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Influence of Freezing and Freeze Drying on Intracellular Enzymatic Activity and Autolytic Properties of Some Lactic Acid Bacterial Strains

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Abstract

Lactic acid bacteria possess several interesting properties of great economic importance. Improvement and stabilization of these industrially important features are an active research area at the present time. The objectives of this work are to study the effect of freezing and freeze-drying on the survival rate, autolytic activity and intracellular enzymatic activity of the main species of lactic acid bacteria used in the dairy industry. The article focused on several characteristics that were not well covered in the past. The obtained results revealed that both preservation methods have a significant effect on viability, autolytic activity and intracellular enzymatic activity. After six months of storage we found that frozen cultures exhibited higher survival rate, higher rate of intracellular enzymatic activity and lower rate of autolysis. The impact of conservation treatments was only strain specific in the case of survival rate. The results obtained lead to the selection of the best preservation method for the selected cultures based on survival rate, autolytic activity and intracellular enzymatic activity.

Keywords

Freeze-Drying, Freezing, Intracellular Enzymes, Lactic Acid Bacteria, Viability, Autolysis

1. Introduction

Lactic acid bacteria (LAB) are indispensable for the manufacture of a wide range of dairy products such as

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cheeses and fermented milks. They show a large number of metabolic activities responsible for flavor development and texture modification in these products; they can also play a role in the acceleration of cheese ripening and/or bitterness reduction [1]-[4].

The industrial use of LAB as starter cultures depends on the technological properties, concentration and preservation technologies, required to maintain high level of viability, acidification activity and enzymatic activity during long-term storage [5]-[8].

Freezing and freeze-drying are commonly used for the preservation and storage of starter cultures used in the dairy industry. These techniques can bring about undesirable side effects, such as membrane injury, protein and enzymes denaturation and DNA damage [9]-[12]. Several factors are relevant for the preparation of bacterial cultures that will have high number of cells and long term viability during storage. These include intrinsic factors, such as cell shape and size, genetic composition, and differences in cell wall and membrane composition, growth conditions, drying medium and storage conditions [13].

Their protective effect during drying and freezing was shown to be strain specific [14] [15]. Cells are usually harvested during the stationary phase of growth because they are reported to be less sensitive to environmental stresses compared to those harvested at the lag and exponential phases [16]-[19].

Drying medium contains cryoprotectants such as dextran; trehalose, sucrose, lactose, glucose, sorbitol, glycerol, proteins and skimmed milk have been demonstrated effective in improving survival of bacteria during freeze-drying and subsequent storage [20]-[23] by inhibiting the intracellular formation of ice, membranes damage, protein denaturation, etc., and decreasing therefore the cells injury. Several studies [13] [24]-[26] demonstrated that reconstituted skimmed milk powder should be selected as a drying medium for LAB.

Previous studies [27]-[29] revealed that cultivability is not enough to completely define viability. It might, therefore, be interesting to assess the changes in the physiological state during storage of frozen and freeze-dried strains by simultaneously measuring other technological parameters such as membrane integrity, esterase activity or proteolytic activity [30] and the autolytic properties which reflect the ability of the strains to liberate their intracellular enzymes [31], showed no correlation with survival rate after freezing or freeze-drying. The faster release of the intracellular proteolytic and lipolytic enzymes will contribute in flavor development during cheese ripening and may lead to acceleration of the ripening process [32] [33].

Work on the impact of the conservation methods on intracellular enzymatic activities and autolytic properties is very limited; therefore, the aim of the present investigation is to study the impact of freezing and freeze-drying on the survival rate, autolytic activity and intracellular enzymatic activities of different lactic acid bacterial species using skimmed milk with glycerol as a cryoprotective media.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

A total of 26 lactic acid bacterial (LAB) strains tested in this study were obtained from the culture collection of the Laboratory of Biochemistry of Dairy Microorganisms (LBDM), Faculty of Agriculture, Alexandria University. All strains originated from traditional Egyptian dairy products; cheeses (Domiatti and Ras) and fermented milk (Zabady and Laban Rayeb). These strains belong to several genera of LAB; *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*. The FAAU numbers and species of tested bacterial strains are given in **Table 1**. The *Lactobacillus* strains were grown on MRS medium (Biolife, Milano, Italy) [34], while *Streptococcus*, *Lactococcus* and *Enterococcus* strains were grown on M17 medium (Biolife, Milano, Italy) [35]. The strains were grown at the optimum growth temperature; 30˚C for *Lactococcus*, 37˚C for mesophilic lactobacilli and 42˚C for thermophilic lactobacilli and streptococci.

2.2. Cryoprotective Medium

The selected strains were frozen and freeze-dried in skimmed milk 10% (w/v) with 2% glycerol.

2.3. Preparation of Cultures for Freezing and Freeze-Drying

The microorganisms were sub-cultured four times in the specific medium for each genus. This medium was inoculated with 10% of the active culture. The growth phase was monitored by measuring the absorbance at 650 nm using Laxco spectrophotometer (model a1202, USA). At the early stationary phase, the medium was divided

Table 1. List of the species and FAAU no. of the tested strains.

No.	Species	FAAU No.	
1	*Lactobacillus delbrueckii* subsp. *bulgaricus*	FAAU 1	FAAU 2
2	*Lactobacillus delbrueckii* subsp. *lactis*	FAAU 7	FAAU 4
3	*Lactobacillus helveticus*	FAAU 11	FAAU 12
4	*Lactobacillus acidophilus*	FAAU 25	FAAU 87
5	*Lactobacillus paracasei* subsp. *paracasei*	FAAU 10	FAAU 9
6	*Lactobacillus plantarum*	FAAU 34	FAAU 36
7	*Lactobacillus rhamnosus*	FAAU 64	FAAU 65
8	*Lactobacillus fermentum*	FAAU 58	FAAU 59
9	*Lactobacillus brevis*	FAAU 60	FAAU 61
10	*Streptococcus thermophillus*	FAAU 16	FAAU 17
11	*Lactococcus lactis* subsp. *lactis*	FAAU 19	FAAU 43
12	*Lactococcus lactis* subsp. *cremoris*	FAAU 47	FAAU 52
13	*Enterococcus faecium*	FAAU 48	FAAU 49

into 2 parts (100 ml and 1000 ml). The cells were then harvested by centrifugation at 4000 rpm for 20 minutes at 4˚C. The resulting pellet was then washed twice with 0.01 M potassium phosphate buffer pH 7.0. The pellet resulting from the 100 ml was used for the determination of the intracellular enzymatic activity and autolytic activity at zero time. While pellet resulting from 1000 ml was dissolved in 100 ml of the cryoprotective medium for the subsequent freezing and freeze-drying treatment.

2.4. Freeze-Drying Conditions

Each bacterial suspension was dispensed into 2 ml vials. Samples were frozen at −80˚C for 24 hrs then subjected to freeze-drying. The freeze-drying process was performed in the Zirbus freeze-dryer (Vaco-5-ll-D, Germany), which was programmed to operate as follows; first primary drying for 20 hrs and 0.6 mbar chamber pressure followed by secondary drying for 2 hrs at 15˚C shelf temperature and 0.4 mbar chamber pressure.

2.5. Storage Conditions

After the end of the freeze-drying cycle, the vials were sealed under vacuum and stored at −20˚C. Frozen samples were stored at −80˚C.

2.6. Determination of Survival Rate

Bacterial counts were determined after 48 hrs of incubation at optimum growth temperature using decimal dilutions of the samples with sterile peptone water. After freeze drying, pellets were rehydrated for 10 min at room temperature to the original volume using peptone water then cell count was determined [36]. Bacterial survival rate (%) was calculated according to the formula

$$\text{Survival rate}(\%) = (N/N_o) \times 100$$

N_o represents the log of colony forming units before freeze-drying.

N represents the log of colony forming units after freeze-drying or freezing.

The survival rates during storage were evaluated at zero time, first day of freeze-drying and after 1, 3 and 6 months.

2.7. Determination of Cell Autolytic Activity

Cells were harvested by centrifugation and washed twice with 0.01 M potassium phosphate buffer pH 7.0. The

resulting pellet was then re-suspended in potassium phosphate buffer (10 mmol/l, pH 5.5) containing 1 mol/l NaCl and diluted to OD_{650} equal 1.0 using Laxco spectrophotometer (model a1202, USA). The rate of autolysis was determined according the method described by Thiboutot *et al.* (1995) [37]. The autolytic properties were determined as the percentage decrease in the absorbance at 650 nm at different time intervals.

2.8. Determination of Intracellular Enzymatic Activity

2.8.1. Preparation of the Crude Cell Free Extract

Cells were harvested by centrifugation and washed twice with 0.01 M potassium phosphate buffer pH 7.0. The resultant pellet was grinded for 20 minutes in mortar using alumina (Sigma Type A-5), the extracted disrupted cells re-suspended in 0.01 M potassium phosphate buffer equivalent to 1/3 of the original volume. The suspension was then centrifuged at 4000 rpm for 20 minutes at 4˚C using a high speed centrifuge (Sigma 6k15-Germany) as described by El-Soda and Desmazeaud (1982) [38].

2.8.2. Aminopeptidase Activity

Aminopeptidase activity in the crude cell free extract was measured using Leucine para-nitroanilide (Sigma, St. Louis, Missouri, USA) as a substrate according to the method of El-Soda and Desmazeaud (1982) [38] A unit of aminopeptidase activity was defined as the variation of 0.01 unit of absorbance at 420 nm in 1 min for 1 ml enzyme of the different strains under previously described assay conditions. The specific activity was defined as the number of activity units/mg of protein.

2.8.3. Esterase Activity

The esterase activity was measured using Para-nitro phenyl butyrate as a substrate as described by Brandle and Zizer (1973) [39]. A unit of esterase activity was defined as the variation of 0.01 unit of absorbance at 420 nm in 1 min for 1 ml enzyme of the different strains under previously described assay conditions. The specific activity was defined as the number of activity units/mg of protein.

2.8.4. Protein Determination

The protein concentration was estimated according to the method of Lowry *et al.*, (1951) [40], using the Folin & Ciocalteu's phenol reagent with bovine serum albumin as standard.

2.9. Statistical Analysis

F-test and analysis of variance of treatments difference was performed in randomized complete design according to Steel and Torrie (1980) [41]. Statistical analysis was done by ANOVA, F-test and L.S.D procedures available within the SAS software package (9.13.2009).

3. Results

3.1. Effect of Freezing and Freeze-Drying on the Survival Rate

Table 2 shows the results of the survival rates of the tested strains at the first day of freeze-drying and during the storage period (1, 3, and 6 months) for both frozen and freeze-dried cultures. There was a significant decrease of the survival rates of frozen and freeze-dried samples. The survival rates at the first day of freeze-drying ranged from 92 to 99.5%, after 6 months of storage, the obtained results revealed that the survival rate was strain related, *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium* had the highest survival rate which ranged from 95% - 98.6%, while *Lactobacillus helveticus* and *Lactobacillus plantarum* showed the lowest survival rate (72.7% - 76.9%). Whereas the survival rates for frozen cultures after one month of storage ranged from 86.2% - 99.4%. After 6 months of storage values decreased gradually to reach 96.5% for *Streptococcus thermophillus* and *Lactobacillus delbrueckii* subsp. *Lactis* and 81% for *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus brevis*.

3.2. Effect of Freezing and Freeze-Drying on the Autolytic Activity

The autolytic properties were evaluated in order to quantify the ability of the strains to liberate their intracellular enzymes which can then play an important role during the ripening process [31].

Table 2. Effect of freezing and freeze-drying on survival rate%.

Treatment	After Freezing (Month)			After Freeze-Drying (Month)				Mean
Time	1	3	6	Zero Time Treatment	1	3	6	
1	97.80	96.80	96.40	99.35	96.10	95.50	94.20	97.615^{g}
2	99.30	97.90	95.40	97.10	94.50	94.40	87.60	96.62^{j}
7	98.90	97.10	95.30	95.60	94.80	94.20	93.30	96.92^{ij}
4	98.80	95.10	92.40	98.50	97.40	95.60	94.50	97.23^{hi}
11	97.90	94.10	91.70	99.10	94.10	84.00	73.50	93.44^{l}
12	95.20	90.70	87.50	93.10	87.10	81.70	72.20	90.75^{m}
25	91.75	90.00	84.50	93.60	93.00	92.35	88.80	93.4^{l}
87	92.00	89.25	84.00	94.20	93.10	92.50	89.45	93.45^{l}
10	97.10	94.50	92.30	96.90	96.50	96.20	96.00	96.95^{ij}
9	99.80	99.20	96.30	99.70	99.50	95.60	95.50	98.56^{cde}
34	99.20	98.00	90.00	98.50	98.40	98.00	98.00	98.0^{1f}
36	99.40	98.20	96.30	98.90	98.80	98.60	98.20	98.84^{abc}
64	90.00	86.00	83.30	99.30	98.10	92.50	83.30	93.25^{l}
65	91.00	86.50	83.00	87.80	86.40	83.30	78.50	89.65^{n}
58	98.50	98.00	97.90	99.50	99.20	98.80	98.10	99^{ab}
59	98.50	97.30	93.50	99.00	99.00	98.80	96.70	98.28^{ef}
60	98.90	98.60	90.60	98.70	98.60	97.90	97.20	98.05^{f}
61	98.20	97.40	97.00	98.50	98.10	97.80	97.30	98.43^{de}
16	98.50	96.70	93.70	97.20	97.00	95.50	94.90	97.35^{gh}
17	99.50	98.20	97.90	99.55	97.80	97.50	96.60	98.705^{bcd}
19	99.50	97.80	95.20	99.50	99.80	99.40	98.60	98.98^{ab}
43	99.90	98.80	98.50	99.25	99.00	98.60	96.70	99.075^{a}
47	97.50	97.20	97.10	98.40	96.80	90.00	84.20	96.12^{k}
52	100.00	99.00	99.00	99.50	97.20	92.00	83.50	97.02i
48	99.20	98.00	96.30	100.00	99.60	99.00	98.50	99.06^{a}
49	99.00	98.80	98.50	99.00	98.50	95.00	91.60	98.04^{f}
Strains (FAAU No.)		97.3^{a}			96^{b}			

$^{a-n}$ Means followed by the same letter's are not significant, but different letters are significant according to LSD procedure ($P < 0.05$).

Table 3 revealed an increase in autolytic activity for frozen and freeze-dried cultures, the obtained results for freeze-dried cultures showed that the lowest autolytic activity after the first day of freeze-drying was 14% for *Enterococcus faecium*, while the highest autolytic activity was 62% for *Lactobacillus plantarum* and *Lactobacillus rhamnosus.* When both treatments were compared the obtained data revealed that freeze-drying had a higher impact on autolytic activity (31.28%) than freezing (27.81%). As a general rule *Lactobacillus* strains exhibited higher rate of autolysis when compared to *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium* strains.

3.3. Effect of Freezing and Freeze-Drying on the Intracellular Enzymatic Activity

The aminopeptidase activity of the tested cultures at the first day of freeze-drying and after 3 and 6 months of the storage period is illustrated in **Figure 1**. The obtained data revealed that *Lactobacillus paracasei* subsp. *paracasei* had aminopeptidase specific activity of 3.18 which was the highest among all the species tested. The

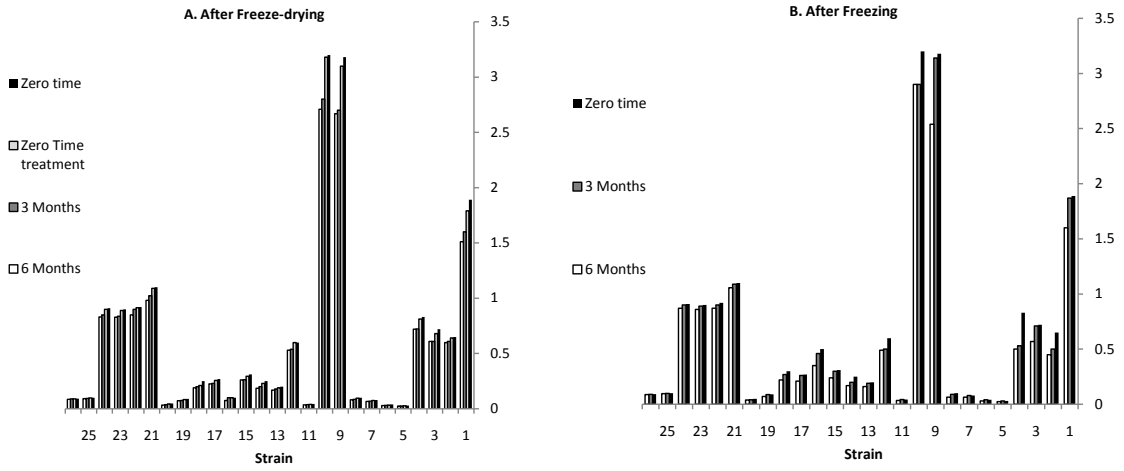

Figure 1. Aminopeptidase activities of tested strains at zero time and after freezing, freeze-drying and during storage period.

Table 3. Effect of freezing and freeze-drying on the autolytic activity.

Treatment		After Freezing (Month)				After Freeze-Drying (Month)				Mean
Time		Zero Time	1	3	6	Zero Time Treatment	1	3	6	
Strains (FAAU No.)	**1**	47.00	69.00	70.00	72.00	62.00	67.00	68.00	70.00	61.9[a]
	2	33.00	51.00	60.00	65.00	40.00	50.00	52.00	53.00	47[d]
	7	40.00	53.00	55.00	57.00	50.00	50.50	51.00	54.00	49.05[c]
	4	35.00	41.00	47.00	55.00	41.10	42.50	47.00	50.80	42.94[f]
	11	20.00	21.50	22.10	24.10	22.10	24.80	27.70	29.60	23.19[o]
	12	40.00	51.00	56.00	58.00	45.00	72.00	73.00	75.00	55[b]
	25	30.00	36.20	42.00	48.20	31.60	32.50	36.20	39.00	35.57[i]
	87	32.00	35.00	39.00	47.00	35.00	45.00	50.00	60.00	40.7[g]
	10	22.00	31.00	32.50	34.90	22.00	21.00	22.50	25.30	26.32[m]
	9	29.00	31.00	33.50	35.50	38.60	39.00	40.70	41.40	34.67[j]
	34	19.00	20.50	22.00	25.00	22.00	23.50	24.60	26.50	22.11[b]
	36	15.00	15.50	16.00	17.20	16.70	17.50	24.00	27.40	17.93[s]
	64	30.00	39.00	44.00	54.50	48.00	55.50	60.00	66.50	45.75[e]
	65	30.00	31.00	31.80	33.90	34.80	40.00	51.90	53.50	36.69[h]
	58	19.00	20.90	28.00	38.70	21.90	34.00	34.60	62.00	29.71[l]
	59	14.00	14.90	28.90	31.70	19.00	19.90	26.00	27.00	20.94[q]
	60	20.00	22.20	28.20	36.20	27.50	31.00	31.30	35.30	27.17[m]
	61	20.50	22.80	25.60	27.50	34.80	37.40	45.80	56.00	31.14[k]
	16	19.00	19.70	30.80	42.60	20.20	22.60	23.00	27.60	24.35[n]
	17	12.00	13.00	13.50	19.40	12.90	22.00	26.00	28.90	17.17[s]
	19	12.00	13.00	14.70	23.40	23.60	24.80	29.00	35.90	20.04[r]
	43	11.00	12.30	18.50	20.50	11.20	12.20	13.20	13.60	13.45[u]
	47	5.00	8.00	10.00	15.90	5.10	9.30	11.10	16.90	9.13[w]
	52	6.00	8.50	11.00	14.80	8.00	9.50	11.00	16.80	9.72[w]
	48	9.50	12.00	17.20	27.50	13.90	14.00	17.20	20.20	15.05[t]
	49	6.00	14.00	16.50	18.30	8.50	10.00	12.00	18.20	11.55[v]
			27.817[b]				31.279[a]			

[a-w]Means followed by the same letter's are not significant, but different letters are significant according to LSD procedure ($P < 0.05$).

aminopeptidase activity of *Lactobacillus* strains was higher when compared to *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium* strains.

As a general rule conservation methods lead to a significant decrease of aminopeptidase activity, which was in favor of freeze-drying. In fact the decrease ranged from 10% - 20% for freeze-dried cultures, while corresponding values in the case of freezing were 7% - 16%. Similar trends was observed for esterase activity (**Figure 2**)

Figure 2 which illustrate the esterase activity of the tested cultures at the first day of freeze-drying and after 3 and 6 months of storage period, show that *Lactobacillus* cultures exhibited lower esterase activity when compared to *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium*.

4. Discussion

Freeze-drying and freezing are commonly used for the preservation and storage of microorganisms for industrial application. The optimal performance of starter cultures should guarantee their potential to survive and the stabilization of their metabolic activity. In the present work we assessed the survival conditions of 26 LAB strains exhibiting technological potential during freeze-drying and freezing over a storage period of six months.

Our data revealed a significant reduction of survival during storage of the frozen and freeze-dried strains. Similar observations were reported for freeze-dried cultures such as *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus para mesenteroides*, *Lactobacillus mesenteroides* ssp. *mesenteroides* [42], *Lactococcus lactis* ssp. *lactis* var. *diacetylactis* [43], *Lactobacillus paracasei* [24], *Streptococcus thermophilus* [44] and *Lactobacillus acidophilus* [45]. As well as frozen cultures such as *Lactobacillus rhamnosus* [46], *Lb. delbrueckii* subsp. *bulgaricus* [8] [29] [47], *Streptococcus thermophilus* [48]. Loss of the viability of freeze-dried cultures is a consequence of cell damage at several target sites, namely the cell wall, the cell membrane and the DNA, as well as a result of membrane lipid oxidation [49]. Also during freeze-drying, bacterial cells are subjected to osmotic stress as a result of decreased water activity of the medium and the external increase of accumulated solutes. While, freezing causes different cellular injuries on LAB [46], damage to cells due to formation of ice crystals and high osmotic pressure brought about by high internal solute concentrations [4] [6] [8]. However resistance to osmotic stress of strains is dependent on their ability to accumulate internal solutes which increases internal osmotic pressure and restores pressure and on changes in the membrane lipid composition [50]. Teixeria *et al.* (1996) [51] suggested that oxidation of the fatty acids of membrane lipids is the most likely cause of death of microbial cells during storage.

Our data revealed that *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis and Lactococcus lactis* subsp. *cremoris* had higher survival rate when compared to lactobacilli. Similar observations were reported by Heckly (1961) [52] who showed that the gram-positive cocci are the most resistant cultures. Also According to-Fonseca *et al.* (2000) [29], the higher the surface area of the cell, the higher the membrane damage owing to extracellular ice crystal formation during freezing. A similar relation also appears to exist for survival during

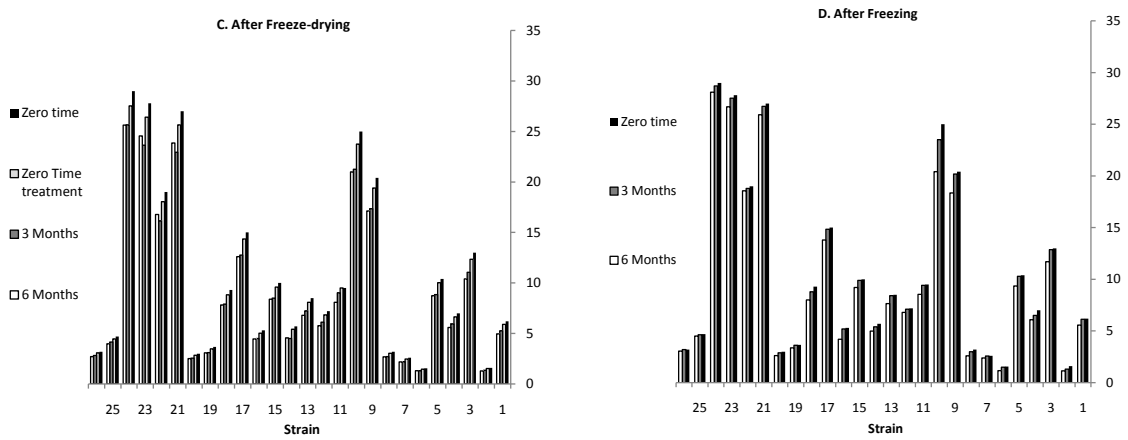

Figure 2. Esterase activities of tested strains at zero time and after freezing, freeze-drying and during storage period.

storage of LAB in the freeze-dried state [6]. The different survival rates of tested *Lactobacillus* strains confirmed that resistance to freeze-drying is strain-specific [20] [36].

Consequence to previous data, specific sections as intrinsic factors, growth factors, sub-lethal treatments, drying medium and storage and rehydration should be controlled for the improvement of LAB survival during freeze-drying and subsequent storage [53]-[56], and several factors during storage affect survival and fermentation activity, including reaction with oxygen, moisture, light, microbial contamination and elevated temperature [19].

Our results showed no significant correlations between autolytic activity and survival rate to freezing or freeze-drying a similar observation were made by Koch *et al.*, 2008 [57].

Intracellular enzymatic activity was significantly decreased by both treatments freezing and freeze-drying. The depression in the intracellular enzymatic activity may be due to several factors; theoretically a cell freeze-dried without injury, and stored under ideal conditions, should rehydrate to its original state, however, cells injured during freezing or freeze-drying, even if stored under perfect conditions, could gradually deteriorate, or could be killed by improper rehydration. Freeze-drying procedure might cause injury of cell envelopes resulting in the release of some intracellular materials [46] [58]. This may explain the correlation that we found between the increase of autolytic activity and the loss of enzymatic activity. These results are comparable data showing that the strains exhibiting higher in autolytic activity are more susceptible to decrease in intracellular enzymatic activity.

The obtained results revealed that *Lactobacillus* strains exhibited higher rate of autolysis than spherical shape strains these results are comparable to the data of other researchers who reported that the ability of strains to lyse and subsequent release of their intracellular enzymes is strain dependent [59]. Autolytic activity is an interesting property of lactic acid bacteria since it allows the liberation of intracellular enzymes into the cheese curd. These enzymes accelerate proteolysis during cheese ripening, which lead to an increase in the rate of free amino acid production that may act as flavor precursors [59] [60]. LAB that are highly autolytic are often used as primary or secondary starters for production of semi-hard and hard cheeses [61]-[63].

It is important to clarify that the presence of cryoprotectant help maintaining good viability and technological properties, which could referred to its ability to modify the dynamics of formation and rupture of intra- and intermolecular weak chemical bonds by mechanisms such as 1) alterations in the viscosity in the bacterial cell; 2) direct participation of the cryoprotectant in the intermolecular interactions; 3) modification of the water activity inside the bacteria; 4) alterations in the nucleation pattern by retarding the formation and/or changing the dimensions of the ice nuclei during solidification..

5. Conclusions

The impact of freezing and freeze-drying on the viability of 26 LAB strains representing 13 different species in presence of skimmed milk as a cryoprotectant was minimal.

Cocci were more resistant to both conservation methods than rod shape bacteria. Freezing was less damaging than freeze-drying. The method used for freezing cycles as well as freeze-drying cycles and cryoprotectant used in the study could be recommended for LAB preservation.

Thirty percent increase in the rate of autolysis could be measured as a consequence of culture conservation. The impact of freeze-drying on the rate of autolysis was slightly higher than freezing. Lactobacilli and more particularly *Lactobacillus plantarum* and *Lactobacillus rhamnosus* were found to be more sensitive than *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium.* Our data in that perspective indicate the possible use of conservation method to induce enzyme release from adjunct cultures during cheese ripening. The impact of freezing cycles and freeze-drying cycles should however be considered.

Intracellular aminopeptidase and esterase activity were affected by the conservation methods. An average reduction of about 14% could be measured. The difference between the impact of both methods is minimal. The decrease in the rate of both enzymatic activities was rather similar.

As a general rule the decrease in intracellular aminopeptidase and esterase activity of *Streptococcus thermophillus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Enterococcus faecium* was less when compared to lactobacilli.

The impact of conservation method on the rate of autolysis and intracellular aminopeptidase and esterase ac-

tivities which was not well investigated in the past can open new perspectives in flavor enhancement and accelerate cheese ripening research.

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