tolerance towards stomach acidity and intestinal bile salts can constitute a limit of the probiotic potential.

Bacteria adaptation increase bacteria resistance to different stresses like acid stress (Jan et al., 2002; Broadbent et al., 2010), bile salt stress (Leverrier et al., 2003, 2004) and drying (Desmond et al., 2001; Carvalho et al., 2004; Silva et al., 2005; Li et al., 2009; Paéz et al., 2012). Cross-protections provided by adaptation are generally strain-dependent (Gaucher et al., 2019b). Concerning *P. freudenreichii* freeze-drying, almost nothing has been published. Acid-adaptation and/or heat-adaptation have however been reported to increase *Lactobacillus casei* (Paéz et al., 2012), *Lactobacillus plantarum* (Paéz et al., 2012), *Lactobacillus bulgaricus* (Li et al., 2009) and *Lactobacillus reuteri* (Palmfeldt and Hahn-Hägerdal, 2000; Koch et al., 2008) viability during freeze-drying as well as *Lactobacillus paracasei* (Desmond et al., 2001), and *Lactobacillus delbrueckii* (Silva et al., 2005) viability during spray drying. During these adaptations, the membrane fluidity of the bacteria will be adjusted by the modulation of the composition of fatty acids, in particularly the amount of saturated (SFA) and unsaturated (UFA) fatty acids (Lanciotti et al., 2001; Machado et al., 2004; Streit et al., 2008; Broadbent et al., 2010). General stress protein like Clps, DnaK, GroES or GroEL are overproduced during these three adaptations (Jan et al., 2001; Leverrier et al., 2004; Savijoki et al., 2005; Dalmasso et al., 2012a; Huang et al., 2016a). During osmoadaptation, *P. freudenreichii* is able to accumulate glutamate, trehalose and glycine betaine (Gaucher et al., 2019a). In hyperosmotic media, bacteria need to accumulate compatible solutes in order to preserve their turgescent pressure and to enable cell growth and division. Compatible solutes can be imported into the cell from the growth medium, or synthesized *de novo* (Csonka and Hanson, 1991). Glycine betaine is a compatible solute accumulated by different bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *P. freudenreichii*, during osmoadaptation (Kunin and Rudy, 1991; Graham and Wilkinson, 1992; Gaucher et al., 2019a). Glutamate has already

been reported as a compatible solute accumulated during osmoadaptation (Empadinhas and Viete-Vallejo, 2008). Trehalose is a compatible solutes accumulated during acid-adaptation and osmoadaptation by *P. freudenreichii* (Cardoso et al., 2007; Huang et al., 2016a; Gaucher et al., 2019a). In the present study, we combined osmoadaptation with acid or heat-adaptation, in order to improve *P. freudenreichii* CIRM-BIA 129 resistance. We investigated the impact of the modulation of the fatty acid composition, compatible solutes accumulation and expression of general stress proteins, on *P. freudenreichii* tolerance towards different stresses and during freeze-drying. Combining two protective pretreatments led to enhance survival of *P. freudenreichii* upon freeze-drying.

5.2 Materials and methods

5.2.1 Strains and pre-culture

Propionibacterium freudenreichii CIRM-BIA 129 (equivalent ITG P20) was provided, stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France). *P. freudenreichii* CIRM-BIA 129 was routinely cultivated in yeast extract lactate (YEL) broth (Malik et al., 1968). YEL medium contained, per liter, 21.4 g DL-lactate (60%), 10 g Yeast extract, 10g tryptone, 0.328 g K_2HPO_4, and 0.056 g $MnSO_4$. This leads to a non-protein nitrogen content of 14.2g. *P. freudenreichii* was grown at 30°C without agitation under microaerophilic condition.

5.2.2 Bacterial adaptation

P. freudenreichii CIRM-BIA 129 was grown under different condition: in YEL medium (0.429 osmol), or in YEL medium with 0.9M NaCl (YEL+NaCl, 1.958 osmol) to induce osmoadaptation. Heat or acid-adaptation where realized at the beginning of the stationary phase. Heat-adaptation was performed by placing 25 mL (in a 50mL Falcon tube) of *P. freudenreichii* cultures in a water bath at 42°C for 1 hour (Anastasiou et al., 2006), and acid-adaptation was performed by adjusting the culture at pH=5 and then incubated during 1 hour at 30°C (Jan et al., 2001)

5.2.3 Stress challenges

Heat, oxidative, bile salts and acid challenge were applied to cultures at the beginning of stationary-phase (when maximal OD was reached), or after adaptations. A strong heat challenge was performed by placing 2mL (in a 15 mL Falcon tube) of *P. freudenreichii* culture in a water bath at 60°C for 10 min (Leverrier et al., 2004). A lower heat stress was performed by placing 2 mL (in a 15 mL Falcon tube) of *P. freudenreichii* culture in a water bath at 55°C for 30 minutes (Leverrier et al., 2004). Oxidative challenge was applied by adding 1.25 mM of hydrogen peroxide (Labogros, France) to 2mL of *P. freudenreichii* culture during 1 hour at 30°C (Serata et al., 2016). Acid challenge was applied by re-suspending *P. freudenreichii* in MMO medium adjusted to pH 2.0 by using HCl at 30°C followed by a 1 hour incubation (Jan et al., 2000). Bile salts challenge was performed by adding 1g.L^{-1} of a bile salts mixture (an equimolar mixture of cholate and deoxycholate; Sigma Chemical, St. Louis, MO, USA) in the culture during 1 hour at 37°C (Leverrier et al., 2003). CFU counting was performed after challenge. In order to calculated survival percentage, a CFU counting was made, with untreated culture left for the same time at 30°C as a control.

5.2.4 Freeze-drying

P. freudenreichii CIRM-BIA 129 were grown in the YEL and YEL+NaCl medium and with or without acid or heat-adaptation. At the beginning of the stationary phase, cultures were harvested (8000g, 10 minutes, 30°C). Pellets were then homogenized in a maltodextrin solution (100g.L^{-1}) (Roquette, France). The bacterial solutions were then freeze-dried (2253-04, Serail, France).

5.2.5 Fatty acids analysis

P. freudenreichii CIRM-BIA 129 was grown in YEL and YEL+NaCl medium with or without acid or heat-adaptation at the beginning of the stationary-phase. Cells were then washed with sterile distilled water. Cells were harvested by centrifugation (8000g, 15 min). Saponification was performed by adding 3 mL of sodium methoxide (3.75 M in methanol) (Sigma Chemical, St. Louis, MO, USA) and shaking vigorously for 10 seconds and incubated during 25 minutes at 100°C. Samples were cooled to performe methylation, a solution of HCL and methanol was added (HCl 3.5 M and methanol 42% final concentration), samples were vortexed 10 seconds and incubated during 10 minutes at 80°C. Before the extraction of BAME (bacterial acid methyl

ester) samples were cooled on ice and a solution of hexane and diethyl ether (hexane 50% and diethyl ether 50%) was added. Samples were agitated during 10 minutes and then decanted, aqueous phases were removed before organic phases washing with NaOH 3 M solution. Samples were then agitated and decanted. Organic phases were then collected for analysis.

The analyses were performed on an Agilent gas chromatograph (7890A) equipped with a BPX70 capillary column (120m X 0,25mm X 0,25µm, SGE, Victoria, Australia) and coupled to a flame ionization detector (Agilent Technologies, Les Ulis, France). Hydrogen was used as carrier gas, and the injection volume was 0.5 µL. Injection was done by a cool on column injector. Detection temperature was 250°C. Agilent MSD ChemStation software was used for data acquisition. Components were identified from the retention time measured from BAME (Bacterial Acid Methyl Ester CP) standards (Merck, France).

Results were expressed as relative percentages of each fatty acid, which were calculated as the ratio of the surface area of the considered peak to the total area of all peaks. The ratio of unsaturated to saturated fatty acids (SFA/UFA) and the ratio branched to saturated fatty acids (BFA/SFA) were determined. Analyses were made in triplicate.

5.2.6 Identification and quantification of compatible solutes accumulated by *P. freudenreichii* CIRM-BIA 129

5.2.6.1 Extraction of accumulated compatibles solutes

P. freudenreichii CIRM-BIA 129 was grown in YEL and YEL+NaCl medium with or without acid-adaptation at the beginning of the stationary-phase. During exponential phase (OD= 0.8), cells were harvested by centrifugation (8000g 10 minutes). Compatible solutes were then extracted as previously described in chapter 3 (Gaucher et al., 2019a).

5.2.6.2 NMR (Nuclear Magnetic Resonance) analyses.

Dried extracts were then analyzed by NMR as previously described in chapter 3 (Gaucher et al., 2019a). The relative concentration of trehalose, glutamate and glycine betaine in the samples was determined by the integration of the peaks' areas of their ^{1}H signals relative to the internal standard TMSP. Results are expressed as NMR relative units (RU).

5.2.7 Label free proteomics

5.2.7.1 Whole-cell protein extraction and protein tryptic digestion

At the beginning of stationary phase or after acid-adaptation, *P. freudenreichii* cells were harvested by centrifugation and washed twice with PBS buffer (NaCl 8g.L^{-1}, KCl 2g.L^{-1} KH_2PO_4 2g.L^{-1}, Na_2HPO_4 $12H_2O$ 35,8g.L^{-1}). The label free proteomics has been conducted as described in chapter 3 (Gaucher et al., 2019a).

5.2.7.2 Nano-LC-MS/MS

Experiments were performed as previously described in chapter 3 (Huang et al., 2018).

5.2.7.3 Protein identification and quantification

Proteins identification and quantification were performed as previously described in chapter 3 (Huang et al., 2018)

5.2.8 SDS-PAGE and Western-blotting

The extraction, the SDS-Page and the Western blot have been conducted as Rabah et al. 2018 (Rabah et al., 2018). Briefly, *P. freudenreichii* was grown in YEL or YEL+NaCl with or without acid or heat-adaptation. Cells were harvested by centrifugation, washed two times with PBS. After centrifugation, supernatants were removed and lysis solution (Tris-HCl 50 mM pH 7.5, SDS 0.3%, DTT200 mM) was added to bacterial pellets and samples were frozen prior to sonication and cell lysis using Precellys® Evolution homogenizer. Resulting SDS extracts were recovered by centrifugation (21,000 ×g, 4 °C, 20 min) and analyzed by one-dimensional electrophoresis SDS-PAGE. The protein extracts from triplicate experiments were pooled and the protein amount was determined using 2-D Quant Kit (GE Healthcare). Protein extracts were separated by 12% SDS–PAGE and then transferred to PVDF membranes (GE Healthcare). A solution with 3% non-fat dry milk diluted in TBS (Tris 10 mM, NaCl 0.15 M, 0.3% tween 20) was added on the PVDF membranes to perform locking. Membranes were washed and then incubated over-night at 4 °C with anti-SlpB primary antibodies purified from rabbit sera (AGRO-BIO, France) at the dilution 1:10,000. After washing, membranes were incubated with secondary antibodies: anti-rabbit IgG conjugated with horseradish peroxidase (1:15,000, AGRO-BIO, France) for 2 h at room temperature. Bound antibodies were visualized with ECL

Plus system (GE Healthcare, Vélizy, France) and blots were scanned using the Syngene GBox (Ozyme, Saint-Quentin-en-Yvelines, France).

5.2.9 Statistical analysis

The data were from triplicate samples. All the results are presented as mean value with standard deviation. Statistical analyses using one-way ANOVA with Tukey *post hoc* analyses for multiple comparison. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows).

5.3 Results

5.3.1 *P. freudenreichii* viability during challenges and freeze-drying

We subjected *P. freudenreichii* to freeze-drying, heat, acid, and bile salts stress challenges, in order to monitor its stress tolerance. *P. freudenreichii* CIRM-BIA 129 viability was determined by numeration (CFU counting) before and after these challenges, which were selected as being relevant to freeze-drying process, Swiss-type cheese making, and to digestive constraints. As shown in Figure 19 A, addition of salt (0.9 M NaCl) to the YEL growth medium increased *P. freudenreichii* CIRM-BIA 129 viability during freeze-drying (43 to 74.4%), yet showing no effect on its viability during lower heat lethal challenge (55°C, 30 min) (Fig. 19 B). This hyperosmotic constraint even had a negative impact on *P. freudenreichii* CIRM-BIA 129 survival during acid (pH 2, 1 h, 36.4 to 1.8%) and bile salt (1 $g.L^{-1}$, 1 h, 38.3 to 0%) challenges (Fig. 19 C and D). Osmoadaptation, acid-adaptation, and the combination thereof led to a viability below the detection threshold during bile salts challenge. A sublethal heat pretreatment (42°C, 1h) had *per se* a positive impact on survival during a subsequent heat lethal challenge (Fig. 19 B, 32.5 to 87.6%), yet a negative impact on survival during acid challenges (Fig. 19 C, 36.4 to 10% respectively). Acid-adaptation (pH 5, 1h) *per se* did not enhance tolerance, neither towards these lethal challenges, nor towards freeze-drying. Heat-adaptation, or acid-adaptation, combined to osmoadaptation, had a negative or no impact on *P. freudenreichii* CIRM-BIA 129 tolerance towards stress challenges drying (Fig. 19 B, C and D). However, such a combination enhanced *P. freudenreichii* CIRM-BIA 129 survival during freeze-drying (Fig. 19 A, 43 to 90.5 and 96.7% respectively). Best viability during freeze-drying was obtained for *P. freudenreichii* CIRM-BIA 129 grown in YEL+NaCl, with an acid-adaptation at the beginning of the stationary phase. Further analyses were then performed on *P. freudenreichii* CIRM-BIA 129, grown in

YEL or in YEL+NaCl, with or without acid or heat-adaptation, in order to elucidate mechanism responsible for enhanced tolerance towards freeze-drying.

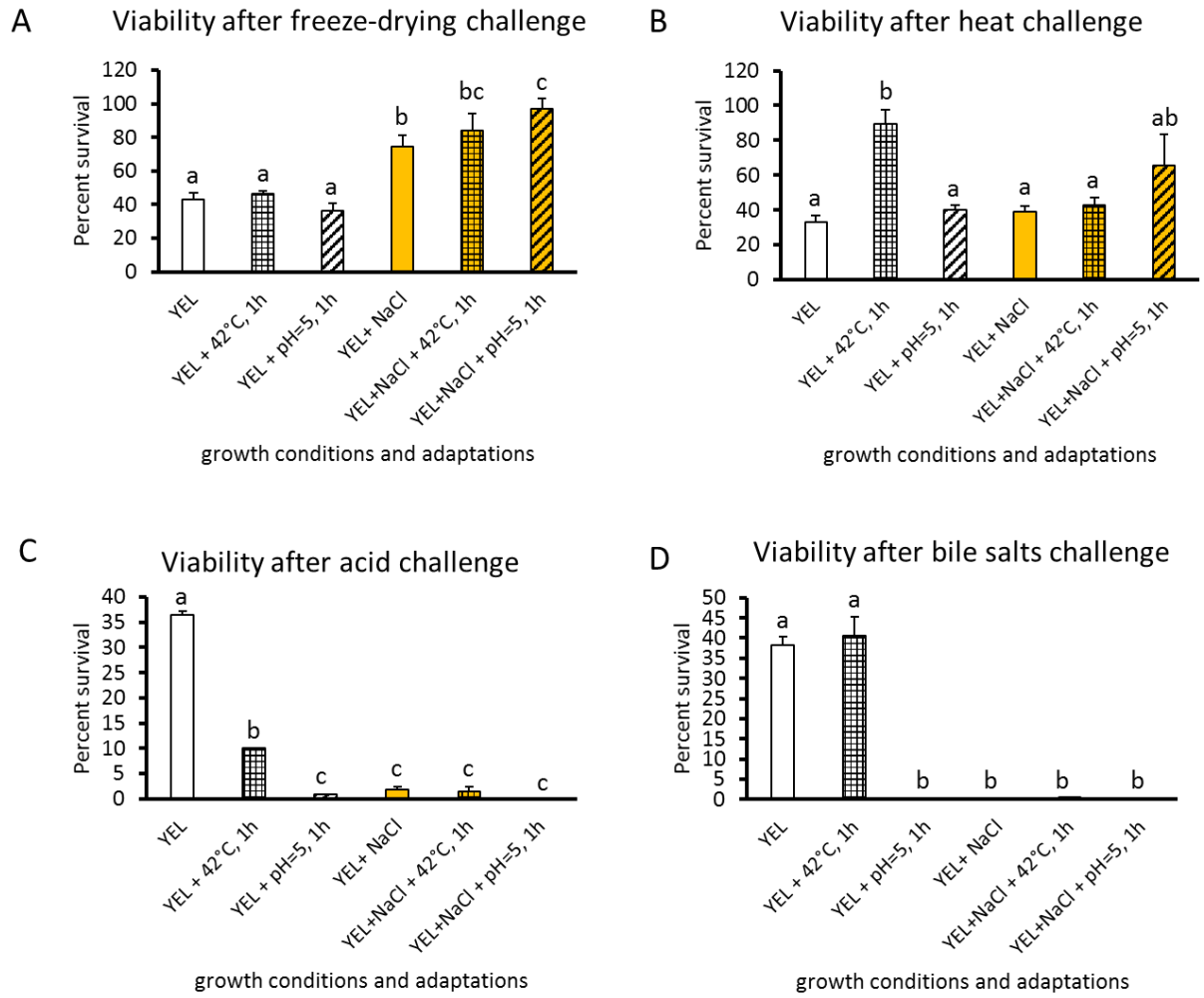


Figure 19: Viability of *Propionibacterium freudenreichii* CIRM-BIA 129 after freeze-drying (A), heat (B), acid (C), and bile salts (D) challenges. The strain was previously grown in YEL and YEL+NaCl broths, and subjected to heat and acid-adaptation. Errors bars represent the standard deviation for triplicate experiments. Significances differences are reported with different letters above columns ($p>0.05$). YEL: Yeast Extract Lactate. 42°C, 1h: heat-adaptation at 42°C during 1 hour. pH=5, 1h: acid-adaptation at pH=5 during 1 hour.

5.3.2 Membrane fatty acids composition is modulated by acid, heat and osmoadaptation

The fatty acid composition of membrane lipids was analyzed to better understand *P. freudenreichii* CIRM-BIA 129 adaptation to high osmotic pressure, to thermal stress or to acidification. As shown in Figure 20, a total of 23 fatty acids were detected in *P. freudenreichii* CIRM-BIA 129 membrane, using gas chromatography, reported in Supplemental table 1. This includes saturated, unsaturated and branched fatty acids. *P. freudenreichii* CIRM-BIA 129

contains a high proportion of branched fatty acids (52.37 to 62.15 %), and lower amounts of saturated (13.54 to 20.97%) and unsaturated ones (4.28 to 16.45%). The anteiso C15:0 was detected as the major fatty acid, which proportion was furthermore highly modulated during *P. freudenreichii* CIRM-BIA 129 adaptations (Fig. 20 A). Osmoadaptation increased the amount of long fatty acids (anteiso C17:0, C17:0 and C19:0) and decreased the amount of short fatty acid (C12:0 and C13:0). Accordingly, the C15-C22/C10-C14 ratio was enhanced as a result of osmoadaptation and of acid-adaptation (Fig. 20 B). The amount of C16;1n7 and C18:1n9c decreased sharply for the bacteria grown in salty medium, by contrast the saturated fatty acid C16:0 and C17:0 are increasing during osmoadaptation (Supplemental Table 2). Such variations led to enhanced saturated/unsaturated ratio upon osmoadaptation (Fig. 20 C). Heat or acid-adaptation, applied after osmoadaptation can modulate this ratio. Saturated and branched fatty acid proportions are modulated in the different conditions, but the ratio saturated/branched did not show significant variations (Fig. 20 D).

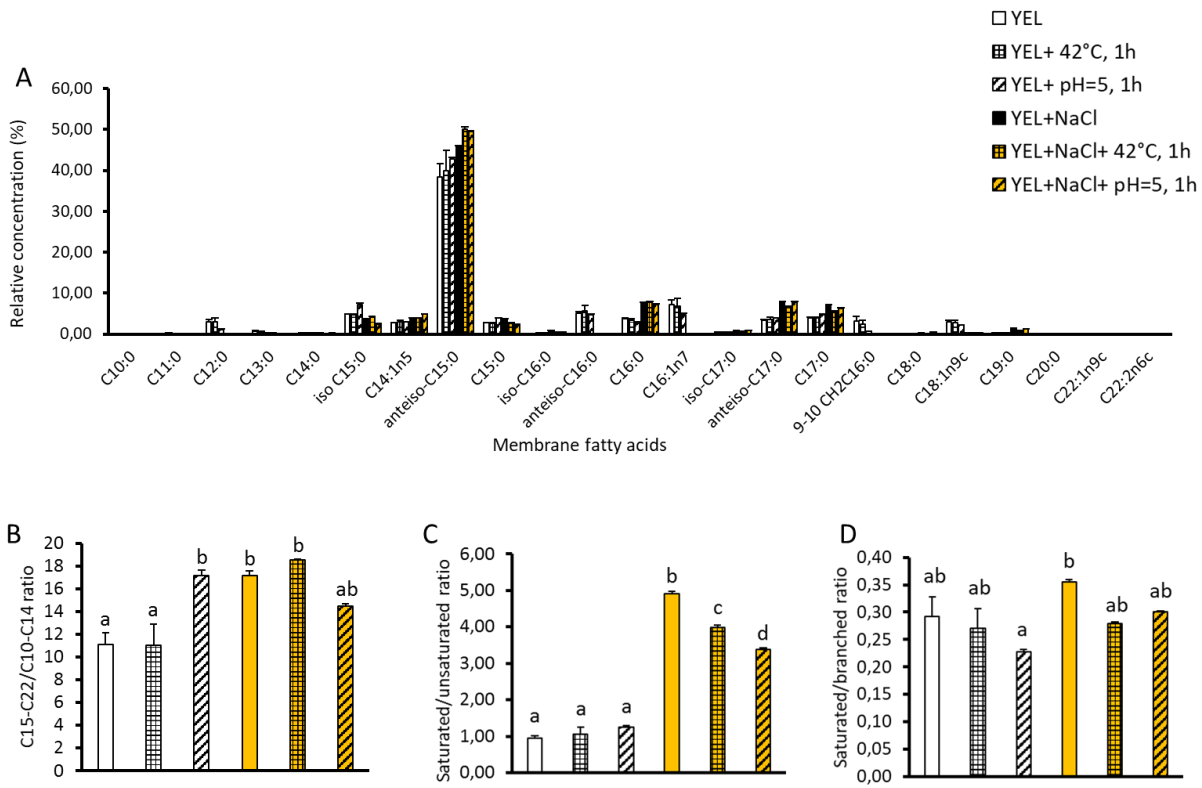


Figure 20: Membrane fatty acids (% of total membrane fatty acids) of *Propionibacterium freudenreichii* CIRM-BIA 129 in different conditions. The fatty acids composition (A), the saturated/unsaturated fatty acids ratios (SFA/UFA) (B), the saturated/branched fatty acids ratio (SFA/BFA) (C) and the C15-C22/C10-C14 ratio (D) are shown for the strain grown in YEL and YEL+NaCl broths and subjected to heat and acid-adaptation. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p>0.05$). YEL: Yeast Extract Lactate. 42°C, 1h: heat-adaptation at 42°C during 1 hour. pH=5, 1h: acid-adaptation at pH=5 during 1 hour.

5.3.3 Compatible solutes accumulation is modulated by the acid and osmoadaptation

When cultivated in the isotonic and rich YEL growth medium in the absence of NaCl and of acid or heat stress, no trehalose, no glutamate and no glycine betaine was detected in the cytoplasm of *P. freudenreichii* CIRM-BIA 129 (Fig. 21). However, acid-adaptation, at the end of growth, at the beginning of stationary phase, triggered glutamate accumulation in the same medium. During growth in rich YEL medium in the presence of NaCl, *P. freudenreichii* CIRM-BIA 129 mainly accumulated glycine betaine, as well as lower amounts of glutamate and of trehalose. In these osmoadapted cultures, acid-adaptation at the beginning of the stationary phase further increased accumulation of glycine betaine and of trehalose, at the expense of glutamate.

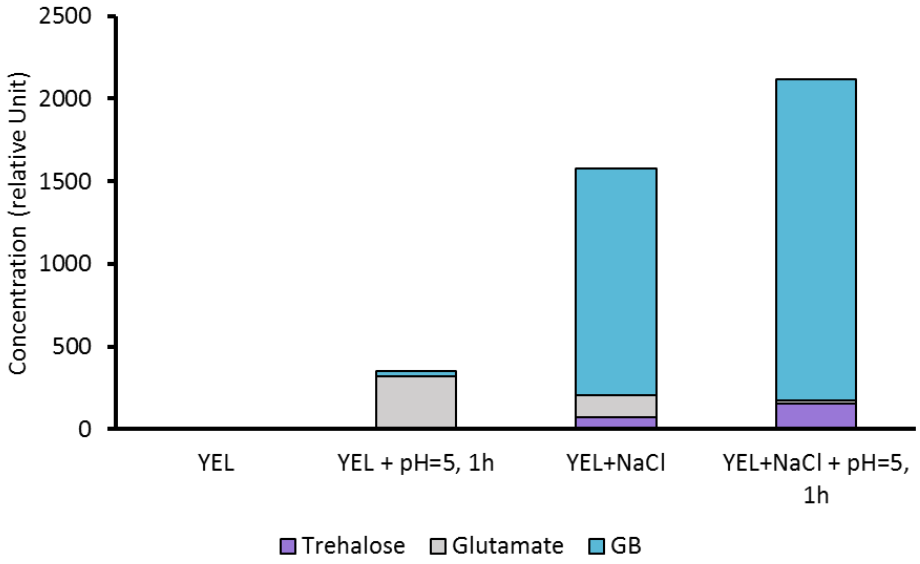


Figure 21: Compatible solutes accumulation (relative units) after growth in YEL and YEL+NaCl broths, with and without acid-adaptation.

5.3.4 Modulation of protein expression during osmoadaptation and acid-adaptation

To understand the respective impact of *P. freudenreichii* CIRM-BIA 129 osmoadaptation and of osmoadaptation coupled to acid-adaptation, we realized a proteomic differential analysis. Theoretically, 2176 proteins are encoded by *P. freudenreichii* CIRM-BIA 129 genome (GCA_000723545.1). In this study, a total of 857 proteins were detected using mass spectrometry. This represents a coverage of 39%. Proteomics data have been deposited into INRA data base (Jardin, 2019). We focused on proteins that were differentially abundant for at least one condition between YEL, YEL+NaCl and YEL+NaCl+pH=5, 1h, with a minimum

ratio of 1.5 or less than 0.66. A total of 216 proteins were significantly modulated, 208 between YEL and YEL+NaCl (see Supplemental Table 3) and 8 proteins between YEL+NACl and YEL+NaCl +pH=5, 1h (Table 9). As shown in Figure 22, the heat map indicated that the two proteomes corresponding to, respectively, YEL+NaCl and YEL+NaCl+pH=5, were very different from the control one corresponding to YEL. By contrast, the proteome corresponding to YEL+NACl+pH=5, 1h is very closed to that of bacteria grown in YEL+NaCl. Salt addition strongly affected *P. freudenreichii* CIRM-BIA 129 proteome, while acid-adaptation after osmoadaptation modulated the expression of few proteins. More precisely, 20 proteins modulated during osmoadaptation were involved in amino acids metabolism and transport, 17 proteins in carbohydrate transport and metabolism, 5 proteins in lipid transport and metabolism, 6 proteins in cell wall-membrane-envelope biogenesis. Another set of 17 proteins belonged to the "post-translational modification, protein turnover and chaperones" category (Table 10). All the other modulated proteins are presented in Supplemental Table 3.

Table 9: Proteins modulated during acid-adaptation after osmoadaptation. Proteins were determined by using a database composed of proteome of *P. freudenreichii* CIRM-BIA 129 (downloaded from NCBI.nlm.nih.gov 2018). Ratio were calculated using the XIC methods and indicate induction (ratio>1.5) or repression (ratio>0.66) by the acid-adaptation.

Accession	Description	YEL+NaCl+pH=5/YEL+NaC ratio
Energy production and conversion		
emb|CDP49838.1|	Sulfite reductase [ferredoxin]	0.53
emb|CDP49142.1|	L-lactate permease	0.49
Amino acid transport and metabolism		
emb|CDP49535.1|	cysteine synthase 2	0.49
Replication, recombination and repair		
emb|CDP49216.1|	Putative endonuclease III	0.61
Inorganic ion transport and metabolism		
emb|CDP49684.1|	ABC-type transport systems, periplasmic component	0.58
emb|CDP48302.1|	Heavy metal transport/detoxification protein	0.54
Secondary metabolites biosynthesis, transport, and catabolism		
emb|CDP49310.1|	Dioxygenase	0.64
Function unknown		
emb|CDP49347.1|	Uridine phosphorylase	0.52

Table 10: Proteins modulated during osmoadaptation belonging to the COG category "amino acid metabolism and transport", "carbohydrate transport and metabolism", "lipid transport and metabolism" and "cell wall-membrane-envelope biogenesis". Proteins were determined by using a database composed of proteome of *P. freudenreichii* CIRM-BIA 129 (downloaded from NCBI.nlm.nih.gov 2018). Ratio were calculated using the XIC methods and indicate induction (ratio>1.5) or repression (ratio>0.66) by NaCl.

Accession	Description	YEL+NaCl/YEL ratio
Amino acid transport and metabolism		
emb|CDP48583.1|	Prephenate dehydrogenase	2.90
emb|CDP47878.1|	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	2.22
emb|CDP48936.1|	Aminopeptidase	2.02
emb|CDP48564.1|	polar amino acid ABC transporter, ATP binding component	1.86
emb|CDP47695.1|	Nucleoside-diphosphate kinase	1.82
emb|CDP48687.1|	Glycine cleavage H-protein (lipoate-binding)	1.79
emb|CDP48582.1|	Cytidylate kinase (CK) (Cytidine monophosphate kinase) (CMP kinase)	1.78
emb|CDP49472.1|	ABC-type choline/glycine betaine transport,ATP-binding protein	1.67
emb|CDP49173.1|	Dihydroxy-acid dehydratase	1.65
emb|CDP49146.1|	L-asparaginase I	1.65
emb|CDP48643.1|	Tryptophan synthase alpha chain (TrpA)	1.55
emb|CDP47760.1|	Kinase ArgK	1.52
emb|CDP49589.1|	Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase related deacylase	1.51
emb|CDP47866.1|	Serine acetyltransferase	1.51
emb|CDP48503.1|	Amidohydrolase (Peptidase M20D) (Putative metal-dependent amidase/aminoacylase/carboxypeptidase)	0.67
emb|CDP48372.1|	binding protein of oligopeptide ABC transporter (OPN : undef : Oligopeptides)	0.65
emb|CDP49606.1|	Shikimate 5-dehydrogenase	0.64
emb|CDP49535.1|	cysteine synthase 2	0.54
emb|CDP49386.1|	Phospho-2-dehydro-3-deoxyheptonate aldolase	0.50
emb|CDP47931.1|	solute binding protein of the ABC transport system	0.49
Carbohydrate transport and metabolism		
emb|CDP49182.1|	iolB (Myo-inositol catabolism IolB protein)	2.04
emb|CDP49834.1|	Ribose-5-phosphate isomerase 3	1.90
emb|CDP48739.1|	Phosphoketolase pyrophosphate	1.87
emb|CDP48882.1|	phosphoglycerate mutase/fructose-2,6-bisphosphatase	1.84
emb|CDP49742.1|	endonuclease	1.68
emb|CDP48781.1|	Alpha-glucan phosphorylase	1.63
emb|CDP48893.1|	Oxidoreductase	1.62
emb|CDP47720.1|	6-phosphogluconate dehydrogenase,decarboxylating	1.56
emb|CDP49513.1|	Alpha-1,4-glucosidase	1.55
emb|CDP49568.1|	Polyphosphate glucokinase	0.66
emb|CDP48902.1|	Fructose-bisphosphate aldolase class I	0.63
emb|CDP49639.1|	Gluconate kinase (Gluconokinase)	0.59
emb|CDP47629.1|	Glycogen debranching enzyme GlgX	0.49

emb|CDP47859.1|	binding protein of ribose ABC transporter	0.46
emb|CDP48824.1|	Dihydroxyacetone kinase	0.43
emb|CDP49430.1|	Glucose-1-phosphate adenylyltransferase (ADP-glucose synthase) (ADP-glucose pyrophosphorylase) (ADPGlc PPase)	0.39
emb|CDP49099.1|	PTS system, mannose/fructose/sorbose family, IIA component subfamily	0.26

Lipid transport and metabolism

emb|CDP49767.1|	inositol-1-phosphate synthase	1.87
emb|CDP47827.1|	Enoyl-CoA hydratase/carnithine racemase CaiD	1.84
emb|CDP49388.1|	Acyltransferase PlsC	1.52
emb|CDP48433.1|	Acyl carrier protein (ACP)	0.59
emb|CDP48431.1|	Carboxylic ester hydrolase	0.57

Cell wall/membrane/envelope biogenesis

emb|CDP47662.1|	GTP-binding protein LepA	2.32
emb|CDP49368.1|	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase (UDP-MurNAc-pentapeptide synthetase) (D-alanyl-D-alanine-adding enzyme)	1.98
emb|CDP49372.1|	S-adenosyl-L-methionine-dependent methyltransferase mraW	1.97
emb|CDP49370.1|	Cell division protein FtsI (penicillin-binding protein 2) (Peptidoglycan glycosyltransferase)	1.88
emb|CDP47743.1|	UDP-N-acetylmuramyl tripeptide synthase (Mur ligase)	1.76
emb|CDP49363.1|	UDP-N-acetylmuramate--L-alanine ligase (UDP-N-acetylmuramoyl-L-alanine synthetase)	1.55

Post-translational modification, protein turnover, and chaperones

emb|CDP48339.1|	Heat shock protein 20 3 (20 kDa chaperone 3)	3.25
emb|CDP49617.1|	thiol peroxidase	2.50
emb|CDP49795.1|	Thioredoxin	2.49
emb|CDP49065.1|	Surface layer protein A (S-layer protein A)	2.24
emb|CDP49048.1|	Thioredoxine	2.15
emb|CDP49400.1|	HesB protein	1.92
emb|CDP48411.1|	SmpB SsrA-binding protein	1.92
emb|CDP48424.1|	Heat shock protein 20 2 (20 kDa chaperone 2)	1.68
emb|CDP48051.1|	Protein GrpE 2 (HSP-70 cofactor 2) (Co-chaperone protein GrpE2)	1.62
emb|CDP49021.1|	Protein GrpE 1 (HSP-70 cofactor 1) (Co-chaperone protein GrpE1)	1.61
emb|CDP48340.1|	Heat shock protein 20 1 (20 kDa chaperone 1)	1.57
emb|CDP47983.1|	Peroxiredoxin/Alkyl hydroperoxide reductase subunit C /Thioredoxin peroxidase/Alkyl hydroperoxide reductase protein C22/General stress protein 22	1.53
emb|CDP49312.1|	FeS assembly protein SufB	0.61
emb|CDP49702.1|	Stomatin/prohibitin	0.60
emb|CDP47885.1|	Putative O-sialoglycoprotein endopeptidase	0.59
emb|CDP48273.1|	Surface layer protein B (S-layer protein B)	0.41
emb|CDP48858.1|	Surface protein with SLH domain	0.15

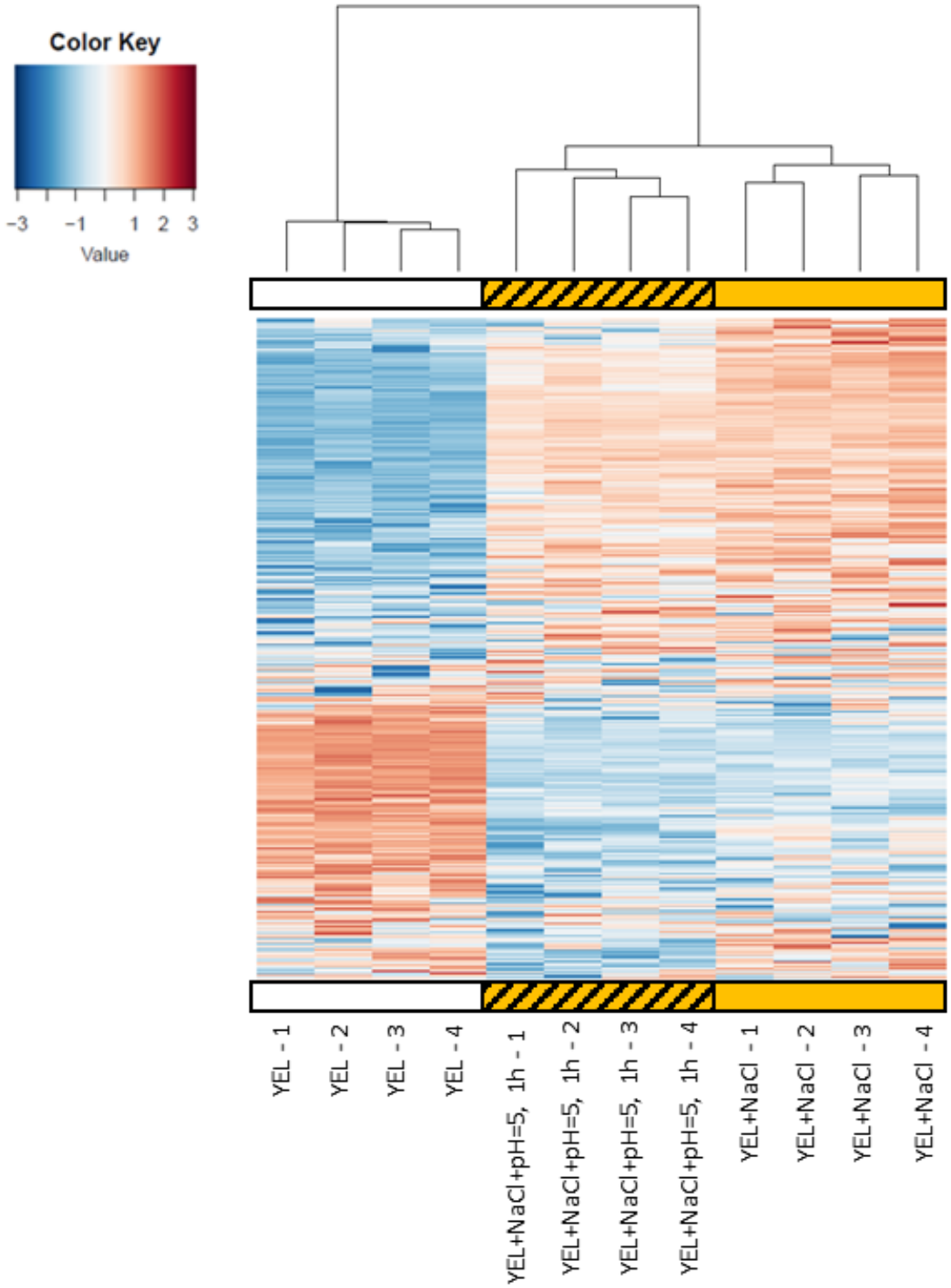


Figure 22: Effects of osmoadaptation and acid-adaptation on *P. freudenreichii* CIRM-BIA 129 proteome

5.3.5 Modulation of S-layer protein B on *P. freudenreichii* cell wall.

Western blotting, using antibodies directed against *P. freudenreichii* CIRM-BIA 129 SlpB protein, allowed detection of this protein in all *P. freudenreichii* whole-cell protein extracts, whatever the growth conditions, but to different extents (Fig. 23 A). Indeed, the amount of detected SlpB was lower for all the cultures performed in YEL with added NaCl. Concerning the culture supernatants, no SlpB was detected in control conditions, i.e. cultures in the rich YEL medium. However, heat and acid-adaptation led to the release of a part of SlpB into the growth supernatant. Less SlpB protein was detected in whole cell extracts when *P. freudenreichii* CIRM-BIA 129 was cultivated in the presence of NaCl, while traces of this protein were detected in the corresponding supernatants.

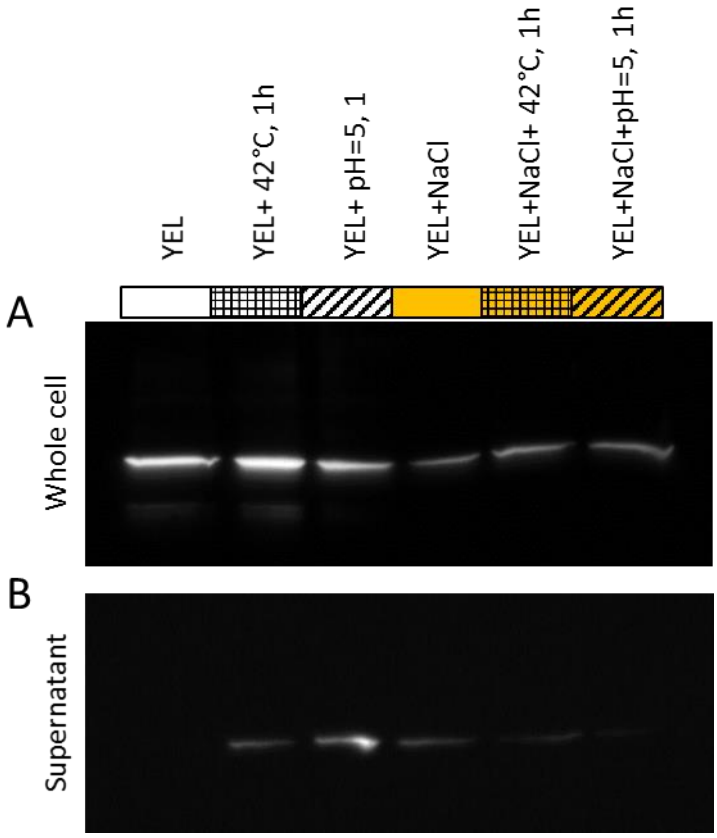


Figure 23: Detection of SlpB protein in Propionibacterium freudenreichii CIRM-BIA 129 whole-cell protein extracts (A) and supernatants (B) for cultures performed in YEL and YEL+NaCl broths, and after heat and acid-adaptations. YEL: Yeast Extract Lactate. 42°C, 1h: heat-adaptation at 42°C during 1 hour. pH=5, 1h: acid-adaptation at pH=5 during 1 hour.

5.4 Discussion

5.4.1 Heat and acid-adaptations, coupled to osmoadaptation, trigger cross-protection to lethal challenges

Osmoadaptation, i.e. growth in the rich YEL medium in the presence of 0.9 M NaCl, triggered enhanced viability of *P. freudenreichii* CIRM-BIA 129 during freeze-drying, as reported previously for *P. freudenreichii* and for *Lactobacillus bulgaricus* and *Lactobacillus buchneri* (Carvalho et al., 2003; Louesdon et al., 2014; Gaucher et al., 2019a). Remarkably, a mild acid-adaptation, coupled to such an osmoadaptation, further enhanced *P. freudenreichii* CIRM-BIA 129 tolerance towards freeze-drying. This opens new perspectives to improve industrial production of this probiotic. However, osmoadaptation, coupled or not to heat or acid-adaptation, dramatically reduced *P. freudenreichii* CIRM-BIA 129 tolerance towards the digestive stresses mimicked by acid and bile salt challenges. By contrast, acid-adaptation reportedly increases acid tolerance in another strain of *P. freudenreichii* and in *Lactobacillus casei* (Jan et al., 2000; Broadbent et al., 2010). Moreover, heat-adaptation increases bile salts and acid tolerance in *P. freudenreichii* (Jan et al., 2000, 2002; Leverrier et al., 2004). In the present study, heat-adaptation alone increased *P. freudenreichii* CIRM-BIA 129 viability during heat challenge, in accordance with the increased thermotolerance of heat-adapted *P. freudenreichii* (Anastasiou et al., 2006), *Lactobacillus paracasei* and *Lactobacillus kefiranofaciens* to heat shock (Desmond et al., 2001). To our knowledge, the present report is the first describing optimal tolerance towards freeze-drying, as a result of osmoadaptation combined with mild acid pretreatment.

5.4.2 Modulation of the membrane fatty acid composition increases the viability during freeze-drying.

Osmoadaptation strongly increased the saturated/unsaturated fatty acids ratio in the membrane of *P. freudenreichii* CIRM-BIA 129, as reported for *L. casei* (Machado et al., 2004). Heat-adaptation reportedly decreases this ratio in the Swiss-type cheese starter *Lactobacilllus helveticus*. Heat or acid-adaptation modulated the increase of the ratio saturated/unsaturated triggered by osmoadaptation. The ratio SFA/BFA (BFA: Branched fatty acid) was not significantly altered whatever the growth and treatment conditions. Osmoadaptation led to enhanced fatty acid chain length. Indeed, salt addition and acid-adaptation, but not heat-adaptation, increased the C15-C22/C10-C14 ratio. Thus, *P. freudenreichii* CIRM-BIA 129

responded to environmental stresses by modulating its membrane fluidity via the saturated/unsaturated ratio, with a profound increase of anteiso-C15:0, the main membrane fatty acid in *P. freudenreichii* (Thierry et al., 2011) and known to play a key role in bacterial stress adaptation (Annous et al., 1997). The membrane fluidity can also be decreased by the cyclopropanation of unsaturated fatty acids, which leads to a higher resistence of *Lactococcus lactis* to freeze-drying (Velly et al., 2015). Anteiso-C15, a fatty acid abundant in halophilic bacteria (Guan et al., 2018; Jang et al., 2018; Ding et al., 2019), is accordingly increased under hyperosmotic conditions in halotolerant Gram-positive bacteria such as *Planococcus sp* (Miller, 1985), *Staphylococcus epidermidis* (Komaratat and Kates, 1975) and *S. aureus* (Kanemasa et al., 1972).

5.4.3 Acid-adaptation, coupled to osmoadaptation, increases compatible solutes accumulation

P. freudenreichii CIRM-BIA 129 accumulated in this study high amounts of glycine betaine during osmoadaptation, as already described (Gaucher et al., 2019a). Glycine betaine is an important compatible solute accumulated by different bacteria such as *Lactobacillus plantarum* and *Lactococcus lactis* during osmoadaptation (Glaasker et al., 1996; Romeo et al., 2003). Only limited amounts of glutamate and trehalose were accumulated by *P. freudenreichii* CIRM-BIA 129 during osmoadaptation. Glutamate is a key molecule of the primary osmoregulation response (Csonka, 1989) and is accumulated by *L. plantarum* (Glaasker et al., 1996; Kets et al., 1996). It should be noted that the rich YEL medium, composed of yeast extract, lactate and peptone, contains non-protein nitrogen (12.4 g), including glutamate and glycine betaine. The *P. freudenreichii* genome allows prediction of at least two glycine betaine import systems, i.e. OpuCA and an ABC-type choline/glycine betaine transporter (see below). Accordingly, *P. freudenreichii* was previously shown to accumulate glycine betaine in hyperosmotic conditions (Boyaval et al., 1999). Trehalose is accumulated in many different conditions such as osmotic, acid, oxidative and cold stresses (Cardoso et al., 2004, 2007; Thierry et al., 2011; Huang et al., 2016b). Trehalose can be synthetized by the OtsA-Otsb and TreS pathway previously described in *P. freudenreichii* (Cardoso et al., 2007). Accumulation of these three compatible solutes, in response to osmotic constraint, is thus a general adaptation mechanism (Csonka and Hanson, 1991). Interestingly, acid-adaptation further enhanced compatible solutes intracellular accumulation and particularly that of glycine betaine. This enhanced accumulation is in accordance with the enhanced tolerance towards the freeze-drying challenge. To better

address mechanisms responsible for such an adaptation, a whole-cell proteomic differential analysis was thus undertaken.

5.4.4 NaCl induces major changes in *P. freudenreichii* CIRM-BIA 129 proteome, while acid-adaptation down-regulates part of the NaCl stress proteome.

Osmoadaptation triggered major modifications in the whole-cell protein content of *P. freudenreichii* CIRM-BIA 129, as illustrated by the heatmap in Figure 22. The patterns corresponding to YEL and to YEL + NaCl exhibit numerous differences, while acid-adaptation coupled to osmoadaptation had little impact on the proteome. The NaCl stress proteome included many proteins involved in amino acid transport and metabolism. In particular, the ABC-type choline/glycine betaine transporter and the ATP-binding protein OpuCA of glycine betaine/carnitine/choline ABC transporter were in higher abundance, in accordance with the observed glycine betaine accumulation in the presence of salt (0.9 M NaCl). Different proteins such as a polar amino acid ABC transporter, an aminopeptidase and enzymes involved in amino acid biosynthesis were also enhanced and this shall lead to an enhanced intracellular pool of amino acids. Prephenate dehydrogenase and L-asparaginase, which liberate ammonia/ammonium from amino acids, take part in intracellular pH homeostasis. L-asparaginase has already been reported as a stress protein in *Salmonella typhimurium* (Sonck et al., 2009).

About proteins involved in the transport and metabolism of carbohydrate, proteins corresponding to the utilization of alternative carbon sources were induced, including Myo-inositol catabolism IolB protein, as it happens during unfavorable conditions in *Corynebacterium glutamicum* (Chen et al., 2019). The pentose phosphate pathway, including the phosphoketolase pyrophosphate and the ribose-5-phosphate isomerase 3, was also enhanced. Stress-induction of this pathway, involved in intracellular homeostasis, has already been described for propionibacteria (Gaucher et al., 2019a), for bifidobacteria (Sanchez et al., 2007) and for bacilli (Goswami et al., 2018). By contrast, PTS (PhosphoTransferase System), is reduced by NaCl as in *Listeria monocytogenes* (Bae et al., 2012), and so is glycolysis (fructose-bisphosphate aldoase 1),, and glycogen utilization (glycogen debranching enzyme GlgX).

Concerning lipid and transport metabolism, the induction of inositol-1-phosphate synthase and of acyltransferase PslC indicates enhanced biosynthesis of phospholipids, in accordance with

the observed effect of salt on membrane fatty acids. These proteins are also involved in stress response in *Serratia plymuthica* and *C. glutamicum* (Nurlinawati et al., 2015; Chen et al., 2019).

Concerning cell wall, enzymes involved in peptidoglycan synthesis, such as UDP-N-acetylmuramate-L-alanine ligase, which adds short polypeptides to UDP-acetylmuramic acid, were enhanced. So is the cell division protein FtsI. By contrast, the S-layer-type proteins anchored into the cell wall via SLH domains, i.e. SlpB, was reduced in the presence of salt, in accordance with the western blot analysis (Fig. 23) showing reduce SlpB. SlpB is known to be involved in immunomodulation by *P. freudenreichii*, with an anti-inflammatory effect (Deutsch et al., 2017). By contrast, *P. freudenreichii* CIRM-BIA 129 surface protein SlpA was enhanced in the presence of NaCl, in accordance with a report showing an impact of osmotic stress on the expression of S-layer proteins in *L. acidophilus* (Palomino et al., 2016).

Adaptation to the osmotic constraint clearly enhanced the amount of general stress proteins involved in protein repair or turnover. This includes chaperones such as the three paralogs of Hsp 20 in *P. freudenreichii* CIRM-BIA 129, which protect other proteins from denaturation, as well as the two paralogs of GrpE, which prevent the aggregation of stress-denatured proteins. NaCl adaptation also enhanced proteins involved in oxidative remediation like thioredoxin, thiol peroxidase and peroxiredoxin. In addition, the enhanced SmpB protein belongs to the superfamily of SsrA-binding proteins, which are RNA-binding proteins involved in translational surveillance (Moore and Sauer, 2007).

Acid-adaptation, after osmoadaptation, did not induce important additional changes in *P. freudenreichii* CIRM-BIA 129 proteome, compared to osmoadaptation *per se*. Only 8 proteins were repressed during the acid-adaptation after the osmoadaptation.

5.5 Conclusion

As a conclusion, this work highlights the positive impact of coupling osmoadaptation with a mild acid pretreatment, prior to freeze-drying. It moreover allows better understanding of adaptation mechanisms in *P. freudenreichii*. During the acid treatment, following an osmoadaptation, no general stress protein is over expressed. The higher *P. freudenreichii* viability during freeze-drying seems correlated to the high accumulation of glycine betaine and to the modulation of membrane fatty acid composition. As seen in the chapter 4, the YEL

medium allows *P. freudenreichii* to accumulate high amounts of glycine betaine, thanks to its high concentration of NPN. In this study, we confirm that higher is the glycine betaine accumulation, higher is *P. freudenreichii* survival.

However, osmoadaptation and osmoadaptation coupled to acid or heat-adaptation do not improve *P. freudenreichii* viability to bile salts and acid challenges. This is a concern for the utilization of live probiotic bacteria. The second concern for industry is the long time needed to *P. freudenreichii* to grow in a salty medium.

Important points of the chapter 5:

- Acid-adaptation and heat-adaptation, after the osmoadaptation, modulate membrane fatty acid composition.
- *P. freudenreichii* osmoadaptation increase the BFA/UFA ratio
- Acid-adaptation after an osmoadaptation increases the amount of glycine betaine accumulated
- Osmoadaptation coupled to acid-adaptation increases *P. freudenreichii* survival to freeze-drying

Chapter 6: Taking advantage of bacterial adaptation in order to optimize industrial production of dry *Propionibacterium freudenreichii* and *Lactobacillus rhamnosus*.

In chapter 4, we saw that growth in the presence of salt and lactose leads to increased *Propionibacterium freudenreichii* viability during spray drying. Through osmoadaptation, *P. freudenreichii* accumulated trehalose and glycine betaine. The lactose/NPN ratio determined *P. freudenreichii* resistance. We also saw that the addition of lactose in the YEL medium, without salt, increased *P. freudenreichii* viability to a lesser extent. It is necessary that *P. freudenreichii* arrives alive in the intestine to guarantee its probiotic effects. However, osmoadaptation, with or without lactose, has a negative impact on *P. freudenreichii* viability during acid and bile salts challenges (Chapter 4 and 5), although the sole addition of lactose in the YEL medium increased survival to acid and bile salts challenges.

Apart from increasing the viability during drying, the industrial concern also meets the slow *P. freudenreichii* growth in the presence of salt, and the formulation of the growth medium with lactose because of lactose intolerance considerations. We therefore continued further experiments with *P. freudenreichii* with the addition of glucose, and salt at the beginning of the stationary phase.

In chapter 5, we saw that the heat-adaptation was a promising way to improve *P. freudenreichii* viability to heat challenges. Heat-adaptation had also the advantage to not decrease *P. freudenreichii* viability during bile salts challenges.

In this chapter, we tried to improve *P. freudenreichii* viability to spray drying using an experimental design. We tried to modulate the glucose concentration, the salt concentration and the time of heat-adaptation. We verified the resistance to digestives stresses and the revivification of dried *P. freudenreichii*.

We investigated optimal growth and adaptation conditions, by monitoring intracellular compatible solutes accumulation. Glucose addition, coupled to heat-adaptation, triggered accumulation of trehalose and of glycine betaine, which further provided high tolerance towards

spray drying and storage. This culture conditions and adaptations permit to obtain high *P. freudenreichii* during acid and bile salts challenges.

We then extended the study to *Lactobacillus rhamnosus*. This type of adaptation did not improve *L. rhamnosus* viability during different drying processes.

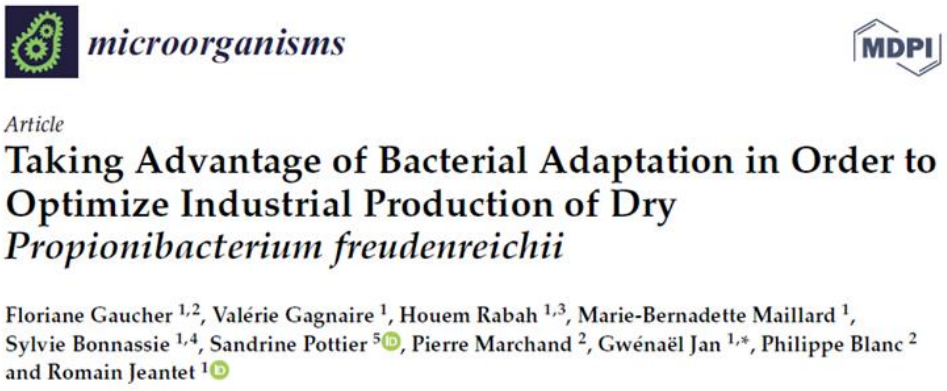
The first part of this chapter will concern *P. freudenreichii* and the second part *L. rhamnosus*.

The aims of this chapter were to:

- Obtain high *P. freudenreichii* and *L. rhamnosus* viability during spray drying with short time of growth and adaptation
- Test different drying processes for the more fragile bacterium, *L rhamnosus*
- Identify the adaptation mechanism of *P. freudenreichii* responsible for high viability during spray drying
- Study *P. freudenreichii* tolerance towards digestives stress after adaptations
- Verify starter efficiency of dried *P. freudenreichii*

The main contents in the first part of this chapter has been published as:

F. Gaucher, V. Gagnaire, H. Rabah, M-B. Maillard, S. Bonnassie, S. Pottier, P. Marchand, G. Jan, P. Blanc, R. Jeantet, Taking advantage of bacterial adaptation in order to optimize industrial production of dry *Propionibacterium freudenreichii*, Microorganisms, (2019) 7, 477. doi: 10.3390/microorganisms7100477.



Part 1

6.1 Introduction

P. freudenreichii should be consumed alive, for an optimal beneficial effect. Starters and probiotics are generally produced under a powder form. Powders allow extended shelf life, and facilitate storage transport of bacteria. Among other drying approaches, spray drying is the most efficient drying process, as it consume less energy and shows a higher productivity than freeze-drying (Huang et al., 2017b, 201). It is furthermore a continuous and fast process, as water evaporation is accelerated by an increased air/product contact surface through spraying into fine droplets. However, bacteria still suffer from heat and oxidative stresses during the spray drying process, which still needs optimization (Huang et al., 2017b). At this end, PolarDry® is a new drying method that has recently been proposed, that consists in using nitrogen rather than air as drying gaz. This normally should supress the oxidative stress during drying, coming with a less stressful processing than classic spray drying as it solely imposes heat stress. This method do not yet exist at the industrial scale but can be promising in order to dry fragile beneficial bacteria.

The ability of bacteria to adapt is usually harnessed to induce homo- or hetero-protections, called cross-protections. Osmoadaptation is well known to increase *P. freudenreichii* tolerance towards heat, acid, and bile salt stresses (Jan et al., 2000; Huang et al., 2016b; Gaucher et al., 2019a). Accordingly, growth in hyperosmotic conditions increases *Lactobacillus paracasei* and *P. freudenreichii* viability during spray drying (Desmond et al., 2001; Huang et al., 2016b). During osmoadaptation, bacteria accumulate compatible solutes, which maintain the turgescent pressure and enable cell growth and division. Compatible solutes may be imported from the growth medium, or synthesized *de novo* (Csonka and Hanson, 1991). As an example, *P. freudenreichii* is able to accumulate glycine betaine, glutamate and trehalose for osmoadaptative purposes. Lactose presence in hyper-concentrated sweet whey leads to trehalose accumulation and further to higher *P. freudenreichii* viability during heat stress and during spray drying (Huang et al., 2016b). Addition of salt in the medium significantly decreases growth rate (Boyaval et al., 1999), that is a problem for industries during starters and probiotics production. Lactose addition in growth medium is also a problem, as industrials aim at banning its utilization because of lactose intolerance issues.

In this study, the osmoadaptation was induced at the end of the stationary phase to avoid reduced growth. Glucose, a food-grade carbohydrate that is more easily available and affordable than

alternative molecules such as trehalose, was chosen. Heat-adaptation has been reported to increase *Lactobacillus paracasei* viability during spray drying (Desmond et al., 2001) and *Lactobacillus plantarum* viability during storage (Paéz et al., 2012). In this work, we thus cultivated *P. freudenreichii* in growth medium with different concentrations of added glucose, we triggered heat and/or osmotic adaptation(s) after the beginning of the stationary phase, and we sought induced protection towards spray drying and storage. We then optimized the conditions of *P. freudenreichii* growth and adaptation with an experimental design, in order to improve cell viability upon spray drying. We quantified compatible solutes to better understand *P. freudenreichii* cross-protections. We further checked the starter capacities of *P. freudenreichii* after adaptations and drying.

6.2 Materials and methods

6.2.1 Strains and pre-culture

Propionibacterium freudenreichii CIRM-BIA 129 (equivalent to ITG P20), *Lactobacillus delbrueckii* CIRM-BIA 209 and *Streptococcus thermophilus* CIRM-BIA 67 were provided by CNIEL. They were stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbienne - Bactéries d'Intérêt Alimentaire, CIRM-BIA, INRA, Rennes, France). *P. freudenreichii, L. delbrueckii* and *S. thermophilus* are routinely cultivated in Yeast Extract Lactate (YEL) medium at 30°C , in Man Rogosa and Sharp (MRS) medium at 37°C and brain heart infusion medium (BHI) at 43°C respectively without agitation (De Man et al., 1960; Malik et al., 1968).

6.2.2 Optimization of *P. freudenreichii* resistance to heat and oxidative challenges

6.2.2.1 Growth and adaptation conditions in the experiment design

The JMP Software from SAS was used to study the impact of glucose addition, heat-adaptation and osmoadaptation on *P. freudenreichii* resistance to heat and oxidative stress. More precisely, glucose was added in YEL medium with concentrations ranging from 0 to 30 $g.L^{-1}$. *P. freudenreichii* was grown at 30°C during 32 h to 46 h with gentle agitations (40 rpm) to decrease the growth rate (Arnaud et al., 1992; Demirtas et al., 2003). At the beginning of the stationary phase, NaCl was added in cultures at a concentration ranging from 0 to 0.9 M during 2 h. Then, the cultures were heated at 42°C during 0, 1 or 2 h. Adaptations were defined by the experimental design (Table 11).

Table 11: Arrangement of the experimental design for the three independent variables used and their level.

	Design matrice			Working matrice		
Trial	NaCl concentration	Glucose concentration	Time of heat-adaptation	NaCl concentration ($g.L^{-1}$)	Glucose concentration ($g.L^{-1}$)	Time of heat-adaptation (h)
1	-1	-1	-1	0	0	0
2	-1	-1	0	0	0	1
3	-1	-1	1	0	0	2
4	-1	0	-1	0	15	0
5	-1	0	0	0	15	1
6	-1	0	1	0	15	2
7	-1	1	-1	0	30	0
8	-1	1	0	0	30	1
9	-1	1	1	0	30	2
10	0	-1	-1	25	0	0
11	0	-1	0	25	0	1
12	0	-1	1	25	0	2
13	0	0	-1	25	15	0
14	0	0	0	25	15	1
15	0	0	0	25	15	1
16	0	0	0	25	15	1
17	0	0	1	25	15	2
18	0	1	-1	25	30	0
19	0	1	0	25	30	1
20	0	1	1	25	30	2
21	1	-1	-1	50	0	0
22	1	-1	0	50	0	1
23	1	-1	1	50	0	2

24	1	0	-1	50	15	0
25	1	0	0	50	15	1
26	1	0	1	50	15	2
27	1	1	-1	50	30	0
28	1	1	0	50	30	1
29	1	1	1	50	30	2

6.2.2.2 Stress challenges

Heat and oxidative challenges were applied to cultures after adaptations as previously described (Gaucher et al., 2019a). Heat challenge was performed by placing 2 mL (in a 15 mL Falcon tube) of *P. freudenreichii* cultures in a water bath at 60°C during 10 minutes. Oxidative challenge was performed by adding 1.25 mM of hydrogen peroxide (Labogros, France) to 2 mL of culture during 1 hours at 30°C. CFU counting was made on untreated bacteria and after challenges in order to calculate survival percentage. The samples tested are reported in the Table 11 and tests were performed in duplicate.

Statistics analyses were done to describe effect of glucose concentration, heat-adaptation and osmoadaptation on *P. freudenreichii* viability during heat and oxidative challenges. The software JUMP was used to fit the second order model to the independent variable. Only variables with a significance higher than 95% ($p < 0.05$) were included in final models. Surface response were drawn to illustrate the main and interactive effects of the independent variables on survival to heat and oxidative challenges. These challenges simulated the stressful spray drying process.

6.2.3 Identification and quantification of compatible solutes accumulated by *P. freudenreichii* CIRM-BIA 129

6.2.3.1 Extraction of accumulated compatible solutes

P. freudenreichii CIRM-BIA 129 was cultivated in YEL medium , YEL medium containing 15g.L^{-1} glucose added with heat-adaptation of 1h at 42°C (YEL+15g glucose+ 42°C, 1h) and YEL with 30 g.L^{-1} glucose added with an heat-adaptation of 2 h at 42°C (YEL+30g Glucose+ 42°C, 2h). Cells were then harvested by centrifugation (8000g 10 minutes) and were washed twice in a NaCl solution with the same osmolality than the culture medium. Compatible solutes were then extracted as previously described in chapter 3 (Gaucher et al., 2019a).

6.2.3.2 NMR (Nuclear Magnetic Resonance) analyses.

Dried extracts were then analyzed by NMR as previously described in chapter 3 (Gaucher et al., 2019a). The relative concentration of trehalose, glutamate and glycine betaine in the samples was determined by the integration of the peaks' areas of their ^{1}H signals relative to the internal standard TMSP. Results are expressed as NMR relative units (RU).

6.2.4 Spray drying and powder storage.

P. freudenreichii was cultivated as previously either in YEL medium, or in YEL medium containing glucose, with 1 or 2 hours of heat-adaptation at 42°C. (YEL+15g glucose+ 42°C, 1h and YEL+30g Glucose+ 42°C, 2h). 20 % (w/w) of maltodextrine (DE = 6-8) was added to the cultures at the beginning of the stationary phase or after heat-adaptation. These different mixtures (~2 L) were shaked for 10 minutes prior to delivery to the dryer by a peristaltic pump (520S, Watson-Marlow, France).

A two-fluid nozzle with a diameter of 0.8 mm was used for atomization. The inlet air temperature was fixed at 160°C. The temperature outlet air was controlled at 60 ± 2°C, by adjusting the feed rate. The bacterial viabilities were estimated by numeration on YEL agar plates before and after spray drying as described previously (Huang et al., 2016a). The powders were collected and sealed in sterilized polystyrene bottles (Gosselin, France), stored at a controlled temperature of 4, 20 or 37 °C, and kept away from light during 145 days. One gram of powder was solubilized in 9 g of sterile water and bacterial viability was tested by numeration on YEL agar plates incubated at 30°C.

6.2.5 Revivification of dried *P. freudenreichii* in a cheese-like dairy medium.

6.2.5.1 Culture with dried *P. freudenreichii* in a cheese-like dairy medium.

Cow milk ultrafiltrate was prepared by milk ultrafiltration (cut-off 8 kDa) as previously described (Cousin et al., 2012). The two major lactic acid starters, *Lactobacillus delbrueckii* CIRM-BIA 209 and *Streptococcus thermophilus* CIRM-BIA 67, were co-cultivated at 43°C during 12 hours in milk ultrafiltrate containing $5g.L^{-1}$ of casein peptone. Cultures were centrifuged in order to eliminate lactic acid bacteria. The supernatants were collected, the pH were readjusted to 7 using a solution of NaOH 5M, then the supernatants were filter-sterilized (Top filter PES, 0.45 µm, Nalgene Company, NY, USA). 1.5 g of *P. freudenreichii* powder dried previously were inoculated in 400 mL of this preconditioned milk ultrafiltrate (named

later cheese-like medium). Control was performed using a fresh liquid *P. freudenreichii* culture realized in the cheese-like medium supplemented by 1.5 g of maltodextrine. Cultures were incubated at 24°C during 14 days in order to mimick growth conditions in the warm room during Emmental cheese making (Gagnaire et al., 2015). Growth curves were established by CFU numeration on YEL agar plates.

6.2.5.2 Organic acids quantification

Supernatants of the different cultures were harvested by centrifugation during exponential phase, the beginning of stationary phase and after 14 days of growth. Culture supernatants were half-diluted with 0.02 M H_2SO_4 and centrifuged at 8000g during 30 minutes at 4°C to discard the protein pellet prior to filter-sterilization and analysis. Organic acids and sugar were separated using a Minex A-6 ion exchange column (Dionex, Sunnyvale, CA) at 55°C with 0.01 M H_2SO_4 as eluent at a flow rate of 1 $mL.min^{-1}$. Both UV (210 nm) and refractometric detectors were used. Appropriate standards of lactose, acetate, propionate and lactate were used as described previously (Aburjaile et al., 2016).

6.2.6 Statistical analysis

The data were from triplicate samples. All the results are presented as mean value with standard deviation. Statistical significance was set at $p<0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows).

6.3 Results

6.3.1 Optimization of *P. freudenreichii* stress tolerance with an experimental design.

We analyzed *P. freudenreichii* survival rates upon heat and oxidative challenges in order to estimate individual and interactive coefficients effect on *P. freudenreichii* tolerance towards heat challenge (Table 12) and oxidative challenge (Table 13). Salt addition after the beginning of stationary phase had a negative impact on *P. freudenreichii* survival during heat and oxidative challenges (Fig. 24) (coefficient estimated: -7% and -10%, in table 12 and 13, respectively), whereas glucose addition and heat-adaptation had a positive impact on *P. freudenreichii* survival during these challenges (Fig. 24). Moreover, glucose addition increased *P. freudenreichii* viability during heat challenge (coefficient estimated: +24%) more

than heat-adaptation (coefficient estimated: +9%). During oxidative challenge, heat-adaptation had a higher impact than glucose addition (coefficient estimated: +19% and +10%, respectively). Best survival during both challenges was obtained when *P. freudenreichii* was grown in the presence of 30 $g.L^{-1}$ of glucose and was heat-adapted at the beginning of stationary phase during 2 hours at 42°C (Fig. 24).

Table 12: Coefficient estimation of the different variables and their interaction (expressed as % survival) on *P. freudenreichii* viability during heat challenge.
NA : not significant

	Estimation	Prob. > |t|
Constant	23	<,0001
NaCl concentration(0,50)	-7	0,0077
Glucose concentration(0,30)	24	<,0001
Time of heat-adaptation(0,2)	9	0,0011
NaCl concentration*Glucose concentration	-13	0,0003
NaCl concentration*Time of heat-adaptation	NS	0,0638
Glucose concentration*Time of heat-adaptation	7	0,0429

Table 13: Coefficient estimation of the different variables and their interaction (expressed as % survival) on *P. freudenreichii* viability during oxidative challenge
NA : not significant

	Estimation	Prob. > |t|
Constant	29	<,0001
NaCl concentration(0,50)	-10	0,0067
Glucose concentration(0,30)	10	0,0068
Time of heat-adaptation(0,2)	19	<,0001
NaCl concentration*Glucose concentration	NA	0,2329
NaCl concentration*Time of heat-adaptation	-14	0,0021
Glucose concentration*Time of heat-adaptation	NA	0,1406

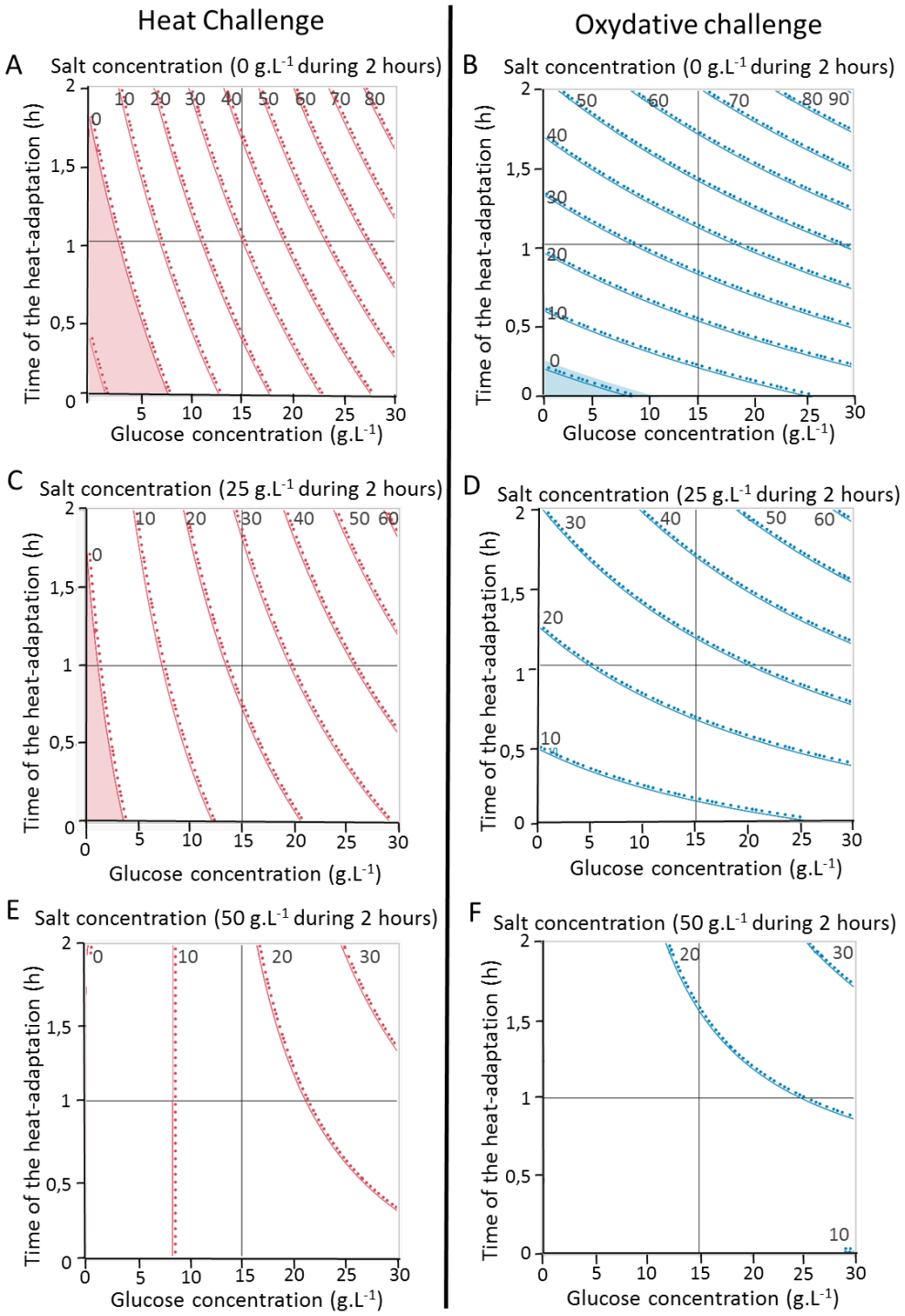


Figure 24: Adaptations and growth medium compositions modulates *P. freudenreichii* stress tolerance. Effect of addition of glucose, heat-adaptation and osmoadaptation were tested on *P. freudenreichii* CIRM-BIA 129 stress tolerance following an experimental design. *P. freudenreichii* was cultivated in YEL medium with 0, 15 or 30 g.L^{-1} of glucose until the beginning of the stationary phase. Cultures were then subjected to osmoadaptation (0, 25 or 50 g.L^{-1} of NaCl) during 2 hours and then heat-adapted at 42°C during 0, 1 or 2 hours. Cultures were subjected to heat challenge (A, C and E, 60°C for 10 min) or oxidative challenge (B, D and F, 1.15 mM H_2O_2 for 1 h) as described in materials and methods. *P. freudenreichii* viability after challenges was determined by CFU counting before and after challenges. Results are expressed as percent survival and reported near the iso-response curves

6.3.2 *P. freudenreichii* viability after spray drying and storage.

Control cultures (YEL), cultures in the best conditions defined by the experimental design (YEL + 30g glucose + 42°C, 2h) and in the intermediate tolerance conditions (YEL + 15g glucose + 42°C, 1h) were spray dried. The measures of the water activity of the powders were 0.19 ± 0.05. *P. freudenreichii* grown in YEL with 15 g of glucose and heat-adapted during 1 hour at 42°C survived better to spray drying than unadapted cells (74% and 30% survival, respectively) (Fig. 25A). A higher glucose concentration in the culture medium with a longer heat-adaptation further increased the survival during spray drying (96.4%). The addition of glucose and/or the heat-adaptation had a positive impact on *P. freudenreichii* viability during spray drying. During storage at 4 and 20°C, *P. freudenreichii* grown with glucose and then heat-adapted displayed enhanced viability (Fig. 25 B and C). The higher the glucose concentration and the longer the heat-adaptation, the lower *P. freudenreichii* death during storage at 4 and 20°C. During storage at 37°C, these adaptations did not have any impact on cells death (Fig. 25 D).

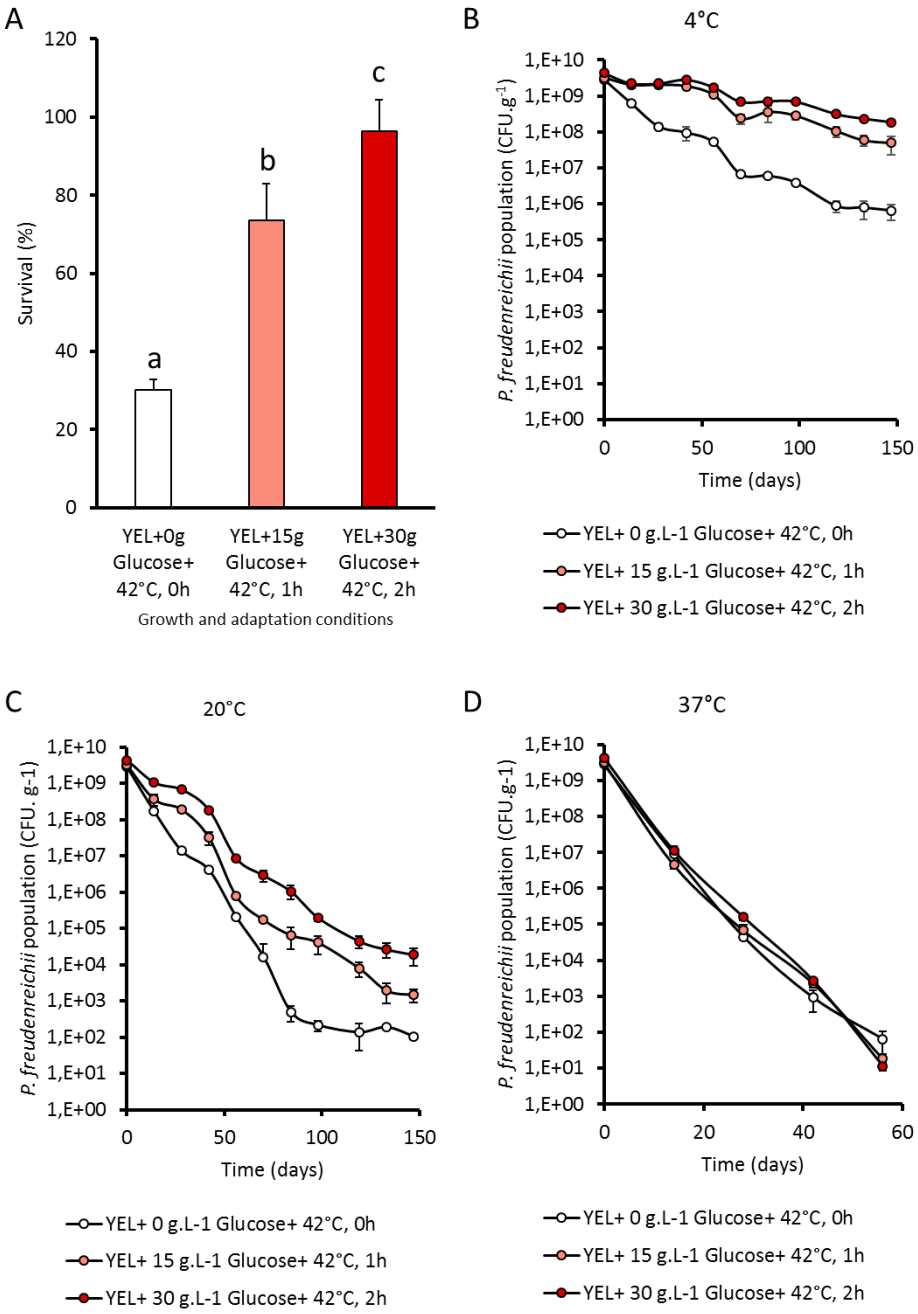


Figure 25: Glucose addition in growth medium and heat-adaptation improve *P. freudenreichii* viability during spray drying and storage. *P. freudenreichii* CIRM-BIA 129 were cultivated in YEL (YEL+ 0g Glucose + 42°C, 0h) , in YEL medium containing 15 $g.L^{-1}$ of glucose and heat-adapted at 42°C during 1 hour (YEL+15g Glucose + 42°C, 1h) or YEL medium containing 30 $g.L^{-1}$ of glucose and heat-adapted at 42°C during 2 hours (YEL+15g Glucose + 42°C, 2h) until the beginning of the stationary phase. Maltodextrine was added in the cultures with a final concentration of 20% (w/w) to increase the dry extract. Cultures were then spray dried and *P. freudenreichii* survival was quantified by CFU counting as described in materials and methods. Results are expressed as percent survival (A). The different powders obtained were stored at 4°C (B), 20°C (C) and 37°C (D) during 145 days. *P. freudenreichii* viability were quantified by CFU counting during storage. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p>0.05$).

6.3.3 Compatible solutes accumulation depends on *P. freudenreichii* growth medium and adaptation conditions.

In the control medium (YEL), *P. freudenreichii* accumulated high amounts of glutamate (66.7 %, Fig. 26), and to a lesser extent proline (24.2 %) and little amounts of glycine betaine (9.1 %); this medium is isotonic with an osmolality of 0.308 osmol.

The addition of 15 g of glucose to this culture medium (increasing its osmolality to 0.433 osmol) as well as heat-adaptation during 1 hour drastically affected compatible solutes accumulation. First, it decreased the total amount of compatible solute accumulated. Second, it changed the proportions of compatible solutes accumulated. Compared to the control medium, glycine betaine and trehalose were accumulated in higher amount (49.4 % and 30.6 % respectively), with lower amounts of proline (13.0 %) and glutamate (7.0 %). Higher concentration of glucose in the YEL medium (increasing YEL medium osmolality to 0.455 osmol at 30 $g.L^{-1}$) and a longer heat-adaptation (2h) further increased the amount of compatible solutes accumulated by *P. freudenreichii*, but with a profile similar to that of *P. freudenreichii* grown with 15 g of glucose and heat-adapted for 1 hour. Indeed, *P. freudenreichii* accumulated in this case trehalose (40 %) and glycine betaine (42.5 %) in high amounts, with lower amounts of proline (10 %) and of glutamate (10 %).

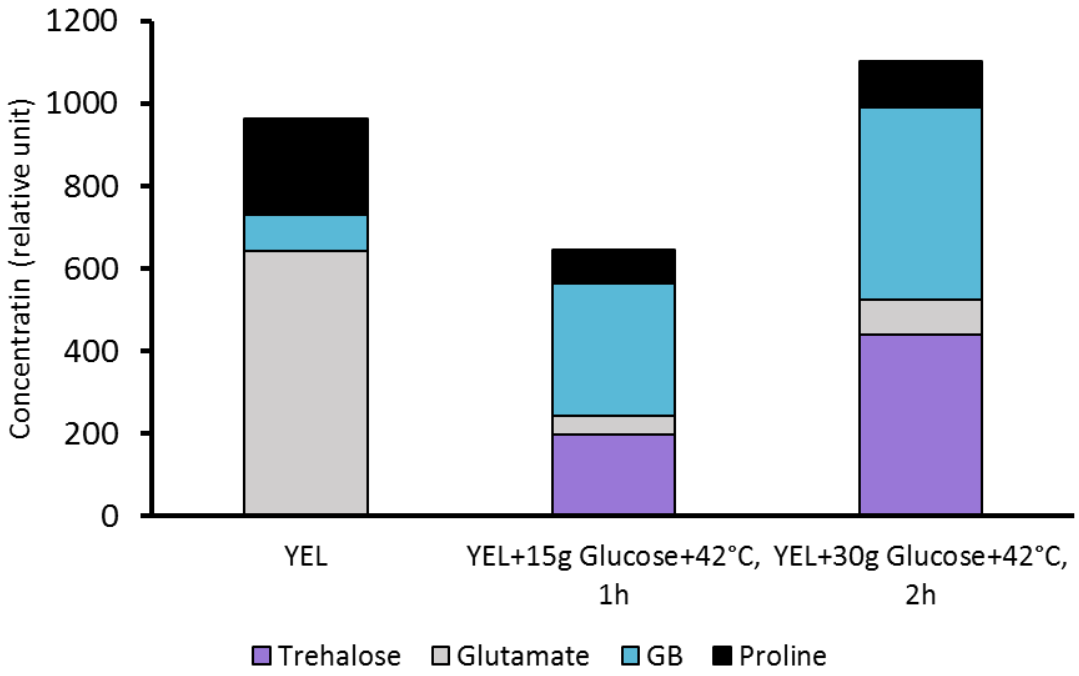


Figure 26: *P. freudenreichii* compatible solutes is modulated by adaptations. *P. freudenreichii* CIRM-BIA 129 were cultivated in YEL (YEL + 0g Glucose + 42°C, 0h) , in YEL medium containing 15 $g.L^{-1}$ of glucose and heat-adapted at 42°C during 1 hour (YEL + 15g Glucose + 42°C, 1h) or YEL medium containing 30 $g.L^{-1}$ of glucose and heat-adapted at 42°C during 2 hours (YEL + 15g Glucose + 42°C, 2h) until the beginning of the stationary phase. Cytoplasmic extracts were made as described in materials and methods. Compatibles solutes were identified and quantified by NMR. Compatible solutes accumulation are expressed in relative units.

6.3.4 *P. freudenreichii* viability after digestive stresses

P. freudenreichii grown in YEL with different glucose concentration with or without heat-adaptation was subjected to acid and bile salt challenges. Glucose addition in the growth medium increased *P. freudenreichii* viability during acid challenge with a dose-response effect (Fig. 27 A). Addition of glucose also increased *P. freudenreichii* viability during bile salts challenges (Fig. 27 B). Heat-adaptation provoked after the beginning of the stationary phase did not increase *P. freudenreichii* viability during these challenges.

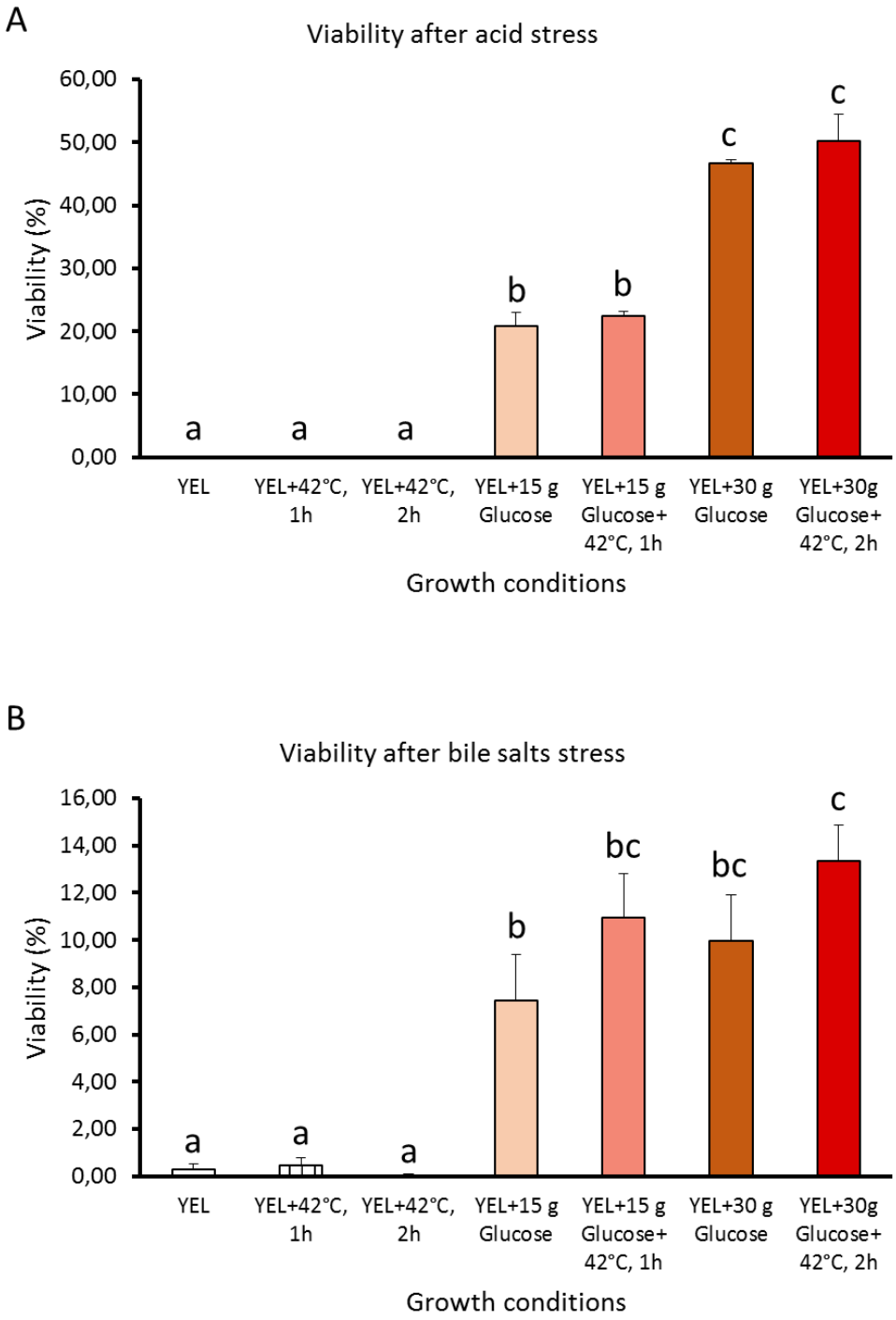


Figure 27: *P. freudenreichii* viability during acid (A) and bile salts (B) challenges after adaptations. *P. freudenreichii* CIRM-BIA 129 were cultivated in YEL with 0, 15 or 30g of glucose and heat-adapted at 42°C during 0, 1 or 2h until the beginning of the stationary phase. Results are expressed as percent survival. Error bars represent the standard deviation for triplicate experiments. Significant differences are reported with different letters above the columns ($p>0.05$).

6.3.5 *P. freudenreichii* revivification

6.3.5.1 Growth of dried *P. freudenreichii* in a cheese-like medium

Powders containing *P. freudenreichii*, or a fresh culture (*P. freudenreichii* in stationary phase) in YEL medium as a control, were inoculated in a cheese-like medium consisting in milk ultrafiltrate previously fermented by a mixture of *L. delbrueckii* and a *S. thermophilus*, the major lactic starters implemented together with *P. freudenreichii* in Emmental cheese. In the absence of adaptation, *P. freudenreichii* was not able to grow in cheese-like medium (data not shown). Conversely, adapted and dried *P. freudenreichii* powders were able to grow in the cheese-like medium (Fig. 28A), indicating revivification of the propionic starter, so that it reached final propionibacteria population comparable to that of control. At the end of the fermentation, all cultures had a pH between 4.72 and 4.95, but the pH decreased more slowly with the fresh culture, when compared to dried propionibacteria (Fig. 28B).

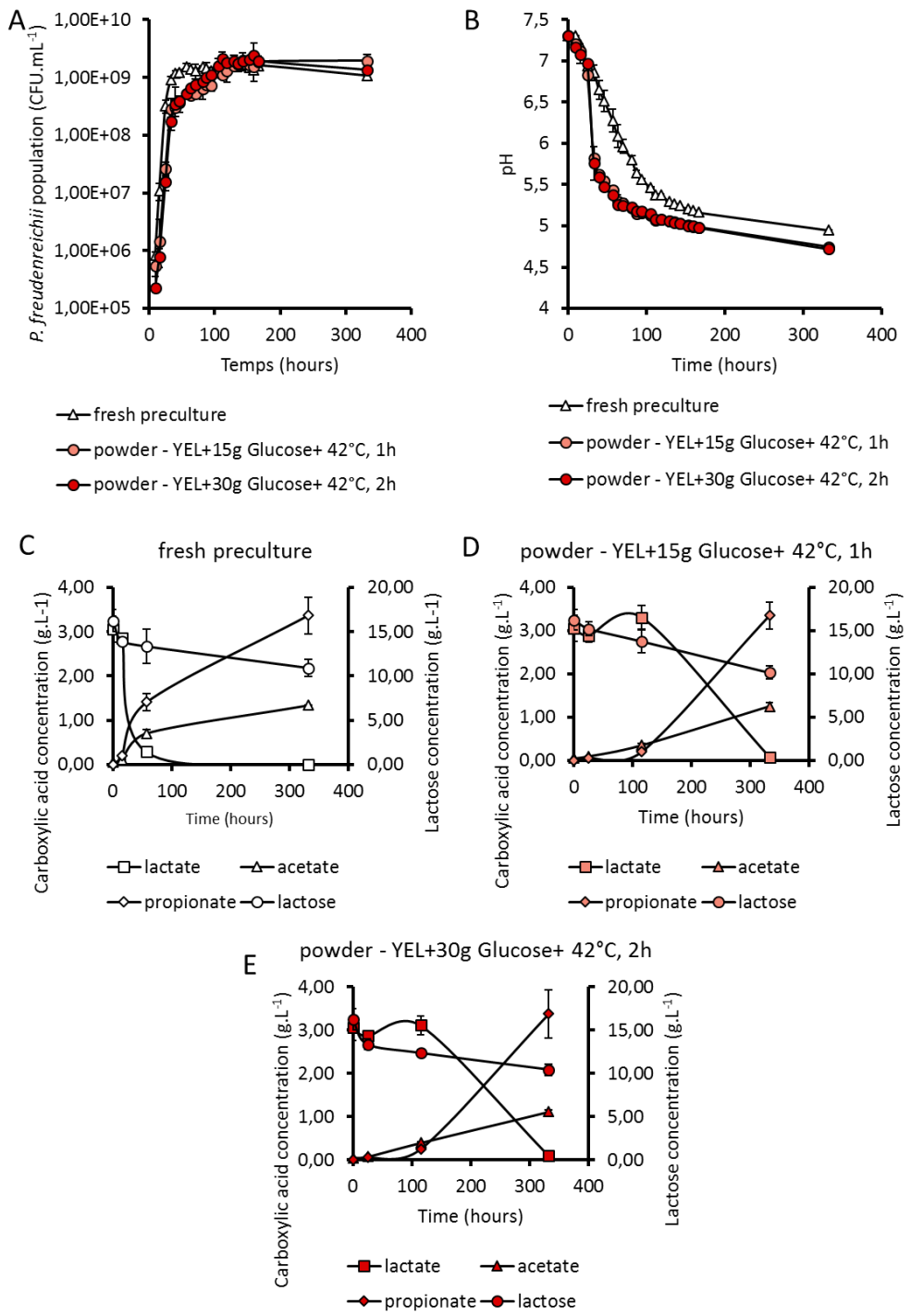


Figure 28: Powders containing *P. freudenreichii* were used to ferment a cheese-like medium. Powders obtained previously were inoculated in a cheese-like medium. *P. freudenreichii* growth were monitored by CFU counting in YEL agar plates (A) and acidification were followed (B). Lactate and lactose consumption, as well as propionate and acetate production, were quantified by HPLC (C, D and E) as described in material and methods. Fresh culture: inoculation with *P. freudenreichii* grown in YEL medium**.** (YEL + 15g Glucose + 42°C, 1h): inoculation with powders containing *P. freudenreichii* cultivated in YEL with 15g.L^{-1} of glucose and heat-adapted at 42°C during 1 hour before spray drying. (YEL + 15g Glucose + 42°C, 1h): inoculation with powders containing *P. freudenreichii* cultivated in YEL with 15g.L^{-1} of glucose and heat-adapted at 42°C during 1 hour before spray drying.

6.3.5.2 Fate of saccharides and organic acids

Utilization of lactose and of lactate, as well as production of propionate and of acetate, the main end products of propionic fermentation, was monitored as described in materials and methods. Lactose utilization was nearly the same between dried *P. freudenreichii* and the fresh culture (Fig. 28 C-E). Dried *P. freudenreichii* consumed lactate and produced propionate and acetate less rapidly than the fresh culture. Final consumption of lactate and lactose and final production of acetate and propionate were non-significantly different between fresh preculture and dried *P. freudenreichii*. Propionate final concentration was twice the acetate final concentration. Interestingly, lactose was consumed as soon as the beginning of growth, while lactate remained first constant before being consumed after 100 hours of growth. In the case of fresh precultures, both lactose and lactate were used throughout the culture.

6.4 Discussion

6.4.1 Addition of glucose and heat-adaptation both induce cross-protections to *P. freudenreichii*

Glucose addition and heat-adaptation both improved *P. freudenreichii* tolerance towards oxidative and heat challenges (Fig. 24). Heat-adaptation was already described to increase *P. freudenreichii*, *Enterococcus faecalis* and *Escherichia coli* thermotolerance (Flahaut et al., 1996; Leverrier et al., 2004; Pleitner et al., 2012). Addition of glucose during growth also improved *P. freudenreichii* viability during heat and oxidative stress. Accordingly, growth in hyper-concentrated sweet whey, a medium rich in the saccharide lactose, was previously shown to induce such cross-protection, in parallel with trehalose accumulation (Huang et al., 2016a, 2016b). We showed here that adding the glucose monosaccharide in combination with a heat-adaptation also triggered cross-protections and the accumulation of trehalose and glycine betaine. Glucose is known to be a trehalose building block in a wide range of microorganisms (Streeter and Gomez, 2006). The experimental design highlighted the positive effect of interactions between the addition of glucose and the heat-adaptation. The conditions allowing the best survival during oxidative and heat challenges were combining the YEL medium containing 30g.L^{-1} of glucose and heat-adaptation during 2 hours at 42°C (Fig. 24). Following glucose addition and heat-adaptation, we also tested addition of NaCl after growth and observed afterwards a reduced tolerance. Growth in the presence of NaCl may induce tolerance (Teixido

et al., 2005; Pleitner et al., 2012; Gaucher et al., 2019a), while NaCl addition after growth did not have such effect in the present work.

6.4.2 Combining glucose addition and heat-adaptation increases *P. freudenreichii* viability during spray drying and storage

Glucose addition in the growth medium and heat-adaptation at the beginning of the stationary phase increased *P. freudenreichii* survival after drying (Fig. 25A). Increasing the glucose concentration and/or the duration of heat-adaptation led to higher survival as predicted by the experimental design. Heat-adaptation was already reported to improve *Lactobacillus paracasei* and *Candida sake* survival during spray drying (Desmond et al., 2001; Cañamás et al., 2008). During storage at 4 and 20°C, dried *P. freudenreichii* grown with glucose and heat-adapted exhibited a better survival rate than the control (Fig. 25 B and C). In other words, a higher glucose concentration and a longer heat-adaptation increased survival during storage as during spray drying. Similarly, heat-adaptation was reported to increase *L. plantarum* viability during storage (Paéz et al., 2012). Sucrose addition can increase *Lactobacillus sakei* survival during storage after spray drying (Ferreira et al., 2005). However, the impact of saccharides on bacteria viability during storage is not well known yet.

6.4.3 Combining glucose addition and heat-adaptation leads to compatible solutes accumulation and increases *P. freudenreichii* viability to spray drying.

Control cultures accumulated high amount of the compatible solutes glutamate and proline. This can be explained by the culture stirring and the presence of oxygen. Indeed, such accumulation was not observed in the same YEL medium in the absence of agitation (Fig. 14). In this study, proline was accumulated in the three different conditions. This amino acid is accumulated by *Brevibacterium flavum* in the presence of dissolved oxygen, and is biosynthesized from glutamate (Akashi et al., 1979). In the presence of glucose and under heat-adaptation, *P. freudenreichii* accumulated high amounts of glycine betaine and of trehalose. Glycine betaine is known to play a role during thermo-protection (Ghazi et al., 1999; Holtmann and Bremer, 2004). Trehalose accumulation is well known during heat-adaptation in yeast (Hottiger et al., 1987; Singer and Lindquist, 1998), and *P. freudenreichii* is known to accumulate trehalose (Cardoso et al., 2007). Increased glucose concentration in the growth medium led to increased trehalose accumulation, as described previously (Cardoso et al., 2007).

Normally, lactose leads to higher trehalose accumulation by *P. freudenreichii* than glucose (Cardoso et al., 2007; Huang et al., 2016b). Addition of glucose coupled to heat-adaptation has yet been shown to increase trehalose accumulation by *Saccharomyces cerevisiae* (Hottiger et al., 1987). When *P. freudenreichii* was grown with high glucose concentration and was heat-adapted, its trehalose accumulation and its resistance to heat challenges increased. Accordingly, trehalose accumulation is determinant for thermo-tolerance for *S. cerevisiae* and *P. freudenreichii* (Hazell et al., 1995; Huang et al., 2016b).

6.4.4 Combining glucose addition and heat-adaptation increases *P. freudenreichii* viability during digestive challenges

Addition of glucose had the advantage to increase *P. freudenreichii* viability during acid and bile salt challenges as the addition of lactose (Chapter 6). The heat-adaptation made after the beginning of the stationary phase did not have negative impact on *P. freudenreichii* during these digestive stresses. Addition of glucose coupled to the heat-adaptation permit to increase viability to spray drying and digestive stresses, which is very important for probiotics production.

6.4.5 Combining glucose addition and heat-adaptation leads to production of efficient propionic starter.

P. freudenreichii, in the cheese-like medium, exhibited similar growth parameters, whether inoculated as a fresh culture, or as a powder obtained from an adapted culture. Compared to the control fresh culture, dried *P. freudenreichii* acidified more and faster the cheese-like dairy medium. Lactate concentration decreased later in cultures inoculated with powders containing adapted *P. freudenreichii,* which delayed the production of propionate and acetate. In all culture, *P. freudenreichii* consumed both lactate and lactose and produced acetate and propionate with a 2:1 ratio, that is characteristic of propionic fermentation according to the Fitz equation (Fitz, 1878). The amount of lactose, lactate, propionate and acetate were similar for all cultures at the end of fermentation. However, *P. freudenreichii* used both lactose and lactate as soon as the beginning of growth in the case of fresh cultures. By contrast, *P. freudenreichii* used preferentially lactose at the beginning of growth, in accordance with faster acidification, in the case of dried bacteria. *P. freudenreichii* grown in presence of glucose and heat-adapted display enhanced resistance to spray drying and enhanced acidification ability. Such powders containing adapted *P. freudenreichii* could thus be profitably used as starter.

6.5 Conclusion

As a conclusion, we modulated the growth medium composition and we used heat-adaptation to increase *P. freudenreichii* viability during spray drying and during storage. In the presence of glucose, *P. freudenreichii* accumulated trehalose and glycine betaine in nearly same proportions as when submitted to heat-adaptation or osmoadaptation. Compared to osmoadaptation, the heat-adaptation is easier to implement in the industry, and permit faster production of *P. freudenreichii* with high viability during spray drying and storage.

Important points of the part 1 of the chapter 6:

- Glucose addition in the growth medium and/or heat-adaptation trigger(s) accumulation of trehalose and of glycine betaine by *P. freudenreichii*.
- Glucose addition in the growth medium and/or heat-adaptation increase(s) *P. freudenreichii* viability during spray drying and storage.
- Glucose addition in the growth medium coupled to heat-adaptation are faster and more applicable for industries than the osmoadaptation coupled to addition of lactose in the growth medium.

Part 2

6.6 Introduction

L. rhamnosus CIRM-BIA 1113 is less robust than *P. freudenreichii* CIRM-BIA 129 (intern tests). This is the reason why we intended to study the potential increase of *L. rhamnosus* CIRM-BIA 1113 viability during different drying processes: freeze-drying as a control and two different spray drying processes. This fragile bacterium is sold by Bioprox as probiotics and bio-preservative, and therefore is routinely produced in a MRS-like medium containing 80 $g.L^{-1}$ of trehalose. Given its sensitivity, obtaining high number of alived *L. rhamnosus* after spray drying seemed a real challenge. This is why an experimental design was build in the same way as for *P. freudenreichii*, to see if the results previously obtained could be generalised to *L. rhamnosus* CIMR-BIA 1113. As before, we intented to increase *L. rhamnosus* resistance thanks to heat and osmoadaptation, and/or the use of different trehalose concentrations. Growth and adaptation conditions, as weel as the heat and oxidative challenges, were adapted for *L. rhamnosus*.

For the experimental design, *L. rhamnosus* was grown in fermenters by Bioprox. Concerning drying steps, freeze-drying was made by Bioprox, spray drying was conducted at the Minor scale on the STLO platform whereas the PolarDry® technology was accessible by the LIS laboratory (Lesaffre Ingrédient Services, Rennes).

6.7 Materials and methods

6.7.1 Strains and pre-culture

Lactobacillus rhamnosus CIRM-BIA 1113 was stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbienne - Bactéries d'Intérêt Alimentaire, CIRM-BIA, INRA, Rennes, France). *L. rhamnosus* is routinely cultivated in MRS medium (De Man et al., 1960).

6.7.2 Optimization of *L. rhamnosus* resistance to freeze-drying and heat and oxidative challenges

6.7.2.1 Growth and adaptation conditions in the experimental design

The JMP Software from SAS was used to study the impact of trehalose addition, heat-adaptation and osmoadaptation on *L. rhamnosus* resistance to heat and oxidative stress. More precisely, trehalose was added in the growth medium used by Bioprox (MRS-like) medium

with concentrations ranging from 80 to 120 g.L^{-1}. *L. rhamnosus* was grown at 35°C during 17 h to 21 h under agitation (300 rpm). At the beginning of the stationary phase, NaCl was added in cultures with concentrations ranging from 0 to 20 g.L^{-1} during 2 h. Then, the cultures were heated at 45°C during 0, 1.5 or 3 h. Adaptations were defined by the experimental design (Table 14).

Table 14: Arrangement of the experimental design for the three independent variables used and their level

	Design matrice			Working matrice		
Samples	NaCl concentration	Trehalose concentration	Time of Heat-adpatation	NaCl concentration (g.L^{-1})	Trehalose concentration (g.L^{-1})	Time of heat-adpatation (h)
1	-1	-1	-1	0	80	0
2	-1	-1	0	0	80	1,5
3	-1	-1	1	0	80	3
4	-1	1	-1	0	120	0
5	-1	1	0	0	120	1,5
6	-1	1	1	0	120	3
7	0	-1	-1	10	80	0
8	0	-1	0	10	80	1,5
9	0	-1	1	10	80	3
10	0	1	-1	10	120	0
11	0	1	0	10	120	1,5
12	0	1	1	10	120	3
13	1	-1	-1	20	80	0
14	1	-1	0	20	80	1,5
15	1	-1	1	20	80	3
16	1	1	-1	20	120	0
17	1	1	0	20	120	1,5
18	1	1	1	20	120	3
19	0	0	0	10	100	0
20	0	0	0	10	100	1,5
21	0	0	0	10	100	3

6.7.2.2 Freeze-drying

Cultures from the experimental design were freeze-dried using Bioprox method. *L. rhamnosus* was cultivated as previously either in MRS-like medium, or in MRS-like medium containing varying trehalose concentrations (MRS-like - 80 g.L^{-1} trehalose and MRS-like - 105 g.L^{-1} trehalose). After being harvested by centrifugation, 20 % (w/w) of maltodextrine (DE = 6-8)

was added. Mixtures were cooled at -25°C in a freeze-dryer pilot. The chamber pressure was decreased to 250µbar. A secondary drying was performed at +25°C during 5 hours, the vacuum was then broken by injecting air. CFU counting was carried out before and after freeze-drying in order to calculate survival.

Statistical analyses were performed to describe effects of trehalose concentration, heat-adaptation and osmoadaptation on *L. rhamnosus* viability during freeze-drying. The software JUMP was used to fit the second order model to the independent variable. Only the variables with a significance higher than 95% ($p < 0.05$) were considered in the final models. Surface response were drawn to illustrate the main and interaction effects of the independent variables on survival to heat and oxidative challenges.

6.7.2.3 Heat and oxidative challenges

The conditions tested are reported in Table 14 and each test was performed in duplicate. These challenges simulated the stressful spray drying process. Heat challenge was performed by introducing 2 mL of *P. freudenreichii* cultures in a 15 mL Falcon tube, then in a water bath at 55°C during 5 minutes. Oxidative challenge was performed by adding 0.0625 mM of hydrogen peroxide (Labogros, France) to 2 mL of culture during 15 minutes at 35°C. CFU counting was made on untreated bacteria and after challenges in order to calculate survival percentage.

Statistical analyses were done to describe effect of trehalose concentration, heat-adaptation and osmoadaptation on *L. rhamnosus* viability during heat and oxidative challenges. As for freeze-drying, the software JUMP was used to fit the second order model to the independent variable.

6.7.3 Spray drying

L. rhamnosus was cultivated as previously either in MRS-like medium, with varying trehalose concentrations (MRS-like - 80 $g.L^{-1}$ trehalose and MRS-like - 105 $g.L^{-1}$ trehalose). After being harvested by centrifugation, 20 % (w/w) of maltodextrine (DE = 6-8) was added. These different mixtures (~2 L) were agitated for 10 minutes prior to delivery to the dryer by a peristaltic pump (520S, Watson-Marlow, France).

6.7.3.1 Spray drying with Minor pilot

A two-fluid nozzle with a 0.8 mm diameter was used for atomization. The inlet air temperature of the Minor pilot was fixed at 150°C. The outlet air temperature was controlled by adjusting the feed rate at 60 ± 2°C or 50 ± 2°C for the first and second experiment, respectively. The bacterial viabilities were estimated by numeration on MRS agar plates before and after spray drying.

6.7.3.2 Spray drying with PolarDry®

During the PolarDry® process, the mixture was sprayed into the drying chamber. In short, this innovative drying process mainly differs from classical spray drying from the spraying step principle and drying gas nature. The droplets are polarized by application of an electrostatic charge (Fig. 29). The water, which is more polar, thus tends to migrate to the outer surface of the droplets, allowing easier water elimination. Besides, nitrogen is used as a drying fluid. Its water vapor pressure being null, it is more hydrophilic than air and this facilitates drying. Overall these modifications make it possible to decrease the drying temperature.

Indeed, the inlet air temperature was fixed at 120°C. The outlet air temperature was controlled at 50 ± 4°C by adjusting the feed rate. The PolarDry® pilot used had an evaporation capacity of 1 $kg.h^{-1}$ and nitrogen maximum flux of 8.5 $m^3.h^{-1}$. The bacterial viabilities were estimated by numeration on MRS agar plates before and after spray drying.

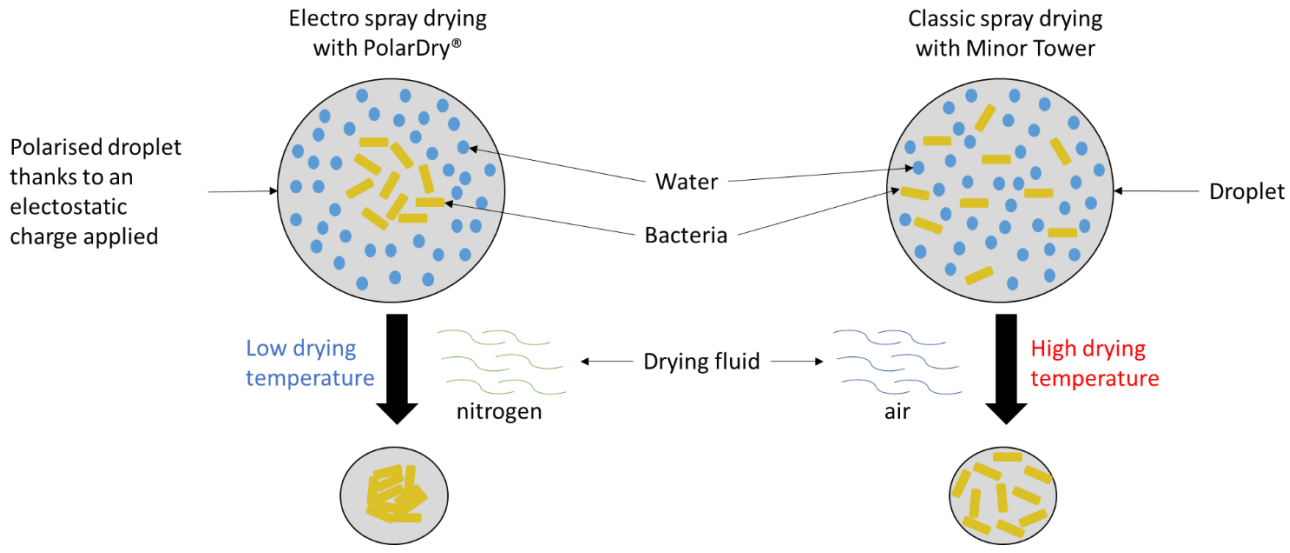


Figure 29: Comparison between the Minor tower and the PolarDry®. Bacteria are represented

in yellow, water molecules in bleu. Schemes highlight the organization of the droplets. The fluid gas (nitrogen and air) used are indicated for each drying process.

6.7.4 Controlling the a_w of spray dried powders

Powders obtained by spray drying and freeze-drying were dehydrated by vacuum drying in order to obtain a constant a_w of 0.025. Powders were introduced in the freeze-drying pilot with a temperature of +25°C. The chamber pressure was decreased to 50µbar, without cooling. After 5 hours of desorption, the vacuum was broken by injecting air.

6.7.5 Storage and post-storage viability assessment

The powders were collected and sealed in sterilized polystyrene bottles (Gosselin, France), then stored at a controlled temperature of 25 °C during 1 month.

6.7.6 Numeration of the freeze-dried and spray dried powders

A 1:100 dilution was performed in sterile tryptone water and bacterial viability was tested by numeration in masse on MRS agar plates incubated at 37°C.

6.8 Results

6.8.1 Optimization of *L. rhamnosus* tolerance to freeze-drying using an experimental design.

Values of freeze-drying viability were analyzed in order to estimate the coefficient of individual and interactive effect on *L. rhamnosus* survival during freeze-drying (Table 15). Salt addition, trehalose addition and heat-adaptation did not have significant influence on *L. rhamnosus* viability during freeze-drying.

Table 15: Coefficient estimation of the different variables and their interactions (expressed as % survival) on *L. rhamnosus* viability during freeze-drying.
NA : not significant

	Estimation	Prob. > |t|
Constant	9	<,0001
NaCl concentration(0,20)	NS	0,8054
Trehalose concentration(80,120)	NS	0,6285
Time of heat-adaptation(0,3)	NS	0,4774
NaCl concentration*Trehalose concentration	NS	0,2078
NaCl concentration*Time of heat-adaptation	NS	0,44
Trehalose concentration*Time of heat-adaptation	NS	0,829

6.8.2 Optimization of *L. rhamnosus* tolerance to heat and oxidative stresses with an experimental design.

Values of heat and oxidative challenges were analyzed in order to estimate the coefficient of individual and interaction effect on *L. rhamnosus* survival during heat challenge (Table 16) and oxidative challenge (Table 17). Salt addition after the beginning of stationary phase seemed to have a negative influence on *L. rhamnosus* survival during oxydative challenges (Fig. 30) (coefficient estimated: -33% table 17), whereas trehalose addition seemed to have a positive influence on *L. rhamnosus* viably during heat challenge (Fig. 30) (coefficient estimated: +10% table 16). Heat-adaptation did not have any significant impact on *L. rhamnosus* viability during heat or oxidative challenge (table 16 and 17).

Overall, the highest survival rates during heat challenge were obtained when *L. rhamnosus* was not heat-adapted nor osmoadapted whatever the trehalose concentration in the growth medium. However, best survival during oxidative challenge were obtained when *L. rhamnosus* was grown with high trehalose concentration (100 to 120 $g.L^{-1}$ of trehalose) and was not heat-adapted or osmoadapted (Fig. 30).

Table 16: Coefficient estimation of the different variables and their interaction (expressed as % survival) on *L. rhamnosus* viability during heat challenge.
NA : not significant

	Estimation	Prob. > |t|
Constant	50	<,0001
NaCl concentration(0,20)	NS	0,1332
Trehalose concentration(80,120)	10	0,246
Time of heat-adaptation(0,3)	NS	0,3651
NaCl concentration*Trehalose concentration	NS	0,9332
NaCl concentration*Time of heat-adaptation	NS	0,4576
Trehalose concentration*Time of heat-adaptation	NS	0,1248

Table 17: Coefficient estimation of the different variables and their interaction (expressed as % survival) on *L. rhamnosus* viability during oxidative challenge.
NA : not significant

	Estimation	Prob. > |t|
Constant	40	0,0004
NaCl concentration(0,20)	-33	0,0137
Trehalose concentration(80,120)	NA	0,1031
Time of heat-adaptation(0,3)	NA	0,3284
NaCl concentration*Trehalose concentration	NA	0,0877
NaCl concentration*Time of heat-adaptation	NA	0,0631
Trehalose concentration*Time of heat-adaptation	NA	0,1445

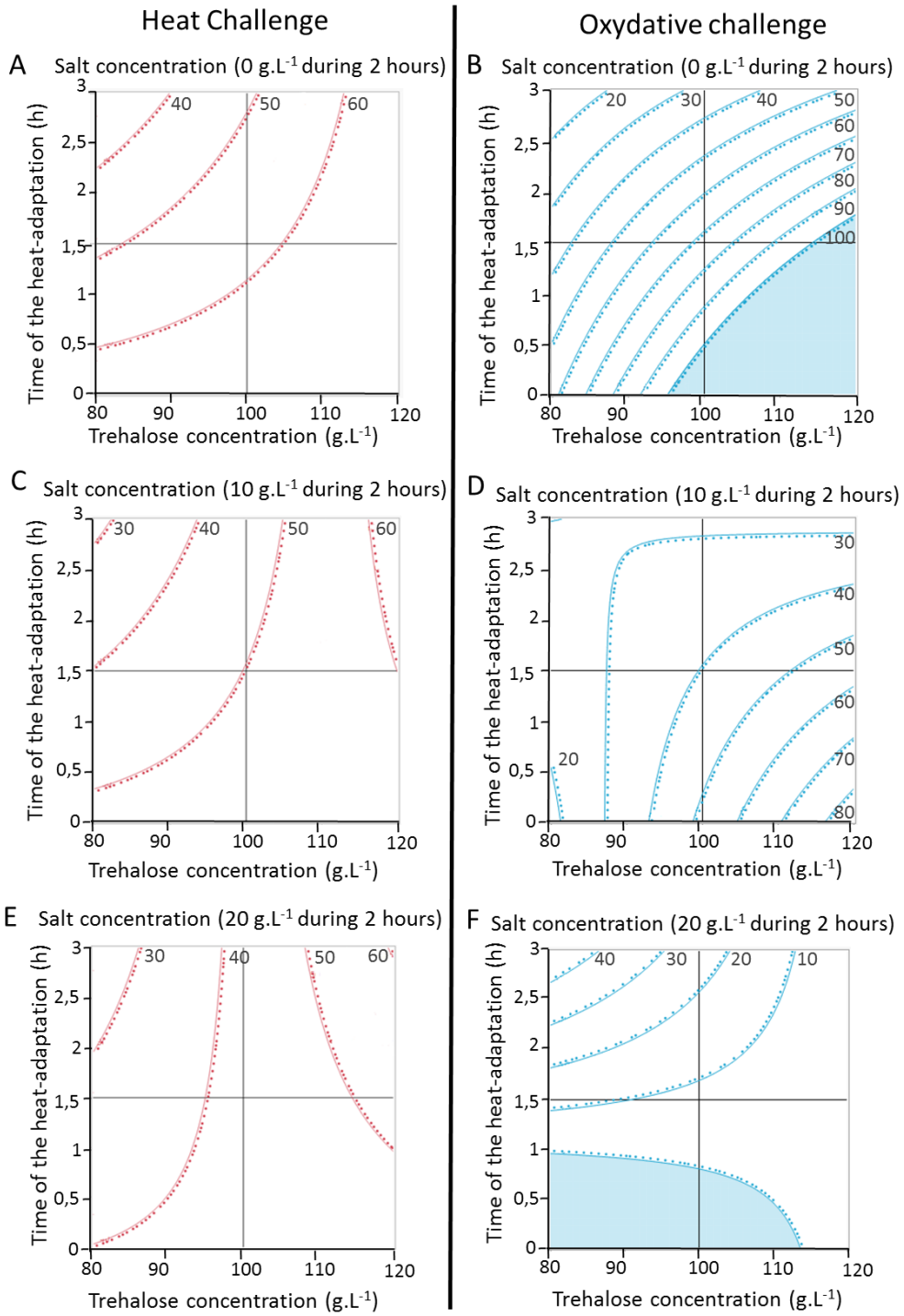


Figure 30: Adaptations and growth medium compositions modulate *L. rhamnosus* stress tolerance. Effect of addition of trehalose, heat-adaptation and osmoadaptation were tested on *L. rhamnosus* CIRM-BIA 1113 stress tolerance following an experimental design. *L. rhamnosus* was cultivated in MRS-like medium with 80, 100 or 120 g.L^{-1} of trehalose until the beginning of the stationary phase. Cultures were then subjected to osmoadaptation (0, 10 or 20 g.L^{-1}) during 2 hours and then heat-adapted at 42°C during 0, 1.5 or 3 hours. Cultures were subjected to heat challenge (A, C and E, 55°C for 5 min) or oxidative challenge (B, D and F, 0.0625 mM H_2O_2 for 15 min) as described in materials and methods. *L. rhamnosus* viability after challenges was determined by CFU counting before and after challenges. Results are expressed as percent survival and reported near the iso-response curves.

6.8.3 *L. rhamnosus* viability after spray drying.

Control cultures (MRS-like - 80 g.L^{-1} trehalose), and the best culture conditions defined though the experimental design (MRS-like – 105 g.L^{-1} trehalose) were then spray dried by the two different process. Regardless of the outlet air temperature, *L. rhamnosus* grown with 80 g.L^{-1} of trehalose better survived to conventional spray drying (Minor) than with PolarDry® (Fig. 31). With an outlet air temperature of 50°C, *L. rhamnosus* grown with 80 g.L^{-1} of trehalose had higher survival during drying than with an outlet air temperature of 60°C (37 and 65% respectively). With an outlet air temperature of 50°C, *L. rhamnosus* showed same range of survival regardless of the drying process. During this study, powders obtained with conventional Minor process had a higher water activity than powders obtained with the PolarDry® tower.

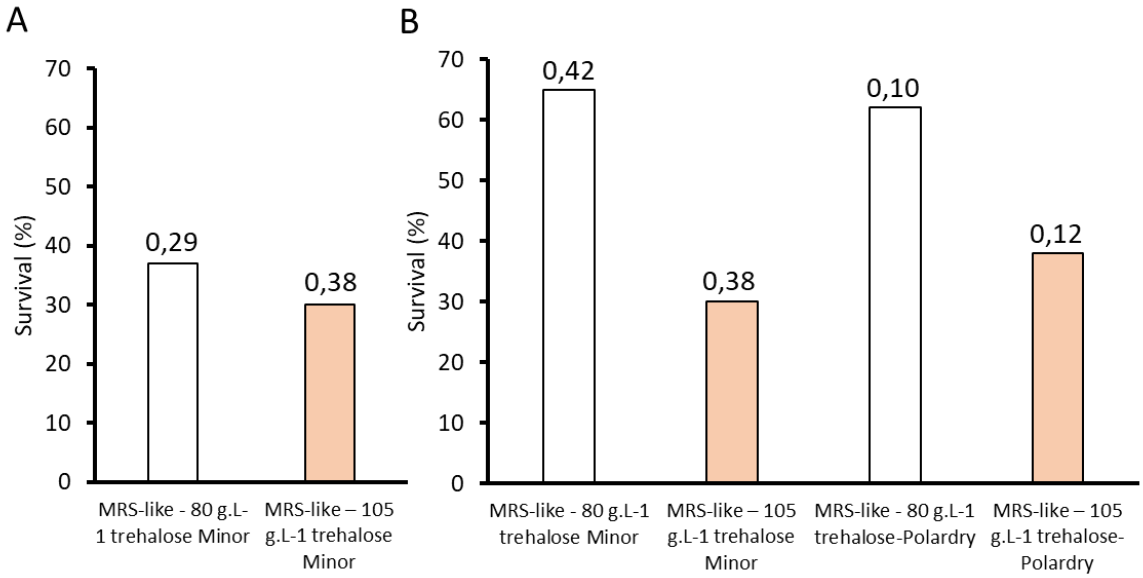


Figure 31: *L. rhamnosus* viability during different drying processes. *L. rhamnosus* CIRM-BIA 1113 was cultivated in MRS-like medium with 80 or 105 g.L^{-1} of trehalose and dried by spray draying with a Minor tour with an outlet temperature of 60°C (A) or 50°C (B) or by a PolarDry® tour with an outlet temperature of 50°C. Results are expressed as percent survival. Water activity (a_w) of each powders are reported on the top of each bar.

6.8.3.1 *L. rhamnosus* viability after controlling the a_w of powders

Powders obtained by the different drying processes (freeze-drying and spray drying) were finally stabilized at an a_w of 0.025 +/- 0.001 thanks to vacuum drying. The water activity reached 0.020 for the powders obtained previously by spray drying and 0.031 for powders previously obtained by freeze-drying (Fig. 32). *L. rhamnosus* grown with 105 g.L^{-1} of trehalose and dried by freeze-drying, survived better to post-drying final dehydration step than the one

grown with 80 g.L^{-1} of trehalose (30 to 66%, respectively). On the contrary, *L. rhamnosus* grown with 80 g.L^{-1} of trehalose and dried by PolarDry® process, survived better to post-drying final dehydration step than the one grown with 105 g.L^{-1} of trehalose (83 and 53%, respectively). The survival of *L. rhamnosus* upon the rehydration of powders produced by the Minor process was not affected by growth conditions.

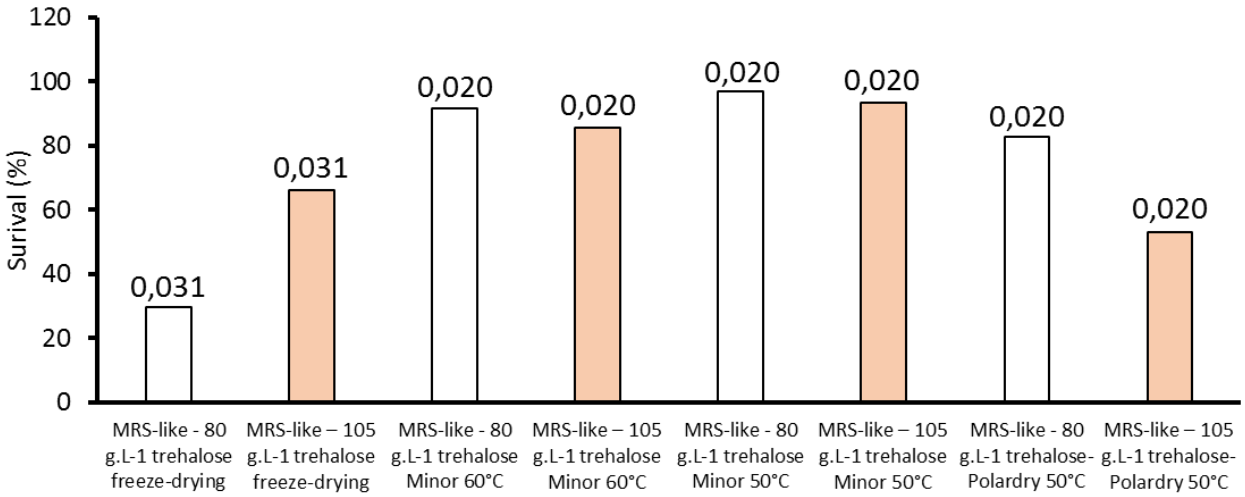


Figure 32: *L. rhamnosus* viability during powder to post-drying final dehydration step using freeze-drying. Powder containing *L. rhamnosus* obtained by freeze-drying, spray drying with a Minor tour with an outlet temperature of 60°C or 50°C or by a PolarDry® tour with an outlet temperature of 50°C were dehydrated using vacuum drying. Results are expressed as percent survival. Water activity (Aw) obtained after dehydration are reported on the top of each bar.

6.8.3.2 *L. rhamnosus* viability during storage

L. rhamnosus grown with 80 g.L^{-1} of trehalose and dried by freeze-drying showed high survival during storage at 25°C during 1 month (Fig. 33). *L. rhamnosus* grown with a higher trehalose concentration and dried freeze-drying had a lower viability during storage (51 compared to 96%, Fig. 33). For the different powders obtained by spray drying (Minor or PolarDry® process), higher viabilities of *L. rhamnosus* were obtained with a growth with 105 g.L^{-1} of trehalose compared with 80 g.L^{-1} of trehalose.

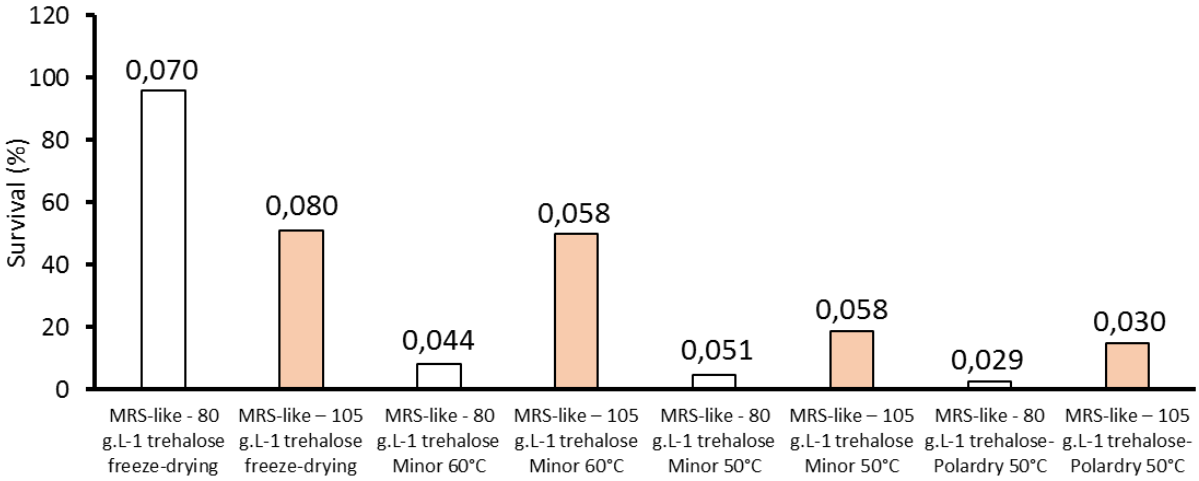


Figure 33: *L. rhamnosus* viability during storage. Dehydrated powders were stored at 25°C during 1 month. Results are expressed as percent survival. Water activity (Aw) obtained after 1 month of storage, are reported on the top of each bar.

6.9 Discussion

6.9.1 High trehalose concentration, heat-adaptation and osmoadaptation did not increase *L. rhamnosus* viability during freeze-drying.

Increasing the trehalose concentration, heat-adaptation or osmoadaptation did not show significant influence on *L. rhamnosus* viability. Addition of trehalose has already been reported to influence on *Lactobacillus salivarius* viability during freeze-drying (Zayed and Roos, 2004). Here, the trehalose concentration in the control condition was already high (80 $g.L^{-1}$), which may explain why increasing this concentration did not influence viability.

On the contrary, osmoadaptation has been previously shown to increase *L. bulgaricus* viability during freeze-drying (Carvalho et al., 2003). However, in this study, the osmoadaptation was made during growth and not after the beginning of the stationary like in our study. Heat-adaptation has been reported to increase *L. bulgaricus, Lactobacillus plantarum* and *Lactobacillus casei* viability to freeze-drying and also to have no impact on *Lactobacillus acidophilus* and *Lactobacillus coryniformis* viability during freeze-drying (Schoug et al., 2008; Li et al., 2009; Paéz et al., 2012). We showed here that combining these adaptations did not impact significantly *L. rhamnosus* viability.

6.9.2 High trehalose concentrations protect *L. rhamnosus* from heat stress

Increasing trehalose concentration did not have significant positive impact on *L. rhamnosus* viability during oxidative challenge but increased *L. rhamnosus* viability during heat challenge. Heat-adaptation did not increase *L. rhamnosus* to both challenges, and osmoadaptation decreased *L. rhamnosus* viability to oxidative challenge. In the first part of this chapter, we showed that heat-adaptation, glucose addition and their combination increased *P. freudenreichii* viability to both challenges. Such cross-protections were not present for *L. rhamnosus*.

The conditions allowing best *L. rhamnosus* survivals during heat challenge were without heat or osmoadaptation whatever the trehalose concentration. The best survival during oxidative challenge was obtained with *L. rhamnosus* grown with high trehalose concentration (100 to 120 $g.L^{-1}$), even if increasing trehalose concentration did not increase significantly *L. rhamnosus* survival during oxidative challenge (Fig. 30).

6.9.3 *High trehalose concentration decreases L. rhamnosus viability during spray drying*

In the control condition, 37 % of *L. rhamnosus* population survived to the conventional spray drying Minor process with an outlet air temperature of 60°C, and 65 % survival with an outlet air temperature of 50°C (Fig. 31). Decreasing temperature permitted to increase *L. rhamnosus* viability. This bacterium can thus be spray dried with higher viability than expected given its fragile characteristics. However increasing trehalose concentration decreased *L. rhamnosus* viability during spray drying. The experimental design realized in this study did not predicted this result.

The use of the PolarDry® process did not increase *L. rhamnosus* viability during drying, despite the use of nitrogen instead of air. One may conclude that the oxidative challenge imposed by the conventional air spray drying (Minor) did not have an important impact on *L. rhamnosus* viability. The *L. rhamnosus* survival during heat stress was decisive, but an adapted heat challenge need to be used to predict *L. rhamnosus* survival during spray drying.

Powders obtain after freeze-drying and spray drying processes were then vacuum dried to decrease the water activity. During the post-drying final dehydration step, a decrease of *L. rhamnosus* viability was observed, especially with the powders originating from freeze-drying (Fig. 32).

6.9.4 High trehalose concentrations impact *L. rhamnosus* viability during storage

When *L. rhamnosus* cultures with increasing trehalose concentration were freeze-dried, *L. rhamnosus* viability decreased among storage (Fig. 33). On the contrary, *L. rhamnosus* cultures that were spray dried survived better during storage when initially grown with a high trehalose concentration. Growth conditions can influence differently bacteria survival during the storage depending on the drying process. Similarly, addition of mannose has also been reported to increase *L. sakei* viability during storage after spray drying, while having no positive impact for the same strain during storage after freeze-drying (Ferreira et al., 2005).

6.10 Conclusion

To conclude with, we modulated the growth medium composition in order to increase *L. rhamnosus* viability during spray drying, freeze-drying and storage. These preliminary results showed that when grown with high trehalose concentration conditions, *L. rhamnosus* had lower survival during spray drying, then survived better during storage. Such results need to be repeated and deepen to understand the adaptation mechanisms of *L. rhamnosus* under different adaptations and especially when it has previously been grown with high trehalose concentration to improve its survival during drying and storage.

Important points of the part 2 of the chapter 6:

- Increasing trehalose concentration increased *L. rhamnosus* viability during heat challenge but not during spray drying.
- Drying *L. rhamnosus* by spray drying was shown to be promising.
- The PolarDry® process did not increase *L. rhamnosus* survival during storage.
- Increasing trehalose concentration increased *L. rhamnosus* viability when powders were produced by spray drying, but decreased *L. rhamnosus* survival when powders were produced by freeze-drying.

General conclusion

The market demand for dried probiotics and starters is growing continually. In order to obtain, freeze-drying is the benchmark as it is considered to provide the milder drying conditions, making it possible to maximise the viability after drying and minimize viability loss during storage. However, freeze-drying still imposes cold and osmotic stresses, while remaining discontinuous and low productive. In contrast, spray drying represents a promising solution for large-scale production of bacteria, although being stressful via heat, oxidative and osmotic stresses. To increase bacteria survival, there are two options:

- the first one consists in decreasing the stresses imposed to bacteria during drying,
- the second is to exploit bacteria adaptation to improve beneficial bacteria resistance among drying and storage.

The innovative spray drying PolarDry® process exclude oxidative stress as it uses nitrogen as carrier gas and therefore permits to dry with lower temperatures than conventional air drying given the null partial vapour pressure of nitrogen. Bacteria can also be protected during drying thanks to encapsulation. Improving viability of beneficial bacteria during drying thus requires more investments in research and development, in order to identify the lever acting on bacteria resistance and to optimize drying processes. In this project we attempted to improve the survival of *Propionibacterium freudenreichii* CIRM-BIA 129 during drying and digestive stresses. We then aimed at generalizing this strategy with regard to *Lactobacillus rhamnosus* viability towards different drying processes. In addition, Figures 34 and 35 highlight the progress gained in knowledge on the role of some adaptive mechanisms that increase *P. freudenreichii* viability toward freeze- and spray drying, respectively. The results obtained provide elements answer to the following questions.

What is the effect of growth conditions on *P. freudenreichii* CIRM-BIA 129 viability during drying processes and digestives stresses?

In the PhD project, we searched cross-protections provided to *P. freudenreichii* by different bacterial adaptations. Bacterial adaptation can be induced by the growth conditions, and more precisely, by modulating the growth medium composition (addition of salts or saccharides) and/or by stirring cultures. Table 18 summarizes the experiments of this PhD research project, showing the impact of the different adaptation ways on key parameters such as final population, growth rate and stress tolerance.

By using a chemically defined medium, we have now a better understanding of *P. freudenreichii* osmoadaptation consequences. For *P. freudenreichii* CIRM-BIA 129, the osmoadaptation in this growth medium was fine-tuned by the presence of compatible solutes. Proteomic profiles of osmoadapted *P. freudenreichii* were different with or without glycine betaine in the growth medium. Some generals stress proteins such as GroES and thiol peroxidase were overexpressed in the presence of salt and without glycine betaine, whereas their expression was back to the baseline when glycine betaine was present. This phenomenon can explain why glycine betaine reduced salt-induced stress tolerance of *P. freudenreichii* towards stress challenges and freeze-drying (Table 18). Conversely, addition of glycine betaine had no impact on *P. freudenreichii* CIRM-BIA 1025. To fine-tune its adaptation, it can be interesting to use another compatible solute.

In Chapter 4, we saw that the osmoadaptation of *P. freudenreichii* CIRM-BIA 129 could also be fine-tuned by the addition of saccharides. As an example, lactose addition enhanced trehalose accumulation, would it be during osmoadaptation or not. Moreover, the growth medium composition, particularly the carbon over NPN (non-protein-nitrogen) ratio, determines the trehalose/glycine betaine ratio accumulated (Chapter 4, Table 18) and the resistance of *P. freudenreichii* CIRM-BIA 129 towards various drying processes. The accumulation of glycine betaine and of trehalose increased *P. freudenreichii* CIRM-BIA 129 viability during spray drying (Fig. 35); However, a high *P. freudenreichii* viability during freeze-drying, requires high accumulation of glycine betaine (Fig. 34). Osmoadaptation in YEL medium leads to high accumulation of glycine betaine thanks to specific transmembrane transporter, to increased SFA/UFA ratio and to over-expression of general stress proteins: these adaptive mechanisms may play a role in the increased of *P. freudenreichii* viability during freeze-drying (Fig. 34, Table 18, Chapter 4 and 5). Unfortunately, *P. freudenreichii* CIRM-BIA 129 osmoadaptation had negative impact on the growth rate, on the biomass yield, and on viability during digestive stresses (Table 18). On the contrary, the addition of lactose had positive effects on all these factors.

The addition of lactose is promising but yet almost banned by industrial because of consumers' demand for lactose-free products. We thus replaced lactose by glucose in the last chapter of the thesis, to respond to industrial needs. Glucose addition led to enhanced biomass, growth rate and viability during digestive stresses (Table 18), as did lactose addition. We also stirred the different *P. freudenreichii* cultures in order to improve *P. freudenreichii* growth rate. This

actually increased the growth rate but without significant effect on the final biomass yield and on *P. freudenreichii* survival during spray drying.

Table 18: Effect of bacteria adaptation on *P. freudenreichii* CIRM-BIA 129 growth parameters and on its viability during technological and digestive stresses

Controls are colored in grey. Positive impacts are colored in green and negatives in red. Colors and color shade represent significant effect ($p < 0.05$). NA: Non Applicable

	Growth parameters							Drying						Bacteria loss during 1 month of storage after spray drying (log)			Digestive stress					
	Adaptation during growth	biomass			µ max (h^{-1})			Adaptation at the beginiing of the stationary phase	survival during freeze-drying (%)			survival during spray drying (%)						survival during acid challenge (%)			survival during bile salt challenge (%)		
Chapter 3	MMO (control)	1,58E+09	±	6,5E+07	0,093	±	0,0032		3,30	±	0,14	NA			NA			4,05	±	0,74	0,48	±	0,04
	osmoadaptation	1,46E+08	±	6,2E+06	0,039	±	0,0012		9,30	±	0,76	NA			NA			7,53	±	0,22	0,00	±	0
	osmoadapatation+ addition of glycine betaine	1,45E+09	±	5,1E+07	0,050	±	0,0012		6,75	±	0,47	NA			NA			1,06	±	0,36	0,00	±	0
Chapter 4 and 5	YEL (control)	1,25E+09	±	7,0E+08	0,091	±	0,0024		43,02	±	5,20	28,04	±	4,25	1,095	±	0,2008	36,39	±	1,21	38,33	±	2,85
	osmoadapation	2,93E+08	±	3,2E+07	0,040	±	0,0018		74,38	±	5,33	13,37	±	3,74	0,76	±	0,2876	1,78	±	0,89	0,00	±	0,00
	addition of lactose	3,93E+09	±	5,4E+08	0,123	±	0,0032		32,75	±	1,58	72,86	±	14,64	0,93	±	0,0088	88,39	±	3,81	57,34	±	3,77
	osmoadapatation + addition of lactose	1,15E+09	±	1,5E+08	0,042	±	0,0031		53,70	±	2,77	102,76	±	3,90	1,39	±	0,0622	0,00	±	0,00	0,02	±	0,03
	YEL	1,25E+09	±	7,0E+08	0,091	±	0,0024	heat-adaptation	46,15	±	2,86	NA			NA			10,00	±	0,00	35,61	±	8,37
	YEL	1,25E+09	±	7,0E+08	0,091	±	0,0024	acid-adaptation	36,28	±	5,92	NA			NA			0,79	±	0,12	0,00	±	0,00
	osmoadapation	2,93E+08	±	3,2E+07	0,040	±	0,0018	+ heat-adaptation	90,50	±	6,65	NA			NA			1,49	±	0,79	0,48	±	0,07
	osmoadapation	2,93E+08	±	3,2E+07	0,040	±	0,0018	+ acid-adaptation	96,70	±	8,36	NA			NA			0,00	±	0,00	0,00	±	0,00
Chapter 6	YEL with agitation (control)	2,26E+09	±	4,5E+08	0,115		0,0027		NA			30,22	±	2,56	2,318	±	0,0913	0,13	±	0,13	0,28	±	0,25
	YEL with agitation	2,26E+09	±	4,5E+08	0,115	±	0,0027	heat-adapatation	NA			NA			NA			0,05	±	0,04	0,05	±	0,05
	YEL with agitation + addition of glucose	9,88E+09	±	2,1E+09	0,121	±	0,0024		NA			NA			NA			46,69	±	0,57	9,95	±	1,96
	YEL with agitation + addition of glucose	9,88E+09	±	2,1E+09	0,121	±	0,0024	+ heat-adaptation	NA			96,37	±	8,13	0,814	±	0,0503	50,28	±	4,20	13,35	±	1,53

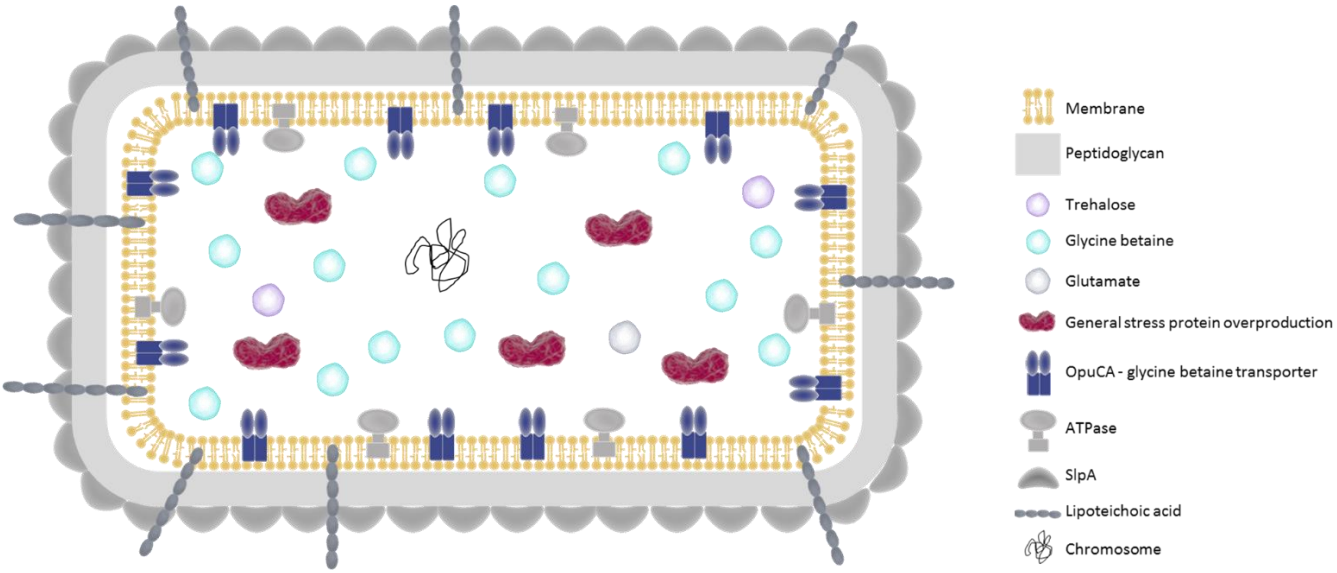


Figure 34: Adaptive mechanisms, enhancing *P. freudenreichii* CIRM-BIA 129 resistance to freeze-drying. Adaptation mechanisms, leading to enhanced freeze-drying tolerance, that were studied during this PhD thesis are indicated in color. The membrane is represented in yellow. Its composition can be modulated to optimize the membrane fluidity. Osmoprotectant uptake systems are represented in blue. Colored circles represent different compatible solutes and energy storage compounds. In the cytoplasm, general stress proteins are represented in red. Other elements that may have an influence, but not studied here are in black/grey: peptidoglycan, S-layer proteins, lipoteichoic acids and ATPase are represented in gray. The chromosome is represented in black.

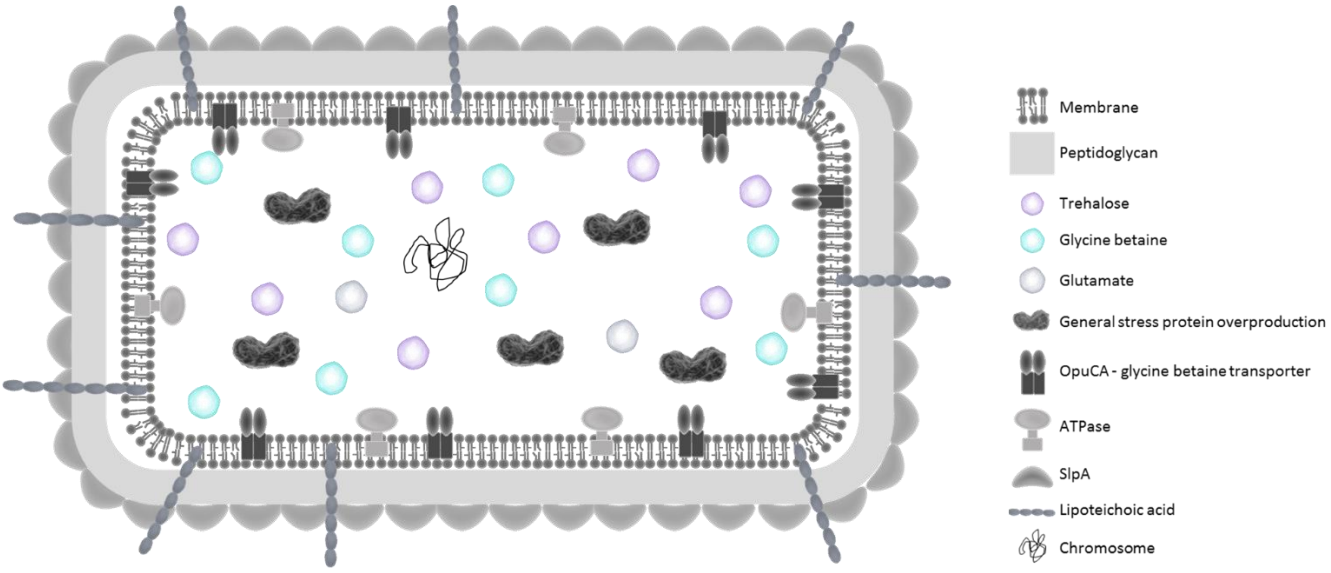


Figure 35: Adaptive mechanisms, enhancing *P. freudenreichii* CIRM-BIA 129 resistance to spray drying. Adaptation mechanisms, leading to enhanced spray drying tolerance, that were studied during this PhD thesis are indicated in color. Colored circles represent different compatibles solutes and energy storage compounds. Other elements that may have an influence, but not studied here are in black/grey: peptidoglycan, S-layer proteins, the membrane, osmoprotectant uptake systems, general stress proteins lipoteichoic acids and ATPase are represented in gray. The chromosome is represented in black.

What is the effect of post-growth adaptations on *P. freudenreichii* CIRM-BIA 129 viability during drying processes and digestives stresses?

Adaptation induced by the modulation of growth conditions, can be coupled with adaptations made at the beginning of stationary phase to increase bacteria resistance. We thus intended to optimize *P. freudenreichii* CIRM-BIA 129 resistance by coupling osmoadaptation with acid-adaptation or to heat-adaptation after growth. The SFA/UFA ratio, which was enhanced by osmoadaptation, was then partially reduced by heat and acid-adaptation. The acid-adaptation performed after the osmoadaptation further increased the glycine betaine accumulation. Enhanced glycine betaine accumulation and moderate increase of the SFA/UFA ratio in membrane fatty acids could be responsible for the increase of *P. freudenreichii* CIRM-BIA 129 survival to freeze-drying (Fonseca et al., 2019).

We thus intended to optimize *P. freudenreichii* resistance to spray drying by coupling the glucose addition with heat-adaptation, and/or with osmoadaptation. The experimental design of the last chapter allowed to optimize *P. freudenreichii* resistance towards heat and oxidative stresses. Glucose addition, coupled to heat-adaptation, increased *P. freudenreichii* CIRM-BIA 129 tolerance to these stresses. Furthers experiments confirmed that *P. freudenreichii*, grown with glucose and heat-adapted, had higher viability during spray drying and accumulated high amounts of trehalose and glycine betaine. Adaptive mechanism can thus be induced through different adaptations, which also lead to increased *P. freudenreichii* tolerance to digestive challenges.

Can we improve *L. rhamnosus* viability during drying by generalizing the previous results?

We intended to optimize *L. rhamnosus* CIRM-BIA 1113 resistance to heat and oxidative stresses thanks to an experimental design around Bioprox growth conditions, was adapted from the one realized for *P. freudenreichii*. As Trehalose is routinely used by Bioprox for *L. rhamnosus* growth, we thus made the experimental design with this saccharide. None of the adaptations showed a significant impact on *L. rhamnosus* viability during freeze-drying. This can be explained by the fact that Bioprox growth conditions are already optimized to increase *L. rhamnosus* viability during freeze-drying.

Trehalose addition was coupled to heat- and/or to osmoadaptation realized at the beginning of the stationary phase. The experimental design showed that none of the three adaptations (heat and osmoadaptation and the addition of high trehalose concentration) had a significant effect on *L. rhamnosus* viability during oxidative challenge. High trehalose concentration can increase *L. rhamnosus* viability during spray drying, as it positively influences viability during heat challenges. However, *L. rhamnosus* grown with high concentration of trehalose was not further protected during spray drying, but was protected during storage. For all the conditions, *L. rhamnosus* viability was higher than expected. This is promising for the utilization of spray drying as an industrial scale drying process.

Can the use of the PolarDry® increase *L. rhamnosus* viability during spray drying?

The PolarDry® process allowed to skip the oxidative challenge during spray drying, as it operates in the absence of oxygen. The resulting powders had a water activity lower than the ones obtained with the classical Minor spray drying process. However, *L. rhamnosus* did not showed higher viability during PolarDry® drying. This process being more expensive and complex than classical spray drying process, it does not represent at this stage a promising alternative for an industrial production.

Perspectives

To achieve the aim of producing alive beneficial bacteria at a large scale, more studies should be conducted to provide insights into the correlations between bacterial physiology, bacteria functionality, and processing.

Optimizing bacterial resistance to drying and storage

To optimize bacteria survival during drying, more knowledge on adaptive mechanisms is needed. For example, the modulation of the membrane fluidity, driven by bacterial adaptation or by protective molecules added in the drying matrix, is promising and needs to be well studied and linked to bacterial resistance during the different drying processes. Different factors have an effect on the membrane fluidity, such as the membrane fatty acids composition, the membrane proteins and the temperature (Fonseca et al., 2019). In our study, we only quantified membrane fatty acids. Further experiments are necessary to correlate treatments, variation in the membrane fluidity, fatty acids composition and stress tolerance. In addition, rigid domains within the membrane can weaken the bacteria during freezing (Meneghel et al., 2017). The global composition of the membrane does not indicate if such rigid domains are present. Furthermore, membrane composition can vary during drying and storage, especially because of lipid oxidation (Coulibaly et al., 2009). In addition, the presence of trehalose in the drying matrix can increase membrane fluidity of bacteria under a dried form (Fonseca et al., 2019).

The cell wall thickness can also be modulated by bacterial adaptation (Palomino et al., 2016; Huang et al., 2018), and may affect bacteria resistance to drying. The role of general stress proteins and of compatible solutes in the increase of bacteria viability during drying, need to be clarified in order to optimize the cross-protections provided by adaptations.

Not all the strains showed the same capacity in the manner of responding to adaptation, as shown by discrepancies in the compatible solutes accumulated by *P. freudenreichii* CIRM-BIA 129 and 1025. An “optimum” bacterium, which can have high survival during spray drying or freeze-drying, can be found for each drying process and each species. Bacteria growth conditions and adaptation have to be adapted to each beneficial bacterium. Furthers studies will allow to identify (strain dependent) mechanisms involved in stress adaptation and tolerance. The genes and proteins, involved, will further allow genetic and proteins tools to optimize the production of beneficial bacteria by predicting the adaptive mechanisms of the strains.

Decreasing the stresses imposed by drying and storage

Drying processes can be optimized in order to decrease the stresses imposed, and particularly the spray drying process. The development of the innovative PolarDry® process shows that there is current research and development on this subject. The normal spray drying is promising and drying with low temperature increased bacteria survival but produce powders with high water activity. The increase of *P. freudenreichii* viability after adaptations needs to be tested at the industrial scale, that makes it possible to implement a secondary/final drying step with a fluidized bed and overall to minimize the drying temperatures.

The composition of the drying matrix is key to protect bacteria during drying and storage. In our study, the drying matrix was solely composed of maltodextrin, and did not well protect during drying and storage. Saccharides addition in the drying matrix may increase the membrane fluidity and decrease bacteria loss during storage. Bacteria encapsulation should protect bacteria during spray drying it can be obtained with the addition of protein in the drying matrix, for example. Encapsulation may prevent membrane fatty acid oxidation during storage.

Producing resistant bacteria with industrial constraints

Bacteria growth conditions and adaptations have to be selected as a function of industrial constraints that include market, cost, environmental and technology considerations. During bacteria production, it is difficult to avoid time out between the end of fermentation, the harvesting, the mixing to the drying matrix and the drying process. Waiting time out can have negative effects on bacteria resistance, by affecting adaptive mechanisms. Decreasing this time out seems important to obtain high bacteria viability during drying.

Drying processes need to be chosen depending on the beneficial bacteria application. In fact, spray drying, which is more economical, can be used for products with limited profit margin, such as probiotics for animals and starters. Products with a high profit margin such as human probiotics can still be produced with freeze-drying, a more expensive process.

Growth medium can be made with different nitrogen and carbon sources. In the case of the production of *L. rhamnosus* by Bioprox, trehalose may be replaced in the growth medium with another less expensive saccharide, providing high biomass production and leading to trehalose accumulation. However, changing sugar is complex because only few carbohydrates are

fermentable and non-reducing. Trehalose can then been added in lower amount in the drying matrix. Trehalose will be present on both sides of the membrane and optimize the protection.

Sugars and protein sources which are currently used in probiotics' growth media are subjected to consumer criticism. Proteins from animal sources can be a problem for the halal, casher, vegetarian and vegan certifications. In addition, environmental issues also appeal for growth media with a vegetal alternative. Proteins from vegetable sources, like soy proteins, can however constitute a problem due to the allergies, and the non-local production. Other vegetables like peas, lupine and faba are yet available in France and should be tested.

Verifying the probiotics and starter capacity of beneficial bacteria

Starters' capacity, like the acidification activity, need to be tested after adaptation, drying and storage. It has been shown that after an addition of high salts concentration during fermentation, the acidification acitivity decreased during storage (Louesdon et al., 2014). Molecular mechanisms responsible for probiotics effects are complex. In the case of the immunomodulatory effects, different action mechanisms are involved. Many metabolites of probiotics can decrease inflammation, such as short chain fatty acids (Machiels et al., 2014; Sun et al., 2017), conjugated fatty acids (Evans et al., 2010; Bassaganya-Riera et al., 2012a, 2012b), vitamins (Thomas and Trust, 1995; Levit et al., 2017, 2018) or vitamin precursors (Okada, 2006). Cell wall is key in the interaction between the host and probiotics. It is composed of different components, including proteins (Sengupta et al., 2013; Ganguli et al., 2015; Lebeer et al., 2018), exopolysaccharides (Laiño et al., 2016; Balzaretti et al., 2017; Verma et al., 2018), lipoteichoic acids and teichoic acids (Kim et al., 2008; Lebeer et al., 2012) which can have immunomodulatory properties. During adaptation, modulation of the composition of the cell wall can affect the presence of key components with immunomodulatory effects. Verifying the presence of key components and the adhesion capacity on host cells after adaptations and after drying, seems essential to guarantee probiotic effects. In the case of probiotics bacteria, which should be alive to exert probiotics effects, the viability of bacteria during digestion should be tested thanks to static or dynamic digestion models.

Producing functional foods

Probiotics bacteria can be produced and then mixed in a food-grade drying matrix. Powders with bacteria encapsulated within a food matrix can be added directly to the food product. This may increase bacteria viability during the food product storage. Food products with probiotics properties can be called functional foods, but their production is still limited, due to the interdiction to use allegation. Functional products for animals can also be produced to feed animals in agriculture and domestic animal. Animal's experiments and clinical studies will further help both scientists and industrials to prove beneficial effects on the host.

References:

Aburjaile, F. F., Rohmer, M., Parrinello, H., Maillard, M.-B., Beaucher, E., Henry, G., et al. (2016). Adaptation of *Propionibacterium freudenreichii* to long-term survival under gradual nutritional shortage. *BMC Genomics* 17, 1007. doi:10.1186/s12864-016-3367-x.

Akashi, K., Shibai, H., and Hirose, Y. (1979). Effect of oxygen supply on L -lysine, L -threonine and L -isoleucine fermentations. *Agricultural and Biological Chemistry* 43, 2087–2092. doi:10.1080/00021369.1979.10863778.

Alcántara, C., Blasco, A., Zúñiga, M., and Monedero, V. (2014). Accumulation of polyphosphate in *Lactobacillus* spp. and its involvement in stress resistance. *Appl. Environ. Microbiol.* 80, 1650–1659. doi:10.1128/AEM.03997-13.

Alcantara, C., and Zuniga, M. (2012). Proteomic and transcriptomic analysis of the response to bile stress of *Lactobacillus casei* BL23. *Microbiology* 158, 1206–1218. doi:10.1099/mic.0.055657-0.

Alp, G., and Aslim, B. (2010). Relationship between the resistance to bile salts and low pH with exopolysaccharide (EPS) production of *Bifidobacterium* spp. isolated from infants feces and breast milk. *Anaerobe* 16, 101–105. doi:10.1016/j.anaerobe.2009.06.006.

Anastasiou, R., Leverrier, P., Krestas, I., Rouault, A., Kalantzopoulos, G., Boyaval, P., et al. (2006). Changes in protein synthesis during thermal adaptation of *Propionibacterium freudenreichii* subsp. *shermanii*. *International Journal of Food Microbiology*, S0168160506000225. doi:10.1016/j.ijfoodmicro.2005.11.015.

Annous, B. A., Becker, L. A., Bayles, D. O., Labeda, D. P., and Wilkinson, B. J. (1997). Critical role of anteiso-C15:0 fatty acid in the growth of *Listeria monocytogenes* at low temperatures. *Applied and Environemental Microbiology*, 3887–3894.

Arnaud, J.-P., Lacroix, C., and Choplin, L. (1992). Effect of agitation rate on cell release rate and metabolism during continuous fermentation with entrapped growing: *Lactobacillus casei* subsp. *casei*. *Biotechnol Tech* 6, 265–270. doi:10.1007/BF02439356.

Arnold, J. W., Simpson, J. B., Roach, J., Kwintkiewicz, J., and Azcarate-Peril, M. A. (2018). Intra-species genomic and physiological variability impact stress resistance in strains of probiotic potential. *Front. Microbiol.* 9, 242. doi:10.3389/fmicb.2018.00242.

Avalljaaskelainen, S., and Palva, A. (2005). Surface layers and their applications. *FEMS Microbiology Reviews* 29, 511–529. doi:10.1016/j.femsre.2005.04.003.

Bae, D., Liu, C., Zhang, T., Jones, M., Peterson, S. N., and Wang, C. (2012). Global gene expression of *Listeria monocytogenes* to salt stress. *Journal of Food Protection* 75, 906–912. doi:10.4315/0362-028X.JFP-11-282.

Balzaretti, S., Taverniti, V., Guglielmetti, S., Fiore, W., Minuzzo, M., Ngo, H. N., et al. (2017). A Novel Rhamnose-Rich Hetero-exopolysaccharide Isolated from *Lactobacillus paracasei* DG Activates THP-1 Human Monocytic Cells. *Appl. Environ. Microbiol.* 83, e02702-16, /aem/83/3/e02702-16.atom. doi:10.1128/AEM.02702-16.

Bampi, G. B., Backes, G. T., Cansian, R. L., de Matos, F. E., Ansolin, I. M. A., Poleto, B. C., et al. (2016). Spray chilling microencapsulation of *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* and its use in the preparation of savory probiotic cereal bars. *Food Bioprocess Technol* 9, 1422–1428. doi:10.1007/s11947-016-1724-z.

Baron, F., Cochet, M.-F., Ablain, W., Grosset, N., Madec, M.-N., Gonnet, F., et al. (2006). Rapid and cost-effective method for micro-organism enumeration based on miniaturization of the conventional plate-counting technique. *Lait* 86, 251–257. doi:10.1051/lait:2006005.

Bassaganya-Riera, J., Viladomiu, M., Pedragosa, M., De Simone, C., Carbo, A., Shaykhutdinov, R., et al. (2012a). Probiotic Bacteria Produce Conjugated Linoleic Acid Locally in the Gut That Targets Macrophage PPAR γ to Suppress Colitis. *PLoS ONE* 7, e31238. doi:10.1371/journal.pone.0031238.

Bassaganya-Riera, J., Viladomiu, M., Pedragosa, M., De Simone, C., and Hontecillas, R. (2012b). Immunoregulatory Mechanisms Underlying Prevention of Colitis-Associated Colorectal Cancer by Probiotic Bacteria. *PLoS ONE* 7, e34676. doi:10.1371/journal.pone.0034676.

Bergenholtz, Å. S., Wessman, P., Wuttke, A., and Håkansson, S. (2012). A case study on stress preconditioning of a *Lactobacillus* strain prior to freeze-drying. *Cryobiology* 64, 152–159. doi:10.1016/j.cryobiol.2012.01.002.

Bergonzelli, G. E., Granato, D., Pridmore, R. D., Marvin-Guy, L. F., Donnicola, D., and Corthesy-Theulaz, I. E. (2006). GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. *Infection and Immunity* 74, 425–434. doi:10.1128/IAI.74.1.425-434.2006.

Blein-Nicolas, M., Albertin, W., da Silva, T., Valot, B., Balliau, T., Masneuf-Pomarède, I., et al. (2015). A systems approach to elucidate heterosis of protein abundances in yeast. *Mol Cell Proteomics* 14, 2056–2071. doi:10.1074/mcp.M115.048058.

Booth, I. R. (2002). Stress and the single cell: Intrapopulation diversity is a mechanism to ensure survival upon exposure to stress. *International Journal of Food Microbiology* 78, 19–30. doi:10.1016/S0168-1605(02)00239-8.

Bove, P., Russo, P., Capozzi, V., Gallone, A., Spano, G., and Fiocco, D. (2013). *Lactobacillus plantarum* passage through an oro-gastro-intestinal tract simulator: Carrier matrix effect and transcriptional analysis of genes associated to stress and probiosis. *Microbiological Research* 168, 351–359. doi:10.1016/j.micres.2013.01.004.

Boyaval, P., Deborde, C., Corre, C., Blanco, C., and Bégué, É. (1999). Stress and osmoprotection in propionibacteria. *Lait* 79, 59–69. doi:10.1051/lait:199914.

Braegger, C., Chmielewska, A., Decsi, T., Kolacek, S., Mihatsch, W., Moreno, L., et al. (2011). Supplementation of infant formula with probiotics and/or prebiotics: A systematic review and comment by the ESPGHAN committee on nutrition: *Journal of Pediatric Gastroenterology and Nutrition* 52, 238–250. doi:10.1097/MPG.0b013e3181fb9e80.

Broadbent, J. R., Larsen, R. L., Deibel, V., and Steele, J. L. (2010). Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. *Journal of Bacteriology* 192, 2445–2458. doi:10.1128/JB.01618-09.

Bron, P. A., Molenaar, D., Vos, W. M., and Kleerebezem, M. (2006). DNA micro-array-based identification of bile-responsive genes in *Lactobacillus plantarum*. *J Appl Microbiol* 100, 728–738. doi:10.1111/j.1365-2672.2006.02891.x.

Brown, D. R., Barton, G., Pan, Z., Buck, M., and Wigneshweraraj, S. (2014). Nitrogen stress response and stringent response are coupled in *Escherichia coli*. *Nat Commun* 5, 4115. doi:10.1038/ncomms5115.

Brusaferro, A., Cozzali, R., Orabona, C., Biscarini, A., Farinelli, E., Cavalli, E., et al. (2018). Is It Time to use probiotics to prevent or treat obesity? *Nutrients* 10, 1613. doi:10.3390/nu10111613.

Caggianiello, G., Kleerebezem, M., and Spano, G. (2016). Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms. *Appl Microbiol Biotechnol* 100, 3877–3886. doi:10.1007/s00253-016-7471-2.

Cañamás, T. P., Viñas, I., Usall, J., Magan, N., Solsona, C., and Teixidó, N. (2008). Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida* sake CPA-1 and viability after spray-drying. *J Appl Microbiol* 104, 767–775. doi:10.1111/j.1365-2672.2007.03590.x.

Candela, M., Centanni, M., Fiori, J., Biagi, E., Turroni, S., Orrico, C., et al. (2010). DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts. *Microbiology* 156, 1609–1618. doi:10.1099/mic.0.038307-0.

Cantero, C., Schrenzel, J., and Darbellay Farhoumand, P. (2018). Current status of probiotics usage for the prevention of a first episode of *Clostridium difficile* infection. *Rev Med Suisse* 14, 1834–1837.

Capozzi, V., Weidmann, S., Fiocco, D., Rieu, A., Hols, P., Guzzo, J., et al. (2011). Inactivation of a small heat shock protein affects cell morphology and membrane fluidity in *Lactobacillus plantarum* WCFS1. *Research in Microbiology* 162, 419–425. doi:10.1016/j.resmic.2011.02.010.

Cardoso, F. S., Castro, R. F., Borges, N., and Santos, H. (2007). Biochemical and genetic characterization of the pathways for trehalose metabolism in *Propionibacterium freudenreichii*, and their role in stress response. *Microbiology* 153, 270–280. doi:10.1099/mic.0.29262-0.

Cardoso, F. S., Gaspar, P., Hugenholtz, J., Ramos, A., and Santos, H. (2004). Enhancement of trehalose production in dairy propionibacteria through manipulation of environmental conditions. *International Journal of Food Microbiology* 91, 195–204. doi:10.1016/S0168-1605(03)00387-8.

Carvalho, A. L., Cardoso, F. S., Bohn, A., Neves, A. R., and Santos, H. (2011). Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance. *Appl. Environ. Microbiol.* 77, 4189–4199. doi:10.1128/AEM.02922-10.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., and Gibbs, P. (2003). Effects of addition of sucrose andsSalt, and of starvation upon thermotolerance and survival during storage of freeze-dried *Lactobacillus delbrueckii* ssp *bulgaricus*. *J Food Science* 68, 2538–2541. doi:10.1111/j.1365-2621.2003.tb07057.x.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., and Gibbs, P. (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal* 14, 835–847. doi:10.1016/j.idairyj.2004.02.001.

Carvalho, A. S., Silva, J., Ho, P., Teixeira, P., Malcata, F. X., and Gibbs, P. (2008). Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freeze-dried *lactobacillus delbrueckii* ssp. *bulgaricus*. *Biotechnol Progress* 20, 248–254. doi:10.1021/bp034165y.

Chen, C., Chen, K., Su, T., Zhang, B., Li, G., Pan, J., et al. (2019). *Myo* -inositol-1-phosphate synthase (Ino-1) functions as a protection mechanism in *Corynebacterium glutamicum* under oxidative stress. *MicrobiologyOpen* 8, e00721. doi:10.1002/mbo3.721.

Chen, M., Sun, Q., Giovannucci, E., Mozaffarian, D., Manson, J. E., Willett, W. C., et al. (2014). Dairy consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis. *BMC Med* 12, 215. doi:10.1186/s12916-014-0215-1.

Chen, M.-J., Tang, H.-Y., and Chiang, M.-L. (2017). Effects of heat, cold, acid and bile salt adaptations on the stress tolerance and protein expression of kefir-isolated probiotic *Lactobacillus kefiranofaciens* M1. *Food Microbiology* 66, 20–27. doi:10.1016/j.fm.2017.03.020.

Chervaux, C., Ehrlich, S. D., and Maguin, E. (2000). Physiological study of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains in a novel chemically defined medium. *Appl. Environ. Microbiol.* 66, 5306–5311. doi:10.1128/aem.66.12.5306-5311.2000.

Collado, M. C., and Sanz, Y. (2007). Induction of acid resistance in *Bifidobacterium* : a mechanism for improving desirable traits of potentially probiotic strains. *Journal of Applied Microbiology* 103, 1147–1157. doi:10.1111/j.1365-2672.2007.03342.x.

Collins, M. D., and Gibson, G. R. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *The American Journal of Clinical Nutrition* 69, 1052s–1057s. doi:10.1093/ajcn/69.5.1052s.

Colliou, N., Ge, Y., Sahay, B., Gong, M., Zadeh, M., Owen, J. L., et al. (2017). Commensal *Propionibacterium* strain UF1 mitigates intestinal inflammation via Th17 cell regulation. *Journal of Clinical Investigation* 127, 3970–3986. doi:10.1172/JCI95376.

Corcoran, B. M., Ross, R. P., Fitzgerald, G. F., Dockery, P., and Stanton, C. (2006). Enhanced survival of GroESL-overproducing *Lactobacillus paracasei* NFBC 338 under stressful conditions induced by drying. *Applied and Environmental Microbiology* 72, 5104–5107. doi:10.1128/AEM.02626-05.

Corcoran, B. M., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2005). Survival of probiotic Lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology* 71, 3060–3067. doi:10.1128/AEM.71.6.3060-3067.2005.

Corona-Hernandez, R. I., Álvarez-Parrilla, E., Lizardi-Mendoza, J., Islas-Rubio, A. R., de la Rosa, Laura. A., and Wall-Medrano, A. (2013). Structural stability and viability of microencapsulated probiotic bacteria: A review. *Comprehensive Reviews in Food Science and Food Safety* 12, 614–628. doi:10.1111/1541-4337.12030.

Coulibaly, I., Amenan, A. Y., Lognay, G., Fauconnier, M. L., and Thonart, P. (2009). Survival of freeze-dried *Leuconostoc mesenteroides* and *Lactobacillus plantarum* related to their cellular fatty acids composition during storage. *Appl Biochem Biotechnol* 157, 70–84. doi:10.1007/s12010-008-8240-1.

Cousin, F. J., Louesdon, S., Maillard, M.-B., Parayre, S., Falentin, H., Deutsch, S.-M., et al. (2012). The first dairy product exclusively fermented by *Propionibacterium freudenreichii*: A new vector to study probiotic potentialities *in vivo*. *Food Microbiology* 32, 135–146. doi:10.1016/j.fm.2012.05.003.

Cousin, F. J., Mater, D. D. G., Foligne, B., and Jan, G. (2010a). Dairy propionibacteria as human probiotics: A review of recent evidence. *Dairy Sci. Technol.* doi:10.1051/dst/2010032.

Cousin, F. J., Mater, D. D. G., Foligne, B., and Jan, G. (2010b). Dairy propionibacteria as human probiotics: A review of recent evidence. *Dairy Science & Technology*. doi:10.1051/dst/2010032.

Crow, V. L. (1987). Citrate Cycle Intermediates in the Metabolism of Aspartate and Lactate by *Propionibacterium freudenreichii subsp. shermanii*. *Appl. Environ. Microbiol.* 53, 2600–2602.

Csonka, L. N. (1989). Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* 53, 121–147.

Csonka, L. N., and Hanson, A. D. (1991). Prokaryotic Osmoregulation: Genetics and Physiology. *Annu. Rev. Microbiol.* 45, 569–606. doi:10.1146/annurev.mi.45.100191.003033.

Curry, B., and Crow, V. (2002). "LACTOBACILLUS spp. | General Characteristics," in *Encyclopedia of Dairy Sciences* (Elsevier), 1479–1484. doi:10.1016/B0-12-227235-8/00238-8.

Dalmasso, M., Aubert, J., Briard-Bion, V., Chuat, V., Deutsch, S.-M., Even, S., et al. (2012a). A Temporal -omic study of *Propionibacterium freudenreichii* CIRM-BIA1T adaptation strategies in conditions mimicking cheese ripening in the cold. *PLoS ONE* 7, e29083. doi:10.1371/journal.pone.0029083.

Dalmasso, M., Aubert, J., Even, S., Falentin, H., Maillard, M.-B., Parayre, S., et al. (2012b). Accumulation of intracellular glycogen and trehalose by *Propionibacterium freudenreichii* under conditions mimicking cheese ripening in the cold. *Appl. Environ. Microbiol.* 78, 6357–6364. doi:10.1128/AEM.00561-12.

Dasari, S., Kathera, C., Janardhan, A., Praveen Kumar, A., and Viswanath, B. (2017). Surfacing role of probiotics in cancer prophylaxis and therapy: A systematic review. *Clinical Nutrition* 36, 1465–1472. doi:10.1016/j.clnu.2016.11.017.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi:10.1038/nature12820.

De Dea Lindner, J., Canchaya, C., Zhang, Z., Neviani, E., Fitzgerald, G. F., van Sinderen, D., et al. (2007). Exploiting *Bifidobacterium genomes*: The molecular basis of stress response. *International Journal of Food Microbiology* 120, 13–24. doi:10.1016/j.ijfoodmicro.2007.06.016.

De Man, J. C., Rogosa, M., and Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* 23, 130–135. doi:10.1111/j.1365-2672.1960.tb00188.x.

Demirtas, M. U., Kolhatkar, A., and Kilbane, J. J. (2003). Effect of aeration and agitation on growth rate of *Thermus thermophilus* in batch mode. *Journal of Bioscience and Bioengineering* 95, 113–117. doi:10.1016/S1389-1723(03)80114-7.

Desmazeaud, M. J., Gripon, J. C., Le Bars, D., and Bergere, J. L. (1976). Etude du rôle des micro-organismes etdes enzymes au cours de la maturation des fromages. (*Streptococcus lactis*, *Penicillium caseicolum* et *P. roqueforti*). *Le Lait*, 379–396.

Desmond, C., Stanton, C., Fitzgerald, G. F., Collins, K., and Paul Ross, R. (2001). Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. *International Dairy Journal* 11, 801–808. doi:10.1016/S0958-6946(01)00121-2.

Deutsch, S.-M., Mariadassou, M., Nicolas, P., Parayre, S., Le Guellec, R., Chuat, V., et al. (2017). Identification of proteins involved in the anti-inflammatory properties of *Propionibacterium freudenreichii* by means of a multi-strain study. *Sci Rep* 7, 46409. doi:10.1038/srep46409.

Dimitrellou, D., Kandylis, P., Petrović, T., Dimitrijević-Branković, S., Lević, S., Nedović, V., et al. (2016). Survival of spray dried microencapsulated *Lactobacillus casei* ATCC 393 in simulated gastrointestinal conditions and fermented milk. *LWT - Food Science and Technology* 71, 169–174. doi:10.1016/j.lwt.2016.03.007.

Ding, Z.-G., Ji, Y., Yin, M., Zhao, Y.-R., Feng, Y.-Z., Chunyu, W.-X., et al. (2019). *Phytoactinopolyspora halophila* sp. nov., a halophilic actinomycete isolated from a saline soil. *International Journal of Systematic and Evolutionary Microbiology* 69, 384–389. doi:10.1099/ijsem.0.003153.

do Carmo, F. L. R., Rabah, H., De Oliveira Carvalho, R. D., Gaucher, F., Cordeiro, B. F., da Silva, S. H., et al. (2018). Extractable bacterial surface proteins in probiotic–host interaction. *Front. Microbiol.* 9, 645. doi:10.3389/fmicb.2018.00645.

Douillard, F. P., Ribbera, A., Kant, R., Pietilä, T. E., Järvinen, H. M., Messing, M., et al. (2013). Comparative Genomic and Functional Analysis of 100 Lactobacillus rhamnosus Strains and Their Comparison with Strain GG. *PLoS Genet* 9, e1003683. doi:10.1371/journal.pgen.1003683.

DuPont, A., Richards, Jelinek, K. A., Krill, J., Rahimi, E., and Ghouri, Y. (2014). Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. *CEG*, 473. doi:10.2147/CEG.S27530.

Ebner, S., Smug, L. N., Kneifel, W., Salminen, S. J., and Sanders, M. E. (2014). Probiotics in dietary guidelines and clinical recommendations outside the European Union. *WJG* 20, 16095. doi:10.3748/wjg.v20.i43.16095.

Elli, M., Zink, R., Rytz, A., Reniero, R., and Morelli, L. (2000). Iron requirement of *Lactobacillus* spp. in completely chemically defined growth media. *J Appl Microbiol* 88, 695–703. doi:10.1046/j.1365-2672.2000.01013.x.

Empadinhas, N., and Viete-Vallejo, O. (2008). Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. *International Microbiology*, 151–161. doi:10.2436/20.1501.01.55.

Ernst, D. C., Christopherson, M. R., and Downs, D. M. (2018). Increased activity of cystathionine β-Lyase suppresses 2-Aminoacrylate stress in *Salmonella enterica*. *J Bacteriol* 200, e00040-18, /jb/200/9/e00040-18.atom. doi:10.1128/JB.00040-18.

Evans, N. P., Misyak, S. A., Schmelz, E. M., Guri, A. J., Hontecillas, R., and Bassaganya-Riera, J. (2010). Conjugated Linoleic Acid Ameliorates Inflammation-Induced Colorectal Cancer in Mice through Activation of PPARγ. *The Journal of Nutrition* 140, 515–521. doi:10.3945/jn.109.115642.

Falentin, H., Deutsch, S.-M., Jan, G., Loux, V., Thierry, A., Parayre, S., et al. (2010). The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1T, a hardy *Actinobacterium* with food and probiotic applications. *PLoS ONE* 5, e11748. doi:10.1371/journal.pone.0011748.

FAO/WHO (2001). Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization expert consultation report.

Feehily, C., and Karatzas, K. A. G. (2013). Role of glutamate metabolism in bacterial responses towards acid and other stresses. *J Appl Microbiol* 114, 11–24. doi:10.1111/j.1365-2672.2012.05434.x.

Ferreira, V., Soares, V., Santos, C., Silva, J., Gibbs, P. A., and Teixeira, P. (2005). Survival of *Lactobacillus sakei* during heating, drying and storage in the dried state when growth has occurred in the presence of sucrose or monosodium glutamate. *Biotechnol Lett* 27, 249–252. doi:10.1007/s10529-004-8351-x.

FIL/IDF (1993). Détermination de la teneur en azote, standard 20B.

Fitz, Alb. (1878). Ueber Spaltpilzgährungen. *Ber. Dtsch. Chem. Ges.* 11, 1890–1899. doi:10.1002/cber.187801102180.

Flahaut, S., Hartke, A., Giard, J., Benachour, A., Boutibonnes, P., and Auffray, Y. (1996). Relationship between stress response towards bile salts, acid and heat treatment in *Enterococcus faecalis*. *FEMS Microbiology Letters* 138, 49–54. doi:10.1111/j.1574-6968.1996.tb08133.x.

Foligné, B., Breton, J., Mater, D., and Jan, G. (2013). Tracking the microbiome functionality: focus on *Propionibacterium* species. *Gut* 62, 1227–1228. doi:10.1136/gutjnl-2012-304393.

Foligne, B., Deutsch, S.-M., Breton, J., Cousin, F. J., Dewulf, J., Samson, M., et al. (2010). Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced *in vitro* and *in vivo*. *Applied and Environmental Microbiology* 76, 8259–8264. doi:10.1128/AEM.01976-10.

Fonseca, F., Pénicaud, C., Tymczyszyn, E. E., Gómez-Zavaglia, A., and Passot, S. (2019). Factors influencing the membrane fluidity and the impact on production of lactic acid bacteria starters. *Appl Microbiol Biotechnol* 103, 6867–6883. doi:10.1007/s00253-019-10002-1.

Fortina, Nicastro, Carminati, Neviani, and Manachini (1998). *Lactobacillus helveticus* heterogeneity in natural cheese starters: the diversity in phenotypic characteristics. *Journal of Applied Microbiology* 84, 72–80. doi:10.1046/j.1365-2672.1997.00312.x.

Frings, E., Kunte, H. J., and Galinski, E. A. (1993). Compatible solutes in representatives of the genera *Brevibacterium* and *Corynebacterium* : Occurrence of tetrahydropyrimidines and glutamine. *FEMS Microbiology Letters* 109, 25–32. doi:10.1111/j.1574-6968.1993.tb06138.x.

Gagnaire, V., Jardin, J., Rabah, H., Briard-Bion, V., and Jan, G. (2015). Emmental cheese environment enhances *Propionibacterium freudenreichii* stress tolerance. *PLoS ONE* 10, e0135780. doi:10.1371/journal.pone.0135780.

Ganguli, K., Collado, M. C., Rautava, J., Lu, L., Satokari, R., von Ossowski, I., et al. (2015). *Lactobacillus rhamnosus* GG and its SpaC pilus adhesin modulate inflammatory responsiveness and TLR-related gene expression in the fetal human gut. *Pediatr Res* 77, 528–535. doi:10.1038/pr.2015.5.

Gao, C., Major, A., Rendon, D., Lugo, M., Jackson, V., Shi, Z., et al. (2015). Histamine H2 receptor-mediated suppression of intestinal inflammation by probiotic *Lactobacillus reuteri*. *mBio* 6, e01358-15. doi:10.1128/mBio.01358-15.

García-Fraile, P., Menéndez, E., Rivas, R., and 1 Laboratory of Fungal Genetics and Metabolism, Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Praha 4, Prague, Czech Republic; (2015). Role of bacterial biofertilizers in agriculture and forestry. *AIMS Bioengineering* 2, 183–205. doi:10.3934/bioeng.2015.3.183.

Gaucher, F., Bonnassie, S., Rabah, H., Leverrier, P., Pottier, S., Jardin, J., et al. (2019a). Benefits and drawbacks of osmotic adjustment in *Propionibacterium freudenreichii*. *Journal of Proteomics* 204, 103400. doi:10.1016/j.jprot.2019.103400.

Gaucher, F., Bonnassie, S., Rabah, H., Marchand, P., Blanc, P., Jeantet, R., et al. (2019b). Review: adaptation of beneficial propionibacteria, lactobacilli, and bifidobacteria improves tolerance toward technological and digestive stresses. *Front. Microbiol.* 10, 841. doi:10.3389/fmicb.2019.00841.

Ge, Y., Gong, M., Colliou, N., Zadeh, M., Li, J., Jones, D. P., et al. (2019). Neonatal intestinal immune regulation by the commensal bacterium, P. UF1. *Mucosal Immunol* 12, 434–444. doi:10.1038/s41385-018-0125-1.

Ghazi, A., Demont-Caulet, N., Richarme, G., and Caldas, T. (1999). Thermoprotection by glycine betaine and choline. *Microbiology* 145, 2543–2548. doi:10.1099/00221287-145-9-2543.

Giulio, B. D., Orlando, P., Barba, G., Coppola, R., Rosa, M. D., Sada, A., et al. (2005). Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. *World J Microbiol Biotechnol* 21, 739–746. doi:10.1007/s11274-004-4735-2.

Glaasker, E., Heuberger, E. H., Konings, W. N., and Poolman, B. (1998). Mechanism of osmotic activation of the quaternary ammonium compound transporter (QacT) of *Lactobacillus plantarum*. *J. Bacteriol.* 180, 5540–5546.

Glaasker, E., Konings, W. N., and Poolman, B. (1996). Osmotic regulation of intracellular solute pools in *Lactobacillus plantarum*. *J. Bacteriol.* 178, 575–582. doi:10.1128/jb.178.3.575-582.1996.

Goh, Y. J., and Klaenhammer, T. R. (2014). Insights into glycogen metabolism in *Lactobacillus acidophilus*: impact on carbohydrate metabolism, stress tolerance and gut retention. *Microb Cell Fact* 13, 94. doi:10.1186/s12934-014-0094-3.

Golowczyc, M. A., Gerez, C. L., Silva, J., Abraham, A. G., De Antoni, G. L., and Teixeira, P. (2011). Survival of spray-dried *Lactobacillus kefir* is affected by different protectants and storage conditions. *Biotechnol Lett* 33, 681–686. doi:10.1007/s10529-010-0491-6.

Goswami, G., Panda, D., Samanta, R., Boro, R. C., Modi, M. K., Bujarbaruah, K. M., et al. (2018). *Bacillus megaterium* adapts to acid stress condition through a network of genes: Insight from a genome-wide transcriptome analysis. *Sci Rep* 8, 16105. doi:10.1038/s41598-018-34221-0.

Gouesbet, G., Blanco, C., Hamelin, J., and Bernard, T. (1992). Osmotic adjustment in *Brevibacterium ammoniagenes*: pipecolic acid accumulation at elevated osmolalities. *Journal of General Microbiology* 138, 959–965. doi:10.1099/00221287-138-5-959.

Gouesbet, G., Jan, G., and Boyaval, P. (2002). Two-dimensionaleElectrophoresis Study of *Lactobacillus delbrueckii* subsp. *bulgaricus* thermotolerance. *Applied and Environmental Microbiology* 68, 1055–1063. doi:10.1128/AEM.68.3.1055-1063.2002.

Graham, J. E., and Wilkinson, B. J. (1992). *Staphylococcus aureus* osmoregulation: roles for choline, glycine betaine, proline, and taurine. *J. Bacteriol.* 174, 2711–2716. doi:10.1128/jb.174.8.2711-2716.1992.

Gray, M. J., and Jakob, U. (2015). Oxidative stress protection by polyphosphate—new roles for an old player. *Current Opinion in Microbiology* 24, 1–6. doi:10.1016/j.mib.2014.12.004.

Grosu-Tudor, S.-S., Brown, L., Hebert, E. M., Brezeanu, A., Brinzan, A., Fadda, S., et al. (2016). S-layer production by *Lactobacillus acidophilus* IBB 801 under environmental

stress conditions. *Appl Microbiol Biotechnol* 100, 4573–4583. doi:10.1007/s00253-016-7355-5.

Guan, H.-L., Zhang, Y.-J., Lu, X.-J., Jia, M., Zhang, Z.-Y., Gao, X.-H., et al. (2018). *Gracilibacillus eburneus* sp.nov., a moderately halophilic bacterium isolated from Xinjiang province, China. *Arch Microbiol* 200, 423–429. doi:10.1007/s00203-017-1450-6.

Guan, N., Liu, L., Shin, H., Chen, R. R., Zhang, J., Li, J., et al. (2013). Systems-level understanding of how *Propionibacterium acidipropionici* respond to propionic acid stress at the microenvironment levels: Mechanism and application. *Journal of Biotechnology* 167, 56–63. doi:10.1016/j.jbiotec.2013.06.008.

Guarner, F., Perdigon, G., Corthier, G., Salminen, S., Koletzko, B., and Morelli, L. (2005). Should yoghurt cultures be considered probiotic? *Br J Nutr* 93, 783–786. doi:10.1079/BJN20051428.

Guillot, A., Obis, D., and Mistou, M.-Y. (2000). Fatty acid membrane composition and activation of glycine-betaine transport in *Lactococcus lactis* subjected to osmotic stress. *International Journal of Food Microbiology* 55, 47–51. doi:10.1016/S0168-1605(00)00193-8.

Hammes, W. P., and Hertel, C. (2015). "*Lactobacillus*," in *Bergey's Manual of Systematics of Archaea and Bacteria*, eds. W. B. Whitman, F. Rainey, P. Kämpfer, M. Trujillo, J. Chun, P. DeVos, et al. (Chichester, UK: John Wiley & Sons, Ltd), 1–76. doi:10.1002/9781118960608.gbm00604.

Hazell, B., Nevalainen, H., and Attfield, P. (1995). Evidence that the *Saccharomyces cerevisiae CIF1 (GGS1/TPS1*) gene modulates heat shock response positively. *FEBS Letters* 377, 457–460. doi:10.1016/0014-5793(95)01392-X.

Heunis, T., Deane, S., Smit, S., and Dicks, L. M. T. (2014). Proteomic profiling of the acid stress response in *Lactobacillus plantarum* 423. *J. Proteome Res.* 13, 4028–4039. doi:10.1021/pr500353x.

Hoffmann, T., Wensing, A., Brosius, M., Steil, L., Volker, U., and Bremer, E. (2013). Osmotic control of opuA expression in *Bacillus subtilis* and its modulation in response to intracellular glycine betaine and proline pools. *Journal of Bacteriology* 195, 510–522. doi:10.1128/JB.01505-12.

Holtmann, G., and Bremer, E. (2004). Thermoprotection of Bacillus subtilis by exogenously provided glycine betaine and structurally related compatible solutes: involvement of Opu transporters. *Journal of Bacteriology* 186, 1683–1693. doi:10.1128/JB.186.6.1683-1693.2004.

Homayouni, A., Azizi, A., Ehsani, M. R., Yarmand, M. S., and Razavi, S. H. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food Chemistry* 111, 50–55. doi:10.1016/j.foodchem.2008.03.036.

Hoseinifar, S. H., Ringø, E., Shenavar Masouleh, A., and Esteban, M. Á. (2016). Probiotic, prebiotic and synbiotic supplements in sturgeon aquaculture: a review. *Rev Aquacult* 8, 89–102. doi:10.1111/raq.12082.

Hottiger, T., Schmutz, P., and Wiemken, A. (1987). Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. *J. Bacteriol.* 169, 5518–5522. doi:10.1128/jb.169.12.5518-5522.1987.

Hou, C., Zeng, X., Yang, F., Liu, H., and Qiao, S. (2015). Study and use of the probiotic *Lactobacillus reuteri* in pigs: a review. *J Animal Sci Biotechnol* 6, 14. doi:10.1186/s40104-015-0014-3.

Hsu, C. A., Yu, R. C., Lee, S. L., and Chou, C. C. (2007). Cultural condition affecting the growth and production of β-galactosidase by *Bifidobacterium longum* CCRC 15708 in a jar fermenter. *International Journal of Food Microbiology* 116, 186–189. doi:10.1016/j.ijfoodmicro.2006.12.034.

Huang, S., Cauty, C., Dolivet, A., Le Loir, Y., Chen, X. D., Schuck, P., et al. (2016a). Double use of highly concentrated sweet whey to improve the biomass production and viability of spray-dried probiotic bacteria. *Journal of Functional Foods* 23, 453–463. doi:10.1016/j.jff.2016.02.050.

Huang, S., Gaucher, F., Cauty, C., Jardin, J., Le Loir, Y., Jeantet, R., et al. (2018). Growth in hyper-concentrated sweet whey triggers multi stress tolerance and spray drying survival in *Lactobacillus casei* BL23: from the molecular basis to new perspectives for sustainable probiotic production. *Front. Microbiol.* 9, 2548. doi:10.3389/fmicb.2018.02548.

Huang, S., Méjean, S., Rabah, H., Dolivet, A., Le Loir, Y., Chen, X. D., et al. (2017a). Double use of concentrated sweet whey for growth and spray drying of probiotics: Towards maximal viability in pilot scale spray dryer. *Journal of Food Engineering* 196, 11–17. doi:10.1016/j.jfoodeng.2016.10.017.

Huang, S., Rabah, H., Jardin, J., Briard-Bion, V., Parayre, S., Maillard, M.-B., et al. (2016b). Hyperconcentrated sweet whey, a new culture medium that enhances *Propionibacterium freudenreichii* stress tolerance. *Appl. Environ. Microbiol.* 82, 4641–4651. doi:10.1128/AEM.00748-16.

Huang, S., Vignolles, M.-L., Chen, X. D., Le Loir, Y., Jan, G., Schuck, P., et al. (2017b). Spray drying of probiotics and other food-grade bacteria: A review. *Trends in Food Science & Technology* 63, 1–17. doi:10.1016/j.tifs.2017.02.007.

Hynönen, U., Kant, R., Lähteinen, T., Pietilä, T. E., Beganović, J., Smidt, H., et al. (2014). Functional characterization of probiotic surface layer protein-carrying *Lactobacillus amylovorus* strains. *BMC Microbiol* 14, 199. doi:10.1186/1471-2180-14-199.

Ikeda-Ohtsubo, W., Brugman, S., Warden, C. H., Rebel, J. M. J., Folkerts, G., and Pieterse, C. M. J. (2018). How can we define "optimal microbiota?": a comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. *Front. Nutr.* 5, 90. doi:10.3389/fnut.2018.00090.

Jahangiri, L., and Esteban, M. (2018). Administration of probiotics in the water in finfish aquaculture systems: a review. *Fishes* 3, 33. doi:10.3390/fishes3030033.

Jan, G., Leverrier, P., Pichereau, V., and Boyaval, P. (2001). Changes in Protein Synthesis and Morphology during Acid Adaptation of *Propionibacterium freudenreichii*. *Applied and Environmental Microbiology* 67, 2029–2036. doi:10.1128/AEM.67.5.2029-2036.2001.

Jan, G., Leverrier, P., Proudy, I., and Roland, N. (2002). Survival and beneficial effects of propionibacteria in the human gut: in vivo and *in vitro* investigations. *Lait* 82, 131–144. doi:10.1051/lait:2001012.

Jan, G., Rouault, A., and Maubois, J.-L. (2000). Acid stress susceptibility and acid adaptation of *Propionibacterium freudenreichii* subsp. *shermanii*. *Lait* 80, 325–336. doi:10.1051/lait:2000128.

Jang, J.-Y., Oh, Y. J., Lim, S. K., Park, H. K., Lee, C., Kim, J. Y., et al. (2018). *Salicibibacter kimchii* gen. nov., sp. *nov.*, a moderately halophilic and alkalitolerant bacterium in the family *Bacillaceae*, isolated from kimchi. *J Microbiol.* 56, 880–885. doi:10.1007/s12275-018-8518-0.

Jardin, J. (2019). Data from *Propionibacterium freudenreichii* CIRM-BIA 129 osmoadaptation coupled to acid-adaptation increases its viability during freeze-drying. doi:10.15454/5cwcll.

Jin, J., Qin, Q., Guo, H., Liu, S., Ge, S., Zhang, H., et al. (2015). Effect of pre-stressing on the acid-stress response in *Bifidobacterium* revealed using proteomic and physiological approaches. *PLoS ONE* 10, e0117702. doi:10.1371/journal.pone.0117702.

Johansson, M. E. V., Jakobsson, H. E., Holmén-Larsson, J., Schütte, A., Ermund, A., Rodríguez-Piñeiro, A. M., et al. (2015). Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host & Microbe* 18, 582–592. doi:10.1016/j.chom.2015.10.007.

Kailasapathy, K. (2006). Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. *LWT - Food Science and Technology* 39, 1221–1227. doi:10.1016/j.lwt.2005.07.013.

Kanemasa, Y., Yoshioka, T., and Hayashi, H. (1972). Alteration of the phospholipid composition of *Staphylococcus aureus cultured* in medium containing NaCl. *Biochim. Biophys. Acta* 280, 444–450.

Kato-Kataoka, A., Nishida, K., Takada, M., Kawai, M., Kikuchi-Hayakawa, H., Suda, K., et al. (2016). Fermented milk containing *Lactobacillus casei* strain *Shirota* preserves the diversity of the gut microbiota and relieves abdominal dysfunction in healthy medical students exposed to academic stress. *Appl. Environ. Microbiol.* 82, 3649–3658. doi:10.1128/AEM.04134-15.

Kets, E., Teunissen, P., and de Bont, J. (1996). Effect of compatible solutes on survival of lactic acid bacteria subjected to drying. *Appl. Environ. Microbiol.* 62, 259–261.

Khaleghi, M., and Kasra, R. (2012). “Effect of environmental stresses on S-Layer production in *Lactobacillus acidophilus* ATCC 4356,” in *Advances in Applied Biotechnology*, ed. M. Petre (InTech). doi:10.5772/28334.

Khaleghi, M., Kermanshahi, R. K., Yaghoobi, M. M., Zarkesh-Esfahani, S. H., and Baghizadeh, A. (2010). Assessment of bile salt effects on s-layer production, slp gene expression and some physicochemical properties of *Lactobacillus acidophilus* ATCC 4356. *J. Microbiol. Biotechnol.* 20, 749–756.

Khalesi, S., Bellissimo, N., Vandelanotte, C., Williams, S., Stanley, D., and Irwin, C. (2019). A review of probiotic supplementation in healthy adults: helpful or hype? *Eur J Clin Nutr* 73, 24–37. doi:10.1038/s41430-018-0135-9.

Kim, H. G., Kim, N.-R., Gim, M. G., Lee, J. M., Lee, S. Y., Ko, M. Y., et al. (2008). Lipoteichoic Acid Isolated from *Lactobacillus plantarum* Inhibits Lipopolysaccharide-Induced TNF-α Production in THP-1 Cells and Endotoxin Shock in Mice. *J Immunol* 180, 2553–2561. doi:10.4049/jimmunol.180.4.2553.

Koch, S., Eugster-Meier, E., Oberson, G., Meile, L., and Lacroix, C. (2008). Effects of strains and growth conditions on autolytic activity and survival to freezing and lyophilization of *Lactobacillus delbrueckii* ssp. *lactis* isolated from cheese. *International Dairy Journal* 18, 187–196. doi:10.1016/j.idairyj.2007.07.009.

Komaratat, P., and Kates, M. (1975). The lipid composition of a halotolerant species of *Staphylococcus epidermidis*. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* 398, 464–484. doi:10.1016/0005-2760(75)90197-6.

Konstantinov, S. R., Smidt, H., de Vos, W. M., Bruijns, S. C. M., Singh, S. K., Valence, F., et al. (2008). S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proceedings of the National Academy of Sciences* 105, 19474–19479. doi:10.1073/pnas.0810305105.

Kunin, C. M., and Rudy, J. (1991). Effect of NaCl-induced osmotic stress on intracellular concentrations of glycine betaine and potassium in *Escherichia coli*, *Enterococcus faecalis*, and staphylococci. *J. Lab. Clin. Med.* 118, 217–224.

Kurtmann, L., Carlsen, C. U., Risbo, J., and Skibsted, L. H. (2009). Storage stability of freeze–dried *Lactobacillus acidophilus* (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology* 58, 175–180. doi:10.1016/j.cryobiol.2008.12.001.

Kwon, M.-S., Lim, S. K., Jang, J.-Y., Lee, J., Park, H. K., Kim, N., et al. (2018). *Lactobacillus sakei* WIKIM30 ameliorates atopic dermatitis-like skin lesions by inducing regulatory T Cells and altering gut microbiota structure in mice. *Front. Immunol.* 9, 1905. doi:10.3389/fimmu.2018.01905.

Laiño, J., Villena, J., Kanmani, P., and Kitazawa, H. (2016). Immunoregulatory Effects Triggered by Lactic Acid Bacteria Exopolysaccharides: New Insights into Molecular Interactions with Host Cells. *Microorganisms* 4, 27. doi:10.3390/microorganisms4030027.

Lan, A., Bruneau, A., Bensaada, M., Philippe, C., Bellaud, P., Rabot, S., et al. (2008). Increased induction of apoptosis by *Propionibacterium freudenreichii* TL133 in colonic mucosal

crypts of human microbiota-associated rats treated with 1,2-dimethylhydrazine. *Br J Nutr* 100, 1251–1259. doi:10.1017/S0007114508978284.

Lanciotti, R., Guerzoni, M. E., and Cocconcelli, P. S. (2001). Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology* 147, 2255–2264. doi:10.1099/00221287-147-8-2255.

Langella, O., Valot, B., Balliau, T., Blein-Nicolas, M., Bonhomme, L., and Zivy, M. (2017). X!TandemPipeline: A tool to manage sequence redundancy for protein inference and phosphosite identification. *J. Proteome Res.* 16, 494–503. doi:10.1021/acs.jproteome.6b00632.

Le Maréchal, C., Peton, V., Plé, C., Vroland, C., Jardin, J., Briard-Bion, V., et al. (2015). Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *Journal of Proteomics* 113, 447–461. doi:10.1016/j.jprot.2014.07.018.

Lebeer, S., Bron, P. A., Marco, M. L., Van Pijkeren, J.-P., O'Connell Motherway, M., Hill, C., et al. (2018). Identification of probiotic effector molecules: present state and future perspectives. *Current Opinion in Biotechnology* 49, 217–223. doi:10.1016/j.copbio.2017.10.007.

Lebeer, S., Claes, I. J. J., and Vanderleyden, J. (2012). Anti-inflammatory potential of probiotics: lipoteichoic acid makes a difference. *Trends in Microbiology* 20, 5–10. doi:10.1016/j.tim.2011.09.004.

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2008). Genes and molecules of Lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reviews* 72, 728–764. doi:10.1128/MMBR.00017-08.

LeBlanc, J. G., Chain, F., Martín, R., Bermúdez-Humarán, L. G., Courau, S., and Langella, P. (2017). Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microb Cell Fact* 16, 79. doi:10.1186/s12934-017-0691-z.

Lee, S. J., Jeong, E.-M., Ki, A. Y., Oh, K.-S., Kwon, J., Jeong, J.-H., et al. (2016). Oxidative defense metabolites induced by salinity stress in roots of *Salicornia herbacea*. *Journal of Plant Physiology* 206, 133–142. doi:10.1016/j.jplph.2016.08.015.

Leverrier, P., Dimova, D., Pichereau, V., Auffray, Y., Boyaval, P., and Jan, G. (2003). Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: Physiological and proteomic analysis. *Applied and Environmental Microbiology* 69, 3809–3818. doi:10.1128/AEM.69.7.3809-3818.2003.

Leverrier, P., Vissers, J. P. C., Rouault, A., Boyaval, P., and Jan, G. (2004). Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. *Archives of Microbiology* 181, 215–230. doi:10.1007/s00203-003-0646-0.

Levit, R., de Giori, G. S., de Moreno de LeBlanc, A., and LeBlanc, J. G. (2017). Evaluation of the effect of soymilk fermented by a riboflavin-producing *Lactobacillus plantarum*

strain in a murine model of colitis. *Beneficial Microbes* 8, 65–72. doi:10.3920/BM2016.0063.

Levit, R., Savoy de Giori, G., de Moreno de LeBlanc, A., and LeBlanc, J. G. (2018). Effect of riboflavin-producing bacteria against chemically induced colitis in mice. *J Appl Microbiol* 124, 232–240. doi:10.1111/jam.13622.

Levri, K. M., Ketvertis, K., Deramo, M., Merenstein, J. H., and D'Amico, F. (2005). Do probiotics reduce adult lactose intolerance? A systematic review. *J Fam Pract* 54, 613–620.

Li, C., Zhao, J.-L., Wang, Y.-T., Han, X., and Liu, N. (2009). Synthesis of cyclopropane fatty acid and its effect on freeze-drying survival of *Lactobacillus bulgaricus* L2 at different growth conditions. *World J Microbiol Biotechnol* 25, 1659–1665. doi:10.1007/s11274-009-0060-0.

Lightfoot, Y. L., Selle, K., Yang, T., Goh, Y. J., Sahay, B., Zadeh, M., et al. (2015). SIGNR 3-dependent immune regulation by *Lactobacillus acidophilus* surface layer protein A in colitis. *EMBO J* 34, 881–895. doi:10.15252/embj.201490296.

Lim, J., Yoon, S., Tan, P.-L., Yang, S., Kim, S., and Park, H. (2018). Probiotic properties of *Lactobacillus Plantarum* LRCC5193, a plant-origin lactic acid bacterium isolated from kimchi and its use in chocolates: properties of chocolate of *L. plantarum*. *Journal of Food Science* 83, 2802–2811. doi:10.1111/1750-3841.14364.

Louesdon, S., Charlot-Rougé, S., Juillard, V., Tourdot-Maréchal, R., and Béal, C. (2014). Osmotic stress affects the stability of freeze-dried *Lactobacillus buchneri* R1102 as a result of intracellular betaine accumulation and membrane characteristics. *J Appl Microbiol* 117, 196–207. doi:10.1111/jam.12501.

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ Microbiol* 19, 29–41. doi:10.1111/1462-2920.13589.

Ma, W., Mao, Q., Xia, W., Dong, G., Yu, C., and Jiang, F. (2019). Gut microbiota shapes the efficiency of cancer therapy. *Front. Microbiol.* 10, 1050. doi:10.3389/fmicb.2019.01050.

Machado, M. C., López, C. S., Heras, H., and Rivas, E. A. (2004). Osmotic response in *Lactobacillus casei* ATCC 393: biochemical and biophysical characteristics of membrane. *Archives of Biochemistry and Biophysics* 422, 61–70. doi:10.1016/j.abb.2003.11.001.

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., et al. (2014). A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 63, 1275–1283. doi:10.1136/gutjnl-2013-304833.

Mahfuz, S. U., Nahar, M. J., Mo, C., Ganfu, Z., Zhongjun, L., and Hui, S. (2017). Inclusion of probiotic on chicken performance and immunity: A review. *International J. of Poultry Science* 16, 328–335. doi:10.3923/ijps.2017.328.335.

Mainville, I., Arcand, Y., and Farnworth, E. R. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *International Journal of Food Microbiology* 99, 287–296. doi:10.1016/j.ijfoodmicro.2004.08.020.

Malik, A. C., Reinbold, G. W., and Vedamuthu, E. R. (1968). An evaluation of the taxonomy of *Propionibacterium*. *Can. J. Microbiol.* 14, 1185–1191. doi:10.1139/m68-199.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., et al. (2017). Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology* 44, 94–102. doi:10.1016/j.copbio.2016.11.010.

Maria-Rosario, A., Davidson, I., Debra, M., Verheul, A., Abee, T., and Booth, I. R. (1995). The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. *Microbiology* 141, 41–49. doi:10.1099/00221287-141-1-41.

marketsandmarkets.com (2014). Starter Culture Market worth $1.0 Billion By 2018.

marketsandmarkets.com (2019). Probiotics market by application (functional food & beverages [dairy products, non-dairy beverages, infant formula, cereals], dietary supplements, feed), ingredient (bacteria, yeast), form (dry, liquid), end user, and region– global forecast to 2023. Available at: https://www.marketsandmarkets.com/Market-Reports/probiotic-market-advanced-technologies-and-global-market-69.html.

Marketwatch.com (2019). Starter cultures market 2025: Global size, key companies, trends, growth and regional forecasts research. Available at: https://www.marketwatch.com/press-release/starter-cultures-market-2025-global-size-key-companies-trends-growth-and-regional-forecasts-research-2019-03-20.

Maus, J. E., and Ingham, S. C. (2003). Employment of stressful conditions during culture production to enhance subsequent cold- and acid-tolerance of bifidobacteria. *J Appl Microbiol* 95, 146–154. doi:10.1046/j.1365-2672.2003.01954.x.

Mehmood, U., Inam-ul-Haq, M., Saed, M., Altaf, A., and Azam, F. A brief review on plant growth promoting rhizobacteria (PGPR): a key role in plant growth promotion. *Plant protection*, 77–82.

Meira, Q. G. S., Magnani, M., de Medeiros Júnior, F. C., Queiroga, R. de C. R. do E., Madruga, M. S., Gullón, B., et al. (2015). Effects of added *Lactobacillus acidophilus* and *Bifidobacterium lactis* probiotics on the quality characteristics of goat ricotta and their survival under simulated gastrointestinal conditions. *Food Research International* 76, 828–838. doi:10.1016/j.foodres.2015.08.002.

Mende, S., Rohm, H., and Jaros, D. (2016). Influence of exopolysaccharides on the structure, texture, stability and sensory properties of yoghurt and related products. *International Dairy Journal* 52, 57–71. doi:10.1016/j.idairyj.2015.08.002.

Meneghel, J., Passot, S., Cenard, S., Réfrégiers, M., Jamme, F., and Fonseca, F. (2017). Subcellular membrane fluidity of *Lactobacillus delbrueckii* subsp. *bulgaricus* under cold and osmotic stress. *Appl Microbiol Biotechnol* 101, 6907–6917. doi:10.1007/s00253-017-8444-9.

Merrick, M. (2015). Post-translational modification of PII signal transduction proteins. *Front. Microbiol.* 5. doi:10.3389/fmicb.2014.00763.

Miller, K. J. (1985). Effects of temperature and sodium chloride concentration on the phospholipid and fatty acid compositions of a halotolerant *Planococcus* sp. *J. Bacteriol.* 162, 263–270.

Miller-Kane, and O'Toole, M. T. (2003). Encyclopedia & Dictionary of medicine, nursing & allied health elsevier eBook on VitalSource. Available at: a href="https://medical-dictionary.thefreedictionary.com/microbiota">microbiota.

Mills, S., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2011). Enhancing the stress responses of probiotics for a lifestyle from gut to product and back again. *Microb Cell Fact* 10, S19. doi:10.1186/1475-2859-10-S1-S19.

Mimura, T. (2004). Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 53, 108–114. doi:10.1136/gut.53.1.108.

Moens, F., Verce, M., and De Vuyst, L. (2017). Lactate- and acetate-based cross-feeding interactions between selected strains of lactobacilli, bifidobacteria and colon bacteria in the presence of inulin-type fructans. *International Journal of Food Microbiology* 241, 225–236. doi:10.1016/j.ijfoodmicro.2016.10.019.

Moore, S. D., and Sauer, R. T. (2007). The tmRNA system for translational surveillance and ribosome rescue. *Annu. Rev. Biochem.* 76, 101–124. doi:10.1146/annurev.biochem.75.103004.142733.

Muller, J. A., Ross, R. P., Sybesma, W. F. H., Fitzgerald, G. F., and Stanton, C. (2011). Modification of the technical properties of *Lactobacillus johnsonii* NCC 533 by supplementing the growth medium with unsaturated fatty acids. *Appl. Environ. Microbiol.* 77, 6889–6898. doi:10.1128/AEM.05213-11.

Nurlinawati, Vanoirbeek, K., Aertsen, A., and Michiels, C. W. (2015). Role of 1-acyl-sn-glycerol-3-phosphate acyltransferase in psychrotrophy and stress tolerance of *Serratia plymuthica* RVH1. *Research in Microbiology* 166, 28–37. doi:10.1016/j.resmic.2014.11.001.

Okada, Y. (2006). *Propionibacterium freudenreichii* component 1.4-dihydroxy-2-naphthoic acid (DHNA) attenuates dextran sodium sulphate induced colitis by modulation of bacterial flora and lymphocyte homing. *Gut* 55, 681–688. doi:10.1136/gut.2005.070490.

Paéz, R., Lavari, L., Vinderola, G., Audero, G., Cuatrin, A., Zaritzky, N., et al. (2012). Effect of heat treatment and spray drying on lactobacilli viability and resistance to simulated gastrointestinal digestion. *Food Research International* 48, 748–754. doi:10.1016/j.foodres.2012.06.018.

Pakdaman, M. N., Udani, J. K., Molina, J. P., and Shahani, M. (2015). The effects of the DDS-1 strain of lactobacillus on symptomatic relief for lactose intolerance - a randomized, double-blind, placebo-controlled, crossover clinical trial. *Nutr J* 15, 56. doi:10.1186/s12937-016-0172-y.

Palmfeldt, J., and Hahn-Hägerdal, B. (2000). Influence of culture pH on survival of *Lactobacillus reuteri* subjected to freeze-drying. *International Journal of Food Microbiology* 55, 235–238. doi:10.1016/S0168-1605(00)00176-8.

Palomino, M. M., Allievi, M. C., Grundling, A., Sanchez-Rivas, C., and Ruzal, S. M. (2013). Osmotic stress adaptation in *Lactobacillus casei* BL23 leads to structural changes in the cell wall polymer lipoteichoic acid. *Microbiology* 159, 2416–2426. doi:10.1099/mic.0.070607-0.

Palomino, M. M., Waehner, P. M., Fina Martin, J., Ojeda, P., Malone, L., Sánchez Rivas, C., et al. (2016). Influence of osmotic stress on the profile and gene expression of surface layer proteins in *Lactobacillus acidophilus* ATCC 4356. *Appl Microbiol Biotechnol* 100, 8475–8484. doi:10.1007/s00253-016-7698-y.

Pandiyan, P., Balaraman, D., Thirunavukkarasu, R., George, E. G. J., Subaramaniyan, K., Manikkam, S., et al. (2013). Probiotics in aquaculture. *Drug Invention Today* 5, 55–59. doi:10.1016/j.dit.2013.03.003.

Papadimitriou, K., Alegría, Á., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem, M., et al. (2016). Stress physiology of lactic acid bacteria. *Microbiol. Mol. Biol. Rev.* 80, 837–890. doi:10.1128/MMBR.00076-15.

Parvez, S., Malik, K. A., Ah Kang, S., and Kim, H.-Y. (2006). Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* 100, 1171–1185. doi:10.1111/j.1365-2672.2006.02963.x.

Peighambardoust, S. H., Golshan Tafti, A., and Hesari, J. (2011a). Application of spray drying for preservation of lactic acid starter cultures: a review. *Trends in Food Science & Technology* 22, 215–224. doi:10.1016/j.tifs.2011.01.009.

Peighambardoust, S. H., Golshan Tafti, A., and Hesari, J. (2011b). Application of spray drying for preservation of lactic acid starter cultures: a review. *Trends in Food Science & Technology* 22, 215–224. doi:10.1016/j.tifs.2011.01.009.

Perdana, J., Fox, M. B., Siwei, C., Boom, R. M., and Schutyser, M. A. I. (2014). Interactions between formulation and spray drying conditions related to survival of *Lactobacillus plantarum* WCFS1. *Food Research International* 56, 9–17. doi:10.1016/j.foodres.2013.12.007.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., and Matuchansky, C. (2005). Review article: bifidobacteria as probiotic agents - physiological effects and clinical benefits. *Alimentary Pharmacology and Therapeutics* 22, 495–512. doi:10.1111/j.1365-2036.2005.02615.x.

Pichereau, V., Bourot, S., Flahaut, S., Blanco, C., Auffray, Y., and Bernard, T. (1999). The osmoprotectant glycine betaine inhibits salt-induced cross-tolerance towards lethal treatment in *Enterococcus faecalis*. *Microbiology* 145, 427–435. doi:10.1099/13500872-145-2-427.

Picot, A., and Lacroix, C. (2004). Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. *International Dairy Journal* 14, 505–515. doi:10.1016/j.idairyj.2003.10.008.

Pilonieta, M. C., Nagy, T. A., Jorgensen, D. R., and Detweiler, C. S. (2012). A glycine betaine importer limits *Salmonella* stress resistance and tissue colonization by reducing trehalose production: Trehalose accumulation limits bacterial colonization. *Molecular Microbiology* 84, 296–309. doi:10.1111/j.1365-2958.2012.08022.x.

Piuri, M., Sanchez-Rivas, C., and Ruzal, S. M. (2005). Cell wall modifications during osmotic stress in *Lactobacillus casei*. *J Appl Microbiol* 98, 84–95. doi:10.1111/j.1365-2672.2004.02428.x.

Plé, C., Breton, J., Richoux, R., Nurdin, M., Deutsch, S.-M., Falentin, H., et al. (2016). Combining selected immunomodulatory *Propionibacterium freudenreichii* and *Lactobacillus delbrueckii* strains: Reverse engineering development of an anti-inflammatory cheese. *Mol. Nutr. Food Res.* 60, 935–948. doi:10.1002/mnfr.201500580.

Pleitner, A., Zhai, Y., Winter, R., Ruan, L., McMullen, L. M., and Gänzle, M. G. (2012). Compatible solutes contribute to heat resistance and ribosome stability in *Escherichia coli* AW1.7. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1824, 1351–1357. doi:10.1016/j.bbapap.2012.07.007.

Poolman, B., Spitzer, J. J., and Wood, J. M. (2004). Bacterial osmosensing: roles of membrane structure and electrostatics in lipid–protein and protein–protein interactions. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1666, 88–104. doi:10.1016/j.bbamem.2004.06.013.

Prasad, J., McJarrow, P., and Gopal, P. (2003). Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. *Applied and Environmental Microbiology* 69, 917–925. doi:10.1128/AEM.69.2.917-925.2003.

Rabah, H., Ménard, O., Gaucher, F., do Carmo, F. L. R., Dupont, D., and Jan, G. (2018). Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during in vitro digestion. *Food Research International* 106, 712–721. doi:10.1016/j.foodres.2018.01.035.

Rabah, H., Rosa do Carmo, F., and Jan, G. (2017). Dairy Propionibacteria: Versatile Probiotics. *Microorganisms* 5, 24. doi:10.3390/microorganisms5020024.

Racioppo, A., Corbo, M. R., Piccoli, C., Sinigaglia, M., Speranza, B., and Bevilacqua, A. (2017). Ultrasound attenuation of lactobacilli and bifidobacteria: Effect on some technological and probiotic properties. *International Journal of Food Microbiology* 243, 78–83. doi:10.1016/j.ijfoodmicro.2016.12.011.

Rios-Covian, D., Nogacka, A., Salazar, N., Hernández-Barranco, A. M., Cuesta, I., Gueimonde, M., et al. (2018). *Bifidobacterium breve* IPLA20005 affects in vitro the expression of *hly* and *luxS* genes, related to the virulence of *Listeria monocytogenes* Lm23. *Can. J. Microbiol.* 64, 215–221. doi:10.1139/cjm-2017-0625.

Robert, H., Le Marrec, C., Blanco, C., and Jebbar, M. (2000). Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus halophila*. *Appl. Environ. Microbiol.* 66, 509–517. doi:10.1128/aem.66.2.509-517.2000.

Roesser, M., and Müller, V. (2001). Osmoadaptation in bacteria and archaea: common principles and differences. *Environ. Microbiol.* 3, 743–754.

Rollan, G., Lorca, G. L., and Font de Valdez, G. (2003). Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough. *Food Microbiology* 20, 313–319. doi:10.1016/S0740-0020(02)00139-9.

Romeo, Y., Obis, D., Bouvier, J., Guillot, A., Fourçans, A., Bouvier, I., et al. (2003). Osmoregulation in *Lactococcus lactis*: BusR, a transcriptional repressor of the glycine betaine uptake system BusA: Regulatory protein BusR of *L. lactis*. *Molecular Microbiology* 47, 1135–1147. doi:10.1046/j.1365-2958.2003.03362.x.

Rong, J., Zheng, H., Liu, M., Hu, X., Wang, T., Zhang, X., et al. (2015). Probiotic and anti-inflammatory attributes of an isolate *Lactobacillus helveticus* NS8 from Mongolian fermented koumiss. *BMC Microbiol* 15, 196. doi:10.1186/s12866-015-0525-2.

Ruiz, L., Margolles, A., and Sánchez, B. (2013). Bile resistance mechanisms in *Lactobacillus*L and *Bifidobacterium*. *Front. Microbiol.* 4. doi:10.3389/fmicb.2013.00396.

Saarela, M., Virkajarvi, I., Alakomi, H.-L., Mattila-Sandholm, T., Vaari, A., Suomalainen, T., et al. (2005). Influence of fermentation time, cryoprotectant and neutralization of cell concentrate on freeze-drying survival, storage stability, and acid and bile exposure of *Bifidobacterium animalis* ssp. *lactis* cells produced without milk-based ingredients. *J Appl Microbiol* 99, 1330–1339. doi:10.1111/j.1365-2672.2005.02742.x.

Saez-Lara, M. J., Gomez-Llorente, C., Plaza-Diaz, J., and Gil, A. (2015). The role of probiotic lactic acid bacteria and Bifidobacteria in the prevention and treatment of inflammatory bowel disease and other related diseases: a systematic review of randomized human clinical trials. *BioMed Research International* 2015, 1–15. doi:10.1155/2015/505878.

Sanchez, B., Champomier-Verges, M.-C., Collado, M. d. C., Anglade, P., Baraige, F., Sanz, Y., et al. (2007). Low-pH adaptation and the acid tolerance response of *Bifidobacterium longum* biotype *longum*. *Applied and Environmental Microbiology* 73, 6450–6459. doi:10.1128/AEM.00886-07.

Sanchez, B., de los Reyes-Gavilan, C. G., and Margolles, A. (2006). The F_1F_0-ATPase of *Bifidobacterium animalis* is involved in bile tolerance. *Environ Microbiol* 8, 1825–1833. doi:10.1111/j.1462-2920.2006.01067.x.

Sánchez, B., Ruiz, L., Gueimonde, M., Ruas-Madiedo, P., and Margolles, A. (2013). Adaptation of bifidobacteria to the gastrointestinal tract and functional consequences. *Pharmacological Research* 69, 127–136. doi:10.1016/j.phrs.2012.11.004.

Santivarangkna, C., Higl, B., and Foerst, P. (2008). Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology* 25, 429–441. doi:10.1016/j.fm.2007.12.004.

Santivarangkna, C., Kulozik, U., and Foerst, P. (2007). Alternative drying processes for the industrial preservation of lactic acid starter cultures. *Biotechnol. Prog.* 23, 302–315. doi:10.1021/bp060268f.

Sarkar, A., and Mandal, S. (2016). Bifidobacteria—Insight into clinical outcomes and mechanisms of its probiotic action. *Microbiological Research* 192, 159–171. doi:10.1016/j.micres.2016.07.001.

Savijoki, K., Suokko, A., Palva, A., Valmu, L., Kalkkinen, N., and Varmanen, P. (2005). Effect of heat-shock and bile salts on protein synthesis of *Bifidobacterium longum* revealed by [^{35}S]methionine labelling and two-dimensional gel electrophoresis. *FEMS Microbiology Letters* 248, 207–215. doi:10.1016/j.femsle.2005.05.032.

Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., et al. (2002). The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proceedings of the National Academy of Sciences* 99, 14422–14427. doi:10.1073/pnas.212527599.

Schoug, Å., Fischer, J., Heipieper, H. J., Schnürer, J., and Håkansson, S. (2008). Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* Si3. *J Ind Microbiol Biotechnol* 35, 175–181. doi:10.1007/s10295-007-0281-x.

Schuck, P., Dolivet, A., and Jeantet, R. (2012). *Les poudres laitières et alimentaires :techniques d'analyse*. Tec et doc, Lavoisier.

Schuck, P., Jeantet, R., Bhandari, B., Chen, X. D., Perrone, Í. T., de Carvalho, A. F., et al. (2016). Recent advances in spray drying relevant to the dairy industry: A comprehensive critical review. *Drying Technology* 34, 1773–1790. doi:10.1080/07373937.2016.1233114.

Sengupta, R., Altermann, E., Anderson, R. C., McNabb, W. C., Moughan, P. J., and Roy, N. C. (2013). The role of cell surface architecture of Lactobacilli in host-microbe interactions in the gastrointestinal tract. *Mediators of Inflammation* 2013, 1–16. doi:10.1155/2013/237921.

Serata, M., Kiwaki, M., and Iino, T. (2016). Functional analysis of a novel hydrogen peroxide resistance gene in *Lactobacillus* casei strain Shirota. *Microbiology* 162, 1885–1894. doi:10.1099/mic.0.000379.

Sheehan, V. M., Sleator, R. D., Fitzgerald, G. F., and Hill, C. (2006). Heterologous expression of BetL, a betaine uptake system, enhances the stress tolerance of *Lactobacillus salivarius* UCC118. *Applied and Environmental Microbiology* 72, 2170–2177. doi:10.1128/AEM.72.3.2170-2177.2006.

Sikorska, H., and Smoragiewicz, W. (2013). Role of probiotics in the prevention and treatment of meticillin-resistant *Staphylococcus aureus* infections. *International Journal of Antimicrobial Agents* 42, 475–481. doi:10.1016/j.ijantimicag.2013.08.003.

Silva, J., Carvalho, A. S., Ferreira, R., Vitorino, R., Amado, F., Domingues, P., et al. (2005). Effect of the pH of growth on the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* to stress conditions during spray-drying. *J Appl Microbiol* 98, 775–782. doi:10.1111/j.1365-2672.2004.02516.x.

Simpson, P. J., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2005). Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage. *J Appl Microbiol* 99, 493–501. doi:10.1111/j.1365-2672.2005.02648.x.

Singer, M. A., and Lindquist, S. (1998). Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *Trends in Biotechnology* 16, 460–468. doi:10.1016/S0167-7799(98)01251-7.

Sollohub, K., and Cal, K. (2010). Spray drying technique: II. Current applications in pharmaceutical technology. *Journal of Pharmaceutical Sciences* 99, 587–597. doi:10.1002/jps.21963.

Sonck, K. A. J., Kint, G., Schoofs, G., Vander Wauven, C., Vanderleyden, J., and De Keersmaecker, S. C. J. (2009). The proteome of *Salmonella Typhimurium* grown under in vivo-mimicking conditions. *Proteomics* 9, 565–579. doi:10.1002/pmic.200700476.

Stack, H. M., Kearney, N., Stanton, C., Fitzgerald, G. F., and Ross, R. P. (2010). Association of beta-glucan endogenous production with increased stress tolerance of intestinal Lactobacilli. *Applied and Environmental Microbiology* 76, 500–507. doi:10.1128/AEM.01524-09.

Steed, H., Macfarlane, G. T., Blackett, K. L., Bahrami, B., Reynolds, N., Walsh, S. V., et al. (2010). Clinical trial: the microbiological and immunological effects of synbiotic consumption - a randomized double-blind placebo-controlled study in active Crohn's disease: Clinical trial: synbiotic therapy in Crohn's disease. *Alimentary Pharmacology & Therapeutics* 32, 872–883. doi:10.1111/j.1365-2036.2010.04417.x.

Streeter, J. G., and Gomez, M. L. (2006). Three enzymes for trehalose synthesis in *Bradyrhizobium* cultured bacteria and in bacteroids from soybean nodules. *Applied and Environmental Microbiology* 72, 4250–4255. doi:10.1128/AEM.00256-06.

Streit, F., Delettre, J., Corrieu, G., and Béal, C. (2008). Acid adaptation of *Lactobacillus delbrueckii* subsp. *bulgaricus* induces physiological responses at membrane and cytosolic levels that improves cryotolerance. *Journal of Applied Microbiology* 105, 1071–1080. doi:10.1111/j.1365-2672.2008.03848.x.

Su, M. S., Schlicht, S., and Gänzle, M. G. (2011). Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation. *Microb Cell Fact* 10, S8. doi:10.1186/1475-2859-10-S1-S8.

Sun, M., Wu, W., Liu, Z., and Cong, Y. (2017). Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *J Gastroenterol* 52, 1–8. doi:10.1007/s00535-016-1242-9.

Taverniti, V., Stuknyte, M., Minuzzo, M., Arioli, S., De Noni, I., Scabiosi, C., et al. (2013). S-Layer protein mediates the stimulatory effect of *Lactobacillus helveticu*s MIMLh5 on innate immunity. *Appl. Environ. Microbiol.* 79, 1221–1231. doi:10.1128/AEM.03056-12.

Teixeira, J. S., Seeras, A., Sanchez-Maldonado, A. F., Zhang, C., Su, M. S.-W., and Gänzle, M. G. (2014). Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*. *Food Microbiology* 42, 172–180. doi:10.1016/j.fm.2014.03.015.

Teixido, N., Canamas, T. P., Usall, J., Torres, R., Magan, N., and Vinas, I. (2005). Accumulation of the compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock cross-protection in the biocontrol agent *Pantoea agglomerans* CPA-2. *Lett Appl Microbiol* 41, 248–252. doi:10.1111/j.1472-765X.2005.01757.x.

Termont, S., Vandenbroucke, K., Iserentant, D., Neirynck, S., Steidler, L., Remaut, E., et al. (2006). Intracellular accumulation of trehalose protects *Lactococcus lactis* from freeze-drying damage and bile toxicity and increases gastric acid resistance. *Applied and Environmental Microbiology* 72, 7694–7700. doi:10.1128/AEM.01388-06.

Thierry, A., Deutsch, S.-M., Falentin, H., Dalmasso, M., Cousin, F. J., and Jan, G. (2011). New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *International Journal of Food Microbiology* 149, 19–27. doi:10.1016/j.ijfoodmicro.2011.04.026.

Thomas, S. R., and Trust, T. J. (1995). Tyrosine phosphorylation of the tetragonal paracrystalline array of aeromonas hydrophila: Molecular cloning and high-level expression of the S-layer protein gene. *Journal of Molecular Biology* 245, 568–581. doi:10.1006/jmbi.1994.0047.

Torino, M. I., Taranto, M. P., Sesma, F., and de Valdez, G. F. (2001). Heterofermentative pattern and exopolysaccharide production by *Lactobacillus helveticus* ATCC 15807 in response to environmental pH. *J Appl Microbiol* 91, 846–852. doi:10.1046/j.1365-2672.2001.01450.x.

Tsvetkova, N. M., Horvath, I., Torok, Z., Wolkers, W. F., Balogi, Z., Shigapova, N., et al. (2002). Small heat-shock proteins regulate membrane lipid polymorphism. *Proceedings of the National Academy of Sciences* 99, 13504–13509. doi:10.1073/pnas.192468399.

Valdés-Varela, L., Hernández-Barranco, A. M., Ruas-Madiedo, P., and Gueimonde, M. (2016). Effect of *Bifidobacterium* upon *Clostridium difficile* growth and toxicity when co-cultured in different prebiotic substrates. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.00738.

Valik, L., Medvedova, A., and Liptakova, D. (2009). Characterization of the growth of *Lactobacillus rhamnosus* GG in milk at suboptimal temperatures. 60–69.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., and Nasrulhaq Boyce, A. (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability—A review. *Molecules* 21, 573. doi:10.3390/molecules21050573.

Velly, H., Bouix, M., Passot, S., Penicaud, C., Beinsteiner, H., Ghorbal, S., et al. (2015). Cyclopropanation of unsaturated fatty acids and membrane rigidification improve the freeze-drying resistance of *Lactococcus lactis* subsp. *lactis* TOMSC161. *Appl Microbiol Biotechnol* 99, 907–918. doi:10.1007/s00253-014-6152-2.

Verma, R., Lee, C., Jeun, E.-J., Yi, J., Kim, K. S., Ghosh, A., et al. (2018). Cell surface polysaccharides of *Bifidobacterium bifidum* induce the generation of Foxp3 $^{+}$ regulatory T cells. *Sci. Immunol.* 3, eaat6975. doi:10.1126/sciimmunol.aat6975.

Vrancken, G., Rimaux, T., Wouters, D., Leroy, F., and De Vuyst, L. (2009). The arginine deiminase pathway of *Lactobacillus fermentum* IMDO 130101 responds to growth

under stress conditions of both temperature and salt. *Food Microbiology* 26, 720–727. doi:10.1016/j.fm.2009.07.006.

Walker, D. C., Girgis, H. S., and Klaenhammer, T. R. (1999). The groESL chaperone operon of *Lactobacillus johnsonii*. *Appl. Environ. Microbiol.* 65, 3033–3041.

Walsh, A. M., Crispie, F., Kilcawley, K., O'Sullivan, O., O'Sullivan, M. G., Claesson, M. J., et al. (2016). Microbial succession and flavor production in the fermented dairy beverage kefir. *mSystems* 1, e00052-16, /msys/1/5/e00052-16.atom. doi:10.1128/mSystems.00052-16.

Wang, X., Xiao, J., Jia, Y., Pan, Y., and Wang, Y. (2018). *Lactobacillus kefiranofaciens*, the sole dominant and stable bacterial species, exhibits distinct morphotypes upon colonization in Tibetan kefir grains. *Heliyon* 4, e00649. doi:10.1016/j.heliyon.2018.e00649.

Weng, Y., Chen, F., Liu, Y., Zhao, Q., Chen, R., Pan, X., et al. (2016). *Pseudomonas aeruginosa* enolase influences bacterial tolerance to oxidative stresses and virulence. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.01999.

Wood, J. M. (1999). Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol. Mol. Biol. Rev.* 63, 230–262.

Yadav, A. K., Tyagi, A., Kaushik, J. K., Saklani, A. C., Grover, S., and Batish, V. K. (2013). Role of surface layer collagen binding protein from indigenous *Lactobacillus plantarum* 91 in adhesion and its anti-adhesion potential against gut pathogen. *Microbiological Research* 168, 639–645. doi:10.1016/j.micres.2013.05.003.

Yoo, D., Bagon, B. B., Valeriano, V. D. V., Oh, J. K., Kim, H., Cho, S., et al. (2017). Complete genome analysis of *Lactobacillus fermentum* SK152 from kimchi reveals genes associated with its antimicrobial activity. *FEMS Microbiology Letters* 364. doi:10.1093/femsle/fnx185.

Young, G. R., Smith, D. L., Embleton, N. D., Berrington, J. E., Schwalbe, E. C., Cummings, S. P., et al. (2017). Reducing viability bias in analysis of gut microbiota in preterm infants at risk of NEC and sepsis. *Front. Cell. Infect. Microbiol.* 7, 237. doi:10.3389/fcimb.2017.00237.

Yu, Y., Zhu, X., Shen, Y., Yao, H., Wang, P., Ye, K., et al. (2015). Enhancing the vitamin B12 production and growth of *Propionibacterium freudenreichii* in tofu wastewater via a light-induced vitamin B12 riboswitch. *Appl Microbiol Biotechnol* 99, 10481–10488. doi:10.1007/s00253-015-6958-6.

Zayed, G., and Roos, Y. H. (2004). Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. *Process Biochemistry* 39, 1081–1086. doi:10.1016/S0032-9592(03)00222-X.

Zhao, G., and Zhang, G. (2005). Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *J Appl Microbiol* 99, 333–338. doi:10.1111/j.1365-2672.2005.02587.x.

Zorriehzahra, M. J., Delshad, S. T., Adel, M., Tiwari, R., Karthik, K., Dhama, K., et al. (2016). Probiotics as beneficial microbes in aquaculture: an update on their multiple modes of action: a review. *Veterinary Quarterly* 36, 228–241. doi:10.1080/01652176.2016.1172132.

Table of figures

Table of tables

Annexes

Matériels & Méthodes

7.1 Réactifs

Lorsque la marque n'est pas spécifiée, les réactifs sont fournis par Sigma Aldrich (Saint-Quentin Fallavier, France). L'extrait de levure et la tryptone proviennent de Biokar Diagnostics, Beauvais, France. Le lactosérum doux déshydraté de Lactalis, Laval, France.

7.2 Microbiologie

7.2.1 Souches bactériennes

7.2.1.1 Origine des souches

Les souches de propionibactéries utilisées lors de ce travail sont conservées au laboratoire UMR STLO (Science et Technologie du Lait et de l'OEuf, INRA-Agrocampus, Rennes, France) dans la collection du CIRM-BIA (Centre International de Ressources Microbiennes - Bactéries d'Intérêt Alimentaire). Les souches CIRM-BIA 129 et CIRM-BIA 1025, propriétées du CNIEL (Centre National Interprofessionnel de l'Économie Laitière), ont été isolées par Actalia de fromages à pâte pressée cuite non-ensemencée (Jean-René Kerjean, Actalia, communication personnelle).

La souche *Lactobacillus rhamnosus* CIRM-BIA 1113 utilisée par Bioprox et étudiée dans cette thèse, est également conservée dans la collection du CIRM-BIA.

7.2.1.2 Conservation et remise en culture des souches

Toutes les souches utilisées dans ce travail sont conservées à -80°C en cryotubes dans du milieu YEL ("Yeast Extract Lactate", (Malik et al., 1968)) pour *P. freudenreichii* et dans du milieu MRS pour *L. rhamnosus*, contenant 15 % de glycérol (v/v). La composition du milieu YEL est la suivante :

- Tryptone[1] 10 $g.L^{-1}$
- Extrait de levure1 10 $g.L^{-1}$
- DL-Lactate de sodium 12,6 $g.L^{-1}$
- MnSO4, H2O2 0,056 $g.L^{-1}$
- K2HPO4, 3H2O3 0,33 $g.L^{-1}$

- Eau distillée Qsp 1 litre

Le pH est ajusté à 7,0 ± 0,2 avant autoclavage (120°C, 15 min).

La composition du MRS est la suivante :

- Peptone 10 $g.L^{-1}$
- Extrait de viande 8 $g.L^{-1}$
- Extrait de levure 4 $g.L^{-1}$
- Glucose 20 $g.L^{-1}$
- Acétate de sodium trihydraté 5 $g.L^{-1}$
- Citrate d'ammonium 2 $g.L^{-1}$
- Tween 80 1 mL
- Hydrogénophosphate de potassium 2 g
- Sulfate de magnésium heptahydraté 0,2 g
- Sulfate de manganèse tétrahydraté 0,05 g
- Eau distillée Qsp 1 L

Le pH est ajusté à 6.7 ± 0,2 avant autoclavage (120°C, 15 min)

Les pré-cultures de *P. freudenreichii* sont réalisées soit en YEL, soit dans les milieux indiqués ci-dessous, à 30°C sans agitation pendant 2 jours (phase stationnaire). Les pré-cultures de *L. rhamnosus* sont réalisées dans du MRS pendant 24h (phase stationnaire). Les pré-cultures sont préparées comme suit : chaque souche est décongelée puis subit trois repiquages à 2 % (v/v) dans le même milieu de culture pendant 2 jours. Ceci permet d'éliminer le glycérol avant utilisation, qui peut être utilisé comme source de carbone par les bactéries propioniques laitières. Un maximum de 5 repiquages successifs est effectué à partir d'un stock congelé. Les pré-cultures et cultures sont incubées à 30°C pour *P. freudenreichii* et 37°C pour *L. rhamnosus*, sans agitation, en tubes ou flacons non-hermétiques à l'air (conditions micro-aérophile). En routine, des tubes de verre contenant 10 mL de YEL ou MRS (la moitié du volume total du tube) sont utilisés pour les pré-cultures. Des flacons de verre de 100 mL sont utilisés pour les cultures de *P. freudenreichii* à l'échelle du laboratoire. Des fermenteurs de 250 mL ont été utilisés dans les laboratoires de Bioprox pour les cultures de *L. rhamnosus*. Pour les cultures de *P. freudenreichii*, des flacons de verre de type « Schott » de 2 L et de 5 L pour les cultures séchées sur la tour Minor. Alors que pour les expérimentations de séchage de *L. rhamnosus*, les cultures ont été réalisées à 35°C dans des fermenteurs de 30 L dans le laboratoire de Bioprox.

La croissance de *P. freudenreichii*, qui s'accompagne d'une fermentation de l'acide lactique en acides propionique et acétique, se fait sans acidification. La croissance de *L. rhamnosus* se fait

avec production d'acide lactique et donc une diminution du pH. Dans les fermenteurs, le pH est donc régulé à 5,7 par ajout d'ammoniac. La croissance est suivie par mesure de la densité optique à 650 nm. Les dénombrements de *P. freudenreichii* (CFU/mL) sont effectués sur milieu gélosé YEL-Agar, après incubation à 30°C en anaérobiose (Anaerocult, Merck, France), pendant 5 jours. Les dénombrements de *L. rhamnosus* (CFU/mL) sont effectués sur milieux MRS-Agar après incubation à 37°C pendant 48 h.

7.2.2 Milieux de culture

7.2.2.1 Les milieux de laboratoire YEL et MRS

Le milieu YEL est un milieu riche de laboratoire spécifiquement adapté à la croissance des bactéries propioniques. Ce milieu sert de référence lors de la comparaison de croissance avec d'autres milieux. La croissance des propionibactéries sur ce milieu se fait sans acidification.

Le milieu MRS est également un milieu riche de laboratoire adapté à la croissance des lactobacilles. Le milieu MRS servira pour les pré-cultures de *L. rhamnosus*. Bioprox utilisant un milieu de culture proche du MRS (qui sera appelé dans le manuscrit MRS-like) pour la production industrielle du *L. rhamnosus* CIRM-BIA 1113. La source de carbone du milieu MRS-like est le trehalose.

7.2.2.2 Milieu chimiquement défini MMO

Le milieu chimiquement défini MMO a été décrit précédemment (Leverrier et al., 2004). Sa composition est la suivante.

Sodium lactate 12,8 g/L, KH_2PO_4 0,6 g/L , Potassium acétate 0,4 g/L, $MgSO_4$.7H2O 50 mg /L, $MnSO_4$.4H2O 5 mg/L, $FeSO_4$.7H2O 2.5 mg/L, CuSO4 2.5 mg/L, Cobalt acétate 0,25 mg/L, $ZnSO_4$ 15 µg/L, H3BO3 1 µg/L ,Na_2MoO_4 1 µg/L, Thiamine 50 µg/L, Pyridoxal 100 µg/L, Calcium pantothénate 50 µg/L, Riboflavine 50 µg/L, Nicotinamide 100 µg/L, p-Aminobenzoic acid 10 µg/L, Biotine 4 µg/L, Folic acid 20 µg/L, Cyanocobalamine 2 µg/L, L-Ala 50 mg/L, L-Arg 160 mg/L, L-Asn 150 mg/L, L-Asp 250 mg/L, L-Cys 140 mg/L, Gly 80 mg/L, L-His 100 mg/L, L-Ile 180 mg/L, L-Leu 300 mg/L, L-Lys 220 mg/L, DL-Met 60 mg/L, L-Phe 170 mg/L, L-Ser 180 mg/L, L-Thr 150 mg/L, L-Trp 50 mg/L, L-Tyr 60 mg/L, DL-Val 480 mg/L

Adenine 5 mg/L, Guanine 5 mg/L, Uracile 5 mg/L, Xanthine 5 mg/L.

Le pH est ajusté à 7.0 et le milieu est ensuite stérilisé par filtration (Top filter PES, 0,2 µm, Nalgene Company, NY, USA).

7.2.2.3 Ultrafiltrat de lait et lactosérum

L'ultrafiltration du lait écrémé cru de vache Holstein a été réalisée comme décrit précédemment (Cousin et al., 2012) avec le pilote TIA (Bollène, France) de la plateforme technologique de l'INRA STLO équipé de membranes spirales (surface: 2x 1,08 m^2; seuil de coupure: 5 kDa). L'ultrafiltrat de lait a été récolté puis stérilisé par filtration à 0,2 µm (Top filter PES, 0,2 µm, Nalgene Company, NY, USA). L'ultrafiltrat de lait est un milieu limpide qui permet un suivi de croissance bactérienne selon les méthodes classiques, notamment par densité optique, ce qui n'est pas possible dans du lait compte tenu de son opacité. Sa composition correspond strictement à celle de la phase soluble du lait (c'est à dire de la fraction du lait exempte de protéines et de matière grasse). Il contient notamment du lactose, des minéraux, des vitamines et de l'azote non protéique (urée). Pour la croissance des bactéries propioniques, il a été supplémenté de 100 mM de lactate (sirop de lactate de sodium 60 %, qualité alimentaire, > 97 % isomère L, galaflow, Société Arnaud, France). Le pH est ajusté à 7.0 et le milieu est ensuite stérilisé par filtration (Top filter PES, 0,2 µm, Nalgene Company, NY, USA).

Un lactosérum doux commercial (Sweet Whey, Lactalis, Laval, France) a également été utilisé comme milieu de culture. Il est reconstitué à 50 $g.L^{-1}$ dans de l'eau distillée et stérilisé par autoclavage (110°C, 15 min). L'autoclavage se fait ici à plus basse température pour limiter les réactions de Maillard.

7.2.2.4 Modifications du milieu de laboratoire YEL

Dans le but de se rapprocher de la composition du lactosérum (Chapitre 4), du lactose a été ajouté au YEL (YEL+Lacose) pour obtenir la même concentration que dans le lactosérum doux (34 $g.L^{-1}$). Dans un secondt temps (Chapitre 6), le lactose a été remplacé par du glucose à la demande de l'industriel pour se rapprocher des conditions industrielles et éviter l'utilisation du lactose qui n'est pas souhaité par les consommateurs. Le glucose a été utilisé à la même concentration que le lactose. Les solutions stock (170 $g.L^{-1}$) de lactose et de glucose ont été préparées et autoclavées (120°C, 15 min) séparément du reste du milieu de culture pour limiter les réactions de Maillard et ajoutées extemporanément à la concentration souhaitée.

7.2.3 Suivi de culture

7.2.3.1 Inoculation

Pour les expérimentations avec *P. freudenreichii*, les différents milieux de culture, thermostatés à 30°C, sont inoculés à 2 % (v/v) avec une pré-culture réalisée dans le même milieu, puis agités doucement. De même, les inoculations de *L. rhamnosus* sont réalisées dans des milieux thermostatés à 35°C, et inoculés à 1% (v/v), avec une pré-culture réalisée dans le même milieu.

7.2.3.2 Suivi de la croissance

Les cultures de *P. freudenreichii* ont été réalisées en flacons alors que les cultures de *L. rhamnosus* ont été réalisées en fermenteurs.

La croissance bactérienne en milieu de culture liquide est suivie :

- par mesure de la densité optique des cultures bactériennes à 650 nm à l'aide d'un spectrophotomètre Beckman (model DU 7400, Beckmann Instruments, Etats-Unis). Lorsque la densité optique est supérieure à 0,8, la culture est systématiquement diluée dans le milieu de culture stérile avant mesure.

Le suivi de la densité optique à 650 nm a permis de déterminer le taux de croissance μ (h^{-1}), durant la phase exponentielle de croissance. Le taux de croissance népérien est le coefficient directeur de la pente obtenue en traçant la courbe ln(DO650nm) = f(temps). Ce taux de croissance népérien permet ensuite de calculer le temps de génération (G, h) $G = \ln(2) / \mu$.

- par mesure de la viabilité cellulaire en utilisant la microméthode de Baron et al., 2006 : des dilutions décimales successives (en cascade) de la culture sont réalisées dans le diluant peptone-sel (peptone Biokar Diagnostics 1,0 $g.L^{-1}$; NaCl 8,5 $g.L^{-1}$, le pH est ajusté à 7,0 ± 0,2 avant autoclavage, norme ISO 8261-FIL 122, 2001), puis ensemencées dans la masse dans du milieu gélosé YEL-Agar ou MRS-Agar (YEL et MRS additionnés d'agar à 10 et 11 $g.L^{-1}$, respectivement, Merck, (Hettinga *et al.*, 1968)). L'incubation des boîtes de dénombrements est réalisée en anaérobiose (Anaerocult, Merck) à 30°C pendant 5 à 7 jours.

- La croissance de *L. rhamnosus* est suivie par une mesure cinétique de la quantité d'ammoniac ajoutée pour ajuster le pH à la valeur consigne de 5,7 pendant la fermentation. Lorsque la chute du pH est stoppée, et donc que l'ajout de d'ammoniac n'est plus nécessaire, les différentes adaptations et analyses sont effectuées.

7.2.4 Adaptations bactériennes

Différentes adaptations ont été testées sur *P. freudenreichii* et *L. rhamnosus*. L'osmoadapatation a eu lieu pendant ou après la croissance, tandis que les adaptations acide et thermique ont eu lieu après la croissance (Figures 5 et 6).

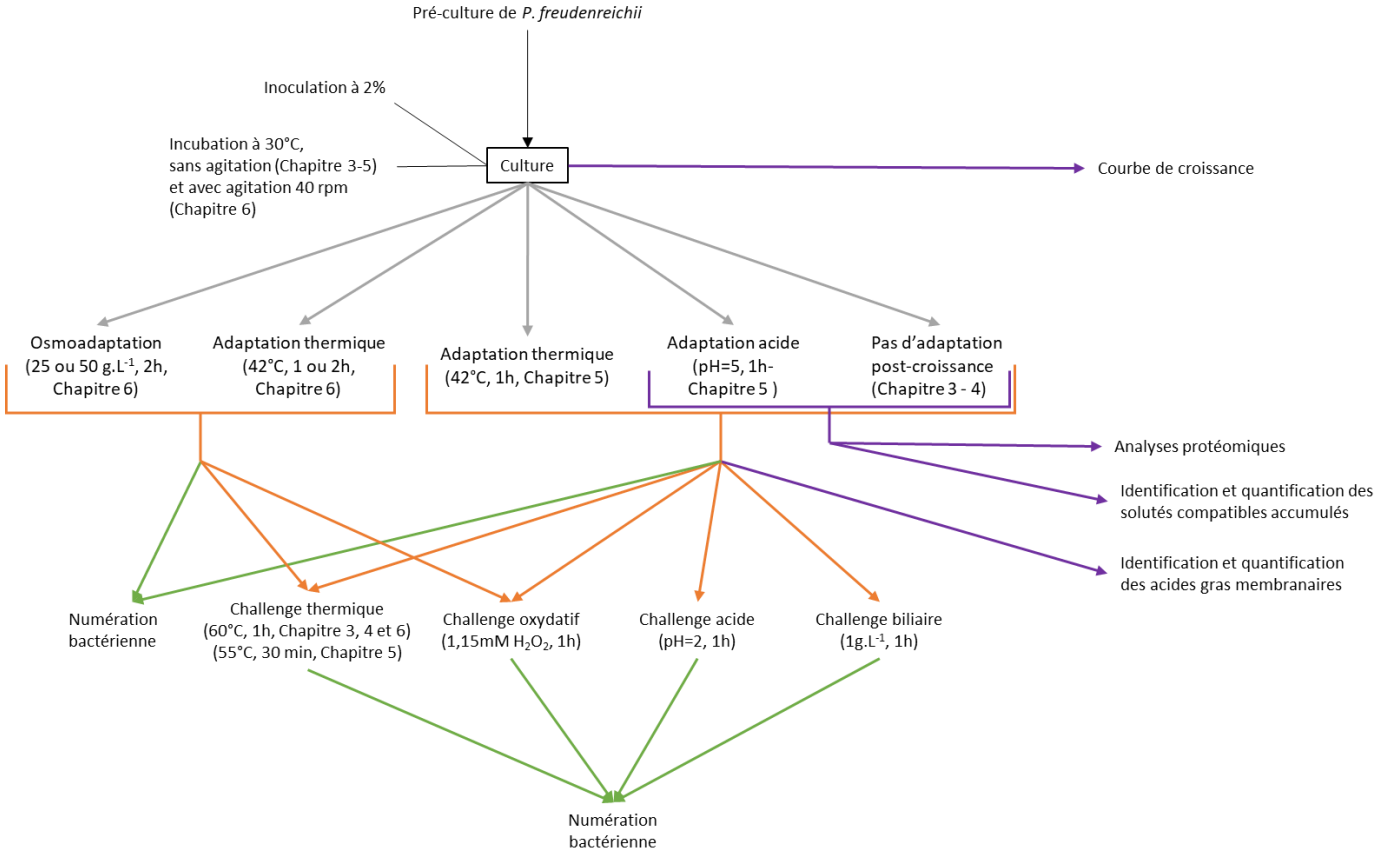


Figure 36 : Schéma représentant les adaptations réalisées sur *P. freudenreichii* et les analyses physiologiques et omiques Les cultures de *P. freudenreichii* ont été réalisées dans différents milieux : MMO, MMO+NaCl, MMO+NaCl+GB (Chapitre 3), YEL, YEL+NaCl (Chapitre 4 et 5), YEL+Lactose, YEL+Lactose+NaCl, MU, MU+NaCl, SW et SW+NaCl (Chapitre 4), YEL+15g Glucose et YEL+30g Glucose (Chapitre 6). *P. freudenreichii* a ensuite subi des adaptations thermique, osmotique et acide en début de phase stationnaire (gris). Les challenges thermique, oxydatif, biliaire et acide ont été réalisés après adaptation (orange). De plus les analyses omiques ont été réalisées au cours de la phase exponentielle (Chapitre 4 et 5) ou au début de la phase stationnaire après adapatation le cas écheant (chapitre 5 et 6).

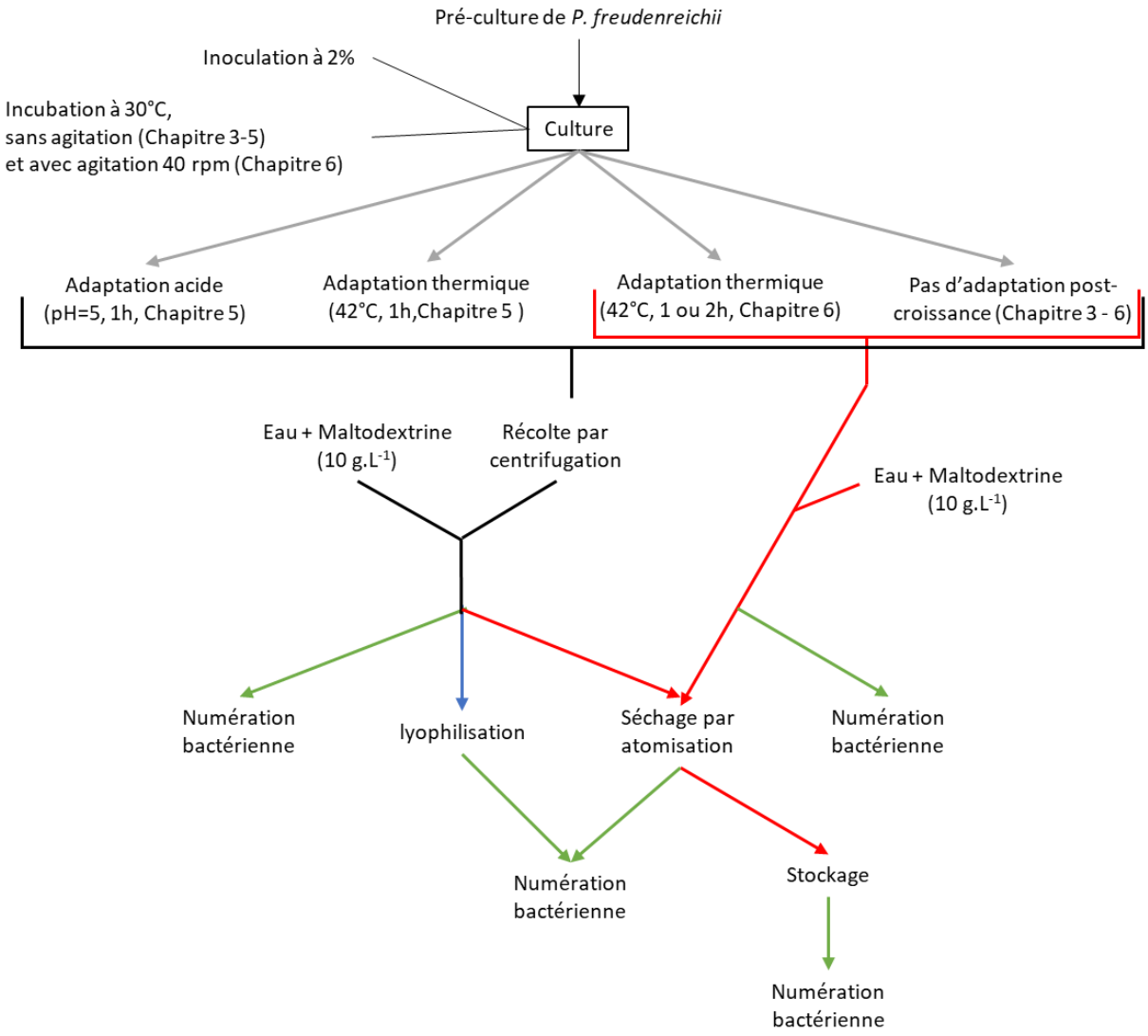


Figure 37 : Schéma représentant les intinéraires technologiques avant séchages par lyophilisation ou pulvérisation *P. freudenreichii* a été cultivé dans différent milieux de culture : YEL, YEL+NaCl (Chapitre 4 et 5), YEL+Lactose, YEL+Lactose+NaCl, (Chapitre 4), YEL+15g Glucose et YEL+30g Glucose (Chapitre 6). Les adaptations acide (Chapitre 5 et 6) et thermique (Chapitre 6) ont ensuite été réalisées si nécessaire. Certaines cultures (Chapitre 4 et 5) ont été centrifugées pour recolter les cellules. Les cellules ont ensuite été homogénéisées dans une solution d'eau contenant de la maltodextrine. Pour les autres cultures, de la maltodextrine a été ajoutée (concentration finale 200 $g.L^{-1}$, Chapitre 6). Les solutions ont ensuite été séchées par lyophilisation ou par séchage par pulvérisation.

7.2.4.1 Croissance en conditions de stress osmotique

P. freudenreichii CIRM-BIA 129 et CIRM-BIA 1025 ont été cultivés dans différentes conditions de stress osmotique : milieu YEL (0,429 osmol), YEL avec 0,9 M NaCl (YEL+NaCl, 2,028 osmol), YEL avec 34 $g.L^{-1}$ de Lactose et 0,9 M NaCl (YEL+Lactose+NaCl, 2,175 osmol), milieu MMO, MMO avec NaCl 0,4 M (MMO+NaCl, 0,958 osmol), MMO avec 0,4 M NaCl et 1 mM de glycine bétaïne (MMO+NaCl+GB 0,960 osmol). Le lactosérum et le perméat d'ultrafiltration ont également été additionnés de 0,7 M NaCl : (SW+NaCl, 1,903 osmol et MU+NaCl, 1,996 osmol). 0,4M NaCl est la plus forte concentration permettant la croissance en milieu MMO. 0,9 M NaCl est la plus forte concentration permettant la croissance en milieu riche YEL, et 0,7 M NaCl est la plus forte concentration permettant la croissance dans le lactosérum et le perméat d'ultrafiltrat de lait. La concentration de glycine betaïne utilisée pour restaurer la croissance en milieu MMO, 1 mM, est celle décrite comme optimale pour les actinobactéries *Brevibacterium ammoniagenes* (Gouesbet et al., 1992) et *Propionibacterium freudenreichii* (Leverrier et al., 2003).

7.2.4.2 Adaptation thermique et acide

P. freudenreichii CIRM-BIA 129 a été cultivé dans du YEL ou YEL+NaCl puis une adaptation thermique ou acide a été réalisée en début de phase stationnaire. L'adaptation thermique a été effectuée à 42°C durant 1h, comme réalisé par Anastasiou et al., 2006. Un Falcon 50 mL contenant 25 mL de culture a été placé dans un bain-marie à 42°C. La durée d'incubation était mesurée lorsque la culture atteignait 42°C. Pour l'adaptation acide, le pH de la culture est ajusté à 5, puis la culture est incubée 1 h à 30°C (Jan et al., 2001).

7.2.4.3 Optimisation des adaptations de *P. freudenreichii*

Dans le dernier chapitre (Chapitre 6), l'osmoadaptation est réalisée en début de phase stationnaire pour ne pas augmenter le temps de génération. Le glucose a été utilisé à la place du lactose, car l'industriel souhaite réaliser des produits « lactose-free ». De plus, l'adaptation thermique a été décrite comme augmentant la survie de *Lactobacillus paracasei* durant le séchage par pulvérisation (Desmond et al., 2001). Le logiciel JMP de SAS a été utilisé pour étudier l'impact de l'addition de glucose, la thermo-adaptation et l'osmoadaptation sur la résistance de *P. freudenreichii* aux challenges thermique et oxydatif. Durant le séchage par pulvérisation, les bactéries souffrent de stress oxydatif et thermique. *P. freudenreichii* a été cultivé à 30°C pendant 32 à 46 h avec une agitation douce (40 rpm) pour diminuer le temps de génération (Arnaud et al., 1992; Demirtas et al., 2003). Au début de la phase stationnaire, le sel (NaCl) a été ajouté aux cultures avec des concentrations comprises entre 0 et 0,9 M, les cultures

ont ensuite été incubées pendant 2 h à 30°C. Puis les cultures ont été chauffées à 42°C pendant 0, 1, ou 2 h. Les adaptations bactériennes ont été définies par les plans d'expériences suivants (Tableau 1).

Tableau 19 : Arrangement du plan expérimental pour les variables indépendantes utilisées et leur niveau

	Matrice de conception			Matrice de travail		
Echantillons	Concentration en NaCl	Concentration en glucose	Durée de l'adaptation thermique	Concentration en NaCl ($g.L^{-1}$)	Concentration en glucose ($g.L^{-1}$)	Durée de l'adaptation thermique (h)
1	-1	-1	-1	0	0	0
2	-1	-1	0	0	0	1
3	-1	-1	1	0	0	2
4	-1	0	-1	0	15	0
5	-1	0	0	0	15	1
6	-1	0	1	0	15	2
7	-1	1	-1	0	30	0
8	-1	1	0	0	30	1
9	-1	1	1	0	30	2
10	0	-1	-1	25	0	0
11	0	-1	0	25	0	1
12	0	-1	1	25	0	2
13	0	0	-1	25	15	0
14	0	0	0	25	15	1
15	0	0	0	25	15	1
16	0	0	0	25	15	1
17	0	0	1	25	15	2
18	0	1	-1	25	30	0
19	0	1	0	25	30	1
20	0	1	1	25	30	2
21	1	-1	-1	50	0	0
22	1	-1	0	50	0	1
23	1	-1	1	50	0	2
24	1	0	-1	50	15	0
25	1	0	0	50	15	1
26	1	0	1	50	15	2
27	1	1	-1	50	30	0
28	1	1	0	50	30	1
29	1	1	1	50	30	2

7.2.4.4 Optimisation des adaptations de *L. rhamnosus*

Le logiciel JMP de SAS a été utilisé pour étudier l'impact de l'addition de tréhalose, la thermo-adaptation et l'osmoadaptation sur la résistance de *L. rhamnosus* aux challenges thermique et oxydatif. De la même façon que pour *P. freudenreichii*, les adaptations de *L. rhamnosus* ont été réalisées au début de la phase stationnaire. Bioprox utilise du tréhalose comme source de carbone pour le *L. rhamnosus*. Durant le plan d'expérience nous avons fait varier la concentration en tréhalose de 80 à 120 $g.L^{-1}$, la concentration en NaCl ajouté en fin de croissance de 0 à 20 $g.L^{-1}$ (incubation 2 h à 35°C), et la durée de l'adaptation thermique entre 0 et 3 h. Les adaptations ont été définies par le plan d'expérience (Table 2).

Table 20 : Arrangement du plan expérimental pour les variables indépendantes utilisées et leur niveau

	Matrice de conception			Matrice de travail		
Echantillons	Concentration en NaCl	Concentration en glucose	Durée de l'adaptation thermique	Concentration en NaCl ($g.L^{-1}$)	Concentration en glucose ($g.L^{-1}$)	Durée de l'adaptation thermique (h)
1	-1	-1	-1	0	80	0
2	-1	-1	0	0	80	1,5
3	-1	-1	1	0	80	3
4	-1	1	-1	0	120	0
5	-1	1	0	0	120	1,5
6	-1	1	1	0	120	3
7	0	-1	-1	10	80	0
8	0	-1	0	10	80	1,5
9	0	-1	1	10	80	3
10	0	1	-1	10	120	0
11	0	1	0	10	120	1,5
12	0	1	1	10	120	3
13	1	-1	-1	20	80	0
14	1	-1	0	20	80	1,5
15	1	-1	1	20	80	3
16	1	1	-1	20	120	0
17	1	1	0	20	120	1,5
18	1	1	1	20	120	3
19	0	0	0	10	100	0
20	0	0	0	10	100	1,5
21	0	0	0	10	100	3

7.2.5 Tolérance au stress

7.2.5.1 Challenges

Les challenges ont été appliqués sur des cultures en début de phase stationnaire, lorsque la DO maximale était atteinte. Pour *P. freudenreichii*, les challenges ont été réalisés sur des cultures de 100 mL de la façon suivante (Fig. 4):

- Le challenge thermique est réalisé en plaçant 2 mL de culture (dans un tube Falcon de 15 mL en polypropylène) à 60°C pendant 10 min.
- Le challenge oxydatif en ajoutant du peroxyde d'hydrogène qsp 1,15 mM (Labogros, France) pendant 1 h à 30°C.
- Le challenge acide est réalisé en remettant en suspension les bactéries dans 2 mL de milieu MMO acidifié à pH 2,0 avant une incubation d'une heure à 30°C.
- Le challenge sels biliaires est réalisé en ajoutant un mélange équimolaire de cholate et désoxycholate (Sigma Chemical, St Louis, USA), sels biliaires naturellement présents dans le duodénum, à raison de 1 $g.L^{-1}$, avant une incubation d'une heure à 37°C (Leverrier et al., 2003).

Pour *L. rhamnosus* seul un challenge thermique et un challenge oxydatif ont été réalisés. Ces challenges ont des barèmes moins élevés, car *L. rhamnosus* est moins résistant que *P. freudenreichii* et nous souhaitions que les challenges soient discriminants. Les challenges ont été réalisés sur des cultures de 200 mL de la façon suivante :

- Le challenge thermique est réalisé en plaçant 2 mL de culture (dans un tube Falcon de 15 mL en polypropylène) à 55°C pendant 5 min.
- Le challenge oxydatif en ajoutant du peroxyde d'hydrogène qsp 0,0625 mM (Labogros, France) pendant 15 min à 35°C.

7.2.5.2 Détermination de la survie

La survie est suivie aux temps indiqués ci-dessus. Les cultures stressées sont diluées rapidement au dixième dans de l'eau peptonée et des dilutions décimales en cascade sont réalisées. Chaque dilution est ensemencée dans la masse dans du milieu gélosé YEL-Agar ou MRS-Agar. La numération des colonies est réalisée après 5 jours d'incubation en anaérobiose à 30°C pour *P. freudenreichii* ou 48h à 37°C pour *L. rhamnosus*. Chaque expérience est réalisée au moins trois fois. Le pourcentage de survie aux stress est obtenu en faisant le rapport entre la population présente en fin de challenge et la population présente initialement à T_0.

7.2.6 Séchage des bactéries par pulvérisation

7.2.6.1 Séchage par pulvérisation de *P. freudenreichii*

Dans un premier temps, nous avons souhaité connaître l'impact des adaptations de *P. freudenreichii* sur la survie au séchage par pulvérisation. Le sel contenu dans les milieux de cultures a un impact durant le séchage par pulvérisation. Nous avons donc choisi de récolter les cellules puis de les re-suspendre dans une matrice de séchage pour enlever l'effet du sel (Fig. 5). Après culture de *P. freudenreichii* en YEL, YEL+NaCl, YEL+Lactose, et YEL+Lactose+NaCl dans des flacons de 5 L, les bactéries ont été récoltées par centrifugation (8000 g, 15 min). Les bactéries ont été re-suspendues dans une solution aqueuse de maltodextrine (DE= 6-8) (20% p/p). Ces suspensions (2 L) ont été agitées pendant 10 min et thermostatées à 20°C avant pulvérisation et séchage à l'échelle pilote (GEA Niro A/S, Mobile Minor Dryer (MMD), Soeborg, Denmark). Une buse bi-fluides, d'un diamètre interne de 0,8 mm, ainsi qu'un pompe péristaltique (débit 25-35 $mL.min^{-1}$, 520S, Watson-Maslow, France) ont été utilisées pour la pulvérisation. La température d'air de sortie a été contrôlée à 60 ± 2°C, en ajustant le débit d'alimentation contrôlé par la pompe péristaltique. La poudre a été immédiatement recueillie et la viabilité déterminée par numération sur milieu YEL-Agar avant et après séchage comme décrit (Huang et al., 2016a). Les poudres ont été collectées et stockées à une température de 4, 20, ou 37°C à l'abri de la lumière pendant 145 jours.

Dans un second temps, les différentes cultures (YEL, YEL+15 g Glucose,+42°C,1 h et YEL+30 g Glucose+42°C, 2 h), en début de phase stationnaire, ont été additionnées de 20% (p/p) de maltodextrine (DE = 6–8). Ces suspensions ont ensuite été homogénéisées et séchées comme précédemment. Dans cette expérimentation les bactéries n'ont pas été récoltées par centrifugation et séparées de leur surnageant de culture, car ces milieux ne contenaient pas de sel (Fig. 5).

L'acivité de l'eau (a_w) a été mesurée comme décrit par Schuck et al., 2012. La viabilité de *P. freudenreichii* est estimée grâce aux numérations avant et après séchage.

7.2.6.2 Séchage par pulvérisation de *L. rhamnosus*

L. rhamnosus a été cultivé dans du MRS-like (Bioprox, France) contenant 80 et 105 $g.L^{-1}$ de tréhalose. En fin de phase stationnaire, les bactéries ont été récoltées par centrifugation puis ont été re-suspendues dans une solution aqueuse de maltodextrine à 20% (DE= 6-8) (p/p). Deux essais de séchage par pulvérisation ont été réalisés pour chaque culture sur le même pilote MMD

(GEA Niro A/S, Soeborg, Denmark) avec une température d'entrée fixée à 150°C: le premier essai a été conduit avec une température de sortie de 60 ± 2°C, et le deuxième essai avec une température de sortie de 50 ± 2°C.

Les échantillons ont également été séchés en utilisant la tour PolarDry®. Durant ce procédé, la solution est pulvérisée dans la chambre. Une charge électrique est alors appliquée aux gouttelettes. Les gouttelettes sont donc polarisées (Fig. 6). L'eau, qui est plus polaire, migre dans la couche externe de la gouttelette. La polarisation des gouttelettes permet une meilleure élimination de l'eau. L'azote a été utilisé comme gaz vecteur. L'azote est plus hydrophile que l'air, ce qui facilite le séchage et permet de diminuer la température de séchage. La température d'entrée a été fixée à 120°C, et la température de sortie est de 50 ± 4°C. La température de sortie est ajustée en jouant sur le débit de la solution à sécher. La tour PolarDry® a une capacité d'évaporation de 1 $kg.h^{-1}$ et un flux maximum d'azote de 8,5 $m^3.h^{-1}$. Cette expérimentation pilote a été réalisée une seule fois, dans les locaux de LIS (Lesaffre Ingredients Services), en concertation avec Bioprox, comme une première tentative.

L'aw finale est contrôlée selon la même méthode que décrit en section 7.2.6.1. La viabilité de *L. rhamnosus* est estimée grâce aux numérations avant et après séchage.

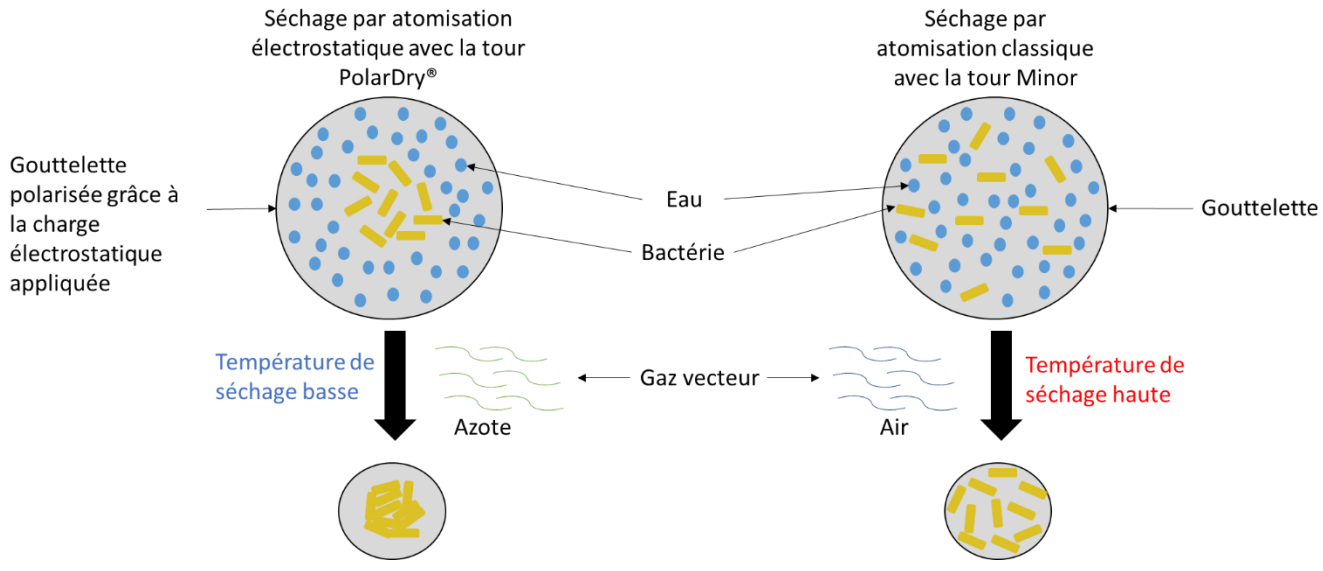


Figure 38: Comparaison entre les tours de séchage minor et PolarDry®. Les bactéries sont représentées en jaune, l'eau en bleu. Les schémas mettent en évidence l'organisation des gouttelettes. Les gaz vecteurs (azote ou air, selon la technologie) sont indiqués pour chaque procédé de séchage.

7.2.7 Séchage des bactéries par lyophilisation

7.2.7.1 Séchage par lyophilisation de *P. freudenreichii*

Les cultures en phase stationnaire de croissance (environ 10^9 propionibactéries par mL), après adaptation s'il y a eu lieu, ont été centrifugées (8000 g, 10 min) et les culots re-suspendus dans une solution de maltodextrine à 100 $g.L^{-1}$ (Roquette, France) (Fig. 5). Lors des premières expérimentations (Chapitre 3 et 4), les solutions contenaient $1x10^9$ $UFC.mL^{-1}$, alors que pour les deuxièmes expérimentations (Chapitre 5), les solutions contenaient $2x10^9$ $UFC.mL^{-1}$. 300 µL de la solution ont été ajoutés dans un eppendorf de 2 mL. L'épaissseur de la couche à lyophiliser était de 1 cm. Ces suspensions ont été congelées immédiatement à -20°C pendant 24 h dans des plateaux en acier inoxydable. Ces plateaux ont été rapidement transférés dans un lyophilisateur à plateaux (SGD Serail type, CIRP, France). Suite à une descente en pression progressive, la lyophilisation a eu lieu sous pression de vide 300-500 µbar, à 25°C, pendant 24 h. Les numérations bactériennes ont été effectuées avant et après la lyophilisation afin de calculer le pourcentage de survie. Les expérimentations ont été réalisées en triplicata.

7.2.7.2 Séchage par lyophilisation de *L. rhamnosus*

La lyophilisation des cultures de *L. rhamnosus* a été réalisée dans le laboratoire de Bioprox. A la fin de la phase stationnaire, et après adaptations si nécessaire, les bactéries ont été récoltées par centrifugation puis homogénéisées dans une solution de maltodextrine à 200 $g.L^{-1}$. Les échantillons ont été placés en barquettes (avec une épaisseur à lyophiliser de 1cm), puis ont été congelés à -25°C dans le pilote de lyophilisation. La pression de la chambre était descendue à -250 µbar. Un second séchage a été réalisé à 25°C pendant 5 h, le vide a ensuite été rompu par injection d'air. Les numérations bactériennes ont été effectuées avant et après la lyophilisation afin de calculer le pourcentage de survie.

7.2.8 Contrôle de l'a_w des poudres contenant *L. rhamnosus*

Les poudres contenant *L. rhamnosus* obtenues par séchage par atomisation et lyophilisation ont été déshydratées par séchage sous vide dans le but d'obtenir une aw constante de 0,025. Les poudres ont été introduites dans le lyophilisateur à une température de 25°C. La pression de la chambre a été diminuée jusqu'à 50 µbar, sans refroidissement. Après 5 h de désorption, le vide est cassé par injection d'air. L'aw finale a été contrôlée selon la même méthode que décrit en section 7.2.6.1.

7.2.9 Revivification des poudres

7.2.9.1 Mesure de la survie de *P. freudenreichii* dans les poudres

Les poudres, à différents temps de stockage, ont été réhydratées à l'aide d'eau distillée stérile de façon à atteindre la concentration (extrait sec) initiale avant séchage. Des dilutions décimales en cascade ont été réalisées dans de l'eau peptonée. Chaque dilution a été ensemencée dans la masse dans du milieu gélosé YEL-Agar. La numération des colonies a été réalisée après 7 jours d'incubation en anaérobiose à 30°C. Chaque expérience a été réalisée au moins trois fois. Le pourcentage de survie aux stress a été obtenu en faisant le rapport entre la population présente en fin de challenge et la population présente initialement à T_0.

7.2.9.2 Mesure de la survie de *L rhamnosus* dans les poudres

Les poudres ont été collectées et scellées dans des flacons en polystyrène stériles (Gosselin, France), puis stockées à une température contrôlée de 25°C pendant 1 mois.

Une dilution au centième a été réalisée dans de l'eau tryptonée stérile. La viabilité bactérienne a été testée par numération sur mileu MRS-Agar. La numération des colonies est réalisée après 7 jours d'incubation en anaérobiose à 37°C. Nous avons ici utilisé le protocole de réhydratation des poudres de Bioprox.

7.2.9.3 Utilisation comme ferment sur milieu de type fromage

Les poudres contenant *P. freudenreichii* réalisées grâce au plan d'expérience ont été utilisées pour ensemencer un milieu modèle de type fromage. Celui-ci a été obtenu de la façon suivante. Un ultrafiltrat de lait de vache a été préparé comme décrit ci-dessus par ultrafiltration de lait (seuil de 8 kDa). Les deux ferments lactiques majeurs, *Lactobacillus delbrueckii* CIRM-BIA 209 et *Streptococcus thermophilus* CIRM-BIA 67, ont été inoculés et co-cultivés, à 43°C, 12 h, dans de l'ultrafiltrat additionné de 5 $g.L^{-1}$ de peptone de caséine. Ces co-cultures ont été centrifugées (8000 g, 10 min), pour éliminer les bactéries lactiques. Le surnageant a été collecté, le pH ajusté à 7,0 à l'aide de NaOH 5 M, avant stérilisation par filtration (Top filter PES, 0,45 µm, Nalgene Company, NY, USA). Ensuite, 1,5 $g.L^{-1}$ de poudre de *P. freudenreichii* ont été inoculés dans cet ultrafiltrat pré-conditionné, milieu modèle mimant le fromage. Un contrôle a été réalisé à l'aide d'une culture fraîche de *P. freudenreichii*, réalisée dans ce même milieu modèle et supplémenté avec 1,5 $g.L^{-1}$ de maltodextrine pour avoir les mêmes conditions. Ces cultures ont été incubées à 24°C pendant 14 jours de façon à mimer les conditions de croissance

des propionibactéries dans la cave chaude lors de la fabrication de l'Emmental (Gagnaire et al., 2015). Les courbes de croissance ont été établies par numération sur milieu YEL-Agar.

La production des acides organiques par les bactéries a été suivie dans ces cultures de la façon suivante. Les surnageants des différentes cultures ont été récoltés par centrifugation en phase exponentielle, au début de la phase stationnaire, et après 14 jours de culture. Ces surnageants ont été dilués au demi dans une solution 0,02 M H_2SO_4 et centrifugés (8000 g, 30 min, 4°C) pour éliminer un culot de protéines, avant filtration 0,2 µm et analyse. Les acides organiques et les sucres ont été séparés par HPLC sur une colonne d'échange d'ions Minex A-6 (Dionex, Sunnyvale, California, USA) à 55°C, avec une solution 0,01 M H_2SO_4 comme éluant, à un débit de 1 mL min^{-1}. Deux détecteurs, UV (210 nm) et réfractomètre, ont été utilisés. Des standards appropriés de lactose, acétate, propionate et lactate ont été utilisés comme décrit précédemment (Aburjaile et al., 2016).

7.3 Méthodes analytiques

7.3.1 Identification et quantification des solutés compatibles accumulés

7.3.1.1 Extraction

Des cultures de *P. freudenreichii* de 1 L, réalisées sur les milieux indiqués ci-dessus, ont été récoltées en phase exponentielle, lorsque les adaptations avaient lieu durant la croissance (chapitre 3 et 4), à une DO de 0,8, par centrifugation (8000 g, 10 min). Dans les deux derniers chapitres (5 et 6) les bactéries ont été récoltées en fin de phase stationnaire et après adaptations s'il y a lieu. Les bactéries ont été lavées deux fois dans une solution saline de la même osmolarité que le milieu de culture utilisé, puis centrifugées (8000 g, 10 min). Elles ont ensuite été resuspendues dans 2 mL d'eau distillée, avant addition de 8 mL d'éthanol absolu. Cette suspension a été homogénéisée puis centrifugée (8000 g, 10 min) pour éliminer les débris cellulaires. Le surnageant a ensuite été évaporé sous vide à l'aide d'un évaporateur rotatif pendant 7 h. L'extrait sec a ensuite été repris dans 1 mL d'eau deutérée (D_2O, Sigma-Aldrich).

Nous avions souhaité identifier et quantifier les osmoprotecteurs accumulés par *L. rhamnosus*. Cependant l'analyse n'a pas été possible car les signaux en RMN étaient brouillés dû à un composant du milieu de culture.

7.3.1.2 Analyses de RMN

Les solutés compatibles ainsi extraits ont été ensuite identifiés et quantifiés par RMN, comme décrit précédemment pour d'autres bactéries (Gouesbet et al., 1992), sur la plateforme de recherche en imagerie et spectroscopie multi-modales PRISM de Rennes. Tous les spectres ^{1}H et ^{13}C RMN ont été enregistrés à 25°C à l'aide d'un spectromètre Bruker Avance 500 équipé d'une cryosonde 5-mm TCI triple-resonance cryoprobe (PRISM Core Facility, Rennes). Les spectres ^{1}H ont été acquis avec une largeur de spectre de 6 kHz, 32 points de données et un temps total de répétition de 6,73 s. Les spectres ^{13}C RMN ont été acquis à l'aide d'une séquence d'impulsions simples avec découplage continu du proton, angle de pulse de 30°, fenêtre spectrale de 30 kHz, 64 K data points, et un temps total de répétition de 3,08 s. Les données ont été traitées avec un logiciel Topspin (Bruker Biospin). Les échantillons ont été solubilisés dans de l'eau deutérée (D_2O, Sigma-Aldrich). Le TSP-d4 (Triméthylsilylpropanoate de sodium deutéré, Sigma-Aldrich) a été utilisé comme référence interne pour les déplacements chimiques ^{1}H et ^{13}C RMN. Les concentrations relatives de tréhalose, de glutamate et de glycine bétaïne ont été déterminées par intégration des surfaces de pics de leurs signaux ^{1}H rapportées au standard interne TSP-d4.

7.3.2 Analyses protéomiques

7.3.2.1 Extraits cellulaires totaux et digestion trypsique

Les analyses protéomiques sans marquage ont été conduites comme décrites par Gaucher et al., 2019a. Au début de la phase stationnaire ou après adaptation acide, les cellules de *P. freudenreichii* ont été collectées par centrifugation et lavées deux fois à l'aide d'un tampon PBS (NaCl 8 $g.L^{-1}$, KCl 2 $g.L^{-1}$ KH_2PO_4 2 $g.L^{-1}$, Na_2HPO_4 $12H_2O$ 35,8 $g.L^{-1}$). Les bactéries lavées ont été centrifugées et re-suspendues dans une solution de lyse (Tris-HCl [pH 7,5] 50 mM, Dodécylsulfate de sodium (SDS) 3 $g.L^{-1}$, dithiothreitol (DTT) 200 mM, fluorure de phénylméthylsulfonyle (PMSF) 0,4 mM) à une DO_{650nm} de 20. Cette suspension a été congelée et décongelée 3 fois, puis soniquée, et les bactéries broyées par des billes de zirconium dans un homogénéisateur (broyeur à billes Precellys Evolution, Bertin Technologie, France). Chaque broyage consiste en 1 mL de suspension bactérienne avec 200 mg de billes (billes zirconia/ silica de 0,1 mm, Biospec) dans un tube Eppendorf Safe-Lock de 1,5 mL. Les extraits sont prélevés après élimination des débris cellulaires par centrifugation (20000 g, 10 min). Les extraits protéiques ainsi collectés ont été purifiés à l'aide du kit 2-D Clean-Up (GE Healthcare) et les protéines quantifiées à l'aide du kit 2-D Quant (GE Healthcare). La digestion trypsique a

été effectuée sur 100 µg de protéines d'extrait cellulaire total pour chaque échantillon. Les 100 µg de protéines sont repris dans 50 µl d'une solution 6 M de Guanidine-HCl, 50 mM Tris-HCl (pH 8), 4 mM DTT. Après addition de 5 µl de solution de trypsine modifiée (Sequencing Grade Modified Trypsin, Promega, Madison, USA) à 0,4 µg/µl pour atteindre un ratio enzyme : protéine de 1 : 50 (w/w). La digestion trypsique est effectuée durant 15 h à 37°C selon les instructions du fabricant et comme décrit précédemment (Huang et al., 2018). De l'acide trifluoroacétique de pureté spectrophotométrique (TFA) (Sigma-Aldrich, USA) a été ajouté qsp 6% (V/V) afin d'arrêter la digestion.

7.3.2.2 Nano-LC-MS/MS

Les analyses ont été effectuées comme précédemment décrit (Huang et al., 2018). Succinctement, les analyses ont été effectuées à l'aide d'un système chromatographique nano RSLC Dionex U3000 couplé à un spectromètre de masse Q-Exactive (Thermo Scientific, San Jose, USA) équipé avec une source d'ions nano-électrospray. Une nano-colonne en phase inverse (PepMap 100 C18, 75 µm i.d., 250 mm length, 3 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands) a permis la séparation des peptides. Leurs spectres obtenus lors de l'élution ont été enregistrés dans un mode Full MS et sélectionnés dans un intervalle de 250-2000 m/z pour les spectres MS avec une résolution de 70 000 à m/z 200. Pour chaque scan, les 10 ions les plus intenses ont été sélectionnés pour être fragmentés. Les spectres MS/MS ont été enregistrés avec une résolution de 17500 à m/z 200 et l'ion parent a ensuite été exclu des fragmentations MS/MS pendant 20 s. Le spectromètre de masse a été calibré à l'aide d'un standard externe conformément aux instructions du fournisseur.

7.3.2.3 Identification des protéines

L'identification des protéines a été effectuée comme décrit précédemment (Huang et al., 2018). Les peptides ont été identifiés à partir des spectres MS/MS obtenus en utilisation le pipeline libre X!Tandem (Langella et al., 2017). La recherche a été effectuée contre le protéome de la souche *P. freudenreichii* CIRM-BIA 129 (ITG P20) (téléchargé à partir du site NCBI.nlm.nih.gov le 23 août 2018). La souche est enregistrée comme "*Propionibacterium freudenreichii* subsp. *freudenreichii* ITG P20" (GenBank: CCBE000000000.1, BioProject PRJEB4826). Les paramètres de recherches sur les banques de données ont été les suivants : utilisation de la coupure trypsique et tolérance en masse du peptide réglé à 10 ppm pour la MS et 0,05 Da pour la MS/MS. L'oxydation de la méthionine et la phosphorylation de la thréonine,

de la sérine et du tryptophane ont été choisies comme modification variable. Pour chaque peptide identifié, un score minimum correspondant à une p-value inférieure à 0,05 a été considéré comme un prérequis pour la validation d'un peptide.

7.3.2.4 Quantification de protéines

La quantification des protéines a été effectuée comme décrite précédemment (Huang et al., 2018). Chaque peptide identifié par spectrométrie de masse en tandem a été quantifié à l'aide du logiciel libre MassChroQ (Valot et al., 2011) avant le traitement des données et leurs analyses statistiques effectuées à l'aide du logiciel R (R 3.2.2, Project for statistical computing). Un paquet R spécifique intitulé "MassChroqR" a été utilisé pour filtrer automatiquement les peptides peu fiables pour lesquels la déviation standard du temps de rétention était supérieure à 30 s et pour relier les données de quantification des peptides aux protéines. En ce qui concerne les analyses en peak counting, les analyses de variance ont été effectuées sur les protéines avec un minimum de rapport des pics de 1,5 entre les différentes conditions de culture. Les protéines avec une p-value ajustée <0,05 ont été considérées significativement différentes.

En ce qui concerne la quantification basée sur les XIC, la normalisation a été effectuée pour prendre en compte les variations quantitatives globales possibles entre les différentes séries de LC-MS. Les peptides partagés entre les différentes protéines ont été automatiquement exclus de l'ensemble des données ainsi que les peptides présents dans moins de 85% des échantillons.

Les données manquantes ont alors été imputées à partir d'une régression linéaire basée sur les intensités des autres peptides pour la même protéine (Blein-Nicolas et al., 2015). L'analyse de variance a été utilisée pour déterminer les protéines avec une abondance significativement différente entre les conditions de culture.

7.3.3 Identification et quantification des lipides membranaires

P. freudenreichii CIRM-BIA 129 a été cultivé sur YEL et sur YEL+NaCl. Puis, au début de la phase stationnaire, les bactéries ont subi ou non une adaptation acide ou thermique. Les cellules ont été récoltées par centrifugation (8000 g, 15 min) puis lavées avec de l'eau distillée stérile. Les bactéries ont été de nouveau centrifugées, puis 3 mL de sodium methoxide (3,75 M dans du méthanol) ont été ajoutés, pour réaliser la saponification. Les tubes ont été mélangés vigoureusement pendant 10 s puis incubés pendant 25 min à 100°C. Pour réaliser la

méthylation, les échantillons ont été refroidis puis 6 mL d'une solution contenant de l'HCl et du méthanol (HCl 3,5 M et concentration finale de méthanol : 42%) ont été ajoutés. Les échantillons ont été vortéxés durant 10 s et incubés pendant 10 min à 80°C. Les échantillons ont ensuite été refroidis dans de la glace et 3,75 mL d'une solution comprenant de l'hexane et du diethyl éther (hexane 50% et diéthyle éther 50%) ont été ajoutés. Les échantillons ont été ensuite agités pendant 10 min puis ont été décantés. La phase aqueuse a été enlevée, puis la phase organique a été lavée avec une solution de soude (NaOH, 3 M). Les échantillons ont de nouveau été agités puis décantés. La phase organique a ensuite été récoltée pour réaliser les analyses.

Les analyses ont été réalisées avec l'Agilent chromatographe gazeux (7890A) équipé avec une colonne BPX70 (120 m x 0,25 mm x 0,25 µm, SGE, Victoria, Australie) et couplé à un détecteur d'ionisation de flamme (Agilent Technologies, Les Ulis, France). L'hydrogène a été utilisé comme gaz vecteur. L'injection a été réalisée dans une colonne d'injection froide. Le volume d'injection était de 0,5 µL. La température de détection était de 250°C. Le logiciel Agilent MSD ChemStation software a permis de traiter les données. Pour l'identification des acides gras, les temps de rétention ont été comparés aux temps de rétention des acides gras du standard BAME (Bacterial Acid Methyl Ester CP, Merck, France).

Les résultats sont exprimés en pourcentage relatif de chaque acide gras, qui représentent le ratio entre la surface du pic considéré et la surface totale de tous les pics. Les ratios des acides gras insaturés/saturés et branchés/saturés ont été déterminés. Toutes les analyses ont été réalisées en triplicata.

7.4 Statistiques

7.4.1 Analyses statistiques des données physiologiques

Les suivis de croissance par densité optique, numération bactérienne et les suivis de pH ont été réalisés en triplicata biologique. Les challenges thermique, oxydatif, biliaire et acide ont également été réalisés en triplicata biologique. Concernant les essais de séchage de *P. freudenreichii*, les essais de pulvérisation avec la tour Minor, ont été réalisés en triplicata biologique, alors que les essais de lyophilisation ont été répliqués 5 fois. La survie de *P. freudenreichii* au cours du stockage a également été suivie en triplicata biologique, tout comme la revivification des poudres dans le milieu mimant la fabrication d'emmental.

Les résultats représentent donc les moyennes avec écart-type. Une analyse de la variance à un facteur a été réalisée avec un test de comparaisons multiples pour déterminer les différences significatives entre les moyennes de groupe.

Les essais de séchage par pulvérisation de *L. rhamnosus* étaient une preuve de concept, et seulement un essai pour chaque condition a pu être réalisé.

7.4.2 Analyses statistiques des plans d'expériences

Les analyses statistiques ont été réalisées pour décrire les effets de la concentration en glucose, de l'adaptation thermique et de l'osmoadaptation sur la survie de *P. freudenreichii* durant les challenges thermique et oxydatif et la survie de *L. rhamnosus* durant la lyophilisation, les challenges thermique et oxydatif. Le logiciel JMP a été utilisé pour adapter le modèle de second ordre à la variable indépendante. Seules les variables avec un effet significatif supérieur à 95% ($p<0,05$) ont été incluses dans les modèles finaux. Les ssurfaces de réponses ont été dessinées pour illustrer les effets principaux et interactifs des variables indépendantes sur la survie aux challenges thermique et oxydatif. Ces challenges simulent les stress du procédé de séchage par pulvérisation.

7.4.3 Analyses statistiques des méthodes analytiques

7.4.3.1 Statistiques de la quantification de l'accumulation des solutés compatibles

Les identifications et les quantifications des osmoprotecteurs ont été réalisées sur trois cultures différentes pour chaque essai. Les résultats correspondent aux moyennes.

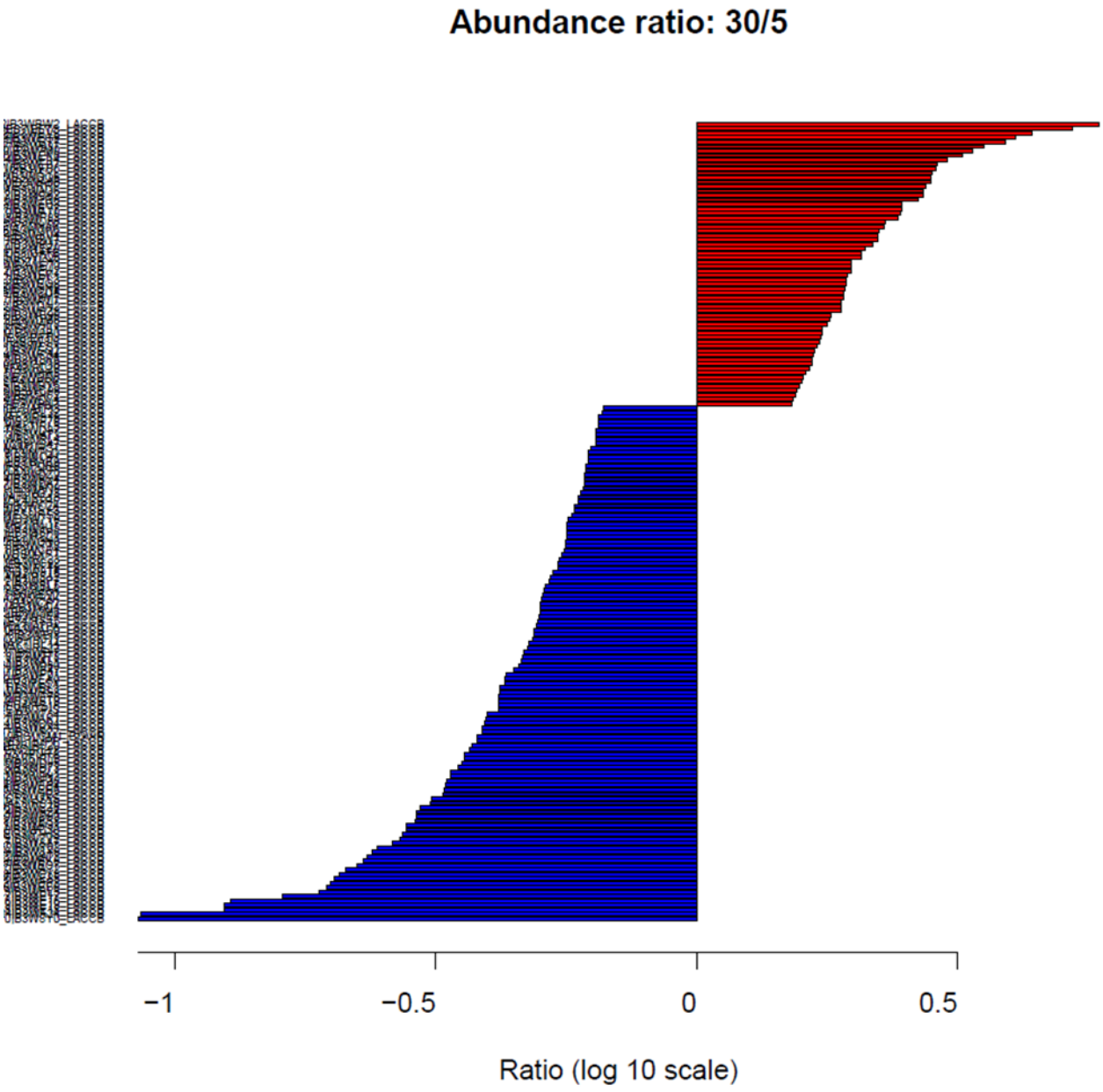
7.4.3.2 Statistiques de l'analyse protéomique

Pour l'analyse protéomique du chapitre 3, 5 réplicats biologiques, et 3 réplicats techniques ont été réalisés pour chaque condition. Dans le chapitre 5, 4 réplicats biologiques et 3 réplicats techniques ont été réalisés.

7.4.3.3 Statistiques de l'analyse des lipides membranaires

L'indentification et la quantification des analyses de lipides membranaires ont été réalisées en triplicata. Les résultats sont présentés sous forme de moyenne avec écart-type. Une analyse de la variance à un facteur a été réalisée avec un test de comparaisons multiples pour déterminer les différences significatives entre les moyennes de groupe.

Supplemental figure



Supplemental figure 1: Abundance ratio of protein expressed by *P. freudenreichii* CIRM-BIA 129 in the hyperconcentrated sweet whey 30% and the hyperconcentrated sweet whey 5%

Supplemental tables

Supplemental Table 1: Salt and glycine betaine-mediated modulation of proteins of the *P. freudenreichii* CIRM-BIA 129 and 1025 strains.

Ratio were calculated with XIC methods, number with stars where calculated with peak counting method when XIC ratio was not available.

a: Determined by using UniProtKB database corresponding to …

b: Ratio of the protein level in the MMO+NaCl medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl.

c: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

d: Ratio of the protein level in the MMO+NaCl+GB medium compared to MMO+NaCl medium. This ratio indicates induction (ratio>1.5) or repression (ratio>0.66) by NaCl+GB.

e: Non significant

		CIRM129			CIRM1025		
accession	**Description**	**MMO+NaCl/ MMO**	**MMO+NaCl+GB/ MMO**	**MMO+NaCl+GB/ MMO+NaCl**	**MMO+NaCl/ MMO**	**MMO+NaCl+GB/ MMO**	**MMO+NaCl+GB/ MMO+NaCl**
RNA processing and modification							
emb|CDP47739.1|	Oligoribonuclease	1,52	NS[e]	NS	NS	NS	NS
Energy production and conversion							
emb|CDP49440.1|	Ferredoxin	0,43	0,45	NS	NS	NS	NS
emb|CDP49591.1|	Cytochrome d ubiquinol oxidase, subunit II	0,53	NS	2,10	NS	NS	NS
emb|CDP49666.1|	Coenzyme A transferase	0,54	NS	NS	NS	NS	NS
emb|CDP47837.1|	Malate dehydrogenase	0,58	NS	NS	0,63	NS	NS
emb|CDP48122.1|	Betaine-aldehyde dehydrogenase	0,59*	NS	NS	NS	NS	NS
emb|CDP48900.1|	Citrate synthase	0,60	NS	1,50	0,53	0,55	NS
emb|CDP49592.1|	Cytochrome d ubiquinol oxidase subunit I	0,65	NS	NS	NS	NS	NS
emb|CDP47594.1|	Succinate dehydrogenase cytochrome B-558 subunit	0,66	NS	NS	NS	NS	NS
emb|CDP48732.1|	L-lactate dehydrogenase	0,66	NS	NS	NS	NS	NS
emb|CDP47815.1|	Pyruvate:ferredoxin oxidoreductase	0,66	NS	NS	NS	NS	NS
emb|CEG99057.1|	Acetate kinase	NS	NS	NS	0,64	NS	NS
emb|CDP49349.1|	Aldehyde dehydrogenase	NS	NS	1,73	NS	NS	NS
emb|CDP48009.1|	electron transfer oxidoreductase	NS	NS	1,61	NS	NS	NS
emb|CDP48084.1|	FAD linked oxidase domain protein	NS	NS	1,67	NS	NS	NS
emb|CDP49268.1|	Glycerol kinase	NS	NS	1,77	NS	NS	NS

emb|CDP48130.1|	Glycerol-3-phosphate dehydrogenase	NS	1,93	2,07	NS	NS	NS
emb|CEG98960.1|	Methylmalonyl-CoA carboxytransferase 5S subunit.	NS	NS	NS	0,63	0,65	NS
emb|CDP48006.1|	NADH-quinone oxidoreductase chain B	NS	1,55	NS	NS	NS	NS
emb|CDP48002.1|	NADH-quinone oxidoreductase chain F	NS	NS	1,65	NS	NS	NS
emb|CDP47853.1|	NADPH:quinone reductase related Zn-dependent oxidoreductase	NS	NS	NS	0,60*	NS	NS
emb|CDP48511.1|	Nitroreductase	NS	NS	NS	1,51	1,80	NS
emb|CEG99056.1|	Phosphate acetyltransferase	NS	NS	NS	0,49	0,50	NS
emb|CEG98002.1|	Putative aldo/keto reductase	NS	NS	NS	1,63	1,52	NS
emb|CDP48449.1|	Succinate dehydrogenase, subunit B	NS	1,52	1,69	NS	NS	NS
emb|CDP47989.1|	Thiamine pyrophosphate (TPP family)	NS	NS	NS	1,56	1,54	NS
emb|CDP48026.1|	Zinc-containing alcohol dehydrogenase superfamily	NS	NS	NS	0,62	0,59	NS
emb|CDP48804.1|	ATP synthase B chain	1,61	1,75	NS	2,17	2,30	NS
emb|CDP49578.1|	Electron transfer flavoprotein-quinone oxidoreductase (FixC protein)	1,61	1,55*	NS	NS	NS	NS
emb|CDP49579.1|	Electron transfer flavoprotein, carnitine metabolism (FixB protein)	1,63	NS	0,60	NS	NS	NS
emb|CEG97844.1|	ATP synthase F1 sector subunit beta	1,63	1,67	NS	NS	NS	NS
emb|CDP48428.1|	pyruvate dehydrogenase E1 component	1,83	NS	NS	NS	NS	NS
emb|CDP48799.1|	ATP synthase F1 sector epsilon subunit	1,85	1,70	NS	1,99	1,94	NS
emb|CDP48899.1|	Oxidoreductase	1,91	1,51	NS	NS	NS	NS
emb|CDP48803.1|	ATP synthase delta chain	1,99	1,93	NS	NS	NS	NS
emb|CDP49838.1|	Sulfite reductase	2,06	1,94	NS	NS	NS	NS
emb|CDP49577.1|	Ferredoxin-like protein fixX	2,06*	NS	NS	NS	NS	NS
Cell cycle control and mitosis							
emb|CDP48414.1|	Cell division protein	0,64	NS	NS	2,48	2,16	NS
emb|CDP48415.1|	ABC transporter, ATP-binding protein	NS	NS	NS	1,56	1,56	NS
emb|CEG97007.1|	DNA translocase FtsK	2,00*	2,08*	NS	NS	2,42*	NS
Amino Acid metabolism and transport							
emb|CDP48506.1|	Alanine dehydrogenase	0,07	NS	11,62	0,30*	0,32*	NS
emb|CDP49711.1|	Nitrogen regulatory protein P-II	0,18*	2,16*	12,1*	4,41*	4,41*	NS
emb|CEG99164.1|	Alanine dehydrogenase	0,36*	NS	2,57*	0,25	0,30	NS
emb|CDP48687.1|	Glycine cleavage H-protein (lipoate-binding)	0,38	0,46	NS	0,51	0,57	NS

emb|CDP49124.1|	Glutamine amidotransferase of anthranilate synthase or para-aminobenzoate synthase	0,45	NS	1,83	NS	NS	NS
emb|CEH00437.1|	Xaa-Pro aminopeptidase I	0,48	NS	1,78	NS	NS	NS
emb|CDP48503.1|	Amidohydrolase	0,53	NS	2,00	NS	NS	NS
emb|CDP48646.1|	anthranilate synthase component I	0,57	NS	NS	NS	NS	NS
emb|CDP49409.1|	Glutamine synthetase	0,59	1,51	2,56	NS	NS	NS
emb|CDP47865.1|	Cysteine synthase 1	0,60	NS	NS	NS	NS	NS
emb|CDP48633.1|	Histidinol-phosphate aminotransferase	0,60	NS	NS	NS	NS	NS
emb|CDP48419.1|	4-aminobutyrate aminotransferase	0,61	NS	NS	NS	NS	NS
emb|CDP49173.1|	Dihydroxy-acid dehydratase	0,62	NS	NS	0,62	0,62	NS
emb|CDP49134.1|	L-serine dehydratase	0,63	NS	NS	0,62	0,62	NS
emb|CDP48561.1|	polar amino acid ABC transporter, binding protein component	0,65	NS	NS	NS	NS	NS
emb|CDP49001.1|	glutamate dehydrogenase	0,65*	NS	NS	NS	NS	NS
emb|CEG98734.1|	Glutamate synthase large subunit (Ferredoxin)	0,65*	NS	NS	NS	NS	NS
emb|CDP48779.1|	3-isopropylmalate dehydrogenase	NS	0,64	NS	NS	NS	NS
emb|CDP48360.1|	3-phosphoshikimate 1-carboxyvinyltransferase	NS	NS	NS	1,52	1,60	NS
emb|CDP47558.1|	Acetylornithine and succinylornithine aminotransferase	NS	NS	1,75	NS	NS	NS
emb|CEG98065.1|	Amino acid permease. membrane protein	NS	NS	2,81*	NS	NS	NS
emb|CEG99028.1|	Amino acid permease-associated region. Membrane protein	NS	NS	NS	1,60	NS	NS
emb|CEG99336.1|	Aminopeptidase N, Lysyl aminopeptidase	NS	1,51	NS	NS	NS	NS
emb|CDP47560.1|	Arginine biosynthesis bifunctional protein ArgJ	NS	1,59	NS	NS	1,70*	NS
emb|CDP49614.1|	Argininosuccinate synthase	NS	1,70	1,65	NS	NS	NS
emb|CDP47562.1|	Aspartate aminotransferase	NS	NS	NS	1,57	1,64	NS
emb|CEG99593.1|	Aspartate ammonia-lyase (Aspartase)	NS	NS	0,63	NS	NS	NS
emb|CDP48567.1|	Bifunctional PLP-dependent enzyme with beta-cystathionase and maltose regulon repressor activities	NS	NS	NS	1,94	2,11	NS
emb|CDP47871.1|	Chorismate mutase	NS	1,60	NS	2,36	3,08	NS
emb|CDP48480.1|	Imidazole glycerol phosphate synthase subunit HisF	NS	NS	NS	1,50	NS	NS
emb|CDP48589.1|	methionine synthase	NS	2,11	NS	NS	NS	NS
emb|CDP47561.1|	N-acetyl-gamma-glutamyl-phosphate reductase	NS	NS	1,81	NS	NS	NS
emb|CEG98743.1|	Phosphoribosyl-AMP cyclohydrolase	NS	NS	NS	0,46	0,44	NS
emb|CDP49050.1|	Phosphoserine aminotransferase	NS	0,54	0,44	NS	NS	NS

emb|CDP48345.1|	Phosphoserine phosphatase/homoserine phosphotransferase bifunctional protein	NS	NS	NS	1,80	2,02	NS
emb|CDP49113.1|	Propanediol utilization protein PduU	NS	NS	NS	NS	12,00*	NS
emb|CEG97416.1|	Protein of unknown function	NS	NS	NS	NS	0,50*	NS
emb|CDP49606.1|	Shikimate 5-dehydrogenase	NS	NS	NS	0,66	0,67	NS
emb|CDP47931.1|	solute binding protein of the ABC transport system	NS	0,63	NS	NS	NS	NS
emb|CEH00744.1|	Thiamine pyrophosphate enzyme	NS	NS	NS	0,55	0,54	NS
emb|CEG97415.1|	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	1,65*	NS	NS	NS	NS	NS
emb|CDP49308.1|	Cysteine desulphurases, SufS	1,66	NS	0,61	NS	NS	NS
emb|CDP48772.1|	3-isopropylmalate dehydratase small subunit	1,66	NS	0,62	NS	NS	NS
emb|CDP47878.1|	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	1,68*	NS	NS	NS	NS	NS
emb|CEG97413.1|	binding protein of choline ABC transporter	2,15*	1,63*	NS	NS	NS	NS
emb|CDP47876.1|	binding protein of choline ABC transporter	2,34*	1,71*	NS	NS	NS	NS

Nucleotide metabolism and transport							
emb|CDP49821.1|	Histidine triad (HIT) protein	0,29	0,54	1,83	0,49	0,52	NS
emb|CDP47814.1|	Dihydroorotate dehydrogenase	0,64	NS	NS	NS	NS	NS
emb|CDP49204.1|	Inosine/uridine-preferring nucleoside hydrolase	NS	NS	NS	1,63	1,71	NS
emb|CDP49245.1|	Phosphoribosylformylglycinamidine synthetase PurS	NS	NS	NS	1,66	1,66	NS
emb|CDP49283.1|	Uridylate kinase PyrH	NS	NS	NS	NS	1,50	NS
emb|CDP47763.1|	Thymidylate synthase	1,52	NS	0,67	NS	NS	NS
emb|CDP48600.1|	Orotidine 5-phosphate decarboxylase	1,75	NS	0,63	NS	NS	NS

Carbohydrate metabolism and transport							
emb|CDP49220.1|	Phosphocarrier, HPr family	0,38	0,53	#N/A	0,49	0,56	NS
emb|CDP48824.1|	Dihydroxyacetone kinase	0,38	NS	2,44	0,39	0,44	NS
emb|CDP48902.1|	Fructose-bisphosphate aldolase class I	0,41	NS	2,72	NS	NS	NS
emb|CDP49430.1|	Glucose-1-phosphate adenylyltransferase	0,53	NS	2,12	0,51	0,57	NS
emb|CDP48965.1|	nucleoside-diphosphate-sugar epimerases	0,55	NS	NS	NS	NS	NS
emb|CDP49639.1|	Gluconate kinase	0,59	NS	1,57*	0,63	NS	NS
emb|CDP49605.1|	3-carboxymuconate cyclase	0,60	0,63	NS	NS	NS	NS

emb|CDP48837.1|	Aldose 1-epimerase	0,60	NS	NS	NS	NS	NS
emb|CDP47731.1|	DhaK PTS-dependent dihydroxyacetone kinase,dihydroxyacetone-binding subunit	0,64	NS	1,65	NS	NS	NS
emb|CDP48996.1|	Galactokinase	0,64	NS	1,80	NS	NS	NS
emb|CDP49431.1|	glycosyltransferase	0,64*	NS	NS	NS	NS	NS
emb|CDP48782.1|	Glycosyl hydrolase, family 13	0,65	NS	NS	NS	NS	NS
emb|CDP49526.1|	Beta-galactosidase	NS	NS	1,55	NS	NS	NS
emb|CDP49788.1|	Galactokinase	NS	NS	NS	1,74	1,82	NS
emb|CEG97971.1|	Inositol-1-monophosphatase	NS	NS	NS	0,58	0,62	NS
emb|CDP48893.1|	Oxidoreductase	NS	1,58	NS	NS	NS	NS
emb|CEG98016.1|	Pyruvate phosphate dikinase	NS	NS	NS	0,52	0,60	NS
emb|CDP49834.1|	Ribose-5-phosphate isomerase 3	NS	NS	NS	2,14	1,99	NS
emb|CDP48129.1|	Xylulokinase protein, Carbohydrate kinase	NS	NS	1,64	0,61	0,66	NS
emb|CDP48974.1|	Enolase 1	1,63	NS	0,60	NS	NS	NS
emb|CDP49189.1|	Fructose-bisphosphate aldolase class II	1,88	NS	0,63	NS	NS	NS
emb|CEG98306.1|	sugar transporter, major facilitator superfamily	2,59*	2,28*	NS	NS	NS	NS

Coenzyme metabolism							
emb|CDP49693.1|	Thiamine-phosphate pyrophosphorylase	0,00*	0,05*	NS	NS	NS	NS
emb|CDP49694.1|	Hydroxyethylthiazole kinase	0,19*	0,27*	NS	NS	NS	NS
emb|CDP48559.1|	pyrazinamidase/nicotinamidase	0,33	0,30	NS	0,36	0,37	NS
emb|CDP48291.1|	thiamine-phosphate diphosphorylase	0,55*	NS	NS	NS	NS	NS
emb|CDP47721.1|	hydroxymethylpyrimidine/phosphomethylpyrimidinekinase	0,63	NS	NS	NS	NS	NS
emb|CEG99409.1|	Thiamine biosynthesis protein	0,65*	NS	NS	NS	NS	NS
emb|CDP47756.1|	CbiF Precorrin-4 C11-methyltransferase	NS	NS	NS	0,61	0,67	NS
emb|CDP47757.1|	CbiL Precorrin-2 C20-methyltransferase	NS	NS	NS	0,65	NS	NS
emb|CDP47754.1|	CysG/CbiX	NS	NS	NS	0,62	NS	NS
emb|CDP49165.1|	Delta-aminolevulinic acid dehydratase	NS	NS	NS	0,44*	0,49*	NS
emb|CDP47762.1|	Dihydrofolate reductase	NS	NS	NS	1,89	1,81	NS
emb|CDP49200.1|	glutamate-1-semialdehyde 2,1-aminomutase	NS	NS	1,84*	1,55*	1,66	NS
emb|CDP47894.1|	Pantothenate kinase	NS	NS	NS	NS	0,59*	NS
emb|CDP49692.1|	Phosphomethylpyrimidine kinase	NS	NS	NS	NS	2,67*	NS

emb|CDP49549.1|	Thiazole biosynthesis family protein	NS	NS	NS	0,54*	NS	NS
emb|CDP47610.1|	Phosphopantetheine adenylyltransferase	1,61	NS	NS	NS	NS	NS
emb|CEG99156.1|	Putative hydroxymethyldihydropteridine pyrophosphokinase	2,01	1,65	NS	1,84	1,78	NS
emb|CDP49852.1|	Riboflavin synthase alpha chain	2,75	2,12	NS	2,00	2,05	NS
emb|CDP49854.1|	6,7-dimethyl-8-ribityllumazine synthase	2,50*	2,29*	NS	3,09*	3,36*	NS
Lipid metabolism							
emb|CDP48790.1|	Methylmalonyl-CoA epimerase	0,62	NS	NS	0,66	NS	NS
emb|CDP48432.1|	3-oxoacyl-(Acyl-carrier-protein) synthase III	NS	NS	0,59	NS	NS	NS
emb|CDP49772.1|	Hypothetical protein PFCIRM12901985	NS	NS	NS	1,73	1,83	NS
emb|CDP49157.1|	Methylmalonyl-CoA carboxytransferase, 1.3S subunit	NS	NS	NS	0,66	NS	NS
emb|CEG97114.1|	Undecaprenyl pyrophosphate synthase	NS	1,74	1,65	NS	NS	NS
emb|CEG97331.1|	Methylmalonyl-CoA mutase small subunit	1,70	NS	NS	NS	NS	NS
emb|CDP48827.1|	Short chain dehydrogenase	1,88	NS	0,62	NS	NS	NS
Translation							
emb|CDP47686.1|	50S ribosomal protein L27	0,53	0,57	NS	0,63	0,65	NS
emb|CDP48981.1|	Ribosomal protein L25	0,56	0,62	NS	NS	NS	NS
emb|CDP47964.1|	30S ribosomal protein S12	0,60	0,63	NS	NS	NS	NS
emb|CDP47687.1|	50S ribosomal protein L21	0,62	0,48	NS	NS	NS	NS
emb|CDP47614.1|	Ribosomal protein L28	0,65	0,54	NS	NS	NS	NS
emb|CDP48765.1|	2-methylthioadenine synthetase MiaB protein	NS	2,09	2,35	NS	NS	NS
emb|CDP47941.1|	30S ribosomal protein S14 type Z	NS	NS	NS	0,56	0,59	NS
emb|CDP47969.1|	50S ribosomal protein L10	NS	NS	NS	1,96	2,15	NS
emb|CDP47915.1|	50S ribosomal protein L17	NS	NS	NS	NS	2,94	NS
emb|CDP47936.1|	50S ribosomal protein L30	NS	0,55	0,61	NS	NS	NS
emb|CDP47568.1|	50S ribosomal protein L35	NS	NS	NS	NS	0,65	NS
emb|CDP48606.1|	Elongation factor P	NS	NS	0,66	NS	NS	NS
emb|CDP49284.1|	Elongation factor Ts (EF-Ts)	NS	NS	0,65	NS	NS	NS
emb|CEG97555.1|	Endoribonuclease L-PSP	NS	NS	NS	NS	1,61	NS
emb|CEG98835.1|	Isoleucyl-tRNA synthetase	NS	NS	NS	0,50	0,50	NS
emb|CDP48811.1|	Methylase	NS	1,54	1,55	NS	NS	NS

emb|CDP48203.1|	Pseudouridine synthase	NS	NS	0,57	NS	NS	NS
emb|CDP49354.1|	Pseudouridylate synthase, Uncharacterized RNA pseudouridine synthase	NS	NS	NS	0,00*	NS	NS
emb|CDP49271.1|	Ribosome-binding factor A	NS	NS	NS	1,56	1,67	NS
emb|CDP47968.1|	50S ribosomal protein L7/L12	1,62	NS	NS	2,29	2,50	NS
emb|CDP49144.1|	Cysteinyl-tRNA synthetase	1,63	NS	NS	NS	NS	NS
Transcription							
emb|CDP48393.1|	Cold shock-like protein CspA	0,41	0,37	NS	0,37	0,32	NS
emb|CDP49615.1|	Cell envelope-related function transcriptional attenuator common domain	0,62	NS	NS	0,51	0,52	NS
emb|CDP49179.1|	GntR-family protein transcriptional regulator	NS	NS	NS	0,65	NS	NS
emb|CDP49689.1|	PadR, Transcriptional regulator PadR-like family	NS	NS	NS	1,62*	1,63*	NS
emb|CEG99499.1|	PadR, Transcriptional regulator PadR-like family	NS	NS	NS	1,74*	1,74*	NS
emb|CDP47608.1|	Ribonuclease III	NS	1,59	NS	NS	NS	NS
emb|CDP48969.1|	tetR-family transcriptional regulator	NS	NS	NS	NS	1,54*	NS
emb|CDP48947.1|	Transcription elongation factor GreA	NS	0,66	NS	NS	NS	NS
emb|CDP48030.1|	Transcriptional regulator, ArsR family	NS	NS	NS	2,06	2,46	NS
emb|CEG97361.1|	Transcriptional regulator, RpiR family	NS	NS	NS	0,65	NS	NS
emb|CDP48357.1|	RNA polymerase sigma-70 factor, sigma-E factor	1,50	NS	NS	NS	NS	NS
emb|CEG99396.1|	Sse9I control protein	1,53	NS	NS	NS	NS	NS
emb|CDP48823.1|	Transcriptional Regulator, GntR family protein	1,56*	NS	NS	NS	NS	NS
emb|CDP48597.1|	DNA-directed RNA polymerase omega chain	1,57	NS	NS	NS	NS	NS
emb|CDP47796.1|	transcriptional regulator TetR family	1,68	2,03	NS	1,77	1,91	NS
emb|CDP49403.1|	Phage shock protein A	1,70	1,63	NS	1,63	1,82	NS
emb|CDP48985.1|	Regulatory protein, TetR	1,96*	NS	NS	NS	NS	NS
emb|CDP49150.1|	transcriptional regulator CarD	2,63	1,59	0,60	1,59	NS	NS
Replication and repair							
emb|CDP48730.1|	DNA gyrase subunit B	NS	NS	NS	0,56	0,51	NS
emb|CEG99330.1|	DNA ligase (NAD+)	NS	NS	NS	1,80	1,90	NS
emb|CDP48941.1|	Exodeoxyribonuclease 7 small subunit	NS	2,14*	NS	2,95*	3,68*	NS
emb|CEG97264.1|	Single-stranded DNA-binding protein 2	NS	NS	NS	5,16*	5,48*	NS
emb|CDP48398.1|	DEAD/DEAH box helicase domain protein	1,52	NS	NS	NS	NS	NS

emb|CEG99882.1|	Excinuclease ABC subunit B	1,58	1,66	NS	NS	NS	NS
emb|CDP49156.1|	Serine 3-dehydrogenase	1,68	NS	0,63	NS	NS	NS
Cell wall/membrane/envelop biogenesis							
emb|CDP48034.1|	cell-wall peptidases, NlpC/P60 family secreted protein	NS	0,46	0,63	NS	NS	NS
emb|CDP48887.1|	MscS transporter, small conductance mechanosensitive ion channel	NS	NS	NS	1,75*	1,92*	NS
emb|CEG99084.1|	Mycoloyl transferase	NS	NS	NS	0,43*	0,62	NS
emb|CDP49504.1|	penicillin-binding protein (peptidoglycan glycosyltransferase)	NS	0,62	NS	NS	NS	NS
emb|CDP49389.1|	Peptidase M23B family / metalloendopeptidase	NS	0,55*	NS	0,33	0,34	NS
emb|CEG99082.1|	UDP-galactopyranose mutase	NS	NS	NS	1,64	1,58	NS
emb|CDP49091.1|	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	NS	NS	2,06	NS	NS	NS
emb|CDP48873.1|	Methyltransferase gidB (Glucose-inhibited division protein B)	2,09*	NS	NS	NS	NS	NS
emb|CEG97947.1|	UDP-glucose 4-epimerase	2,89	1,72	0,60	NS	NS	NS
Post-translational modification, protein turnover, chaperone functions							
emb|CDP48273.1|	Surface layer protein B	0,14	0,26	1,83	#N/A	NS	NS
emb|CDP48952.1|	peptide-methionine (S)-S-oxide reductase	0,41	0,57	NS	0,47*	0,54*	NS
emb|CDP48879.1|	Thioredoxin	0,49	0,58	NS	NS	NS	NS
emb|CDP49261.1|	Secreted protein	0,57*	NS	NS	0,35*	0,41*	NS
emb|CDP48858.1|	Surface protein with SLH domain	0,61*	NS	NS	NS	NS	NS
emb|CDP48588.1|	SppA, Periplasmic serine proteases	0,61	NS	NS	NS	NS	NS
emb|CEH00247.1|	Surface layer protein B	0,65*	NS	NS	NS	NS	NS
emb|CDP49020.1|	Chaperone protein dnaJ 1	0,66	NS	NS	0,55	0,57	NS
emb|CDP49048.1|	Thioredoxine	0,67	NS	NS	NS	NS	NS
emb|CDP47983.1|	Alkyl hydroperoxide reductase subunit C	NS	NS	NS	1,78	1,79	NS
emb|CDP47657.1|	Chaperone protein dnaJ 3	NS	NS	0,65	0,62	0,62	NS
emb|CEH00703.1|	Co-chaperone protein GrpE2	NS	NS	NS	1,91	2,08	NS
emb|CDP47874.1|	groEL protein 1	NS	NS	0,63	NS	NS	NS
emb|CDP49125.1|	groEL protein 2	NS	NS	0,62	NS	NS	NS
emb|CDP47875.1|	groES protein 1	NS	NS	NS	1,96	1,94	NS
emb|CEG97258.1|	Heat shock protein 20 kDa 1	NS	NS	NS	1,85	1,82	NS
emb|CEG99654.1|	Heat shock protein 20 kDa 2	NS	NS	NS	1,56	NS	NS

emb|CDP49400.1|	HesB protein	NS	NS	NS	0,66	0,63	NS
emb|CDP47586.1|	Magnesium chelatase, subunit ChlI	NS	NS	NS	3,59*	3,59*	NS
emb|CDP47990.1|	Metalloprotease (Peptidase family M13)	NS	NS	NS	1,62	1,70	NS
emb|CDP49595.1|	peptidyl-prolyl cis-trans isomerase	NS	0,51	0,64	NS	NS	NS
emb|CDP48201.1|	peptidyl-prolyl cis-trans isomerase	NS	0,64	0,59	NS	NS	NS
emb|CDP49391.1|	Probable peptidyl-prolyl cis-trans isomerase A	NS	0,65	NS	NS	NS	NS
emb|CEH01092.1|	thiol peroxidase	NS	NS	NS	2,58	2,46	NS
emb|CDP48789.1|	Thioredoxin	NS	NS	0,65	NS	NS	NS
emb|CDP49795.1|	Thioredoxin	NS	0,44	0,41	0,58	0,56	NS
emb|CDP47709.1|	Trigger factor	NS	NS	0,66	NS	NS	NS
emb|CDP47745.1|	groES protein 2	1,51	NS	NS	NS	NS	NS
emb|CDP49311.1|	FeS assembly protein SufD	1,51	NS	NS	NS	NS	NS
emb|CDP48411.1|	SmpB SsrA-binding protein	1,71	NS	NS	NS	NS	NS
emb|CDP47705.1|	ATP-dependent Clp protease proteolytic subunit 2	1,78	NS	NS	1,54	1,61	NS
emb|CDP49309.1|	ABC-type transport system involved in Fe-S cluster assembly, ATPase component, SufC	1,85	NS	0,58	NS	NS	NS
emb|CDP49617.1|	thiol peroxidase	1,88	NS	0,59	NS	NS	NS
emb|CEG97257.1|	Heat shock protein 20b 3 (20 kDa chaperone 3)	1,90*	1,90*	NS	NS	1,63	NS
emb|CDP48339.1|	Heat shock protein 20 kDa 3	1,97*	1,90*	NS	NS	NS	NS
Inorganic ion transport and metabolism							
emb|CEG98403.1|	Iron transport system substrate-binding protein ABC transporter	0,52	0,53	NS	0,37	0,45	NS
emb|CDP48057.1|	Cobalt transport protein CbiN	0,62	NS	1,53	0,56	NS	NS
emb|CDP48457.1|	ABC-transporter metal-binding lipoprotein	NS	NS	NS	1,98*	1,90*	NS
emb|CDP48215.1|	ABC-type transporter, ATPase component	NS	NS	NS	2,78	3,03	NS
emb|CDP49519.1|	Catalase	NS	NS	0,66	NS	NS	NS
emb|CDP49221.1|	Cation transporting p-type ATPase 3.A.3.5.4	NS	NS	NS	1,68	1,68	NS
emb|CDP48059.1|	Cobalt import ATP-binding protein CbiO	NS	NS	NS	NS	1,61	NS
emb|CEH00551.1|	Copper-exporting ATPase	NS	NS	NS	1,65	1,56	NS
emb|CEG97448.1|	Iron/Manganese superoxide dismutase	NS	NS	NS	NS	1,50	NS
emb|CDP48458.1|	Manganese/zinc transport system ABC transporter ATP-binding protein	NS	NS	NS	2,38*	2,44*	NS
emb|CDP49458.1|	Membrane protein, Transporter, MFS superfamily	NS	NS	NS	0,49*	0,44*	NS

emb|CEG99680.1|	Periplasmic substrate-binding component of ABC-type sulfonate/nitrate/taurine transport system	NS	NS	NS	7,45	7,24	NS
emb|CDP49660.1|	Starvation-inducible DNA-binding protein	NS	NS	1,64	NS	NS	NS
emb|CDP49835.1|	Sulfate adenylyltransferase large subunit	NS	1,53	NS	NS	NS	NS
emb|CEH00264.1|	Thiosulfate sulfurtransferase	NS	NS	NS	1,84	1,85	NS
emb|CDP49153.1|	Putative phosphate transport system protein	1,53*	NS	NS	1,88*	1,90*	NS
emb|CDP49762.1|	Polyphosphate kinase	1,61	NS	NS	NS	NS	NS
emb|CDP48302.1|	Heavy metal transport/detoxification protein	1,78	NS	0,57	1,53	1,72	NS
emb|CDP49464.1|	Thiosulfate sulfurtransferase	1,80	NS	0,58	NS	NS	NS
emb|CDP47833.1|	Hypothetical protein PFCIRM12907625	1,87*	NS	NS	NS	NS	NS
emb|CDP49760.1|	binding protein of phosphate ABC transporter	2,06*	NS	0,63*	3,93*	4,00*	NS
emb|CDP49842.1|	Sulfate/thiosulfate import ATP-binding protein CysA	2,30*	1,66*	NS	NS	NS	NS
emb|CDP49756.1|	Phosphate import ATP-binding protein pstB	3,85	2,50*	NS	2,90*	2,62*	NS
emb|CDP49684.1|	ABC-type transport systems, periplasmic component	6,68	4,24	0,63	2,45	2,26	NS
Secondary Structure							
emb|CDP48919.1|	Protein-disulfide isomerase (DSBA oxidoreductase)	0,53*	NS	NS	NS	NS	NS
emb|CDP49310.1|	Dioxygenase	NS	0,37	0,55	0,26	0,28	NS
emb|CDP49496.1|	Isochorismatase hydrolase	NS	NS	NS	0,37	0,40	NS
emb|CEG99686.1|	SDS hydrolase SdsA1	NS	NS	NS	1,70*	1,71*	NS
emb|CEH00126.1|	Metallo-dependent hydrolases, subgroup A	1,56	NS	NS	NS	NS	NS
Function Unknown							
emb|CDP49561.1|	Metallophosphoesterase	0,27*	NS	NS	NS	NS	NS
emb|CDP49343.1|	Beta-lactamase-like	0,36	0,51	NS	0,66	NS	NS
emb|CDP49687.1|	Surface protein with SLH domain	0,37*	NS	NS	NS	NS	NS
emb|CEG97262.1|	S-layer protein	0,40*	NS	NS	NS	NS	NS
emb|CDP48018.1|	PRC-barrel	0,42	0,48	NS	0,53	0,62	NS
emb|CDP49497.1|	internaline A	0,42*	0,56*	NS	0,23*	0,25*	NS
emb|CEG98197.1|	Putative minor extracellular serine protease	0,43*	0,57*	NS	0,24*	0,26*	NS
emb|CDP49418.1|	Surface protein D with SLH domain	0,48	0,24	0,50	0,19	0,22	NS
emb|CDP48655.1|	LMBE-related protein	0,50	0,31	0,62	NS	NS	NS

emb|CDP48618.1|	Beta-lactamase-like	0,52	NS	NS	NS	NS	NS
emb|CEH00950.1|	Methylase	0,56	NS	NS	NS	NS	NS
emb|CDP48374.1|	acetyltransferase	0,59	NS	NS	NS	NS	NS
emb|CDP48400.1|	Predicted metal-dependent phosphoesterase	0,61	NS	1,77	NS	NS	NS
emb|CEH00728.1|	NADH-flavin reductase	0,62	NS	NS	NS	NS	NS
emb|CDP49531.1|	Putative purine phosphoribosyltransferase	0,65	NS	1,55	1,55	1,73	NS
emb|CDP47980.1|	R hydratase like, (R)-hydratase	0,65	NS	1,67	0,56	0,52	NS
emb|CEG97303.1|	Putative uncharacterized protein	0,65	NS	NS	NS	NS	NS
emb|CDP49435.1|	Lysine decarboxylase	0,66	0,62	NS	NS	NS	NS
emb|CEG98544.1|	Methionine synthase, vitamin-B12 independent	0,66	0,61	NS	NS	NS	NS
emb|CDP49338.1|	ABC transporter, ATP-binding protein	NS	NS	NS	0,61	0,60	NS
emb|CDP48609.1|	Aminodeoxychorismate lyase	NS	NS	NS	0,58*	0,58*	NS
emb|CEG97606.1|	GCN5-related N-acetyltransferase	NS	NS	NS	1,67	1,83	NS
emb|CEG99725.1|	Gp207	NS	NS	NS	NS	1,55	NS
emb|CDP48292.1|	Hypothetical protein PFCIRM12900795	NS	NS	NS	NS	NS	NS
emb|CDP49211.1|	Metallophosphoesterase	NS	2,97*	NS	NS	NS	NS
emb|CDP49064.1|	NUDIX hydrolase	NS	NS	1,56	NS	NS	NS
emb|CEH01047.1|	secreted transglycosydase	NS	NS	0,47*	2,10	1,96	NS
emb|CDP49444.1|	secreted transglycosydase	NS	NS	0,63*	NS	NS	NS
emb|CEH00684.1|	UPF0210 protein	NS	NS	NS	0,64	0,64	NS
emb|CDP48042.1|	UPF0210 protein	NS	NS	NS	0,65	0,63	NS
emb|CDP48438.1|	Von Willebrand factor, type A	NS	NS	NS	4,17*	3,67*	NS
emb|CDP47784.1|	Phosphomannomutase (PMM)	1,57	NS	#N/A	NS	NS	NS
emb|CDP49193.1|	transmembrane protein	1,76	NS	NS	NS	NS	NS
emb|CDP47852.1|	Possible stress-response transcriptional regulator protein PspC	1,82	NS	NS	NS	NS	NS
emb|CDP48441.1|	ATPases MoxR family	1,82*	NS	NS	1,63*	1,71*	NS
emb|CDP49799.1|	TRNA processing ribonuclease BN	1,85	NS	NS	1,93	1,93	NS
emb|CDP49373.1|	Protein mraZ	2,07	NS	0,56	1,85	1,68	NS
emb|CDP48895.1|	drug exporters of the RND superfamily	2,30	1,77	NS	1,96	1,86	NS

Signal Transduction							
emb|CDP48041.1|	UPF0237 protein	0,15	0,26	1,69	NS	NS	NS

emb|CDP47898.1|	GTP phosphohydrolase (mRNA-translation-assisting)	0,43	NS	3,01	NS	NS	NS
emb|CDP48686.1|	Forkhead-associated protein	0,65	NS	NS	NS	NS	NS
emb|CEG98018.1|	Hypothetical protein PFCIRM1025 10550	NS	NS	NS	2,03	2,26	NS
emb|CDP48332.1|	Low molecular weight protein-tyrosine-phosphatase	NS	NS	NS	NS	1,51	NS
emb|CDP48394.1|	PP2Cc, Serine/threonine phosphatases	NS	NS	NS	1,51	NS	NS
emb|CDP49681.1|	Sensor protein, ATP-binding region	NS	NS	NS	0,60	0,61	NS
emb|CDP49077.1|	S-ribosylhomocysteine lyase (Autoinducer-2 production protein luxS)	NS	NS	NS	1,94	2,02	NS
emb|CDP49682.1|	two component system response regulator	NS	NS	1,71	NS	NS	NS
emb|CEG99898.1|	two-component response regulator	NS	NS	NS	NS	1,50	NS
emb|CDP49398.1|	Response regulator receiver protein	1,53	NS	NS	NS	NS	NS
emb|CDP49793.1|	Forkhead-associated protein	1,69	NS	NS	NS	NS	NS
emb|CDP49152.1|	Sensor-like histidine kinase	2,33*	2,25*	NS	1,88*	2,00*	NS
Intracellular trafficking and secretion							
emb|CDP48622.1|	Protein-export membrane protein secF	NS	NS	NS	1,57	NS	NS
emb|CDP48197.1|	Redox protein export	NS	1,85*	NS	NS	NS	NS
emb|CDP48310.1|	secretory protein	NS	NS	NS	2,89*	2,91*	NS
emb|CDP48624.1|	Preprotein translocase subunit YajC	1,73	NS	NS	1,57	1,60	NS
Defense mechanism							
emb|CEH01110.1|	ABC transporter permease	NS	NS	NS	1,90*	1,77*	NS
emb|CDP49602.1|	ATP binding protein of ABC transporter	NS	NS	NS	1,55*	1,56*	NS
emb|CDP48425.1|	Putative ABC transporter ATP-binding protein	NS	1,73	2,41	NS	NS	NS
emb|CDP49067.1|	Restriction endonuclease PvuRts1 I	NS	NS	1,62	NS	NS	NS
emb|CDP48012.1|	ABC transporter ATP-binding protein	1,70	NS	NS	NS	NS	NS

Supplementary Table S1: Membrane fatty acids composition after different adaptations. The strain was previously grown in YEL and YEL+NaCl broths, and subjected to heat and acid-adaptation. Relative membrane fatty acids concentration are expressed in percentage.

	YEL	YEL+ 42°C, 1h	YEL+ pH=5, 1h	YEL+NaCl	YEL+NaCl+ 42°C, 1h	YEL+NaCl+ pH=5, 1h
C10:0	0,00 ± 0,00	0,04 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C11:0	0,14 ± 0,05	0,14 ± 0,09	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C12:0	3,00 ± 0,55	2,99 ± 0,94	1,05 ± 0,14	0,03 ± 0,01	0,08 ± 0,00	0,15 ± 0,01
C13:0	0,65 ± 0,18	0,50 ± 0,21	0,24 ± 0,00	0,27 ± 0,01	0,12 ± 0,00	0,13 ± 0,00
C14:0	0,32 ± 0,03	0,35 ± 0,05	0,32 ± 0,00	0,27 ± 0,04	0,18 ± 0,01	0,32 ± 0,01
iso C15:0	4,93 ± 0,05	4,71 ± 0,16	7,27 ± 0,27	3,67 ± 0,10	4,37 ± 0,04	2,46 ± 0,08
C14:1n5	2,82 ± 0,25	2,94 ± 0,38	3,01 ± 0,02	3,93 ± 0,05	3,90 ± 0,04	4,88 ± 0,06
anteiso-C15:0	38,38 ± 3,22	39,86 ± 5,06	42,80 ± 0,34	45,86 ± 0,13	50,05 ± 0,57	49,37 ± 0,20
C15:0	2,72 ± 0,03	2,66 ± 0,13	3,92 ± 0,04	3,80 ± 0,04	2,67 ± 0,05	2,31 ± 0,02
iso-C16:0	0,19 ± 0,03	0,20 ± 0,03	0,27 ± 0,01	0,81 ± 0,01	0,44 ± 0,02	0,53 ± 0,01
anteiso-C16:0	5,13 ± 0,26	5,66 ± 1,29	4,69 ± 0,14	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C16:0	3,78 ± 0,18	3,40 ± 0,28	2,82 ± 0,14	7,69 ± 0,12	7,75 ± 0,20	7,27 ± 0,05
C16:1n7	7,24 ± 1,11	6,78 ± 1,89	4,80 ± 0,36	0,08 ± 0,01	0,15 ± 0,01	0,13 ± 0,01
iso-C17:0	0,39 ± 0,04	0,44 ± 0,04	0,49 ± 0,03	0,86 ± 0,02	0,72 ± 0,03	0,94 ± 0,03
anteiso-C17:0	3,35 ± 0,25	3,65 ± 0,44	3,92 ± 0,03	5,26 ± 0,98	6,58 ± 0,21	7,70 ± 0,22
C17:0	3,95 ± 0,14	3,94 ± 0,16	4,80 ± 0,10	7,06 ± 0,16	5,45 ± 0,20	6,31 ± 0,08
9-10 CH2C16:0	3,23 ± 1,09	2,43 ± 1,02	0,72 ± 0,04	0,00 ± 0,00	0,05 ± 0,00	0,04 ± 0,00
C18:0	0,16 ± 0,01	0,15 ± 0,02	0,12 ± 0,01	0,23 ± 0,03	0,15 ± 0,02	0,41 ± 0,02
C18:1n9c	3,08 ± 0,34	2,78 ± 0,68	2,26 ± 0,06	0,26 ± 0,02	0,26 ± 0,00	0,33 ± 0,00
C19:0	0,29 ± 0,03	0,27 ± 0,04	0,28 ± 0,02	1,47 ± 0,09	0,83 ± 0,05	1,24 ± 0,07
C20:0	0,04 ± 0,02	0,01 ± 0,01	0,00 ± 0,00	0,15 ± 0,03	0,15 ± 0,06	0,16 ± 0,02
C22:1n9c	0,01 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
C22:2n6c	0,07 ± 0,01	0,03 ± 0,01	0,04 ± 0,00	0,01 ± 0,01	0,00 ± 0,00	0,05 ± 0,00
% saturated	15,06 ± 1,02	14,45 ± 0,96	13,54 ± 0,18	20,97 ± 0,23	17,39 ± 0,43	18,30 ± 0,16
% unsaturated	16,45 ± 2,28	14,96 ± 3,20	10,82 ± 0,39	4,28 ± 0,04	4,36 ± 0,03	5,42 ± 0,06
% branched	52,37 ± 3,28	54,52 ± 4,37	59,45 ± 0,37	56,46 ± 0,98	62,15 ± 0,87	61,00 ± 0,12
saturated/ unsaturated	0,92 ± 0,08	0,97 ± 0,22	1,25 ± 0,04	4,90 ± 0,07	3,99 ± 0,07	3,37 ± 0,04
saturated/ branched	0,29 ± 0,04	0,27 ± 0,04	0,23 ± 0,00	0,37 ± 0,01	0,28 ± 0,00	0,30 ± 0,00
unstaurated/ branched	0,31 ± 0,06	0,27 ± 0,08	0,18 ± 0,01	0,08 ± 0,00	0,07 ± 0,00	0,09 ± 0,00

Supplementary Table S2: Proteins modulated during osmoadaptation.
Proteins were determined by using a database composed of proteome of *P. freudenreichii* CIRM-BIA 129 (downloaded from NCBI.nlm.nih.gov 2018). Ratio were calculated using the XIC methods and indicate induction (ratio>1.5) or repression (ratio>0.66) by NaCl.

Accession	Description	YEL+NaCl/YEL
Energy production and conversion		
emb|CDP49203.1|	Lactaldehyde dehydrogenase	5.59
emb|CDP48732.1|	L-lactate dehydrogenase	3.53
emb|CDP49604.1|	2,5-diketo-D-gluconate reductase A	3.13
emb|CDP48297.1|	NADH-dependent flavin oxidoreductase	2.64
emb|CDP47932.1|	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase	2.57
emb|CDP49671.1|	Putative aldo/keto reductase (oxidoreductase)	2.53
emb|CDP47725.1|	oxidoreductase	2.40
emb|CDP48479.1|	Aldo/keto reductase	2.24
emb|CDP48511.1|	Nitroreductase	1.87
emb|CDP49123.1|	Aldo/keto reductase	1.79
emb|CDP47999.1|	NADH-quinone oxidoreductase subunit I (NADH dehydrogenase I subunit I) (NDH-1 subunit I)	1.78
emb|CDP48899.1|	Oxidoreductase	1.77
emb|CDP48001.1|	NADH-quinone oxidoreductase chain G (NADH dehydrogenase I, chain G)	1.72
emb|CDP47998.1|	NADH-quinone oxidoreductase chain J (NADH dehydrogenase I, chain J)	1.69
emb|CDP48908.1|	D-lactate dehydrogenase	1.69
emb|CDP48804.1|	ATP synthase B chain (F0F1 ATP synthase subunit B)	1.67
emb|CDP48428.1|	pyruvate dehydrogenase E1 component (2-oxo-acid dehydrogenase E1 subunit, homodimeric type)	1.63
emb|CDP48006.1|	NADH-quinone oxidoreductase chain B	1.62
emb|CDP48801.1|	ATP synthase gamma chain (ATP synthase F1 sector gamma subunit)	1.61
emb|CDP49140.1|	iron-sulfur protein	1.60
emb|CDP49141.1|	Oxidoreductase	1.58
emb|CDP49579.1|	Electron transfer flavoprotein, carnitine metabolism (FixB protein)	1.57
emb|CDP48003.1|	NADH-quinone oxidoreductase chain E	1.57
emb|CDP48002.1|	NADH-quinone oxidoreductase chain F (NADH dehydrogenase I, chain F) (NDH-1, chain F)	1.52
emb|CDP48444.1|	Dihydrolipoyl dehydrogenase (E3 component of alpha keto acid dehydrogenase complexes) (Dihydrolipoamide dehydrogenase)	1.51
emb|CDP49268.1|	Glycerol kinase (ATP:glycerol 3-phosphotransferase) (Glycerokinase) (GK)	0.67
emb|CDP48122.1|	Betaine-aldehyde dehydrogenase	0.66
emb|CDP48900.1|	Putative aldo/keto reductase (oxidoreductase)	0.65
emb|CDP49717.1|	Inorganic pyrophosphatase	0.55
emb|CDP48543.1|	Acetate kinase	0.54
emb|CDP48544.1|	Phosphate acetyltransferase	0.45
emb|CDP49838.1|	Sulfite reductase [ferredoxin]	0.36

Cell cycle control, cell division, chromosome partitioning		
emb|CDP49426.1|	Protein mrp homolog (ATP-binding protein)	1.74
emb|CDP49357.1|	Hypothetical protein PFCIRM129_11455	1.59

Amino acid transport and metabolism		
emb|CDP48583.1|	Prephenate dehydrogenase	2.90
emb|CDP47878.1|	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	2.23
emb|CDP48936.1|	Aminopeptidase	2.02
emb|CDP48564.1|	polar amino acid ABC transporter, ATP binding component	1.86
emb|CDP47695.1|	Nucleoside-diphosphate kinase	1.82
emb|CDP48687.1|	Glycine cleavage H-protein (lipoate-binding)	1.79
emb|CDP48582.1|	Cytidylate kinase (CK) (Cytidine monophosphate kinase) (CMP kinase)	1.78
emb|CDP49472.1|	ABC-type choline/glycine betaine transport,ATP-binding protein	1.67
emb|CDP49173.1|	Dihydroxy-acid dehydratase	1.65
emb|CDP49146.1|	L-asparaginase I	1.65
emb|CDP48643.1|	Tryptophan synthase alpha chain (TrpA)	1.55
emb|CDP47760.1|	Kinase ArgK	1.52
emb|CDP49589.1|	Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase related deacylase	1.51
emb|CDP47866.1|	Serine acetyltransferase	1.50
emb|CDP48503.1|	Amidohydrolase (Peptidase M20D) (Putative metal-dependent amidase/aminoacylase/carboxypeptidase)	0.67
emb|CDP48372.1|	binding protein of oligopeptide ABC transporter (OPN : undef : Oligopeptides)	0.65
emb|CDP49606.1|	Shikimate 5-dehydrogenase	0.64
emb|CDP49535.1|	cysteine synthase 2	0.54
emb|CDP49386.1|	Phospho-2-dehydro-3-deoxyheptonate aldolase	0.50
emb|CDP47931.1|	solute binding protein of the ABC transport system	0.49

Nucleotide transport and metabolism		
emb|CDP47763.1|	Thymidylate synthase	1.92
emb|CDP49821.1|	Histidine triad (HIT) protein	1.53
emb|CDP48844.1|	ribose-phosphate pyrophosphokinase (RPPK) (Phosphoribosyl pyrophosphate synthetase) (P-Rib-PP synthetase) (PRPP synthetase)	0.59

Carbohydrate transport and metabolism		
emb|CDP49182.1|	iolB (Myo-inositol catabolism IolB protein)	2.04
emb|CDP49834.1|	Ribose-5-phosphate isomerase 3	1.90
emb|CDP48739.1|	Phosphoketolase pyrophosphate	1.87
emb|CDP48882.1|	phosphoglycerate mutase/fructose-2,6-bisphosphatase	1.84
emb|CDP49742.1|	endonuclease	1.68
emb|CDP48781.1|	Alpha-glucan phosphorylase	1.63
emb|CDP48893.1|	Oxidoreductase	1.62
emb|CDP47720.1|	6-phosphogluconate dehydrogenase,decarboxylating	1.56
emb|CDP49513.1|	Alpha-1,4-glucosidase	1.55
emb|CDP49568.1|	Polyphosphate glucokinase	0.66

emb|CDP48902.1|	Fructose-bisphosphate aldolase class I	0.63
emb|CDP49639.1|	Gluconate kinase (Gluconokinase)	0.59
emb|CDP47629.1|	Glycogen debranching enzyme GlgX	0.49
emb|CDP47859.1|	binding protein of ribose ABC transporter	0.46
emb|CDP48824.1|	Dihydroxyacetone kinase	0.43
emb|CDP49430.1|	Glucose-1-phosphate adenylyltransferase (ADP-glucose synthase) (ADP-glucose pyrophosphorylase) (ADPGlc PPase)	0.39
emb|CDP49099.1|	PTS system, mannose/fructose/sorbose family, IIA component subfamily	0.26

Coenzyme transport and metabolism		
emb|CDP49852.1|	Riboflavin synthase alpha chain	11.88
emb|CDP49854.1|	6,7-dimethyl-8-ribityllumazine synthase (Riboflavin synthase beta chain)	5.57
emb|CDP49853.1|	GTP cyclohydrolase II protein, Riboflavin biosynthesis protein	5.41
emb|CDP47769.1|	Nicotinate phosphoribosyltransferase	1.99
emb|CDP47762.1|	Dihydrofolate reductase	1.89
emb|CDP47610.1|	Phosphopantetheine adenylyltransferase (Pantetheine-phosphate adenylyltransferase) (PPAT) (Dephospho-CoA pyrophosphorylase)	1.83
emb|CDP49449.1|	Hypothetical protein PFCIRM129_11950	1.56
emb|CDP47839.1|	Methylenetetrahydrofolate dehydrogenase (Bifunctional protein)	1.55
emb|CDP47757.1|	CbiL Precorrin-2 C20-methyltransferase	1.51
emb|CDP48767.1|	Thiamine monophosphate kinase	0.65
emb|CDP49317.1|	Glutamine amidotransferase subunit pdxT (Glutamine amidotransferase glutaminase subunit pdxT)	0.31

Lipid transport and metabolism		
emb|CDP49767.1|	inositol-1-phosphate synthase	1.87
emb|CDP47827.1|	Enoyl-CoA hydratase/carnithine racemase CaiD	1.84
emb|CDP49388.1|	Acyltransferase PlsC	1.52
emb|CDP48433.1|	Acyl carrier protein (ACP)	0.59
emb|CDP48431.1|	Carboxylic ester hydrolase	0.57

Translation, ribosomal structure and biogenesis		
emb|CDP48703.1|	Ribonuclease D (3-5 exonuclease)	2.13
emb|CDP48586.1|	tRNA (adenine-N1-)-methyltransferase	2.00
emb|CDP47563.1|	Phenylalanyl-tRNA synthetase beta chain (Phenylalanine--tRNA ligase beta chain) (PheRS)	1.61
emb|CDP49392.1|	Hypothetical protein PFCIRM129_11635	1.59
emb|CDP47564.1|	Phenylalanyl-tRNA synthetase alpha chain (Phenylalanine--tRNA ligase alpha chain) (PheRS)	1.55
emb|CDP49673.1|	rRNA (guanine-N2-)-methyltransferase	1.53
emb|CDP47688.1|	Ribonuclease, Rne/Rng family	1.52
emb|CDP48399.1|	Tryptophanyl-tRNA synthetase	1.51
emb|CDP48614.1|	Aspartyl-tRNA synthetase (Aspartate--tRNA ligase) (AspRS)	0.66
emb|CDP47699.1|	Valyl-tRNA synthetase (Valine--tRNA ligase) (ValRS)	0.64
emb|CDP48886.1|	tRNA nucleotidyltransferase PcnB	0.58
emb|CDP47921.1|	Translation initiation factor IF-1	0.55

Transcription		
emb|CDP49615.1|	Cell envelope-related function transcriptional attenuator common domain	2.08
emb|CDP48823.1|	Transcriptional Regulator, GntR family protein	1.79
emb|CDP49019.1|	Heat shock protein transcriptional repressor HspR1 (Hspr1 protein)	1.77
emb|CDP49403.1|	Phage shock protein A	1.53
emb|CDP49049.1|	Iron-dependent repressor	0.66
emb|CDP49150.1|	transcriptional regulator CarD	0.57
emb|CDP48876.1|	chromosome partitioning protein	0.56

Replication, recombination and repair		
emb|CDP48328.1|	DNA polymerase III, delta prime subunit	2.43
emb|CDP49216.1|	Putative endonuclease III	1.86
emb|CDP49351.1|	Putative DNA polymerase I	1.73
emb|CDP47716.1|	DNA ligase (NAD+)	1.55
emb|CDP49156.1|	Serine 3-dehydrogenase	1.54
emb|CDP48988.1|	DNase	0.60
emb|CDP48861.1|	DNA polymerase III, beta chain	0.60

Cell wall/membrane/envelope biogenesis		
emb|CDP47662.1|	GTP-binding protein LepA	2.32
emb|CDP49368.1|	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase (UDP-MurNAc-pentapeptide synthetase) (D-alanyl-D-alanine-adding enzyme)	1.98
emb|CDP49372.1|	S-adenosyl-L-methionine-dependent methyltransferase mraW	1.97
emb|CDP49370.1|	Cell division protein FtsI (penicillin-binding protein 2) (Peptidoglycan glycosyltransferase)	1.88
emb|CDP47743.1|	UDP-N-acetylmuramyl tripeptide synthase (Mur ligase)	1.76
emb|CDP49363.1|	UDP-N-acetylmuramate--L-alanine ligase (UDP-N-acetylmuramoyl-L-alanine synthetase)	1.55

NA		
emb|CDP48292.1|	Hypothetical protein PFCIRM129_00795	4.28
emb|CDP48978.1|	Hypothetical protein PFCIRM129_06090	3.88
emb|CDP49476.1|	protein PFCIRM129_12125	3.05
emb|CDP49779.1|	Hypothetical protein PFCIRM129_02030	2.16
emb|CDP49700.1|	Hypothetical protein PFCIRM129_01610	2.14
emb|CDP49087.1|	Hypothetical protein PFCIRM129_09745	1.98
emb|CDP47734.1|	Hypothetical protein PFCIRM129_07095	1.73
emb|CDP49445.1|	Hypothetical protein PFCIRM129_11930	1.71
emb|CDP49043.1|	Hypothetical protein PFCIRM129_06445	1.59
emb|CDP48185.1|	Protein of unknown function	0.65
emb|CDP48289.1|	Hypothetical protein PFCIRM129_00780	0.57
emb|CDP48151.1|	Hypothetical protein PFCIRM129_09035	0.55
emb|CDP48183.1|	Putative uncharacterized protein	0.50
emb|CDP49235.1|	Putative uncharacterized protein	0.48
emb|CDP49067.1|	Restriction endonuclease PvuRts1 I	0.47
emb|CDP48840.1|	Hypothetical protein PFCIRM129_05365	0.45

emb|CDP48275.1|	Protein of unknown function	0.36
emb|CDP49138.1|	Hypothetical protein PFCIRM129_10170	0.25

Post-translational modification, protein turnover, and chaperones		
emb|CDP48339.1|	Heat shock protein 20 3 (20 kDa chaperone 3)	3.25
emb|CDP49617.1|	thiol peroxidase	2.50
emb|CDP49795.1|	Thioredoxin	2.49
emb|CDP49065.1|	Surface layer protein A (S-layer protein A)	2.24
emb|CDP49048.1|	Thioredoxine	2.15
emb|CDP49400.1|	HesB protein	1.93
emb|CDP48411.1|	SmpB SsrA-binding protein	1.92
emb|CDP48424.1|	Heat shock protein 20 2 (20 kDa chaperone 2)	1.68
emb|CDP48051.1|	Protein GrpE 2 (HSP-70 cofactor 2) (Co-chaperone protein GrpE2)	1.62
emb|CDP49021.1|	Protein GrpE 1 (HSP-70 cofactor 1) (Co-chaperone protein GrpE1)	1.61
emb|CDP48340.1|	Heat shock protein 20 1 (20 kDa chaperone 1)	1.57
emb|CDP47983.1|	Peroxiredoxin/Alkyl hydroperoxide reductase subunit C /Thioredoxin peroxidase/Alkyl hydroperoxide reductase protein C22/General stress protein 22	1.53
emb|CDP49312.1|	FeS assembly protein SufB	0.61
emb|CDP49702.1|	Stomatin/prohibitin	0.60
emb|CDP47885.1|	Putative O-sialoglycoprotein endopeptidase	0.59
emb|CDP48273.1|	Surface layer protein B (S-layer protein B)	0.41
emb|CDP48858.1|	Surface protein with SLH domain	0.15

Inorganic ion transport and metabolism		
emb|CDP49080.1|	transporter	3.17
emb|CDP49660.1|	Starvation-inducible DNA-binding protein	1.77
emb|CDP48854.1|	Metal binding protein	1.61
emb|CDP48059.1|	Cobalt import ATP-binding protein CbiO	0.63
emb|CDP49684.1|	ABC-type transport systems, periplasmic component	0.62
emb|CDP49458.1|	Membrane protein, Transporter, MFS superfamily	0.61
emb|CDP48888.1|	Iron transport system substrate-binding protein ABC transporter	0.47
emb|CDP49835.1|	Sulfate adenylyltransferase, large subunit/ ATP-sulfurylase, subunit 1 (ATP:sulfate adenylyltransferase)	0.44
emb|CDP48302.1|	Heavy metal transport/detoxification protein	0.40
emb|CDP48261.1|	Putative uncharacterized protein	0.29

Secondary metabolites biosynthesis, transport, and catabolism		
emb|CDP47718.1|	DSBA-like thioredoxin	2.05
emb|CDP49510.1|	Cytochrome P450 (Heme-thiolate monooxygenase)	1.82

Function unknown		
emb|CDP48021.1|	Protein of unknown function	3.58
emb|CDP48426.1|	Hypothetical protein PFCIRM129_03130	2.18
emb|CDP48618.1|	Beta-lactamase-like	2.11
emb|CDP48018.1|	PRC-barrel	2.07
emb|CDP48395.1|	Hypothetical protein PFCIRM129_02970	2.03

emb|CDP49561.1|	Metallophosphoesterase	1.98
emb|CDP49540.1|	Hypothetical protein PFCIRM129_00415	1.977
emb|CDP48751.1|	FeoB, Ferrous iron transport protein B	1.95
emb|CDP47930.1|	ABC2 protein of oligopeptide ABC transporter (OPN:undef)	1.82
emb|CDP47717.1|	Hypothetical protein PFCIRM129_07010	1.75
emb|CDP48684.1|	Putative uncharacterized protein	1.70
emb|CDP47923.1|	Hypothetical protein PFCIRM129_08080	1.70
emb|CDP48064.1|	NADH-flavin reductase	1.69
emb|CDP48994.1|	Hypothetical protein PFCIRM129_06175	1.69
emb|CDP48378.1|	Hypothetical secreted and membrane protein	1.62
emb|CDP48956.1|	Hypothetical protein PFCIRM129_05975	1.59
emb|CDP47744.1|	(glutamine amidotransferase) (adenosylcobyric acid synthase (glutamine-hydrolysing))	1.57
emb|CDP48721.1|	Hypothetical protein PFCIRM129_04715	1.54
emb|CDP47616.1|	Acetyltransferase	1.53
emb|CDP47639.1|	Hypothetical protein PFCIRM129_06595	1.53
emb|CDP49667.1|	Hypothetical protein PFCIRM129_01445	1.52
emb|CDP48651.1|	ABC transporter substrate-binding protein	0.67
emb|CDP48701.1|	YbaK / prolyl-tRNA synthetases	0.65
emb|CDP48279.1|	Protein of unknown function	0.65
emb|CDP49334.1|	ATPase	0.60
emb|CDP49444.1|	secreted transglycosydase	0.58
emb|CDP48901.1|	Hypothetical protein PFCIRM129_05690	0.47
emb|CDP49497.1|	internaline A	0.20

Signal transduction mechanisms		
emb|CDP48332.1|	Low molecular weight protein-tyrosine-phosphatase (protein-tyrosine-phosphatase)	6.63
emb|CDP47742.1|	Protein-tyrosine phosphatase	2.77
emb|CDP49789.1|	Serine/threonine protein kinase	2.48
emb|CDP49682.1|	two component system response regulator	1.97
emb|CDP49715.1|	phosphohistidine phosphatase	1.77
emb|CDP48394.1|	PP2Cc, Serine/threonine phosphatases	1.73
emb|CDP49816.1|	Two component transcriptional regulator, LuxR family	1.51
emb|CDP49793.1|	Forkhead-associated protein	1.50

Intracellular trafficking, secretion, and vesicular transport		
emb|CDP48622.1|	Protein-export membrane protein secF	1.55

Defense mechanisms		
emb|CDP48863.1|	transporter ATP-binding protein	1.86
emb|CDP48250.1|	Protein of unknown function	1.69

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Titre : Les adaptations bactériennes améliorent la survie de Propionibacterium freudenreichii durant le séchage et le stockage

Mots clés : Probiotique, ferment, séchage par pulvérisation, adaptation bactérienne, protections-croisées

Résumé : Les probiotiques et les ferments sont stabilisés sous forme de poudre pour faciliter leur stockage et leur utilisation. Ils sont généralement séchés par lyophilisation, procédé à la fois coûteux énergétiquement et à faible rendement. Le séchage par pulvérisation représente donc une alternative durable. Cependant, il impose des stress osmotique, chaud et oxydatif, et entraine par suite la mort d'une part importante des bactéries.

Dans ce travail, les adaptations de Propionibacterium freudenreichii durant différents traitements ont été étudiées pour mieux comprendre les protections-croisées induites. Les mécanismes d'adaptation comprennent l'accumulation de solutés compatibles, la surproduction des protéines générales de stress et la modulation de la composition en acides gras membranaires. Pendant l'osmoadaptation, l'accumulation des solutés compatibles a été modulée par la quantité d'azote non-protéique et de glucides du milieu de culture. De grandes quantités de glycine bétaïne accumulées ont permis d'obtenir un fort taux de survie pendant la lyophilisation. En revanche, l'accumulation conjointe de glycine bétaïne et de tréhalose a permis d'obtenir un fort taux de survie pendant le séchage par pulvérisation. De plus, l'addition de glucide combinée à l'osmoadaptation ou à une thermo-adaptation provoque l'accumulation de trehalose et de glycine bétaïne.

L'adaptation peut donc être optimisée pour obtenir des bactéries plus résistantes aux procédés de séchage, de stockage et de la digestion. Ces travaux offrent de nouvelles solutions pour la production de levains et de probiotiques avec des applications plus nombreuses.

Title : Bacteria adaptations improves the survival of Propionibacterium freudenreichii during drying and storage

Keywords : Probiotic, starter, spray drying, bacteria adaptation, cross-protections

Abstract : Probiotic and starters are produced in powder form to facilitate storage, delivery and usage. These beneficial bacteria are usually dried by freeze-drying, which is expensive with low productivity. From this point of view, spray drying is an alternative and sustainable process to produce beneficial bacteria. Unfortunately, this process imposes osmotic, heat and oxidative stresses: therefore, it can lead to an important bacterial death.

In this work, Propionibacterium freudenreichii adaptation to different treatments were studied to better understand their impact on cross-protections. Adaptation mechanisms included compatible solutes accumulation, general stress protein over-production and modulation of membrane fatty acids composition. During osmoadaptation, compatible solutes accumulation was fine-tuned by the medium. composition and especially by the amount of non-protein nitrogen sources and carbohydrates. Accumulation of high amounts of glycine betaine during osmoadaptation led to high survival during freeze-drying. By contrast, accumulation of high amounts of glycine betaine and trehalose led to high viability during spray drying.

Different adaptations can trigger the same adaptation mechanisms. Accumulation of high amounts of trehalose and of glycine betaine was triggered by the addition of saccharides, combined to osmoadaptation or to heat-adaptation.

Adaptation can be optimized to confer bacteria enhanced resistance during drying processes, storage and digestion. This work opens new avenues for the production of starters and probiotics with enhanced robustness.