Spray drying of probiotic bacteria: From molecular mechanism to pilot-scale productio

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Séchage par atomisation des bactéries probiotiques : des mécanismes de protection à la production à l'échelle pilote

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欲穷千里目，更上一层楼。
- 王之涣 《登鹳雀楼》
Désirant embrasser toute l'étendue de ce paysage,
je gravis un étage supplémentaire de la tour.
De Zhihuan WANG (688-742)

Acknowledgement

Three years have gone by since the first time I arrived Rennes in 2014. France (Bretagne) is the first country (region) I reach abroad, and Rennes is the first city I live out of China. It seems not so far away from the first time I saw the amazingly huge green farms and clean blue sky here. As the first and only child who got the “college-level” education in my big family, it is hard to imagine this experience of doing a PhD in Europe. I am very grateful to this joint PhD project supported by Soochow University and Agrocampus Ouest. I also thank the joint research unit (STLO) between INRA and Agrocampus Ouest, and the Suzhou Key Lab of Green Chemical Engineering for providing the world-class laboratories.

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Figure without number. The logo of the office of Elise, Samira and Song, 2016-2017. "FRAZINA" symbolizes the friendship among France, Brazil and China.

Thesis Outputs

Review article

Huang, S., Vignolles, M., Chen, X.D., Le Loir, Y., Jan, G., Schuck, P., and Jeantet, R., 2017. Spray drying of probiotics and other food-grade bacteria: A review. Trends in Food Sci. Technol. 63, 1-17.

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Huang, S., Cauty, C., Dolivet, A., Le Loir, Y., Chen, X.D., Schuck, P., Jan, G., Jeantet, R., 2016a. Double use of highly concentrated sweet whey to improve the biomass production and viability of spray-dried probiotic bacteria. J. Funct. Foods 23, 453–463.

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Patents

Method for preparing a probiotic powder using a two-in-one whey-containing nutrient medium. Patent application filed in Europe on 21 September 2015, EP no. 15 306465.4. Inventors: Jeantet, R., Huang, S., Jan, G., Schuck, P., Le Loir, Y., Chen, X.D.

Papers in Preparation

Huang, S., et al., Concentrated sweet whey induces multistress tolerance of *Lactobacillus casei* BL23.

Huang, S., et al., Long term survival of *Lactobacillus casei* BL23 in sweet whey medium.

Huang, S., et al., Probiotic efficacy of spray-dried *Propionibacterium freudenreichii* in piglet model.

Oral Presentations

Huang, S., Le Loir, Y., Chen, X. D., Jan G., Schuck, P., & Jeantet, R. Armed probiotics with 2-in-1 medium: The whey is a way. The 2nd CHE-SIS, 11-13 October 2015, Suzhou, China.

Huang, S., Cauty, C., Dolivet, A., Mejean, S., Le Loir, Y., Chen, X. D., Schuck, P., Jan G. & Jeantet, R. From growth to spray drying: two-in-one use of sweet whey to improve the biomass production and spray drying viability of probiotics. IDF Concentration and Dried Milk Products Symposium, 11-13 April 2016, Dublin, Ireland.

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Huang, S., Jan, G., Le Loir, Y., Schuck, P., Jeantet, R., & Chen, X. D. A novel strategy to increase energy efficiency and productivity for probiotic production: Combination of molecular adaptation, protectant application and processing optimization. The 5th International Congress on Sustainability Science & Engineering, 24-27 October 2016, Suzhou, China

Chen, X. D., Huang, S., Jeantet, R., Wu, P., Liu, M., Chen, L., Zhang, X., Liao, Z. Progressive construction of more realistic species specific in vitro digestion bio-mechatronic models. The 5th International Conference on Food Digestion, 04-06 April 2017, Rennes, France

Supervision

Lab-supervision of two master students (Yi Yang, Xiamen University, from 01/06/2014 to 01/06/2015; Juan Wang, Soochow University, from 01/06/2014 to 01/06/2017)

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List of Abbreviations

CFU – Colony forming unit

CAGR – Compound annual growth rate

GA – Gum Arabic

GIT – Gastricintestinal tracts

LAB – Lactic acid bacteria

LCBL23 – *Lactobacillus casei* BL23

LCZ – *Lactobacillus casei* Zhang

LGG – *Lactobacillus rhamnosus* GG

LP – *Lactobacillus plantarum* P-8

M_d – Droplet mass

MRS – de Man, Rogosa and Sharpe broth

PF129 – *Propionibacterium freudenreichii* CIRM-BIA129

PSD – Particle size distribution

RSM – Reconstituted skim milk

SGF – Simulated gastric fluid

SIF – Simulated intestinal fluid

SW – Sweet whey

TS – Total solid content

T_2 – Transverse relaxation time

T_d – Droplet temperature

T_g – Glass transition temperature

WPI - Whey protein isolate

YEL - Yeast Extract Sodium Lactate broth

SCFAS - Short chain fatty acids

Abstract

Probiotics are defined by FAO/WHO as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'. Having the dried, preserved and ready-to-use form of probiotics offers a wider range of applications for a substantially longer period of time as compared to its unpreserved liquid or slurry form. Probiotic ingredients market was valued at 33.6 Billion USD in 2015 (Global Market Insights, 2016), would reach 64.4 Billion USD by 2020 (Markets and Markets, 2015). Freeze drying is currently a popular drying method to produce probiotic products due to its reliability in term of cell viability. However, its high production-cost, low energy-efficiency and productivity have become a stumbling block with regard to the increasing market demand. Spray drying is expected to be an alternative method for production of probiotic powders. The low production cost and high productivity are highly desirable by industry. The challenge consists in the considerable inactivation of probiotics caused by high temperature and dehydration during the process.

In this thesis, we present a novel spray drying process for continuous production of probiotics. Sweet whey is used as 2-in-1 highly concentrated medium (up to 30% w/w dry matter) for both culturing and spray drying of probiotics. This process simplifies the operation units between culturing and drying (e.g. harvesting, washing, and re-suspending steps), increases the cell population yield during growth. It improves probiotic viability and productivity during spray drying and probiotic stability during storage.

The mechanisms were explored from bacterial stress resistance (intrinsic) and drying process conditions (extrinsic). The hypertonic stress led to overexpression of key stress proteins, accumulation of intracellular compatible solutes, which contributed to the enhanced multistress tolerance acquisition. The presence of protein aggregates and increased concentration of Mg^{2+} in the drying matrix may also be responsible for the enhanced probiotic protection in highly concentrated sweet whey.

The feasibility of scaling up this process was validated with a semi industrial pilot scale spray dryer. A multi-stage mild-conditions drying process, coupling spray drying with belt drying and fluid-bed drying, was applied to further improve the probiotic viability to

approximately 100% (> 10^9 CFU g^{-1}). The storage stability and digestion resistance of these probiotic powders were investigated.

This work opens new perspectives for the sustainable development of new starter and probiotic preparations with enhanced robustness.

General Introduction

Scientific and Economic Context of Probiotics

The term "probiotics" has been originally defined by FAO/WHO, and recently endorsed by ISAPP (The International Scientific Association for Probiotics and Prebiotics) as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001; Hill et al., 2014). The overview of microorganisms considered as probiotics and potential 'next generation probiotics' are shown in Table 1.

Table 1. Microorganisms commonly proposed as probiotics or potential probiotics (Concluded from Cani and Van Hul, 2015; Felis et al., 2009; Foligné et al., 2013b; Hill et al., 2014; Holzapfel et al., 2001).

Lactobacillus species	*Bifidobacterium* species	Other LAB	Non lactic acid bacteria	Potential next generation probiotics
L. acidophilus	*B. adolescentis*	*Enterococcus Faecalis*	*Bacillus cereus var.* toyoi	*Akkermansia muciniphila*
L. cmylovorus	*B. animalis*	*Enterococcus faecium*	*Bacillus clausii*	*Clostridium butyricum*
L. casei	*B. bifidum*	*Lactooccus. lactis*	*Bacillus coagulans*	*Eubacterium hallii*
L. crispatus	*B. breve*	*Leuconstoc mesenteroides*	*Bacillus licheniformis*	*Faecalibacterium prausnitzii*
L. bulgaricus	*B. infantis*	*Pediococcus acidilactici*	*Bacillus mesentericus*	*Roseburia* spp.
L. lactis	*B. lactis*	*Pediococcus pentosaceus*	*Bacillus subtillis*	
L. fermentum	*B. longum*	*Sporolactobacillus inulinus*	*Clostridium butyricum*	
L. gallinarum		*Streptococcus thermophilus*	*Escherichia coli* strain nissle	
L. gasseri			*Propionibacterium freudenreichii*	
L. helveticus			*Saccharomyces cerevisiae*	
L. johnsonii			*Saccharomyces boulardii*	
L. kefiri				
L. paracasei				
L. plantarum				
L. reuteri				
L. rhamnosus				
L. sakei				
L. salivarius				

The mechanisms by which probiotics provide these beneficial effects on the host may be mainly classified in three categories: probiotic metabolic effects, microbiota composition normalization and molecular interaction between probiotics and host. Based on these underlying mechanisms, probiotic effects are mostly dependent on strain (Figure 1), albeit some effects are widely spread at the species level or even across different taxonomic groups (Hill et al., 2014).

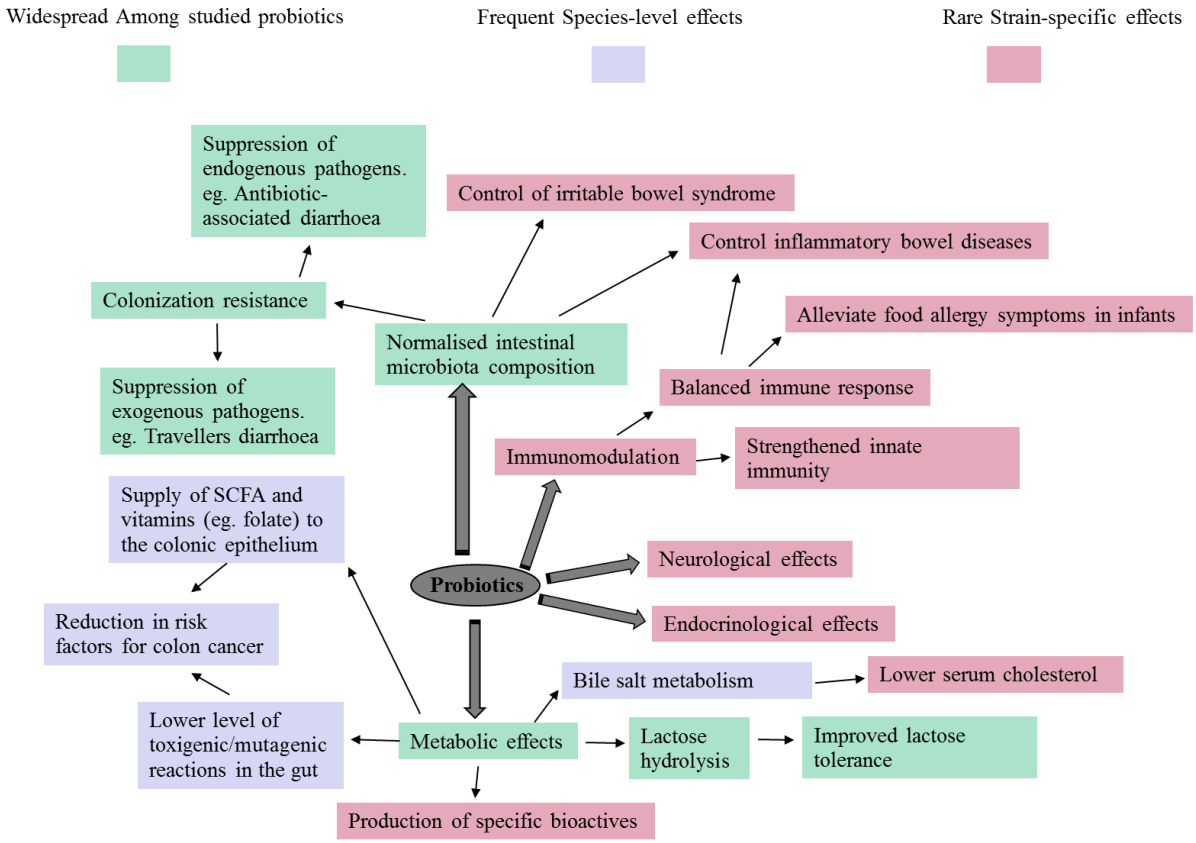


Figure 1. Various health benefits of probiotics and its possible distribution of mechanisms. Some benefits might be widespread among commonly studied probiotic genera; others might be frequently observed among most strains of a probiotic species; others may be rare and present in only a few strains of a given species (Concluded from Hill et al., 2014; Parvez et al., 2006).

The benefits of probiotics are also dependent on adequate dose administered (Johansson et al., 2015; Mimura et al., 2004). Although it differs from different regulators and countries, the recommended minimum dose of orally administered probiotics is typically ranging from 10^6 to 10^7 colony-forming units (CFU)/g of product and 10^9 CFU intake per day. However and from a clinical point of view, no standardised dose of probiotic bacteria generalized to any bacterial species can be recommended to date as ensuring a beneficial effect. The duration of

probiotic intake can range from days to years depending on different cases and probiotic strains (Deshpande et al., 2011; Vouloumanou et al., 2009).

Since the increasing evidences on these benefits, products including live probiotics are highly demanded by current food and pharmaceutical market. According to the report of Global Market Insights, Inc., (2016), the global probiotics market size was valued at USD 36.6 Billion in 2015, in which the human-use probiotics was over USD 34 Billion. This market is expected to grow continuously with over 7.4% CAGR (Compound Annual Growth Rate) from 2016 to 2023, and is projected to 64.6 Billion USD by 2020 due to the growing standard of living and health awareness at a global scale (Global Market Insights, 2016; Markets and Markets, 2015).

Currently, the global market of probiotics is highly competitive and fragmented between food companies. In 2015, the top five players in probiotics market, Danone, Yakult, Nestle, Chr. Hansen and Dupont Danisco, occupied less than 40% of the global revenue (Global Market Insights, 2016). The latter is expected to grow during the following 10 years in all the world regions. Figure 2A shows the potential increase of probiotic market revenue in the United States. Globally speaking, the Asia-Pacific is the largest probiotics market accounting approximately for 38% in 2014, in which Japan forms the largest market with a share of 45% (Mordor Intelligence, 2016). However, China appears likely to be the fastest growing market, with a forecast at 13% CAGR from 2014 to 2020 (Mordor Intelligence, 2016). Driven by this potential of Chinese and Indian market, the Asia-Pacific should remain the largest regional industry for participants (Figure 2B) (Global Market Insights, 2016).

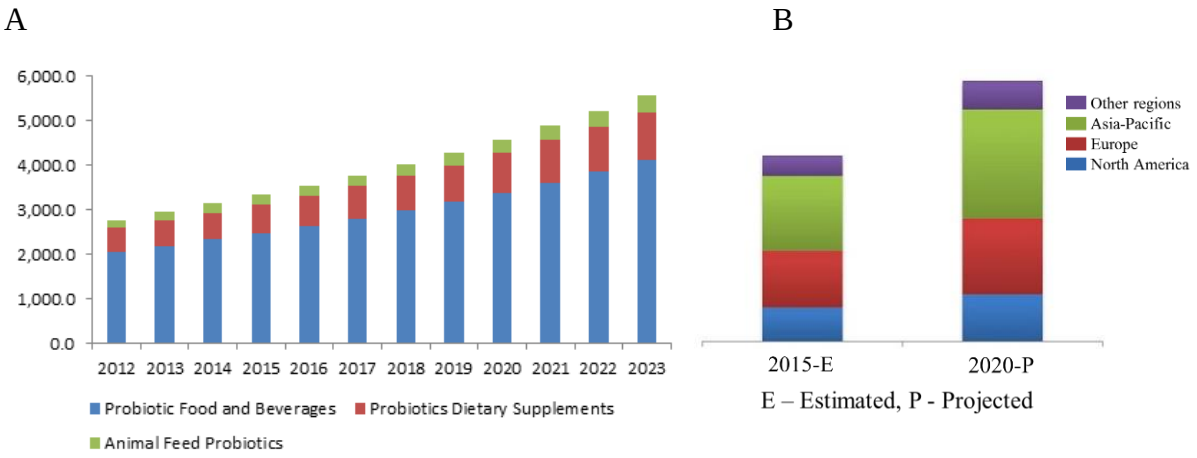


Figure 2. (A) U.S. Probiotics market revenue by product, 2014 - 2023 (USD Million) and (B) probiotic ingredients market size, by region, 2015 vs 2020 (USD Million) (Adapted from Global Market Insights, 2016; MarketsandMarkets, 2015).

Spray Drying of Probiotics: New Opportunities and Challenges

The first detailed description of spray drying was given in United States patent in 1872 (Anandharamakrishnan and S, 2015), as "*the process of simultaneously atomizing and desiccating fluid and solid substances, and its application to the purpose of the exhaustion of moisture from such substances, and for the prevention of destructive chemical change*." (Samuel R., 1872).With more than 140 years' growth and development, the spray drying process has nowadays evolved as one of the most widely-used and industry-friendly drying technique that is spread in dairy industries all over the world.

Briefly, spray drying consists in spraying (or atomizing) the liquid feed in fine droplets (10 to 150 µm) into a flow of hot and dry air (usually 150°C to 250°C; Figure 3). The subsequent increase in the air-liquid interface area dramatically increases the drying kinetics, and it is generally admitted that drying occurs within a few seconds.

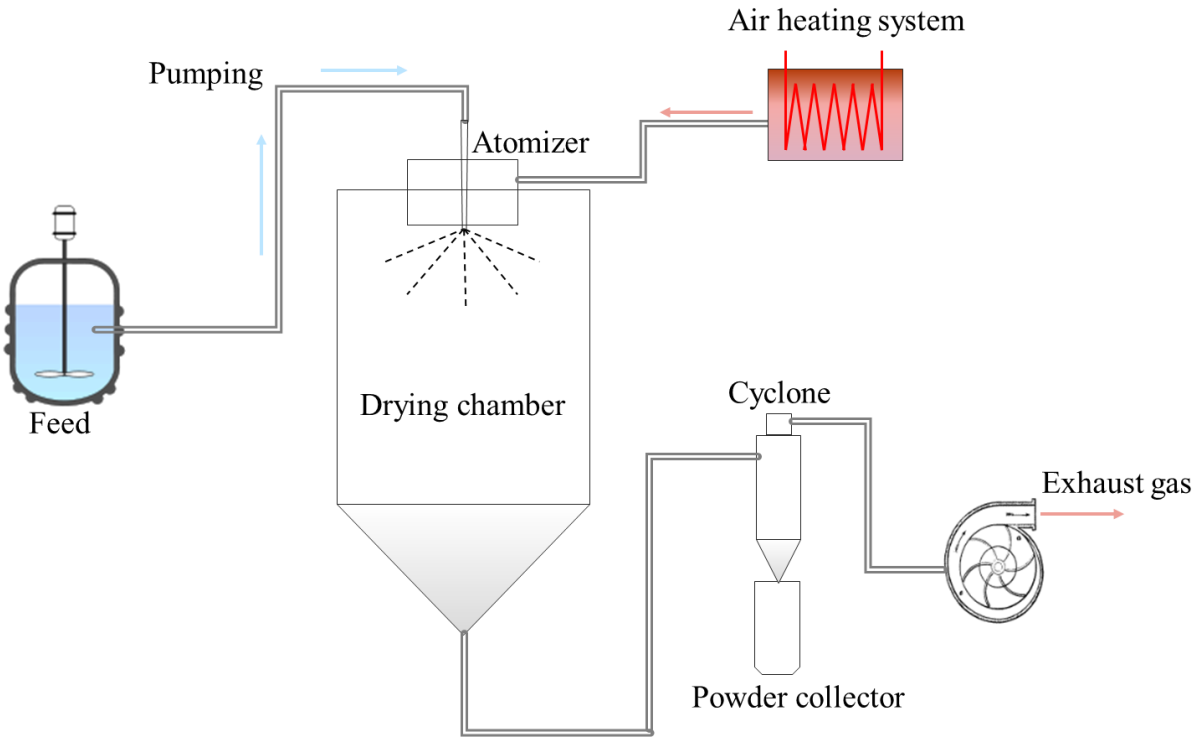


Figure 3. Schematic of a typical spray dryer and its operation process.

The atomizer is the heart of a spray dryer. To date, several types of atomizers have been developed in order to deal with various feed material and thus achieve satisfactory atomization. Among these atomizers, pressure nozzle atomizer, two-fluid nozzle atomizer and rotating

atomizer are the most frequently used. Besides, several innovative atomizers have gradually been applied to obtain powders with distinct particle properties, piezoceramics-driven glass nozzle and ultrasonic nozzle for instances (Tatar Turan et al., 2015; Wu et al., 2014).

Using these technical innovations and multidisciplinary knowledge, it is possible nowadays to tailor via spray drying powders with various surface/internal morphology (Figure 4), having therefore various encapsulating abilities (Nandiyanto and Okuyama, 2011).

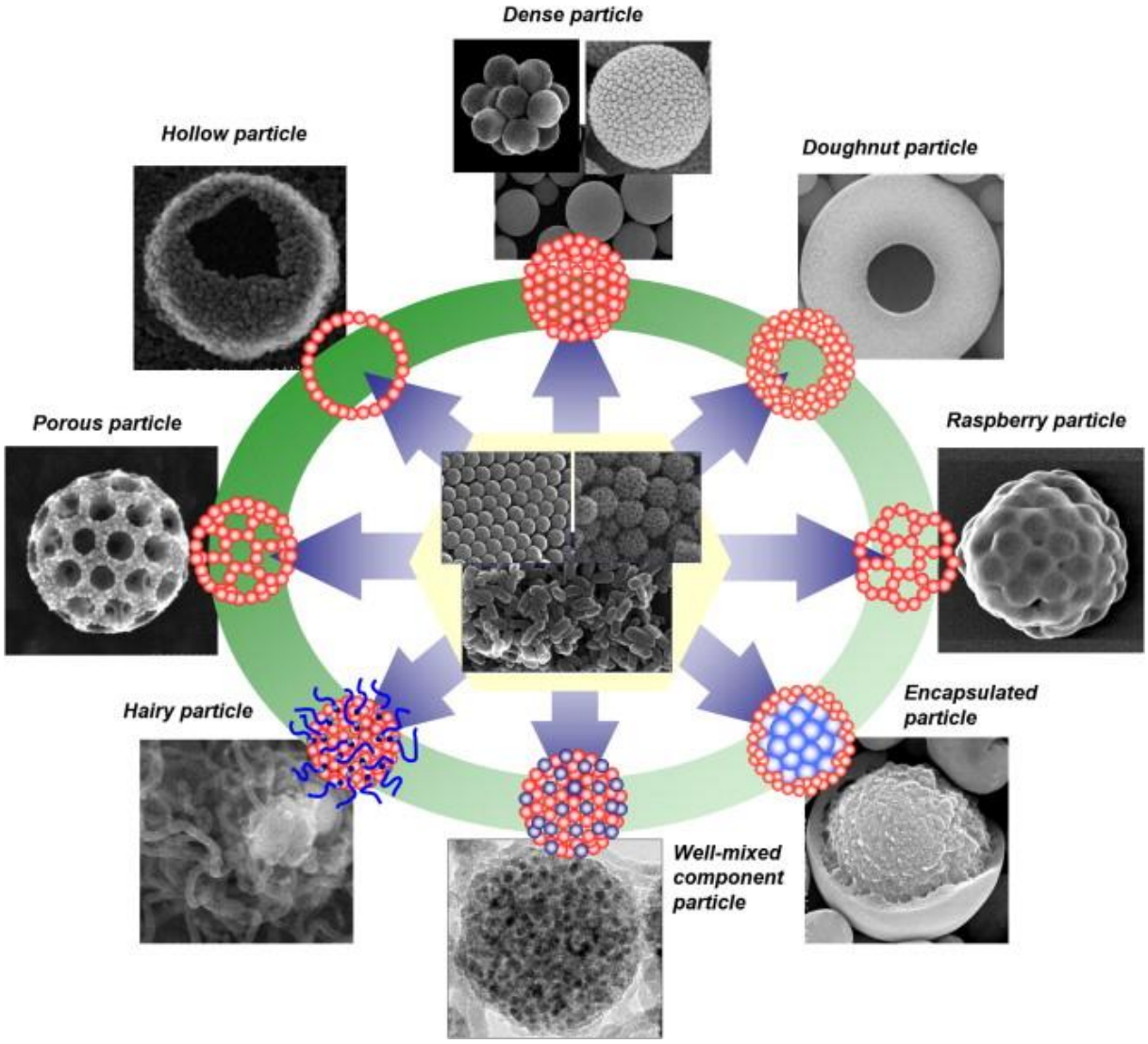


Figure 4. Various particle morphologies prepared using the spray drying method (adapted from Nandiyanto and Okuyama, 2011).

On the other hand, the application of spray drying technique is expected to extend from dairy industry to other emergent and increasing market of food ingredients, as probiotic bacteria for instance. Indeed, drying is a widely-used process for probiotic preservation, given the

longer and stable shelf-life and lower transportation cost of dried probiotics. Nowadays, freeze drying remains the preferred technique to preserve probiotic bacteria; but it is a time-consuming and expensive process (Table 2). Among the drying techniques, spray drying is one of the most promising methods in substitution to freeze drying with regards to drying of probiotics (Schuck et al., 2016). When compared to freeze drying, spray drying exhibits a lower specific energy cost and higher productivity (Table 2).

Table 2. General production pattern and specific energy consumption of commonly used drying processes. (Modified from Huang et al., 2017)

Processes	Production pattern		Specific energy consumption (kJ.kg^{-1} water)	Productivity (ton.year^{-1})	Disadvantages
Spray drying	Liquid ↓ Solid	Continuous	5,300	~50000[a]	High temperature involvement High investments Inapplicable for small sample size
Freeze drying	Liquid ↓ Solid	Batch	18,000	~10000[a]	High residence time (24-48h) High production cost Low energy efficiency
Fluidized-bed drying	Solid ↓ Solid	Continuous	11,400	n.a.	High residence time High investments Low energy efficiency Inapplicable for liquid sample

n.a.: not available
[a] An approximate value for a large scale dryer from personal communication (The productivity actually depends on equipment, production scale and market demand).

However, the use of spray drying to produce viable cultures, especially with "sensitive" probiotic strains, is still challenging (Broeckx et al., 2016; Liu et al., 2015; Peighambardoust et al., 2011). Extensive investigations have been carried out in the recent two decades all over the world in order to find strategies to protect probiotics during spray drying (Figure 5), in particular based on:

- **Inducing bacterial stress adaptation to enhance cellular intrinsic tolerance upon spray drying (Microbiological strategy)**
- **Application of protective agents to minimize cell damage during process (Material strategy)**
- **Optimization of process to moderate the harsh conditions (Processing strategy).**

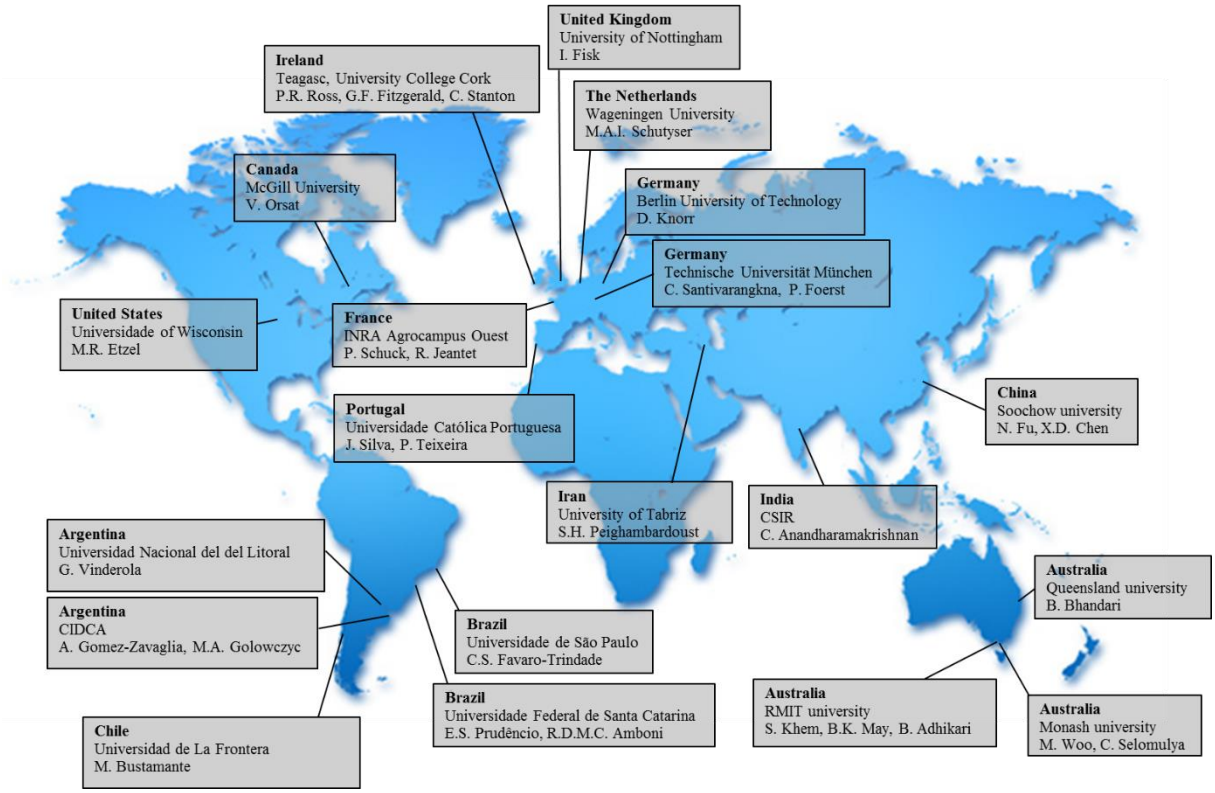


Figure 5. Overview of main research groups focusing on the application of spray drying in probiotic production.

Chapter 1. Literature Review

This chapter aims at providing the literature review of the current strategies used to improve probiotic viability upon spray drying process. These strategies were categorized into three parts according to the proceeding of spray drying probiotics: the influence of bacterial culture (i.e. feed material) preparation before spray drying, the drying parameters and operating conditions during drying, and the storage and application of powders after drying. The studies and investigations made it possible to introduce the strategies for improving quality of spray dried probiotics, mainly based on knowledge from microbiology, material science, engineering and processing disciplines.

The PhD project is introduced at the end of this chapter with its aim and innovation. It has benefited from the multidisciplinary knowledge due to the joint direction between China (Chemical Engineering) and France (Food Science and Technology), and between processing (SMCF) and microbiology team (MICROBIO) in the INRA-Agrocampus Ouest joint laboratory (UMR1253 STLO).

The literature review has been published in Trends in Food Science & Technology as:

Huang, S., Vignolles, M., Chen, X.D., Le Loir, Y., Jan, G., Schuck, P., and Jeantet, R., 2017. Spray drying of probiotics and other food-grade bacteria: A review. Trends in Food Sci. Technol. 63, 1-17.

1.1 Spray drying of probiotics: pre-drying stage

1.1.1 Selection of bacterial Strains

To date, most efforts have focused on the drying of *Lactobacillus*, *Lactococcus* and various *Bifidobacteria* species. These probiotic bacteria generally do not survive well after spray drying because of the harsh conditions which prevail during the process. The resistance characteristics of a bacterial strain should thus constitute an important criterion when selecting probiotic bacteria, in order to improve the final probiotic viability of the spray-dried powders.

Heat, osmotic, oxidative and desiccation stresses are usually considered to be the main mechanisms which cause the inactivation of bacteria during and after spray drying (Santivarangkna et al., 2008b). It has been shown that different bacterial species, or even strains, may display variable tolerance towards such stresses.

By comparison with *Lactobacillus*, *Lactococcus* and *Bifidobacteria*, *Propionibacteria* usually display higher tolerance due to their greater abilities for environmental adaptation, either through their metabolism or a multitolerance response (Leverrier et al., 2004). However, compared to *Lactobacillus* (*Lb.*) and *Bifidobacteria*, studies involving the drying of *Propionibacteria* (*P.*) are still rare.

Streptococcus (*S.*) is usually more resistant than *Lactobacillus* to spray drying; for instance, *S. thermophilus* was shown to survive better than *Lb. delbrueckii* ssp. *bulgaricus* in spray-dried yoghurt (Kumar and Mishra, 2004), and *S. thermophilus* CCRC14085 survived better than *Lb. acidophilus* CCRC 14079 in spray-dried fermented soymilk (Wang et al., 2004). The threshold temperature at which damage is caused to microbial cells is usually within the range of the upper limit of growth temperature of the microbial species (Foerst and Kulozik, 2011). Thus the spray drying resistance of *S. thermophilus* is probably linked to its greater thermotolerance. In another observation, *Lb. paracasei* NFBC 338 was however found to survive as successfully as *S. thermophilus* (Kearney et al., 2009). This finding indicates that certain strains within a usually fragile species may be as resistant as bacteria from a generally robust species.

When compared within the *Lactobacillus* genus, *Lb. plantarum* is a species with relatively robust stress tolerance (Ferrando et al., 2015). Mille, Beney, and Gervais (2005) showed that the osmotic tolerance of *Lb. plantarum* was generally greater than that of *Escherichia coli*, *Bradyrhizobium japonicum* and *Lb. bulgaricus*, and in some cases even higher than that of yeasts (*Saccharomyces cerevisiae* and *Candida utilis*). Other species frequently studied in the

context of spray drying include *Lb. rhamnosus*, *Lb. casei*, *Lb. paracasei*, *Lb. salivarius* and *Lb. acidophilus*. However, the stress tolerance of these species appears to be strain-dependent (Table 3).

Bifidobacterium longum ssp. *longum* B6 survived spray drying better than *Bifidobacterium longum* ssp. *infantis* CCRC 14633 when fermented soymilk and reconstituted skim milk were used as the drying media, while both *B. longum* ATCC15708 and CCRC 14634 survived better than *B. infantis* CCRC14661 when using gelatin as the spray drying medium (Lian et al., 2002; Wang et al., 2004). In a comparison of 17 strains from the *Bifidobacterium* genus, it was found that the greater the resistance to heat and oxidative stresses, the better was survival after spray drying (Simpson et al., 2005). This relationship has also been reported in the *Lb. rhamnosus* and *Lactococcus lactis (Lc. Lactis)* species (Dijkstra et al., 2014; Lavari et al., 2015).

Although the species may generally reflect the apparent resistance of different bacteria, the robustness of probiotic bacteria during spray drying seems more likely to be strain-dependent. Such variability is likely related to the environment of the original source, the presence of specific genes, interactions with the extracellular matrix, or an ability for intracellular polyphosphate accumulation and exopolysaccharide production, etc. (Lebeer et al., 2008; Papadimitriou et al., 2016). Given that susceptibility to both process and environment is strain-dependent, drying conditions need to be adapted while considering the latter and not directly transposed from one strain to another.

Table 3. Review of key factors determining viability of probiotic bacteria (*Lb.* refers to *Lactobacillus*, *Lc.* refers to *lactococcus*, *S.* refers to *Streptococcus*, *B.* refers to *bifidobacteria*, *P.* refers to *propionibacteria*) upon spray drying (Modified from Huang et al., 2017).

Bacteria	Growth parameters		Cell concentration before drying (CFU mL^{-1})	Drying parameters			Devices	Powder moisture content (%)	Survival (%)	Reference
	Medium	T (°C)		Medium	T_{inlet} (°C)	T_{outlet} (°C)				
S. thermophilus MK-10	Condensed non fat milk (18% w/w)	42	9.5×10^{8}	Fermented growth medium (18% w/w)	n.a.[1]	60	"Luwa" type industrial spray dryer	10.2	**69.5**	(Bielecka and Majkowska, 2000)
						80		4.4	**12.7**	
Lb. bulgaricus 151						60		10.2	**22.1**	
						80		4.4	**8.0**	
B. infantis CCRC14633	MRS broth with 0.05% cysteine ‡	37	n.a.	Gelatin (10%)	100	50	Buchi B-191 Mini dryer, Switzerland	10.0	**1.3**	(Lian et al., 2002)
B. infantis CCRC14661									**0.2**	
B. longum ATCC15708									**54.3**	
B. longum CCRC14634									**39.4**	
B. longum B6								7.8	**63.7**	
Lb. rhamnosus E800	MRS broth	37	3.2×10^{9} *	RSM (20% w/v)	170	85~90	Buchi B-191 Mini dryer, Switzerland	3.8	**~30 ***	(Corcoran et al., 2004)
Lb. rhamnosus GG			1.6×10^{9} *					3.7	**~50 ***	
Lb. salivarius UCC500			6.3×10^{9} *					2.7	**~1 ***	

Table 3 continued

17 strains of *Bifidobacterium* species	MRS broth with 0.05% cysteine ‡	37	0.1~3.2×10^9	RSM (20% w/v)	170	85~90	Buchi B-191 Mini dryer, Switzerland	2.5~4.2	**12~102** [a]	(Simpson et al., 2005)
B. animalis ssp. *lactis* BB12 [b]			5.0×10^8					3.2	**79**	
B. breve NCMB8807 [b]			6.3×10^8					3.2	**38**	
Lb. rhamnosus GG	MRS broth supplemented with trehalose (1.25% w/v) ‡	37	1.8×10^9	Trehalose (20% w/w)	n.a.	65~70	Buchi B-191 Mini dryer, Switzerland	3.8	**69**	(Sunny-Roberts and Knorr, 2009)
				Trehalose (20% w/w) + Monosodium glutamate					**80.8**	
Lb. rhamnosus E800				Trehalose (20% w/w)				4.1	**23**	
				Trehalose (20% w/w) + Monosodium glutamate					**89.3**	
Lb. paracasei A12	MRS broth	37	4.0×10^8	RSM (20% w/v)	170	85	Buchi B-290 mini spray drier, Switzerland	< 4	**~100 ***	(Páez et al., 2013) †
Lb. casei Nad			4.0×10^8							
Lb. acidophilus A9			5.0×10^9							
P. acidipropionici	Acid whey permeate (10% w/v) with corn steep (2.5% w/v) and yeast extract (1% w/v)	30	10^{10} [e]	SW (~50% w/w)	130	60	GEA pilot Bionov spray dryer, France	~3 [d]	**100**	(Schuck et al., 2013)

Table 3 continued

14 strains of *Lc. lactis* species	M17 broth with 0.5% w/v glucose	30	n.a.	RSM (20% w/v)	200	100	Buchi B-290 mini spray drier, Switzerland	< 4	[c]	(Dijkstra et al., 2014)
Lb. acidophilus La-5	MRS broth	37	~10^9 *	RSM (30% w/w)	180	85~95	SD-05 lab-scale spray-dryer, Lab-Plant, UK	4.3	**77.7**	(Maciel et al., 2014)
				SW (30% w/w)				4.8	**75.4**	
Lb. plantarum WCFS1	MRS broth	30	~10^8	Maltodextrin (20% w/w)	135	90	Buchi B-290 mini spray drier, Switzerland	4~8	**~10 ***	(Perdana et al., 2014)
Lb. plantarum 299v	MRS broth	37	n.a.	Orange juice (1.5% w/v) + Maltodextrin (2% w/v)	150	70	Niro spray dryer, Denmark	a_w = 0.42	**~100 ***	(Barbosa et al., 2015a)
Lb. casei 431	MRS broth	37	5.0×10^8	WPI and GA (3:1 mixture, ~5% w/w)	180	80	Buchi B-290 mini spray drier, Switzerland	2.90	**37.6**	(Eratte et al., 2015)
			2.0×10^9	WPI, tuna oil and GA (3:2:1 mixture, ~10% w/w)				3.19	**56.2**	
Lb. plantarum CNRZ 1997	MRS broth	37	~10^8	Phosphate-buffered saline (~1% w/v)	145	70	Buchi B-290 mini spray drier, Switzerland	a_w = 0.3	**~50 ***	(Iaconelli et al., 2015)
Lb. zeae CNRZ 2268									**~1 ***	
B. bifidum CIP 56.7	MRS broth with 0.05% cysteine ‡								**~10 ***	

Table 3 continued

Lb. zeae LB1	MRS broth	37	~10^{10}	Sodium caseinate and vegetable oil (1:1 mixture, 20% w/w)	170	80	Laboratory-scale ADL 310 spray dryer, USA	3.25	**~15**	(Liu et al., 2015)
				Sodium caseinate and low melting point fat (1:1 mixture, 20% w/w)				3.68	**~65**	
Lb. acidophilus LA-5	MRS broth	37	3.2×10^{8} *	Goat's milk (20% w/v)	195	85	Buchi B-290 mini spray drier, Switzerland	2.9	**~1 ***	(Ranadheera et al., 2015)
B. animalis subsp. *lactis* BB-12	RC medium ‡		10^{8} *						**< 1 ***	
P. jensenii 702	SL broth	30	10^{9} *						**~8 ***	
Lb. plantarum A17	MRS broth	37	$3\sim4\times10^{9}$	WPI (pH 7.0, 10% w/w)	110	68-70	Laboratory LabPlant SDBasic FT30MKIII, spray dryer, UK	5.6	**69.0**	(Khem et al., 2016)
				WPI (pH 4.0, 10% w/w)				5.3	**39.3**	
				Denatured WPI (pH 7.0, 78 ºC for 20 min, 10% w/w)				5.4	**25.0**	
B. infantis ATCC 15679	MRS broth with 0.05% cysteine ‡	37	$10^{8}\sim10^{9}$	Maltodextrin (15% w/v)	110	75-80	LabPlant SD-05 spray dryer, England	a_w~0.24	**91.6**	(Bustamante et al., 2017)
Lb. plantarum ATCC 8014	MRS broth								**83.0**	

Table 3 continued

Lb. rhamnosus GG	MRS (pH controlled at 6.8 by 6 M NaOH)	37	~3.2×10^{7} [e]	Micellar caseins with denatured whey proteins (90:10 v/v mixture, 12.5% w/w)	195	85	Anhydro MicraSpray 150, Danmark	6.3	**~50 ***	(Guerin et al., 2017)
B. lactis Bb12	MRS broth with 0.1% cysteine ‡	37	1.3×10^{10}	RSM (20% w/v)	137	75-90	Buchi B-290 mini spray drier, Switzerland	4.2	**~98**	(Burns et al., 2017) †

[1]n.a.: not available

* means data were calculated from the figures or information from the paper

† *In vivo* study of effect of spray drying on probiotic functionality

‡ Growth under anaerobic condition

[a] Survival ranged from 12% to 102%, depend on species and strain, closely related strains exhibiting superior heat and oxygen tolerance performed best after spray drying

[b] representitive examples from the 17 strains

[c] *Lc. lactis* subsp. *lactis* strains generally displayed more robust phenotypes than *Lc. lactis* subsp. *cremoris* strains. The most robust strains displayed a more-than-200-fold-better survival during spray drying than the most sensitive strains.

[d] Data was calculated from total solid content of spray dried powders after delivery by a crystallizer

[e] The unit of bacterial population is CFU g^{-1}

1.1.2 Response to cellular stress

Growth conditions are widely accepted as key determinants for the acquisition of bacterial stress tolerance. Indeed, like other bacteria, probiotics are able to withstand and adapt to different adverse environmental stresses (e.g. high temperatures or osmotic stress) by activating the cellular stress-response system (Table 4), which determines their tolerance during spray drying. This system is generally induced by exposure to a sub-lethal dose of the stress itself. For instance, significant improvements in heat and spray drying tolerance were reported for heat-adapted strains of *Lb. salivarius* (Zhang et al., 2016).

In addition to heat adaptation, the exposure of bacteria to sub-lethal osmotic, H_2O_2 (0.003 M for 30 min) or bile salts (0.1% w/v for 30 min) could also improve bacterial viability during spray drying (Desmond et al., 2001). Edwin, Ananta and Knorr (2003) also found that *Lb. rhamnosus* GG pre-exposed to high pressure (100 MPa) displayed greater resistance to heat. Furthermore, starvation (mostly glucose starvation) can also induce bacterial tolerance of osmotic and heat stresses (Guchte et al., 2002).

Table 4. Examples of genes and proteins involved in probiotics adaptation towards spray drying-related stress (Adapted from Huang et al., 2017).

Stress*	Gene/Protein	Description and function	Example strain	Reference
General	*rpoS*/σ^{38} or σ^{S}	Transcription initiation factor that enables specific binding of RNA polymerase to gene promoters at stationary phase	*E. coli* Nissle 1917	(Coldewey et al., 2007)
Heat Osmotic	*dnaK, dnaJ, grpE*/DnaK, DnaJ, GrpE	Heat shock proteins folding chaperones to prevent misfolding and to promote the refolding or degradation of unfolded polypeptides	*Lb. casei* Zhang	(Wu et al., 2011)
	groL, groS/GroEL,GroES		*Lb. paracasei* NFBC 338	(Corcoran et al., 2006)
	hsp20/Hsp20		*B. longum* NCC2705	(Khaskheli et al., 2015)
	clp genes / ClpB, ClpC, ClpE, ClpP etc.	Clp ATPase family members act as chaperones and regulators of proteolysis	*Lc. cremoris* MG1363	(Frees and Ingmer, 1999)
	htrA/HtrA	High temperature requirement protein with dual chaperone-protease activities	*Lb. plantarum FS5-5*	(Wu et al., 2016)

Table 4 continued

Heat	*ftsH*/FtsH	ATP-dependent zinc metalloprotease with dual chaperone-protease activities and involving in protein quality control	*Lb. plantarum* WCFS1	(Bove et al., 2012)
Osmotic	*opu* genes/OpuA, OpuB, OpuC etc.	ATP-binding ABC transporter in charge of compatible solute transport	*Lc. cremoris* MG1363	(Romeo et al., 2003)
	ptsH/HPr	Phosphocarrier protein family catalyzes the phosphorylation of incoming sugar substrates concomitantly with their translocation across the cell membrane.	*L. rhamnosus* HN001	(Prasad et al., 2003)
Oxidative	*ahpC*/AhpC	Alkyl hydroperoxide reductase C22 protein destroys toxic radicals normally produced within cells to maintain cell redox homeostasis	*B. longum* NCC2705	(Zuo et al., 2014)
	katA/KatA	Catalase decomposes hydrogen peroxide into water and oxygen, to maintain cell redox homeostasis	*Lb. brevis* CGMCC1306	(Lyu et al., 2016)
	nox,npr/NOX, NPR	NADH oxidase and NADH peroxidase promote NADH cycling to maintain cell redox homeostasis	*Lb. plantarum* C17	(Zotta et al., 2013)
	sod*A/SodA*	Superoxide dismutase destroys toxic radicals normally produced within cells to maintain cell redox homeostasis	*Lb. casei* BL23	(Lee et al., 2015)

* Cross-protection possibly emerges between different stresses

Apart from the adaptation to environmental conditions, modest improvements to stress tolerance can also be achieved through genetic engineering. For instance, a genetically engineered *Lb. paracasei* strain was able to overproduce the heat shock protein GroESL, and thereby exhibited a 10-fold higher thermotolerance compared to its parent wild-type strain (Desmond et al., 2004). Given its heat robustness, the GroESL-overproducing *Lb. paracasei* strain survived 10-fold better than the wild-type strain during spray drying (Corcoran et al., 2006). Cells appear to be more robust and intact in GroESL-overproducing bacteria and are sometimes huddled together. However, the overexpression of GroESL did not enhance survival during storage of the lactic acid bacteria (LAB) powder. Moreover, the thermotolerance of this GroESL overproducing strain can be further improved up to 50-fold after sub-lethal heat

adaptation, which indicates the involvement of other heat stress response mechanisms (e.g., contributions from other heat shock proteins or general stress proteins) (De Angelis et al., 2016; Lebeer et al., 2008). Hence, the induction of the whole battery of heat stress tolerance mechanisms by sub-lethal heat treatment may lead to greater viability. A genetically engineered *Lb. salivarius* UCC 118, which can overexpress the listerial betaine uptake system BetL, was also reported to have a 5-fold improvement in survival following spray drying (Sheehan et al., 2006). Indeed, betaine protects (LAB) from desiccation (Table 5).

To sum up, in most cases, bacterial spray drying tolerance may be affected by how the strain or culture has been produced. Furthermore, Teixeira, Castro, Malcata and Kirby (1995) reported that protein synthesis was not observed in *Lb. bulgaricus* during its recovery from spray drying injury. As concluded by Morgan, Herman, White and Vesey (2006), this result suggests that such injured bacteria do not need to synthesize new proteins during recovery from drying injury, or that they are unable to synthesize proteins. In this case, the overproduction of stress response proteins before drying may constitute an effective means of protecting bacteria from drying injury, and particularly those proteins or chaperones responsible for preventing or repairing misfolding polypeptides.

1.1.3 Growth conditions and preparation of feed concentrate

1.1.3.1 Growth media

Only a few published studies have addressed how the growth medium affects the viability of probiotics after drying. Most studies have focused on the media used in laboratories, such as De Man Rogosa and Sharpe medium (MRS broth) which is non-food grade, and relatively expensive for industrial application. As an example, the presence of 1 $mol.L^{-1}$ or 1.25 $mol.L^{-1}$ NaCl in MRS during the growth of *Lb plantarum* resulted in decreased residual activity after drying in a fluidized bed or by convection. The viability of *Lb. plantarum* after drying was also greater when the bacteria were grown in diluted MRS medium, despite lower betaine and carnitine uptake in this case (Linders et al., 1997).

As well as these studies based on MRS medium, several others have explored the effects of other media on growth and the subsequent drying or storage of bacteria. Dairy media, such as skimmed milk and whey, were mostly used during these studies (Table 3). By comparison with MRS, these media are inexpensive, easily used for mass production and edible. Moreover, they may improve following production processes such as drying (Lavari et al., 2014).

When facing a reduction in water activity, bacteria tend to compensate for the concomitant osmotic pressure and thus maintain their viability by accumulating compatible solutes such as amino acids, quaternary amines or carbohydrates, etc. (Table 5) (Papadimitriou et al., 2016; Wood, 2011). The accumulation of compatible solutes from media during growth thus can be used as a strategy to increase the viability of probiotics during drying. However, it has been suggested that the uptake of compatible solutes may not necessarily guarantee tolerance towards drying, depending on the bacterial strains studied (Morgan et al., 2006). Furthermore, it is worth noting that the synthesis of compatible solutes in LAB was seen to be dependent on the presence of their precursors in the medium (Wood, 2011). The transport and accumulation of these compatible solutes is also an energy-dependent process (Romantsov et al., 2009). Because of the short timescale of the spray drying process, it is suggested that compatible solutes or their precursors should be added during the growth of bacteria rather than before spray drying.

Table 5. Examples of compatible solute accumulation on improvement of bacterial stress tolerance (Adapted from Huang et al., 2017).

Compatible solute	Example bacterial strain	Stress response	Application in spray drying	Reference
Proline	*Lb. acidophilus* IFO 3532	Enhance osmotic tolerance	not to date	(Jewell and Kashket, 1991)
Carnitine Acetylcarnitine Propionylcarnitine	*Lb. plantarum* P743	Enhance osmotic tolerance	not to date	(Kets and Bont, 1997)
Glutamate	*Lb sakei* CTC 494	No significant effect on heat tolerance	Improved the survival	(Ferreira et al., 2005)
Glycine betaine Ectoine	*Pantoea agglomerans* CPA-2	Enhance osmotic, water and heat tolerance	not to date	(Teixidó et al., 2005)
Listerial betaine	*Lb. salivarius* UCC118	Enhance osmo-, cryo-, baro- and chill tolerance	Improved the survival	(Sheehan et al., 2006)
Glycogen	*P. freudenreichii* ITG 20	Enhance osmotic, cold tolerance, and long-term survival	Not to date	(Dalmasso et al., 2012)
Trehalose	*Lb. salivarius* NRRL B-30514	Enhance osmotic, heat, desiccated tolerance and long-term survival	Improved the survival	(Zhang et al., 2016)
Polyphosphate	*Lb. casei* BL23	Enhance heat, oxidative tolerance and long-term survival	Not to date	(Alcántara et al., 2014)

1.1.3.2 pH of growth

The effects of the pH of the growth medium on bacterial viability during drying remain a matter of discussion, depending on the processing scheme implemented. Linders et al. (1997) reported a two-fold increase in *Lb. plantarum* viability after drying when the pH was controlled during growth. By contrast, the viability of *Lb. bulgaricus* during spray drying and heating was found to be greater when the pH was not controlled during growth (Silva et al., 2005). It was shown that in this case, enhanced protection was due to the overexpression of Hsp70, GroES and GroEL, induced by acid stress. However, cells may lose their viability and activity as being unable to maintain a near neutral intracellular pH in a low pH environment for a long period. The ability to maintain intracellular pH homeostasis differs between strains (Baker-Austin and Dopson, 2007). This may condition strain dependence relative to acid adaptation and thus induced cross-protection against drying. Further, because acid stress influences cells in a dynamic manner, the final pH of cultures, the time of harvest or feed storage before drying should be taken into account when investigating the effects of pH of growth.

1.1.3.3 Growth phase

Probiotics are generally harvested during either the late exponential phase or the early stationary phase, at which a maximal yield is reached. However, (early) stationary phase is mentioned more frequently as being optimal and used than the (late) exponential phase, because the cells collected also exhibit higher viability during drying (Peighambardoust et al., 2011). The viability of *Lb. rhamnosus* was 2%, but over 14% and 50% when cells were harvested in the lag, early exponential and stationary phases, respectively (Corcoran et al., 2004). Indeed, resistance to various types of stress is increased during the stationary phase, as its challenging conditions trigger a stringent response that leads to multitolerance which requires general stress proteins and alternative sigma factor (Alcantara et al., 2011; Hussain et al., 2009; Upadrasta et al., 2011).

1.1.3.4 Harvesting techniques

Centrifugation is still the most widely used harvesting technique. It has long been considered to be efficient in concentrating cells. The usual harvesting temperature is 4°C. However, the effects of these centrifugal conditions on bacterial viability after drying have rarely been reported. As well as centrifugation, other harvesting techniques, such as membrane

filtration, were mentioned in the review by Santivarangkna, Kulozik, & Foerst (2007), but to the best of our knowledge, they have not been reported in the context of the spray drying of bacteria.

Separating bacteria from growth medium can cause considerable wastage of materials and energy. It is therefore preferable to keep the growth medium and directly spray-dry the probiotic culture during large-scale production. Apart from drying probiotic cells, some beneficial metabolites such as short chain fatty acids, vitamins and bacteriocin are also retained, possibly improving the use value of powders. This process requires the dual use of one medium for both bacterial growth and spray drying. A food grade medium is highly desirable because of application considerations. However, a key problem of this process is that the solid content of bacterial growth isotonic media is typically low (5%~10%), while a high solid content in the feed concentrate is required to enable productivity and energy savings. This is usually achieved by adding more powders to the culture, or by implementing concentration techniques (Schuck et al., 2013). However, these intermediate steps may cause a loss of viability and increase the risk of contamination.

1.2 Spray drying probiotics: During drying

1.2.1 Bacteria may be injured during spray drying

Damage to bacteria during spray drying is not only ascribed to the thermal effect, but also to a loss of bound water at the cell surface. The cytoplasmic membrane is highly susceptible to cellular injury, and this is exacerbated during spray drying (Santivarangkna et al., 2008b). Indeed, the removal of water leads to a state transition of the phospholipid bilayer, from lamellar to gel phase, or even a hexagonal one (Figure 6A). This results in the phospholipid chains gaining a rigid and fully extended structure (Crowe et al., 1998; Wolfe and Bryant, 1999). It was indeed shown that fine holes at the surface of *Lc. cremoris* were observed by scanning electron microscopy in droplet drying with skimmed milk at 90°C and 110°C, while the cell surface remained intact when drying at 70°C (Figure 6B) (Fu et al., 2013a).

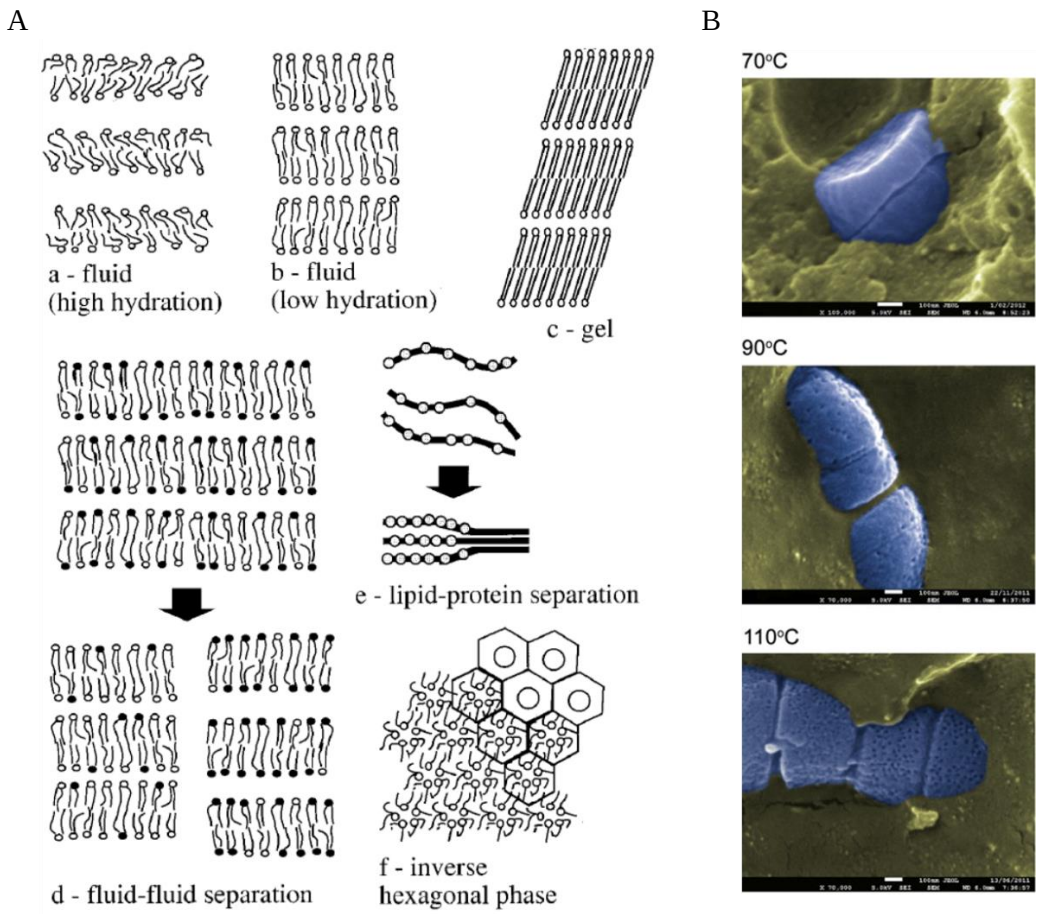


Figure 6. (A): The strains produced by dehydration-induced stress (source from (Wolfe and Bryant, 1999)): (a) The lamellar fluid phase at high hydration. (b) The geometric strain produced at lower water content. (c) Transition to gel phase at lower water contents. (d) The mixed phase of lipid species with high (top) and low (bottom) hydration. (e) Separation of hydrophilic and hydrophobic molecules. (f) tHE topological response emerged at very low hydration. (B): The surface morphology of *Lc. cremoris* cells dried with different air temperature (Adapted from Fu et al., 2013a).

Other dehydration stress targets include the nucleic acids (where the mechanism remains unclear) and ribosomes, which are probably injured as a result of the escape of Mg^{2+} from the heat-compromised cell membrane (O'Connor et al., 2009).

1.2.2 Drying Devices

1.2.2.1 Spray drying scale

To date, little attention has been paid to the effects of spray drying devices on the viability of probiotics in powders. The main influence of different devices on probiotic powders is probably the residence time of particles in the drying chamber: The longer the residence time, the longer the bacteria are exposed to stress and consequently the poorer the viability. Another factor worth noting is that industrial scale spray dryers are normally suitably equipped with pneumatic devices to enable the continuous collection and cooling of the powders, thus maximizing viability.

Table 3 presents the drying equipment used during different studies, which mainly focused on laboratory-scale spray dryers. It is worth noting that only two studies mentioned experiments on a larger semi-industrial to industrial scale, i.e., with a water evaporation capacity in the range 100 to 1645 kg h^{-1} (Bielecka and Majkowska, 2000; Schuck et al., 2013). It is therefore still difficult to conclude as to the influence of scaling-up on the residual probiotic viability of the resulting powders.

1.2.2.2 Atomization

The cell inactivation caused by the atomization process *per se* is usually suggested to be negligible. Fu, Suen and Etzel (1995) reported a complete viability of *Lc. lactis* ssp. *lactis* after the spraying step, despite the shear force experienced in the nozzle. By contrast, Riveros, Ferrer and Bórquez (2009) found that the viability of *Lb. acidophilus* increased by around 2 log CFU g^{-1} when lowering the two-fluid spray nozzle pressure from 1.5 bar to 1 bar. However, it should be noted that a lower spray pressure might first of all increase the droplet size, and hence affect the drying kinetics and subsequent temperature increase. Besides, Guerin et al. (2017) showed that the increase in bacteria population after atomization might be caused by cell reorganization during atomization, or more specifically, the *Lb. rhamnosus* GG distributed in linear chains before atomization and in individual cells after atomization (Figure 7). In another study, a significant reduction in survival was reported for a strain of *Lc. lactis* ssp. *cremoris* after atomization using both a rotary wheel and two-fluid nozzle atomizer (Ghandi et al., 2012). Furthermore, the addition of ascorbic acid (as an anti-oxidant) to the drying medium was found to reduce the damage caused by atomization. These results indicate that a loss of cell viability

during atomization may be strain-dependent, and the inactivation mechanism could be triggered by both shear force and oxidative stress.

Figure 7. Effect of spraying-induced shear stress on cellular organization (Adapted from Guerin et al., 2017).

1.2.3 Protective agents

A strategy that is commonly employed to enhance cell protection during drying consists in adding specific components, known for their protective properties, to the surrounding medium.

1.2.3.1 Carbohydrates

Among protective agents of a carbohydrate type, trehalose has by far been the one most frequently investigated, given the established role of its accumulation in the acquired survival of some microorganisms facing anhydrobiosis. It has been suggested that this mechanism is due to the stabilizing effect of trehalose on membranes and proteins, by replacing the water around polar residues within these macromolecular structures (i.e. water replacement hypothesis), and thus depressing the membrane phase transition temperature (T_m) (Figure 8A) (Crowe et al., 1998; Santivarangkna et al., 2008a). Moreover, Conrad, Miller, Cielenski and De Pablo (2000) demonstrated a synergistic effect of trehalose and borate ions in protecting bacteria: indeed, the glass transition temperature (T_g) of the dry medium is increased in this case due to crosslinking of the trehalose molecules.

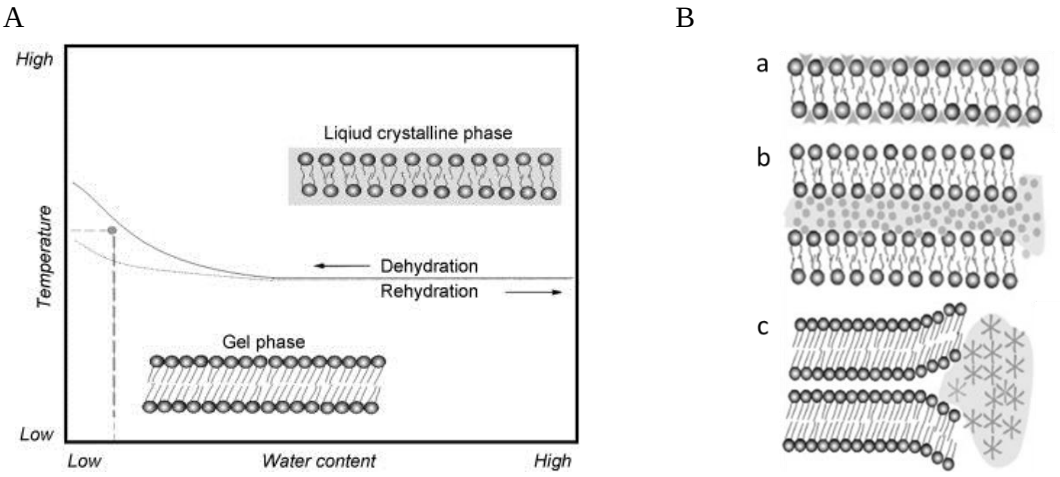


Figure 8. (A) a simplified phase diagram of a phospholipid associated with drying and rehydration processes: During drying at a given temperature until cells reach a relatively low moisture content (closed circle), cell membrane may remain in liquid phase in the presence of a sugar at this same point. (B) According to the water replacement hypothesis: Sugars depress the membrane phase transition temperature (a) by specifically interacting with phospholipid headgroups, or (b) by vitrification of sugars between the membranes. (c) Polymeric sugars with a very high molecular weight are not capable of depressing the T_m because they have fewer osmotic effects and are excluded from the intermembrane space during the removal of water. (Adapted from Santivarangkna et al., 2008a)

In a recent work, carbohydrates that included trehalose, sorbitol, mannitol, xylose, glucose, sucrose, maltose, lactose, maltodextrin with dextrose, inulin, fructo-oligosaccharides, galacto-oligosaccharide and potato starch were used as drying matrices in order to compare the protection they endowed on probiotic *Lb plantarum* WCFS1 during convective droplet drying (Perdana et al., 2014). It was found that a carbohydrate-rich formulation with a low molecular weight and high glass transition temperature (T_g) procured the highest degree of protection during drying. It may be explained by the exclusion effect of bacterial membrane on the high molecular weight sugars (Figure 8B).

1.2.3.2 Reconstituted skimmed milk

Reconstituted skimmed milk (RSM) appears to be another suitable medium to enable the efficient spray drying of probiotic cultures (Table 3). In view of the water replacement hypothesis, the lactose in RSM may play an important role in the same way as the non-reducing disaccharides trehalose and sucrose in cell protection. However and unlike the trehalose and sucrose, the effect of the reducing disaccharide lactose in protecting bacterial cells during drying remains doubtful, because of the possibility of its interaction with proteins (Maillard reaction) during drying and storage. When using whey permeate (which mainly consisted of lactose) as a carrier for the spray drying of *Lb. acidophilus*, its survival was only half of that achieved with RSM under similar conditions (Riveros et al., 2009). Nevertheless, whether or not the lactose in RSM plays the main role in stabilizing LAB during storage remains undetermined.

The proteins in RSM can prevent cellular injury by stabilizing cell membrane constituents. Furthermore, they may form a protective coating on the bacterial cell wall, when interacting with milk calcium (Huang and Chen, 2013; Zheng et al., 2016). The calcium in milk can also cause milk protein aggregation during heat treatment, forming aggregates that protect by their structure the microorganisms: indeed the protection of *Lb. rhamnosus* GG was higher in pre-aggregated milk than that of untreated milk during heat treatment (Huang et al., 2014). Interestingly, innovative spray-dried microparticles with different reconstitution behaviors have also been developed based on milk protein aggregation controlled by chymosin (Guerin et al., 2017). The microparticles displayed an encapsulation effect on *Lb. rhamnosus* GG when rehydrating at 40°C, while a releasing effect was seen at 8°C. During another laboratory-scale spray drying experiment, RSM (20% w/v) was found to confer superior protection on *Lb.*

paracasei Nad, *Lb. casei* A13 and *Lb. acidophilus* A9, with around 100% survival for all three strains under the same drying conditions (Páez et al., 2013). As well as this well-preserved viability, the resistance to digestion and immunomodulation capacity of these three strains within the powders was also improved significantly when compared to the fresh culture.

1.2.3.3 Other protective agents

Other protective agents have also been used in the spray drying of probiotics: as such, dairy-based materials have often been reported because of their compatibility with probiotics and the possibility to promote their efficiency (Lee et al., 2015b). **Whey has also been reported as being an efficient protective agent for the spray drying of probiotics (Maciel et al., 2014). Its advantages are linked to its source as a by-product of cheese manufacturing and to its potentially good powder quality (e.g. solubility, flowability, dispersibility, etc.) when compared with casein-based products (Lavari et al., 2014)**.

Low melting point fat (LMF) has also been reported to protect *Lactobacillus* cells from damage when it is added to sodium caseinate during spray drying (Liu et al., 2015). This protection may be attributed to the ability of LMF to absorb some of the thermal energy present during spray drying.

As well as dairy-based materials, other reported protective agents include gelatin, gum arabic, fruit juice, etc. (Table 3), used either alone or in a mixture to achieve synergistic effects. For instance, the spray drying of probiotics with fruit juice as a medium or additive was recently reviewed by Barbosa and Teixeira (2016). The incorporation of prebiotics in such protective agents is also of interest because of the possible "synbiotic" effect. For example, the addition of Omega-3 fatty acids to a whey protein-gum Arabic complex significantly improved the viability of *Lb. casei* 431 after spray drying (Eratte et al., 2015). The viability of *Lb. plantarum* after spray drying was also higher when incorporating galacto-oligosaccharides (GOS) in maltodextrins, or fructooligosaccharide (FOS) in whey protein isolate (WPI) (Rajam and Anandharamakrishnan, 2015; Sosa et al., 2016). Recently, mucilage and soluble proteins from chia seed and flaxseed were found to protect *Lb. acidophilus*, *Lb. plantarum* and *B. infantis* during spray drying (Bustamante et al., 2017, 2015). However, Corcoran et al. (2004) and Pinto et al. (2015) reported that the presence of the prebiotics inulin and polydextrose in whey concentrate did not enhance probiotic viability during spray drying or powder storage.

1.2.3.4 Total solids content

The total solids contents of the drying media reported in the literature are usually 20~30% (w/v): this value has been considered as being optimal to ensure the high residual viability of different LAB strains (Table 3). An increase in the feed concentration above this point using gelatin, gum arabic and soluble starch resulted in lower viability of bifidobacteria (Lian et al., 2002). Indeed, it should be noted that the stress adaptation to hyper-osmolality that drives the increase in viability after drying may not be acquired in the case of too short an exposure to osmotic stress before spray drying, leading to controversial results on the effect of the total solid contents.

Apart from residual bacterial viability, the process energy costs, powder quality and subsequent product applications should also be taken into account. More specifically, a high total solids content is usually desired by industry because of the benefits for drying process productivity, lower energy costs and a better encapsulation effect. By contrast, a low total solids content may make it possible to maintain an isotonic environment for the bacteria before drying and obtain fine powders with a high cell count (a higher cell/medium ratio) after spray drying.

1.2.4 Drying parameters

1.2.4.1 Drying temperature

Drying temperature, in combination with drying time, is the key factor that influences the final probiotic viability of powders because of the general heat sensitivity of these bacterial cells. As such, the drying kinetics mainly determines bacterial inactivation during drying, and particular attention should be paid to the drying curve, i.e. temperature as a function of time. Briefly, the drying curve generally exhibits two stages:

- During the first stage, the temperature of the droplets is limited to the wet bulb temperature by an almost constant evaporation rate of water, and bacterial inactivation is therefore limited.
- During the second falling rate stage, the temperature of droplets rises toward the outlet air temperature (T_{outlet}), depending on the residence time. The latter depends on both the drying temperatures applied and the expected moisture content and water activity of the dried product.

It has thus been shown extensively that the lower the T_{outlet}, the higher the post-drying viability (Table 3). T_{outlet} is therefore considered to be the principal drying parameter that affects the viability of spray-dried LAB, and any lack of monitoring and control of the latter may be markedly detrimental (Peighambardoust et al., 2011). For instance, it was seen that relatively small changes in T_{outlet} appeared to have significant effects on the survival of *Lb. salivarius* (Zhang et al., 2016).

However, apart from bacterial viability, the T_{outlet} can also influence powder quality: if the T_{outlet} is too low, the residual water activity and moisture content in powders should exceed the values required for prolonged powder storage, e.g. around 0.2 of water activity or 4% of wet basis moisture content (Abe et al., 2009; Vesterlund et al., 2012). The reduction in viability caused by an increased T_{outlet} may also vary with the drying medium used. A greater reduction in *B. longum* and *B. infantis* viability was observed in soluble starch compared to other carriers such as gelatin, gum arabic and skimmed milk (Lian et al., 2002). Further, industrial spray drying at a lower T_{outlet} may also result in improved storage stability (Desmond et al., 2002). Therefore, spray drying temperatures are of considerable significance for the preservation of bacteria, and probably need to be optimized individually for any new application.

1.2.4.2 Drying rate

The influence of the drying rate on the viability of post-drying probiotics is still a matter of debate. At the laboratory scale, it was found that slow drying kinetics led to significant inactivation of the dehydration of *Lb. plantarum*, while a rapid drying rate could instantly stabilize the cells and thereby prevent this inactivation (Perdana et al., 2013). In addition, the influence of a slow drying rate on the viability of *Lc. lactis* bv. diacetylactis may have arisen from the detrimental reactions favored by higher water mobility above an a_W of 0.84-0.88 (Santivarangkna et al., 2007). Conversely, and as already stated in this review, a high drying rate during the first stage of drying, when facilitated by hydraulic membrane permeability, may limit bacterial adaptation because of too short exposure to the gradual withdrawal of moisture (Linders et al., 1996).

1.2.4.3 Process optimization

In order to obtain probiotic powders with high bacteria viability and good powder quality, the process should be optimized in order to simultaneously achieve a reduction in drying temperature and an acceptable moisture content in the powders. A multi-stage drying process is the strategy most frequently employed for this purpose. For example, a process was proposed by Schuck et al. (2013) for pilot-scale spray drying which enabled a reduction in T_{outlet} during the spray drying of *Propionibacterium acidipropionici*, with sweet whey as the carrier. A crystallizer was used after the spray drying chamber to enable a low T_{outlet} at 60°C along with a high moisture content: indeed, lactose crystallization reduced the powder hygroscopicity and improved the flowability of the powder. Finally, an external fluid-bed dryer was used to remove the excess of moisture. **However, the applicability of this multi-stage drying process should be validated on more bacterial strains**.

1.2.5 "In-process" investigations

1.2.5.1 Single droplet drying

During a real-life spray drying process, billions of droplets are sprayed into a relatively large chamber. It is therefore only possible to analyze the microbial viability and drying status of samples at the start and/or at the end of the process, without being able to address the complexity of 'in-process' droplet-particle conversion and the related mechanism of probiotic inactivation. To circumvent these difficulties, single droplet drying (Figure 9) has therefore been developed to map the drying behavior of bacteria at the droplet level during the drying process (Schutyser et al., 2012).

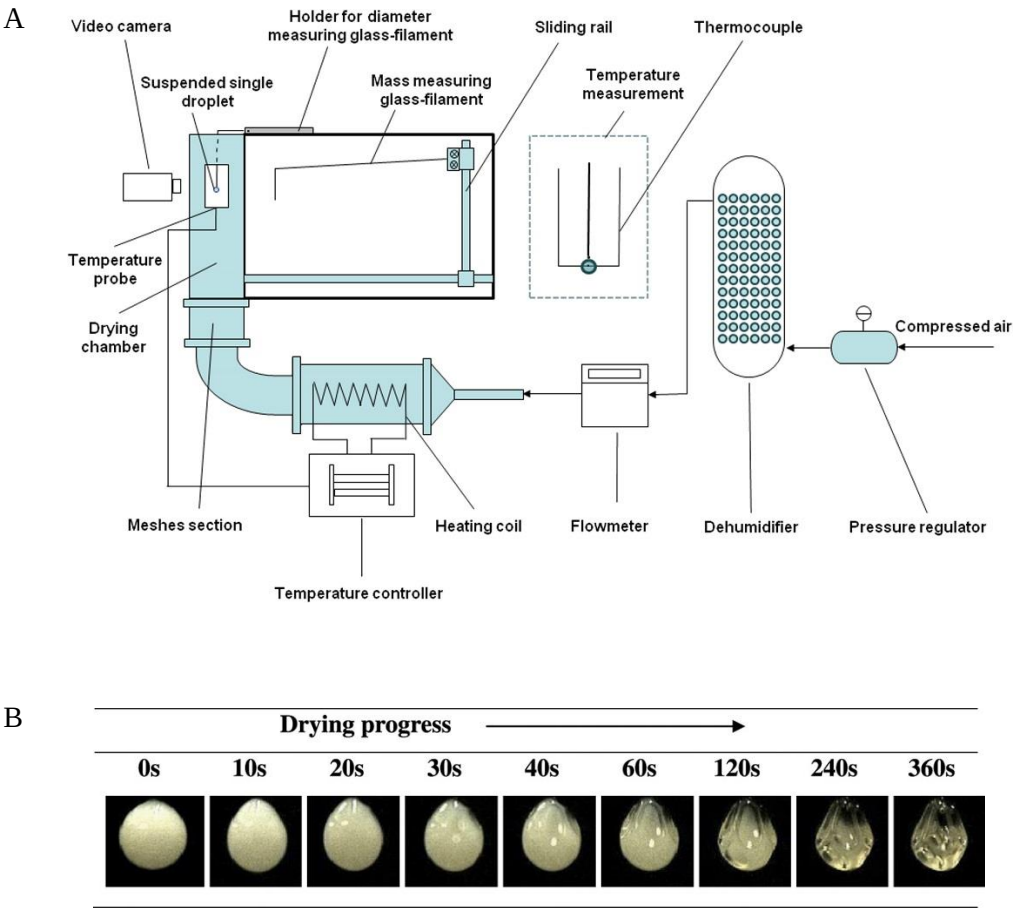


Figure 9. (A) Schematic diagram of a glass-filament single droplet drying experimental set-up (droplet diameter, weight, and temperature were measured in separate runs). (B) An example of the observation of morphology evolution during the single droplet drying (Adapted from Wang et al., 2014).

Under such a protocol, and controlling the drying conditions, the protective effect of the medium can easily be compared without running spray drying at a larger scale. On the other

hand, the effect of some drying parameters on bacterial inactivation can also be correlated by comparing drying kinetics and bacterial inactivation using the same drying medium (Fu et al., 2013a; Perdana et al., 2013). Further, the drying rate may be relatively slow when applying mild conditions, thus enabling the observation and analysis of phase transition phenomenon such as crystallization, sol-gel transition or skin formation. These observations may offer new avenues for the design of functional probiotic particles via spray drying. For example, lactose crystallization during droplet drying was found to be related to bacterial inactivation, which is probably dependent on the crystal shape formed by different media (Perdana et al., 2014). In addition, the milk protein type and its denaturation degree are important factors in determining powder morphology (Khem et al., 2015; Sadek et al., 2013). This formation of particle morphology was recently found to be a factor that triggered bacterial inactivation, probably due to the mechanical stresses in play (Khem et al., 2016).

Based on the single droplet drying technique and the development of an *in situ* analytical technique, it is also possible to gain insights into the interaction between drying conditions and the structure of bacterial cell components, thus suggesting specific strategies for the protection of bacterial cells, or even cellular components at the molecular level.

1.2.5.2 Modelling

Based on the "in-process" data obtained using the single droplet drying technique, mathematical models have been developed to predict the inactivation of probiotics during thermal convective drying.

A first order kinetics model was reviewed by Chen and Patel, (2007). The inactivation kinetics of microorganisms during drying was expressed as:

$$\frac{\mathrm{d}(N/N_0)}{\mathrm{dt}} = -k_d(N/N_0) \qquad \text{(Eq 1)}$$

where N and N_0 are the concentrations of live bacteria (CFU mL^{-1}) after and before drying, respectively, and k_d is the inactivation rate constant (s^{-1} for fast drying).

The Arrhenius equation is used to correlate the temperature of the microbial medium with the inactivation rate of the microorganisms. Therefore, k_d is usually expressed as:

$$k_d = k_0 \exp(-\frac{E_d}{R_g T}) \qquad \text{(Eq 2)}$$

where k_0 is the pre-exponential factor and E_d is the deactivation energy which represents the energy required to deactivate living cells, and R_g is the universal gas constant (approximately 8.314 J $mol^{-1}K^{-1}$).

To incorporate the moisture content factor (X), Meerdink & Riet (1995) proposed an attractive approach containing just four parameters (a, b, k_0 and E_d) which must be determined from experiments. The inactivation rate constant (k_d) is then expressed as:

$$k_d = k_0 \exp(aX - \frac{E_d + bX}{R_g T}) \quad \text{(Eq 3)}$$

(Eq 3) was then further developed by incorporating the influence of average drying rate and average heating rate as:

$$k_d = k_0 (1 + b \cdot \left|\frac{dX}{dt}\right|) \exp(-\frac{E_d}{R_g T}) \quad \text{(Eq 4)}$$

$$k_d = k_0 (1 + a \cdot \left|\frac{dT}{dt}\right|)(1 + b \cdot \left|\frac{dX}{dt}\right|) \exp(-\frac{E_d}{R_g T}) \quad \text{(Eq 5)}$$

$$k_d = k_0 (1 + a \cdot \left|\frac{dX}{dt}\right| + b \cdot \left|\frac{dX}{dt}\right|^2) \exp(-\frac{E_d}{R_g T}) \quad \text{(Eq 6)}$$

The prediction of these three equations was found to be more accurate when compared with the traditional non-rate models (Eq 2) and (Eq 3) (Li et al., 2006).

As well as these first-order-kinetics-based models, a statistical model was also developed based on the principle of Weibull distribution (Perdana et al., 2013). This hypothesized the death of microbial cells during spray drying as probabilities rather than determinism. This led therefore to a distribution of inactivation times, which means the survival curve of bacteria is a cumulative form of the inactivation times. Temperature and moisture content were incorporated as two factors determining the inactivation of probiotic *Lb. plantarum* WCFS1.

Overall, the predictions of these models agreed well with the experimental data on bacterial inactivation during single droplet drying. However, the predictions cannot be considered precise, particularly in the context of real-life spray drying. This may be due to the much faster drying rate in spray drying than in droplet drying, and because factors such as oxidative stress, osmotic stress and the protective capacity of the medium, must also be taken into account. Furthermore, the intrinsic tolerance of bacteria varies significantly over different species and strains, which may lead to problems in applying these models to different cases.

1.3 Spray drying probiotics: Post-drying stage

1.3.1. Storage stability of bacteria in powder form

1.3.1.1 Injury-induced loss of viability and strain-dependent shelf-life

The numbers of viable bacterial cells tend to decrease during storage (generally less than 6 months), and particularly during the early stages (up to one month), possibly due to cell injury during spray drying (Wang et al., 2004). For instance, good survival was found to depend on selecting the best survivors following spray drying (G.E. Gardiner et al., 2000). The stability of a freeze-dried culture was reported as being better than that of a spray-dried culture, suggesting the damage caused by heat stress (Wang et al., 2004).

The storage conditions of powders may also have a significant influence on the survival of probiotics. Key parameters which must be controlled include: storage temperature, moisture content or water activity, exposure to oxygen and light, powder composition and storage materials (Morgan et al., 2006). These conditions may cause heat, desiccation, oxidative and starvation stress to bacteria, which can lead to a considerable loss of the viability of probiotics during long-term storage. The intrinsic tolerance of bacterial strains is known to play a critical role in overcoming their inactivation due to either spray drying injury or storage-related adverse stresses. The shelf-life of probiotic powders is therefore dependent on the bacterial strains concerned.

1.3.1.2 Storage temperature and glass transition temperature (T_g)

Probiotic viability in a powder is inversely related to storage temperature. This has been demonstrated extensively in probiotic-containing powders obtained from spray drying (Barbosa et al., 2015b; Wang et al., 2004). However, prolongation of the shelf-life of probiotics by reducing the storage temperature is not favored by industry because of the cost of chilled or frozen storage, even though storage at room temperature still poses an overwhelming challenge to the stability of probiotic powders.

During storage, the viability of probiotics is affected in particular by temperature in relation to the T_g of the dried sample, which in turn is mainly dependent on its moisture content (Passot et al., 2012). Indeed, dried biological matrices stored at a temperature below their T_g have been shown to display greater stability because of the low molecular mobility and reaction kinetics induced by the high viscosity of the glassy state (Buitink et al., 2000). In this respect,

monitoring the moisture content during storage may be of considerable value to controlling the detrimental influence of aging on viability, although this has rarely been reported in the literature. Another strategy consists in supplementing the growth medium with specific components (e.g. high-molecular-weight maltodextrins) in order to increase the overall T_g of the final dried product and thus better maintain viability during storage under given conditions (Conrad et al., 2000).

1.3.1.3 Moisture content: the influence of water activity

It is well known that the lower a_w limit for bacterial growth is around 0.6 (Beuchat, 1981). This suggests that a high a_w might lead to a reduction of probiotic viability and an increase in the risk of contamination during storage. The survival rate is not related linearly to a_w. In most studies, the optimal range of a_w values for the storage of probiotics was lower than 0.2. For example, the relatively poorer survival of *S. thermophilus* CCRC 14085 and *B. longum* B6 detected in spray-dried fermented soymilk after storage could partially be attributed to the high a_w of this dried product (Wang et al., 2004). Spray-dried *Lb. paracasei* CRL 431 was found to survive better when the a_w was lower than 0.33 (Poddar et al., 2014). It was also found in the context of vacuum-drying that *Lb. paracasei* maintained significantly higher survival at an a_w of 0.07 when compared with that seen at 0.22 and 0.33 following storage at 20°C and 37°C for two months, respectively (Foerst et al., 2012). Another study performed on *Lb. rhamnosus* GG also showed that the viability of bacteria in dried crushed flaxseed dropped rapidly, with an a_w at 0.43 and 0.22 during long-term storage, while a loss of viability of only 0.29 $\log_{10}$ units was found with an a_w at 0.11 (Vesterlund et al., 2012).

1.3.1.4 Exposure to oxygen: lipid oxidation

During storage, the oxidation and subsequent saturation of membrane lipids exert a negative impact on viability (Teixeira et al., 1996). Changes to the degree of lipid unsaturation, which increased over time, were shown to markedly affect the passive permeability of the membrane. Furthermore, the products of lipid peroxidation have been shown to induce damage to the bacterial cell wall, cell membrane and DNA during storage (Teixeira et al., 1995; Zotta et al., 2017).

In order to limit these detrimental effects, the drying medium can be supplemented with an antioxidant. However, contradictory results have been reported in the literature regarding this

strategy. The addition of ascorbic acid and monosodium glutamate during spray drying improved culture viability during powder storage (Sunny-Roberts and Knorr, 2009), although other studies found that they had detrimental effects on culture stability during storage. For example, the addition of ascorbic acid and monosodium glutamate protected *Lb. bulgaricus* cells, but only during storage at 4°C. At 20°C, the death rate of the culture was even higher in the presence of these compounds than in the control sample (Teixeira et al., 1995). This could be explained by the pro-oxidant properties of ascorbic acid as a metal ions reducer, in addition to its antioxidant function as a radical scavenger.

Barbosa, Borges and Teixeira (2015) also reported that the exposure of *Lb. plantarum* 299v to sub-lethal thermal, acid or oxidative stress before spray drying could enhance bacterial survival during storage for 180 days at room temperature. However, these sub-lethal stresses did not improve the storage stability of *Pediococcus acidilactici* HA-6111-2 during the same study, which indicates the strain-dependence of this strategy.

1.3.1.5 Powder composition and protective agents

Spray drying is a method used at an industrial scale to encapsulate various ingredients, including microorganisms. Encapsulated bacterial cells have been widely reported as being more stable than free cells (De Prisco and Mauriello, 2016). Song, Cho and Park (2003) showed that the protective effect of encapsulation during storage increased as the storage temperature rose. The effects of encapsulation on the shelf-life of bacteria are also closely linked to the encapsulating carrier (drying medium).

The protective agents used during drying (i.e. efficient drying medium) may also protect bacteria during storage. For example, non-reducing disaccharides (trehalose and sucrose) provided good protection for *Enterococcus faecium* and *Lb. plantarum* during both fluidized-bed drying and storage (Strasser et al., 2009). However, a medium suitable for drying may be not effective in protecting bacteria during powder storage, and vice versa. For instance, addition of polydextrose and oligofructose to skimmed milk was found to decrease the stability of *Lb. rhamnosus* during powder storage when compared to skimmed milk alone, although this did not affect protection during spray drying (Ananta et al., 2005). The addition of chitosan to the drying medium was found to decrease viability of *Lb. acidophilus* NCIMB 701748 during spray drying, but provided excellent protection for the bacterial cells during long-term storage (Yonekura et al., 2014).

1.3.1.6 Storage materials

Options for packaging include different types of barriers to the aforementioned reactive agents such as humidity, oxygen and light. High barrier plastic bags and blister packs have been reported (Morgan et al., 2006). It was found that laminated pouches ensured better protection during the storage of *S. thermophilus* CCRC 14085 and *B. longum* B6, when compared to glass or PET bottles.

Modifying the composition of the surrounding atmosphere by means of a vacuum or nitrogen generally enhances the storage stability of foods. However, the improvement to the storage stability of probiotic powders procured using this method appears to be slight or insignificant (Clementi and Rossi, 1984; Espina and Packard, 1979). Ambiguous results have thus been obtained regarding the effects of the storage atmosphere on bacterial stability; so they are rarely reported.

1.3.2. Applications of dried bacteria

1.3.2.1 Rehydration capacity

After drying, a number of functionalities are expected to be retained in probiotics in powders, such as metabolic activity, tolerance towards human gastrointestinal juices, adherence to epithelial surfaces, antagonistic activity against pathogens and immunoregulatory capacities. To operate these probiotic functionalities, rehydration is generally the first step for products in a powder form.

The recovery of spray-dried probiotics increased with increase in the rehydration temperature, and was seen to be a strain-dependent process (Mille et al., 2004). At the same time, this temperature should not be as high as the heat-lethal temperature (i.e. 50°C for most LAB). Rehydration of spray dried *S. thermophilus* and *B. longum* resulted in enhanced recovery of live cells as much as the temperature increased, within the range 5 to 50°C (Wang et al., 2004). When the rehydration of *Lb. bulgaricus* was performed at 4°C, the cell concentration was only 2.1 x 10^9 CFU g^{-1}, whereas it reached 1.5 x 10^{10} CFU g^{-1} at 30°C or 37°C. Between these values, the live bacteria recovery increased steadily in line with the rehydration temperature: 3 x 10^9 CFU g^{-1}, 3.5 x 10^9 CFU g^{-1}, 5.2 x 10^9 CFU g^{-1}, and 1.1 x 10^{10} CFU g^{-1} at 10°C, 15°C, 20°C and 25°C, respectively (Mille et al., 2004). Conversely, temperature had no significant effect on the final bacterial concentration when dried *Lb. plantarum* samples were rehydrated at 30°C or 37°C (1.5 x10^{10} CFU g^{-1} and 1.3 x 10^{10} CFU g^{-1}, respectively). This could be explained by the higher cellular resistance of *Lb. plantarum*. Interestingly, the difference in the optimum rehydration temperature observed when recovering freeze-dried (20°C) and spray-dried (35°C to 50°C) bacteria further demonstrated that a physiological difference does exist between freeze-dried and spray-dried cells of *S. thermophilus* CCRC 14085 and *B. longum* B6 (Wang et al., 2004). Furthermore, the reconstitution behavior of freeze-dried yoghurt was better than that of spray-dried yoghurt, which may be due to the thermal cellular injury of spray-dried bacteria (Kumar & Mishra, 2004).

Regarding the effect of the rehydration medium, Teixeira, Castro, & Kirby (1995), and Teixeira, Castro, Malcata (1995) demonstrated that there were no significant differences between the recovery rates of dried *Lb. bulgaricus* when using skimmed milk, MRS broth, deionized water or phosphate buffer as the rehydration medium. Only minor differences (of a maximum 0.5 log CFU mL^{-1}) were observed between the recovery rates of spray-dried *Lb. paracasei* NFBC338 powders in various rehydration media, which included AM buffer (0.01 mol/L K_2HPO_4 and 0.01 mol/KH_2PO_4), AM buffer containing 20% (w/v) sucrose, maximum

recovery diluent (MRD), 10% (w/v) RSM, and sterile water (Desmond et al., 2001). Muller et al., (2010) reported that the pH of rehydration medium, presence of L-arabinose or maltodextrin and the rehydration duration time could also exert significant effects on the rehydration behavior of spray-dried probiotics.

1.3.2.2 Changes to bacterial metabolism

Unlike freeze-drying, spray drying has been reported to delay lactic acid production in *Lc. cremoris*, *Lactobacillus casei* ssp. *pseudoplantarum* and *S. thermophilus* (To and Etzel, 1997). The higher the outlet air temperatures, the longer the lag time before the acid production began. For example, the lag time for *Lb. pseudoplantarum* was shorter (2h) at T_{outlet} 65°C than at T_{outlet} 90°C (10h). Apart from its influence on lactic acid fermentation, more attention needs to be paid to how lag time affects the beneficial effects of spray-dried probiotics. For instance, it has been documented that bacteria retain their ability to produce bacteriocin peptides following spray drying: this has been shown for nisin (Nisaplin®; Danisco A/S, Copenhagen, Denmark), lacticin 3147 (Morgan et al., 2006), and other bacteriocins produced by both lactobacilli and lactococci (Mauriello et al., 1999; Zhang et al., 2015). Pérez-Chabela, Lara-Labastida, Rodriguez-Huezo and Totosaus (2013) reported that the incorporation of spray-dried LAB in meat batters enhanced the initial LAB population and a concomitant reduction of Enterobacteria levels in the meat was seen during storage. *Lb. salivarius* UCC 118 was also reported to retain its ability to produce bacteriocin after spray drying, even at high outlet air temperatures (up to 95°C) (G. E. Gardiner et al., 2000). In another study, the spray drying process did not affect the antagonistic activity of *Lb. sakei* and *Lb. salivarius* against *Staphylococcus aureus*, *Listeria innocua* and *Listeria monocytogenes* (Silva et al., 2002).

Overall, the residual viability of probiotics after spray drying is an important indicator when characterizing probiotic activity within spray dried powders. Low viability is more a reflection of the cellular injury of probiotics during the drying process. Severe cellular injury may significantly influence the lag time of bacterial growth and primary metabolism-related functionalities, such as lactic acid production. However, the culture contains a sufficient population of new generation bacteria when regrowth reaches the late log phase or early stationary phase. These new generation bacteria and their secondary metabolism-related functionalities (such as bacteriocin-producing ability and enzyme activity) are not influenced by the drying process.

1.3.2.3 Resistance to food manufacturing processes

Due to the convenience of powder-form products, spray-dried bacteria can be expected to be used as an ingredient to be added in other foods. Conversely, this necessitates the heat resistance of spray-dried probiotics because of the presence of a thermal process in most food manufacturing (Dianawati et al., 2015; Zhang et al., 2014). Spray-dried bacteria generally display higher heat resistance when compared to free bacteria. For example, the survival of spray-dried *Lactobacillus reuteri* DSM 17938 (with co-cross-linked alginate and chitosan as drying medium) was significantly better than that of free bacteria during the baking of a chocolate soufflé (Malmo et al., 2013). The reduction in the bacterial population of spray-dried *Lb. plantarum* BM-1 was only 0.08 log CFU mL^{-1} after exposure to 60°C, whereas the reduction in free cells reached 2.58 log CFU mL^{-1} (Zhu et al., 2016). This can be explained by three mechanisms: first, the encapsulation effect of the drying medium (De Prisco and Mauriello, 2016; Sabikhi et al., 2010); second, the lower water activity surrounding bacterial cells (Laroche et al., 2005), and third, the possible induction of a heat shock response due to the heat stress experienced during spray drying (Rossi et al., 2016). In addition to the thermal process, probiotics or LAB cells (which are used as a starter) are often exposed to an acidic environment in foods because of the drop in pH during fermentation. Spray-dried bacteria can often survive better in an acidic environment during their shelf-life. For example, Dimitrellou et al. (2016) showed that spray-dried *Lb. casei* ATCC393 had an increased survival rate in fermented milk during refrigerated storage, by comparison with free bacteria.

However, it is also possible that the cellular injury caused during spray drying may lead to a higher bacterial death rate during subsequent processing (Wu, 2008). Hence, the adequate bacterial viability and activity in spray-dried powders constitute important criteria for food applications.

1.3.2.4 Resistance to gastrointestinal conditions

Survival is essential for bacteria that are targeted to populate the human gut: this is one of the most important issues when procuring health benefits with probiotics (De Prisco and Mauriello, 2016).

The challenge is to maintain probiotic viability during delivery through the digestive tract. More specifically, a considerable loss of viability was observed when bacterial cells were

exposed to acid and bile stresses, gastric or intestinal enzymes, and a mechanical shearing force during consumption.

In vitro studies have shown that an appropriate spray drying medium can protect probiotics against stress during digestion. For example, a loss of viability was limited by using gum acacia (GA) as a carrier during spray drying: the viability of GA-treated bacteria was 100-fold higher than the control following exposure for 120 min to porcine gastric juice at 37°C (Desmond et al., 2002). Such protection may be reliant on the resistance of GA to digestion (Arslan et al., 2015). As well as GA, it has also been suggested that a dairy matrix might act as a buffering agent, thereby protecting ingested bacteria during transit through the upper gastrointestinal tract (Würth et al., 2015). The anionic polysaccharide alginate, and the cationic polysaccharide chitosan, are also known for their biocompatible resistance to digestion and their mucoadhesive properties (Kim et al., 2014). Rajam et al. (2012) showed that a combination of sodium alginate and denatured WPI as the drying medium could improve the survival rate and controlled core release behavior of spray-dried *Lb. plantarum* during simulated acidic and bile conditions. By comparison with sodium alginate, chitosan was reported to achieve better protection of *Bifidobacterium breve* during fluid-bed drying, and of *Lb. acidophilus* during simulated digestion (Cook et al., 2011; Yonekura et al., 2014). This could be explained by the attractive electrostatic interactions between chitosan (cationic polysaccharide) and bacterial cells (negative surface charge) (Anselmo et al., 2016).

To date, only a few studies have been performed *in vivo*. It has been shown that milk-protein-based microcapsules display a protective effect on probiotics during simulated *in vitro* digestion, but not in the mouse gastrointestinal system (*in vivo*) (Würth et al., 2015). Therefore, the results of simple *in vitro* assays cannot be extrapolated directly to living organisms. It is problematic to perform numerous digestion trials in the context of ethically and technically challenging animal and human studies. The novel dynamic gastrointestinal models now available, which can better reflect the complex digestive systems of living organisms, now need to be applied to testing the behavior during digestion and efficiency of delivery of probiotic products (Chen et al., 2016; Cordonnier et al., 2015).

1.3.2.5 Adhesion to epithelial cells

The importance of the adhesion of probiotics to epithelial cells or the intestinal mucus has been well documented. However, very few publications have reported the effects of spray

drying on the adhesion capacity of probiotics to intestinal epithelial cells. *Lb. plantarum* 83114 and *Lactobacillus kefir* 8321 did not lose their ability to adhere to intestinal Caco-2/TC-7 cells, while *Lb. kefir* 8348 displayed a significant loss of adhesion capacity after spray drying under the same conditions (Golowczyc et al., 2011). This result is in agreement with the sensitivity of bacterial strains to spray drying. *Lb. kefir* 8348 had the poorest survival after spray drying (~30%), while the survival of *Lb. plantarum* 83114 and *Lb. kefir* 8321 was between 80% and 90%.

Improved survival (approximately higher than 50%) may guarantee the retention of the adhesion capacity of probiotics because of the insignificant injury of cell surface structures. Further, probiotics adhesion capacity may also be improved after spray drying by using prebiotics in the drying medium (Brink et al., 2006).

1.3.2.6 Immunomodulation ability

Immunomodulation is one of the most important probiotic functionalities and has been extensively investigated during the past decade. However, there have been very few studies reporting the effects of spray drying on the efficacy of probiotic immunomodulation. The effects of air drying, freeze-drying and spray drying on the immunomodulation ability of the probiotics *Lb. plantarum* CNRZ 1997 and *Lactobacillus zeae* CNRZ 2268 and *B. bifidum* CIP 56.7 were compared with peripheral blood mononuclear cells (PBMC) *in vitro*. The probiotic functionality was not directly linked to cell survival, and displayed strain-dependent sensitivity to each of the drying methods. After different drying processes, the probiotic powders might positively or negatively modify the bacterial immunomodulation capacity, and spray drying appeared to be the best drying process because of its effect on decreasing the production of PBMC IL-12 (Iaconelli et al., 2015). Furthermore, during an *in vivo* study performed with *Lb. acidophilus* A9, *Lb. paracasei* A13 and *Lb. casei* Nad, spray drying was carried out using 20% (w/v) skimmed milk as the drying medium, and the probiotic powders were administered to mice for 5 and 10 days. A significantly higher number of Immunoglobulin A (IgA)-producing cells in the small intestine were induced by spray-dried cultures when compared with fresh cultures (Páez et al., 2013).

Immunomodulation by probiotics is mainly due to molecular interactions between the surfaces of the bacterial cell and host cell (Bron et al., 2011). The spray-dried probiotics are encapsulated by the drying medium. The barrier effect of the drying medium may therefore

modify the interaction between the microbes and the host, and this effect is probably dependent on the digestion behavior of the matrix. The advantage of spray drying probiotics may be linked to the barrier effect of the drying medium in such way it can protect the bacterial surface structure from the digestion process, including pH-induced conformation changes, enzyme catalysis and the shearing force of gastrointestinal tracts. However, severe cell damage caused by spray drying may also lead to a diminished adhesion capacity of bacteria, heat or osmotic-induced conformation changes to the surface structure or a shield effect of the drying medium. However, this remains a hypothesis to be addressed by future research.

1.4 Context, aim and innovation of the PhD Project

1.4.1 Context of the PhD project

The food and pharmaceutical industries are currently faced to a new opportunity with respect to the drying of probiotics by spray drying. By far, it is clear that the final quality of spray dried probiotic powders is determined by several factors ranging from bacteria preparation to final product application (Figure 10).

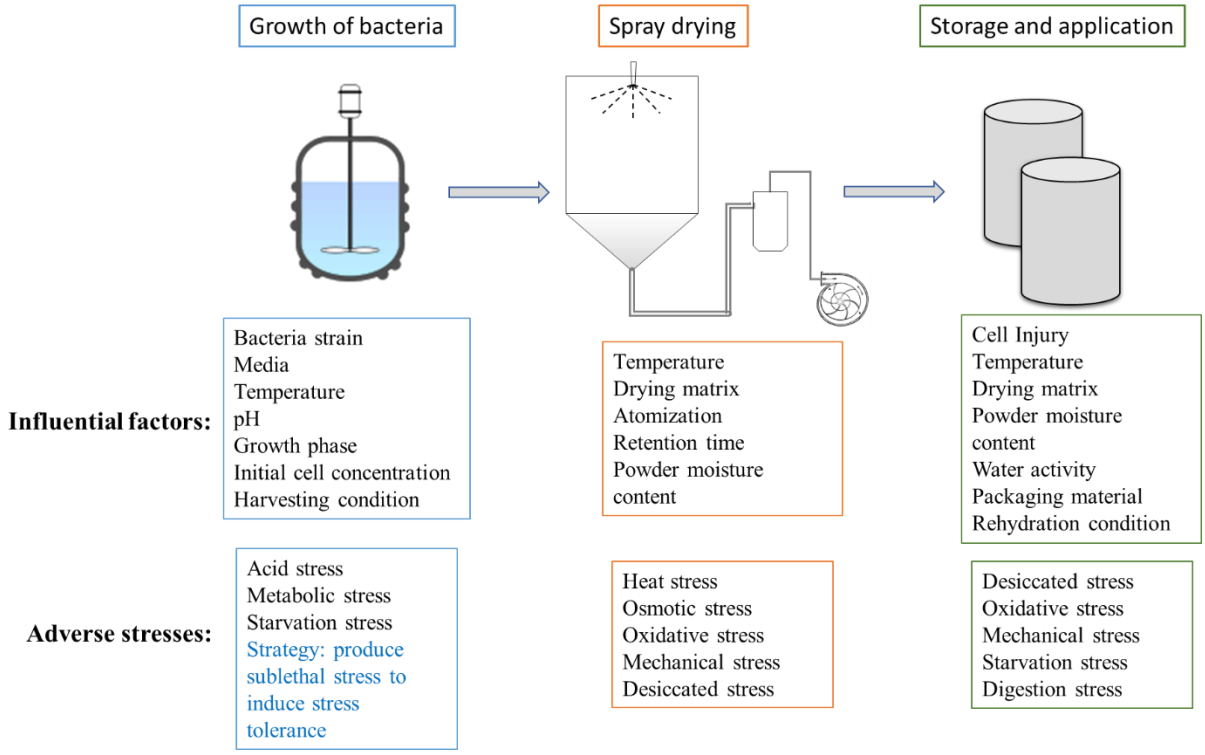


Figure 10. The most influential factors and adverse stresses experienced during the growth, spray drying, storage and application of probiotics (Adapted from Huang et al., 2017).

In this thesis, we firstly introduce a novel simplified process used for spray drying of probiotics (**Chapter 2**). The advantages of this process will be described from both microbiological and processing perspectives. This novel process results in a high bacterial viability in final spray-dried powders. The underlying mechanisms of the viability improvement are then investigated and presented (**Chapter 3**). The scale-up feasibility of the novel process is validated in a semi industrial scale dryer (**Chapter 4**). This novel process is further coupled with a multi-stage drying process to present an industry-friendly approach for production of high quality probiotic powders.

1.4.2 Aim of the PhD project

The aim of this PhD project is to explore an industry-friendly process for spray drying of high quality probiotic powders. The "quality" should not be restricted to the viability/survival of probiotics in the final powders, but also their remaining activity and functionality. To achieve this aim, it is necessary to have a better understanding of the influences of process conditions (including growth, drying and storage conditions) and material properties (mainly including growth and drying media) on the stress tolerance physiology of probiotics throughout the whole process (growth-drying-storage).

Through this project, we attempt to solve the following pending issues in the field of spray drying probiotics:

- **Unsatisfactory bacterial viability after spray drying**
- **Complex operation steps in comparison to freeze drying**
- **High cost of effective protective agents when scale-up drying process**
- **Possibility of probiotic functionality loss**

With the results presented in this thesis, we hope to add the value of spray drying in terms of probiotic production when comparing to freeze drying.

1.4.3 Innovation of the PhD project

Involvement of multidisciplinary knowledges is required when tackling with increasingly scientific questions and technical challenges in spray drying of probiotics. This project has benefited from the first joint PhD project between Soochow University (Suzhou, China) and Agrocampus Ouest (Rennes, France). The collaboration between chemical engineering lab (China) and UMR STLO lab (Processing & Microbiology teams, France) provided access to more instruments and analytical tools, and also allowed us to understand fundamental and experimental issues with a broader view.

As reviewed earlier in this chapter, different strategies to improving bacterial viability during spray drying have been investigated to date. However, these studies are mostly limited in the pre-drying unit (bacterial growth, stress response physiology, etc.) or the drying unit (protective agents, drying parameters, etc.). In comparison with previous studies, the innovation of this project mainly concludes:

- **This project proposed a strategy with the merits throughout the entire production process, particularly targeting at high bacterial growth before spray drying and high remaining probiotic viability in the final powders**
- **The operation steps were simplified compared to the conventional spray drying process in terms of probiotic production**
- **The mechanisms were investigated from different aspects including bacterial stress response physiology, droplet drying behavior, droplet-particle transition, etc.**
- **The cost efficiency was targeted both from processing and material perspectives and the scale-up feasibility was validated in a semi-industrial scale experiment**
- **Apart from the bacterial viability, the storage stability and digestion resistance of probiotic powders were investigated.**

Chapter 2. A novel 2-in-1 Process: Double Use of Highly Concentrated Sweet Whey

In this chapter, a novel process is proposed in order to produce probiotics simply and sustainably via spray drying. In this novel and now patented process, the highly concentrated sweet whey was first used as a 2-in-1 medium for growth and spray drying of probiotics. Probiotic strain *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* CIRM-BIA129 were used as test strains. Compared to the usual process, the intermediate operation steps from growth to spray drying (e.g. cell washing, harvesting and re-suspending) were avoided in this novel process. Meanwhile, probiotic metabolites could be retained in the final powders as the food-grade feature of the culture medium.

To achieve such a 2-in-1 process, the growth of bacteria is a prerequisite: given the hypertonic condition in highly concentrated sweet whey, "Fight or Flight" of bacteria thus become a determining factor with regard to the feasibility of the process.

The total solids (TS) of the sweet whey medium was increased from 5% to 40%, and the optimal range of TS was explored in relation to its effects on biomass production after growth and viability after spray drying and during storage. The physiology of bacteria under hypertonic conditions in the highly concentrated sweet whey was shown to drastically influence both the resistance to drying and the final quality of probiotic powders.

The main contents in this Chapter have been published[1] or prepared[2] as:

[1] Method for preparing a probiotic powder using a two-in-one whey-containing nutrient medium. Patent application filed in Europe on 21 September 2015, EP no. 15 306465.4. Inventors: Jeantet, R., Huang, S., Jan, G., Schuck, P., Le Loir, Y., Chen, X.D.

[1] Huang, S., Cauty, C., Dolivet, A., Le Loir, Y., Chen, X.D., Schuck, P., Jan, G., Jeantet, R., 2016a. Double use of highly concentrated sweet whey to improve the biomass production and viability of spray-dried probiotic bacteria. J. Funct. Foods 23, 453–463.

[2] Huang, S., et al. Long term survival of *Lactobacillus casei* BL23 in sweet whey medium.

2.1 Introduction

Why do we need a 2-in-1 drying process?

As reviewed in Chapter 1, the challenge of utilizing spray drying to produce probiotic powders is mainly related to the use of high temperatures during the process. Extensive investigations have been carried out in order to improve the remaining viability of probiotic bacteria during spray drying and subsequent storage, in which the strategies mainly include enhancement of cellular resistance, addition of protectants to the drying medium and process optimization. **However, these strategies have usually focused only on the drying stage, and rarely considered the overall process from growth to drying of bacteria**.

Standard laboratory culture media (e.g. the most frequently used de Man, Rogosa and Sharpe–MRS broth) have been used to grow bacteria in most studies (Table 3). **Since they are non food-grade, the subsequent operations required additional harvesting, rinsing and re-suspension of bacteria before reaching the drying unit (**see Figure 11**). In addition to the waste of material and beneficial probiotic metabolites, the removal of these culture media may lead to the loss of bacteria viability and increase the risk of contamination during rinsing, centrifugation and re-suspension operations. The possible residual components from the culture media on the bacteria pellets may also interfere with subsequent operations and further applications.**

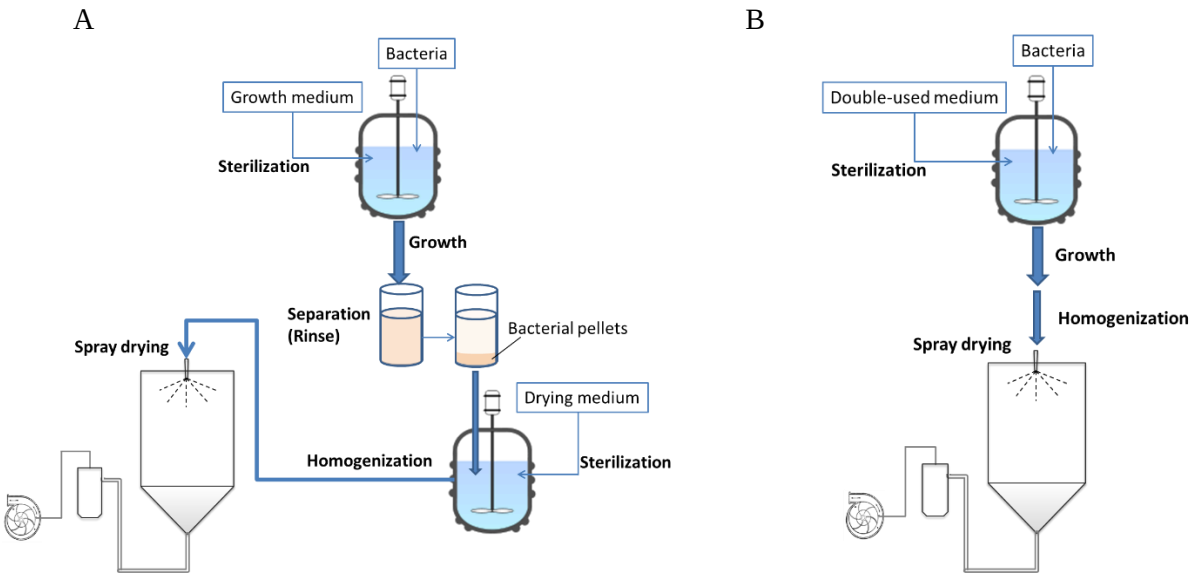


Figure 11. Diagrams of (A) the conventional process used in spray drying of probiotic bacteria and (B) the innovative 2-in-1 drying process.

Why did we use sweet whey?

A proper food-grade medium is a key factor to achieve the one-step spray drying without intermediate operations.

The food-grade culture media based on MRS medium have been proposed for biomass production of probiotic bacteria (Hwang et al., 2012; Lavari et al., 2014; Sawatari et al., 2006, 2006). However, probably due to the high cost of MRS, the feasibility of double using these MRS based media for both biomass production and spray drying has rarely been reported.

Whey is an abundant food grade byproduct of the cheese industry. On a global scale, most of the cheese production is realized in Europe and United states, leading to large byproduction of whey (Table 6): 40 to 50% is exported from the EU, currently at a relatively low price after a continuous increase from 2010 due to infant formula gold rush. The large amount of lactose and whey proteins in sweet whey, obtained from the manufacturing of rennet-type cheese, makes it an ideal medium for growing dairy bacteria. Moreover, several reports have demonstrated that the **lactose, whey proteins and calcium could have protective effects on probiotics against adverse stresses during spray drying, storage and digestion** (Huang et al., 2014; Huang and Chen, 2013; Mattila-Sandholm et al., 2002; Mäyrä-Mäkinen and Bigret, 1993; Picot and Lacroix, 2004; Rajam et al., 2012). In view of all of this, sweet whey can be considered as an excellent candidate to be used in the 2-in-1 process (Figure 11B).

Table 6. The production, exportation and price of whey in the European Union. (Data source: CNIEL, 2011~2016)

Year		2010	2011	2012	2013	2014	2015
Production (×1000 tons)		1939	1976	2097	2218	2229	-
Exportation (×1000 tons)		917	973	932	917	913	888
Exported fraction (%)		47	49	44	41	41	-
Price[a] (Euros/ton)	Whey	669	845	911	960	884	652
	Whole milk	2716	3143	2758	3541	3067	2422
Relative whey to whole milk price (%)		25	27	33	27	29	27

[a] Data were from the quotations of milk products recorded on the internal market in France

Why did we increase whey concentration (total solid content)?

To achieve one-step drying from a bacterial culture, the total solid content (TS) of the double-used medium, i.e. the concentration of sweet whey in this project, has a critical role in the overall process and the final product. Indeed, the TS values of MRS and Yeast Extract Sodium Lactate (YEL) broth (Malik et al., 1968), respectively the standard culture media of *Lactobacillus* and *Propionibacterium*, are around 5% (w/w). This low value, in the same range as that of sweet whey, has several adverse consequences when using these culture media as raw materials in industrial spray drying: in particular, low powder flow rate, large amount of water to evaporate and subsequent high drying temperature needed, and potential occurrence of undesirable caking or sticking in these relatively fine powders (Stoklosa et al., 2012; Wu et al., 2014). Therefore, **considering the energy efficiency and productivity of spray drying, the feed with high TS is generally desired by industry** (Figure 12). In the aim of improving bacterial survival, **the feed with high TS also has higher potential in term of moderating the drying condition (especially temperature).**

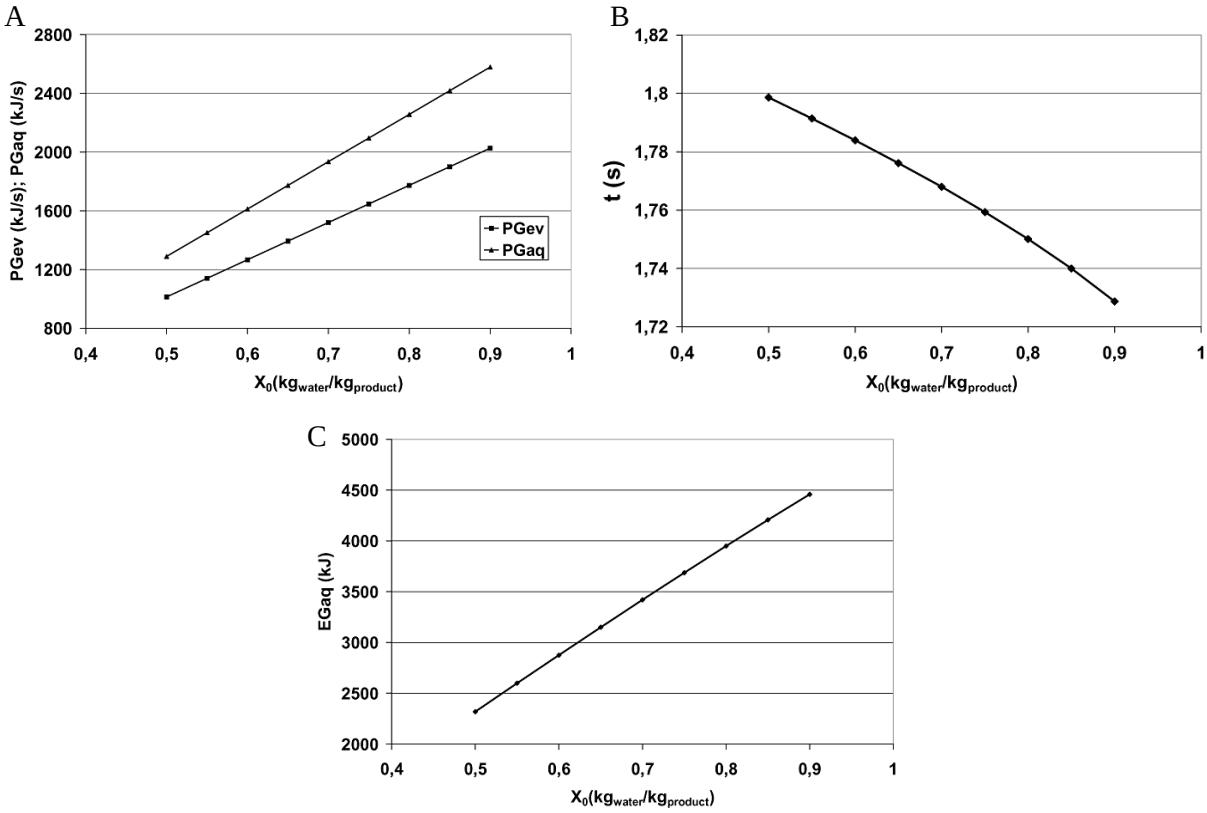


Figure 12. (A) Evaporation power (PGev, power required to evaporate water) and heating power (PGaq, the power required to heat the drying air), (B) Total drying time and (C) Total drying energy as a function of feed moisture content. (adapted from Kajiyama and Park, 2011)

Why did we choose the two probiotic strains?

In this project, the well-documented probiotics *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* CIRM-BIA129 (also known as ITG P20) were used as the model probiotic strains.

L. casei is an important species of lactic acid bacteria which has been widely used in dairy production, especially as the nonstarter bacteria in cheese ripening. Besides, *L. casei* species also forms the *lactobacillus* flora inhabiting the human mouth, intestine and vagina, which plays significant role in human health and disease (Ahrné et al., 1998; Guarner and Malagelada, 2003). Increasing *L. casei* strains have been reported to confer beneficial effects on the hosts through different action mechanisms, hence being recognized as potential probiotics used as functional foods or adjuncts in treatment or prevention of certain diseases (Aktas et al., 2016; Aoyagi et al., 2016; Ca et al., 1999; Kato-Kataoka et al., 2016). *Lactobacillus casei* BL23 is an anti-inflammatory strain, also known to attenuate colitis (Rochat et al., 2007; Watterlot et al., 2010). The ability of attenuating colitis of the *L. casei* BL23 strain was found to depend on the dairy delivery matrix (Lee et al., 2015b), which makes it an ideal target to be encapsulated by the sweet whey medium used in this work.

P. freudenreichii, the main species of dairy propionibacteria, is consumed in high amounts both in traditional fermented foods and in probiotic supplements. Its promising probiotic properties include beneficial modulation of several gut parameters (Cousin et al., 2010). Consumption of *P. freudenreichii* indeed leads to increased bifidobacterial intestinal population in humans (D. Bouglé, 1999). In an animal model of carcinogenesis, such consumption reduces proliferation while enhancing apoptotic depletion of colon cancer cells (Lan et al., 2008) in accordance with the pro-apoptotic effect of its major metabolites, the short chain fatty acids (SCFAs) propionate and acetate (Cousin et al., 2012a; Jan et al., 2002; Lan et al., 2007). This cytotoxic effect, killing cancer but not healthy human colon epithelial cells, relies on survival and activity of *P. freudenreichii* in the ingested product and in the gut, key prerequisites for *in situ* release of SCFAs (Cousin et al., 2016). Selected strains of *P. freudenreichii* CIRM-BIA129 furthermore modulate gut inflammation through strain-specific surface layer proteins (Foligné et al., 2013a, 2010), which induce the production of the immunomodulatory cytokine IL-10 by human immune cells and which are expressed in fermented dairy products (Plé et al., 2015). This anti-inflammatory effect may be further enhanced by *P. freudenreichii* ability to release folic acid and the menaquinone biosynthesis precursor 1.4-dihydroxy-2-naphthoic acid (DHNA) (Mitsuyama et al., 2007; Rossi et al., 2011).

Similarly with *L. casei* BL23, the dairy matrix was also demonstrated to display protective effects for the probiotic potential of *P. freudenreichii* (Cousin et al., 2012a; Gagnaire et al., 2015; Mitsuyama et al., 2007; Plé et al., 2015).

Altogether, these two strains were selected as model strains in this work in terms of their potent beneficial effects and their compatibilities with the sweet whey medium.

Based on all the above considerations, sweet whey was used for the first time as a double-used medium in a simplified one-step spray drying process in this work.

2.2 Materials and Methods

2.2.1 Strains and culture conditions

The probiotic strain *Lactobacillus casei* BL23 was kindly provided by UMR1219 MICALIS, (INRA-AgroParisTech, Jouy-En-Josas, France) and *Propionibacterium freudenreichii* subsp. *shermanii* CIRM-BIA 129 was maintained and pre-cultured by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France). *L. casei* was activated by inoculation (1% inoculum size) in MRS Broth and static cultivation at 37ºC for 16 h. *P. freudenreichii* was inoculated (1% inoculum size) in YEL broth and cultivated statically at 30ºC for 50 h.

2.2.2 Growth in sweet whey culture media

Sweet whey powder (Lactalis ingredients, Mayenne, France) was used to prepare the sweet whey culture media with different levels of TS. Composition of the sweet whey powder was analyzed by the procedures described by Gaucher et al. (2008) (Table 7).

Table 7. Physical and chemical analysis of the sweet whey powder (composition expressed as w/w%).

Total solids (%)	94.81
pH	6.52
Total nitrogen (%)	11.81
Non-protein nitrogen (%)	3.11
Lactose (%)	67.91
Ash (%)	6.85
Calcium (%)	0.37
Magnesium (%)	0.11
Sodium (%)	0.59
Potassium (%)	2.27
Chloride (%)	1.72
Phosphate (%)	1.14
Citrate (%)	2.26

The sweet whey powder was dissolved in deionized water to obtain culture media with final total solids content (TS, w/w) of 5%, 10%, 20%, 30% and 40%, respectively. The osmolality of the medium (Figure 13) was measured with a freezing-point osmometer (Osmomat 030-D, Gonotec, Berlin, Germany) calibrated with standard solutions.

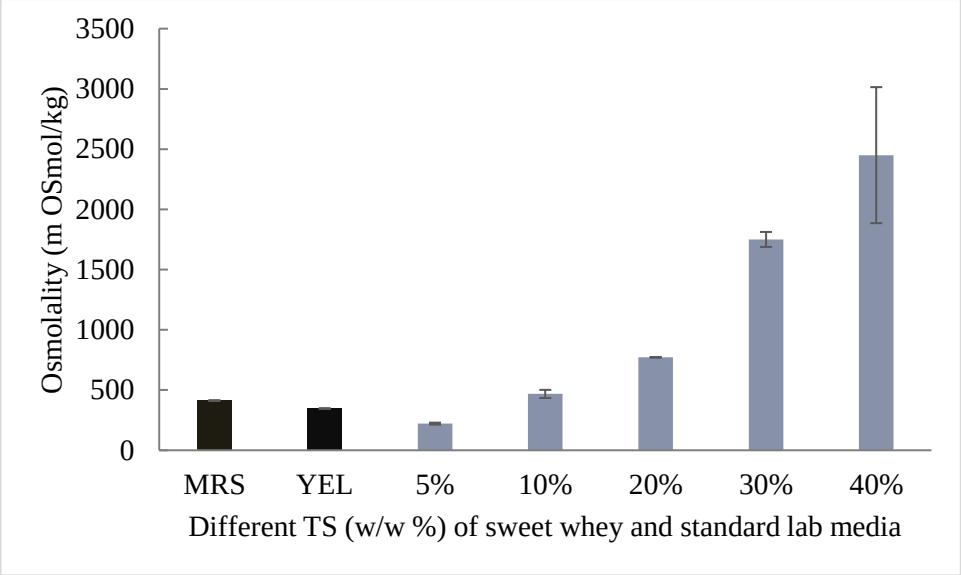


Figure 13. Osmolality of sweet whey media and standard lab media (MRS and YEL) as a function of the TS (w/w %)

The culture media with casein peptone were prepared by adding casein peptone plus (Organotechnie, France) to the above sweet whey culture media at a concentration of 0.5% w/w (Cousin et al., 2012b). All of these culture media were autoclaved at 100 ºC for 30 min before inoculation of probiotic bacteria. *L. casei* was inoculated at 1% inoculum size in the different sweet whey culture media (i.e. with different TS of sweet whey with/without casein peptone) with the MRS preculture. The inoculated culture media were incubated statically at 37 ºC for 48 h. *P. freudenreichii* was inoculated in the sweet whey culture media using a YEL preculture and incubated statically at 30 ºC for 120 h (5 days).

2.2.3 Monitoring of bacterial growth and acidification curves

The growth curves and pH changes of *L. casei* were monitored for the cultures grown in the MRS broth, 5% sweet whey with casein peptone supplementation and 30% sweet whey without casei peptone supplementation. The growth curves were expressed by the bacterial population in the culture. Bacterial population was determined by CFU counting on MRS agar plates. The *L. casei* culture was diluted serially (1 mL to 9 mL) in peptone water (0.1% w/v) prior to pouring onto MRS agar plates. The agar plates were then incubated at 37^{o} C for 48 h (aerobic conditions) before counting the bacterial colonies.

2.2.4 Spray drying

The probiotic cultures grown in the casein peptone-supplemented culture media were used for spray drying. In addition, further samples were prepared by growing bacteria in 5% casein peptone-supplemented medium but increasing the TS value to 30% by adding the sweet whey powder in order to dry these bacteria cultures directly.

Spray drying was carried out using a pilot-scale Mobile Minor^{TM} spray dryer (GEA Niro A/S, Denmark) at the maximum evaporation rate of 5 kg water h^{-1}. A two-fluid spray nozzle with an orifice diameter of 0.8 mm was used coupled to a peristaltic pump (Watson-Marlow, France) for feeding and atomization. The absolute humidity of the inlet air was maintained at around 1g kg^{-1} of water in the air by a dehumidifier (Munters, Sweden). Spray drying parameters were monitored by SD^{2}P® software (Schuck et al., 2009). All the sample-contact parts of the dryer, including the nozzle, chamber etc., were washed with hot water at 90ºC. Inlet temperature of 200ºC was used to dry the dryer and inactivate any possible contaminating microorganisms 2 h before drying the probiotic culture.

All the culture media (1 L) were agitated moderately for 10 min before spray drying. In view of the intrinsic resistance of both strains, the inlet temperature of spray drying was set at 140ºC for *L. casei* (fragile strain) and 180ºC for *P. freudenreichii* (robust strain). The outlet temperature was 63 ± 2ºC for *L. casei* and 73 ± 2ºC for *P. freudenreichii*, and the relative humidity of outlet air was maintained at 10 ± 1% by adjusting the feed rate. The water content and water activity of the powders were tested according to the methods described by Schuck, Dolivet, & Jeantet (2012).

2.2.5 Size distribution measurement

The size distribution of powders was measured by laser light scattering with a MasterSizer 2000 equipped with a 5-mW helium-neon laser (Malvern Instruments, Malvern, UK).The dry powder feeder attachment was coupled to the standard optical model presentation for dispersion of powders in the air. The $D_{0.5}$ and *span* parameters were used to characterize the size distribution of powders, $D_{0.5}$ being the maximum diameter of 50% of the particles, and the *span* of powders being calculated as:

$$\boldsymbol{Span = \frac{D_{0.9} - D_{0.1}}{D_{0.5}}} \quad \text{(Eq 7)}$$

where $D_{0.9}$ and $D_{0.1}$ are the maximum diameters of 90% and 10% of the particles, respectively.

The result represented the mean of two independent samplings, each measured three times successively.

2.2.6 Scanning electron microscopy

The *L.casei* powders from culture media with 5% and 30% TS were fixed on carbon tape and then sputter-coated with gold-palladium. These powder samples were observed by scanning electron microscopy (JSM 7100F. JEOL. Tokyo. Japan) at 5 kV.

2.2.7 Storage

The powders were collected and sealed in sterilized polystyrene bottles (Gosselin, France), stored at a controlled temperature of 4° C and kept away from light. The samples were analyzed at 30 day intervals for 120 days (4 months).

2.2.8 Enumeration and quantification of bacteria viability

The number of viable cells was first measured after growth (i.e. before spray drying) by colony forming unit (CFU) counting as follows. After 10 min agitation, the bacteria culture was diluted serially (1 mL to 9 mL) in peptone water (0.1% w/v). After spray drying or during storage, the powder samples were rehydrated by dissolving 1 g of powder in 9 mL of peptone water before serial dilutions. Each diluted sample of *L. casei* was poured onto MRS agar and incubated at 37° C for 48 h (aerobic conditions). *P. freudenreichii* dilutions were poured into YEL agar and incubated at 30° C for 6 days under anaerobic conditions (Anaerocult®, Merck KgaA, Germany).

The dependency of bacteria final population on the casein peptone supplementation was calculated according to:

$$Dependency = \frac{N_+ - N_-}{N_-} \quad \text{(Eq 8)}$$

where N_+ is the bacteria population (CFU/mL) in the medium with the casein peptone supplementation, and N_- the bacteria population without casein peptone supplementation.

The survival of bacteria after spray drying (%) was calculated according to:

$$Survival = \frac{N_d}{N_0} \times 100 \quad \text{(Eq 9)}$$

where N_d refers to the bacteria population (CFU/g) in powders after spray drying. The initial population N_0 (CFU/g) was calculated from N_+ and the total solid content (TS) of medium as follows:

$$N_0 = N_+ \times \frac{1-TS}{TS} \quad \text{(Eq 10)}$$

The log reduction of bacteria after 120 days' storage was calculated as:

$$Log\ reduction = Log\ (N_d) - Log\ (N_{120}) \quad \text{(Eq 11)}$$

where N_{120} is the bacteria population in the powders following storage for 120 days.

2.2.9 Statistical analysis

All the experiments were repeated at least three times. The results are presented as mean value with standard error. Significant differences ($p < 0.05$) between the mean values were determined by Tukey's test. The statistical analysis was carried out using R 3.2.1 with the 'Rcmdr' package (R Development Core Team).

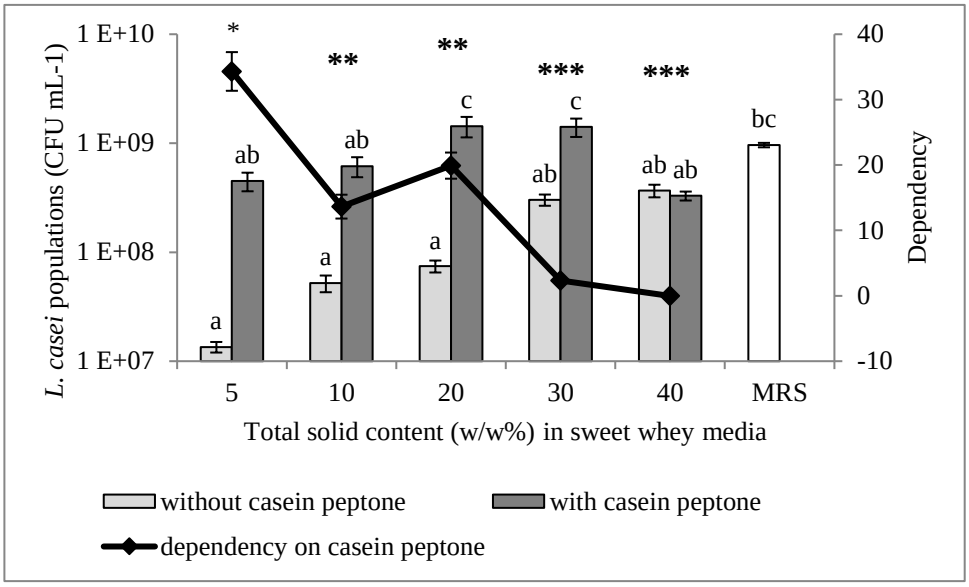
2.3 Results and Discussion

2.3.1 Effect of sweet whey concentration on bacterial biomass production

The growth of bacteria was compared in sweet whey culture media at different TS, with or without casein peptone supplementation. As shown in Figure 14A, the final population of *L. casei* in sweet whey culture media without casein peptone increased as TS increased from 5% to 40%. Moreover, casein peptone supplementation significantly enhanced the final *L. casei* population at 5% to 30% TS, but not that at 40%. In these casein peptone supplemented culture media, a final *L. casei* population of approximately 2×10^9 CFU mL^{-1} was reached for culture media with 20%, 30% TS, and 1×10^9 CFU mL^{-1} for that in MRS broth. The dependency of *L. casei* final population on casein peptone decreased when the TS of sweet whey increased.

Propionibacterium freudenreichii subsp. *shermanii* CIRM-BIA 129, used in this study, is able to use various substrates as carbon and energy sources for growth. Carbohydrates, including sucrose, lactose, glucose, galactose, inositol, erythritol, adonitol, and esculine, may be used (Loux et al., 2015), as well as amino acids, glycerol, or its preferred carbon source, which is lactate (contained in the reference YEL growth medium). Within dairy products, it can therefore utilize either lactose, the major milk carbohydrate, or lactate, resulting from lactic acid fermentation. Sweet whey and acid whey, which may contain either lactose or lactate or a mixture thereof, should thus sustain *P. freudenreichii* growth. The final population of *P. freudenreichii* also showed a similar trend to that of *L. casei* when the TS of sweet whey increased (Figure 14B). Specifically, the population increased as the TS increased from 5% to 30%, but with a considerable reduction at 40%. The optimal TS values also ranged from 20% to 30%, with larger populations than those obtained in YEL broth (~ 1.0×10^9 CFU mL^{-1}). The casein peptone-supplemented medium with 30% TS produced the highest population of *P. freudenreichii* (~ 2.5×10^9 CFU mL^{-1}) among all the culture media tested in this study. However, compared to *L. casei*, the final population of *P. freudenreichii* in sweet whey culture media was less influenced by the casein peptone supplementation. The maximum improvement was obtained when adding casein peptone to sweet whey with 20% TS. However, the dependency was around 1.0, which means that the *P. freudenreichii* population was only doubled after supplementation with casein peptone.

A



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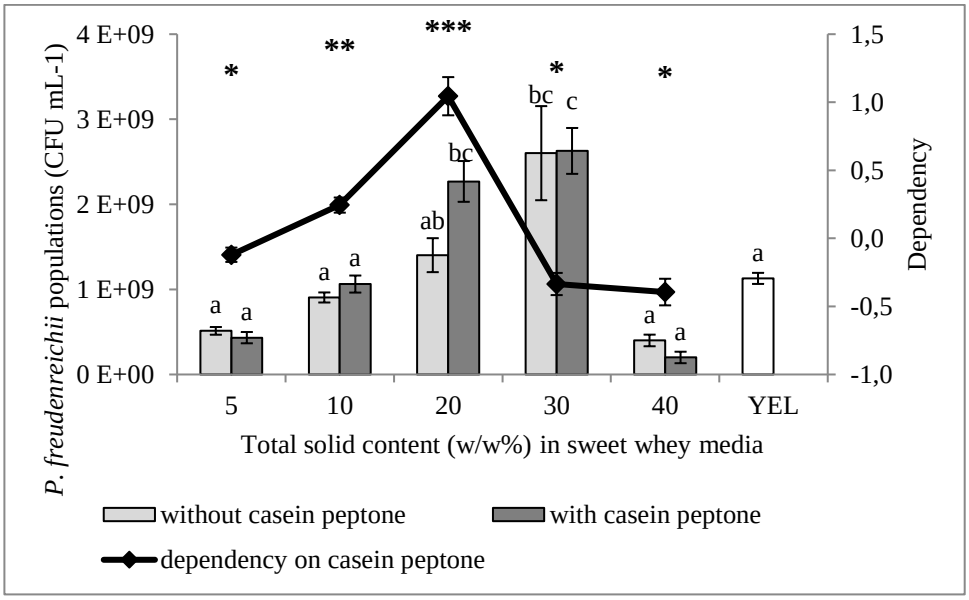

Figure 14. Final bacteria populations (left Y axis) and the dependency (right Y axis) of bacteria final population on casein peptone of (A) *L. casei* 48-h culture and (B) *P. freudenreichii* 120-h culture in the sweet whey culture media with different TS, with or without casein peptone supplementation (compared to the standard culture media, i.e. MRS broth for *L. casei* and YEL broth for *P. freudenreichii*). Mean ± SEM, n = 6. Different letters mean significant difference between bacteria populations ($p < 0.05$). Different number of * means significant difference in dependency ($p < 0.05$).

The results indicate that increasing the TS of sweet whey to values ranging between 20% and 30% was able to improve the biomass production of both probiotic strains. As mentioned above, these higher TS values would be beneficial for subsequent spray drying. The improvement in the final bacteria populations may result from the richer nutrients in the culture media with higher TS values. Although the osmotic pressure was also higher in these culture

media, the time of growth may have been long enough to trigger the stress response of these two strains to adapt to the high-osmolality environments (Wood, 2011). For example, it has been reported that these two strains are able to accumulate intracellular polyphosphate which is related to the improvement in bacterial stress tolerance (Alcántara et al., 2014). The presence of large amounts of phosphate in the culture media with 20% or 30% TS may facilitate the accumulation of polyphosphate by uptake of phosphate from the extracellular environment. However, when increasing the TS of sweet whey to 40%, the inhibition effect caused by the extreme osmolality and related energy consumption in osmoregulation began to be deleterious for the growth of both strains.

2.3.2 Long-term survival of L.casei BL23 during growth in sweet whey media

The growth curves of *L. casei* was monitored and expressed by the bacterial population in Figure 15. They were significantly different in sweet whey medium from that in MRS broth, either at 5% or 30% TS. The bacterial population in MRS peaked at 12 h with a population around 2.5×10^{9} CFU mL^{-1}, then maintained a constant for around 1 day prior to a gradient drop from 10^{9} to 10^{3} CFU mL^{-1} during the following 4 days (i.e. 10^{5} CFU drop from 48 h to 192 h). Interestingly, the alive bacterial population in sweet whey media maintained a constant after peaking at 12 h for 8 days, albeit the highest population is around 1 log lower than that of MRS culture (~ 5×10^{8} CFU mL^{-1}). Moreover, this long term survival of bacteria existed both in isotonic (5% TS) and hypertonic (30% TS) sweet whey.

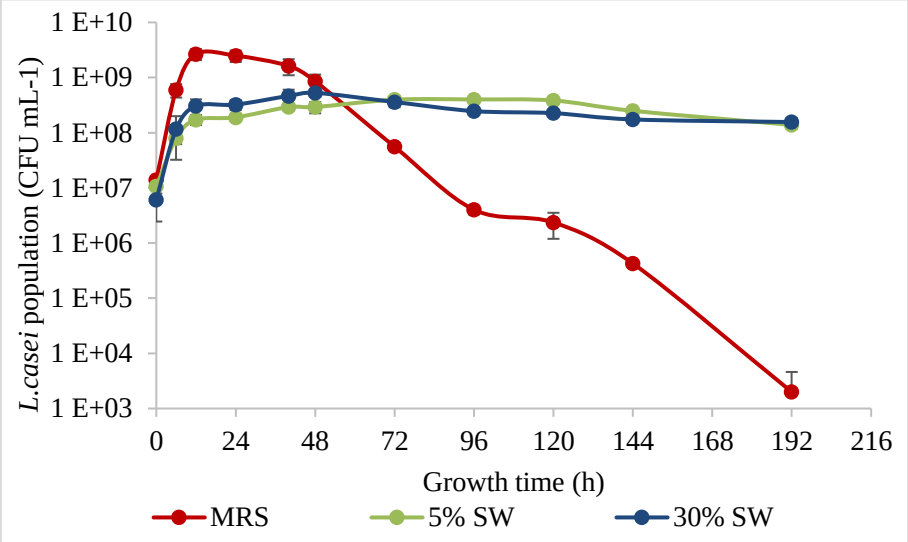

Figure 15. Growth curve (CFU) of *L. casei* BL23 in MRS broth, 5% sweet whey with casein peptone supplementation and 30% sweet whey without casein peptone supplementation.

Long term survival of food-grade bacteria within the food matrix is of interest from both practical and scientific perspectives, especially for the probiotics with beneficial claims. Although we cannot propose a confirmed mechanism for the long term survival of *L. casei* in sweet whey medium, we can offer a number of hypotheses.

Growth in MRS resulted in a higher bacterial population (~ 1 log more). It indicated that nutrients in MRS may be consumed completely, leading to starvation stress on bacteria in the late stationary phase. Similarly, the rapid fermentation in MRS may lead to faster and harsher acid stress towards *L. casei* than in sweet whey medium (Figure 16). This limited acid stress in

sweet whey medium can also be caused by the buffering capacity of the whey proteins and phosphate salts (Salaün et al., 2005). Overall, the severe starvation and acid stress may be the reason of bacterial inactivation in MRS culture in the late stationary phase.

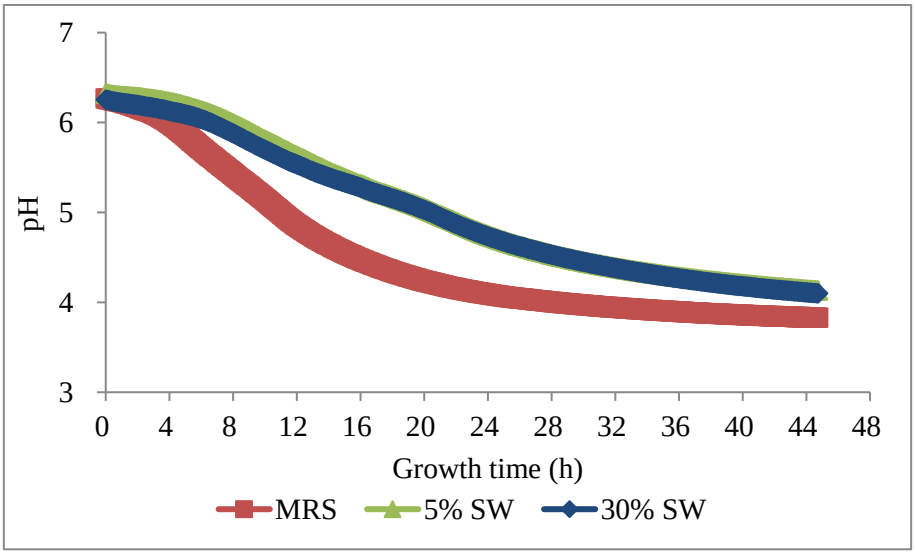

Figure 16. Acidification curve of *L. casei* BL23 in MRS broth, 5% sweet whey with casein peptone supplementation and 30% sweet whey without casein peptone supplementation.

Apart from the stress-induced bacterial death in MRS, it is also possible that some elements in sweet whey medium suppress those lethal factors. The buffering capacity of sweet whey medium on the decreasing of pH may affect the autolysis of *L. casei* to some extent (Kang et al., 1998; Martínez-Cuesta et al., 1997), that is although an important matter for cheese ripening. Besides, the lower bacterial population in sweet whey culture could also avoid quorum-sensing-dependent programmed cell death which may emerge in the MRS culture when the population reached up to 2.5×10^{9} CFU mL^{-1} (Heurlier et al., 2005; You et al., 2004). However, these hypotheses warrant further investigation.

2.3.3 Improved probiotic viability in spray drying application

The remaining viability of probiotics was expressed as the population and as the percentage of survival of bacteria after spray drying (Figure 17). The water content and water activity values of all powders were 6±1% and 0.2 ± 0.05, respectively.

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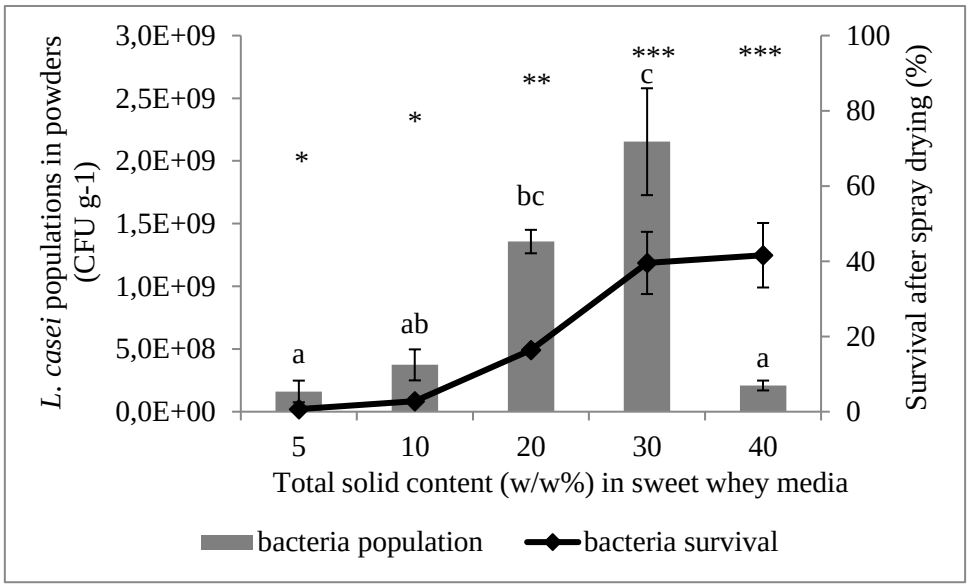

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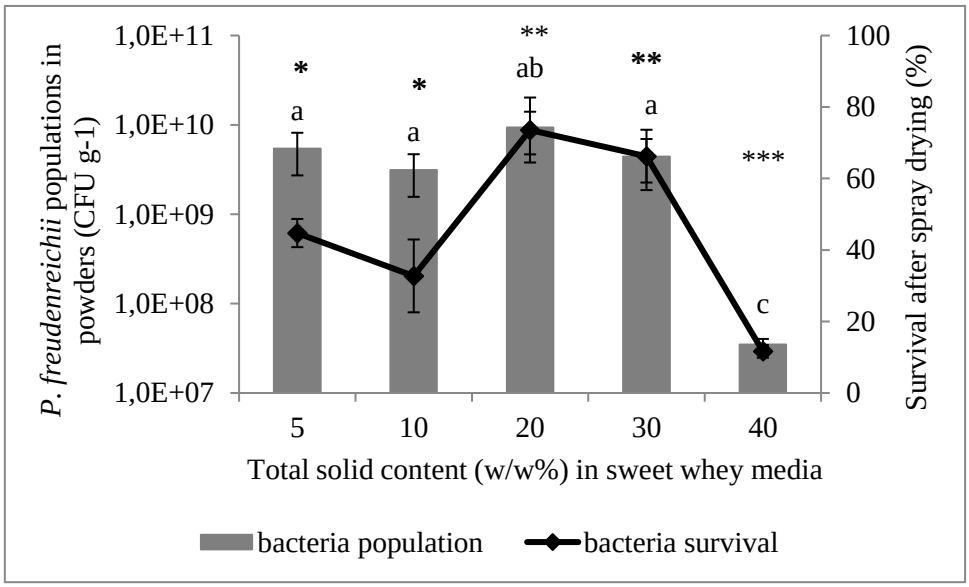


Figure 17. Remaining bacteria populations (left Y axis) and survival (right Y axis) of (A) *L. casei* and (B) *P. freudenreichii* in the sweet whey culture media with different TS after spray drying. Mean ± SEM, n = 3. Different letters mean significant difference between bacteria populations ($p < 0.05$). Different number of * means significant difference between survival rates ($p < 0.05$).

The survival of *L. casei* in powders after spray drying increased with TS from 5% to 40%, with a maximum survival of 40% for TS values of 30% and 40%. In comparison with the powder from the 5% TS medium, the survival in the powder from the 30% TS medium was improved approximately 60-fold (~0.6% survival for 5% TS and ~40% survival for 30% TS). The population of *L. casei* in powders also increased as the TS increased from 5% to 30%, but decreased at 40% TS due to the relatively low *L. casei* population in the 40% TS culture before drying. The maximum survival rates for the *P. freudenreichii* strain (ca. 70%) after spray drying occurred in the culture media with 20% and 30% TS. Similarly to *L. casei*, the low TS culture resulted in lower *P. freudenreichii* survival close to 40% after spray drying. The populations of viable bacteria in the powders from the 5%, 10%, 20% and 30% TS cultures were all above 10^9 CFU g^{-1}. The highest population was obtained for the 20% TS culture, which reached 10^{10} CFU g^{-1}. The *P. freudenreichii* growing in 40% TS culture displayed the lowest survival and remaining population after spray drying.

When comparing the two probiotic strains, the survival of *P. freudenreichii* was generally higher than that of *L. casei* after spray drying, despite the higher drying temperature used. This suggested that the *P. freudenreichii* strain was more tolerant to spray drying stress than the *L. casei* strain. It is known that *P. freudenreichii* is generally a heat resistant species which is often used for making Emmental-type cheeses in which the curd is heated at 50 ~ 55ºC. It has been reported that *P. freudenreichii* accumulates intracellular trehalose and glycogen in addition to polyphosphate, which are involved in improving bacterial tolerance of heat and/or desiccation stress (Falentin et al., 2010b).

In relation to both the survival and final viable population of the probiotics in powders, the optimal TS value of sweet whey was between 20% and 30%. In this range, both strains also presented optimal biomass production. The improvement in survival may have resulted from the robust cellular tolerance induced by higher osmolality in the 20% to 30% TS culture media. However, it may also have been due to the higher levels of dry matter (i.e. TS values). Indeed, relatively fine droplets might be formed from low TS culture media due to their lower viscosity and solid content, leading to shorter evaporation time and potentially to longer residence time of the resulting particles within the dryer (Jeantet et al., 2008). The consequence would be a longer exposure to higher temperature.

Moreover, a lower solid content in the medium also indicates that less wall material can be used to encapsulate the bacteria cells. In other words, the bacteria in the lower TS medium may be more exposed to the hot air (Perdana et al., 2014).

Lower solid content in the feed seems therefore to be disadvantageous for production of powders with live probiotic bacteria. This can also be deduced from the particle size distribution (PSD) of the powders (Figure 18 and Table 8). For both strains, the powder PSD right shifted as the TS in the culture media increased. This result was concordant with the observation by scanning electron microscopy, shown in Figure 19. Powders dried from high TS (30%) culture had a wider size distribution (*Span* > 2) coming, with $D_{0.5}$ larger than 10 µm. Powders from 5% TS culture were finer, with a narrow size distribution (*Span* between 1 and 1.5) peaking below 10 µm. However, it was difficult to find the bacteria cells on the surface of powders in the SEM observation, even for the powders obtained from the medium with 5% TS. This indicated that the 5%TS sweet whey was already sufficient for full microencapsulation of bacteria during spray drying.This coincided with the results for spray dried powders of *Lactobacillus plantarum* with trehalose as drying medium (Perdana et al., 2014).

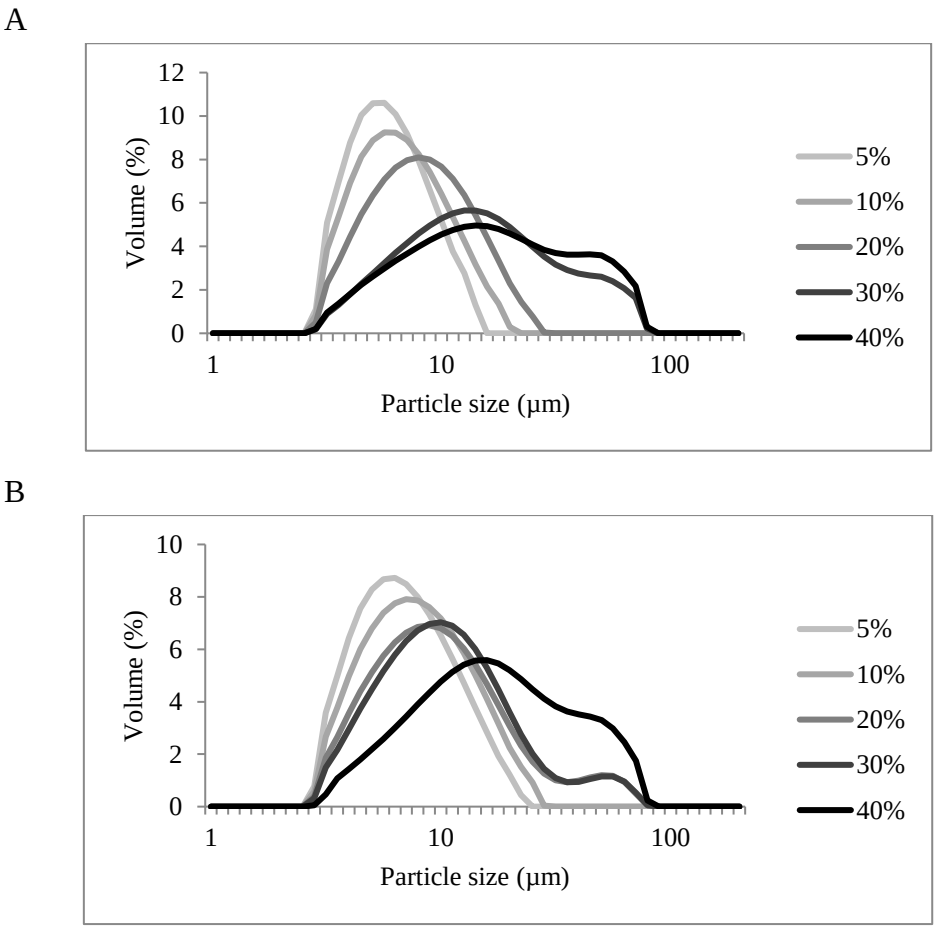


Figure 18. Particle size volume of spray dried powders of (A) *L. casei* and (B) *P. freudenreichii* from sweet whey culture media with different TS. Curves were obtained from the average values of duplicate experiments. The size range was 0.1 to 2000 µm; the volume values in the range 0.1 to 1 µm and 200 to 2000 µm were all 0.

Table 8. Size distribution of spray dried powders from culture media with different TS content.

	5%	10%	20%	30%	40%
			L. casei		
$D_{0.5}$ (µm)	6.1 ± 0.1	6.9 ± 0.1	8.5 ± 0.1	15.3 ± 0.5	17.1 ± 0.1
Span	1.1 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
			P. freudenreichii		
$D_{0.5}$ (µm)	7.2 ± 0.1	8.2 ± 0.1	10.0 ± 0.1	10.7 ± 0.1	17.6 ± 0.1
Span	1.4 ± 0.1	1.5 ± 0.1	2.2 ± 0.1	2.0 ± 0.1	2.6 ± 0.1

Size distribution values are significantly different from each other in lines ($p < 0{\cdot}05$).

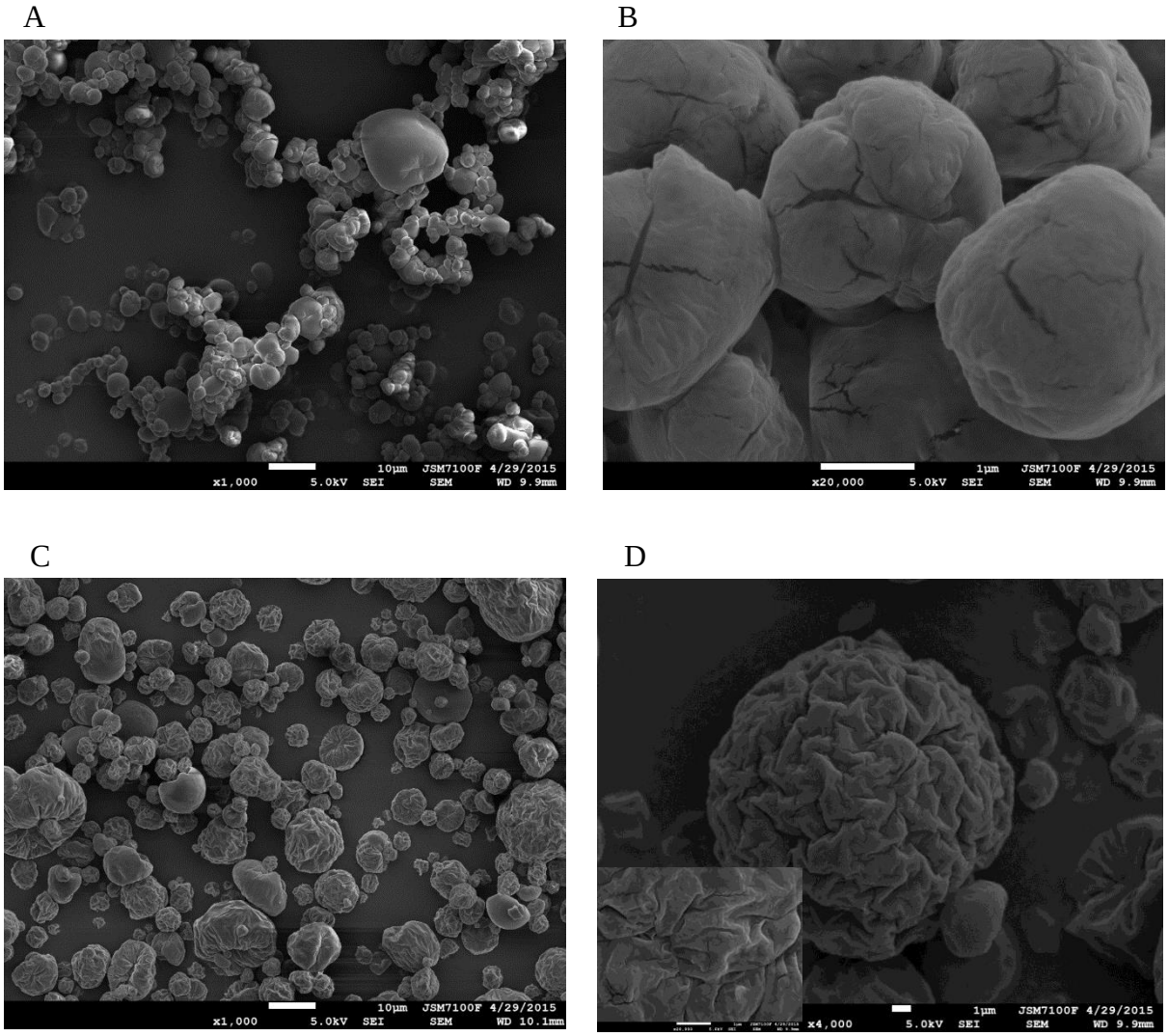


Figure 19. SEM observation of spray dried powders of *L. casei* obtained from (A,B) sweet whey culture media with 5% TS and (C,D) sweet whey culture media with 30% TS. D: the size of the object is 5× bigger than in B (Image with same magnification as B inserted in D).

In order to investigate the contribution of increased TS on the improved survival of probiotics after spray drying, a drying experiment was conducted by growing bacteria in 5% TS casein peptone-supplemented medium but increasing the TS to 30% immediately before drying (Figure 20). After increasing the TS, the survival of *L. casei* following spray drying was improved to around 6% compared to that of growing and drying at 5% TS. However, it was still significantly lower than that of growing and drying at 30% TS. In contrast, the survival of *P. freudenreichii* decreased slightly (from 45% to 26%) when TS was increased from 5% to 30% before drying, while growth in 30% TS significantly enhanced spray drying survival to 70%.

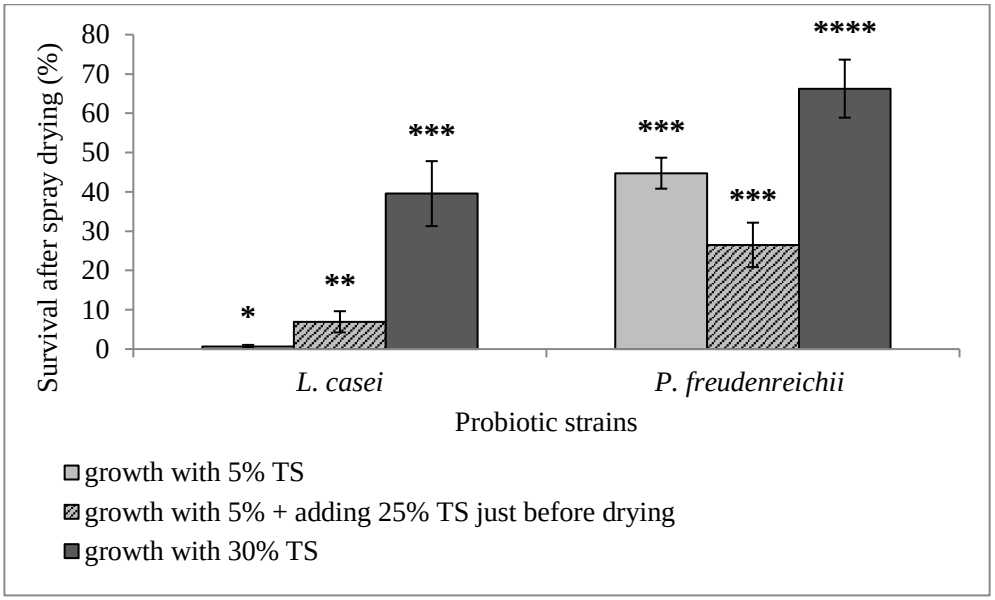


Figure 20. Survival of bacteria grown and spray dried in 5% medium, grown in 5% medium but 30% added before drying, grown and spray dried in 30% medium. Mean ± SEM, n = 3. Different number of * means significant difference between survival rates ($p < 0.05$).

These results indicate that the improvement in probiotic survival after spray drying was not a direct consequence of higher TS levels (thus lower water content) at the time of drying. The enhanced intrinsic bacterial tolerance triggered by high osmolality during growth may play a decisive role in the enhanced survival following spray drying. It has been reported that salt-adaptation of *Lactobacillus paracasei* with high osmolality before spray drying (by 0.3 M NaCl) can improve bacteria survival following heat treatment and spray drying (Desmond et al., 2001). In our study, the two bacteria strains were exposed to high osmolality during the growth time (2 days for *L. casei* and 5 days for *P. freudenreichii*). It is known that reaching cellular

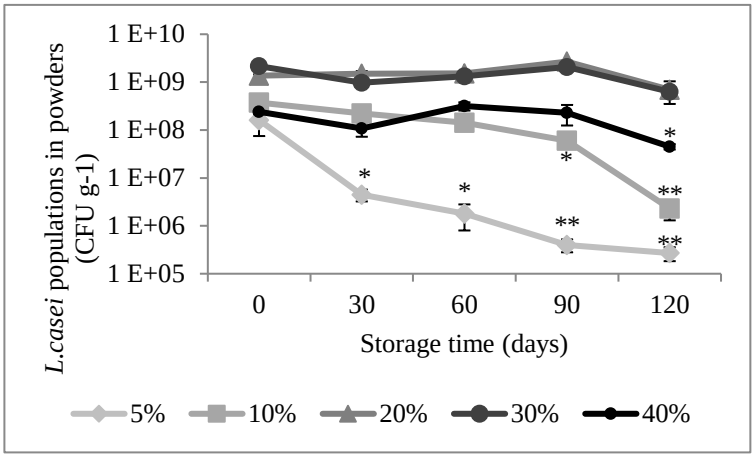
homeostasis by accumulation of compatible solutes is an energy-dependent bacterial osmoregulation process (Papadimitriou et al., 2016; Wood, 2011). In other words, an induction time is required for bacteria to adapt to the adverse environments and trigger the stress response. In contrast to bacteria growing in normal osmolality but exposed to osmolality before drying, growing bacteria in a medium with high osmolality may have enough time to accumulate the compatible intracellular solutes either by synthesis *de novo* or by uptake from the environment.

2.3.4 Improved storage stability of probiotic powders

The changes in bacteria population in powders were monitored during storage at 4ºC for 120 days (Figure 21). The viability of both *L. casei* and *P. freudenreichii* strains in the powders dried from the 20% ~ 40% TS culture remained constant, with a maximum log reduction of 0.89. After storage for 120 days, the *L. casei* population in the powders from 20% and 30% TS culture remained the highest at around 7×10^8 CFU g^{-1}, while the *P. freudenreichii* population was around 2×10^9 CFU g^{-1}. Since the initial bacteria populations in the powders from 40% TS culture were relatively low, the *L. casei* and *P. freudenreichii* populations were around 4×10^7 and 1×10^7 CFU g^{-1} after 120 days, respectively. In comparison, the viable *L. casei* population in the powder from 5% medium decreased significantly over the entire storage time, with a final population below 10^6 CFU g^{-1} at the end of storage (~ 2.8 log reduction at 120 days). Moreover, the *L. casei* in the powder from 10% medium remained unchanged during the first 90 days (viability loss < 1 log), but decreased considerably after 90 days (~ 2.2 log reduction at 120 days). The *P. freudenreichii* powders from the 5% and 10% culture media exhibited very steady viability during the first 60 days. Although there was subsequently reduction in viability after storage (~1.5 and 1 log reduction for 5% and 10%, respectively), the final viable bacteria population still remained above 10^8 CFU g^{-1} at the end of 120 days. The ability of *P. freudenreichii* to accumulate polyphosphate, trehalose and glycogen as energy and carbon storage compounds, as well as compatible solutes, might lead to higher survival rate throughout long-term storage (Boyaval et al., 1999; Cardoso et al., 2007; Leverrier et al., 2004).

It was obvious that the two probiotic strains in the powders from the lower TS culture (i.e. 5% to 10%) less survived well than those in the powders from higher TS culture (20% to 40%). Three reasons can be proposed. First, although the bacteria in powders from lower TS culture were also encapsulated in the drying matrix (Figure 19), the lower solid content led to a thinner encapsulating wall layer, which may not be effective enough to protect bacteria from oxidation stress in long term storage (Elversson et al., 2003; Perdana et al., 2014). Secondly, the bacteria in the powders from lower TS culture had lower survival levels after spray drying. This means that bacteria in these powders may be initially more damaged from the spray drying step, and might die upon storage if not able to recover from these damage (Ananta et al., 2005; Teixeira et al., 1995). Thirdly, the bacteria in culture media with higher TS were more tolerant due to the cellular response triggered by the high osmolality during growth (e.g., accumulation of compatible intracellular solutes) (Kets et al., 1996; Selmer-Olsen et al., 1999; Sunny-Roberts and Knorr, 2009).

A



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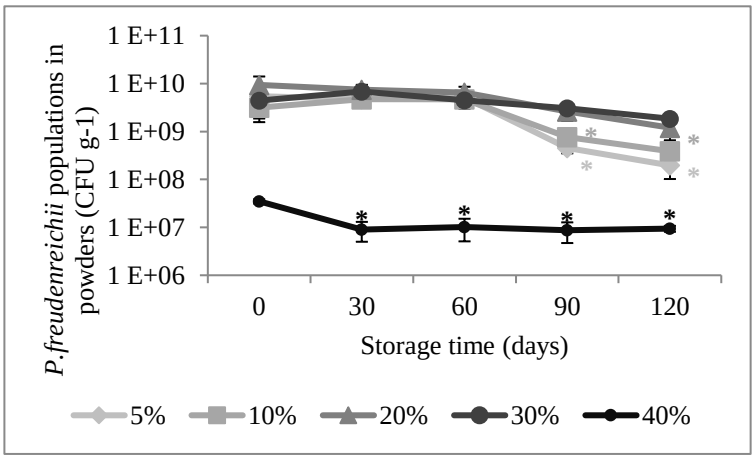

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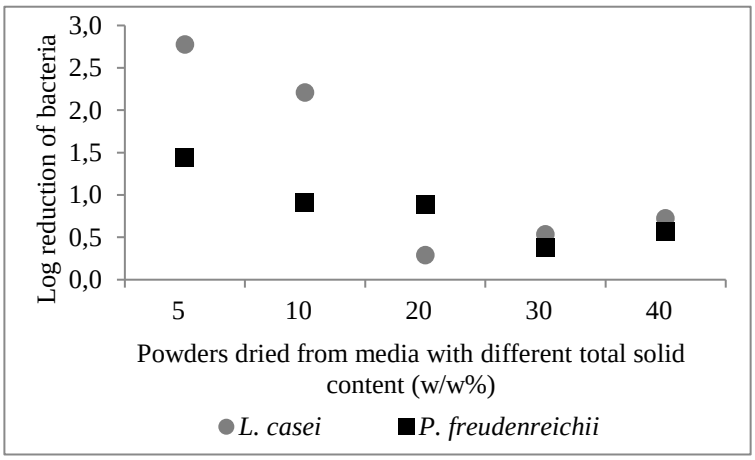

Figure 21. Change in bacteria populations in spray dried powders of (A) *L.casei* and (B) *P.freudenreichii* from sweet whey culture media with different TS during storage at 4°C for 120 days. Mean ± SEM, n = 3. (C) Final log reduction of two probiotic strains when stored for 120 days. Different number of * means significant difference between survival rates ($p<0.05$)

2.3.5 Additional results

The bacterial population of other *Lactobacillus* species grown in 5% and 30% TS sweet whey media were also tested (Table 9). Among the four species investigated, *L. acidophilus*, *L. plantarum and L. rhamnosus* showed a higher bacterial population in 30% TS sweet whey culture compared to the control 5% TS sweet whey culture. *L. reuteri* population in the 30% TS culture was slightly lower (~0.25 log) than that in the 5% TS culture, but still with a final bacterial population above 10^8 CFU mL^{-1}. This result suggests that the 30% TS sweet whey may be used for *Lactobacillus* species in a more general way.

Table 9. The bacterial population of different lactobacillus strains grown in 5% or 30% TS sweet whey media for 48 hours.

	L. acidophilus CIRM-BIA 1674	*L. plantarum* CIRM-BIA 466	*L. reuteri* CIRM-BIA 522	*L. rhamnosus* CIRM-BIA 607
5% sweet whey (log CFU mL^{-1})	7.96±0.19	8.61±0.19	8.32±0.03	9.24±0.01
30% sweet whey (log CFU mL^{-1})	8.38±0.10	9.00±0.21	8.07±0.10	9.31±0.10

Further, *L. acidophilus* CIRM-BIA 1674 cultures in 5%, 25%, 30% and 35% TS sweet whey were submitted to spray drying (Figure 22). The 30% TS *L. acidophilus* culture survived best (~15%) among all the four groups test in this study, with a survival approximately 7-fold higher than that of the 5% TS culture. It suggests the potential genericity of the 2-in-1 process to *Lactobacillus* species

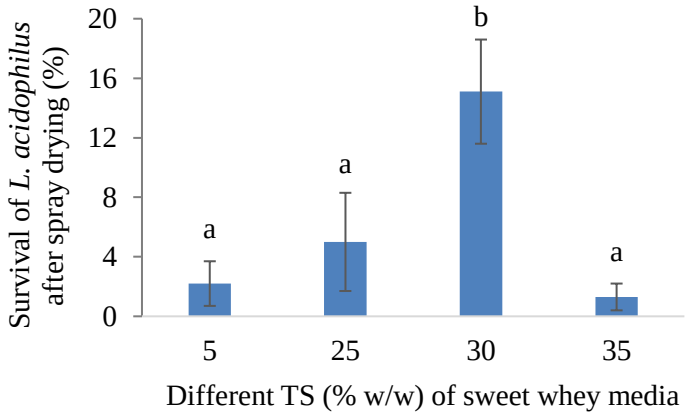

Figure 22. Survival of *L. acidophilus* CIRM-BIA 1674 grown in different TS sweet whey after spray drying at inlet temperature 185°C and outlet temperature 75°C. Different letters above the column mean significant difference between bacterial survivals ($p < 0.05$).

2.4 Conclusion

In this chapter, to the best of our knowledge, we proposed for the first time the feasibility of simplifying the process of spray drying bacteria by utilizing a double-used medium with increased total solid content.

From our results, it was clearly showed that culturing *L. casei* and *P. freudenreichii* in sweet whey with 20% ~ 30% TS resulted in greater biomass production and sustained viability after spray drying and following a long-term storage. Besides, it is interesting to note that the growth of *L. casei* was less dependent on casein peptone supplementation in the sweet whey with high TS. More remarkably, sweet whey medium allowed *L. casei* to survive in the liquid culture for long term at 37°C.

The results add to the value of sweet whey and may indicate a new route to producing probiotic powders with higher process efficiency and final probiotic viability. However, the protective mechanisms of this process on probiotics during spray drying and storage warrant a further investigation at a molecular level. Moreover, the feasibility of scaling-up this process should also be explored on an industrial scale.

Chapter 3. What are the Underlying mechanisms of the enhanced Protection on Probiotics?

In chapter 2, we showed a 2-in-1 process for growth and drying of probiotics using highly concentrated sweet whey. This process affords an exceptional protection on probiotics during spray drying and storage. Therefore, we tried here to reveal the probiotic protection mechanisms enhanced by the 2-in-1 process.

These latter were proposed and studied on three aspects: Bacterial stress response on the one side, and specific composition and structure of drying matrix on the other side. The results suggest that the protection on probiotics may result from the synergistic effects of these three aspects during the process.

The main contents in this Chapter have been published as:

Huang, S., Rabah, H., Jardin, J., Briard-Bion, V., Parayre, S., Maillard, M.-B., Loir, Y.L., Chen, X.D., Schuck, P., Jeantet, R., Jan, G., 2016b. Hyperconcentrated sweet whey, a new culture medium that enhances *Propionibacterium freudenreichii* stress tolerance. Appl. Environ. Microbiol. 82, 4641–4651.

Wang, J., Huang, S., Fu, N., Jeantet, R., Chen, X.D., 2016. Thermal aggregation of calcium-fortified skim milk enhances probiotic protection during convective droplet drying. J. Agric. Food Chem. 64, 6003–6010.

Yang, Y., Huang, S., Wang, J., Jan, G., Jeantet, R., Chen, X.D., 2017. Mg^{2+} improves the thermotolerance of probiotic *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8. Lett. Appl. Microbiol. (in press)

3.1 Introduction

What could make bacteria stronger?

As reviewed in Chapter 1, the ability of bacteria to adapt to sub-lethal stresses may later induce their tolerance towards the same type of stress (stress adaptation) or different types of stresses (multistress response, also termed cross-protection). Extensive studies have shown that such cross-protection could be induced in lactic acid bacteria and propionibacteria (Boyaval et al., 1999; Guchte et al., 2002). Therefore, the cross-protection can be used as an effective strategy to guarantee probiotic viability and activity. For examples, heat adaptation of bacteria has been mostly employed to improve probiotic thermotolerance and thereby help probiotics to better survive during spray drying (Desmond et al., 2001).

Apart from heat stress, osmotic stress (also described as dehydration stress elsewhere) is another stressing factor for bacteria during drying. In our innovative process introduced in Chapter 2, bacteria face osmotic stress during growth in the highly concentrated sweet whey culture (see the osmolality of 30% sweet whey, Figure 13, 2.2.2). However, the large final population (Figure 14, 2.3.1) suggests that this osmotic stress is probably at a sub-lethal level for the two bacterial strains used in this work. In other words, bacteria adapt to the osmotic stress during growth in 30% sweet whey, instead of being inhibited by this latter. This osmotic adaptation *per se* has been reported to protect bacteria during drying (Prasad et al., 2003). It could also induce multistress tolerance, i.e. cross-protection, against different stresses existing in spray drying process (e.g. heat and oxidative stresses) (Wood, 2011). The investigations aiming at confirmation of these hypotheses were carried out with proteomic analysis, enzymatic and microscopic techniques in this Chapter.

Why did we study aggregation effect with Ca^{2+}-induced aggregated milk as the model?

In the 2-in-1 process, the highly concentrated sweet whey was used as bacterial growth and drying medium. The sterilization step is thus necessary in order to guarantee the quality of bacterial culture and final powders. In comparison to the natural whey, the concentrated whey induces mineral-protein aggregation during autoclave or pasteurization due to the increasing concentration of mineral salts and whey proteins (Havea et al., 2002; Oldfield et al., 2005). Moreover, during fermentation of lactobacilli, the pH of whey medium will decrease to a value close to 4.5, which will lead to even more protein aggregation within the medium prior to

drying step (Alting et al., 2000; Britten and Giroux, 2001). Therefore, from both practical and scientific perspectives, it is of importance to explore the potential influence of these aggregates on probiotic protection by the medium during drying process.

However, in order to study the independent effect of the aggregates, the influence of dry matter content should be excluded. In other words, it is necessary to design a comparison between the media with and without aggregates, but with same level of TS content. To this purpose, induction of aggregation in skim milk by very low concentration of calcium salts could be a solution due to their well-known interaction with casein micelles. Indeed, among all the mineral salts in milk, calcium (especially free Ca^{2+}) is known to be the most efficient one in terms of inducing thermal aggregation of milk proteins (Gaucheron, 2005; Varunsatian et al., 1983). Besides, calcium-fortified milk is of value from both nutrition and marketing perspectives. Even more remarkably, the combination of calcium supplementation with probiotics has been reported to improve both the absorption of calcium and the efficacy of probiotics (Scholz-Ahrens et al., 2007; Trautvetter et al., 2012). On the other hand, although the characteristics of thermal aggregation of milk proteins induced by calcium supplementation has been extensively studied, the feasibility of applying calcium-fortified milk to microencapsulate probiotics by drying has rarely been reported.

Therefore, we employed the single droplet drying technique to map the drying behavior of Ca^{2+}-induced aggregated skim milk. This latter was used to protect commercial probiotic strain *Lactobacillus rhamnosus* GG during droplet-particle conversion.

Why did we focus on Mg^{2+}?

Food-grade carbohydrates and proteins are extensively employed as protective agents to improve bacterial viability upon heat treatment and spray drying (Table 3). However, the effect of inorganic salts on thermotolerance of probiotics has rarely been reported. Previously, Ca^{2+} was found to influence the thermotolerance of lactic acid bacteria (Huang and Chen 2013). Apart from Ca^{2+}, Mg^{2+} is generally present in most of dairy products including the sweet whey ingredients used in this project (Table 7).

In bacteria, Mg^{2+} is the second-most abundant cation (Romani and Scarpa 2000). The roles of bacterial Mg^{2+} in homeostasis, sensing and transport were extensively investigated (mostly in gram-negative bacteria *Salmonella enterica* serovar *typhimurium* and *Escherichia coli*), including acting as a cofactor in ATP-dependent phosphorylation and a variety of other

enzymatic reactions, stabilizing ribosome and membranes, influencing RNA folding, the nucleic acid-proteins interactions and bacterial virulence (Groisman et al. 2013). O'Connor et al. (2009) first reported the function of Mg^{2+} in the regulation of bacterial thermotolerance. Increased expression of Mg^{2+} transport proteins was found to enhance survival of *Salmonella enterica* upon heat treatment. However, the role of Mg^{2+} in the regulation of thermotolerance in other organisms still remains unclear.

Therefore, we preliminarily investigated in this work the influence of Mg^{2+} on thermotolerance of three well-documented probiotic lactobacilli strains, *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8.

3.2 Materials and Methods

3.2.1 Strains, culture conditions and bacteria-milk matrix preparation

P. freudenreichii CIRM-BIA 129 was cultivated in YEL broth or sweet whey media with different TS described in Item 2.2.1 and 2.2.2. *Lactobacillus* strains were routinely activated and cultivated statically at 37°C for 24 h in MRS broth prior to further experiment. Among these strains, *Lactobacillus rhamnosus* GG (LGG) was obtained from a commercial product (Culturelle, CVS Pharmacy). *Lactobacillus casei* Zhang (LCZ) and *Lactobacillus plantarum* P-8 (LP) were provided by the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, China.

The effect of milk aggregates on protective capacity of milk matrix was studied with LGG strain. The Ca^{2+}-aggregated milk was prepared as described previously (Huang et al., 2014). Briefly, UHT skim milk (Devondale, Australia) was added by 10 mmol l^{-1} $CaCl_2$ (Sinopharm, Shanghai, China) and then heated at 90°C for 30 min to cause the protein aggregation. Apart from the untreated UHT skim milk, the 10 mmol l^{-1} $CaCl_2$-added milk (without heating) was also used in this work as a control to exclude the effects of both $CaCl_2$ addition and higher dry matter (Ca-added milk, in brief). All of the milk matrices were fully vortexed and then placed at 25°C for 30 min by a thermal mixer (Eppendorf, Germany) before being mixed with LGG cells. LGG cells were harvested in the 2 mL Eppendorf tube from the 24 h culture by centrifuging at 8000×g for 15 min (Heal Force, Hong Kong, China). The cell pellets were washed by 0.5% (w/v) peptone water in the same centrifuging conditions and then suspended in a 1:1 volume of the above milk matrix. All of the bacteria-suspended matrices were fully vortexed and placed at 4°C before single droplet drying.

3.2.2 Stress challenges

(Multistress challenges to P. freudenreichii)

Acid, bile salts, and heat challenges were applied to stationary-phase cultures of *P. freudenreichii* according to standardized procedures at pH 2 and 37°C for 60 min in YEL broth adjusted to pH 2.0 using HCl (Jan et al., 2001, 2000) or in the presence of 1 g L^{-1} of bile salts (an equimolar mixture of cholate and deoxycholate; Sigma Chemical, St. Louis, MO, USA) in YEL at 37°C for 60 min (Leverrier et al., 2003) or at 60°C for 10 min (Leverrier et al., 2004) prior to CFU counting on YEL agar to determine survival percentages. As a control, cultures

were left untreated for the same time in order to determine the population corresponding to 100% survival. The viable and cultivable population of bacteria was determined by CFU counting on plates for maximal recovery of both treated and untreated cultures. Percent survival was then determined by comparing stressed and unstressed cultures at the end of the same time period.

(Heat challenge to Lactobacillus)

The effect of Mg^{2+} on thermotolerance of *Lactobacillus* was investigated with LCZ, LGG and LP strains. Preparation of bacterial suspension and heat challenge experiments were performed as described previously (Huang and Chen, 2013). Lactose solution (10% w/v) was used as the medium due to its excellent drying behavior and potential protection on bacteria during spray drying. Briefly, 500 µL of bacterial culture after 24 h incubation was washed with peptone water and then re-suspended in 500 µL 10% w/v lactose solution with 0, 5, 10, 20, 50, 100 and 500 mmol l^{-1} of $MgCl_2$, respectively. Besides, the same concentrations of NaCl and $ZnCl_2$ were also used as a control to compare with the effects of Mg^{2+}. The suspensions were moderately shaken at 25ºC for 30 minutes prior to a heat treatment at 70ºC for 2 mins. The viable and cultivable population of *Lactobacillus* was determined by CFU counting, on plates of MRS medium, solidified by 10 g l^{-1} agar, for both heated and unheated cultures. The survival was thus expressed as log N/N_0, where N is the population following the heat treatment and N_0 the population before the heat challenge.

(Recovery of Lactobacillus from heat injury)

Similarly as in the heat challenge experiment, 1 mL of bacterial suspension was prepared by re-suspending bacterial pellets in 1 mL 10% w/v lactose solution. Heat treatment was performed at 75ºC for 1 min. After the heat treatment, the heated suspension was inoculated (1% inoculum) into the MRS broth supplemented with different concentrations of inorganic salts in the 48-wells plate. The bacterial regrowth was monitored by measurement of the optical density change (OD_{600}) at 37ºC for 30 h using a microplate reader (SpectraMax M5, Molecular Devices). The modified Gompertz model was used to fit the OD_{600} curves (Zwietering et al. 1990). The growth lag phase (λ) and asymptotic value (A, indicates the final biomass yield) were calculated to characterize the regrowth activity of bacteria (an example of fitting is shown in Figure 23 and Table 10). The R-square values between all experimental curves and fitting equations were calculated by Origin Pro 8 SR02 (OriginLab, USA). The goodness of fitting of all curves were found to be higher than 0.995. To compare the effects of salts on the regrowth

activity of bacteria, the difference between heat-induced changes of lag phase and asymptotic value were compared by $\lambda-\lambda_0$ and A-A_0. λ_0 and A_0 refer to the lag phase and asymptotic value of bacteria regrowth in MRS without inorganic salt supplementation after heat treatment, while λ and A refer to that of in MRS with inorganic salt supplementation after heat treatment, respectively.

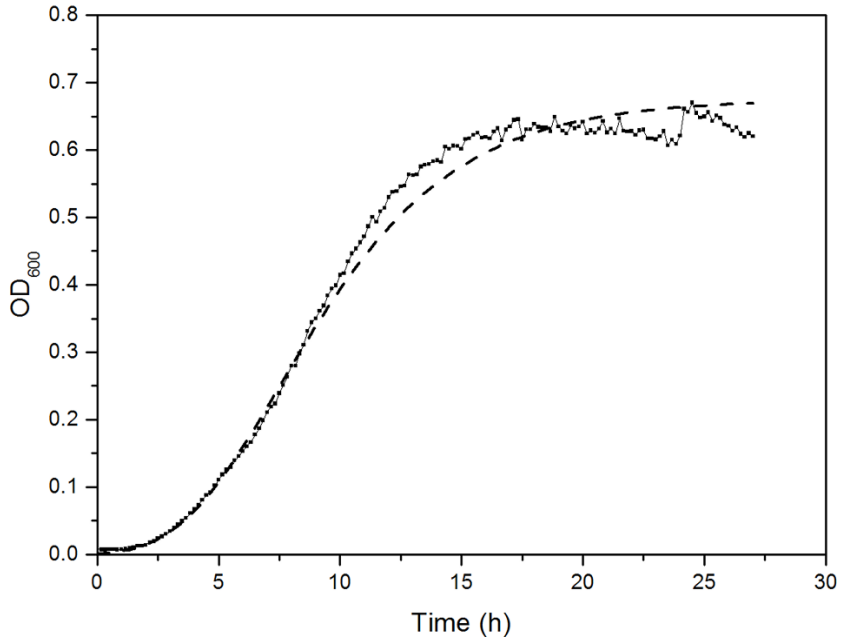

Figure 23. An example to describe the growth curve of LGG at 37°C fitted with the Gompertz model. The solid curve is experimental curve, while the dotted one represents the fitted curve.

Table 10. The values of λ and A from the curve fitted by Gompertz model in Figure 23

L. rhamnosus GG	Equation	Y=a×exp(-exp(-k×(x-x×c)))
Fitted value	a	0.67503
	x×c	7.48637
	k	0.24523
Formula	λ=(k×xvc-1)/k	λ=3.963933
	A=a	A=0.64727

(Drying challenge of L. rhamnosus GG via single-droplet-drying technique)

Single droplet drying was carried out with the SDPA MARK II equipment (Dong-Concept New Material Technology Co. Ltd.). The working principles of this equipment have been described in detail elsewhere (Fu et al., 2011; Lin and Chen, 2002). In brief, the system consists of an air supply unit used to provide laminar air flow with controllable temperature, humidity, and velocity and a droplet-drying unit in which an isolated droplet can be suspended at the tip of a specially made glass filament for drying in the conditioned air stream. A total of three

droplet-suspension modules were equipped in the droplet-drying unit for measuring the changes of droplet temperature, mass, and diameter, respectively. A high-resolution video camera was coupled with the droplet-drying unit to monitor and record the change of droplet morphology during drying. Droplet moisture content on a dry basis (X_d, kg kg^{-1}) was calculated with the following equation:

$$X_d = (M_d - M_s)/M_s \quad \text{(Eq 12)}$$

where M_d is the mass of droplet, and M_s is the total solids mass of the matrix (mg).

The single droplet was generated from the above bacteria suspended milk matrixes with a gas chromatograph microsyringe (SGE Analytical Science Pty Ltd.). The control consisted of the drying of sterilized Milli-Q water droplets. For each drying run, the syringe was rinsed by Milli-Q water, 75% (v/v) ethanol, and sterilized Milli-Q water subsequently. The remaining LGG viability within droplets was measured as described before (Fu et al., 2013a; Zheng et al., 2015). Briefly, the drying process was stopped at a defined time by blocking the air flow with a cold, humid barrier. The droplet was then immediately rehydrated in 1 mL of 0.5% peptone water and subsequently serially diluted for the measurement of the viable bacteria population by pouring it on an MRS agar plate. The agar plates were incubated at 37°C for 48 h. The initial bacteria population in three milk matrixes was also measured before drying (~10^9 CFU mL^{-1} for all three matrixes). The drying conditions used in this work were set as follows: air temperature, 65 ± 0.2°C; air velocity, 0.91 ± 0.02 m s^{-1}; air moisture content, 1 ± 0.01 g kg^{-1}; and droplet volume, 2 ± 0.05 μL.

3.2.3 Physico- physicochemical properties of bacteria-milk matrix and particle

The size distribution of dispersed elements in milk matrixes were measured by laser-light diffraction (Horiba LA-960). The size distribution of LGG cells suspending in the peptone water (0.5% w/v) was also measured after washing twice by the peptone water.

The viscosity of milk matrixes was measured using an oscillatory rheometer (Kinexus Pro+, Malvern, UK). The samples were first equilibrated at 25°C and then quickly pipetted onto the bottom plate of the rheometer. A 40 mm cone (4°) geometry was used with the sheer rate at 50 s^{-}1, and the operation temperature was controlled at 25 ± 0.5°C.

The content of milk whey proteins in the serum phase of different milk matrixes was compared by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Milk

matrixes were first centrifuged at ×8000g for 15 min. The supernatant was collected and then mixed with the LDS sample buffer (NuPGE, ThemoFisher Scientific) at the volume ratio of 1:1 prior to heat denaturation at 75°C for 10 min. One-dimensional polyacrylamide gel electrophoresis was conducted on the XCell SureLock Mini-Cell system (ThermoFisher Scientific) with 12% Bis−Tris Precast gel (NuPAGE, ThermoFisher Scientific). The gel was stained by Bio-Safe Coomassie blue reagent (Biorad) prior to photograph with the imaging system (ChemiDoc XRS+, Biorad).

To characterize the mobility of water in bacteria-suspended milk matrixes, the transverse relaxation time (T_2) was measured using a 23 MHz ^{1}H nuclear magnetic resonance (NMR) spectrometer (Suzhou Niumai Electronic Technology Co., Ltd.) at 32 ± 0.1°C. The equilibrated samples were placed in the 15 mm diameter tube. The tube was sealed with parafilm to prevent the water loss during measurement. The T_2 distributions were measured using the Carr−Purcell −Meiboom−Gill (CPMG) pulse sequence, with the 90−180° pulse spacing varied between 25 and 50 μs. The echo number was set to 4000, the echo time was 0.5 s, the waiting time was 2 s, and 16 scans were acquired for each measurement. The data were collected and analyzed by the instrument MultiExp Invfit-T Analysis software.

The particles after drying at 65°C for 420 s (by single-droplet drying) were collected and cut by a surgical knife blade. The particle pieces were placed on the carbon tape and then sputter-coated by gold − palladium. These samples were observed by scanning electron microscopy (Hitachi Ltd.).

3.2.4 P. freudenreichii proteomic analysis and indentification

In order to understand the bacterial stress response in highly concentrated sweet whey, a proteomic analysis was performed with *P. freudenreichii*. Two-dimensional electrophoresis was carried out as described previously (Leverrier et al., 2004). Briefly, propionibacteria were harvested by centrifugation, together with medium-insoluble materials; washed in 0.1 M Tris buffer, pH 8, containing 2% sodium citrate; centrifuged; washed twice in 0.1 M Tris buffer, pH 8, containing 0.001 M EDTA and then in phosphate-buffered saline (PBS); pelleted; frozen; and then resuspended in SDS lysis buffer prior to sonication and cell lysis using a Retsch MM301 mixer mill. The resulting SDS extracts were recovered by centrifugation (21,000 × *g*; 20°C; 20 min) and analyzed by one-dimensional SDS-PAGE (27) prior to protein precipitation using a two-dimensional (2D) cleanup kit (GE Healthcare Bio-Sciences AB, Uppsala,

Sweden). Protein pellets, as well as immobilized pH gradient (IPG) dry strips, were rehydrated in destreaking solution (GE Healthcare Bio-Sciences AB) supplemented with IPG buffer, pH 4 or 7. For each two-dimensional gel, 300 µg of cellular proteins, whatever the treatment, were loaded onto the acid end of a linear-gradient IPG dry strip (pH 4 to 7) prior to isoelectric focusing on a MultiphorII up to 60,000 V·h according to a standardized procedure (Jan et al., 2001). The second dimension was SDS-PAGE (12.5% polyacrylamide; 16 by 16 by 0.1 cm; Protean II; Bio-Rad, Hercules, CA). The gels were stained using Bio-Safe Coomassie G-250 stain (Bio-Rad) prior to scanning on an ImageScanner III (GE Healthcare Bio-Sciences AB). Image analysis, gel matching, and quantification of the protein amounts in individual spots, calculated as average normalized volumes, were performed using Progenesis SameSpot software 3.1 (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). Molecular weights and isoelectric points were calculated with the software using molecular markers (LMW; GE Healthcare Bio-Sciences AB) and assuming a linear 4-to-7 pH gradient, as indicated by the supplier. Gel subgroups corresponding to growth in 5% SW and in 30% SW were compared to compute fold and *P* values of all spots using one-way analysis of variance (ANOVA) and principal-component analysis (PCA), as described previously (Morris et al., 2010). The spots of interest, significantly upregulated in 30% SW compared to 5% SW (above a 1.2-fold change), were selected and spotted for further identification, as previously described (Gagnaire et al., 2015). The fold changes are indicated.

Gel pieces were washed with acetonitrile and ammonium bicarbonate solution and then dried under vacuum in a Speed-Vac concentrator (SVC100H-200; Savant; Thermo Fisher Scientific, Waltham, MA, USA). In-gel trypsin digestion was performed overnight at 37°C and stopped with spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma-Aldrich, Saint Quentin Fallavier, France) as described previously (Gagnaire et al., 2015). The supernatants containing peptides were then vacuum dried in a Speed-Vac concentrator and stored at -20°C until mass spectrometry (MS) analysis. Nanoscale liquid chromatography coupled online with tandem mass spectrometry (NanoLC–MS-MS) analysis was performed with an on-line liquid chromatography (MS-MS) setup using a Dionex U3000-RSLC NanoLC system fitted to a QStar XL (MDS SCIEX, Ontario, Canada) equipped with a nano-electrospray ion (ESI) source (Proxeon Biosystems A/S, Odense, Denmark). Samples were first concentrated on a PepMap 100 reverse-phase column (C18; 5-µm particle size; 300-µm inside diameter [i.d.] by 5-mm length) (Dionex, Amsterdam, The Netherlands). Peptides were separated on a reverse-phase PepMap 100 column (C18; 3-µm particle size; 75-µm i.d. by 150-mm length; Dionex) at 35°C,

using solvent A (2% [vol/vol] acetonitrile, 0.08% [vol/vol] formic acid, and 0.01% [vol/vol] TFA in deionized water) and solvent B (95% [vol/vol] acetonitrile, 0.08% [vol/vol] formic acid, and 0.01% [vol/vol] TFA in deionized water). A linear gradient from 10 to 40% solvent B in 17 min was applied for elution at a flow rate of 0.3 µl min^{-1}. The eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode. A full continuous MS scan was carried out followed by three data-dependent MS-MS scans. Spectra were collected in the selected mass range of 300 to 2,000 m/z for MS and 60 to 2,000 m/z for MS-MS spectra. Charged ions (2+ to 4+) were considered for the MS-MS analysis when the ion intensity was above 10 cps. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS-MS acquisition using Analyst QS 1.1 software. The instrument was calibrated by multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein (β-CN) (193 to 209). To identify peptides, all data (MS and MS-MS) were submitted to X! Tandem using the X! Tandem pipeline developed by Plateforme d'Analyze Protéomique de Paris Sud-Ouest (PAPPSO), INRA, Jouy-en-Josas, France (http://pappso.inra.fr). The search was performed against a database composed of (i) a homemade database containing all the predicted proteins of *P. freudenreichii* strain CIRM-BIA 129 used in this study and (ii) a portion of the UniProtKB database corresponding to *P. freudenreichii*. Database search parameters were specified as follows: trypsin cleavage was used, and the peptide mass tolerance was set to 0.2 Da for both MS and MS-MS. Oxidation of methionine and deamidation of asparagine and glutamine residues were included as variable modifications. Protein identifications were automatically validated when they showed at least two unique peptides with an E value below 0.05.

3.2.5 P. freudenreichii cellular compatible solutes

Polyphosphate granule staining was done as recently described for lactobacilli (Alcántara et al., 2014) for the detection of polyphosphate granules using the procedure of Neisser. Bacterial cultures were smeared on microscope slides and heat fixed. The slides were covered for 1 min with a freshly prepared mixture of 1 volume of Neisser's methylene blue solution (Fluka Analytical, France) and 2 volumes of 0.33% crystal violet solution in 10% ethanol. Excess dye was absorbed using blotting paper, and the slides were covered for 1 min with a 0.3% solution of chrysoidin G solution (Sigma-Aldrich, Saint Quentin Fallavier, France) prior to extensive rinsing with water. The slides were observed on an Olympus BX51 microscope at a ×1,000 magnification. In addition, polyphosphate granules were visualized by DAPI (4',6'-

diamidino-2-phenylindole) staining as previously described (Günther et al., 2009) and later modified (Mukherjee et al., 2015) for cyanobacteria. Briefly, bacteria were washed in McIlvaine's buffer (Mukherjee et al., 2015), fixed in 4% formaldehyde, permeabilized in 0.3% Triton X-100, and stained in 20 µg mL^{-1} DAPI in the same buffer. The stained cultures were observed on an Olympus BX51 epifluorescence microscope equipped with a U-MWU2 fluorescence filter cube (excitation filter, 330 to 385 nm; emission filter, 480 to 800 nm) and an Olympus plan 100×/1.25 oil objective. For maximal resolution, bacteria were further imaged using a confocal Leica SP8 and a 63×/1.4 oil HC PL APO CS objective. Images were acquired using LAS-AF (Leica, Wetzlar, Germany) software. DAPI was excited with a 405-nm laser. Photomultiplier detector parameters were set to collect fluorescence between 412 and 450 nm and between 553 and 598 nm. Polyphosphate granules were further visualized by transmission electron microscopy as described previously for lactobacilli (Alcántara et al., 2014). Briefly, propionibacteria, grown in different media until stationary phase, were washed in PBS and in sodium cacodylate buffer (0.1 M, pH 7.3) prior to fixation with 2.5% glutaraldehyde for 3 h. The bacterial pellet was embedded in agarose prior to being cut into 1-mm pieces and fixed with 1% sodium tetroxide for 1 h. The agar pieces were rinsed with cacodylate buffer and dehydrated in ethanol prior to inclusion in Epon-Araldite-DMP30 resin (polymerized at 60°C for 48 h). Thin sections (90 nm) were cut (Leica ultra microtome Ultracut E), collected on copper grids, and then stained with uranyl acetate. Samples were observed with a Jeol 1400 electron microscope (Jeol Co. Ltd., Tokyo, Japan), and images were digitally captured with a Gatan Orius camera (Digital Micrograph Software). Cellular glycan detection was done by using the periodic acid-Schiff (PAS) staining method, as previously described (Moats, 1959). The fixed bacterial smears on slides were stained with 1% periodic acid solution for 5 min and then transferred into absolute ethanol. The slides were rinsed with water, air dried, and subsequently stained with Schiff's reagent (Sigma-Aldrich, France), consisting of 1% pararosaniline HCl and 4% sodium metabisulfite in 0.25 mol L^{-1} hydrochloric acid. The slides were washed with flowing water for 5 min and then air dried before observation. For scanning electron microscopy (SEM) examination of the powder grains, powders were attached to SEM stubs using a two-sided carbon adhesive tape and then sputter coated with gold-palladium. Samples were analyzed at 5 kV at room temperature with a scanning electron microscope (JSM 7100F; Jeol Co. Ltd., Tokyo, Japan).

Trehalose and glycogen were assayed as previously described for *Saccharomyces cerevisiae* (Parrou and François, 1997) and adapted for propionibacteria (Dalmasso et al.,

2012). Briefly, propionibacteria were washed twice in PBS, resuspended in acetate buffer (40 mmol l^{-1}, pH 5.2), heat inactivated for 5 min at 95°C, and disrupted using zirconium beads in a Retsch MM301 mixer mill prior to centrifugation of cellular debris. The resulting extract was divided into two parts, further digested using amyloglucosidase (from *Aspergillus niger* [no. 10115; Sigma-Aldrich]) or trehalase (from porcine kidney [no. T8778; Sigma-Aldrich]) for the hydrolysis of glycogen and trehalose at 57°C and 37°C, respectively. Samples were quickly frozen, and the resulting glucose was quantified using a glucose hexokinase assay kit (Sigma). The results were expressed as the concentration of generated glucose per unit weight of cellular wet matter.

3.2.6 Statistical Analysis

All experiments were independently repeated three times or performed with samples in triplicate (biological replicates). The results are presented as means ± standard deviations. Significant differences ($P < 0.05$) between the mean values were determined by Tukey's test. Statistical analysis was performed using R 3.2.1 with the Rcmdr package (R Development Core Team). The features of bacterial and particle morphology, particle size distribution and SDS-PAGE were repeatedly observed in triplicate samples.

3.3 Results and Discussion

3.3.1 Osmotic adaptation induces multistress tolerance of P. freudenreichii

3.3.1.1 Multitolerance response is triggered during growth

As shown in Chapter 2 (Figure 14B in Item 2.3.1), the use of highly concentrated sweet whey sustained *P. freudenreichii* growth. The final populations of propionibacteria were around 10^9 CFU mL^{-1}, whatever the growth medium, as determined by CFU counting. Hyperconcentrated sweet whey, 20 and 30% TS, allowed growth yields above that of the normal YEL reference medium, with a larger final population of *P. freudenreichii*, in accordance with the larger amount of substrates present in the medium. In contrast, growth was inhibited at 40% dry matter, suggesting inhibition at a concentration that was too elevated. As a matter of fact, osmolality increased with the concentration (Figure 13 in Item 2.2.2) to levels far above that of YEL medium; as hyperosmotic stress is a major stress for bacterial elongation and growth (Empadinhas and da Costa, 2008). *P. freudenreichii* was thus able to adapt to osmolality up to about 1,500 but not above 2,500 mosmol kg^{-1}.

We then looked at the effect of such stress, undergone during growth, on tolerance for other stresses, imposed after growth. Growth in 20 and 30% TS sweet whey led to significant increases of stress tolerance in *P. freudenreichii* (Figure 24). The most striking example was bile salts challenge survival, with 60.9% survival in 30% TS sweet whey compared to 2.6% in 5% TS sweet whey. This result strongly suggests that the growth conditions imposed sublethal doses of stress responsible for stress adaptation; hyperosmotic stress being the main stimulus in hyperconcentrated sweet whey and known to trigger bile salts tolerance in other bacteria (Flahaut et al., 1996; Zhang et al., 2011).

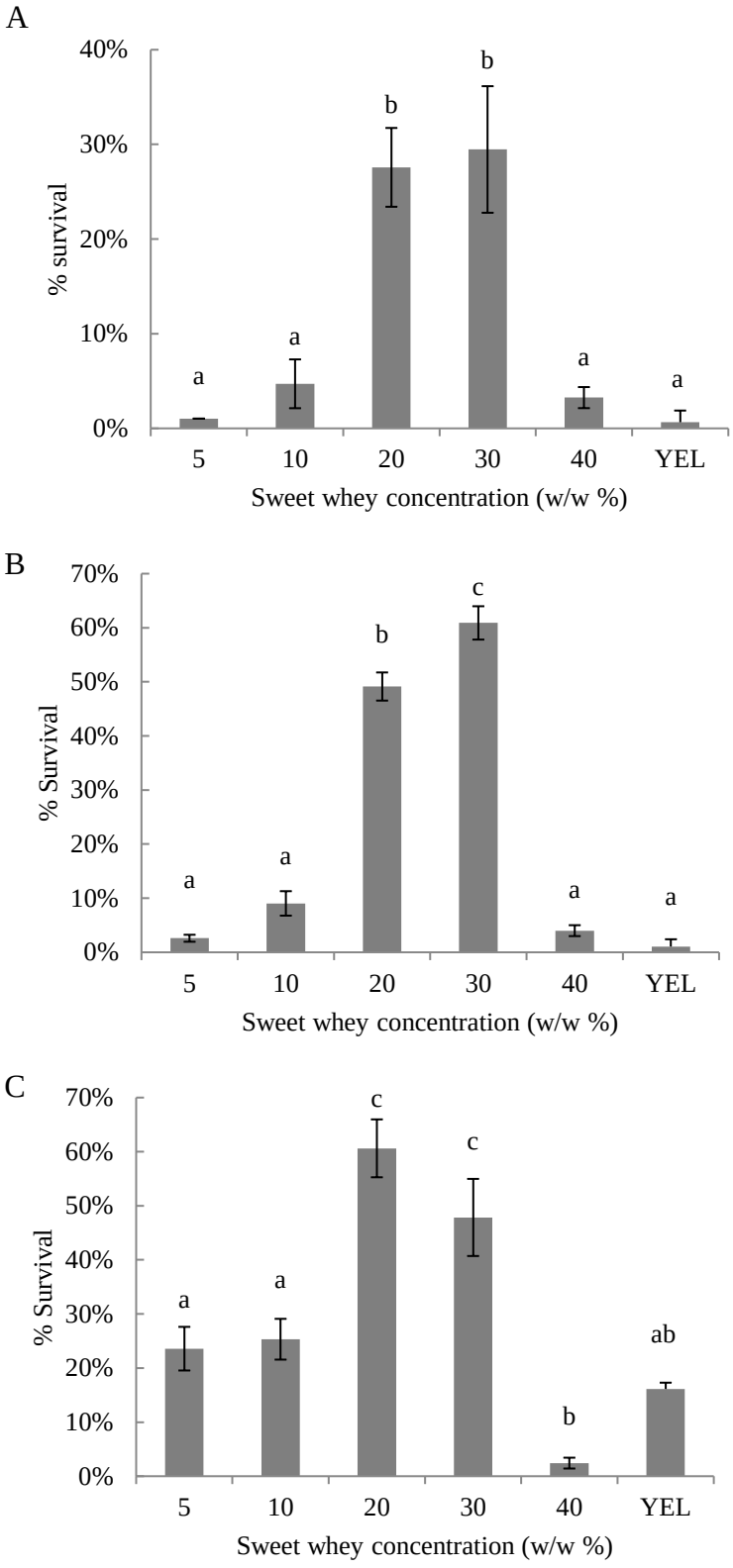

Figure 24. *P. freudenreichii* were cultivated for 72 h in the indicated growth media until stationary phase, then subjected to (A) heat (60°C for 10 min), (B) acid (pH 2.0 for 1 h), or (C) bile salts (1 g l^{-1} for 1 h) challenge. Viable propionibacteria were enumerated by plate counting in treated and control cultures, and then expressed as percent survival. Different letters above the columns indicate significant differences ($P < 0.05$).

3.3.1.2 The multitolerance response involves key stress proteins

To investigate the molecular mechanisms responsible for such adaptation, we performed a proteomic analysis in 5% versus 30% sweet whey culture media. As shown in Figure 25, at least 41 protein spots were enhanced in the pH range from 4 to 7 in 30% sweet whey. They were also enhanced in this hypertonic medium compared to the reference rich and isotonic YEL medium (data not shown).

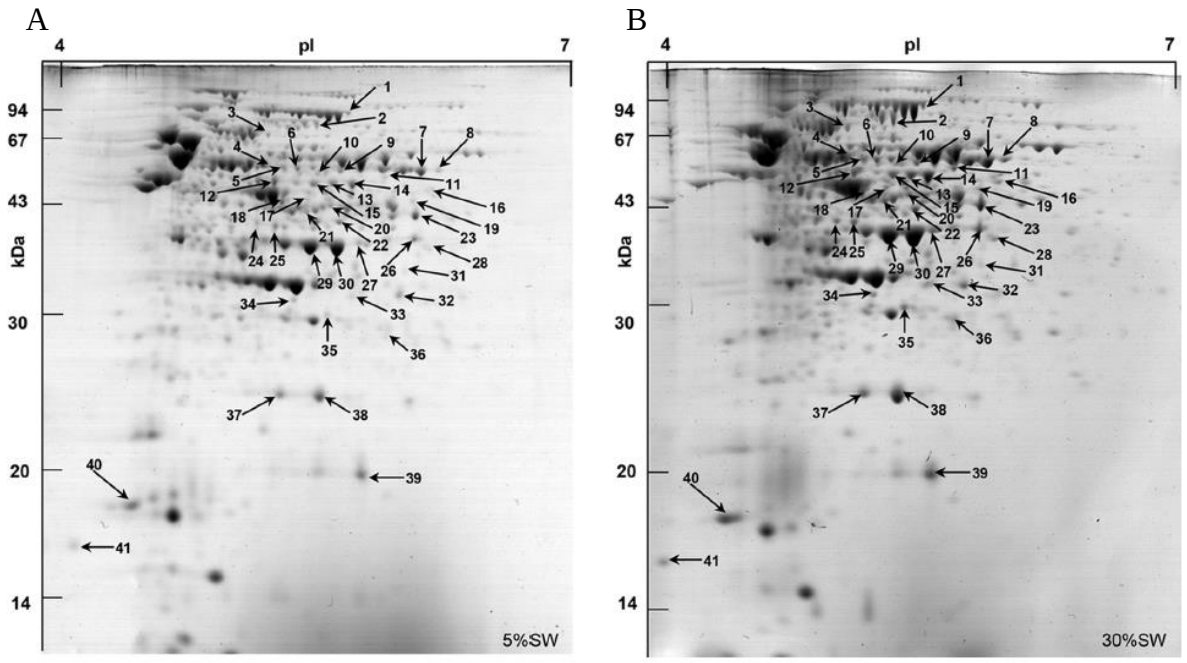

Figure 25. Hyperconcentrated sweet whey culture triggers overexpression of stress proteins. Propionibacteria were cultivated for 72 h in isotonic 5% (A) or hyperconcentrated 30% (B) sweet whey. The proteins accumulated in the cellular compartment were then analyzed by 2D electrophoresis (pH 4 to 7 isoelectric focusing and then SDS-12.5% PAGE) prior to differential analysis using the SameSpot software. The proteins induced in 30% sweet whey are indicated by arrows. Their identities, revealed by trypsinolysis and NanoLC–MS-MS, are shown in Table 11.

The induced protein spots were then picked and subjected to in-gel trypsinolysis as previously described (Leverrier et al., 2004), and the resulting peptides were subjected to NanoLC–MS-MS as described in Materials and Methods. For each protein spot, the major identified protein is presented in Table 11.

Table 11. *P. freudenreichii* CIRM BIA 129 proteins overexpressed in hyperconcentrated 30% sweet whey culture, compared to isotonic 5% sweet whey culture, as identified by NanoLC–MS-MS

No.[a]	M_{exp}[b]	PI_{exp}[c]	Locus tag	Description	Log E value[d]	Cover %[e]	M_{theor}[f]	PI_{theor}[g]	No. of Spectra[h]	Fold[i]
1	93.2	5.71	PFCIRM129_06355	Chaperone clpB 2	-524.4	75	94.2	5.33	220	1.5
2	82.5	5.55	PFCIRM129_07240	Methylmalonyl-CoA mutase large subunit mutB	-426.4	75	80.1	5.21	160	1.5
3	72.1	5.30	PFCIRM129_10415	Myo-inositol catabolism iolD protein	-472.7	78	69.7	5.04	160	1.7
4	56.8	5.28	PFCIRM129_11225	FeS assembly protein SufB	-471.8	86	53.8	5.02	214	1.5
4	56.8	5.28	PFCIRM129_10420	Myo-inositol catabolism IolA protein	-369.2	77	52.7	5.00	132	1.4
5	55.5	5.38	PFCIRM129_00340	Aspartate ammonia-lyase	-215.0	65	53.0	5.03	93	1.3
6	55.2	5.45	PFCIRM129_11435	Pyruvate kinase 1	-432.7	83	53.9	5.17	202	1.4
7	54.6	6.07	PFCIRM129_01440	Coenzyme A transferase	-695.6	87	55.6	5.53	261	1.6
8	54.2	6.18	PFCIRM129_01440	Coenzyme A transferase	-526.4	81	55.6	5.53	177	1.4
9	53.9	5.68	PFCIRM129_10235	Glycine hydroxymethyltransferase precursor	-432.3	80	51.8	5.28	187	1.6
10	53.5	5.56	PFCIRM129_11790	Xaa-Pro aminopeptidase I	-302.5	74	54.4	5.32	127	1.7
11	51.9	5.90	PFCIRM129_07855	ATP-binding protein opuCA of osmoprotectant ABC transporter	-352.3	70	47.9	5.51	134	1.3
12	49.7	5.33	PFCIRM129_09770	Dihydrolipoyl dehydrogenase	-395.4	75	49.5	5.08	179	1.4
13	49.1	5.63	PFCIRM129_11895	Fumarate hydratase. class-II	-611.8	78	51.1	5.34	261	1.6
14	48.6	5.74	PFCIRM129_11895	Fumarate hydratase. class-II	-636.2	90	51.0	5.34	311	1.4
15	48.6	5.54	PFCIRM129_07025	6-phosphogluconate dehydrogenase. decarboxylating	-398.4	84	52.5	5.25	191	1.5
16	47.2	6.15	PFCIRM129_06185	Galactokinase	-328.8	63	41.8	5.57	99	1.4
17	44.5	5.50	PFCIRM129_04835	Anaerobic glycerol-3-phosphate dehydrogenase subunit B	-321.2	89	45.8	5.27	118	1.9
18	44.5	5.34	PFCIRM129_08275	Elongation factor Tu	-344.6	68	43.6	5.05	138	1.4
19	44.4	6.05	PFCIRM129_04290	Tryptophan synthase beta chain (TrpB)	-250.1	72	44.5	5.47	72	1.4
20	43.6	5.63	PFCIRM129_06815	Glutamate-5-semialdehyde dehydrogenase	-243.3	75	43.5	5.20	109	1.5
21	42.9	5.50	PFCIRM129_07640	Putative isocitrate/isopropylmalate dehydrogenase	-330.8	78	44.7	5.20	178	1.4
22	42.5	5.70	PFCIRM129_02990	Tryptophanyl-tRNA synthetase	-227.9	77	40.7	5.45	113	1.2

Table 11 continued

23	42.2	6.06	PFCIRM129_03745	Acetate kinase	-600.6	89	42.4	5.50	303	1.5
24	40.6	5.23	PFCIRM129_10390	Transaldolase 2	-300.4	78	39.3	5.10	150	1.7
25	39.9	5.32	PFCIRM129_10390	Transaldolase 2	-295.4	79	39.3	5.10	173	2.8
26	39.5	6.05	PFCIRM129_07545	zinc-binding dehydrogenase	-522.9	83	37.1	5.52	201	1.2
27	39.1	5.78	PFCIRM129_03400	Aldo/keto reductase	-301.6	80	36.9	5.36	140	1.7
28	38.6	6.11	PFCIRM129_05680	Oxidoreductase	-274.2	89	38.7	5.57	142	1.6
29	37.9	5.52	PFCIRM129_11300	Glyceraldehyde-3-phosphate dehydrogenase	-424.3	85	36.0	5.46	306	1.2
30	37.6	5.66	PFCIRM129_11300	Glyceraldehyde-3-phosphate dehydrogenase	-412.2	78	37.7	5.46	313	1.4
31	35.3	6.07	PFCIRM129_01130	NADPH:quinone reductase	-171.3	74	33.6	5.59	64	1.9
32	32.6	5.98	PFCIRM129_01465	Putative aldo/keto reductase (oxidoreductase)	-233.0	81	31.1	5.48	135	1.4
33	32.3	5.78	PFCIRM129_01465	Putative aldo/keto reductase (oxidoreductase)	-182.0	79	30.4	5.48	122	1.4
34	31.2	5.45	PFCIRM129_07790	Cysteine synthase 1	-175.6	87	31.6	5.11	62	1.8
35	30.1	5.61	PFCIRM129_08515	NADH-quinone oxidoreductase chain C	-227.1	80	28.6	5.36	138	1.8
36	28.3	5.91	PFCIRM129_10185	Oxidoreductase	-104.0	54	28.8	5.56	52	1.3
37	24.5	5.41	PFCIRM129_08020	Superoxide dismutase [Mn/Fe] sodM	-452.1	89	22.8	5.28	209	1.4
38	24.0	5.57	PFCIRM129_08020	Superoxide dismutase [Mn/Fe] sodM	-533.7	93	22.8	5.28	319	1.6
39	19.1	5.79	PFCIRM129_05095	Methylmalonyl-CoA epimerase	-214.1	90	16.6	5.40	203	1.1
40	18.0	4.71	PFCIRM129_02555	Heat shock protein 20 1 (20 kDa chaperone 1)	-139.6	77	17.3	4.73	119	1.4
41	16.2	4.28	PFCIRM129_10610	Phosphocarrier. HPr family	-146.0	95	8.9	4.22	106	1.8

[a] The numbers correspond to the spots in Figure 25.

[b] M_{exp}, molecular mass (kDa) evaluated from bidimensional electrophoresis migration.

[c] pI_{exp}, isoelectric point evaluated from bidimensional electrophoresis migration.

[d] The E value is the probability that a given peptide score will be achieved by incorrect matches from a database search. Protein identifications were automatically validated when they showed at least two unique peptides with E values below 0.05, corresponding to a log(E value) of less than -1.3.

[e] Cover %, the percentage of the protein amino acid sequence covered by tandem mass spectrometry identification of the peptide.

[f] M_{theor}, protein molecular mass (kDa) automatically predicted from the corresponding gene on the Agmial annotation platform.

[g] pI_{theor}, isoelectric point automatically predicted from the corresponding gene on the Agmial annotation platform (http://genome.jouy.inra.fr/agmial/).

[h] Number of MS-MS spectra with an individual E value of <0.05 matched to a peptide sequence from the protein.

[i] Fold change of protein expression in 30% SW compared to 5% SW as determined using Progenesis SameSpot software.

[j] CoA, coenzyme A.

Several of the identified proteins are known to participate in stress adaptation of *P. freudenreichii* and/or in other microorganisms. As an example, the ClpB 2 chaperone (spot no. 1 in Table 11), involved in acid stress adaptation in *P. freudenreichii* (Leverrier et al., 2004), is heat and salt inducible in *Bifidobacterium breve* (Ventura et al., 2005). The protein synthesis elongation factor EF-Tu (spot no. 18) was also induced here. EF-Tu is known to alleviate the negative effect of heat stress by acting as a molecular chaperone (Caldas et al., 1998). It is induced by acid stress in *P. freudenreichii* (Leverrier et al., 2004) and in *Lactobacillus delbrueckii* (Zhai et al., 2014) and plays a role in osmotic adaptation in *Escherichia coli* (Berrier et al., 2000). Citrate synthase (spot no. 16) is involved in stress tolerance, and its disruption leads to multistress susceptibility in *Pseudomonas syringae* (Sengupta et al., 2015), while its overexpression leads to aluminum tolerance in alfalfa (Barone et al., 2008), confirming a role in multistress tolerance response. We also reported the implication of cysteine synthase (no. 34), superoxide dismutase (no. 37), methylmalonyl-coenzyme A (CoA) mutase (no. 2), and aspartate ammonia-lyase (no. 5) in *P. freudenreichii* stress adaptation (Leverrier et al., 2004).

3.3.1.3 The multitolerance response is linked with osmoadaptation

Our observations suggest that *P. freudenreichii*, in the 30% sweet whey culture medium, adapted via osmoadaptation mechanisms leading to intracellular accumulation of compatible solutes. Likewise, previous studies have evidenced that some strains of *P. freudenreichii* possess efficient osmoadaptation mechanisms using glycine betaine, dimethylsulfoniopropionate, and dimethylsulfonioacetate (Boyaval et al., 1999) and involving the synthesis of trehalose via the OtsA-OtsB pathway, induced by osmotic, acid, and oxidative stresses (Cardoso et al., 2007). Here, we observed the induction of the OpuCA compatible solute transport system (no. 11 in Table 11) involved in glycine betaine uptake and osmoadaptation (Du et al., 2011), which suggests adaptation of *P. freudenreichii* to hyperconcentrated sweet whey by increased transport of such compatible solute molecules. Compatible solutes, in addition to those transported by the OpuCA system, may also be synthesized *de novo*, as suggested here by the induction of serine hydroxymethyltransferase, also called glycine hydroxymethyltransferase (no. 9 in Table 11; EC 2.1.2.1), which is involved in accumulation of choline precursors and thus in osmoadaptation in the halotolerant cyanobacterium *Aphanothece halophytica* (Waditee-Sirisattha et al., 2012). Furthermore, induced proteins also comprised Xaa-Pro aminopeptidase (no. 10), which is involved in

osmoprotection by proline-containing peptides in *Bacillus subtilis* (Zaprasis et al., 2013), and gamma-glutamyl phosphate reductase (no. 20, encoded by *proA*), involved in osmoadaptive proline production in *B. subtilis* (Brill et al., 2011). They also comprised glycerol 3-phosphate dehydrogenase (no. 17), which is involved in osmoadaptation in yeasts, where glycerol plays a key role, while its mutational inactivation results in reduced salt tolerance (Yan et al., 2008). Altogether, these data strongly suggest that *P. freudenreichii* triggers an osmoadaptive response during growth in hyperconcentrated sweet whey.

We also investigated on the accumulation of trehalose and glycogen, two carbohydrates known to participate in stress tolerance and in long-term survival in *P. freudenreichii* (Cardoso et al., 2007; Dalmasso et al., 2012). As shown in Figure 26, both compounds were below the limit of detection when *P. freudenreichii* was grown in YEL medium. In contrast, both were accumulated in 30%, and to a lesser extent in 5%, sweet whey cultures.

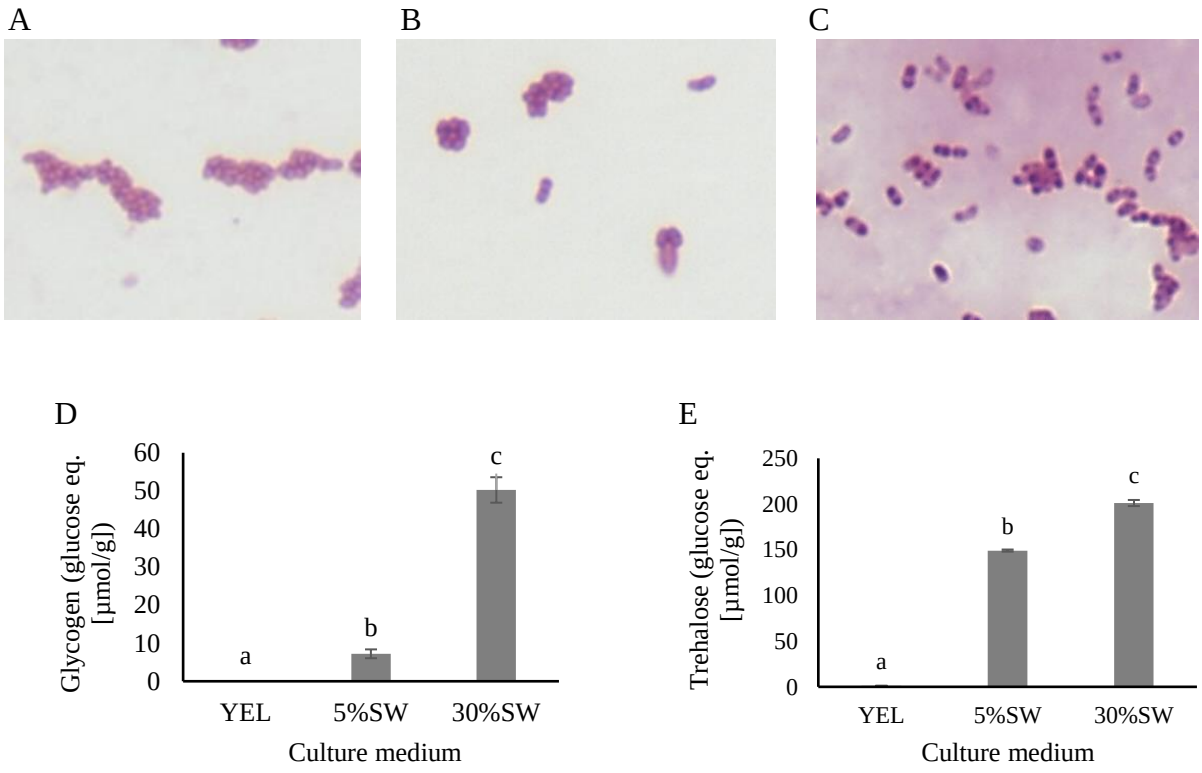

Figure 26. Hyperconcentrated sweet whey culture triggers intracellular accumulation of carbohydrates. (A to C) Propionibacteria were cultivated for 72 h in YEL medium (A) or in isotonic 5% (B) or hyperconcentrated 30% (C) sweet whey. Periodic acid-Schiff staining was applied to smears of the cultures. (D and E) Glycogen (D) and trehalose (E) were then enzymatically quantified in whole-cell extracts. The results are expressed as concentrations of generated glucose per unit weight of cellular wet matter. Different letters above the bars indicate significant differences (*P*_0.05).

Such accumulation is known to confer enhanced osmotolerance and long-term survival under cheese-ripening conditions (Dalmasso et al., 2012). Accordingly, periodic acid-Schiff staining of carbohydrates in smeared cultures of *P. freudenreichii* evidenced the presence of deeply stained round structures at both ends of the bacteria in 30% sweet whey that were not present in YEL cultures.

Inorganic polyphosphate was also reported to play a key role in stress tolerance by acting as a protein-protecting chaperone and by regulating sigma factors involved in stress response in bacteria (Gray and Jakob, 2015). Polyphosphate accumulation in the form of volutin granules has been evidenced in various *Lactobacillus* species (Alcántara et al., 2014), in *Corynebacterium glutamicum* (Klauth et al., 2006), and in *P. freudenreichii* (Clark et al., 1986). In our study, we revealed the characteristic intracellular dark-stained granules in *P. freudenreichii* by the Neisser staining used by the above-mentioned authors (Figure 27A to C). Granules were present in sweet whey cultures—more abundant in 30% than in 5% sweet whey—while they were not detected in YEL-cultured propionibacteria. In addition to Neisser staining, the fluorescent DAPI probe was also used to visualize polyphosphate (green) and DNA (blue). Green-light-emitting bacteria were evidenced in sweet whey cultures using epifluorescence microscopy, where emission wavelengths of 420 to 800 nm were seen together (Figure 27D to F). This was further confirmed by confocal microscopy (Figure 27G to I), where blue (412 to 450 nm) and green (553 to 598 nm) images were acquired and then merged. Furthermore, transmission electron microscopy (Figure 27J to L) showed, in sweet whey cultures, circular and oval holes that had already been described when these granules were chipped or torn out during cutting of thin sections (Alcántara et al., 2014; Auling et al., 1991; Bode et al., 1993).

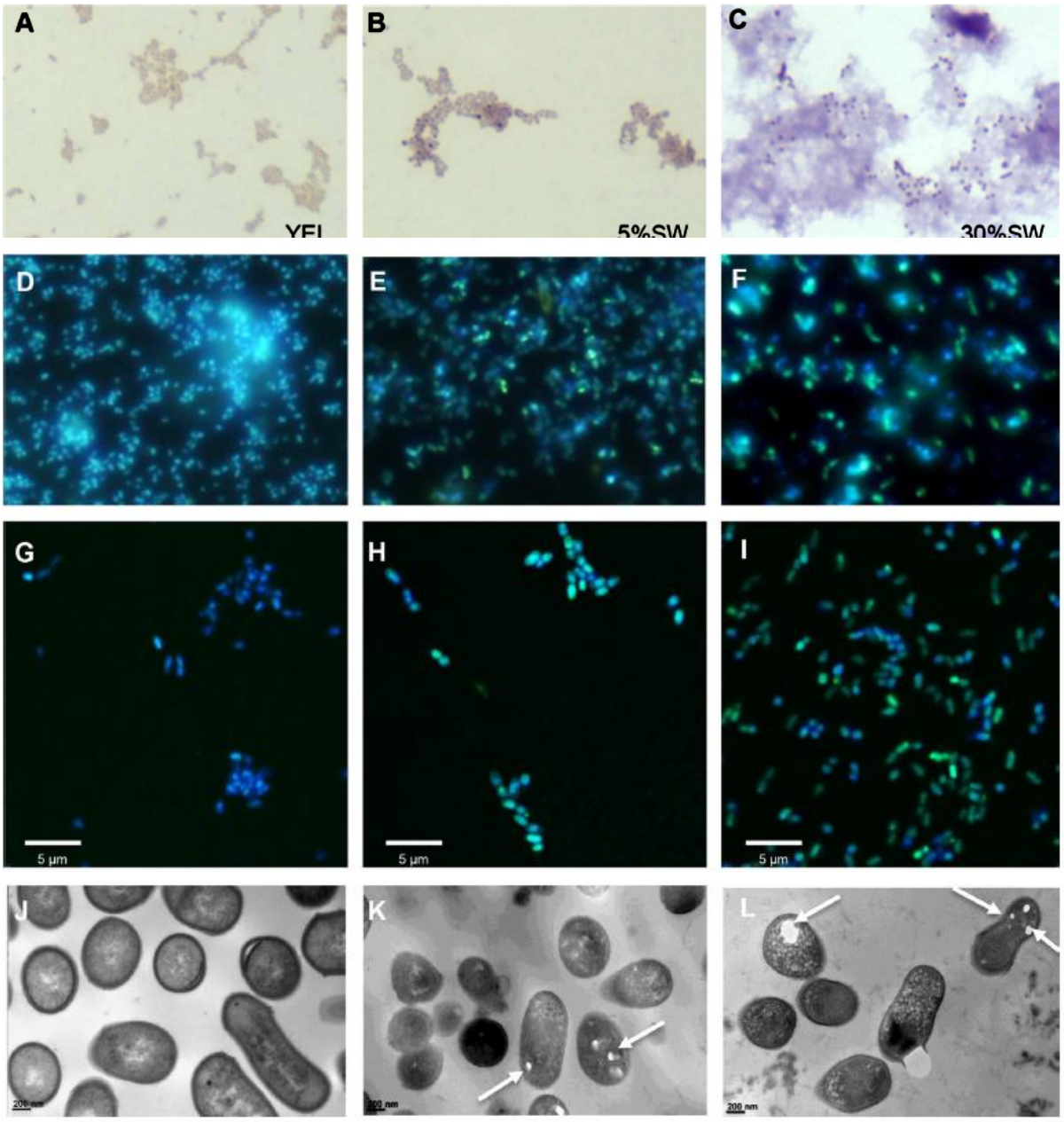

Figure 27. Propionibacteria were cultivated in YEL (A, D, G, and J) or in 5% (B, E, H, and K) or 30% (C, F, I, and L) sweet whey. (A to C) Polyphosphate metachromatic granules were evidenced by Neisser staining and observed at ×1,000 magnification. (D to I) Polyphosphate was visualized by DAPI staining prior to epifluorescence microscopy (D to F) or confocal microscopy (G to I) observation. Green fluorescence indicates cytosolic poly(P), and blue indicates DNA. (J to L) Transmission electron micrographs of propionibacteria. The positions that were occupied by granules appear as holes in the bacterial cytoplasm (examples are indicated by the arrows).

Altogether, these data indicate that such granules are accumulated in *P. freudenreichii* CIRM-BIA 129 under the growth conditions used in the present study. In lactobacilli, accumulation of intracellular polyphosphate in the form of polyphosphate granules depends on the species considered, the strain within the species, and the culture conditions (Alcántara et

al., 2014). The presence of inorganic phosphate in the culture medium is a prerequisite for polyphosphate accumulation. In the sweet whey used here, inorganic phosphate accounts for 1.14% of the dry matter, so its concentration is close to 6 mM and 36 mM for 5% and 30% sweet whey, respectively. This is close to the 37 mmol l^{-1} KH_2PO_4 concentration in malic enzyme induction (MEI) medium (0.5% yeast extract, 0.5% tryptone, 0.4% KH_2PO_4, 0.5% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4$, 1 mL Tween 80 $liter^{-1}$, 0.05% cysteine, 0.5% glucose), favoring polyphosphate accumulation in lactobacilli, which coincides with enhanced stress tolerance, including salt and low pH (Alcántara et al., 2014). Interestingly, abolishment of polyphosphate accumulation ability by mutational inactivation of the *ppk* gene leads to reduced stress tolerance (Alcántara et al., 2014). We propose that osmotically induced accumulation of polyphosphates, used as both energy storage molecules and compatible solutes by *P. freudenreichii*, participates in osmoinduced enhanced multitolerance.

3.3.1.4 Summary

In summary, highly concentrated sweet whey, as used as a culture medium in the novel 2-in-1 process, triggers different stress tolerance pathways, including overexpression of chaperones and accumulation of key compatible solutes. It leads to live and stable probiotics with induced multitolerance. This can partially explain the reason why bacteria survived better during spray drying and among storage.

Considering these adaptation processes, we can even expect that probiotic powders produced from the 2-in-1 process may display enhanced tolerance towards digestive stresses, when compared to fresh culture. This would lead to better delivery efficiency of probiotic health effects. However, this remains an interesting topic for further investigation.

3.3.2 Calcium-protein aggregates protect probiotics against droplet drying

A gentle reminder

In comparison to the natural whey, the concentrated whey induces more aggregation during autoclave, pasteurization and fermentation. In order to study the independent effect of the aggregates on probiotic protection (excluding the influence of high TS content as already proven in the previous sections), protein aggregation was induced in non-concentrated skim milk (ca-aggregated milk in brief) by adding very low concentration of calcium salts (10 mmol l^{-1}). The untreated UHT skim milk (skim milk in brief) and the 10 mmol l^{-1} $CaCl_2$-added skim milk (without heating, Ca-added skim milk in brief) were used as controls to exclude the effects of $CaCl_2$ addition and high TS content.

3.3.2.1 Physical and chemical properties of aggregated milk

The size distribution of *L. rhamnosus* GG (LGG) and the dispersed elements in the three LGG-suspended milk matrixes is shown in Figure 28. The size of LGG cells ranged from 1 to 10 µm (Figure 28A). This size range appears in all the three LGG-suspended milk matrixes (Figure 28B). Specifically, the dispersed elements in untreated skim milk and Ca-added milk showed a very similar size distribution, consisting mostly in elements ranging approximately from 70 to 500 nm and, conversely, in a smaller proportion of elements with a large diameter (from 3 to 10 µm). These later might correspond, respectively, to the casein micelles and LGG cells in the milk (Kim et al., 2009; McGann et al., 1980). Therefore, the addition of 10 m mol^{-1} $CaCl_2$ had no influence on the solid size distribution in skim milk. By contrast, most of dispersed elements in the Ca-aggregated milk had diameters ranging from 5 to 50 µm. In addition, the Ca-aggregated milk contained a portion of elements with diameter larger than 100 µm. The protein aggregation and increasing of the casein micelle size emerged in the skim milk with additional Ca^{2+} upon the heat treatment taking place.

Besides this, the content of proteins in the serum phase of Ca-aggregated milk was largely decreased (Figure 28C) due to the formation of whey protein-casein micelle aggregates during

heat treatment in the presence of added Ca^{2+} (Anema and Li, 2003; Dalgleish, 1990; Gaucheron, 2005).

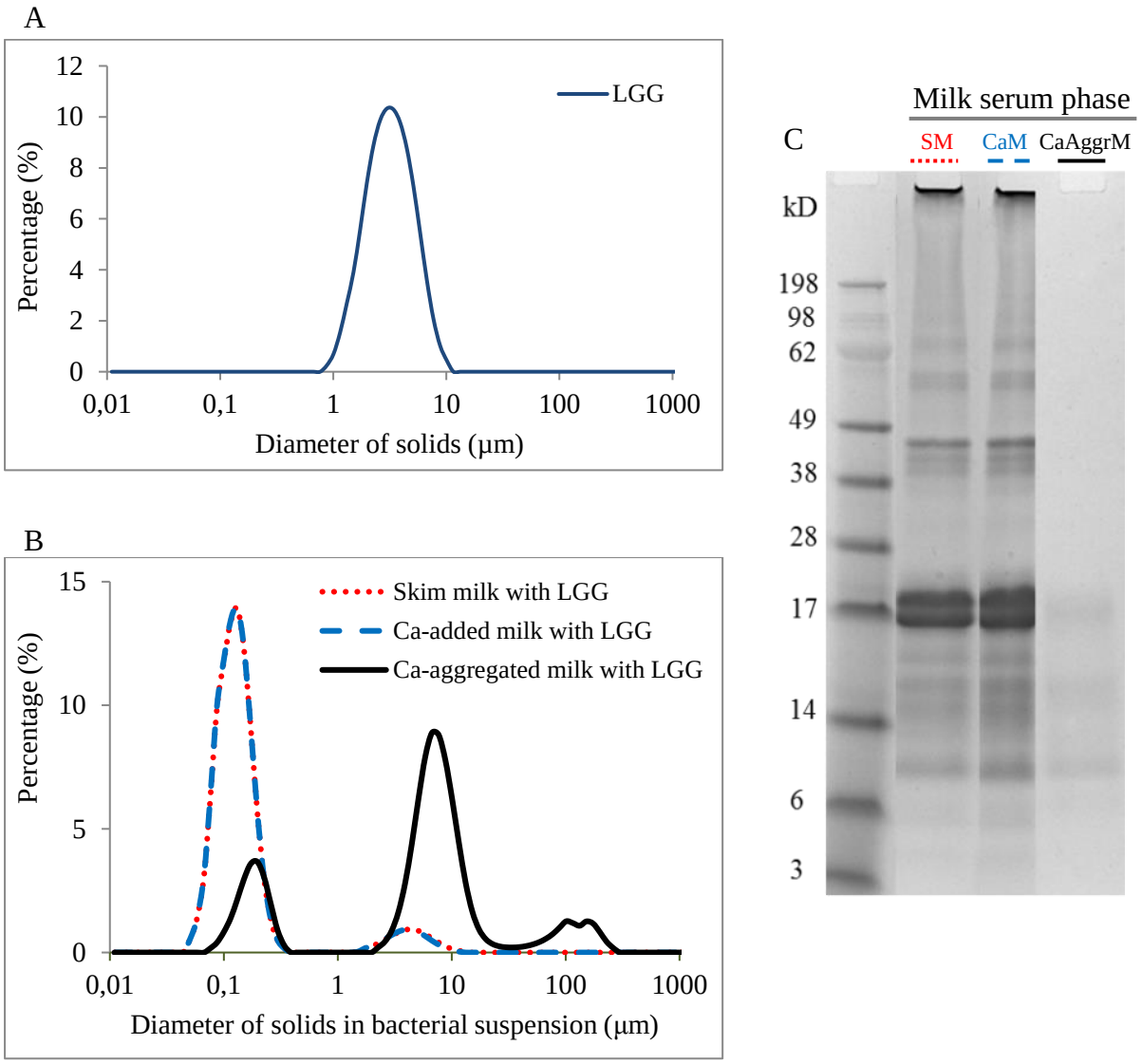

Figure 28. (A) The size distribution of dispersed LGG cells in the 0.5% w/v peptone water. (B) The size distribution of dispersed elements in three LGG-suspended milk matrixes. (C) The milk serum protein content of the three bacteria-suspended milk matrixes indicated by SDS-PAGE. SM: UHT skim milk, CaM: Ca-added skim milk, and CaAggrM: Ca-aggregated skim milk.

The Ca-aggregated milk displayed a considerably higher viscosity when compared to that of the skim milk and Ca-added milk (Table 12). The higher viscosity of Ca-aggregated milk was caused by the heat treatment, leading to denaturation of whey proteins and further aggregation, either in the soluble milk phase or with the casein micelles (Jeurnink and De Kruif, 1993). In addition, the untreated and Ca-added skim milk showed a similar T_2 relaxation times distribution, as shown in Figure 29 and Table 12. However, the Ca-aggregated milk displayed a

left shift of the T_2 distribution with a smaller area under the peak, which indicated a decrease of T_2 (Li et al., 2012). It can be concluded that water had a lower mobility in the Ca-aggregated milk than in untreated and Ca-added skim milk. It could result from the aggregation process, leading to the entrapping of water into the protein network (Belloque and Ramos, 1999; Lambelet et al., 1992).

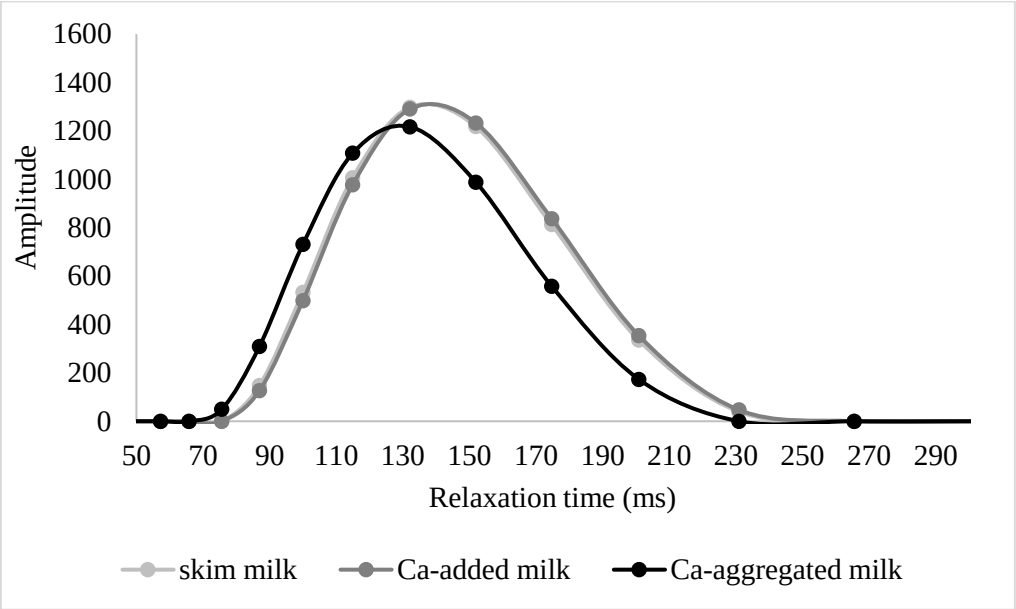

Figure 29. Distribution of transverse relaxation time (T_2) of the three bacteria-suspended milk matrixes measured by a low-field ^1H nuclear magnetic resonance (NMR) spectrometer.

Table 12. Viscocity and distribution of T_2 relaxation time of the three bacteria-suspended milk matrixes

Milk matrixes	Viscocity (mPa·s)	T_2 distribution				
		Start (ms)	Peak (ms)	End (ms)	Area	Area proportion (%)
Untreated skim milk	1.4±0.1[a]	75.6±0.1[a]	141.0±0.0[a]	265.6±0.1[a]	5440±110[a]	98.3±0.6[a]
Ca-added milk	1.3±0.1[a]	75.6±0.0[a]	141.0±0.1[a]	265.6±0.0[a]	5339±27[ab]	98.5±0.2[a]
Ca-aggregated milk	28.9±0.5[b]	65.8±0.1[b]	131.2±0.1[b]	231.0±0.2[b]	5187±100[b]	98.2±1.0[a]

[a] Different roman letters (by column) refer to significant difference ($p < 0.05$).

3.3.2.2 Drying kinetics of milk matrixes in single droplet drying

The drying kinetics of three different bacteria-suspended milk matrixes during convective drying are shown in Figure 30 through the description of temperature and mass changes. The features of droplet temperature (T_d) and mass (M_d) were consistent with previous studies (Sadek et al., 2013; Zheng et al., 2015). For the temperature curve, four stages can be identified during drying: in the first stage, the T_d was rapidly preheated to the wet-bulb range (~ 23°C); in the second stage, the T_d was maintained as nearly constant for tens of seconds; in the third stage, the T_d curve experienced a progressive increase with time and tended toward a final plateau with a sigmoid shape; in the fourth and last stage, the T_d approached the air temperature at around 65°C. At the same time, the curve of droplet mass M_d decreased progressively with time. Considering the temperature-curve stages previously mentioned, the decrease of M_d was almost linear with time at the first and second stages. The mass loss rate slowed gradually from the third stage until it approached zero at the fourth stage. The untreated skim milk and Ca-added milk showed similar T_d and M_d behaviors during drying. The addition of 10 mmol l^{-1} $CaCl_2$ without further thermal aggregation had no significant influence on the drying kinetics of skim milk. However, Ca-aggregated milk displayed distinct drying kinetics, with a longer second stage and a shorter time to reach the final fourth-stage plateau.

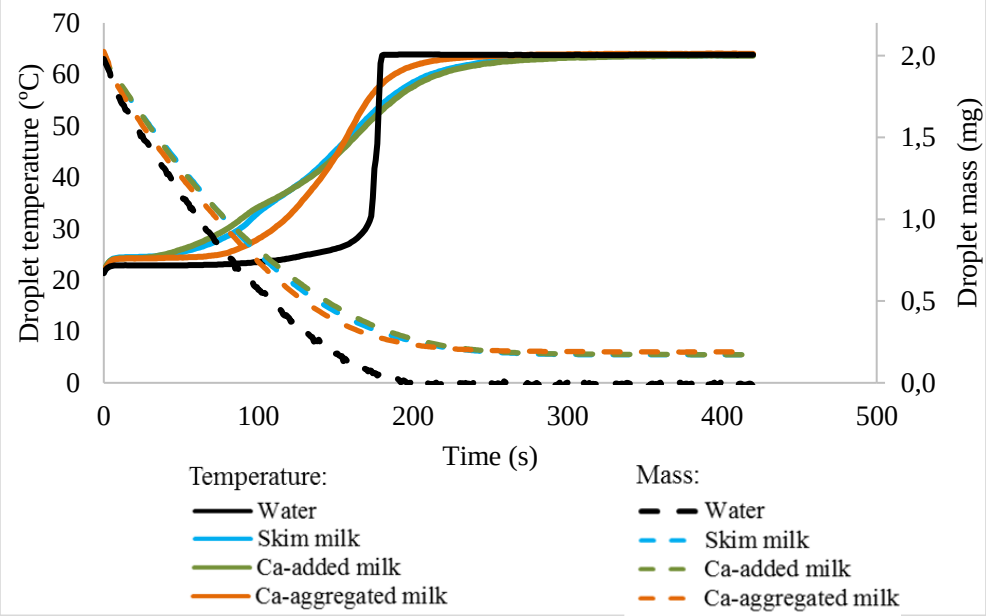

Figure 30. Changes of droplet temperature (solid curves, corresponding to the left ordinate) and mass (dotted curves, corresponding to the right ordinate) in three bacteria-suspended milk matrixes (UHT skim milk, Ca-added skim milk, and Ca-aggregated skim milk) and pure water during single-droplet drying.

The features of Ca-aggregated milk were closer to those of pure water droplets under the same drying conditions. It is known that the evaporation of pure water can be considered as diffusion-controlled and has the highest dehydration rate under the same conditions compared to those of other materials (Sadek et al., 2013). Thus, the Ca-aggregated milk experienced longer time of diffusion-controlled evaporation in comparison with those of untreated and Ca-added skim milk. The overall drying efficiency was thus the highest among the three milk matrixes tested in this work. It can be assumed that the water in the serum phase of the Ca-aggregated milk was bound to a lesser extent due to its lower protein content (Figure 28C). It may therefore lead to the more water-like drying behaviour previously observed, with a longer second stage and faster drying rate during final convective drying.

3.3.2.3 Droplet morphology evolution during drying

The evolution of surface morphology of droplets during drying was recorded. The droplet morphology with the corresponding drying time for each milk matrix is shown in Figure 31.

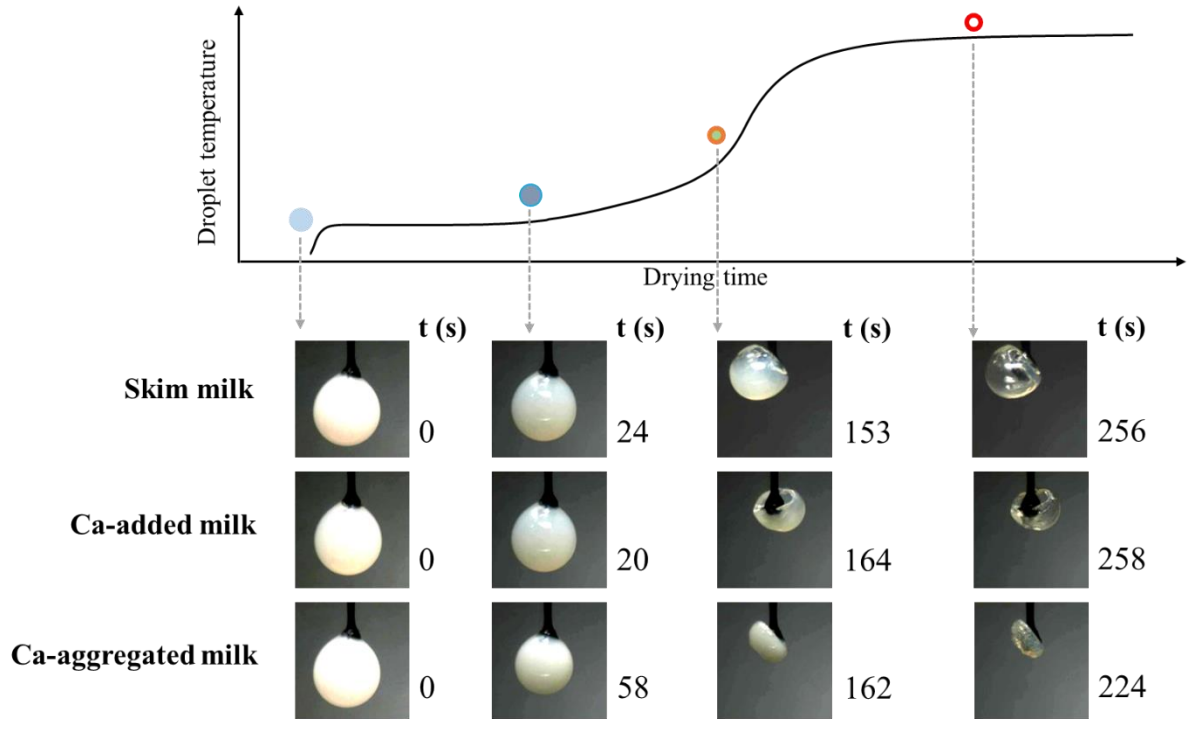

Figure 31. Droplet morphology and the corresponding drying time of three bacteria-suspended milk matrixes (UHT skim milk, Ca-added skim milk, and Ca-aggregated skim milk) at four representative events during single-droplet drying.

Regardless of the milk matrixes, the droplets had a similar shape and size at the beginning of drying. They shrank homogeneously from the beginning of drying to the end of second stage, which can be assumed as diffusion-controlled (Arcamone et al., 2007). By the end of second stage, the spherical shape of droplets were maintained, but the droplet size of Ca-aggregated milk was smaller than that of untreated and Ca-added milk. When the drying proceeded to the third stage, the droplet began to distort due to the skin formation on the surface and progressively lost its spherical shape (Fu et al., 2013b; Pauchard and Allain, 2003). During this droplet-particle conversion stage, the water diffused through the droplet surface because of the porous properties of the skin. However, as the sol-gel transition took place over the free surface of droplet, the droplet skin developed into a thickened shell that impeded the shape of droplet from further change and progressively hindered the evaporation (Arai and Doi, 2012; Sadek et al., 2013). In this stage, a vacuole progressively formed inside the particle, as indicated by the transparent feature of droplets. This could be explained by the pressure change across the droplet skin, which made it possible for the air to enter the droplet at the same time as water diffused through the shell (Arai and Doi, 2012; Sadek et al., 2013). This transparent feature was particularly apparent on the final skim milk and Ca-added milk particles.

Interestingly, the particle formed by the Ca-aggregated milk was hemispherical (lower height) compared to that of from the skim milk and Ca-added milk during the droplet-particle conversion. The morphology observed by the SEM displayed an even more remarkable different structure of the Ca-aggregated milk dried particle (Figure 32). Specifically, this latter presented a wrinkled surface, and the surface of skim milk and Ca-added milk particles were relatively smooth (Figure 31). It has been reported in the convective droplet drying that the particles formed by the whey proteins displayed a relatively smooth surface and spherical shape, and the casein micelles would form preferentially wrinkled particles (Sadek et al., 2016, 2015, 2014). We suggest that the smooth and spherical particle surface of the untreated skim milk and Ca-added milk could be attributed to the predominant participation of whey proteins in the shell formation due to their small size and thereby faster diffusion rate compared to that of the casein micelles. As discussed above, the whey proteins in Ca-aggregated milk were associated with the casein micelles to form the large size of aggregates. Hence, the wrinkled particle surface of the Ca-aggregated milk was due to the main participation of casein micelles in the shell formation.

Besides, it is also interesting to note that in Chapter 2, we have displayed the surface morphology of powders dried from 5% and 30% sweet whey (Figure 19 in Item 2.3.3). To some

extent, the features (smooth surface) displayed by the 5% sweet whey powders is similar with that of the skim milk particle, while the features (wrinkled surface) displayed by the 30% sweet whey powders is similar with that of the Ca-aggregated milk particle.

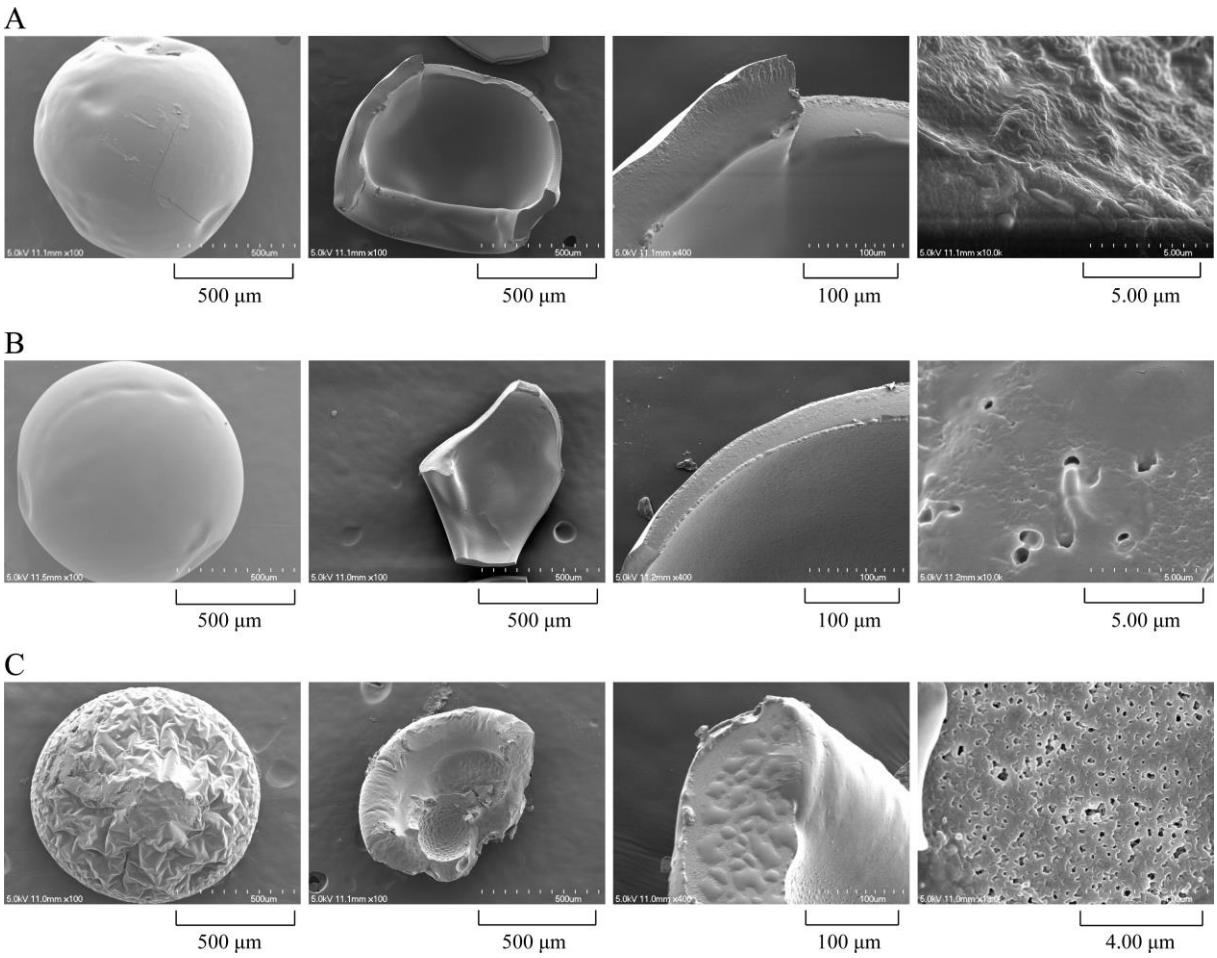

Figure 32. Micromorphology of particles dried from (A) UHT skim milk, (B) Ca-added skim milk, and (C) Ca-aggregated skim milk. Pictures were observed by scanning electron microscopy. For each milk matrix, four pictures were used to display the particle morphology from different focus sites (from left to right): surface of intact particle at ×100 magnification, vacuole structure at ×100 magnification, shell section at ×400 magnification, and shell section at ×10000 magnification.

Last, the inner-morphology of dried particles is also shown in Figure 32. The vacuole structure was indeed formed inside the particles regardless of the matrix type. However, the vacuole size of the Ca-aggregated milk particles was smaller than that of untreated and Ca-added skim milk particles. In a comparison of the particle shell section, the skim milk and Ca-added milk showed a similar compact and thin shell, and the Ca-aggregated milk displayed a dense shell with the bubble-like structure. It supports our above hypothesis that the casein micelles played the main role in the shell formation of the particles dried from Ca-aggregated

milk (Sadek et al., 2015, 2014). In the figures with ×10000 magnification, the network-like structure in the shell was clearly observed in the particle of Ca-aggregated milk. It was significantly different from that of matrix-filled structure displayed in the particle shell of untreated and Ca-added skim milk. This porous property of shell may also explain the distinct drying kinetics of the Ca-aggregated milk. As evidence, it should be easier for water to diffuse across a porous shell, leading thereby to the longer second stage and faster drying rate observed.

3.3.2.4 Bacteria viability during single droplet drying

The inactivation kinetics of LGG is shown in Figure 33A. The initial bacterial population was around 1.0×10^9 CFU g^{-1} before drying, whatever the milk matrix. The viability of LGG in the three milk matrixes maintained constant within the first 200 s of drying, corresponding to the period from the first stage to the end of the third stage in Figure 30. Viability of bacteria dropped when the drying proceeded to the fourth stage (~210 s); in skim milk and Ca-added milk, it decreased dramatically to a level lower than 10^6 CFU g^{-1} within 240 s. In comparison, the Ca-aggregated milk showed a remarkable protection on LGG. Specifically, the LGG population in Ca-aggregated milk decreased significantly slower than that in the skim milk and Ca-added milk from 210 s to 420 s. At the end of drying, the remaining bacterial population in the particle of Ca-aggregated milk was still higher than the level of 10^7 CFU g^{-1}, which made the final viability around 2 logs higher than that of in the skim milk or Ca-added milk.

Inactivation of LGG in the droplets was also plotted as a function of droplet temperature (Figure 33B). It was found that the viable LGG population in the three milk matrixes all remained at the initial level of 10^9 CFU g^{-1} for temperatures lower than 50°C. However, as the droplet temperature increased continually, the bacterial population in the skim milk and Ca-added milk droplets decreased by approximately 1 log CFU g^{-1}, from around 53 to 60°C, and then dropped sharply when the temperature overcame 60°C. In contrast, the Ca-aggregated milk-matrix better-protected LGG bacteria in the temperature range from 53 to 62°C. For this matrix, a significant loss of bacterial population was only observed during the fourth stage, when the droplet temperature approached the air temperature (~63°C). In addition, the bacterial inactivation as a function of droplet moisture content is shown in Figure 33C. The reduction of bacterial population cropped up when the droplet moisture content decreased to approximately 0.5 (kg kg^{-1}). Similarly as in Figure 33B, the Ca-aggregated milk exhibited better LGG

protection at the end of drying, i.e., when the droplet moisture content was lower than 0.3 kg kg^{-1}.

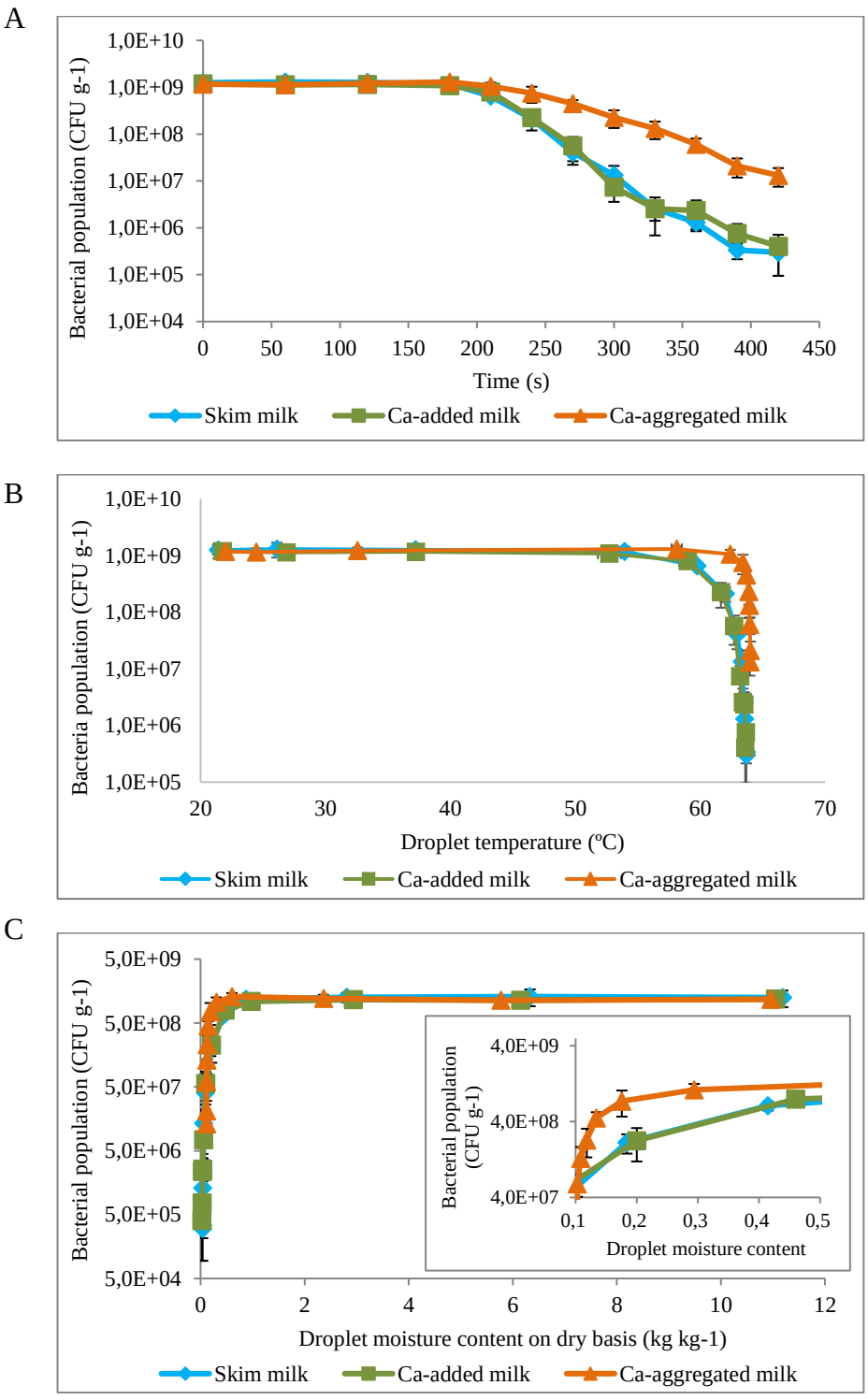

Figure 33. Inactivation of LGG in three bacteria-suspended milk matrixes (UHT skim milk, Ca-added skim milk, and Ca-aggregated skim milk) during single-droplet drying. (A) The bacteria population vs the drying time, (B) the bacteria population vs the droplet temperature, and (C) the bacteria population vs the droplet moisture content.

The inactivation of LGG during single droplet drying mainly rose when the droplet moisture content was lower than 0.5 kg kg^{-1} and the temperature reached above 60ºC. The Ca-aggregated milk also exerted its protective effects on LGG during this period. It is known that the inactivation of bacteria during single droplet drying is due to the thermal and dehydration stress (Schutyser et al., 2012). Previously, the Ca-aggregated milk has been found to protect LGG under thermal stress due to the better interaction between milk solids and bacterial cells and a more-stable extracellular environment during heat treatment (Huang et al., 2014). Such a mechanism could explain the protection of LGG bacteria by Ca-aggregated milk observed here at the end of thermal convective drying. In our previous work, we reported that the 10 mmol l^{-1} Ca^{2+} could improve the heat resistance of lactic acid bacteria (Huang and Chen, 2013). However, the skim milk with 10 mmol l^{-1} Ca^{2+} addition did not increase the survival of LGG in the present work. It should be noted that the Ca^{2+} added in milk was subjected to salt equilibrium between the soluble phase and casein micelles, ruled by HPO_4Ca solubility; it was then partially transferred to the casein micelles calcium phosphate nanoclusters instead of acting on the bacterial cells (Arifin et al., 2014; Mekmene et al., 2009).

The improved survival of LGG in the Ca-aggregated milk may also be attributed to its physicochemical properties and distinct drying behavior. The Ca-aggregated milk matrix before drying presented a higher viscosity compared to the untreated and Ca-added skim milk (Table 12). It has been assumed that the high viscosity of media could retard molecular mobility and reaction rate, hence stabilizing the biological system (Buitink et al., 2000). The Ca-aggregated milk was also relatively inhomogeneous and consisted of a network of large and insoluble aggregates that incorporated denatured whey proteins and entrapped part of water. This matrix may provide a local protective environment for the embedded bacterial cells under the dehydration and thermal stress (Hansen and Riemann, 1963; Potts, 1994). In addition, the droplet of Ca-aggregated milk had a longer second stage during evaporation (Figure 30); that is to say, it was maintained at a lower temperature. Conversely, it was faster to reach the final fourth stage to form the dried particle. It has been reported that the fast drying may induce instant fixation of the cells and thereby prevent the dehydration inactivation of bacteria (Perdana et al., 2013). The bacteria cells hence accumulated less dehydration inactivation within the droplet of Ca-aggregated milk in comparison to that of untreated and Ca-added skim milk. Besides, it has been observed that the bacteria cells are more heat-resistant in the dry state than in a hydrated environment (Xing et al., 2014; Zhang et al., 2014). When the temperature reached the harmful range (mostly the upper limit of the growth temperature, i.e., ~40ºC for

Lactobacillus), the Ca-aggregated milk provided a drier extracellular environment for LGG, likely to explain the better survival of LGG at the high-temperature range. Besides this and according to (Mezzenga et al., 2005; Sadek et al., 2015), the mechanical properties of the porous structure in the Ca-aggregated particle (Figure 32) should be soft and ductile, which may indicate that the bacteria has experienced less mechanical stress during the sol-gel transition and shrinkage of droplet. It might also explain the reason of the probiotic protection by the 30% sweet whey in the 2-in-1 process due to the similar morphology between powders dried from 30% sweet whey and the Ca-aggregated milk particle. This remains a hypothesis for further research.

3.3.2.5 Summary

To conclude, the protein aggregation occurring in the Ca-aggregated milk led to lower protein concentrations in the milk-serum phase and, conversely, protein enrichment of the casein micelle phase. This microstructure resulted in a more-waterlike drying kinetics, different feature of droplet-particle conversion (especially regarding the porosity of the shell formed), and a higher protective capacity on *L. rhamnosus* GG during single droplet drying.

This work may open new avenues for development of probiotic products with high bacterial viability and calcium enrichment, as well as a novel strategy for the optimization of drying efficiency and powder morphology in the dairy industry. The rehydration and digestion behaviour of powders from the Ca-aggregated milk await further investigation. On the other hand, it also provides a possible mechanism to explain the protection of the 2-in-1 process on probiotics by the presence of protein aggregates in highly concentrated sweet whey culture.

3.3.3 Contribution of minerals: a preliminary exploration with magnesium effect

A gentle reminder

In comparison to carbohydrates and proteins, the influence of inorganic salts on thermotolerance of probiotics has rarely been reported. Mg^{2+} is generally present in most of dairy products including the sweet whey ingredients used in this project. In bacteria, Mg^{2+} is the second-most abundant cation. It has been reported to influence thermotolerance of *Salmonella enterica* serovar *typhimurium*. However, the role of Mg^{2+} in the regulation of thermotolerance in other organisms still remains unclear. We thus address the impact of Mg^{2+} on thermotolerance of *Lactobacillus*.

3.3.3.1 Mg^{2+} improves bacterial survival after heat challenge

The survival of three probiotic *Lactobacillus* strains, *L. casei* Zhang (LCZ), *L. rhamnosus* GG (LGG) and *L. plantarum* P-8 (LP), after heat challenge, is shown in Figure 34. Treatment of bacteria with low concentration of $MgCl_2$ (5 ~ 100 mmol l^{-1}) in a 10% lactose solution was found to improve bacterial thermal tolerance. More specifically, 20 mmol l^{-1} $MgCl_2$ was the optimal concentration to enhance thermotolerance of LGG and LP (Figure 34A and C), while 10 mmol l^{-1} for LCZ (Figure 34B). In LGG, 20 mmol l^{-1} $MgCl_2$ addition led to a 100 fold higher survival rate upon heat challenge. However, a negative effect on thermotolerance of all three probiotic strains was observed when the concentration of $MgCl_2$ was higher than 500 mmol l^{-1}. By contrast, treatment of bacteria with $ZnCl_2$ and NaCl did not enhance the bacterial thermotolerance despite the use of high or low concentration. Moreover, the bacterial thermotolerance dropped sharply as a result of high concentrations of $ZnCl_2$ (> 100 mmol l^{-1}).

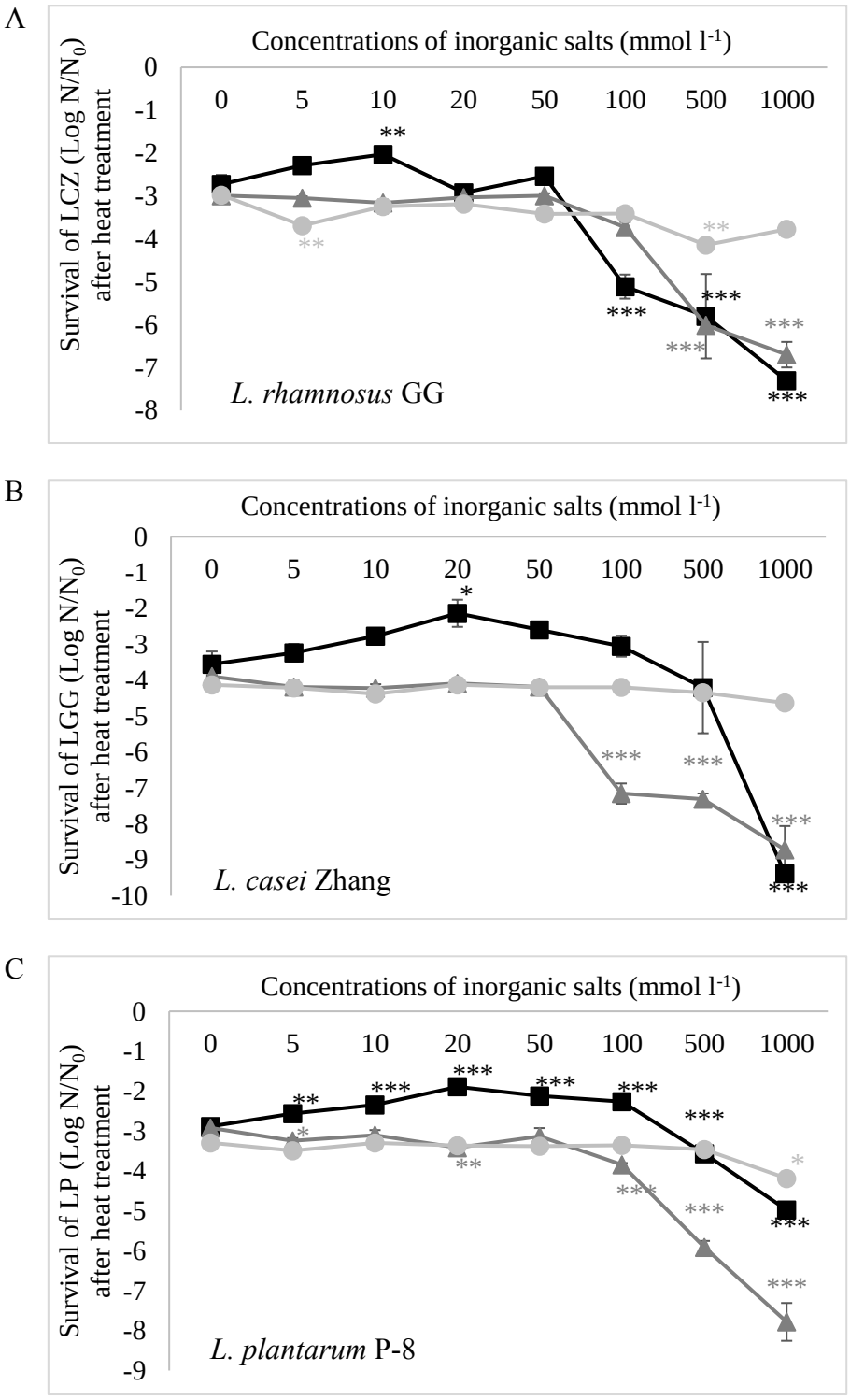

Figure 34. Survival (log N/N_0) of (A) *L. rhamnosus* GG, (B) *L. casei* Zhang and (C) *L. plantarum* P-8 in lactose solutions supplemented with different concentration of $MgCl_2$ (-■-), $ZnCl_2$ (-▲-) and NaCl (-●-) after heat treatment. Significant difference was compared between the salt-treated samples and the samples without adding salt, i.e. 0 mmol l^{-1} added (*: 0.01<P<0.05, **: 0.001<P<0.01, ***: P<0.001).

The results in Figure 34 show the different effects of $MgCl_2$, $ZnCl_2$ and NaCl on the survival rate of three bacterial strains after heat challenge. The comparison suggests the effects of $MgCl_2$ on bacterial thermotolerance should be attributed to the cation Mg^{2+}, instead of the anion chloride. The osmolality caused by salt supplementation (mostly by NaCl) may trigger thermotolerance in bacteria in lactose solution (De Angelis and Gobbetti 2004). However, this cross protection phenomenon was not induced by high concentration of salts in this work. It may be explained by the insufficient duration of the 30-minute salt treatment to induce the cellular stress response (Huang et al. 2016). Moreover, high concentration of salts (500 mmol l^{-1}) displayed negative effects on the survival of bacteria upon heat treatment. This may be caused by the extra osmotic stress apart from heat stress. Since there was only lactose in the suspension, the bacteria were not able to uptake the compatible solutes from the environment to reach cellular homeostasis.

The enhanced thermotolerance of *Lactobacillus* strains in this work is in agreement with the speculation proposed by O'Connor et al. (2009), who found that overproduction of the *B. subtilis* MgtE protein lead to enhanced thermotolerance of *Salmonella*. The genes encoding similar magnesium-transport proteins can be found in most of lactobacilli species and strains, *mgtA*, *mgtC* and *corA* in LGG for instance (Morita et al. 2009). It indicates that the Mg^{2+} effect on bacterial thermotolerance may be of interest for more other lactobacilli species and strains used as starter and adjunct cultures for many food fermentations. However, the possible interactions between the magnesium transport, homeostasis and heat shock response in *Lactobacillus* require further transcriptomics and proteomics investigation.

Besides, the effect of Mg^{2+} on improving bacterial thermotolerance observed here was similar to the Ca^{2+} effect reported previously regarding to the close range of effective concentration (Huang and Chen 2013). Indeed, Mg^{2+} is similar to Ca^{2+} regarding their roles in lactic acid bacteria, especially for their interactions with enzyme activity, cell division, surface proteins and nucleic acids (Boyaval 1989). This indicates that the distinct physicochemical properties of Mg^{2+} and Ca^{2+} may play significant roles in thermotolerance of *Lactobacillus*, particularly their abilities in binding and stabilizing biomolecules such as nucleic acids, proteins and phospholipid bilayer, and their influence on cell adhesion on environmental matrices or cell mutual adhesion (Burgain et al. 2014). These physicochemical properties may allow the effect of Mg^{2+} to be applicable to other bacterial species.

3.3.3.2 Mg^{2+} improves bacterial recovery of heat-injury

The regrowth activity of bacteria is characterized by lag phase duration (λ), and asymptotic value (A) from the regrowth curves (Table 13 and Figure 35). The definition of regrowth corresponds to the ability of *Lactobacillus* to grow after being exposed to a heat treatment of 1 min at 75°C. The methods of obtaining λ and A explained in the *Materials and Methods* (Figure 23 and Table 10, 3.2.2). Heat treatment caused extended λ and decreased A in the absence of salt addition in MRS. Interestingly, as a reference, MRS supplementation with $MgCl_2$ or NaCl can significantly shorten the heat-induced extension of lag phase duration $\lambda–\lambda_0$ (Table 13 and Figure 35). The optimal concentrations differed depending on the strain. For $ZnCl_2$ supplementation, only a low concentration (5 mmol l^{-1}) showed a positive effect on $\lambda–\lambda_0$ of LP strain, but not for LGG and LCZ strains. Regarding the asymptotic value (A) in the regrowth curves, the effects of inorganic salts differed largely depending on different strains. Specifically, the MRS supplementation with 5 to 50 mmol l^{-1} $MgCl_2$ and 10 to 20 mmol l^{-1} $MgCl_2$ showed a significantly enhanced $A-A_0$ values for LCZ and for LP, respectively, whereas no effect of $MgCl_2$ was shown on LGG. By contrast, low concentration of $ZnCl_2$ increased $A-A_0$ values of LGG and LP, while the NaCl supplementation increased $A-A_0$ values of LGG (5 mmol l^{-1}) and LCZ (5, 10 and 100 mmol l^{-1}).

Recovery of bacteria from sub-lethal injury requires de novo synthesis ribosomes and membrane (Wu 2008). Mg^{2+} can influence the nitrogen metabolism and enzyme activity of bacteria, which may relate to the protein synthesis after thermal injury (Boyaval 1989; O'Connor et al. 2009). Besides, Mg^{2+} can stabilize bacterial cell morphology and membrane integrity, which was found to relate to the heat shock response in *L. plantarum* WCFS1 (Capozzi et al. 2011). For instance, Rayman and MacLeod (1975), found that Mg^{2+} strengthen the bacterial cell wall by influencing the structure of peptidoglycan.

Mg^{2+} was also reported to promote the division of Gram-positive bacteria at certain concentrations, which may explain the better regrowth after being treated with Mg^{2+} (Hayek et al. 2013). However, the effect of Mg^{2+} on bacterial regrowth was strain-dependent as shown in Table 13. Moreover, certain concentrations of Na^+ and low concentration of Zn^{2+} also displayed a strain-dependent effect on promoting the regrowth of heat-injured bacteria. We speculated that the reason may be the interactions between Na^+ and Zn^{2+}, and bacterial enzyme activity and membrane integrity, which possibly affect the metabolism and transmembrane transport of nutrients in bacteria as suggested by Korkeala et al. (1992) and Omburo et al. (1992).

Table 13. The heat-induced changes of lag time duration $\lambda-\lambda_0$ and asymptotic value $A-A_0$ between the regrowth curves of *L. rhamnosus* GG, *L. casei* Zhang and *L. plantarum* P-8 in MRS with and without inorganic salt supplementation.

	Concentrations of mineral ions (mmol l^{-1})						
	0	5	10	20	50	100	500
				***L. rhamnosus* GG**			
				$\lambda-\lambda_0$ (h)			
$MgCl_2$		0.1±0.1^{c}	0.2±0.0^{cd}	**-0.2±0.1^{d}**	**-0.5±0.1^{b}**	0.3±0.1^{a}	ND
$ZnCl_2$	0	0.6±0.1^{a}	5.0±0.1^{b}	ND	ND	ND	ND
NaCl		**-0.5±0.1^{a}**	0.5±0.0^{d}	0.2±0.1^{c}	**-0.2±0.1^{b}**	**-0.3±0.1^{b}**	1.6±0.1^{e}
				$A-A_0$			
$MgCl_2$		0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	-0.2±0.0^{a}	ND
$ZnCl_2$	0	**0.1±0.0^{b}**	0.0±0.0^{a}	ND	ND	ND	ND
NaCl		**0.1±0.0^{c}**	-0.1±0.0^{a}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	-0.1±0.0^{a}
				***L. casei* Zhang**			
				$\lambda-\lambda_0$ (h)			
$MgCl_2$		**-0.7±0.1^{b}**	**-0.6±0.1^{b}**	**-2.5±0.2^{a}**	**-0.8±0.1^{b}**	1.7±0.1^{c}	ND
$ZnCl_2$	0	0.9±0.1^{a}	2.5±0.1^{b}	ND	ND	ND	ND
NaCl		**-1.2±0.2^{c}**	**-0.6±0.2^{d}**	**-2.4±0.3^{a}**	**-1.7±0.2^{b}**	**-1.4±0.2^{bc}**	**-0.6±0.2^{d}**
				$A-A_0$			
$MgCl_2$		**0.2±0.0^{c}**	**0.3±0.0^{d}**	**0.1±0.0^{b}**	**0.1±0.0^{b}**	-0.1±0.0^{a}	ND
$ZnCl_2$	0	0.0±0.0^{b}	-0.1±0.0^{a}	ND	ND	ND	ND
NaCl		**0.2±0.0^{e}**	**0.2±0.0^{e}**	-0.1±0.0^{a}	0.0±0.0^{c}	**0.2±0.0^{d}**	-0.1±0.0^{b}
				***L. plantarum* P-8**			
				$\lambda-\lambda_0$ (h)			
$MgCl_2$		0.0±0.1^{d}	**-0.8±0.1^{a}**	**-0.5±0.1^{b}**	**-0.3±0.1^{c}**	**-0.1±0.1^{d}**	ND
$ZnCl_2$	0	**-0.6±0.1^{a}**	5.2±0.1^{b}				
NaCl		**-0.5±0.1^{a}**	**-0.3±0.1^{bc}**	**-0.4±0.1^{ab}**	**-0.2±0.1^{c}**	**-0.5±0.1^{a}**	0.5±0.1^{d}
				$A-A_0$			
$MgCl_2$		-0.1±0.0^{a}	**0.1±0.0^{c}**	**0.2±0.0^{d}**	0.0±0.0^{b}	0.0±0.0^{b}	ND
$ZnCl_2$	0	**0.1±0.0^{b}**	0.0±0.0^{a}	ND	ND	ND	ND
NaCl		0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	0.0±0.0^{b}	-0.1±0.0^{a}

Different superscript letter represents significant difference from the values in the same lines, i.e. the samples treated with the same salts ($P < 0.05$).

'ND' represents undeterminable due to non-growth of bacteria after heat treatment

Data in bold means positive effect in regrowth (i.e. less extended lag time and less decreased biomass yield

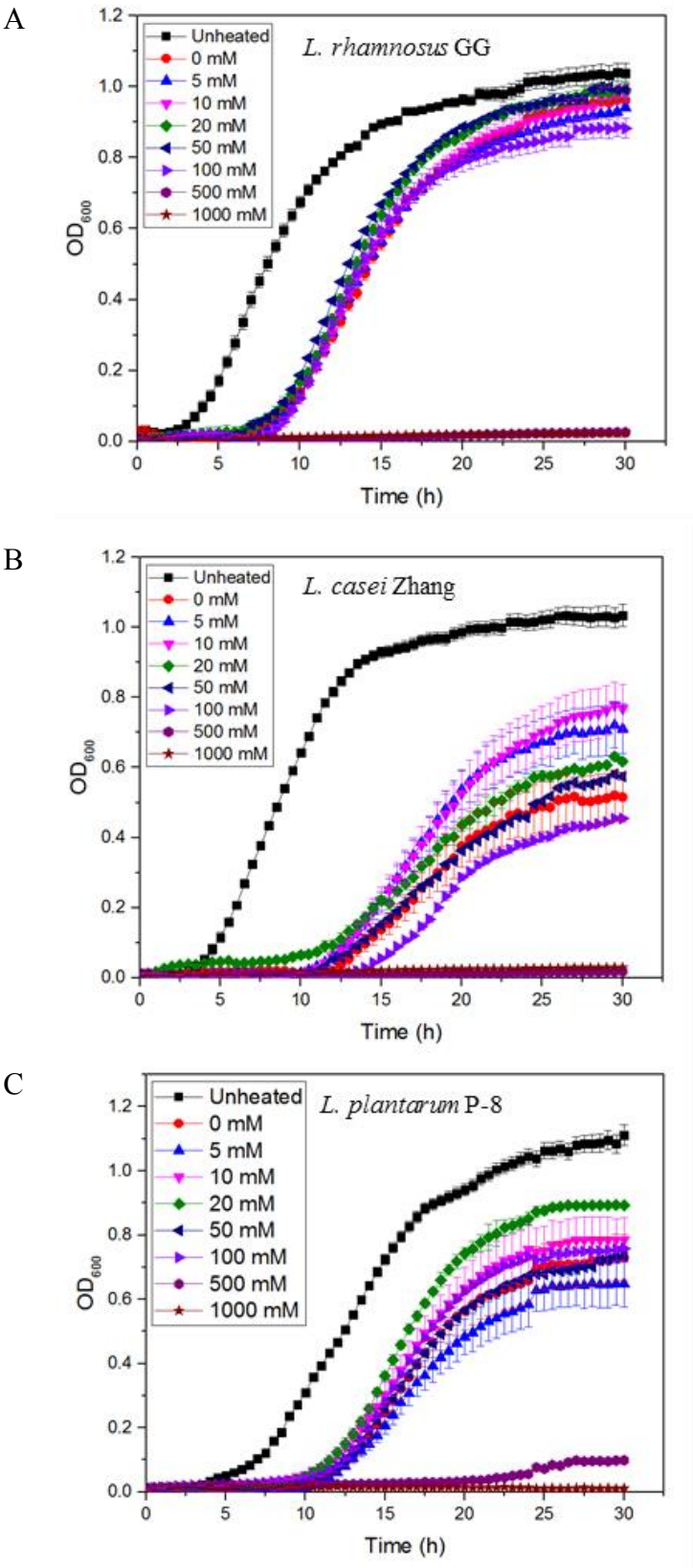

Figure 35. The regrowth curves of (A) *L. rhamnosus* GG, (B) *L. casei* Zhang and (C) *L. plantarum* P-8 grown in MRS broth containing different concentrations of $MgCl_2$ after heat challenge. The curve of bacteria grown in MRS broth before heat challenge (i.e. -■- Unheated) is provided as a reference.

3.3.3.3 Summary

To conclude, Mg^{2+} ranging from 10 to 50 mmol l^{-1} exerted a general improvement on the thermotolerance of the three lactobacilli strains. The reason may be the effect of Mg^{2+} on the recovery of bacteria from heat injury. These results confirm the important roles of mineral salts in probiotic physiology. It should be noticed that, apart from carbohydrates and proteins, certain mineral salts existing in foods could also be used as potential protectants for probiotics during thermal processing, such as spray drying. Besides, this work indicates that the effect of Mg^{2+} may also attribute to the probiotic protection of 2-in-1 process because of the improved concentration of Mg^{2+} in highly concentrated sweet whey culture (specifically, the concentration of total magnesium in 30% sweet whey is around 20 mmol l^{-1} as calculated from the value in Table 7).

3.4 Conclusion

In this Chapter, we attempted to get insight into the mechanisms of the probiotic protection effect of the 2-in-1 process.

Firstly, the stress response of bacteria during growth in highly concentrated sweet whey could contribute to the enhanced tolerance of bacteria against harsh conditions during spray drying. More specifically, cellular osmoregulation occurs when bacteria adapt to the hypertonic condition in highly concentrated sweet whey. This osmoregulation includes the uptake or synthesis of compatible solutes inside cells and the induction of osmotic and general stress proteins, leading to cross-protection. Hence, the bacteria in highly concentrated sweet whey display a multistress tolerant phenotype in comparison to the bacteria grown in isotonic medium, leading to enhanced survival under the heat, osmotic, oxidative and dehydration stresses during spray drying. Besides, we also explored the effect of magnesium salt ($MgCl_2$) on the thermotolerance of lactobacilli. The concentration of Mg^{2+} ranging from 10 to 50 mmol l^{-1} was found to improve the bacterial survival under heat stress and to promote the bacterial recovery from heat injury. It suggests that the influence of mineral salts on bacterial physiology should not be ignored during thermal processing. Given the range of Mg^{2+} concentration in highly concentrated sweet whey, it may be another factor which contributes to the protection in the 2-in-1 process.

In addition to the effect of matrix composition, the presence of protein aggregates in medium also showed a positive effect on improving bacterial survival during thermal convective single-droplet drying, which highlights the importance of matrix sturcture. These aggregates may moderate the excellular environment of bacterial cells during droplet-particle transition through influencing the drying kinetics and/or particle formation.

Overall, the 2-in-1 process introduced in Chapter 2 is a complex system combining bacterial growth and thermal convective drying. Therefore, the final viability of probiotics in powders is thus influenced by multiple factors including growth conditions, feed physiochemical properties, droplet-particle transition.

Chapter 4. Scale-up of the 2-in-1 Process in Semi-Industrial Scale Spray Drying

In this chapter, the feasibility of scaling up the 2-in-1 process was validated with a semi industrial pilot scale spray dryer. A multi-stage mild-conditions drying process, coupling spray drying with belt drying and fluid-bed drying, was therefore applied in this work, in which the final probiotic survival was improved to approximately 100% (> 10^9 CFU g^{-1}).

Probiotic viability in the powders was monitored during a 6-months storage, which indicated that storage temperature and moisture content of powders both played crucial roles in the stability of probiotic powders. Moreover, the functionalities of spray-dried probiotic powders were also investigated. Spray drying afforded a strain-dependent enhancement of bacterial survival in simulated intestinal fluid, in comparison with fresh cultures.

The main contents in this Chapter have been published as:

Huang, S., Méjean, S., Rabah, H., Dolivet, A., Le Loir, Y., Chen, X.D., Jan, G., Jeantet, R., Schuck, P., 2017. Double use of concentrated sweet whey for growth and spray drying of probiotics: Towards maximal viability in pilot scale spray dryer. J. Food Eng. 196, 11–17.

Huang, S., Cauty, C., Dolivet, A., Le Loir, Y., Chen, X.D., Schuck, P., Jan, G., Jeantet, R., 2016a. Double use of highly concentrated sweet whey to improve the biomass production and viability of spray-dried probiotic bacteria. J. Funct. Foods 23, 453–463.

4.1 Introduction

Why did we investigate the scale-up feasibility of the 2-in-1 process?

Scale-up is a term in Chemical Engineering, which generally means the migration of a process from the laboratory scale to the pilot-scale, and further to the industrial scale. To validate the scale-up feasibility an innovation process is of prime importance for its industrial application and further development. As scaling factors and laws are generally poorly known, this represents often the bottleneck of innovation.

The studies on spray drying of probiotics, that were mostly performed on laboratory-scale spray dryers, have been reviewed in Chapter 1 (Table 3). To the best of our knowledge, two studies mentioned experiments on a larger scale with a water evaporation capacity in the range 100 to 1645 kg h^{-1} (Bielecka and Majkowska, 2000; Schuck et al., 2013). In comparison to laboratory-scale spray drying, spray drying at pilot-scale or industrial scale should pay more attention to concerns about the mixing and homogeneity of feed, pumping, atomization, contamination, etc. On the other hand, the conditions of large scale drying, such as the generally longer residence time may also differ the results of probiotic viability in powders which are obtained from lab-scale drying. However, it is still ambiguous to conclude on the influence of scaling-up on probiotic viability since the very few studies are available at larger scale drying.

Regarding the 2-in-1 process, to investigate the scale-up feasibility will help to address the following questions:

1. Do the bacteria grow in highly concentrated sweet whey in large scale (i.e. concerns on the mass transfer of nutrients during static fermentation)?
2. Can bacteria maintain stability during large scale drying (as residence time distribution of particles is dependent of the dryer type and scale)?
3. Does the presence of aggregates impede the pumping and atomization?
4. Overall, does scaling up influence the bacterial viability in the final powders?

Why did we incorporate multi-stage drying in the process?

In order to minimize the heat inactivation of bacteria during spray drying, the drying temperature can be moderated through technical innovation or process optimization, multi-

stage drying process for instance Schuck et al., (2013) has previously designed a multi-stage drying process for the production of *Propionibacterium acidipropionici* powders. In this process, the bacteria were cultivated in 13.5% (w/v) acid whey medium and then concentrated by membrane ultrafiltration and powder addition prior to the spray drying. The spray dryer was coupled with a crystallizer belt followed by a vibro-fluidizer. Due to the contribution of the crystallizer belt and vibro-fluidizer stages to the overall water elimination, the first drying stage could be subsequently limited, which allowed the setting of spray drying inlet temperature at 130°C and outlet temperature at 60°C, thus minimizing bacteria death.

However, it is still a prerequisite to provide the concentrated feed material for the first spray drying stage in order to form the semi-dried but shell-formed particles, which are further dried on the crystallization belt and fluidized bed. Regarding the 2-in-1 process, the probiotics were already cultivated in highly concentrated sweet whey with 30% TS. Use of this culture with high dry matter provided the possibility of skipping the intermediate concentration step. In other words, the 2-in-1 process allowed probiotic fermentation to be directly coupled with the multi-stage drying process, which may achieve the sustainable continuous production of high-viability probiotics.

Is viability the only criteria to assess the powder quality?

The main output and challenge of probiotics spray drying is generally considered as the preservation of bacterial viability during the drying process. In most studies, the viability after spray drying refers to the culturable cell counts on the growth agar medium within a certain time (Papadimitriou et al., 2016). Although this definition of viability can well represent the amount of residual viable probiotics in the powders, it should not be regarded as the only criterion to assess the quality of probiotic powders.

Probiotics within the powders should be able to pass through a long-term storage at low temperature (2~8°C for most of commercial dairy products), or even at more challenging temperature (ambient temperature at 25°C for instance) (Tripathi and Giri, 2014). Last but not least, the probiotics rehydrated from powders should be resistant to the harsh stress during digestion for several hours from gastric to intestinal tract to guarantee the delivery efficiency and expected functionalities of probiotics (Cook et al., 2012). Moreover, the probiotics rehydrated from powders should be challenged on their effective beneficial properties to the host before validation of a drying processing sheme for their obtention. This point is of outmost

importance, although rarely investigated and reported to date in the literature (only 2 papers mentioning this point, see Perspectives).

4.2 Materials and Methods

4.2.1 Bacterial strains and starter preparation

Lactobacillus casei BL23 and *Propionibacterium freudenreichii* CIRM-BIA 129 were used in this work. *L. casei* was activated in MRS Broth and static cultivation at 37ºC for 16 h. *P. freudenreichii* was activated in YEL broth and cultivated statically at 30ºC for 50 h.

Sweet whey medium with 30 wt% total solid content was prepared by rehydration of sweet whey powder (Lactalis ingredients, Mayenne, France) in deionized water. For preparation of starter culture, the 5 L sweet whey was autoclaved at 100°C for 30 min before inoculation of *L. casei* or *P. freudenreichii*. The inoculation (1 v/v% inoculum size) was made from the above-mentioned pre-culture of *L. casei* in MRS broth or of *P. freudenreichii* in YEL broth. The inoculated sweet whey culture of *L. casei* was incubated statically at 37°C for 30 h, and *P. freudenreichii* at 30°C for 72 h. The obtained culture was used as starter for further fermentation.

4.2.2 Fermentation with sweet whey

Sweet whey medium for fermentation at the semi industrial pilot scale was prepared in a steel tank (Goavec, Alençon, France) by rehydrating 150 kg sweet whey powders in 350 kg water to obtain 30 wt% total solid content. The medium was then pumped through a scraped surface heat exchanger (HRS Heat Exchangers, France) for heat treatment. The heating temperature and the residence time of sweet whey medium within the heat exchanger were 120°C and 1 min, respectively. The heated sweet whey medium was then transferred into a 500 L bio-reactor (Goavec, Alençon, France). The pipes and the bio-reactor were previously treated by steam. The above-mentioned 5 L starter culture was inoculated after the temperature of sweet whey had been cooled down to the setting growth temperature (i.e. 37°C for *L. casei* and 30°C *P. freudenreichii*). The sweet whey with *L. casei* was fermented statically at 37 °C for 48 h, and *P. freudenreichii* at 30°C for 96 h.

4.2.3 Spray drying

Before spray drying, the sweet whey probiotic culture was agitated moderately for 20 min. Three processes of spray drying were performed for each probiotic strain (Figure 36).

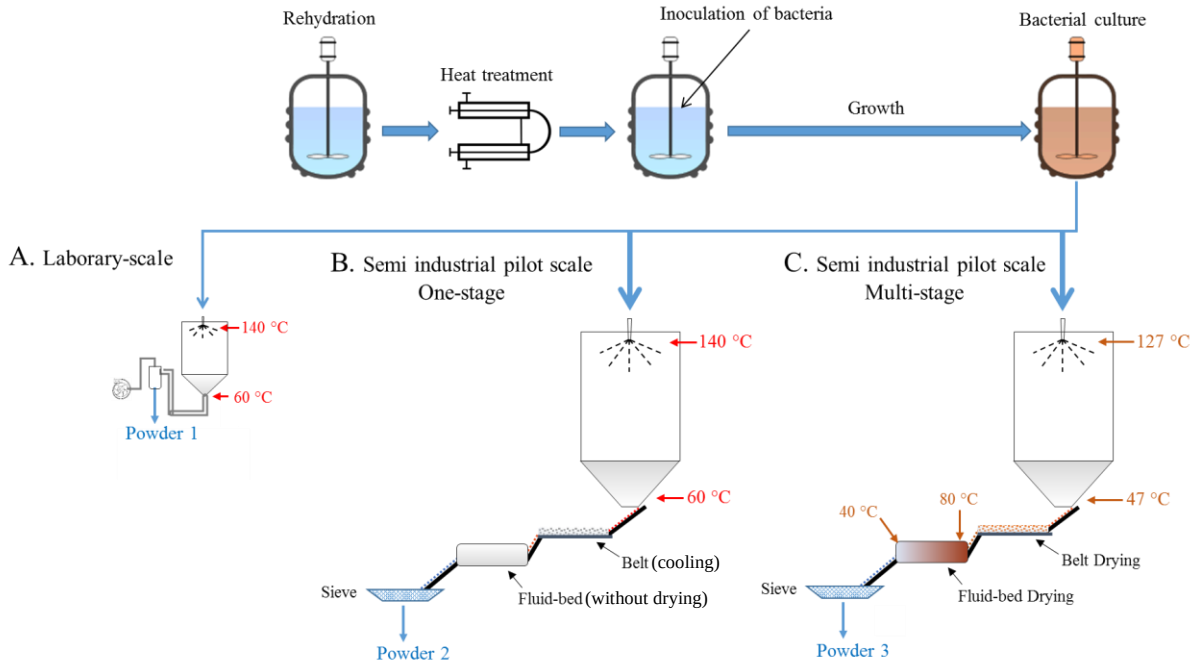

Figure 36. The schematic diagram of three drying process: (A) Lab-scale spray drying (for obtaining Powder 1), (B) Semi industrial pilot scale one-stage spray drying (for obtaining Powder 2) and (C) Semi industrial pilot scale multi-stage spray drying (for obtaining Powder 3).

For the lab-scale spray drying (Figure 36A), 1 L sweet whey culture was pumped to a Mobile Minor™ spray dryer (GEA Niro A/S, Denmark). A two-fluid spray nozzle with an orifice diameter of 0.8 mm was used. The evaporation rate of this dryer was approximately 3 kg h^{-1}. The inlet air temperature was at 140 ± 1°C, and the outlet air temperature 60 ± 3°C. The one-stage semi industrial pilot scale spray drying (Figure 36B) was performed in the Bionov spray dryer pilot workshop (Niro Atomizer, GEA, Saint Quentin en Yvelines, France) based in Rennes (France). A pressure nozzle with an orifice diameter of 0.73 mm was used. The evaporation capacity was approximately 80 kg h^{-1}. The drying temperatures were set to the same values as in minor dryer, namely inlet air temperature at 140 ± 5°C and outlet air temperature at 60 ± 3°C. A belt (GEA, Saint Quentin en Yvelines, France) and a vibro-fluidizer (GEA, Saint Quentin en Yvelines, France) were used following the spray drying step (Figure 36B), but respectively for conveying (turn-off status) and powder cooling purposes in this configuration. The multi-stage semi industrial pilot scale spray drying (Figure 36C) was also performed using the Bionov spray dryer pilot workshop, as described before (Schuck et al., 2013). Briefly, the inlet air temperature of spray dryingwas decreased to 127 ± 3°C, and the outlet air temperature 47 ± 2°C. After spray drying, the partially dried powder was delivered through the belt dryer at ambient temperature for crystallization purpose. The residence time

of powders on belt was approximately 5 min. Fluid-bed drying was then carried out in a vibro-fluidizer (VF) with inlet temperature at 80 ± 2°C and outlet temperature at 40 ± 2°C.

4.2.4 Analysis of physical and chemical properties

For liquid samples, the pH value was measured with a pHmeter (Ecolab, Issy-l_es-Moulineaux, France). Viscosity measurements were performed using an AR 2000 rheometer (TA instruments, Guyancourt, France) equipped with coaxial cylindrical geometry (stator inner radius: 25 mm; rotor outer radius: 23 mm; immersed cylinder height: 30 mm; bottom cap: 4000 mm). Apparent viscosity was determined at 20°C using the HerscheleBulkley model at a shear rate of 1 s^{-1}.

The water content of liquid or powder samples was measured according to the method described by Schuck et al. (2012): the sample (respectively 5 g for liquid samples and 1 g for powder samples) was mixed with 25 g pre-dried sand and then dried at 105°C for 7 h (liquid samples) or 5 h (powder samples). The water activity of powders was determined at 25°C using an a_w-meter (Novasina, aw-center 92T0003) immediately after drying and cooling.

4.2.5 Storage of powders

The powders were collected and aliquoted in PA/PE plastic vacuum bags (La Bovida, France). The bags were then sealed in the presence of air or under vacuum conditions (Britek, France) respectively. These powders were stored at the controlled temperature of 4°C and 25°C and kept away from light.

4.2.6 In vitro simulated digestion

The effect of spray drying on the probiotic tolerance against digestion stress was investigated in the *in vitro* simulated digestion experiment. The powders obtained from multi-stage drying process (Figure 36C; powder 3) were used to compare with the fresh probiotic culture in 30% sweet whey. The powders were rehydrated to reconstitute the 30% probiotic suspension. The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to Minekus et al. (2014). In the gastric phase, the probiotic samples were mixed with SGF (1:1 v/v) containing 2000 U mL^{-1} porcine pepsin, and incubated at 37°C for 3 h under agitation. In the intestinal phase, the gastric digesta were mixed with SIF (1:1 v/v)

containing 10 mmol l^{-1} porcine bile extract and porcine pancreatin with 100 U mL^{-1} trypsin activity, and incubated at 37°C for 3 h under agitation. The pH for the gastric and intestinal phases were adjusted to 3.0, and 7.0 respectively. The viability of probiotics during simulated gastric or intestinal digestion were tested in 1 h interval.

4.2.7 Enumeration and quantification of bacteria

The enumeratioin and quantification of bacteria were performed as described in Item 2.2.8. Briefly, the populations of probiotics in liquid samples were serial diluted with peptone water (0.1% w/v) and then poured onto MRS or YEL agar plates. The powder samples were rehydrated by dissolving 3 g of powder in 7 g of peptone water prior to serial dilution. *L. casei* was incubated at 37 °C for 48 h and *P. freudenreichii* at 30 °C for 6 days under anaerobic conditions for colony counting (Anaerocult®, Merck KgaA, Germany). Percent survival was then determined by comparing bacteria population before and after spray drying or simulated digestion treatment.

Besides, total thermophilic and coliform flora populations were evaluated according to the methods described previously (Schuck et al., 2013).

4.2.8 Statistical analysis

All the results displayed in this work were obtained from triplicate samples. The results are presented as mean value with standard deviation. The analysis of variance (ANOVA) followed by Tukey test was performed using R software with the ‘Rcmdr’ package (R Development Core Team). Differences between mean values were considered significant when $p < 0.05$.

4.3 Results and Discussion

4.3.1 Validation the feasibility of scaling up the 2-in-1 process

The bacterial populations of *L. casei* and *P. freudenreichii* after fermentation in 500 L 30% sweet whey were $(1.6 \pm 0.1) \times 10^9$ and $(5.0 \pm 0.6) \times 10^9$ CFU mL^{-1}, respectively. These results are in agreement with our previous work at laboratory-scale described in Item 2.3.1 (1–2 L): it validates that the two probiotic strains are able to yield high biomass production at a semi industrial pilot scale when cultivated in 30% sweet whey without casein peptone supplementation. Besides, the viscosity of sweet whey significantly decreased after being fermented by both probiotic strains (Table 14), due to the lactose consumption during fermentation, subsequent mass transfer to the biomass and last biomass decantation. More specifically, the large amount of lactose, which contributes considerably to the viscosity of sweet whey, was mainly metabolized into lactic acid and propionic acid by *L.* casei and *P. freudenreichii*, respectively. The control of the viscosity of feed at a given total solid content is generally desired by industries in order to facilitate atomization during spray drying (Cal and Sollohub, 2010).

Table 14. The physical properties of feed concentrates and powders.

Sample	pH	Viscosity (mPa·s)	Water Content w/w%	Water activity
Heat treated media	6.3±0.1	33.0±0.2	70.7±0.1	-
L. casei culture	4.4±0.1	22.0±0.4	71.0±0.0	-
Powder 1	-	-	5.3±0.2[a]	0.27±0.02[a]
Powder 2	-	-	5.2±0.2[a]	0.27±0.01[a]
Powder 3	-	-	5.5±0.1[a]	0.28±0.01[a]
P. freudenreichii culture	5.7±0.0	23.0±0.1	71.2±0.0	
Powder 1	-	-	5.0±0.2[a]	0.27±0.01[a]
Powder 2	-	-	5.2±0.1[a]	0.27±0.01[a]
Powder 3	-	-	6.0±0.2[b]	0.30±0.01[a]

The different superscript indicates the significant difference ($p < 0.05$).

Spray drying was carried out in three different processes as illustrated in Figure 36. The powders obtained from the semi industrial pilot scale process and lab-scale drying were non-significantly different regarding water content and water activity, except for the water content of *P. freudenreichii powder 3* (Table 14). It may be caused by the variation of drying parameters during process (Písecký, 2012). Nevertheless, the water contents and water activities were at the whole within a same range, so that the viability results could be compared between different processing shemes.

For the two probiotic strains, the remaining viabilities after lab-scale and one-stage semi industrial pilot scale spray drying (Figure 36A and B) were not significantly different, the survival being at around 60% for *L. casei* (remaining population ~1.0 × 10^9 CFU g^{-1}) and 100% for *P. freudenreichii* (remaining population ~4.5 × 10^9 CFU g^{-1}) (Figure 37). The comparison between two strains indicated that the robustness of *P. freudenreichii* upon spray drying was higher than that of *L. casei*. It is known that *P. freudenreichii* can accumulate intracellular trehalose, glycogen and polyphosphate, which are involved in improving bacterial tolerance against heat and/or desiccation stress (Anastasiou et al., 2006; Leverrier et al., 2004). This thermotolerance acquisition ability is in agreement with the ability of *P. freudenreichii* to withstand harsh conditions of Swiss-type cheese making, including thermal treatment (52°C, 30–60 min) and saline stress caused by immersion (48–72 h) in saturated brine. These results were fully consistent with the bacterial survival previously obtained at lab-scale spray drying (Item 2.3.3), albeit survival was further increased due to the lower drying temperature used in this work. Therefore, the feasibility of scaling up the 2-in-1 process (i.e., using highly concentrated sweet whey to grow and spray probiotics) is validated by this work.

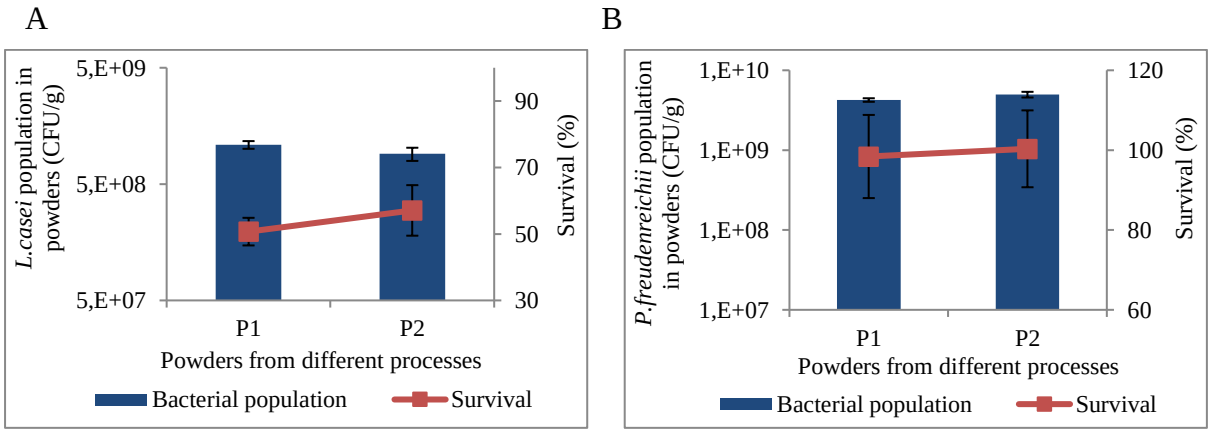

Figure 37. Survival of probiotics after spray drying at lab-scale or semi industrial scale. Bacterial population (refers to column, left Y-axis) and survival (refers to curve, right Y-axis) of (A) *L.casei* BL23 and (B) *P.freudenreichii* ITG20 after spray drying. P1 refers to the powders from lab-scale spray drying, and P2 refers to that of from semi industrial pilot scale.

4.3.2 Improvement of probiotic viability through multi-stage drying process

When using the multi-stage semi industrial pilot scale spray drying (Figure 36C), the probiotic survival was significantly improved to approximately 100% ($p < 0.05$) for *L. casei*, and slightly improved for *P. freudenreichii* ($p < 0.2$) (Figure 38). This process was previously conducted on two *P. acidipropionici* stress-tolerant strains (Schuck et al., 2013). However and given the robustness of the two strains, the survival of bacteria was shown to be 100% throughout the whole process. In this work, the multi-stage semi industrial pilot scale spray drying was performed for the first time on *L. casei*, which represent commonly commercialized and more fragile probiotics. The survival of *L. casei* was increased from 60% to 100%. Compared to the single-stage drying mode, the particles containing probiotics suffered here less heat stress at the first stage of spray drying as the outlet temperature was lowered from around 13°C. In accordance, the viability of bacteria was higher at the end of spray drying (Zhang et al., 2016). Although the bacteria experienced another heat stress at the second stage of fluid-bed drying (80°C inlet temperature and 40°C outlet temperature), the cells were already embedded in the partially dried powders. Moreover, it has been reported that the heat resistance of bacteria in a dry state, especially in the a_w range of 0.3–0.5, was higher than that of the same bacteria in a wet state (Laroche et al., 2005). Therefore, this process is more suitable for such heat sensitive bacteria, in comparison with the one-stage semi industrial pilot scale spray drying.

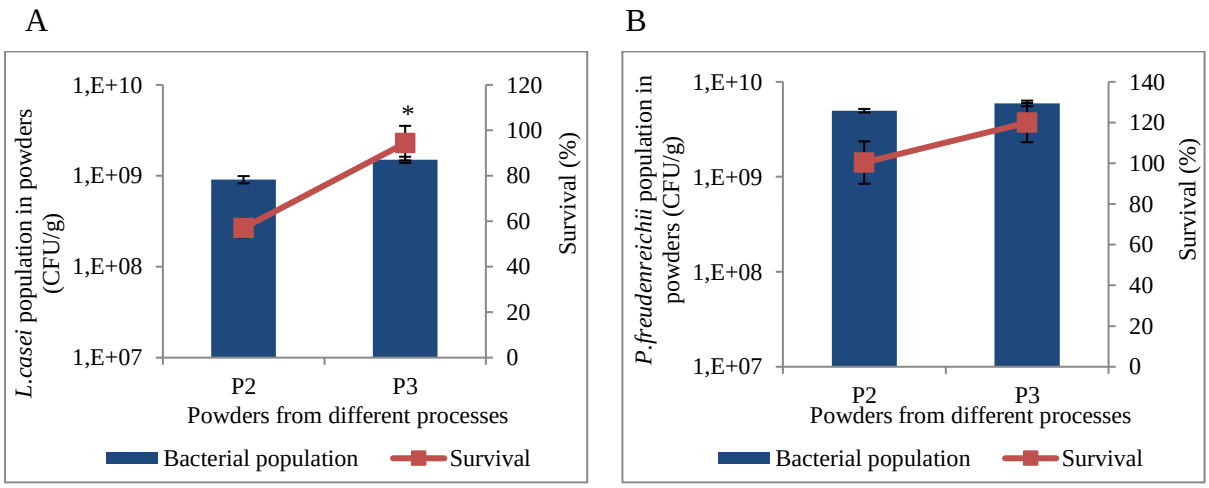

Figure 38. Survival of probiotics after one-stage spray drying or multi-stage drying at semi industrial scale. Bacterial population (refers to column, left Y-axis) and survival (refers to curve, right Y-axis) of (A) *L.casei* BL23 and (B) *P.freudenreichii* ITG20 after spray dryingin the semi industrial pilot scale. P2 refers to the powders from one-stage spray drying, and P3 refers to that of from multi-stage drying process. * means significant difference between the survival rates ($p<0.05$)

In addition to the targeted probiotic strains, thermophillic and coliform flora populations were also evaluated in the powders after spray drying as indicators of contamination. Thermophilic and coliform floras represent common microbiological spoilage in dairy powders and constitute a major concern in controlling the safety of foods at industrial scale production (Breeuwer, 2014; Chandan et al., 2015). As shown in Table 15, low amount of thermophilic and coliform floras initially contaminated the sweet whey medium, despite the heat treatment applied and the previous disinfection of the equipment: the conditions may not have been strict enough to achieve a fully aseptic medium. However, the bacterial population of thermophilic and coliform floras kept constant without break out during probiotic fermentation, probably due to the competitive inhibition (Verschuere et al., 2000; Wilderdyke et al., 2004). After spray drying, the coliform flora was undetectable in the powders but the thermophilic flora still remained. This indicates that the coliform flora is sensitive to spray drying stress, while the thermophilic flora is more resistant during spray drying probably due to its higher heat resistance. Therefore, more attention should be paid on controlling the thermophilic flora before spray drying.

Table 15. The enumeration of contaminating flora (thermophillic and coliform floras)

Sample	Thermophillic flora (log CFU g^{-1})	Coliform flora (log CFU g^{-1})
Heat treated media	2.6±0.1	2.8±0.0
L. casei culture	2.4±0.0	2.8±0.1
Powder 1	2.6±0.0	ND
Powder 2	2.4±0.1	ND
Powder 3	2.2±0.2	ND
P. freudenreichii culture	1.9±0.6	2.0±0.2
Powder 1	1.4±0.6	ND
Powder 2	1.4±0.6	ND
Powder 3	0.9±0.2	ND

ND means undetectable in plate agar counting method

4.3.3 Storage stability of probiotic powders

Probiotics stability upon storage was compared among the powders obtained from different drying processes. For *L. casei* at 4°C (Figure 39A), the viability kept constant during the first 2 months. A one log CFU g^{-1} reduction of viability was reached after 6 months storage. In contrast, the *L. casei* viability decreased gradually after 7 days at 25°C (Figure 39B). Within one month, the viability loss was around 2 logs CFU g^{-1} whatever the drying process and scale considered. After 6 months storage, the *L. casei* viability remained at approximately 4 logs CFU g^{-1}. Moreover, the conditioning environment (vacuum or atmosphere) had no significant effect on the stability of *L. casei* powders at both 4°C and 25°C (Figure 39).

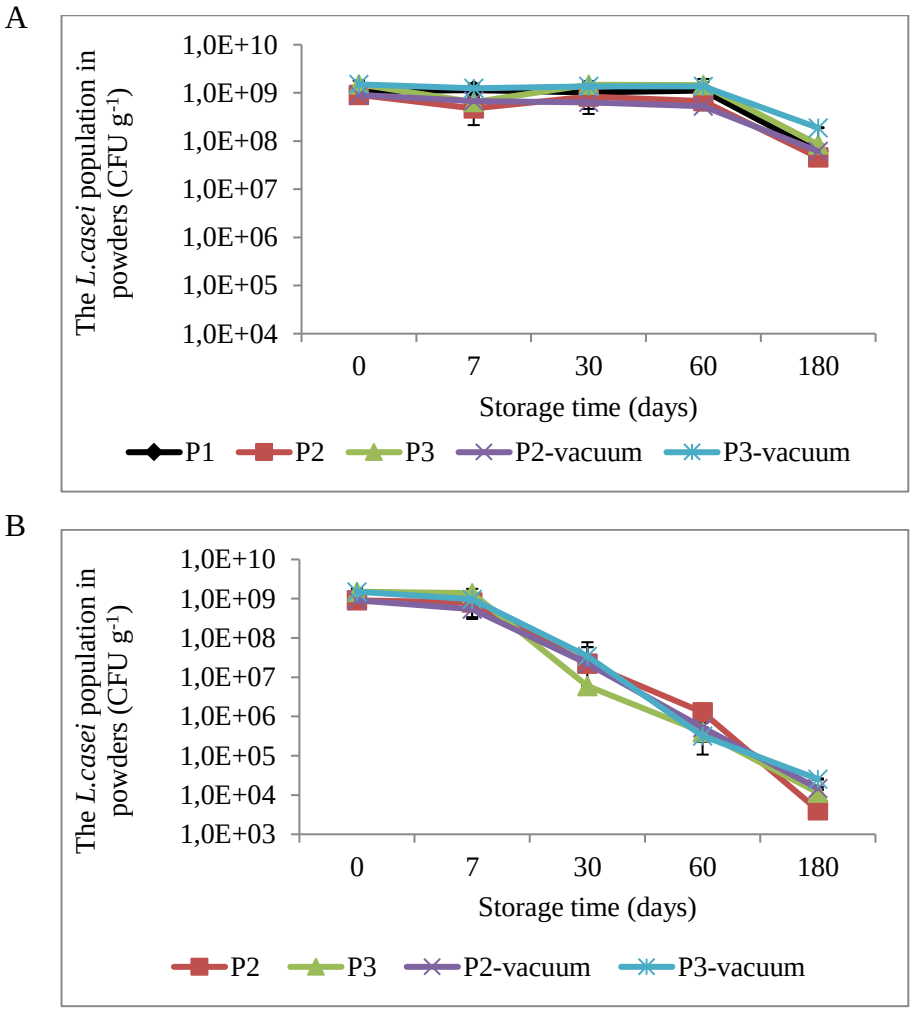

Figure 39. Storage stability of *L.casei* BL23 powders from different drying processes (expressed as reduction of survival) during 6 months storage at (A) 4°C and (B) 25°C. P1 refers to the powders from lab-scale spray drying, P2 refers to that of from one-stage spray drying at semi industrial pilot scale, and P3 refers to that of from multi-stage drying process at semi industrial pilot scale. P2-vacuum and P3-vacuum mean the powders stored under vacuum.

Similarly, the viability of *P. freudenreichii* in powders also kept constant at 4°C for the first 2 months and reduced by around 1 log CFU g^{-1} after 6 months storage whatever the drying process considered (Figure 40A). However, at this time, the powders from pilot scale one-stage drying process (P2) displayed slightly better stability when conditioned under vacuum. At 25°C, a larger viability loss was also observed in all powders, reaching around 5 logs CFU g^{-1} after 6 months storage under these conditions (Figure 40B).

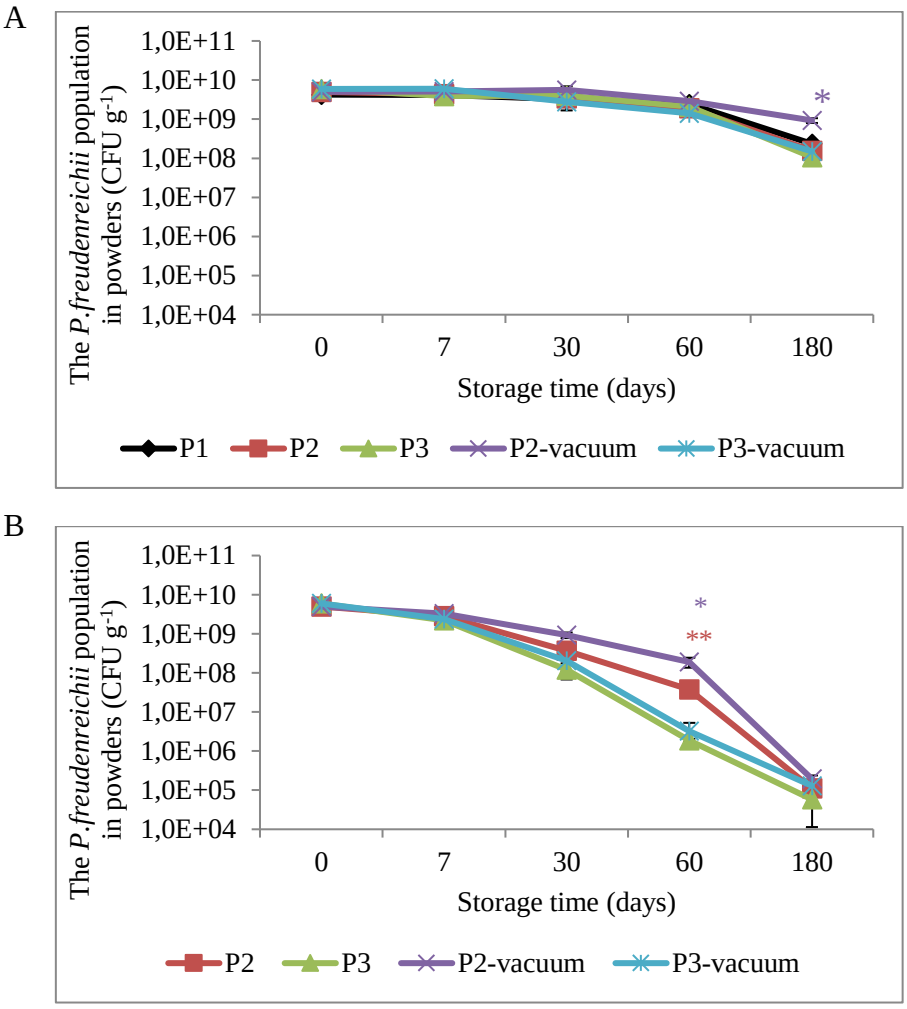

Figure 40. Storage stability of *P. freudenreichii* ITG20 powders from different drying processes (expressed as reduction of survival) during 6 months storage at (A) 4°C and (B) 25°C. P1 refers to the powders from lab-scale spray drying, P2 refers to that of from one-stage spray drying at semi industrial pilot scale, and P3 refers to that of from multi-stage drying process at semi industrial pilot scale. P2-vacuum and P3-vacuum mean the powders stored under vacuum. Presence of * means significant difference in population compared to other groups at the same storage time ($p < 0.05$). Different number of * indicates significant difference between each other ($p < 0.05$).

This result highlights the predominant influence of the storage temperature on the stability of probiotic powders. Moreover, the *P. freudenreichii* P2 powder stored under vacuum at 25°C showed a better stability in the first 2 months, compared to same powder stored under regular atmosphere: indeed, the corresponding viability losses at this time were lower than 1 log CFU g^{-1} and approximately 2 logs CFU g^{-1}, respectively. By comparison, the viability of *P. freudenreichii* in powders was more stable than that of *L. casei* in powders. Such a difference may be attributed to the better ability of *P. freudenreichii* cells to accumulate energy and carbon storage compounds such as polyphosphate, trehalose, glycogen, glycine betaine (previously discussed in Item 3.3.1), as well as the absence of their lysis during stationary either dormant phases, which allows long-term survival even under carbon starvation (Aburjaile et al., 2016; Falentin et al., 2010a).

Last, we observed a significant better stability of *P. freudenreichii* in P2 powders compared to P3 powders at 6 months storage under 4°C and at 2 months storage under 25°C. This could be explained by the detrimental effect of higher water content in P3 powders (Table 14) (Vesterlund et al., 2012). Besides, storage under vacuum improved the stability of *P. freudenreichii* powders at both 4 and 25°C, but not that of *L. casei* powders. The reason may be the facultative anaerobic phenotype of *L. casei*, leading to oxidative stress tolerance. However, storage under vacuum did not make it possible to enhance the *P. freudenreichii* viability in the P3 powder. It may indicate that the influence of moisture is more detrimental than the oxidation on the stability of probiotic powders in long-term storage.

4.3.4 Resistance of rehydrated probiotics to simulated digestion

The simulated digestion of probiotic powders from pilot scale multi-stage drying process (P3 powders) was investigated in comparison with the fresh 30 w/w % sweet whey culture (Figure 41). The results showed that both strains were resistant to the simulated gastric fluid (SGF) within 3 h regardless of the rehydrated or fresh culture type. It may be explained by the large amount of whey proteins in concentrated sweet whey (around 2.6 w/w %), which can protect bacteria from digestion stress through its buffer effect (Chen et al., 2006; Doherty et al., 2011; Tavares et al., 2014). Besides, it has also been previously reported that the osmolality of concentrated sweet whey could trigger the multi-stress tolerance of bacteria during growth, leading to the higher survival of bacteria under acid and bile salt stresses (discussed in Item 3.3.1).

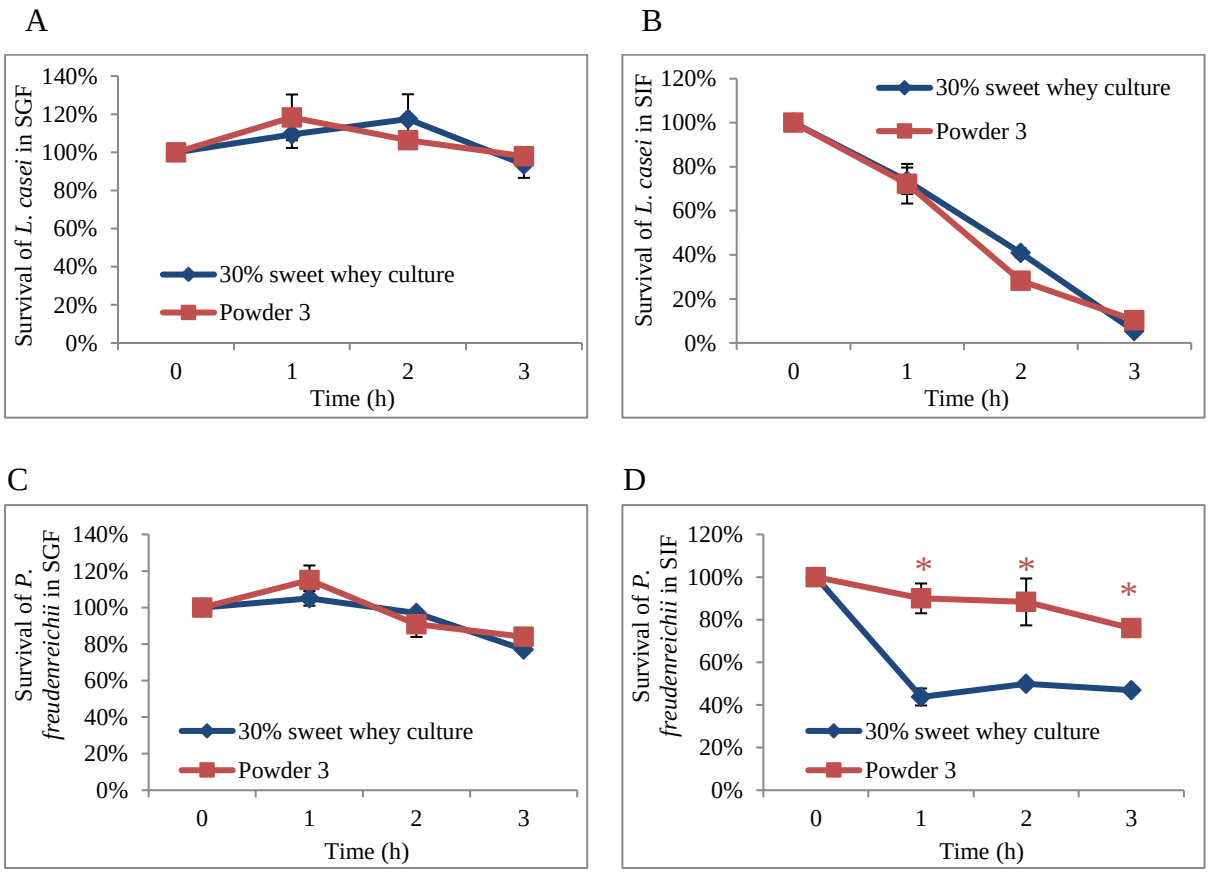

Figure 41. The survival of probiotic in 30 wt% fresh culture and powders from Multi-stage drying process (P3), (A) *L.casei* BL23 in simulated gastric fluid (SGF), (B) *L.casei* BL23 in simulated intestinal fluid (SIF), and (C) *P. freudenreichii* ITG20 in simulated gastric fluid (SGF), (D) *P. freudenreichii* ITG20 in simulated intestinal fluid (SIF).

However, the viability of two probiotics both decreased gradually in the simulated intestinal fluid (SIF) within 3 h. It may be caused by the effects of bile salt and trypsin, since there is barely lipid in the sweet whey to buffer the bile salt and the whey proteins would be digested to a large extent. Interestingly, *P. freudenreichii* powders showed a stronger tolerance, compared to the fresh culture, during simulated intestinal digestion (Figure 41D). However, this enhanced tolerance did not emerge on the *L. casei* strain (Figure 41B). It indicates that the heat, osmotic or oxidative stress underwent during spray drying or the desiccate environment in the spray-dried powders may further trigger the stress tolerance of bacteria and this cellular stress response may be strain-dependent. This hypothesis however remains further research.

4.4 Conclusion

In this chapter, the 2-in-1 process (i.e., use of highly concentrated sweet whey for growth and spray drying of probiotics) was successfully scaled up at a semi industrial pilot scale (500 L fermentation and spray drying) for two well-documented probiotic strains. Indeed, a high viability of probiotics was obtained after spray drying.

The multi-stage drying process was shown to further improve the probiotic survival rate, compared to conventional one-stage spray drying. This multi-stage drying process can represent a win-win strategy with the 2-in-1 process due to the benefits of utilizing highly concentrated sweet whey (30% TS). This couping could thus be used in protecting more heat sensitive microorganisms in the future.

The storage temperature and powder moisture content were found to be the key factors influencing the stability of probiotic powders. This may suggest that the viability of probiotics after drying should not be considered as the only criteria driving the quality of probiotic powders. From a long-term stability point of view, it may be worth to increase the drying temperature to some extent, hence obtaining probiotic powders with ideal water content/activity while sacrificing a minimum viability during drying.

In addition, spray drying was found to improve the tolerance of *P. freudenreichii* against simulated intestinal fluid. It suggests the possibility of cross protection from spray drying stress to digestion stress, which would be bacterial strain dependent.

General Conclusion

The market demand for functional probiotics-containing foods is facing an exponential growth due to the growing standard of living and health awareness at a global scale. Spray drying holds great promises for sustainable and large-scale production of probiotic powder ingredients. Although it has been widely applied in dairy and pharmaceutical industries at a global scale, its use for producing powders of probiotics or other food-grade microorganisms remains a major challenge due to the concomitant undesired loss of cell viability. Improving the quality of spray-dried probiotics thus warrants more investments in research and development to gain better understanding of the process-probiotics compatibility and probiotic physiology-functionality interaction. In this project, we attempted to design an industry and environment-friendly process for spray drying of probiotics, and provide insights into the interaction between bacterial physiology and processing. The obtained results can be considered as the answers to the following questions:

What are the advantages of the novel 2-in-1 process?

In the present project, a simplified process from growth to spray drying of probiotics was developed, using a dairy by-product (sweet whey) in a concentrated form (~30% dry matter) as a 2-in-1 medium to both culture probiotic bacteria and protect them from spray drying injury. This innovative process, briefly named 2-in-1 process, has been patent-protected.

In the 2-in-1 process, the usual intermediate steps between bacterial growth and spray drying (such as harvesting, washing, re-suspending) were skipped and the probiotic metabolites were retained. In fact, instead of being inhibited by the hypertonic conditions in 30% sweet whey, probiotic *L. casei* and *P. freudenreichii* were found to yield higher biomass than that in isotonic 5% sweet whey. The growth of *L. casei* was less dependent on casein peptone supplementation when increasing the dry matter of sweet whey from 5% to 30%. As a liquid culture medium, sweet whey also enabled longer survival of *L. casei* in stationary phase than in MRS broth at 37°C. More importantly, the survival of probiotics after spray drying and their stability during storage at 4°C were significantly improved after growth in concentrated (hypertonic) sweet whey in the 2-in-1 process, in comparison to the 5% isotonic sweet whey.

What contributes to the improvement of probiotic survival in the 2-in-1 process?

Use of highly concentrated sweet whey as a bacterial growth medium is one of the most important features for the 2-in-1 process. The stress response of bacteria during growth in this hypertonic medium was hypothesized to be a main reason for the enhanced bacterial multistress tolerance (i.e. multistress tolerance conferred by osmotic adaptation). In this work, we conducted an experiment with *P. freudenreichii* to demonstrate this hypothesis. The osmoregulation was proven in the *P. freudenreichii* sweet whey culture (30% dry matter) by evidencing the accumulation of compatible solutes (trehalose, glycogen, polyphosphate) and the upregulation of several proteins in charge of osmoregulation (e.g., ATP-binding protein OpuCA of osmoprotectant ABC transporter). At the same time, the induction of multistress tolerance was demonstrated through the improved bacterial survival upon heat, acid, bile-salt, spray drying treatments, and the upregulation of universal stress proteins (e.g., heat shock proteins 20, Chaperone ClpB 2).

Besides, Mg^{2+} ranging from 10 to 50 mmol l^{-1} also showed a positive effect on the thermotolerance of bacteria, which may contribute to the protection of highly concentrated sweet whey displayed in the 2-in-1 process.

In addition to cross protection and Mg^{2+} effect, the calcium-induced milk protein aggregates also displayed a significant effect on improving the protective capacity of milk on bacteria during thermal convective drying. This improved protection is suggested to link to the distinct drying kinetics and droplet-particle transition of the aggregates-containing milk. It highlights the contribution of matrix structure to probiotic protection during drying.

Although the correlation between Mg^{2+} or calcium-induced protein aggregates with the highly concentrated sweet whey requires further investigations, these results clearly suggest that, apart from the well-known protection effects of sugar and proteins, the mineral salts may also play important roles in probiotic survival during drying, either through influencing the bacterial physiology directly (i.e., composition effect), or through changing the protective capacity of matrixes (i.e., structure effect).

How to scale up the 2-in-1 process, and for which powder quality?

The feasibility of scaling up the 2-in-1 process was validated at a semi industrial pilot scale (500 L fermentation and spray drying). A high residual viability of probiotics was obtained in the spray-dried powders. Besides, the 2-in-1 process is easy to couple with other industrial

processes for the purpose of decreasing drying temperature given the high solid content of the probiotic sweet whey culture. For example, we showed that the 2-in-1 process can be well coupled with a multi-stage drying process, resulting in a further improvement of probiotic viability in the final powders. However, it is suggested that the viability after spray drying should not be considered as the only criteria of the quality of probiotic powders. Given the importance of moisture content and water activity for the probiotic storage stability, it may be more reasonable to sacrifice a minimum viability of probiotics through increasing drying temperature, in order to obtain powders with ideal moisture content (<4%) and water activity (<0.2).

As another important property of probiotics, the digestion resistance of spray dried probiotics was also investigated. *P. freudenreichii* rehydrated from spray dried powders was found to be more resistant against the simulated intestinal fluid. However, this improvement was not found in *L. casei*. This suggests the potential strain-dependent cross protection between spray drying stimuli and digestion stress tolerance.

Overall, this work will open a new perspective for large-scale production of probiotics in the future. It offers an alternative to the currently used freeze drying with the following main advantages:

- Continuous production of probiotics via spray drying
- Use of the food-grade dairy byproduct (sweet whey) throughout the process
- Further improved energy efficiency and productivity of spray drying probiotics
- Producing probiotic powders with high bacterial viability and resistance phenotype
- Avoid the intermediate steps between fermentation and drying
- Keep the probiotic metabolites in final powders

Future Perspective

To achieve the aim of using spray drying for probiotic production/encapsulation, more studies should be conducted to provide insights into the correlations of processing, bacterial physiology and probiotic functionality.

Surrounding the 2-in-1 process *(short-term perspective)*

Does osmoregulation influence probiotic functionality?

Whether or not spray drying process influences the probiotics functionality remains a key concern. To the best of our knowledge, only two *in vivo* studies investigated this potential influence using mice model: A higher number of Immunoglobulin A (IgA)-producing cells in the small intestine were induced in mice by spray-dried cultures, when compared with fresh cultures (Páez et al., 2013). Another recent study also reported that spray drying process preserved the immunomodulatory properties of *B. lactis* strain on acute and chronic colitis in mice, as no significant differences were observed between fresh and spray-dried bacteria (Burns et al., 2017). However, it should be noticed that the probiotic powders in these two studies were both produced with a lab-scale spray dryer (Buchi B290, Flawil, Switzerland) with probiotic survivals close to 100%. This high survival is generally unexpected in lab-scale spray drying due to the intensive energy required to evaporate the moisture. It may not be representative of the influence of real industrial spray drying process on bacterial physiology. Besides, a closer to human animal model, porcine for instance, should be considered in order to better simulate the human digestion.

We have highlighted in our study the enhanced acid and bile-salt resistance in the 30% sweet whey culture in comparison to the 5%. It indicates that the probiotics grown in 30% sweet whey may survive better during delivery to gastrointestinal tract. However, considering the change of bacterial physiology during osmoregulation, more studies should be carried out *in vitro* and *in vivo* to investigate in detail the potentially modified functionalities of probiotics obtained from the 2-in-1 process. For example, does the 2-in-1 process influence the expression of *P. freudenreichii* CIRM-BIA 129 surface proteins, hence affecting its immunomodulatory effects (Le Maréchal et al., 2015)? Similarly, does it influence the expression and activity of *L. casei* BL23 sortases, hence affecting its adhesive and immunomodulatory effects (Muñoz-Provencio et al., 2012)?

Free combination of elements to create a new 2-in-1 process

The applicability of the 2-in-1 process could be further extended. The key innovation of the 2-in-1 process is utilizing bacterial osmoregulation to link bacterial growth with drying process. Four key elements (i.e., microorganisms, medium, stress, production) can be extracted from the 2-in-1 process as shown in Figure 42:

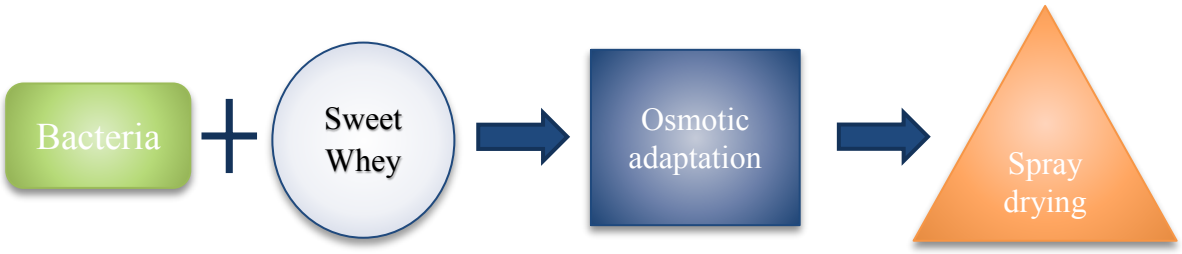

Figure 42. The key four elements of the current 2-in-1 process. To further extend the applicability of this process, each element is a matter of investigation to form a new technical route in terms of probiotic production.

First, more probiotic species and strains should be tested with regard to this process in order to extend its applicability and demonstrate its potential genericity. The challenge remains to explore whether or not this process could be efficient in encapsulating more fragile bacteria such as bifidobacteria. It would be interesting to understand the difference of osmoregulation-pattern among different bacterial species/strains. Moreover, the possibility of cultivating multi-species bacteria instead of single strain should be explored. The species-species interaction and quorum sensing issue may exert significant effects on bacterial physiology during growth, stress adaptation and drying.

Second, the 2-in-1 medium could be replaced by other food or pharmaceutical-grade materials, the only constraint being the food-grade feature of the medium and the ability of its nutrients to sustain the growth of bacteria. However, it is also possible to add some basic nutrients in a given material to form a 2-in-1 medium. In order to support the choice and formulation of medium, it is of prime importance to better understand the role of each component: is it possible to identify a key component (or group of components) to sustain bacterial growth? What component(s) is(are) able to trigger osmotic stress, overall to provide protection during drying? On the contrary, what may induce negative effects on bacterial biomass yield or survival during drying? A clear understanding of the role of each component will provide great help in developing a chemically defined food grade medium, which may

promote the improvement of biomass yield, bacterial resistance before drying, and maximize the bacteria viability after drying.

Third, in the 2-in-1 process, concentrated medium promotes bacterial tolerance in the growth unit, and also provides high dry matter and production yields for the drying unit. In other words, osmotic adaptation links growth and drying on the basis of its benefits for both sides. It would be interesting to explore the possibility of other cross benefits apart from osmotic adaptation, for instance on thermal, oxidative or acid adaptation. Take thermal adaptation as an example: Bacteria could be cultivated in an isotonic 2-in-1 medium with the purpose of producing maximal bacterial population. Then this bacterial culture could be delivered to a concentration unit with sublethal heating to trigger the thermal adaptation (falling film evaporator for instance). Once dry matter achieves the desirable range (15~40%), this thermal adapted culture could be delivered to spray drying. Moreover, exploring the possibility to trigger oxidative stress resistance seems at this point of higher interest.

Last but not least, as the induced cross-protection is not specific to spray drying, the 2-in-1 process could be also efficient when applied to freeze drying for probiotic production purposes. The high dry matter is also desirable for freeze drying from the energy efficiency point of view. It is also interesting to explore the possibility of cross protection from osmotic stress to cold stress, as well as the glass transition behavior of concentrated 2-in-1 medium during freeze drying.

From probiotic-delivery to functionality-delivery *(Long-term perspective)*

Tailor the powders: Making every cell matter

Nowadays, extensive studies have been carried out in the aim of maximal delivery of viable probiotics to the gastrointestinal tract (GIT). Spray drying is one of the methods used to encapsulate probiotics within a drying matrix, hence protecting probiotics cells from digestion stress. The protection here mostly refers to maintenance of bacterial viability within the GIT. However, **does the viability of probiotics guarantee its probiotic functionality**?

It should be noted that, in most studies, the viability of probiotics refers to the metabolism and cell-division abilities of bacteria in their favored growth medium (Papadimitriou et al., 2016). These abilities may not completely reflect the successful delivery of probiotic functionality. For example, most of immunomodulatory effects of probiotics are induced by the interaction between probiotic surface molecules and host intestinal mucosa (Bron et al.,

2011; Lebeer et al., 2010). The loss or inactivation of these molecules during digestion may not destroy the metabolism and cell-division abilities of probiotics, but it would result in the silencing of beneficial effects conferred by probiotics. In contrast, encapsulated probiotics may also lose the interaction with host mucosa due to the shield effect of drying matrix. This latter could also alleviate the beneficial effects of probiotic metabolites, such as bacteriocin, SCFAs, vitamin and secreted proteins. Therefore, controlling the delivery of probiotic functionality is the next key step in order to tailor the probiotics chain. **To guarantee the successful delivery of probiotic functionality, an encapsulation strategy should be designed on the basis of a better understanding of the specific probiotic strains and of the action mechanisms of their probiotic effects.** For example, core-shell particles or porous particles could constitute ideal vehicle to solve the above-mentioned concerns in probiotic-functionality delivery (Figure 43).

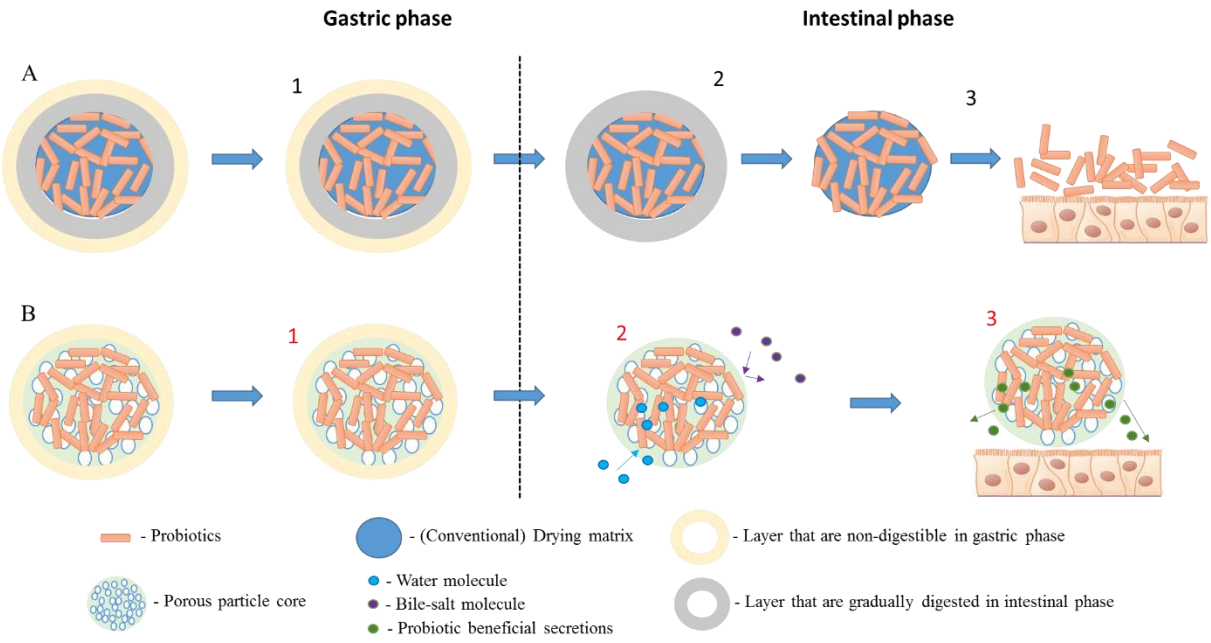

Figure 43. Schematic diagram of two hypothetic particles used to encapsulate and deliver probiotics which have two different action mechanisms. (A) Layer-by-layer encapsulation for delivery of probiotics that exert their beneficial effects through the interaction between their surface molecules and host intestinal cells or mucosa (inspired by Anselmo et al., 2016): (1) The first layer of shell protects the particle through gastric phase; (2) The second layer of shell is gradually digested in intestinal phase to partly protect the particle core from the bile and gradually release the particle core; (3) The particle core is collapsed and release the probiotics into the intestinal phase. (B) Encapsulation of porous core particle for delivery of probiotics that exert their beneficial effects through secretion of beneficial molecules (inspired by Mahbubani et al., 2014 and Nandiyanto and Okuyama, 2011): (1) The encapsulated layer protects the particle core through gastric phase; (2) The porous particle core allows water to penetrate inside to rehydrate and to activate probiotics, but hinders the bile salt to penetrate inside; (3) The porous particle core allows the release of probiotic beneficial secretions.

Although it is possible to produce powders with different particle morphologies and structures (Figure 4) via spray dying (Nandiyanto and Okuyama, 2011), and thus theroretically to optimize the encapsulation features with regard to the susceptibility of probiotics, tailoring such powders in terms of probiotic encapsulation remains practically challenging. Besides, the other difficulty relates to the range of available materials, which should be food-grade and affordable. From this point of view, milk-protein based (eg, WPI) materials seem promising (Tavares et al., 2014), but in the same time are expensive for large-scale production. Therefore, this requires more innovative studies with assistance of multidisciplinary knowledge in the future.

An ideal in vitro system for investigation of shaping microbiota

The gut microbiota, considered as our "forgotten organ", plays a major role in human health (Clemente et al., 2012). As the definition of "healthy microbiota" becomes increasingly clear in the future, it is possible to shape the gut microbiota through oral delivery of probiotics in the aim of improving human health (Gareau et al., 2010).

Due to the ethics issues, it is difficult to obtain the *in vivo* information on the interaction of oral probiotic supplementation and gut microbiota (not fecal microbiota). An *in vitro* digestion system that can reflect the real human GIT environment is expected (Figure 44).

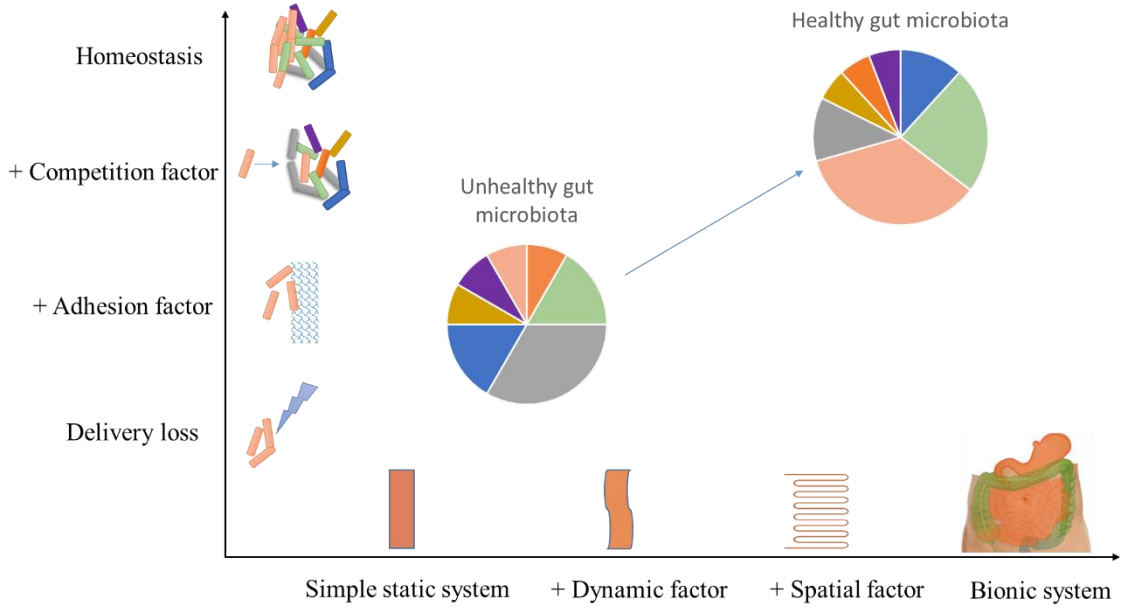

Figure 44. Schematic diagram of expected development *in vitro* digestion system for study of probiotics-microbiota interaction.

X axis in Figure 44 shows the expected technological development of an *in vitro* digestion system: First, simple static system refers to conventional beaker/tube-based system with the use of simulated digestive juice, which is most commonly used *in vitro* system by far (Minekus et al., 2014). Second, dynamic factor refers to the flow-rate and time-dependent changes of digestive juice, which has been reported in increasing literatures in the recent decade (Minekus et al., 2014). Third, spatial factor includes the morphological, mechanical and physico-chemical properties of different sections of GIT. Yet simulating these complexity of real human GIT remains a crucial challenge (Guerra et al., 2012). The final bionic system refers to the near real digestive tracts that can well reflect lumen-side environment, mucosal penetration, GIT peristaltic movements, hormonal and nervous control, feedback mechanisms, and involvement of the local immune system (Guerra et al., 2012). It requires more interdisciplinary and multidisciplinary endeavours in the future.

Y axis in Figure 44 is the expected increasing understanding of biologically influential factors during probiotic delivery: First, delivery loss means the loss of bacteria due to the physico-chemical effect of digestive juice, which nowadays mostly refers to the effects of gastric juice (acid stress) and bile-salt (Cook et al., 2012). Second, adhesion factor mainly includes the effects of GIT movement and dynamic flow of digestive juice on the bacteria adhesion to GIT, which has been investigated *in vitro* in a few studies in the recent decade (Van Tassell and Miller, 2011). However, it should be noted that most of these studies are conducted in a simple static *in vitro* digestion model, which may not reflect the real dynamic and mechanical effects of digestive flow, and the interaction between probiotics and mucosal layer. Third, competition factor refers to the effects of existing gut microbiota on the adhesion and colonization of the delivering bacteria at a targeted niche. Similarly with the second "adhesion factor", the investigations in this aspect is largely limited by the authenticity of *in vitro* model (Payne et al., 2012). Besides, the unculturability of most gut bacteria in lab also himders a more insightful understanding of the competition factor within the GIT (Browne et al., 2016). The final homeostasis means colonization and proliferation of delivering bacteria result in a desired homeostatic ecosystem. The developments of "omics" analytical approach, sequencing, and *in situ* imaging technologies are expected to promote the breakthoughs in this axis in the future.

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RÉSUMÉ *ABSTRACT*

Séchage par atomisation des bactéries probiotiques : des mécanismes de protection à la production à l'échelle pilote

Les probiotiques sont des microorganismes vivants qui, ingérés en quantité suffisante, exercent des effets positifs sur la santé. La lyophilisation est aujourd'hui questionnée quant à sa consommation d'énergie et son caractère discontinu. S'il offre une alternative pour produire massivement des poudres probiotiques à faible coût, le séchage par atomisation induit quant à lui des stress thermiques et oxydatifs conduisant à des pertes de viabilité rédhibitoires.

Dans ce travail, un procédé innovant de séchage par atomisation est proposé. Du lactosérum doux concentré (jusqu'à 30% p/p) est utilisé à la fois comme support de culture et de séchage de *P. freudenreichii* et *L. casei*. Ce procédé élimine les étapes intermédiaires à risque de contamination élevé, accroît la biomasse et améliore la viabilité des bactéries.

Les mécanismes sous-jacents ont été explorés au plan de la résistance bactérienne et des conditions de séchage. Le milieu concentré induit une osmoadaptation des bactéries par expression de protéines de stress et accumulation de solutés compatibles, conduisant à une tolérance accrue des probiotiques à différents stress. La présence d'agrégats et la concentration en Mg^{2+} du milieu concentré pourraient également être impliquées.

Le scale-up du procédé a été étudié : un schéma technologique semi-industriel impliquant séchage par atomisation, sur bande et en lit fluidisé a permis d'atteindre une viabilité de 100% ($> 10^9$ CFU g^{-1}). Par ailleurs, la fonctionnalité des poudres probiotiques a été évaluée in vitro and in vivo sur modèle porcelet. Ce travail ouvre de nouvelles perspectives pour l'obtention à grande échelle et selon un procédé sobre et durable de préparations probiotiques.

Mots-clés : probiotiques ; séchage par atomisation ; osmorégulation ; tolérance ; viabilité

Spray drying of probiotic bacteria: From molecular mechanism to pilot-scale production

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Freeze drying, the reference drying method, is currently challenged because of its low energy-efficiency and productivity. Therefore, spray drying is expected to be an alternative and sustainable method for producing probiotic powders. The issue remains in the considerable inactivation of probiotics caused by high temperature and dehydration during the process.

In this work, a novel spray-drying process for continuous production of probiotics was challenged. Concentrated sweet whey (up to 30% w/w dry matter) was used to both culture and spray dry P. freudenreichii ITG P20 *and* L. casei *BL23. This process cuts down the steps between culturing and drying (e.g. harvesting, washing), increases the cell population after growth and improves spray drying productivity and probiotic viability.*

The mechanisms were explored from bacterial physiology and drying process conditions. The hypertonic stress led to overexpression of key stress proteins and accumulation of intracellular compatible solutes, which enhanced multistress tolerance. The presence of protein aggregates and optimal concentration of Mg^{2+} in matrix may also be involved.

The feasibility of scaling up this process was validated. A multi-stage semi industrial drying process, coupling spray-drying with belt drying and fluid-bed drying, was applied to further improve the probiotic viability to 100% ($> 10^9$ CFU g^{-1}). Moreover, the functionality of these probiotic powders was investigated in vitro and in vivo with piglet model. This work opens new avenues for the sustainable development of new starter and probiotic preparations with enhanced robustness.

Keywords: *Probiotics; spray drying; osmoregulation; stress tolerance; viability*

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