Lyophilization (Drying Method) Cause Serious Damages to the Cell Viability of Lactic Acid Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Author IC designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author EKK and EN managed the analyses of the study. Author JD managed the literature searches. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Preservation of industrial's lactic acid bacteria (probiotics) by freeze-drying. Lactic acid bacteria have important nutritional needs and do not have resistance against the environmental conditions surrounding their production (drying, storage, etc.) and their use *in vivo* (physico-chemical properties of the digestive tract). In this condition, industrials and microbiologists develop regularly research

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projects of new lactic bacteria able to support the whole of the processes of production, storage and formulation without losing their functional properties. Among various methods of drying (atomization, fluidization and freeze-drying), freeze-drying makes it possible to obtain a thorough dehydration compatible with very long storage times. This method involves changes in product temperature and cause damage to microorganisms because it requires freezing that is not without consequences for cells. On the other side, it causes cellular (peroxydation of the fatty-acids) and genetic (proteins's modifications) deteriorations. Using cryoprotectants and antioxidants during freeze-drying storage increases appreciably the rate of viability of these cells.

1. GENERAL INFORMATION ON THE DRYING OF MICROORGANISMS

1.1 Introduction

It is common knowledge that processing may partially or totally affect the quality of a food product. Various changes may occur in physical, chemical and/or biological characteristics of foodstuffs during processing, storage and distribution [1]. Industrial strain development requires system-wide engineering and optimization of cellular metabolism while considering industrially relevant fermentation and recovery processes (Sang et al., 2015).

Commercialization of microbial strains, whether bacteria, yeast or mold, requires conditioning in a stable, stable form [2]. Indeed, because of their physiology, the microbial cells do not preserve themselves in the native state in their culture medium. Their development is often carried out in a fermenter in a liquid substrate. The cells conserved in this medium after their growth, consume the last available nutrients and for the most part express a fermentation metabolism which impairs the quality of the product (pH modification, odor emission, etc.). In addition, the cells, after burning their reserves, die in large numbers [3]. It is therefore essential to stabilize the microbial population. The drying of the cells then appears as a practical solution. By eliminating water, the cell metabolism is blocked, the cells are managed in a determined physiological state. In addition to the storage of micro-organisms, drying facilitates handling and storage and reduces costs. The methods of drying will be reviewed, especially the methods applied on an industrial scale, namely atomization, fluidization and freeze-drying. They are quite aggressive towards micro-organisms because they involve large variations in the temperature of the product [4]. The use of cryoprotectants during lyophilizing and antioxidants during storage significantly increases the viability of the cells (Rampino et al., 2013).

1.2 Types of Drying in Agro-Food Industries

Concentration, desiccation, drying, dehydration, all these terms will be grouped under the general term of water elimination. All these operations obey, basically, the same laws and their objectives are the same. While the concentration treats a liquid product to result in a liquid, the drying starts from a liquid or solid product, resulting in a solid. Drying is an extremely old method of preservation which, depriving the food of free water, prohibits any microbial or enzymatic activity. Concentration gives rise to only partial water removal, but it produces a product whose osmotic pressure is sometimes sufficient to impede any microbial development [5,6]. Thus, the removal of water makes it possible to buffer the seasonal of certain agricultural (haymaking) or industrial activities (apple juice concentrates in cider plants). Dry products, such as powdered milk, are kept for years. Freeze-drying has become a standard processing tech- nique in the bio-industry sector, where it enables stable products of high quality to be manufactured. Yet until quite recently, no monograph has been published that would analyse various aspects, scientific, engineering, economic, and regulatory ones, which make effective freeze-drying such a complex operation [7].

Energy must be provided to compensate both solid-liquid binding energy (due to Van Der Waals forces) and the latent heat of vaporization of the solvent [8]. . This contribution can be made by the gas phase (known as convection drying) or by an external source (heating by Joule effect, by infrared radiation or by high-frequency currents). Solvent saturation of the gas phase must also be avoided, which is ensured by

a sweeping of the atmosphere, which maintains the solvent partial pressure in the vicinity of the solid, which is lower than the saturating vapor pressure. The purpose of the fluidization is to increase in particular gas-solid contact to facilitate drying. There are three mains types of fluidized bed : single fluidized bed, air assures both fluidization, heat supply and evacuation of desorbed vapors ; fluidized bed vibrates, the supply of heat is still provided by the air, but the fluidization is done this time by a mechanical process ; As air is more likely provided with an internal heat exchanger, which only provides fluidization and evacuation of the vapors. The heat is supplied by an external heat exchanger placed inside the bed, [9].

1.2.1 Atomization

In agri-food, atomization is a method of dehydrating a liquid (juice, milk, etc.). During spray dehydration, the liquid foods are sprayed into a heating chamber in the form of droplets, thereby evaporating the water and collecting the dehydrated powdered food at the base of the apparatus. This method is used in particular for the dehydration of milk. It takes place in four mains stages : nebulization or dispersion into a homogeneous spray of the liquid, contact of the spray with hot air (150°C), evaporation of water from the droplets and separation of the dehydrated product (50-60°C) and air. It has the disadvantage of causing food losing their flavors which are entrained with water vapor and also, as a result of heating, denaturing certain components because, when the cells are dried with a small water content, a number of cellular components, such as DNA or RNA, and proteins are assayed [10,11].

1.2.2 Fluidization

Fluidization is a process whereby a granular material is converted from a static solid-like state to a dynamic fluid-like state. The process occurs when a fluid (liquid or gas) is passed up through the granular material (powder). In a fluidized bed gas is passing upwards through a bed of particles. The earliest applications of fluidization were for the purpose of carrying out chemical reactions. Since that time there have been a number of successful chemical processes involving fluidized bed reactors, but also other applications.

If the current is sent at a sufficient velocity, it causes a disintegration of the solid ; On the other hand, if this velocity is not too high, the grains, instead of being trained, have a disorderly agitation comparable to molecular agitation [12]. This is called a fluidized bed. The purpose of the fluidization is in particular to increase the gas-solid contact surface to make easy drying. There are three main types of fluidized bed: simple fluidized bed, air assures both fluidization, heat supply and evacuation of desorbed vapors; Fluidized bed, the supply of heat is still provided by the air, but the fluidization is done this time by a mechanical process; Fluidized bed with internal exchanger, the air only provides fluidization and the evacuation of the vapors, the heat is supplied by an external exchanger placed inside the bed.

1.2.3 Freeze-drying

Freeze-drying is a dehydration process especially suited to the conservation of biological products. In comparison with other drying processes, freeze-drying is considered as a reference for manufacturing high-quality dehydrated product. The direct transition of water from solid to vapor (sublimation), without a liquid phase, helps to preserve most of the initial raw material's properties such as appearance, shape, taste, color, and flavor. As an important functional property, the freeze-dried product has a high rehydration capacity. The main limit to the industrial development is its cost due to the low productivity. Consequently, except the application for biological active material (bacteria, vaccine), the use of freeze-drying is restricted in food industry to high added-value products like coffee, ingredients for ready-to-eat foods (fruits and vegetables, meat and fish), and aromatic herbs. [13]. The rate of freezing and the temperature level, depending on the nature and the composition of the product, define the number (nucleation) and size (growth) of the ice crystals. It may occur naturally (drying in the mountains) or, more rapidly, in a lyophilizer [14]. Freeze-drying generally involves three steps: freezing, sublimation and secondary drying.

2. LYOPHILIZATION OF LACTIC BACTERIA

Freeze-drying developed during the Second World War for the storage of blood plasma (Pegg, 2002). Thus, for vaccines, as for a large number of drugs and microorganisms, freeze-drying is the only technics for long-term preservation of dry active ingredients [15]. In the food industry, lactic ferments (probiotics and starters) are increasingly used in lyophilized form.

2.1 Description and Principles of Freeze-Drying

The first phase of the operation consists of freezing the product, that is to say bringing the water it contains into the solid state; This involves removing energy from it. The second phase consists in extracting water from the product by volatilization by supplying it with thermal energy [16]. This second operation takes place in an enclosure where there is a pressure that is very much less than atmospheric pressure (of the order of 0.1 to 1 mBar).

2.1.1 Freezing

During freezing, the starting liquid solution is assumed to be homogeneous, part of the water separates from the dissolved substances to crystallize in the pure state. The remainder of the water then solidifies gradually in a mixture with the solutes to form an interstitial material (between the ice crystals) which adopts an amorphous (non-crystalline or vitreous) structure. In general, dissolved substances do not crystallize from a complex solution [17] An important parameter to be taken into account for the freezing step is the freezing rate which determines the size of the ice crystals. Indeed, the larger the ice crystals formed, the larger the pore diameter and, consequently, the higher the desiccation rate. It is therefore important from a technological point of view to produce small crystals of ice by rapid freezing, resulting in small intracellular crystals that are not harmful to the cells [18,19].

2.1.2 Drying

It takes place in two stages: first, the sublimation of the crystallized water (primary drying, the water passes directly from the solid to the gaseous state), then the desorption and evaporation phase of the water, Which has adopted a non-crystalline or vitreous form (secondary drying) during freezing. This results from the solidification without rearrangement of the molecules due to the very large increase in viscosity [20]. These states (crystalline and glassy) can coexist in variable proportions for the same sample, the proportion of water in the vitreous state being the greater the higher the rate of freezing and viscosity [21].

The cavities left by the crystals after the sublimation of the water give the porosity necessary for the evaporation of the water situated in the interstitial space [22].

The water present in the vitreous mass must first undergo a molecular diffusion within the material before reaching the cavities and the channels that will lead it towards the outside. It is therefore understood that the way in which freezing is carried out determines the quality of the drying. Crystals of large size and joints give rise to a network of cavities which communicate with each other and which conduct the water well to the outside. In addition, rehydration is also facilitated with less risk of obtaining "otors", ie, suspended solids. According to [23], a high degree of crystallization (ie, a high proportion of the total water is in crystalline form) promotes junctions between the crystals and reduces the thickness of the glass partition walls separating the crystals, as shown in Fig. 1.

2.1.3 Sagging / collapse

Schematically, the frozen structure may be considered as a porous substance initially filled with ice. The walls of this pore consist of the cryo-concentrated phase (solute and "bound" water). When the ice is removed by sublimation, the walls of the pore are no longer supported by ice. If the temperature of the product is sufficiently low, the cryo-concentrated phase is maintained in an amorphous solid state and structural retention is observed (the walls do not undergo viscous flow and remain in their initial form). On the contrary, if the temperature rises, the material constituting the walls of the pore can undergo a glass transition and the resulting viscous flow leads to the collapse of the structure. According to [24]. the collapse of the product is observable when the viscous flow is induced by the surface tension forces which tend to minimize the specific surface area of the product.

2.1.4 Sagging/ glass transition temperature

According to [25], the glass transition temperature, defined as the temperature at which the transition between two forms of the amorphous state takes place, the "rigid" and "rubbery" forms (Fig. 2) For the characterization of a product. Indeed, amorphous materials, such as food products, are more stable in their vitreous state well above the glass transition temperature (Tg). This is due to the high viscosity and much lower molecular mobility in the vitreous state than in the rubbery state [22].

By convention, the glass transition temperature (Tg) is the place where the viscosity value reaches 1012 Pa.s^{-1} [25,1]. Sustainability and the activity of the bacteria generally decreases during the various stages of manufacture and storage. Composed mainly of carbohydrates and proteins, frozen bacteria are susceptible to the phenomena of glass transition and collapse. The molecular mobility and physico-chemical properties of frozen biological materials are strongly related to water activity and temperature. The thermal analysis technique [differential scanning calorimetry (DSC)] consists of maintaining a zero temperature difference between the test substance and an inert reference substance when the two samples are subjected to the same linear temperature variation and the same environment, And to measure the necessary energy supplied either to the sample studied or to the reference (generally by the Joule effect). These diagrams are usually established with measurements performed by Differential Scanning Calorimetry (DSC). Associated with water sorption isotherms of dry products, they are often used to explain the stability of these products during storage (Hausman et al., 2005). On the other hand, the phenomenon of subsidence intervenes in the dry layer, during desiccation. The study of the aforementioned physical properties carried out on lactic acid bacteria is likely to explain some changes that occur during their processing and storage.

2.2 Factors Affecting the Conservation of Dried Cells

The control of the processes of freezing or freeze-drying must not be limited to the conditions of Changes in the state of water ; Success is intimately linked to the state of the biological product at the time of treatment. The many factors directly affecting the survival of strains are shown in Fig. 3 : physical factors of cooling, freezing and thawing or desiccation, but also biological factors of adaptability of the microorganism to the treatment [26]. The number and diversity of these factors explain the difficulty of obtaining reproducible results from the survival of a micro-organism, especially since it is difficult to study them in isolation. Other factors such as U.V. or solar radiation and oxygen promote oxidation reactions. For optimal storage, packaging under vacuum or under inert atmosphere (pure nitrogen) in sealed or sealed acon is recommended [27]. The loss of viability of dry cells is a consequence of the damage caused to cells at different targets, namely cell wall, cell membrane and DNA [28,29]. Drying is responsible for different forms of cellular damage mainly due to changes in the physical state of membrane lipids and the structure of sensitive proteins that often lead to severe loss of bacterial viability [30]. Desiccation imposes severe stress on microorganisms by removing water and induces changes in the conformation of proteins
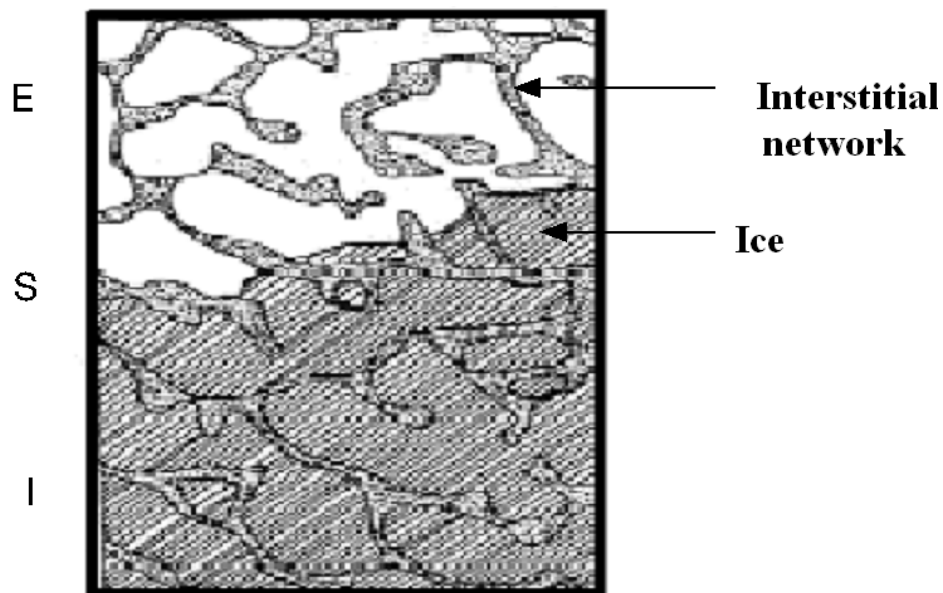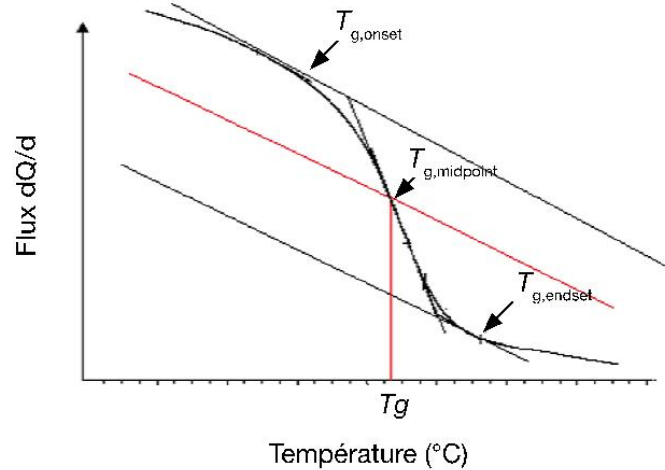

Fig. 1. Diagrammatic representation of a product in the course of freeze-drying (Simato et al., 1994) ; E = porous external zone - S = interface of sublimation – I = frozen internal zone

Fig. 2. Evolution of heat flow (dQ/dt) according to the temperature (T) measured by differential calorimetric analysis. The temperature correspond of vitreous transition (Tg) coresponds to the point of inflexion of curve
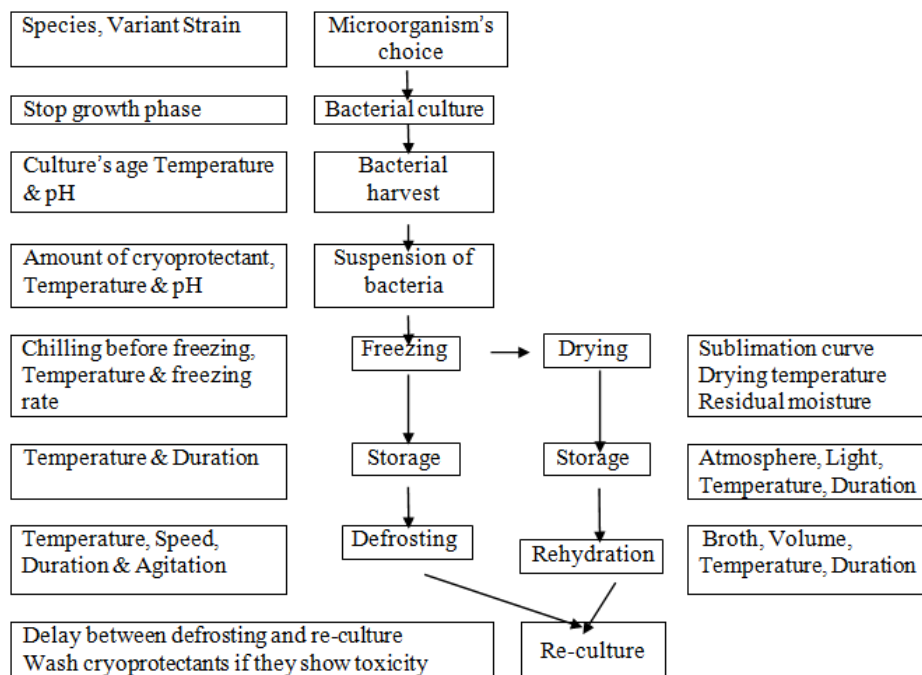
Fig. 3. Factors influencing the survival of the freeze-drying bacteria

and cell membrane [31]. To preserve the freeze-dried product and ensure its long shelf life, it is sometimes desirable to restore the atmospheric pressure in the storage chamber using an inert dry gas (N2, Ar). On the other hand, the lower the storage temperature, the better the conservation [32]. at the level of lactic starters.

Lyophilized products are characterized by properties that are important for their conservation, handling or use : residual moisture, specific mass, color or luminance (light intensity emitted / reflected in a given direction per unit area), their friability And their rehydratability [33,34].

3. ADDITION OF CRYOPROTECTANTS

3.1 Cryoprotection of Lactic Acid Bacteria

There are protective substances that allow cells to withstand slow freezing or thawing and storage at temperatures above -50°C called cryoprotectants [35]. The cryoprotectants are grouped into two classes which are not necessarily exclusive (Table 1). In this way, the substances that penetrate the cells (intracellular cryoprotectants, CPI) and those that remain outside the cells (extracellular cryoprotectants, CPE) are distinguished. Bacteria naturally contain cryoprotectants: sugars (sucrose and trehalose), in gram + bacteria [36,37] , Polyols (glycerol, sorbitol and mannitol), which are found in algae, fungi, yeasts, plants and certain bacteria [38], amino acids and derivatives (betaine and carnitine) In several groups of bacteria [39] and tetrahydroxypyrimidine carboxylic acid (hydroxyectoine) in halophilic bacteria [40], glutamate, glutamine, proline and alanine in *Escherichia coli*, *Staphylococcus Aureus*, *Pseudomonas* and other microorganisms and β-amino acids in methanogenic bacteria.

3.2 Mechanisms of Cryoprotectants Used

The mechanisms of cryoprotection are the subject of many studies today. The most likely modes of action are: an increase in the volume of interstitial liquid phase, at a given temperature, by depression of the starting point of freezing and thus reduction of the effects associated with slow cooling; A reduction in the size of the ice crystals by lowering the nucleation temperature, a reduction in their growth rate and stabilization of the native conguration of the proteins [31]. When the first ice crystals appear, the CPEs only concentrate outside the cells, unlike other solutes that concentrate inside and outside the cells. The

loss of water from the cells therefore leads to a concentration of solutes. This is lower in the absence of CPE, thus minimizing the effects of increased interstitial fluid concentration (Fonseca et al., 2001) [22]. At the level of dried cell membranes, sugars such as trehalose and sucrose can replace water molecules in their binding with polar groups of phospholipids, thus preventing damage during rehydration (Leslie et al., 1995) [30]. Trehalose and sucrose stabilize the structure of intracellular proteins by this phenomenon of water substitution (Carpenter et al., 1993). Other studies have shown that cryoprotectants are indispensable during freezing, regardless of the cooling curves. Not all cryoprotectants enter the cells [41]. The diffusing cryoprotectants will substitute for part of the intracellular water and thus allow partial dehydration of the cells, in addition to limiting the formation of intracellular ice crystals. They also reduce the growth rate of these crystals, lower the temperature of solidification of the intracellular water, such as an antifreeze, and modify the shape of ice crystals [42,43]. As for penetrating (non-diffusing) cryoprotectants, they also protect during freezing and thawing by reducing the size of the ice crystals and inducing less traumatic crystal forms. They allow, by increasing the viscosity of the medium, the reduction of the speed of the movements of the water and of osmotic shocks in addition, they increase the osmotic pressure by reducing the amount of penetrating cryoprotectants required for good conservation (Mille et al., 2005) [44].

4. FATTY ACIDS AND CELLULAR OXIDATION

4.1 Roles of Fatty Acids in Bacteria and Mechanisms of Lipid Oxidation

Membrane lipids are subject to intense modi cations in order to maintain uidity and thus

Table 1. Some characteristics and substances used as intracellular (ICP) and extracellular cryoprotectants (ECP), according to *Anchordoguy et al., 1987*.

Caracteristics	Intracellulars (ICP)	Extracellulars (ECP)
Molecular weight (g.mol-1)	< 400	> 1000
Activity at a concentration of the order	The mole (M)	The millimole (mM)
Examples of molecules used	Glycerol, Dymethylsulfoxide, Methanol, Ethanol, PolyEthylene Oxide (PEO-400), Dimethyl 1-2 acetamide propanediol	Polyvinyl pyrolidone, Hydroxyethyl starch, Dextran

ensure the permeability of cell membranes. This activity is essential for the proper functioning of the cells in case of stress. The increase in incubation temperature in *Lb. plantarum* (Table 2) has an impact on the increase in the synthesis of unsaturated fatty acids (mainly $C_{18:1}$) on the one hand and on the other hand, Increase in $C_{16:0}$ synthesis, to regulate membrane velocity [45,46]. Under the same conditions, for *Lb. Monocytogenes*, there was a decrease in $C_{15:0}$ synthesis and an increase in $C_{17:0}$ and $C_{18:1}$ synthesis [45]. During storage, the phenomena of oxidation of the membrane lipids are responsible for the degradations of the cells resulting from the freeze-drying and the decrease of viability. With unsaturated fatty acids as substrates, the degree of oxidation and velocity will depend on the unsaturation [47]. The fundamental difference between saturated fatty acids and polyunsaturated fatty acids exists at temperature, while saturated fatty acids oxidize at a temperature above 60°C, polyunsaturated fatty acids even oxidize, Storage of frozen food [48]. Several natural mechanisms of oxidation control take place at the cellular level and Are aimed at preventing the oxidative destruction of membrane lipids, proteins and nucleic acids [49]. The regulation mechanisms of pro-oxidant and antioxidant systems are put in place to maintain the balance between the factors involved in the oxidation reactions. However, this regulation of pro and antioxidant factors is disrupted during product transformation and storage processes, which favors the development of oxidation reactions [50]. According to the work done by [51], the factors that induce or initiate the oxidation of lipids are of two types :

- intrinsic factors such as fatty acid composition of lipids (number and position of unsaturations), presence of pro-oxidants (metal ions, enzymes, etc.) or natural antioxidants (tocopherols, carotenoids, etc.)
- external factors such as temperature, light, oxygen partial pressure, water activity, storage and processing conditions (Steinberg et al., 1989) [51].

The classi cation of oxidation is a function of the initiating agents, so three types of lipid oxidation are distinguished :

- auto-oxidation catalyzed by temperature, metal ions and free radicals,
- photo-oxidation, initiated by light in the presence of photosensitizer,
- enzymatic oxidation initiated by the presence of oxidation enzymes.

4.2 Auto-Oxidation

In nature, there are a number of self-oxidations, including that of dioxygen (O_2) which gives rise to ozone (O_3). In the organism, the self-oxidation, or spontaneous oxidation of certain components, essentially peroxides degraded subsequently by specific enzymes : peroxidases. With regard to lipids, oxidation is an auto-catalytic reaction. It is a chain of radical reactions taking place in three stages. A first reaction produces a free radical by removal of a hydrogen from the fatty acid (initiation stage). The reaction is self-sustaining, followed by reactions to produce several free radicals (Phase of propagation), the unsaturated lipids gradually disappear and the hydroperoxide concentration increases to reach its maximum in the middle of the propagation phase [49]. The reactions of auto-oxidations lead to the formation of free radicals and then of hydroperoxides, intermediate compounds, which in turn decompose giving rise to volatile compounds, furan compounds and especially saturated and unsaturated aldehydes. Hydroperoxides, the first products of lipid oxidation, are unstable [52,53].

Table 2. Effect of culture's temperature on *Lactobacillus plantarum* fatty acids composition (%), *Russell et al., 1989*.

| Fatty acids	Commun name	Scientific name	Compistion in fatty acids		
			10°C	30°C	40°C
$C_{16:1}$	Palmitoleic acid	Acid 9-hexadecanoïc	11	18	2
$C_{16:0}$	Palmitic acid	Acid hexadecanoïc	15	30	56
$C_{17:0cyc}$	Margaric acid	Acid heptadecanoïc	<1	trace	trace
$C_{18:1}$	Oleic acid	Acid 9-octodecanoïc	56	34	11
$C_{18:0}$	Stearic acid	Acid octadecanoïc	10	4	13
$C_{18:0cyc}$	Nonadecyclic acid	Acid nonadecanoïc	6	8	16

Cyc : cyclopropane fatty acid

This instability generates a series of complex reactions that result in a myriad of compounds with variable molecular weights.
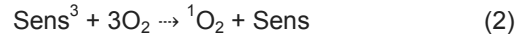
4.3 Photo-Oxidation

As evoked for auto-oxidation, photooxidation, which is an oxidation induced in large part by the presence of light and photosensitizers such as hemoproteins, chlorophyll or ribo avin, is also an important route of production of Hydroperoxides in the presence of oxygen. As for the mechanism, the photosensitizers (Sens) would absorb the light energy to reach a state of excited triplet (Sens3). These photosensitizers are involved in the oxidation of lipids according to two types of mechanisms [52].

The first type of mechanism is induced by photosensitizers (type I), such as ribo avine, which act as initiating free radicals. In their triplet state, they tear a hydrogen atom or an electron from the lipid molecules to form a radical capable of reacting with oxygen :

$$\text{Sens}^3 + \text{RH} \dashrightarrow \text{Sens H} + \text{R}° \qquad (1)$$
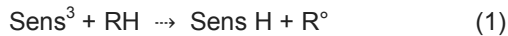
According to the second mechanism, the type II photosensitive molecules, such as chlorophyll and erythrosine, react in their excited state (Sens3) with the triplet oxygen to which they transfer their energy to give singlet oxygen ($^1O_2$) :

$$\text{Sens}^3 + 3O_2 \dashrightarrow {}^1O_2 + \text{Sens} \qquad (2)$$

The singlet oxygen thus formed is highly electrophilic and can react directly with an unsaturated fatty acid (RH) thus forming a hydroperoxide (ROOH) :

$$^1O_2 + \text{RH} \dashrightarrow \text{ROOH} \qquad (3)$$

The radical reactions occur in a chain by the subsequent of this auto-oxidation. According to the work done by Laine [54] , the hydroperoxides thus formed are different from those formed by autoxidation.

4.4 Enzymatic Oxidation

After autoxidation and photooxidation, a third type of oxidation takes place at the level of the lipids generally derived from the plants. This

Fatty acid

↓ Acyl hydrolase

Linoleic acid
Linolenic acid

↓ Lypoxygenase

9- and 13- hydroxyperoxydes

Allene oxyde synthase → Cethol and jasmonic acid lyase

Dimet hyl ether syntha → Dimethyl esther of fatty acid

Hydroxyperoxyde lyase → Aldehydes, Alcanes or Alchools, Oxo-acids
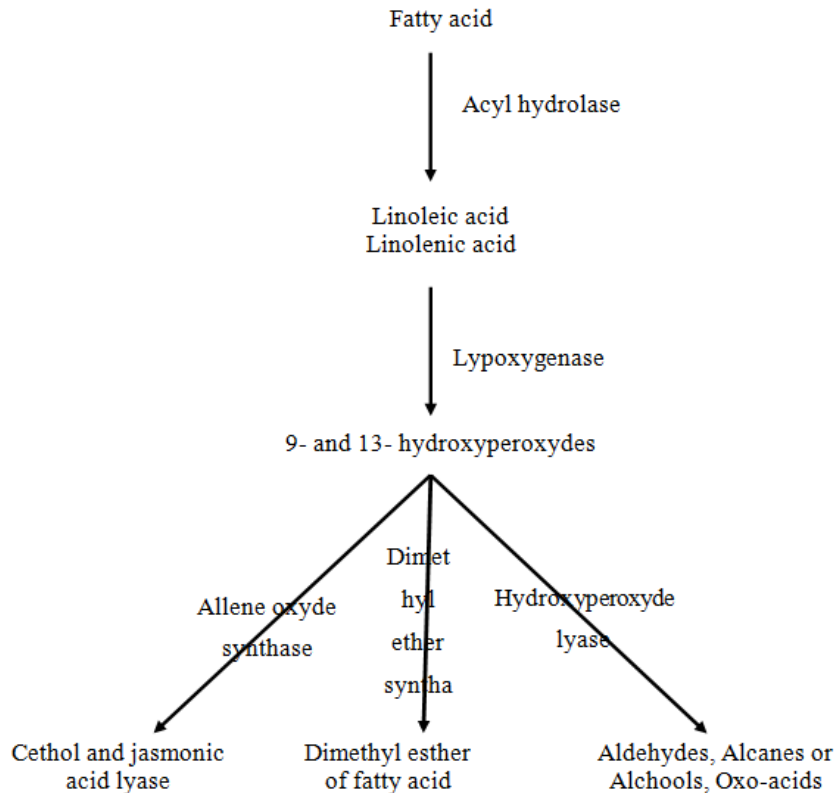
Fig. 4. The main lypoxygenase pathways, *Fauconnier et al., 1997*

oxidation is initiated by enzymes, hence the phenomenon of oxidation of the unsaturated fatty acids of enzymatic origin. The two enzymes mainly involved are lipoxygenase and cyclooxygenase. The lipoxygenase catalyzes the insertion of an oxygen molecule onto an unsaturated fatty acid by stereospecific reaction and results in the formation of hydroperoxides [49]. It acts specifically on non-esterified fatty acids. Its activity is therefore often coupled with that of lipases and phospholipases as indicated in Fig. 4 [55]. Cyclooxygenase is a lipoxygenase which incorporates two molecules of oxygen at an unsaturated fatty acid to form specific hydroperoxides. Enzymatic oxidation occurs even at low temperatures. During storage, in the frozen state, the enzymatic activity is slowed down. At -40°C., the enzymatic oxidation of the lipids is completely stopped. However, as soon as thawing has begun and the temperatures reached from 0°C to 4°C., this activity is resumed and accentuated.

5. PRINCIPAL FACTORS OF OXIDATION OF LIPIDS AND ITS IMPACTS ON CELL VIABILITY

Several factors are involved in lipid oxidation during food processing and preservation processes. These include temperature, pH, water activity (a_w) and oxygen partial pressure (PO_2) [56]. A rise in temperature promotes the oxidation of lipids. This oxidation is the more rapid as the temperature is high. Thus, cooking operations, for example, are well known to have a marked pro-oxidant effect [57]. As for freezing, on the other hand, it proves to be a good means of increasing the shelf-life of foodstuffs, since the rate of oxidation of lipids is significantly reduced at low temperature. The influence of pH in the course of oxidation is manifested through several mechanisms.

First, for oxidation-reduction reactions involving protons (H^+), the redox potential decreases linearly with pH. An acidic pH therefore favors the oxidation reaction, in particular when the water-soluble pro-oxidizing (transition metal ions) or antioxidant (ascorbic acid, BHT and BHA) species are present. The water activity of a system in uence the lipid oxidation reactions. Indeed, water allows the mobilization of pro-oxidant or antioxidant substances [58]. In general, a water activity (a_w) of between 0.2 and 0.3 corresponds to the lowest oxidation rates. These values correspond to the formation of a monomolecular layer of water around the constituents. On the other hand, an aw between 0.6 and 0.8 corresponds to the highest oxidation rates. The oxidation of lipids generates hydroperoxides. Damage caused to LABs during storage is also attributed to changes in the fatty acid profile (saturated fatty acid and unsaturated fatty acid content) of the membrane, for which lipid oxidation is an important cause [59,60]. Lipid oxidation can lead to physical changes in membrane functions and structures [61,62]. The rapid loss of viability tends to occur at the beginning of the conservation period [63], While changes in lipid composition of the cell membrane increase during storage [64]. Moreover, it is now established that the consequence of numerous biological oxidations is the formation of free radicals, which are one of the main causes of cell death [65]. The attack of fatty acids by free radicals decreases the hydrophobicity due to the introduction of hydrophilic groups and consequently weakens the hydrophobic interaction of membrane proteins [66]. For example, a decrease in membrane ATPase activity was suggested as a consequence of the change in this interaction [67]. The changes also affect the membrane in certain control mechanisms linked to the regulation and replication of DNA since the initiation of DNA synthesis and its maintenance require the attachment of the DNA complex to the cytoplasmic membrane [68]. In addition, free radicals can directly cause damage to the DNA [69,70,71]. Although it is plausible to add an antioxidant to reduce oxidation of lipids, the addition should be exercised with care. These methods and compounds will serve in the following to define the different approaches to inhibit cellular oxidation.

6. DIFFERENTS APPROACHES TO INHIBIT THE OXIDATION OF LYOPHILIZED LACTIC STARTERS DURING THE STOCKAGE

The control of the oxidation in concern of lipids is based on the control of some parameters: temperature, pH, aw and oxygen concentration [72]. According to Buettner (1999), antioxidants capable of protecting lipids from oxidation can be divided into two types : preventive antioxidants that prevent the formation of reactive oxygen species or intercept the species responsible for initiating the oxidation. Lipoperoxidation and chainbreaking antioxidants which intercept the propagating radicals of lipid peroxidation and retard peroxidation (induction period).

6.1 Mechanisms Action of Preventive Antioxidants

6.1.1 Transition metal chelators

The redox cycle of certain metals, responsible for the production of free radicals, is blocked by metal chelators. Proteins such as transferrin, ferritin, and lactalbumin sequester iron, ceruloplasmin and albumin which sequester copper [73]. We note that avonoids are good chelators of iron, which is one of the mechanisms of their antioxidant activity [74,75].

6.1.2 Deactivators (quencher) of singlet oxygen

According to Buettner, they can act by chemical deactivation on a molecule such as a fatty acid to give a hydroperoxide : $^1O_2$ + LOH → LOOH, or else by physical deactivation eliminating the excitation energy without chemical change : $^1O_2$ + β-carotene → O_2 + β-carotene. Carotenoids are particularly effective. Lycopene is the most reactive.

6.1.3 Elimination of hydroperoxides

Hydroperoxides generate free radicals and their elimination prevents oxidation of biomolecules. Hydroperoxides (LOOH) can be reduced by enzymes. Glutathione peroxidase (GPx) removes organic hydroperoxides and hydrogen peroxide (H_2O_2).

6.1.4 The oxygen scavengers

They are molecules such as sulphites or ascorbic acid.

6.2 Mechanisms of Antioxidants' Action on the "Chain Breaking"

This category of antioxidants will most often react with the peroxyl (LOO°) or alkoxyl (LO°) radicals thus interrupting the peroxidation propagation reaction. This class of antioxidants will most often react with the peroxyl or alkoxyl radicals, thus interrupting the peroxidation propagation reaction. It should be noted that these antioxidants will therefore not inhibit the autoxidation of the lipids by singlet oxygen. These antioxidants can act according to two mechanisms, [76].

6.2.1 The hydrogen donors

These antioxidants are mainly mono- or polyhydroxylated phenolic compounds (tocopherols, tocotrienols, BHT, BHA, avonoids, etc.). They are capable of yielding a hydrogen to the alkoxyl radical and to the peroxyl radical. After oxidation's reaction, the antioxidant is transformed into a radical which must be sufficiently stable to inhibit the formation of another radical and thus stop the propagation of the radical chain. It must then evolve towards a stable oxidation product, which leads to the consumption of the antioxidant. Tocopherol will give a tocopherocyl radical which will evolve towards a non-radical oxidation compound such as tocopherylquinone or a higher dimerization or polymerization compound [77].

6.2.2 The "sacrificed" antioxidants

Qualitative "chain breaking antioxidant sacrificied" used by Buettner concerns molecules, themselves radical, which react with peroxyl or alkoxyl radicals to give non-radical products, thus interrupting the propagation of peroxidation. Two radicals are known to combine with peroxyl radicals, nitrogen monoxide (NO) and superoxide anion (O_2-) :

$$LOO + NO \dashrightarrow LOONO \text{ and } LO + NO \dashrightarrow LONO$$
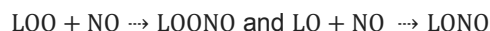
6.2.3 Mixed action mechanisms of antioxidants

Some antioxidants have mixed modes of action. Two examples can illustrate these multiple mechanisms. Ascorbic acid's example which is a singlet oxygen deactivator and removes molecular oxygen. It is also a hydrogen donor to lipid radicals and tocopheryl radicals to regenerate tocopherol. As for avonoids such as anthocyanins, catechins, avones, avonols, iso-avones and proanthocyanidins, they are metal chelators, superoxide anion scavengers and hydrogen donors. Antioxidants use like as (tocopherols, polyphenols, avonoids, vitamin E, vitamin C, etc.) is often the most common method in the food industry to inhibit lipid oxidation. The antioxidants used are either preventive agents which block the initiation phase by reacting with the initiators of the reaction (O_2, light, metals, etc.) or terminating agents which block the continuation of the propagation phase by Reacting with free radicals and converting them into stable compound [50,78].

7. CONCLUSION

It's possible to preserve by freeze-drying lots of lactic acid bacteria, according to laboratory's

scale, and also industrial way. Conditions in which it possible to obtain good preservation of the metabolic activity of bacteria are relatively well known. The mechanisms of their alteration is areel problem. The resolution of difficulties, such as the poor preservation of certain lyophilized strains and the often too slow recovery of ferments intended for direct seeding, certainly requires a better understanding of these mechanisms. To optimize conservation, several technics and ways of research must be carried out in concert. Among the most promising are the study of changes in membrane structures during conservation treatments, the search for a formulation incorporating more effective cryoprotectants, the induction of mechanisms of resistance to these treatments (by adaptation Bacteria, by culturing them under suitable conditions or possibly by using antioxidants to block the peroxidation phenomenon).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Agnieszka C, Andrzej L. Freeze-drying – application in food processing and biotechnology – A review. Pol. J. Food Nutr. Sci. 2011;61:165-171.
2. Leslie SB, Lighthart IB, Crowe JH, Crowe LM. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl. Environ. Microbiol. 1995;61:3592-3597.
3. Zhao G, Zhang G. Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. J. Appl. Microbiol. 2005;99:333-338.
4. Mille Y, Beney L, Gervais P. Compared tolerance to osmotic stress in various microorganisms: Towards a survival prediction test. Biotechnol. Bioeng. 2005; 92:479-484.
5. Thonart PH. Les applications des bacteries lactiques. In: Seminaire sur la selection, la production et le conditionnement des ferments lactiques, compte-rendu des allocutions et des conferences, école superieure des industries alimentaires de Tunis et Centre Wallon de Biologie Industrielle-liege. 2004;8-31.
6. Fan W, Yan W, Xu Z, Ni H. Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. Colloids Surf. B. 2012;90:21–27.
7. Topcu A, McKinnon I, Mc., Sween PHL. Measurement of the oxidation-reduction potential of cheddar Cheese. J. Food. Sc. 2008;73:198–203.
8. Bayrock D, Ingledew WM. Fluidized bed drying of baker's yeast: Moisture levels, drying rates and viability changes during drying. Food. Res. Int. 1997;30:407-415.
9. Russell NJ. Functions of lipids: Structural roles and membranes functions. *In:* Ratledge C. & Wilkinson S.C., eds. *Microbial lipids.* London: Academic Press, 1989;2:279-365.
10. Santivarangkna C, Kulozik U, Foerst P. Inactivation mechanisms of lactic acid starter cultures Incidences de la lyophilisation preserved by drying processes. J. Appl. Microbiol. 2008;105:1-13.
11. Coulibaly I, Dubois-Dauphin R, Danthine S, Majad L, Mejoub T, Destain J, Béra F, Wathelet JP, Thonart P. Techniques de séchage des starters lactiques et mécanismes affectant la viabilité cellulaire suite à la lyophilisation. Biotechnol. Agron. Soc. Environ. 2011;15:287-299.
12. Rasul MG, Rudolph V, Wang FY. Particles separation using fluidization techniques. Int. J. Mineral Process. 2000;60:163-179.
13. Marin A. Freeze-drying: Structure and flavor (flavour). Encyclopedia of food and nutrition, 2nd Edition. 2003;2701-2705.
14. Chouvenc P, Vessot S, Andrieu J, Vacus P. Optimization of the freeze-drying cycle: A new model for pressure rise analysis. Drying Technol. 2004;22:1577-1601.
15. Tang XD, Garcia ML, Heinemann SH, Hoshi T. Reactive oxygen species impair Slo1 BK channel function by altering cysteine-mediated calcium sensing. Nat. Struct. Mol. Biol. 2004;11:171-178.
16. Shalev U, Grimm JW, Shaham Y. Neurobiology of relapse to heroin and cocaine seeking: A review. Pharmacol. Rev. 2002;54:1-42.
17. Simatos D, Blond G, Le Meste M, Morice M. Conservation des bacteries lactiques par congelation et lyophilisation. In: De Roissart H. & Luquet F.M., eds. Bacteries lactiques, aspects fondamentaux et technologiques. Uriage, France: Edition Lorica. 1994;1:555-573.

18. Pegg DE. The history and principles of cryopreservation. Semin. Reprod. Med. 2002;20:5-13.

19. Bolla PA, Serradell MA, Urraza PJ, De Antoni GL. Effect of freeze-drying on viability and *in vitro* probiotic properties of a mixture of lactic acid bacteria and yeasts isolated from kefir. J. Dairy Research. 2011;78:15-22.

20. Caillet A, et al. Crystallization of monohydrate citric acid. Part 1: *In situ* monitoring through the joint use of raman spectroscopy and image analysis. Cryst. Growth Des. 2007;7:2080-2087.

21. Hausman DS, Cambron RT, Sakr A. Application of on-line Raman spectroscopy for characterizing relationships between drug hydration state and tablet physical stability. Int. J. Pharm. 2005;299:19-33.

22. Fonseca F, Corrieu G. Cryoprotection and freezing in uence on acidi cation activity of thermophilic lactic acid bacteria. Relationships between biologic and thermodynamic properties. Thèse de doctorat: Institut national agronomique Paris-Grignon (France); 2001.

23. Wang R, Zhang M, Mujumdar AS. Effect of food ingredient on microwave freeze-drying of instant vegetable soup. LWT. 2010;43:1144-1150.

24. Shah A, Munir K, Khan S, Abbas Z. Can large industries lead the market? World. App. Sci. J. 2011;11(3):443-448.

25. Matthieu M. Rigidity transitions and constraint counting in amorphous networks: Beyond the mean-eld approach. Europhys. Lett. 2002;58:830-836.

26. Romeo HE. The glossopharyngeal nerve as a new pathway in immune-to-brain interactions: Relevance to neuroimmune surveillance of the oral cavity. J. neuroimmunol. 2001;115:91-100.

27. Carpenter JF, Prestrelski SJ, Arakawa T. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-speci c stabilization. I. Enzyme activity and calorimetric studies. Arch. Biochem. Biophys. 1993;303:456-464.

28. Teixeira PC, Castro MH, Malcata FX, Kirby RM. Survival of *Lactobacillus delbruckii* ssp. bulgaricus following spray drying. J. Dairy. Sci. 1995;78:1025-1031.

29. Hoobin P, Burgar I, Zhu S, Ying D, Sanguansri L, Augustin MA. Water sorption properties, molecular mobility and probiotic survival in freeze dried protein-carbohydrate matrices. Food. Funct. 2013; 9:1376-86.

30. Leslie, Andrew B, Jeremy M. Beaulieu, Hardeep S. Rai, Peter R. Crane, Michael J. Donoghue, Sarah Mathews. Hemisphere-scale differences in conifer evolutionary dynamics. Proceedings of the Nat. Acad. Sci. 2012;109:16217-16221.

31. Strasser S, et al. In uence of lyophilization, uidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. J. Appl. Microbiol. 2009;107:167-177.

32. Gardiner GE, et al. Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. Appl. Environ. Microbiol. 2000;66: 2605-2612.

33. Abdel Gawad AM, Nassar AM, Hilali M. Isolation of *Toxoplasma gondii*, *Isospora felis* and *Isospora rioolfa* from the meat of some farm animals. J. Egypt. Vet. Med. Assoc. 1989;49:405-414.

34. Ying DY, Phoon MC, Sanguansri L, Weerakkody R, Burgar I, Augustin MA. Microencapsulated *Lactobacillus rhamnosus* GG powders: relationship of powder physical properties to probiotic survival during storage. J. Food Sci. 2010;75:1-9.

35. Fonseca F, Cenard S, Passot S. freeze-drying of lactic acid bacteria. Cryopreservation and Freeze-Drying. Protocols. 2014;477-488.

36. Buettner G. Singlet oxygen toxicity is cell inedependant: A study of lipid peroxidation in nine leukemia cell lines. Photochem. Photobiol. 1999;70:858-867.

37. Li B, Tian F, Liu X, Zhao J, Zhang H, Chen W. Effects of cryoprotectants on viability of *Lactobacillus reuteri* CICC6226. Appl. Microbiol. Biotechnol. 2011;92:609-616.

38. Korobkina GS, et al. Development of dried *L. acidophilus* milk formulae for infant feeding. Dairy Sci. Abst. 1982;44:396.

39. Castro HP, Teixeira P, Kirby R. Storage of lyophilized cultures of *Lactobacillus bulgaricus* under different relative humidities and atmospheres. Appl. Microbiol. Biotechnol. 1995;44:172-176.

40. Del Moral A, et al. Compatible solutes in new moderately halophilic isolates. FEMS Microbiol. Lett. 1994;12:165-172.

41. Pegg DE, Diaper MP. On the mechanism of injury to slowly frozen erythrocytes. Biophys. J. 1988;54:471-488.

42. O'byrne CP, Booth IR. Osmoregulation and its importance to food-borne microorganisms. Int. J. Food Microbiol. 2002;74:203-216.

43. Tymczyszyn EE, Gerbino E, Illanes A, Gómez-Zavaglia A. Galacto-oligosaccharides as protective molecules in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus.* Cryobiology. 2011;62:123-9.

44. Mille Y, Beney L, Gervais P. Compared tolerance to osmotic stress in various microorganisms: Towards a survival prediction test. Biotechnol. Bioeng. 2005; 92:479-484.

45. Russell NJ, et al. Membrane as a target for stress adaptation. Int. J. Food Microbiol. 1995;28:255-261.

46. Tachon S, Brandsma JB, Yvon M. NoxE NADH Oxidase and the electron transport chain are responsible for the ability of *Lactococcus lactis* to decrease the redox potential of milk. Applied Environ. Microbiol. 2010;76:111-123.

47. Rousch JM, Scott EB, Milton RS. Changes in fatty acid pro les of thermo-tolerant marine diatoms during temperature stress. J. Exp. Mar. Biol. Ecol. 2003;295:145-156.

48. Niki E, et al. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochim. Biophys. Res. Commun. 2005;338:668-676.

49. Pereira EDJ, Panek AD, Eleutherio ECA. Protection against oxidation during dehydratation of yeast. Cell Stress Chaperone. 2003;8:120-124.

50. Niki E, et al. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochim. Biophys. Res. Commun. 2005;338:668-676.

51. Steinberg D, Parthasarathy S, Carew TE, Witztum JL. Beyond cholesterol. Modi cations of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. 1989;320:915-924.

52. Noguchi N, et al. The speci city of lipoxygenase- catalyzed lipid peroxidation and the effects of radical- scavenging antioxidants. Biol. Chem. 2002;383:619-626.

53. Peiren J, Buyse J, De Vos P, Lang E, Clermont D, Hamon S. Improving sur- vival and storage stability of bacteria recalcitrant to freeze-drying: A coordinated study by European culture collections. Appl. Microbiol. Biotechnol. 2015;99:3559–3571.

54. Laine G, et al. Study of precursors responsible for off- avor formation during storage of potato akes. J. Agric. Food Chem. 2006;54:5445-5452.

55. Fauconnier ML, Perez AG, Sanz C, Marlier M. Puri cation and characterization of tomato leaf (*Lycopersicon esculentum* Mill.) hydroperoxide lyase. J. Agric. Food Chem. 1997;45:4232-4236.

56. Chatterjee MT, Seunath A, Khalawan SA, Curran BPG. Cellular lipid composition in uences stress activation of the yeast general stress response elements (STRE). Microbiology. 2000;146:877-884.

57. Howlett N, Avery SV. Induction of lipid peroxidation during heavy metal stress in *Saccharomyces cerevisiae* and in uence of plasma membrane fatty acid unsaturation. Appl. Environ. Microbiol., 1997;63:2971-2976.

58. Chen ZH, et al. Adaptive response induced by lipid peroxidation products in cell cultures. FEBS Lett. 2006;580:479-483.

59. Hendrickson A, McKinstry LA, Lewis JK, Lum J, Louie A, Schellenberg GD, Hatsukami TS, Chait A, Jarvik GP. *Ex vivo* measures of LDL oxidative susceptibility predict carotid artery disease. Atheroscl. 2005;179:147-153.

60. Andersen AB, Fog-Peterson MS, Skibsted LH. Storage stability of freeze-dried starter cultures (*Streptococcus thermophilus*) as related to physical state of freezing matrix. Food Sci. Technol. 1999;32:540-547.

61. Hirano R, Kondo K, Iwamoto T, Igarashi O, Itakura H. Effects of antioxidants on the oxidative susceptibility of low-density lipoprotein. J. Nut. I Sci. Vitamin., 1997;43:435-444.

62. Borst JW, Visser NV, Kouptsova O, Visser AJ. Oxidation of unsaturated phospholipids in membrane bilayer mixtures is accompanied by membrane fluidity changes. Biochim. Biophys. Acta. 2000;1487:61-73.

63. Ito M, Ohishi K, Yoshida, Y, Yokoi W, Sawada H. Antioxidative effects of lactic acid bacteria on the colonic mucosa of iron-overloaded mice. J. Agri. Food Chem. 2003;51:4456-4460.

64. Teixeira PC, Castro MH, Kirby RM. Evidence of membrane lipid oxidation of spray-dried *Lactobacillus bulgaricus* during storage. Lett. Appl. Microbiol., 1996;22:34-38.

65. Van de Guchte M, et al. Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek. 2002;82:187-216.

66. Santivarangkna C, Aschenbrenner M, Kulozik U, Foerst P. Role of glassy state on stabilities of freeze-dried probiotics. J. Food. Sci., 2011;8:384-391.

67. Castro HP, Teixeira PM, Kirby R. Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. Biotechnol. Lett. 1996;18:99-104.

68. Larsen N, Werner BB, Vogensen FK, Jespersen L. Effect of dissolved oxygen on redox potential and milk acidification by lactic acid bacteria isolated from a DL-starter culture. J. Dairy Sci. 2015; 98:1640–1651.

69. Inouye S. Site-speci c cleavage of double-strand DNA by hydroperoxide of linoleic acid. FEBS Lett. 1984;172:231-234.

70. Akasaka S, Inactivation of transforming activity of plasmid DNA by lipid peroxidation. Biochim. Biophys. Acta, 1986;867:201-208.

71. Hrynkiewicz K, Baum C. The Potential of Rhizosphere Microorganisms to Promote the Plant Growth in Disturbed Soils. In A. Malik, E. Grohmann (eds.), Environmental Protection Strategies for Sustainable Development, Strategies for Sustainability. 2011;15912:35-64.

72. Coulibaly I, et al. Survival of freeze-dried *Leuconostoc mesenteroides* and *Lactobacillus plantarum* related to their cellular fatty acids composition during storage. Appl. Biochem. Biotechnol. 2009;157:70-84.

73. Morel I, Lescoat G, Cogrel P. Antioxidant and iron-chelating activities of the avonoids catechin, quercetin and diosmetinon iron-loaded rat hepatocytes cultures. Biochem. Pharmacol. 1993;45:13-19.

74. Van Acker S, Bast A, Van Der Vijgh W. Stuctural aspects of antioxidant activity of avonoids. In: Rice- Evans C. & Packer L., eds. Flavonoids in health and disease. New York, USA: Marcel Decker. 1998;221-251.

75. Morel I, Lescoat G, Cogrel P. Antioxidant and iron-chelating activities of the avonoids catechin, quercetin and diosmetinon iron-loaded rat hepatocytes cultures. Biochem. Pharmacol. 1993;45:13-19.

76. Vitali B, Minervini G, Rizzello CG, Spisni E, Maccaferri S, Brigidi P. Novel probiotic candidates for humans isolated from raw fruits and vegetables. Food Microbiol. 2012;31:116–125.

77. Gansäuer A, Justicia J, de Cienfuegos LÁ, Camapaña Miguel D, Jakoby V, Cuerva J. M 2011. Bioinspired terpene synthesis: A radical approach, Chem. Soc. Rev. 2011;40:3525-3537.

78. Suganya T, Fumio I, Siriporn O. Antioxidants active principles isolated from Psidium guajava grown inThailand. Sci Pharm., 2007;75:179-193.