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***Ligilactobacillus salivarius* functionalities, applications and manufacturing challenges**

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Abstract

Ligilactobacillus salivarius is a lactic acid bacteria that has been gaining attention as a promising probiotic. Numerous strains exhibit functional properties with health benefits such as antimicrobial activity, immunological effects, and the ability to modulate the intestinal microbiota. However, just a small number of them are manufactured at an industrial scale and included in commercial products. The under exploitation of *L. salivarius* strains that remain in the freezer of companies is due to their incapacity to overcome the environmental stresses induced by production and stabilization processes.

The present study summarizes the functionalities and applications of *L. salivarius* reported to date. It aims also at providing a critical evaluation of the literature available on the manufacturing steps of *L. salivarius* concentrates, the bacterial quality after each step of the process, and the putative degradation and preservation mechanisms. Here we highlight the principal issues and future research challenges for improving the production and long-term preservation at the industrial scale of this microorganism, and probably of other probiotics.

Key points:

- *L. salivarius* beneficial properties and commercialized products
- Production conditions and viability of *L. salivarius* after stabilization processes
- Prospects for identifying preservation mechanisms to improve *L. salivarius* stability

Keywords: *L. salivarius*, functionality, probiotic, stabilization, freeze-drying, spray-drying

Introduction

Ligilactobacillus salivarius, formerly named *Lactobacillus salivarius*, is a homofermentative gram positive bacteria producing only lactic acid from carbohydrates metabolism. It is a lactic acid bacteria (LAB) with the status of Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA Panel on Biological Hazards (BIOHAZ) et al. 2020) and naturally found in the human tract, human milk, human vagina, and human oral cavities, among other sources (Martín et al. 2006; Barfod et al. 2011; Pino et al. 2019). In recent years, *L. salivarius* has been gaining attention as a promising probiotic microorganism with an increasing number of studies that explore the variety of interesting applications of different strains (Neville and O'Toole 2010; Messaoudi et al. 2013; Chaves et al. 2017).

Probiotic microorganisms have been defined as “viable microorganisms that, when administered in adequate amounts, promote or support a health benefit on the host” (FAO/WHO 2006). Lactic acid bacteria and *Bifidobacterium* genus are the largest and most studied groups of probiotics. For centuries, LAB have been involved in food microbiology and human nutrition because of their fermentative properties. The *Lactobacillus* genus, first described in 1901 with *Lactobacillus delbrueckii*, has been expanding extremely fast over the last two decades (261 species in March 2020) (Zheng et al. 2020). The whole genome sequence analysis of species performed in the past few years has shown a wide heterogeneity of the genus at the phenotypic, ecological and genotypic levels, which was almost unobserved in bacterial taxonomy. To provide a stable and unambiguous taxonomy, essential for academic research but also medical, nutritional, and environmental purposes, the classification of LAB has recently been revised (Zheng et al. 2020). The new *Lactobacillaceae* family contains 30 genera, resulting in the creation of 23 new genera and the reduction of the original genus of *Lactobacillus* to only 38 species around the type species *L. delbrueckii*. *L. salivarius* strains have been removed from the *Lactobacillus* genus to be included in a novel genus *Ligilactobacillus* (composed of 16 species), which name means uniting (*L. v. ligare* = to tie; unite), with a host adapted life style (vertebrate host for *Ligilactobacillus salivarius*) (Zheng et al. 2020).

Probiotic bacteria are currently sold as frozen pellets or frozen cans of highly concentrated cultures to food companies, for producing fermented probiotic products (yoghurt, fermented milk, fermented fruit juice, etc.) or as a powder that is the preferred form to be included in dietary supplements and medicines (capsules, sachets, or tablets) (Fenster et al. 2019). The manufacturing of probiotic concentrates requires the application of successive operations that generate potential cellular damage and loss of viability and functionalities (Cunningham et al. 2021), in particular following the stabilization operation (i.e. freezing, freeze-drying, spray-drying) (Santivarangkna et al. 2008; Béal and Fonseca 2015; Broeckx et al. 2016). To limit the cellular damage, probiotic bacteria, can also be prepared to cope with the different environmental stresses encountered during the stabilization processes by varying the growth conditions (Carvalho et al. 2004; Fonseca et al. 2019) and by applying pre-stabilization treatments such as moderate stress pre-treatments (e.g. acidic conditions, heat-shock) (Huang et al. 2017). Furthermore, protective molecules and particularly sugars are added to the concentrated culture before stabilization (Teng et al. 2017; Broeckx et al. 2017; Archacka 2019; Broeckx et al. 2019; Fonseca et al. 2021).

The most health beneficial promising strains of *L. salivarius* will have thus no commercial value if the viability and the functional properties could not be maintained all along with the product shelf life (not only after stabilization

but also after long-term storage) (World Gastroenterology Organisation Global Guidelines 2017), and will remain unexploited in the freezer of laboratories or companies, unable to deliver to the society their expected health benefits.

This review aims at examining the published information on *L. salivarius*, focusing on the production, stabilization, and storage of concentrated cultures. Through an in-depth analysis of the information reported to date, we highlight the achievements but also the critical points and problems of *L. salivarius* stabilization. It is structured in three main parts: i) the health beneficial properties, the potential applications of *L. salivarius*, as well as the commercial products available in the market containing this microorganism; ii) the manufacturing process of highly concentrated cultures of *L. salivarius* and the influence of the operations on the bacterial viability; and iii) future prospects for research on *L. salivarius*. The novelty of this mini-review lies both in the update on *L. salivarius* health benefits and applications previously reported (Neville and O'Toole 2010; Messaoudi et al. 2013; Chaves et al. 2017) and the focus on the production of dried *L. salivarius* concentrates, when most review articles relate the production and stabilization of LAB in general (Carvalho et al. 2004; Santivarangkna et al. 2007; Meng et al. 2008; Broeckx et al. 2016; Huang et al. 2017; Fenster et al. 2019) or another micro-organism such as *L. lactis*, (Archacka 2019)

***Ligilactobacillus salivarius*: a probiotic bacteria with recognized health benefit**

Functional properties *L. salivarius* for improving human health

Table 1 summarizes the research works that have evidenced human health benefits for 42 strains of *L. salivarius*. The scientific studies involved *in vitro* (31 studies) and *in vivo* (46 studies) experiments. Among the *in vivo* experiments, 59 % were carried out on human. The functional properties, as well as the modes of administration and the potential medical applications of the probiotic strain, are also included. Three main functionalities have been reported for explaining the health benefits of *L. salivarius*: i) antimicrobial activity (AMA) ii) immunological effects (IME); and iii) modulation of the intestinal microbiota and barrier function (MIMB). The dried form of the probiotic has been used in 43 % of the reported studies.

The functional properties exhibited by *L. salivarius* are sometimes shared with other members of the *L. salivarius* clade and with other LAB (e.g. *L. reuteri*, *L. rhamnosus* GG, *L. casei*, *L. acidophilus*) (Neville and O'Toole 2010). The recent reclassification of the *Lactobacillaceae* family indicates a great diversity within the *L. salivarius* group that could be explained by horizontal gene transfer from the diverse lifestyles of the group (Zheng et al. 2020).

Furthermore, recent studies suggest that *L. salivarius* strains represent an emerging option for cancer prevention and treatment (Zhang et al. 2013; Zhang et al. 2015; Wang et al. 2016b; Yenuganti et al. 2021).

The antibacterial activity of some strains of *L. salivarius* has also interest for animal nutrition. Intensification of animal agriculture with consequent imprudent use of antibiotic growth promoters implies risks to human and animal health due to the antibiotic resistance developed in the pathogenic microorganism. Probiotics are an alternative to antibiotic growth promoters in animal production. Several probiotics have been found effective in improving animal performance and preventing diseases (Bajagai et al. 2016). Certain *L. salivarius* strains have also antimicrobial

activity in animals, preventing *Salmonella enteritidis* colonization in chicken (Pascual et al. 1999; Lone et al. 2021), and reducing and preventing enteropathogen infections in piglets (Kumar et al. 2010; Zhou et al. 2020; Moturi et al. 2021) and calves (Maldonado et al. 2018).

Probiotic products available on the market including strains of *L. salivarius*

The global market for probiotics was evaluated at 662 million US\$ in 2020 and is projected to reach 887 million US\$ by the end of 2026, growing at 5 % during the forecast period 2021-2026 (WiseGuy Reports 2020). Europe is the main producer of probiotics, with 436.58 MT produced in 2016, accounting for about 48 % of the total amount, followed by North America, with 24.5 %. Leading actors in the probiotics industry are Chr. Hansen, Lallemand, Nestle®, DuPont™ Danisco®, and China-Biotics. These five companies occupied about 61 % share of the market in 2016; being Chr. Hansen the largest manufacturer with a sales market share of 16 % in 2016 (WiseGuy Reports 2020).

Among the 42 strains of *L. salivarius* exhibiting probiotic properties reported in **Table 1**, eight are present in products commercialized for specific applications such as intestinal health (treatment of chronic inflammatory diseases, prevent gastrointestinal problems, maintain gut flora balance) and other health care claims (treatment of candidiasis, oral health maintenance, preventing of otitis and allergic rhinitis) (**Table 1**).

Manufacturing processes of highly concentrated cultures of *Ligilactobacillus salivarius*

The manufacturing of a probiotic product involves two main steps: i) the production of the probiotic microorganism, as a highly concentrated culture stabilized by freezing or drying (orange-colored part of **Fig. 1**), and ii) the production of the commercial probiotic product using the probiotic microorganism as an ingredient (grey-colored part of **Fig. 1**).

The probiotic microorganism can be incorporated in the food matrix either in a metabolically active state requiring thawing or rehydrating steps (e.g. fermented food such as yogurt, fermented milk, or non-dairy foods such as juices) or in a dry and metabolically inactive state (e.g. powdered infant formula or dietary supplements delivered as capsules, tablets or sachets) (Fenster et al. 2019). In the latter case, the probiotic microorganism will become metabolically active in the human gut.

When considering the production of fermented food, frozen or dried starters could be used. Frozen starter's utilization has however some drawbacks. The very low temperatures required for their transport and storage are costly and limit their use to countries or regions where the management of low temperatures is well established. For the production of fermented food, the probiotic microorganism viability and acidifying activity have to be maintained during the intermediate storage step (orange-colored storage step), and the probiotic activity should not be compromised by the manufacturing conditions of the fermented food.

Conversely, the manufacture of infant milk powder or a dietary supplement requires the use of a dried form of the probiotic microorganism. The viability of the dried cell concentrate has to be maintained during the two steps of

storage (the intermediate and the final steps), with a total duration of which can easily exceed one year. However, storage stability is often lower in dried compared to frozen probiotics and has to be overdose when included as a functional ingredient in foods. The intermediate storage of the microorganism is generally performed at a temperature below 4 °C or lower (-20°C or -45 °C), and the final storage step is usually carried out at ambient temperature.

Only those probiotic microorganisms that exhibit stable viability and functional properties for long periods can be sold and included in commercial products. This review will therefore focus on the production and stabilization of the probiotic cell concentrate, the first step of the probiotic product manufacture, but it will not address the second step of elaboration and storage of the commercial product.

To get stable cell concentrates is often a great challenge because during manufacturing cells go through several processes (fermentation, cooling, concentration, freezing or drying, storage, and transport to the end-user) (**Fig. 1**) which are sources of stress and affect their viability and functional properties (Santivarangkna et al. 2008; Béal and Fonseca 2015; Fenster et al. 2019). During the fermentation step, cell multiplication and growth take place as well as the elaboration of the functionality of interest that cells will exhibit when probiotic products are consumed. During the stabilization and the storage steps, viability and functional properties usually decline due to different types of stress cells are exposed to (cold, thermal, osmotic, mechanical, and oxidative). Specific compounds are currently added before the stabilization process (protection step before freezing, drying, **Fig. 1**), to protect bacteria and deliver to the final user a product with maximal functional properties. The main objective of all steps after fermentation (from harvesting to product elaboration) is to minimize cell death and losses of functionality, to reach long-term stability compatible with market requirements.

However, before going to the market, the strain selected for its potential probiotic benefits for human health, and identified as safe, will have to follow the industrial development itinerary illustrated in **Fig. 2**. The first step is the laboratory scale, to identify relevant fermentation conditions (medium composition, temperature, pH, dissolved oxygen, harvesting time, fermentation duration) for biomass production. The cell concentrates produced are analyzed to confirm the expected health benefit, *in vitro*, *in vivo*, and finally, through clinical trials. Before the well-defined laboratory-scale processes can be transferred to the industrial scale, pilot scale tests are required (Crater and Lievens 2018). Many parameters that are tightly controlled at the laboratory scale and affect process performance (amount of biomass, strain's physiological state upon harvesting, and financial return), significantly change and are not easily controlled when scaling up the processes. The holding times during each step of the process can greatly increase at an industrial scale (e.g. cells separation from culture medium), thus leading to different stresses (usually heat and shear stress) (Fenster et al. 2019). The fermentation conditions (raw material, utilities, and investments) can also significantly differ between lab and industrial scales. Since the fermentation scale-up generates important costs and affects stabilization processes (Crater and Lievens 2018), it is important to scale up to an intermediate volume (pilot scale, **Fig. 2**). Moreover, the transfer from the laboratory freeze-drier to the industrial freeze-drier may also be challenging. The freeze-drier cycle will have to be defined considering the changes in heat and mass transfers, condenser capacity, and condensation rate, between the down-sized and the industrial freeze-drier.

We have identified 17 published works on the production and the stabilization of highly concentrated cultures of *L. salivarius*. The experimental conditions and main results of all those published papers were organized in tables (<https://doi.org/10.15454/DK0B4K>, and **Tables S2 and S3**) designed according to the different steps reported in **Fig. 1** (orange-colored steps) to identify the levers to improve the long-term stability of dried samples of *L. salivarius*.

Among the reported studies, 65 % were performed at a laboratory-scale, the first step in the industrial development of a probiotic microorganism (**Fig. 2**). Pilot scale studies are thus lacking, and are needed to evaluate process conditions closer to industrial ones before proceeding to industrial scale-up.

Production of concentrated cultures of *L. salivarius*

The first stage that has an important impact on the final probiotic viability and functionality is the production of a concentrated bacterial suspension. It involves several unit operations: fermentation, harvesting and cooling, concentration, and optional washing. Unfortunately, none of the reported works on *L. salivarius* has investigated the effect of the operating conditions applied for biomass production on the quality of bacteria after storage.

Fermentation

Fermentation is a key step for the biomass production and the elaboration of bacterial functionalities. Furthermore, modulating the fermentation conditions, in particular the composition of the culture medium, the temperature, and the pH, enables the improvement of the bacterial resistance to stabilization processes and storage (Fonseca et al. 2019).

L. salivarius strains are generally grown in MRS Broth (**Table S2**), a rich medium containing carbon (dextrose) and nitrogen (proteose peptone, beef, and yeast extracts) sources, as well as salts (sodium acetate, magnesium, and manganese sulfate, and dipotassium phosphate) and growth factors (Tween 80). The culture temperature is usually 37 °C (**Table S2**) that is the temperature of a healthy adult human from which some strains of *L. salivarius* are isolated (**Table 1**). Frequently, the pH at the laboratory scale is set free, due to the difficulty to control it in Erlenmeyer flasks, and it is controlled at pilot within the pH range 5.5 -7 (**Table S2**).

The reported works on *L. salivarius* that investigated the effect of fermentation parameters on the bacterial properties after fermentation or stabilization mainly involved the composition and the pH of the culture medium (**Table S2**). Among the 10 works, only 3 analyzed the viability recovery (or survival rate) after the stabilization process (freeze-drying).

Even if the MRS broth medium is commonly used to cultivate lactic acid bacteria in laboratory scale studies, it is not used for industrial production due to its high cost and non-food grade quality (Kasuga et al. 2017). Some authors developed a food-grade culture medium for *L. salivarius* based on the composition of the MRS medium (Lim et al. 2007; Dong et al. 2014; Yeo et al. 2018). The following ingredients were identified as crucial for the growth of *L. salivarius* BBE 09-18: lactose or maltose, yeast extract, sodium acetate/acetic acid, MnSO₄, and Tween 80; galactose, sucrose, fructose, or glucose can also be used as a carbohydrate source (Dong et al. 2014). The concentration of the yeast extract in the culture medium had a high effect on cell density compared to the

carbohydrate content or the initial pH on *L. salivarius* I24 (Lim et al. 2007) and *L. salivarius* W13 (Yeo et al. 2018). Increasing the content of yeast extract up to 80 g/L was retained by Dong et al. (2014) and Yeo et al. (2018), whereas a sugar concentration between 20 and 40 g/L seemed adequate.

An optimized culture medium (LDTM) was defined for *L. salivarius* W13 (Yeo et al. 2018), which was composed of 85 g/L of yeast extract, 21.64 g/L of lactose, 1.21 g/L of Tween 80, 6g/L of sodium acetate, 0.2 g/L of MgSO₄, 0.02 g/L of MnSO₄, 1.5 g/L of KH₂PO₄, 1 g/L of K₂HPO₄, 0.01 g/L of FeSO₄ and 1 g/L of sodium citrate. When considering culture at laboratory scale (50 mL flask), the use of this medium resulted in an 11 fold increase of cell count compared to MRS medium. When moving to pilot scale with culture performed in a 5-liter fermentor, the efficiency of the LDTM medium remains higher than the MRS medium, but the final cell concentration obtained was two times lower than the value obtained at the laboratory scale (2.10⁹ CFU/mL and 4.6.10⁹ CFU/mL, respectively). Furthermore, the authors validated the use of the LDTM medium on 6 other strains of *L. salivarius*. However, its efficiency was not confirmed for the growth of 5 commercial probiotics of other *Lactobacillus* spp.

For reducing the cost of probiotic microorganisms' production, low-cost medium components are sometimes searched. Cheap sources of carbon and nitrogen such as molasses, corn steep liquor, and malt sprout have been proposed for the culture medium of *L. salivarius* NRRL B1950 (Montelongo et al. 1993), *L. salivarius* L29 (Lee et al. 2013), and *L. salivarius* CM-CIDCA 1231B and CM-CIDCA 1232Y (Cejas et al. 2017).

At the laboratory scale, two works compared different values of culture temperature and initial pH and confirmed 37°C and initial pH between 6 and 6.5 as the optimal growth conditions for *L. salivarius* CRL 1328 and *L. salivarius* I24 (Juarez Tomás et al. 2002; Lim et al. 2007). At the pilot scale, the pH of the culture medium seemed to have a moderate effect on bacterial metabolism when it was controlled during bacterial growth (Montelongo et al. 1993; Valenzuela et al. 2015). A higher ATP production following bacterial growth was observed when the pH was controlled at 6 (instead of 7) (Valenzuela et al. 2015). However, controlling the fermentation pH (pH 6.1) is an important factor for improving the survival rate after freeze-drying for *L. salivarius* I24 (Ming et al. 2009).

Harvesting and cooling

After the fermentation step, the biomass is harvested and cooled to a temperature below 20 °C (8-15 °C) to stop bacterial growth and metabolism. The cell suspension is maintained at this temperature for a duration of 15 minutes to several hours, before the concentration step (Béal and Fonseca 2015). In all the works on *L. salivarius* strains (<https://doi.org/10.15454/DK0B4K>), cells were harvested in the stationary phase of growth. No study investigated the effect of the harvesting time or the cooling step on the survival rate after the stabilization step.

The harvesting time has been reported to strongly affect the resistance to the stabilization process of other lactic acid bacteria (Corcoran et al. 2004; Li et al. 2009; Velly et al. 2014). The improved resistance observed in the stationary phase is often attributed to the physiological changes that occur at the membrane and cytosolic level (Béal and Fonseca 2015), such as, the expression of specific proteins (Lorca and de Valdez 2001; Cohen et al. 2006). At the membrane level, the increased freeze-drying survival and storage stability of *L. lactis* ssp. *lactis* TOMSC161 observed when harvesting 6 hours after the beginning of the stationary phase, were ascribed to the modulation of

membrane lipid composition for obtaining a high ratio of CFA/UFA (cyclic fatty acids/unsaturated fatty acids) and the concurrent membrane rigidification (Velly et al. 2015).

Concentration

The goal of the concentration step is to separate the bacterial cells from the culture medium to obtain high cell concentrates comprised between 10^9 to 10^{12} CFU/g (Béal and Fonseca 2015), while also contributing to reducing the concentration of fermentation metabolites. Two technologies can be applied: i) centrifugation that is the commonly used method, and ii) ultrafiltration or microfiltration less frequently applied.

In the reported studies on *L. salivarius* (<https://doi.org/10.15454/DK0B4K>), centrifugation was applied at 4°C, for 5-15 min in a wide range of centrifugation speed values, between 3,000 and 13,000 g.

The concentration step, performed either by centrifugation or filtration induces mechanical stress on bacteria, which in turn can induce cellular damage and thus modify the bacterial resistance to the stabilization process. However, to our knowledge, no study has been reported on the effect of the concentration step on the quality of *L. salivarius* concentrates.

Washing

A washing step of the bacterial pellet obtained after concentration is sometimes applied to neutralize the solution and to remove the residual fermentation compounds. The washing solution may contain mineral salts (sodium or potassium phosphate, sodium glycerophosphate, magnesium sulfate, sodium chloride), amino acids (sodium glutamate), and vitamins (sodium ascorbate). Despite the absence of concentration and washing steps, two *L. salivarius* strains (CM-CIDCA 1231B and CM-CIDCA 1232Y) cultivated in malt sprout medium in uncontrolled pH condition, exhibited improved survival after freeze-drying when the fermentation (and dehydration media) was neutralized (Cejas et al. 2017). Cell washing was performed in less than 50 % of *L. salivarius* reported studies (<https://doi.org/10.15454/DK0B4K>). The washing solutions included: saline solution, Ringer's solution, phosphate buffer, phosphate buffer saline solution (PBS), sterile water, or sterile peptone water. However, the effect of the washing step on the bacterial resistance to freeze-drying was not evaluated.

The addition of a washing step to remove the residual fermentation metabolites such as lactic acid is sometimes necessary to make possible the drying process or improve its productivity (Fonseca et al. 2004; Fonseca et al. 2021). Washing the bacterial pellet before the addition of the protective solution allowed to increase the collapse temperature of the bacterial suspension (Fonseca et al. 2004), which represents the maximum product temperature that could be applied during the sublimation step of the freeze-drying process.

Among the first steps of the manufacturing process of *L. salivarius* concentrates that can affect the viability and functionality recovery after stabilization and storage, the fermentation is the most studied. Two levers can be identified to improve the viability of concentrated cultures: i) the concentration of the yeast extract in the fermentation medium, and ii) the control of the pH during bacterial growth. However, among the ten studies focusing on the fermentation step, only two evaluated the bacterial quality after the stabilization step (freeze-drying process) and none of them after storage. The effect of the fermentation parameters is usually studied on the

physiological state of cells at the end of the fermentation (eight studies). Moreover, no information has been reported on the effect of harvesting, cooling, concentration, and washing on *L. salivarius* stability.

Stabilization of concentrated cultures of *L. salivarius*

After the production of the highly concentrated *L. salivarius* suspensions, a stabilization stage is required to increase the microorganisms' shelf life for several months or years (before their incorporation in a probiotic product or consumed) (**Fig. 1**). This stage is composed of two steps: the addition of the protective agents and the stabilization process itself. In this review we will focus on drying methods since even more challenging, they enable the utilization of probiotic concentrates in all applications (fermented foods and probiotic products).

Drying strategies are based on the decrease of water activity to inhibit or strongly slow down degradation reactions. The water activity decrease is obtained by water removal from the bacterial suspension, which results in a dry product. Freeze-drying is the most widely used drying technology for stabilizing compounds or microorganisms that are thermally sensitive and predisposed to oxidation since it involves high vacuum and low temperatures (Fonseca et al. 2015; Vass et al. 2019; Verlhac et al. 2020). However, other conventional methods such as spray-drying, fluidized bed drying, and vacuum-drying are also industrially used technologies to stabilize probiotics (Mille et al. 2004; Santivarangkna et al. 2007; Broeckx et al. 2016; Huang et al. 2017). Microwave-vacuum drying is a less known drying method that is emerging as a viable alternative for the dehydration of bacterial cultures (Ambros et al. 2018). Radiant energy under vacuum is a form of vacuum microwave dehydration that can also achieve a good survival of dried *L. salivarius* (Ahmad et al. 2012) and *L. rhamnosus* (Noorbakhsh 2013). In the works investigating the stabilization of *L. salivarius* (**Table S3**), freeze-drying (5 studies) and spray drying (5 studies) were applied.

Table 2 summarizes the main characteristics of the freeze-drying and spray drying processes, as well as the associated physical events and environmental modifications. Freeze-drying is a technique that comprises three steps that operate at different temperatures and pressures. It involves freezing of the aqueous solution containing bacteria, followed by primary drying to sublimate ice to water vapor and, finally, secondary drying to remove unfrozen water by desorption (Muller et al. 2009; Fonseca et al. 2015; Fonseca et al. 2021). The final product is a dry cake that requires a further grinding step to obtain individual particles. Spray-drying is a rapid and cost-efficient drying method (Santivarangkna et al. 2007; Broeckx et al. 2016). It produces a dry powder by the atomization of the bacterial suspension into fine droplets (10-200 μm), directed at high velocity into a hot air stream.

Regardless of the preservation process (freeze-drying or spray-drying), the bacterial cells are exposed to osmotic and mechanical stress, which can cause cellular damage resulting in the loss of probiotics' viability and functionality (Ananta et al. 2005; Santivarangkna et al. 2008; Meng et al. 2008; Iaconelli 2015). The physical mechanisms at the origin of the stress will however differ. The crucial difference between both processes is the thermal history of the product, which corresponds to the temperature and time of each step of the stabilization process. During freeze-drying, cells are exposed to low or mild temperature (lower than 0 °C) for several hours, whereas in the spray drying process, the temperature largely exceeds the ambient temperature (higher than 50-60 °C) for few minutes. Oxygen exposure may also have a detrimental effect on probiotics, particularly during stabilization and storage.

Two strategies are generally applied to limit the negative effects of the stresses encountered during the stabilization processes and to improve bacterial survival and functionality recovery:

- The addition of protective molecules to the cell concentrates
- The choice of the operating conditions of the stabilization processes, such as thermal history, dehydration kinetics, or residual water content.

Few works reported on the effect of the protection step (eight studies) and stabilization conditions (six studies) on the survival of *L. salivarius* after stabilization and storage (**Table S3**). Stabilization conditions investigations mainly relate to spray drying (five studies) with only one study on the freezing parameters during freeze-drying.

Protection / The choice of the protective molecules

Protective agents have an important role during the drying process and storage. They minimize adverse effects of freeze-drying or spray-drying and therefore allowing better preservation of cells' viability and functionality. The studies on the effect of different protective agents for preserving *L. salivarius* following freeze-drying and spray-drying are summarized in **Table S3**. The evaluation of the efficiency of the protective molecules was mostly based on the measurement of bacterial viability before and after the stabilization process (survival rate).

Among the different media tested to protect *L. salivarius* strains, skim milk has been the most largely used one (80 % of the works). Furthermore, the freeze-drying process seems to result in lesser loss of viability than spray drying. For example, the strain UCC500 exhibited a survival rate of 22 % after freeze-drying in the presence of skim milk (Zayed and Roos 2004), while the survival rate decreased to 1 % after spray drying (Corcoran et al. 2004). The viability recovery of *L. salivarius* appeared to be also dependent on the strain considered. Survival rates higher than 90 % were reported after freeze-drying and spray drying of *L. salivarius* CFR 2158, regardless of the protective agents used (Reddy et al. 2009b; Reddy et al. 2009a).

The comparison of different protective agents' efficiency remains delicate since their concentration changed between reported works, and the ratio between the biomass and the protective agent (in a dry matter weight basis) is often unknown. Nevertheless, in **Figure 3** we attempted to compare the efficiency of several protective solutions for preserving the viability of *L. salivarius* UCC500, W13, I24, CM-CIDCA 1231B, and CM-CIDCA 1232Y after freeze-drying (Zayed and Roos 2004; Ming et al. 2009; Cejas et al. 2017; Yeo et al. 2018). The strain CFR 2158 studied by Reddy et al. (2009a) was not included in the comparison due to its particular high drying resistance. When only one component is included in the protective solution, survival rates lower than 20 % were reported, regardless of the nature of the molecule (sugar, skim milk, yeast extract). Survival rates higher than 80 % were obtained when two or three components were used in the protective solution. The associations of trehalose with sucrose or skim milk appeared to be the most effective ones for preserving *L. salivarius* during freeze-drying. The addition of sodium glutamate to skim milk and sucrose also had a beneficial impact on preserving *L. salivarius* W13 (Yeo et al. 2018).

Only one work investigated the recovery of functional properties such as acid and bile tolerance, and cholesterol assimilation in addition to the bacterial viability after freeze-drying (Reddy et al. 2009a). Among the 11 tested formulations, only 4 enabled a recovery rate higher than 80 % compared to active fresh cells for the three functional

properties. These are the formulations containing maltodextrin, skim milk + glycerol + CaCO₃, fructooligosaccharides (FOS), and maltodextrin + FOS.

Regards to the spray drying process, few studies have reported the effect of the protective solution composition on the survival rate of *L. salivarius*. The cell concentration (1 – 5 %) of *L. salivarius* CFR 2158 in a protective solution composed of skim milk or maltodextrin had no significant impact on the bacterial viability and functional properties recovery (Reddy et al. 2009b). **Figure 4** illustrates the efficiency of skim milk (SM) and maltodextrin (MD) for preserving the functional properties of *L. salivarius* CFR 2158 after spray drying. The recovery rates obtained after freeze-drying (Reddy et al. 2009a) were also included. Maltodextrin appeared as an efficient protective agent, better than skim milk. Furthermore, the survival rate of *L. salivarius* NRRL B-30514 after spray drying was slightly improved when lactose and trehalose were combined to skim milk, but the results appeared dependent on the water activity of the dried samples (Zhang et al. 2016).

Mechanisms of protection

Skim milk is a widespread complex protective agent that contains lactose, proteins, and salts. However, the mechanisms underlying the protective effect of skim milk are still under debate. Early studies attributed the protection of skim milk to lactose for its possible interaction with the cell membrane which could contribute to maintaining membrane integrity (Khem et al. 2015; Zhang et al. 2015). Another hypothesis is that milk proteins prevent cellular injury by forming a protective coating around the cell wall (Zayed and Roos 2004; Ming et al. 2009; Yeo et al. 2018) and stabilizing the cell membrane constituents (Castro et al. 1995). Besides, the use of skim milk as a protective agent can lead to stability problems because, during the manufacturing and storage of probiotic microorganisms, lactose may crystallize or participate in early Maillard reaction (nonenzymatic browning) (Morgan et al. 2005).

Trehalose and sucrose are sugars widely used (**Table S3**) for their ability to replace water (Golovina et al. 2009), maintain biological structures, and prevent fusion and loss of membrane integrity associated with dehydration (Crowe et al. 1988). They also contribute to form a glassy state that is mainly characterized by extremely high viscosity. In a glassy state, the probiotic microorganisms are immobilized in a viscous matrix where diffusive damaging reactions are prevented due to the low molecular mobility characterizing this state (Crowe et al. 1998; Passot et al. 2012). The protective ability of different disaccharides for preserving dried probiotic microorganisms was positively correlated to the glass transition temperatures and glass-forming ability and a low water activity (< = 11.4 %) (Miao et al. 2008).

Among glassy forming molecules, polymers such as maltodextrins are very interesting protective agents because they ensure physical stability and increases the efficiency of the manufacturing process (Fonseca et al. 2021). They exhibit high glass transition temperatures (T_g) in the liquid (Fonseca et al. 2021) and dry states (Roos and Karel 1991). Maltodextrins, therefore, provide a glassy stable matrix over a wide range of temperatures in the frozen and dried states.

However, polymers are less efficient than disaccharides to replace water because of the molecular steric hindrance inherent to their size. As could be thus expected, complementary or synergic protective effects of polymers and

disaccharides were evidenced when using them in combination for drying stabilization (Oldenhof et al. 2005) or dry storage (Teng et al. 2017) of probiotic strains.

Oxygen may have adverse effects on the survival of dried probiotic microorganisms that depend on the water activity and storage temperature, but also on strain intrinsic sensitivity (Castro et al. 1995; Teixeira et al. 1996; Talwalkar and Kailasapathy 2004; Simpson et al. 2005; Kurtmann et al. 2009b). Oxidative processes during drying and subsequent storage may cause viability losses due to the presence of free radicals that may oxidize membrane lipids (Teixeira et al. 1996; Borst et al. 2000), cell wall, cell membrane, and DNA affecting the viability of dried cultures (Lieveense and Verbeek 1994). Sodium ascorbate is the antioxidant most currently added to bacterial concentrates to avoid oxidative cell deterioration caused by the drying process (Kurtmann et al. 2009a), or by the intermediate storage before freezing or frozen storage at high subzero temperatures (-20°C) (Fonseca et al. 2003). It is noteworthy that no antioxidant has been used in the *L. salivarius* reported studies (**Table S3**).

Drying processes / The choice of the operating conditions

The drying processes, either freeze-drying or spray drying, by exposing bacterial cells to important environmental changes (temperature, solute concentration), can induce significant cellular damage (**Table 2**). The thermal stress induced by spray drying appeared to be highly detrimental to the survival of some sensitive strains of *L. salivarius*. Whereas one log of viability loss is commonly observed after freeze-drying in the presence of skim milk, the losses can reach 4 logs after spray drying (**Table S3**). During the drying process, product parameters such as temperature and residual moisture content can affect the probiotic viability recovery (Passot et al. 2012; Behboudi-Jobbehdar et al. 2013; Perdana et al. 2013; Verlhac et al. 2020). However, these parameters are not directly controlled during the processes, but result from the operating conditions applied (air temperature, product feed rate for spray drying, shelf temperature, chamber pressure and time for freeze-drying)

The published studies on *L. salivarius* drying were carried out with laboratory scale dryers (**Table S3**). This can make it difficult to precisely control the operating conditions and thus limits the scope of the studies.

The impact of freezing conditions on the viability of freeze-dried cells has however been studied in *L. salivarius* I24. The freezing protocol, in particular the freezing temperature, affected the survival rate of bacteria (Ming et al. 2009). Lowering the freezing temperature from -30°C to -80°C improved by 20 % (45 % to 65 %, respectively) the survival rate of *L. salivarius* I24 freeze-dried with a mixture of sucrose and skim milk. Furthermore, the addition of a holding step at 4°C for 2 hours before freezing reduced the survival rate, in particular for the freezing temperature of -30°C . Since no antioxidant was added to the protective solutions, oxidation reactions during the holding step could explain these results (Fonseca et al. 2003).

The outlet temperature of the air is the main studied operating condition of the spray drying process of *L. salivarius* (2 out of 5 studies). **Figure 5** illustrates the effect of the outlet air temperature on the survival rate of two strains of *L. salivarius*: i) *L. salivarius* UCC188 spray-dried directly after fermentation without the addition of protective molecules (**Fig. 5a**) (Gardiner et al. 2000), and *L. salivarius* NRRL B-30514 (**Fig. 5b**) protected with skim milk (in blue), or skim milk + lactose + trehalose (in green), or skim milk + sucrose + trehalose (in yellow) (Zhang et al. 2016). The values of the water content or the water activity of the spray-dried samples were also included since the

modifications of the outlet air temperature changed the residual moisture content of the sample. Increasing the outlet temperature of the air in the spray dryer resulted in decreasing the survival rate, as well as reducing the residual moisture content. Consequently, it is difficult to dissociate the effect of the two factors, namely air temperature and residual moisture content, on the bacterial survival rate. The moisture content of the freeze-dried samples is usually lower than 3 % while negative temperatures are applied to remove water. We can thus speculate that the outlet air temperature had a major effect on the viability of *L. salivarius* after spray drying, and values of outlet air temperature lower than 70 °C might be appropriate.

Radiant under vacuum is an emerging drying technique that has been reported as an alternative for the dehydration of *L. salivarius* 417 (Ahmad et al. 2012). Drying *L. salivarius* 417 by radiant energy under vacuum at laboratory scale, resulted in a better survival (89 %) than after freeze-drying (36 %). Operating conditions such as power and absolute pressure levels have a strong effect on bacterial survival after drying (Ahmad et al. 2012) and need to be optimized.

Two works investigated the effect of adding a thermal treatment to the protected *L. salivarius* bacterial suspensions just before the stabilization process. A cold shock treatment consisting of maintaining the samples at 8°C for 1-5 hours before freeze-drying was applied to *L. salivarius* CFR2158 (Reddy et al. 2009a). A slight improvement of the functional properties (bile tolerance and cholesterol assimilation) in the freeze-dried samples was observed. A heat shock treatment (50 °C for 15 min) applied before the spray-dried process (**Fig. 6**) resulted in an improvement of survival rate of *L. salivarius* NRRL B-30514 when values of outlet air temperature lower than 90 °C were applied (Zhang et al. 2016). The exposition of bacterial cells to moderate environmental stress that will be encountered during the freeze-drying (exposure to low temperature) or spray drying (exposure to high temperature), made it possible to activate some cellular responses, such as the production of heat or cold shock proteins (Reddy et al. 2009a), to improve the resistance of *L. salivarius* to the stabilization processes.

Storage of dried concentrated cultures of *L. salivarius*

After the stabilization process, the dried product is usually packed in aluminium bags and stored for several months at refrigerated temperature. The storage stability of *L. salivarius* is a key point for its commercialization and unfortunately remained little investigated.

The effect of the residual moisture content (or water activity) on the viability of *L. salivarius* during storage was studied by (Zayed and Roos 2004). The authors modulated the residual moisture content of freeze-dried samples of *L. salivarius* UCC500 and investigated the viability evolution following 49 days of storage at ambient temperature. The best viability preservation appeared to be associated with an optimal value of residual moisture content.

The storage stability of spray-dried *L. salivarius* NRRL B-30514 was assessed at 21°C, for different drying conditions (Zhang et al. 2016). Various outlet air temperatures leading to different water activity values (between 0.25 and 0.12) were investigated. The highest loss of bacterial viability after storage was observed for the samples spray-dried at the lowest outlet air temperature (below 84 °C), corresponding to a water activity higher than 0.19, whereas these operating conditions exhibited the highest survival rate immediately after freeze-drying. The optimal value of water content of a dried bacterial sample is determined by the nature of the protective medium used, the

storage temperature, and may differ depending on whether the target is a high survival rate after drying or low inactivation during storage.

Low storage temperatures generally lead to the highest bacterial survival because they slow down chemical reactions responsible for cell damage (Foerst et al. 2012). Accordingly, spray-dried samples of *L. salivarius* UC500 and *L. salivarius* UCC118 exhibited a higher survival after storage when stored at refrigerated temperatures than at room and high temperatures (Gardiner et al. 2000; Corcoran et al. 2004) (**Table S3**).

The storage stability of dried bacteria is affected not only by storage temperature and the residual moisture of the samples but also by the physical state of the dried product, represented by the glass transition temperature (T_g) (Kurtmann et al. 2009b; Fonseca et al. 2021). T_g is a threshold temperature for stability that is determined by the protective agents added before drying and the final water activity and water content. At storage temperatures lower than T_g, the bacteria are embedded highly viscous matrix (glassy state) that where molecular diffusion and deleterious reactions are limited, thus maintaining cell survival. The T_g may change during the storage, depending on the combination of water activity (relative humidity) and storage temperature. Water uptake during storage lowers the T_g of the powder and the transition from a glassy state to a rubbery unstable state can occur and lead to degradation of viability.

Furthermore, several studies on *L. salivarius* included skim milk in the formulation of the protective solution. Lactose crystallization and early Maillard reaction can occur when the protective agents are composed of skim milk. Such reactions lead to increased water activity, resulting in the acceleration of degradation reactions (Morgan et al. 2005; Thomsen et al. 2005), decreasing the survival of the cells. Maillard reactions can also take place if fermentation medium's residues such as amino acids and reducing sugars (e.g. glucose, galactose, lactose, and maltose) (Kurtmann et al. 2009c) are present in the bacterial concentrate (e.g. no washing step and low biomass concentration).

Rehydration

For optimum utilisation of probiotics as food ingredients, it is essential to identify the most appropriate reconstitution or rehydration conditions for recovering viability and functionality of dried probiotics powders. Parameters such as the powder quality and composition (protective agents, particle size, and water activity), the rehydration medium (composition, osmolarity, and pH), and the rehydration conditions (temperature, duration, stirring) may affect the rate of recovery to the viable state and thus influence survival rates (Muller et al. 2010). The effect of rehydration temperature differs among strains and drying processes. However, the tendency is to exhibit a linear increase in viability recovery of lactic acid bacteria and bifidobacteria with rehydration temperatures (5-40°C) (Wang et al. 2004; Mille et al. 2004). To our knowledge, no study has been reported about the appropriate reconstitution conditions for *L. salivarius* dried concentrates.

Future prospects for improving the quality and long-term stability of *Ligilactobacillus salivarius* concentrates

In this review, we highlighted the interest of *L. salivarius* as a probiotic for different health applications and we summarized the data available on the manufacturing process of cell concentrates, in particular in the dried form. Quite extensive information is available about the functional properties of nearly twenty different strains of *L. salivarius*. However, few (around 20 %) of these promising strains are included in probiotic commercialized products. Maintaining the viability and functionalities for long periods appears not easy but challenging.

To date, most studies focused on the fermentation step, but the effect of the fermentation conditions on *L. salivarius* survival is rarely evaluated after stabilization, and not at all upon storage (<https://doi.org/10.15454/DK0B4K>). Stabilization processes and storage conditions are crucial for cell survival since they are sources of different types of stress. Moreover, most studies reported viability losses incompatible with storage at ambient temperature that is a major challenge for most probiotic applications. It appears essential to have a holistic approach, identifying the steps of the whole production chain leading to the degradation of the cell viability and functionalities, namely the hot spot(s). Once the hot spot has been identified, the next step is to understand the factors (or associated operating conditions) governing the recovery of cell viability and to modify them to improve the final quality and product shelf life. Furthermore, due to the cost of *in vitro* and *in vivo* trials, the functionality of the probiotic is often assessed by measuring the viability recovery after rehydration of the powder and/or after exposure to environmental stresses encountered during probiotic consumption (bile and acid stresses). Alternative approaches based on the research and quantification of biomarkers of the probiotic benefits (e.g. antioxidant and anti-inflammatory capacities, cholesterol and triglycerides reduction) need to be further developed to be sure that any modification of the manufacturing operating conditions does not alter the functionality.

Our review of the manufacturing conditions of *L. salivarius* pointed out several strategies for improving the viability recovery after drying and storage.

Genome comparison for identifying markers of functionality and resistance to environmental stresses

Genome sequencing and genomic comparative approach currently contribute to the identification of probiotic characteristics and the prediction of probiotic behavior in the human gastrointestinal tract. For instance, analysis of endogenous plasmids of *Lactobacillus* enabled the identification of genes encoding bacteriocin production (Flynn et al. 2002; Cho et al. 2011; Langa et al. 2012), exopolysaccharides synthesis (Liu et al. 2009; Ham et al. 2011; Cho et al. 2011), and genes involved in adherence (Ham et al. 2011; Cho et al. 2011).

The mechanisms that allow probiotic strains to cope with the deleterious consequences of exposure to environmental stresses are likewise controlled by well-regulated genetic systems, including DNA damage, protein misfolding, and loss of cell wall/membrane integrity (Arnold et al. 2018). Genomic and functional characterization of resistant and sensitive strains of *L. salivarius* could help to understand the cellular mechanisms that affect survival during the manufacturing process or product consumption.

Cell adaptation to stressful environments during fermentation and post-fermentation steps

Application of moderate stressful treatment during fermentation and/or post fermentation to probiotic bacteria may result in improving survival recovery after the stabilization processes and storage. The treatment applied to probiotics include high (Broadbent and Lin 1999; Desmond et al. 2001) and low temperature (Broadbent and Lin 1999), high osmolarity (Panoff et al. 2000; Desmond et al. 2001; Louesdon et al. 2014; Huang et al. 2018), low pH (Palmfeldt and Hahn-Hagerdal 2000; Saarela et al. 2009), oxydative conditions (Desmond et al. 2001). However, the degree of stress needs to be adequately selected to avoid negative effects on the cell growth rate, cell concentration, or even survival after storage (Desmond et al. 2001; Schoug et al. 2008; Mozzetti et al. 2013).

These cell adaptation treatments induce activation of cellular pathways of stress sensing defenses that in turn result in the production of specific proteins (Desmond et al. 2004) and modification of the membrane (Fonseca et al. 2019) and wall composition. With the exponential advances in genomic, transcriptomic, and proteomic approaches it has been possible to identify genes involved in *Lactobacillus* stress responses (Prasad et al. 2003; Walter et al. 2003). Bacterial membrane being the main target of cell injury of stabilization and storage processes, the systematic characterization, at different steps of the production of stabilized probiotics, of the lipid composition and biophysical properties of membranes associated with bacterial viability will enable the identification of optimal strategies for designing the fermentation operation.

Development of new protective formulations and stabilization processes

The addition of protective molecules to the cell concentrates appears as a promising way to reduce viability and functionality losses during stabilization and storage. Promising mixtures should contain polymers, non-reducing disaccharides and antioxidants, to limit diffusion degradation reactions, membranes injury/disorganization, and oxidation, respectively. The effect of antioxidants, so far not studied for *L. salivarius*, deserves to be explored. The replacement of skim milk by maltodextrin should improve productivity by increasing the Tg value of the cell concentrates and avoiding deterioration reactions during storage associated to skim milk.

Oligosaccharides such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS) have been reported as alternative protective compounds during drying, dehydration process, and subsequent storage (Corcoran et al. 2004; Romano et al. 2016; Cejas et al. 2017). Since these oligosaccharides are also prebiotics (functional compounds that induce growth or activity of beneficial microorganisms), their addition to probiotic cell concentrates can add value for probiotic product development.

Encapsulation of probiotic strains to be dried by freeze-drying or spray-drying appears as an interesting option to protect the cells against diverse stresses to which they are subjected during drying (e.g. thermal, mechanical). Different encapsulation techniques have been developed (Picot and Lacroix 2004; Burgain et al. 2011; Dianawati et al. 2013; Wang et al. 2016a; Yao et al. 2021). Recently, the survival of *L. salivarius* added to a food matrix was improved after 30 days of storage when it was encapsulated (alginate based matrix) before hot air drying (Betoret et al. 2019) or freeze-dried (Burca-Busaga et al. 2020). Furthermore, the direct mixture of a *L. salivarius* concentrated cell suspension with amorphous lactose powder at a suspension: lactose ratio (v:w) of 1:15 and 1:25,

led to low loss of viability ($\sim 0.5 \log \text{CFU.g}^{-1}$) after 3 months of storage at 4°C, while no survival was detected after 2 months at room temperature (Wang et al. 2020).

Finally, in a world context of sustainable awareness, the development of a new process has to include the environmental dimension. Stabilization processes are known for being energy-consuming. The environmental assessment of the production of *Lactobacillus delbrueckii subsp. bulgaricus* CFL1 concentrates (frozen and freeze-dried) revealed that freeze-drying, frozen storage, and fermentation were the environmental hotspots of the system (Pénicaud et al. 2018). However, the choice of the stabilization process (freezing vs freeze-drying) depended on the intended storage duration. Regardless of the preservation strategy, increasing the LAB quality appears a key parameter for improving the environmental performance of the system.

Conclusion

Despite a plethora of interesting properties, potential health benefits, and applications of *Ligilactobacillus salivarius*, few strains have reached the market. This can be explained by the sensitivity of this microorganism to stabilization and storage processes. However, much information remains unknown about their survival at the different steps of the manufacturing process, and particularly after storage. Moreover, most works relate to probiotic production in laboratory conditions and the assessment of viability recovery only (not probiotic functionality). Research on other probiotic microorganisms can give guidelines for future research, but it is well known that the degradation and preservation mechanisms are dependent on the species and even on the strains.

Different future research strategies have been identified in this review to increase knowledge on the mechanisms of resistance, and therefore to improve the viability and long-term stability of *L. salivarius*. To work on formulation with a rationalized approach appears as the simplest and most straightforward strategy. According to the literature on bacterial preservation mechanisms, such as medium vitrification, membrane protection, and antioxidant activity, alternative formulations can be proposed. Formulation and stabilization operating conditions (freeze-drying, drying) should be investigated and optimized together/coupled, and in relevant scales for further industrial transfer.

The cell adaptation to moderate environmental stress leading to high resistance to the stabilization and storage processes appears as a promising approach. Likewise, the encapsulation of bacterial cells before drying appears as an appealing method in particular for stabilizing sensitive strains. Both strategies deserve further investigation, to adapt them to pilot and industrial conditions, and the specificity of each strain.

The systematic characterization of the biological, biochemical, and biophysical properties of *L. salivarius* at each step of the process and until its end-use is crucial for identifying the preservation mechanisms and biomarkers of resistance, as well as the operating conditions for optimizing production and stabilization processes. The integration of the environmental dimension in such a holistic approach would be of great value for developing environmentally friendlier ways of preserving probiotics.

Authors' contribution

MGS searched and organized the selected literature. SP and FF conceived the review. MGS, SP and FF wrote the manuscript with input from MO and SC. All authors read and approved the final version of the manuscript.

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Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Table 1 Demonstrated health benefits and applications of *Ligilactobacillus salivarius* strains of different origins

Strain and Origin	Demonstrated health benefit effect			Application	Reference
	Functionalities	Type of study	Mode of administration		
FV2 in Probiotic blend (Human vagina)	AMA	<i>In vivo</i> (human)	Vaginal tablet	Bacterial vaginosis	(Mastromarino et al. 2009)
UCM572 (Human vagina)	AMA	<i>In vitro</i>	B. suspension	Urinary tract infections	(De Llano et al. 2017)
CRL1328 (Human vagina)	AMA	<i>In vitro</i>	B. suspension	Urogenital tract infections	(Zarate and Nader-Macias 2006)
MG242 (Human vagina)	AMA	<i>In vitro</i>	B. suspension	Vulvovaginal candidiasis	(Kang et al. 2018)
ATCC11742 in Probiotic blend	AMA	<i>In vitro</i>	B. suspension	Vaginal infections	(Cavaliere Ved. Vesely and De Simone)
CNU1334 (Oral cavity)	AMA	<i>In vitro</i>	B. suspension	Chronic infections	(Kang et al. 2017)
NK02 (Oral cavity)	AMA	<i>In vitro</i> / <i>In vivo</i> (human)	B. suspension / Dry powder	Periodontitis	(Sajedinejad et al. 2018)
SGL03 in a dietary supplement	AMA	<i>In vitro</i> / <i>In vivo</i> (human)	B. suspension	Periodontitis	(Kucia et al. 2020)
MG4265 (Human)	AMA	<i>In vitro</i>	B. supernatant	Periodontitis and dental caries	(Jung et al. 2021)
WB21 (from WB 1004)	AMA	<i>In vivo</i> (human)	Capsule of FD powder	Periodontitis	(Shimauchi et al. 2008; Mayanagi et al. 2009)
	AMA	<i>In vivo</i> (human)	Capsule of FD powder	Halitosis	(Iwamoto et al. 2010; Suzuki et al. 2014)
	AMA	<i>In vitro</i>	B. suspension	Periodontitis, halitosis and dental caries	(Higuchi et al. 2019)
K35 / K43 (Human saliva)	AMA	<i>In vitro</i>	B. suspension	Dental caries	(Wu et al. 2015)
TI2711 (LS1)	AMA	<i>In vivo</i> (human)	Oral capsule	Dental caries	(Nishihara et al. 2014)
AR809 (Human pharynx)	AMA	<i>In vitro</i> / <i>In vivo</i> (mouse)	B. suspension	Pharyngitis	(Jia et al. 2020)
PS7 (Human breast milk)	AMA	<i>In vitro</i> / <i>In vivo</i> (rat, human)	B. suspension	Acute otitis media	(Cárdenas et al. 2019)
AP-32 in probiotic blend	AMA	<i>In vitro</i> / <i>In vivo</i> (human)	Lozenges	Oral diseases	(Lin et al. 2021)
UCC118 (Human ileal-caecal region)	AMA	<i>In vivo</i> (mouse)	B. suspension	Bacterial infection	(Corr et al. 2007)
MM1 (Healthy infant feces)	AMA	<i>In vitro</i>	B. supernatant	Diarrheal illness	(Tsai et al. 2008)
FP25 / FP35 (Healthy infant feces)	AMA	<i>In vitro</i>	B. suspension	Colon cancer	(Thirabunyanon and Hongwittayakorn 2013)
UO.C003 / UO.C027 (Chicken caecal mucosa)	AMA	<i>In vitro</i>	B. suspension	Foodborne illnesses outbreaks	(Lone et al. 2021)
CTC2197 (Chicks gastro-intestinal tract)	AMA	<i>In vivo</i> : chicken	FD powder	Foodborne salmonellosis outbreaks	(Pascual et al. 1999)

Probio-37 (Porcine gastro-intestinal tract)		AMA	<i>In vitro</i>	B. supernatant	Intestinal infections in piglets	(Kumar et al. 2010)
CRL1702 in probiotic blend (Calf feces)		AMA	<i>In vivo</i> (calf)	Fermented milk	Diarrhea in new-born calves	(Maldonado et al. 2018)
PS7 (Human breast milk)		AMA	<i>In vitro</i> / <i>In vivo</i> (rat, human)	B. suspension	Acute otitis media	(Cárdenas et al. 2019)
TUCO-L2 (llama milk)		AMA & IME	<i>In vitro</i> / <i>In vivo</i> (mouse)	B. suspension	Intestinal infections	(Albarracin et al. 2020)
Probiotics blend		AMA & IME	<i>In vivo</i> (human)	Capsule of FD powder	Ulcerative colitis	(Palumbo et al. 2016)
PS2 (Human breast milk)		AMA & IME	<i>In vivo</i> (human)	Capsule of FD powder	Lactational mastitis	(Fernández et al. 2016; Jiménez et al. 2021)
FXJXJ7-2 (Human feces)		IME	<i>In vitro</i> / <i>In vivo</i> (mouse)	NA / B. suspension	Inflammatory bowel diseases	(Zhai et al. 2020)
Ls-33		IME	<i>In vitro</i> / <i>In vivo</i> (mouse)	B. suspension	Inflammatory bowel diseases	(Daniel et al. 2006)
LA 302 in Probiotic blend		IME	<i>In vitro</i> / <i>In vivo</i> (mouse)	B. suspension / FD powder	Inflammatory bowel diseases	(Drouault-Holowacz et al. 2006)
UBL S22		IME	<i>In vivo</i> (human)	Capsule of FD powder	Cholesterol	(Rajkumar et al. 2015)
		IA	<i>In vitro</i>	B. suspension	Colorectal cancer	(Yenuganti et al. 2021)
W57 in Probiotics blend		IME	<i>In vivo</i> (human)	Rehydrated FD powder	Allergic disorders	(Watts et al. 2016)
PM-A0006		IME	<i>In vivo</i> (human)	Rehydrated powder	Allergic disorders	(Lin et al. 2013)
		IME	<i>In vivo</i> (mouse)	Rehydrated FD powder	Allergic disorders	(Li et al. 2010)
LS01 (Feces or vaginal brushes)	Strain alone	IME	<i>In vivo</i> (human)	Sachets of FD powder	Skin disorders	(Niccoli et al. 2014)
		IME	<i>In vitro</i> / <i>In vivo</i> (human)	B. suspension / Rehydrated FD powder	Skin disorders	(Drago et al. 2011)
	Probiotic blend	IME	<i>In vitro</i> / <i>In vivo</i> (human)	B. suspension / Rehydrated FD powder	Skin disorders	(Iemoli et al. 2012)
		IME	<i>In vivo</i> (human)	Rehydrated powder	Skin disorders	(Nettis et al. 2016)
FFIG58 (Intestine of pigs)		IME	<i>In vitro</i>	B. suspension	Bacterial infections in pigs	(Zhou et al. 2020)
LA307 (Animal)		IME & MIMB	<i>In vitro</i> / <i>In vivo</i> (mouse)	B. suspension / FD powder	Inflammatory bowel diseases	(Alard et al. 2018)
E4191 (Infant feces)		IME & MIMB	<i>In vitro</i>	Rehydrated FD powder	Inflammatory bowel diseases	(Oh et al. 2012)
BP121 (Infant feces)		IME & MIMB	<i>In vitro</i> / <i>In vivo</i> (rat)	B. supernatant	Acute kidney injury	(Lee et al. 2020)
LI01 (Healthy human)		IME & MIMB	<i>In vivo</i> (rat)	B. suspension	Liver disorders	(Lv et al. 2014; Shi et al. 2017)
LS144 (Feces of pigs)		MIMB	<i>In vivo</i> (piglet)	Rehydrated FD powder	Bacterial infections in pigs	(Moturi et al. 2021)
W24 in Probiotics blend		MIMB	<i>In vivo</i> (human)	Rehydrated FD powder	Pouchitis	(Persborn et al. 2013)
		MIMB	<i>In vivo</i> (human)	FD powder	Sad mood	(Steenbergen et al. 2015)
REN (Human feces)		MIMB	<i>In vivo</i> (rat)	B. suspension	Colon cancer	(Zhang et al. 2015)
		Protective effect	<i>In vivo</i> (rat)	NA / B. metabolites	Oral cancer	(Zhang et al. 2013)

		IA	<i>In vitro</i>	B. metabolites	Oral cancer	(Wang et al. 2016b)
CECT5713 (Human breast milk)	Strain alone	AMA	<i>In vitro / In vivo</i> (mouse)	B. suspension	Bacterial infections	(Olivares et al. 2006)
		IME	<i>In vivo</i> (mouse)	B. suspension	Inflammatory bowel diseases	(Arroyo et al. 2010)
		IME	<i>In vitro</i>	NA	Inflammatory bowel diseases	(Díaz-Ropero et al. 2007)
		IME	<i>In vivo</i> (rat)	B. suspension	Inflammatory bowel diseases	(Peran et al. 2005)
		IME & MIMB	<i>In vivo</i> (human)	Oral capsule	Gut microbiota composition and immune response	(Sierra et al. 2010)
		MIMB	<i>In vivo</i> (human)	FD powder	Infantile formula	(Maldonado et al. 2010)
		IME & MVM	<i>In vivo</i> (human)	FD powder	Infertility	(Fernández et al. 2021)
	Heat- inactivated	AMA	<i>In vitro</i>	B. suspension	Dental caries	(Krzyściak et al. 2017)
		AMA	<i>In vitro / In vivo</i> (human)	B. suspension / FD Powder	Dental caries	(Sañudo et al. 2017)
	Probiotic blend	AMA & IME	<i>In vivo</i> : human	Capsule of FD powder	Lactational mastitis	(Jiménez et al. 2008)

NA: not available, AMA: Antimicrobial activity, IME Immunological effects, IA: induction of apoptosis; MIMB: Modulation of the intestinal microbiota and barrier function, MVM: Modulation of the vaginal microbiota, B.: Bacterial, FD: Freeze-dried

Table 2: Comparison of freeze-drying and spray-drying

Characteristics	Freeze-drying	Spray-drying
Operating conditions	Shelf temperature, chamber pressure, time	Air inlet temperature, air flow rate, feed flow rate Relative humidity of the inlet air, speed (or pressure) of pulverization
Product temperature	Not exceed 30 °C	65-120 °C
Drying time Process Productivity	Days Batch process / Low productivity	Seconds / Minutes Continuous process / High productivity
Physical events (environmental changes) And associated bacterial stress	<p>Freezing Low product temperature (~ -50 °C) => Cold stress Ice crystal formation and growth => Mechanical stress Freeze-concentration of solutes => Osmotic stress Cell dehydration and cell volume reduction => Mechanical stress</p> <p>Primary Drying Ice sublimation Under vacuum (< 30 Pa) Low product temperature (~ -30 / -20 °C) => Cold stress</p> <p>Secondary drying Moderate product temperature (~25 / 30 °C) Under vacuum (< 10 Pa) Removal of unfrozen water (H-bonds breakage) => Mechanical stress</p>	<p>Pulverization of the solution => Mechanical stress Evaporation of water: High product temperature (~ 65 – 120 °C) => Thermal stress Solute's concentration => Osmotic stress Cell dehydration and cell volume reduction => Mechanical stress Removal of bound water (H-bonds breakage) => Mechanical stress Exposure to air => Oxidative stress</p>
Energy consumption	6 000 -9 000 kJ/ kg of water	5 000 kJ/kg of water*
Form of the dried product	Cake to be broken to obtain powder	Powder

* (Bimbenet et al. 2002)

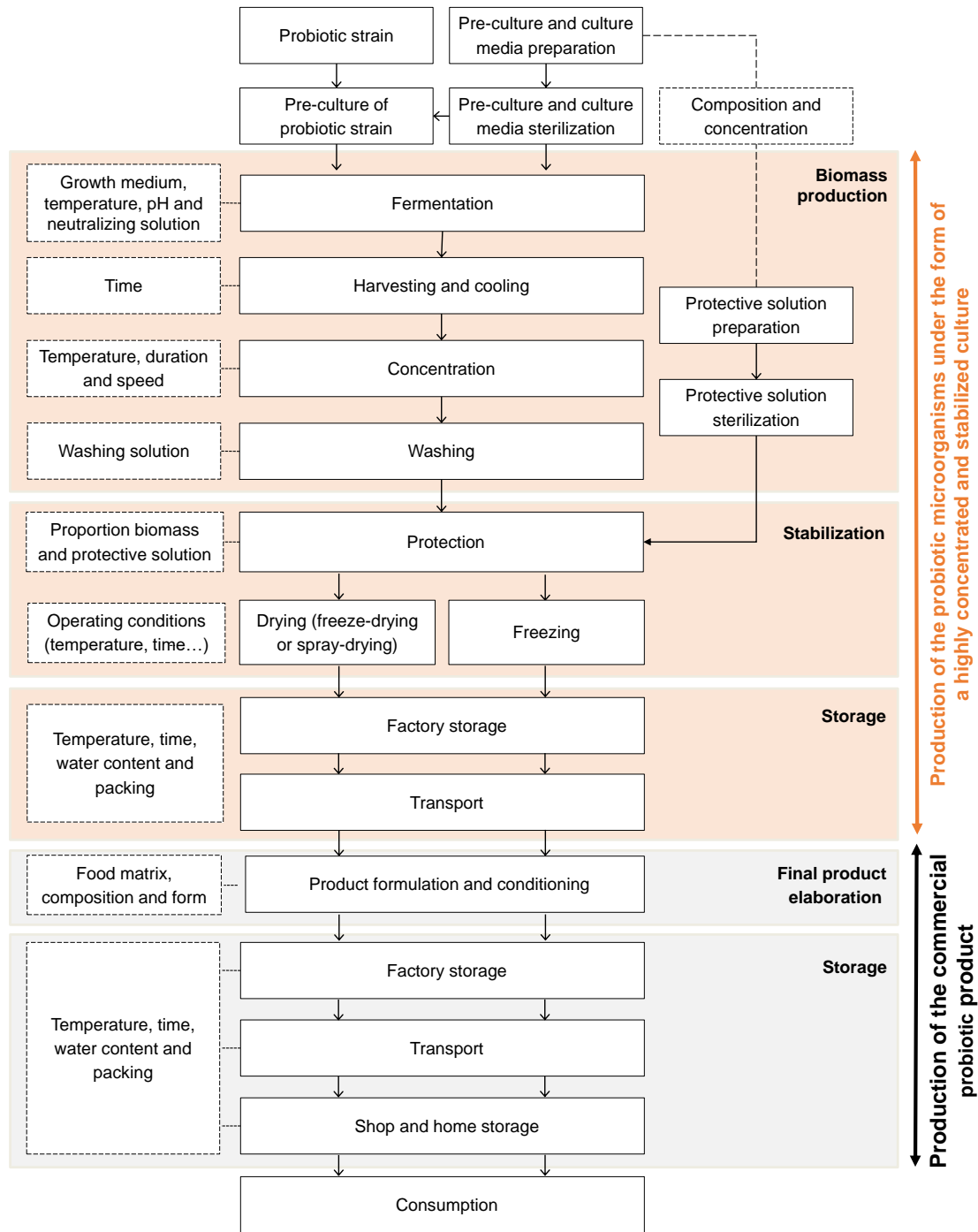


Figure 1. Flowchart showing the main operations of the production, stabilization, formulation and storage processes applied for manufacturing probiotic microorganisms until their final use

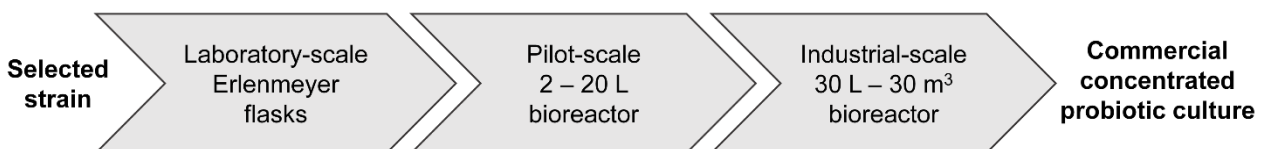


Figure 2. Basic itinerary of the industrial development of a probiotic microorganism

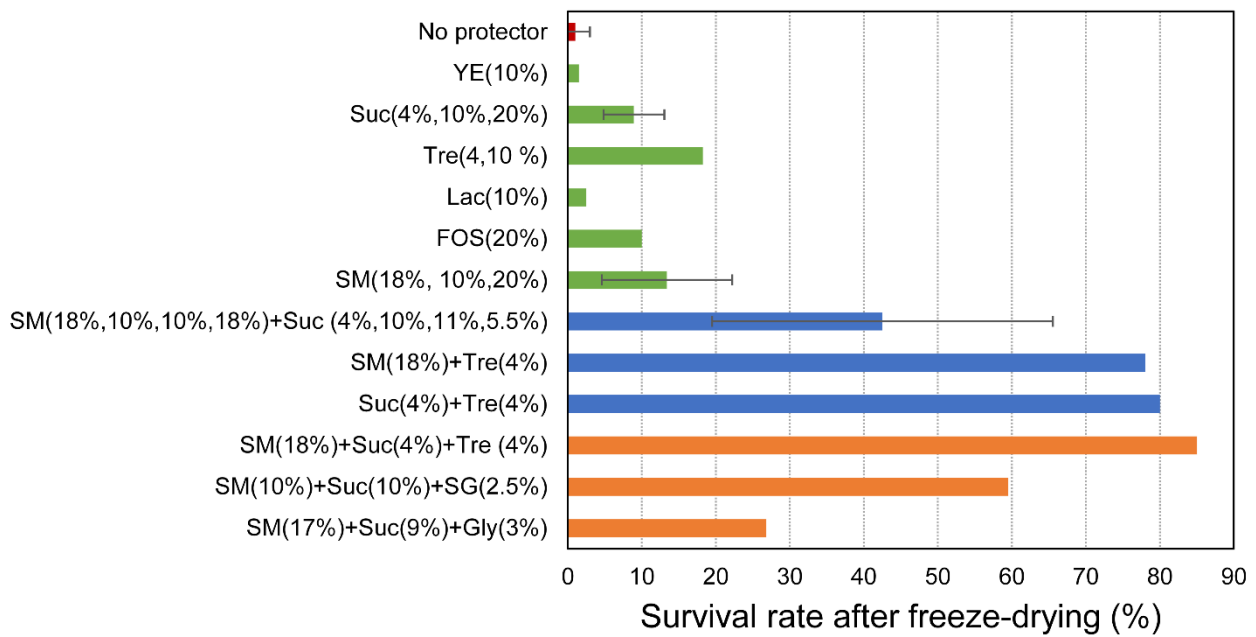


Figure 3. Effect of different protective solution on the survival rate of *L. salivarius* after freeze-drying

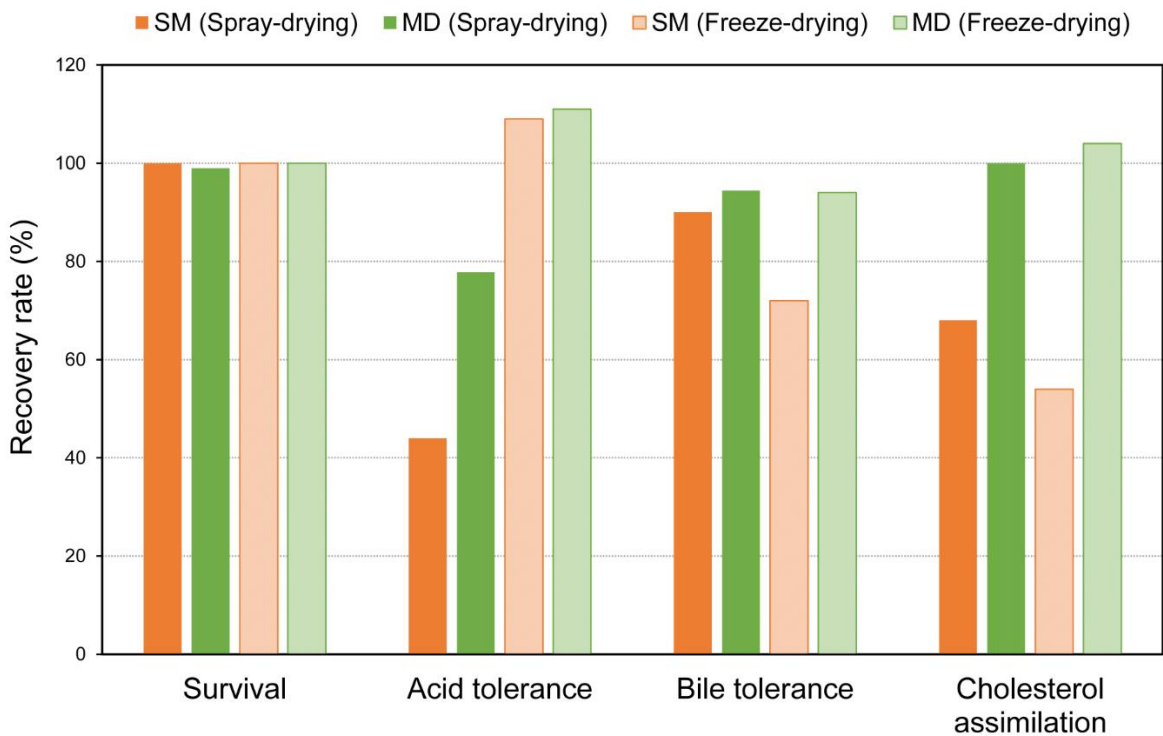


Figure 4. Effect skim milk (SM) and maltodextrine (MD) as protective solutions, on the recovery of functional properties of *L. salivarius* CFR 2158 after spray drying and freeze-drying (adapted from Reddy et al. 2009a and b). The recovery rates were estimated as the percentage of the viability or functional property determined after rehydration of dried samples in relation to the viability or functional property of the active cells.

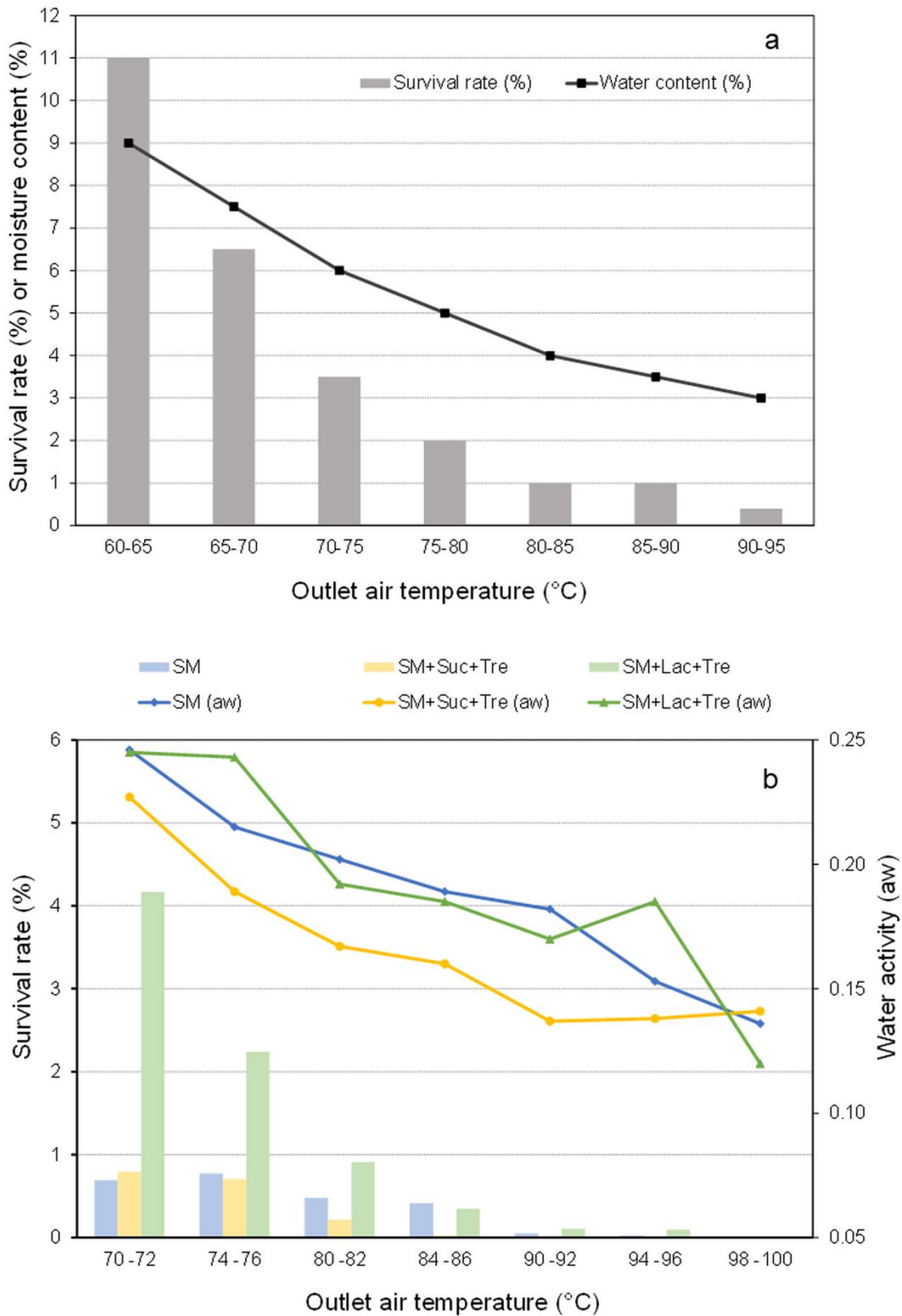


Figure 5. Effect of the outlet air temperature applied during the spray drying process on the survival rate, water activity (aw) and water content of *L. salivarius* UCC118 samples without addition of protective molecules (5b) (adapted from Gardiner et al. 2000), and *L. salivarius* NRRL B-30514 samples protected with either skim milk (SM), a mixture of skim milk, sucrose and trehalose (SM+Suc+Tre), or a mixture of skim milk, lactose and trehalose (SM+Lac+Tre) (5a) (adapted from Zhang et al. 2016).

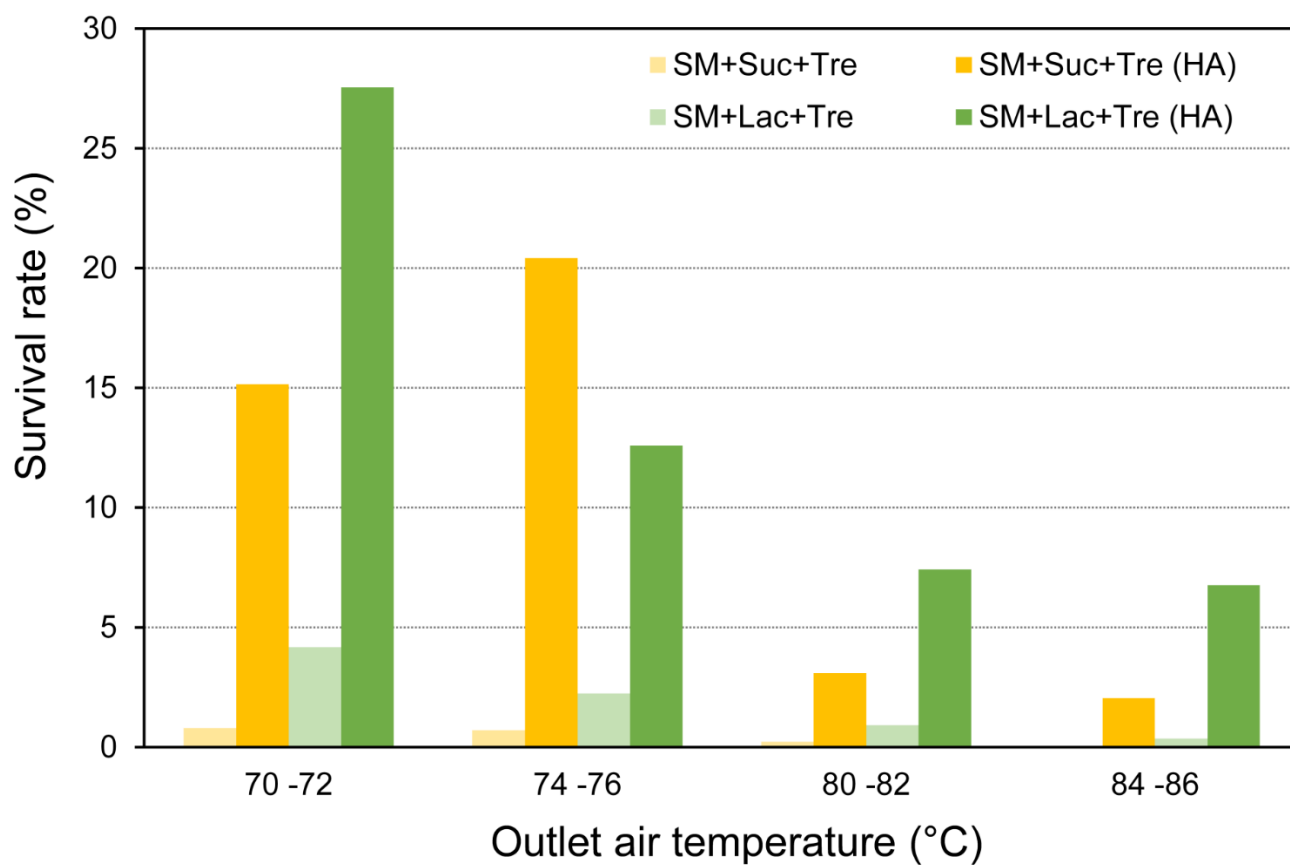


Figure 6. Effect of a heat treatment (50 °C for 15 min) (HA) before spray drying on the survival rate of *L. salivarius* NRRL B-30514 samples protected with either a mixture of skim milk, sucrose and trehalose (SM+Suc+Tre), or a mixture of skim milk, lactose and trehalose (SM+Lac+Tre) (adapted from Zhang et al. 2016).

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