Lyophilisation of Lactic Bacteria with Probiotic Effect for Production of Starter Cultures

Vintilă Teodor[1], Sava Camelia[2*], Moț Daniela[1], Gaspar Eniko[2], Barbu Horia[2], Julean Călin[1]

[1]BUASVM "King Michael I of Romania" from Timișoara, Faculty of Bioengineering and Animal Resources, Calea Aradului, nr. 119, Timișoara, Romania.
[2]Lucian Blaga University, Faculty of Agricultural Sciences, Food Industry and Environmental Protection, Str. Dr. Ion Rațiu, no. 7-9, Sibiu, Romania.

Abstract
Selection of lactic acid bacteria (LAB) for probiotic effect and preservation of LAB by lyophilized have been studied. The two LAB strains belonging to *Lactobacillus plantarum* and *Lactobacillus pentosus* producing bacteriocins in inhibitory concentration for colibacilli were selected from a total of 17 tested strains. The selected strains were further tested for their ability to survive the preservation process by lyophilisation. Two methods of preserving LAB by lyophilisation were tested and the viability of cells in lyophilized products was monitored in order to obtain products used as starter cultures. The presence of inhibition zones in mixt cultures indicates the ability of LAB to inhibit the growth of *E. coli* bacteria due to the synthesis of bacteriocins. During three years period, the viability of lyophilized LAB was monitored. Obtained data indicate that starter cultures of LAB can be stored in the lyophilized state for at least three years in dark and cool place, and the inoculation rate can be increased after this period by 50% if the starter culture was preserved and stored by applying the described techniques.

Keywords: lactic acid bacteria, probiotic, preservation, lyophilisation

1. Introduction

In applied microbiology, one of the main factors for successful application of laboratory techniques in practice and scaling up a biotechnological process from laboratory to pilot and industrial scale is viability and stability of the inoculums. In this respect, it is crucial to establish effective method for preservation of microorganisms to ensure the maintenance of metabolic activities of microorganisms. One of the most used and widely recognized method of storing bacterial strains is lyophilisation [1]. Lyophilisation causes a slight decrease in the mass of the lyophilized material, which allows to obtain a perfectly soluble product, easily rehydrated. The treatment of a freeze-dried product comprises the following processes: preliminary treatments, freezing, sublimation (primary drying), secondary drying, conditioning of the freeze-dried product and storage of the lyophilizate [2]. The success of lyophilisation is conditioned by both the chosen freezing method and the final temperature reached. In general, the formation of ice crystals and the increase of electrolyte concentrations, as a result of the removal of water after freezing, affect the living material subjected to lyophilisation. It appears that the damage is mainly due to the removal of water from proteins and nucleic acids. The rapid freezing of the cell suspension induces the formation of small ice crystals, while low-rate freezing facilitates the appearance of large ice crystals [3]. To allow effective lyophilisation, it is recommended to cool the suspensions until the eutectic mixtures (solvents with high concentrations caused by water removal) are completely frozen [4,5].

* Corresponding author: Sava Camelia, 0745256095, Email: camelia.sava@ulbsibiu.ro

The aims of the research conducted in this study are: selection of lactic acid bacteria (LAB) with probiotic affect and preservation of viable LAB by lyophilisation. We have focused our study on LAB with probiotic effect that are part of the dominant microaerophilic flora, able to inhibit the development of microorganisms that are part of the facultative anaerobic subdominant flora (*E. coli*), in order to maintain balance of the intestinal microflora.

Two methods of preserving lactic bacteria by lyophilisation were tested and the viability of cells in lyophilized products was monitored in order to obtain products containing living LAB to be used as starter cultures or inoculants.

2. Materials and methods

Culture media used in this laboratory research are: MRS (for culturing lactic acid bacteria) and Mueller Hinton – a special medium for testing susceptibility to antibiotics. The lactic acid bacteria are provided by the Collection of Industrial Microorganisms from Timișoara (CMIT) [6]:

1. *Lactobacillus sp.* 1.45. (ex. CMIT1, ex. 80) - Isolated from 2-week-old infant calf ruminal fluid. Provenance: USAMVB Timișoara, Faculty of Bioengineering and Animal Resources, Discipline of Applied Microbiology.
2. *Lactobacillus plantarum* 1.46. (ex. CMIT2, ex 1188). Isolated from fermented vegetal material (corn silage). Provenance: USAMVB Timișoara, Faculty of Bioengineering and Animal Resources, Discipline of Applied Microbiology.
3. *Lactobacillus sp.,* 1.47. (Ex CMIT3, probably acidophilus). Isolated from 2-week-old infant calf ruminal fluid. Provenance: USAMVB Timișoara, Faculty of Bioengineering and Animal Resources, Discipline of Applied Microbiology.
4. *Lactobacillus rhamnosus* 1.51. (R0011). Provenance: U.C. Dublin, Corcionivoschi Nicolae.
5. *Lactobacillus rhamnosus* 1.58. (CMGB 34). Working name: 15b. Provenance: University of Bucharest, Faculty of Biology.
6. *Lactobacillus pentosus* 1.60. (DSM 20314). Other collection no. or WDCM no .: ATCC 8041, DSM 43, NCDO 363, NCIB 8026. Type strain, Bacteria; Firmicutes; Bacilli; Lactobacilli; Lactobacillaceae; Lactobacillus. whole genome shotgun sequencing project - 60 rc DNA linear BCT 06-NOV-2015.
7. *Escherichia coli* isolated from calf ruminal fluid. Provenance: USAMVB Timișoara, Faculty of Veterinary Medicine, Department of Microbiology.

Determining the ability of lactic acid bacteria to inhibit colibacilli

In this study we have applied spot inoculation of lactic bacteria in Petri dishes with MRS and MH medium. Lactic bacteria were inoculated in 3-4 wells per plate, and *Escherichia coli* was inoculated by scattering over the entire surface of the plate after inoculation of lactic bacteria in wells. After incubation of plates at 37°C for 48 hours, the existence of inhibition areas of *E. coli* bacterium growth around the well with lactic acid bacteria is analysed. The presence of inhibition zones with a diameter of a few millimetres was observed, which indicates the ability of lactic acid bacteria to inhibit the growth of *E. coli* bacteria due to the synthesis of bacteriocins. Inhibition areas of up to 1 mm appear because of the acidity around lactic acid bacteria due to the secretion of lactic acid.

Figure 1. Well formation (left) and Petri dish inoculated with lactic acid bacteria and *E. coli* (right)

Lyophilisation of lactic acid bacteria

The equipment used to lyophilize lactic acid bacteria is UNICRYO MC4-L lyophilizer (Uniequip – Germany). In condensing chamber of the lyophilizer, the lowest reachable temperature is -60ºC. The equipment consists of freezer unit; stainless steel condensing area; acrylic chamber with trays and vacuum pump. To measure temperature during lyophilisation, a thermometer was inserted into the freeze-drying unit, in condensation area and in acrylic chamber. The lactic bacteria were placed in 20 ml vials and freeze-drying process was performed applying two methods:

Method 1:

In the first method applied, the suspensions of lactic bacteria in a protective medium with skimmed milk were placed in the upper area of the lyophilizer (acrylic chamber), where the temperature rises above 0ºC during vacuum drying, we register the temperature of + 20ºC.

Method 2:

In the second method applied, the suspensions of lactic bacteria were placed in the lower area of the lyophilizer (condensation area), where the temperature is maintained around -30ºC during vacuum drying. We recorded the temperature of -33ºC.

Determination of the viability of microorganisms

The lyophilized products containing LAB were stored at + 4ºC and were periodically verified by rehydration with sterile saline solution. The LAB suspensions were counted by CFU method (making serial decimal dilutions and inoculation in Petri dishes containing MRS medium). After incubation of the inoculated plates at 37ºC for 48 hours, the colonies were counted and the viability of the cells in the lyophilized product was determined [7].

3. Results and discussion

Antagonistic effects of LAB against colibacilli

All 17 strains of LAB listed above have grown in plate wells, both in MRS and Mueller-Hinton medium formulas. LAB were inoculated in 3-4 wells per plate and *E. coli* was inoculated by dispersion after inoculation of lactic acid bacteria in the wells (Figure 2).

Figure 2. Petri dishes with LAB and *E. coli*. Left: *Lactobacillus sp*. CMIT 1.45, *Lactobacillus plantarum* CMIT 1.46, *Lactobacillus acidophilus* CMIT 1.47 inoculated in wells and *Escherichia coli* inoculated by dispersion on MRS medium. Right: *Lactobacillus rhamnosus* CMIT 1.51, *Lactobacillus rhamnosus* CMIT 1.58. and *Streptococcus sp.* CMIT 1.48. and *E. coli* on Mueller-Hinton medium.

After 48 hours of incubation, the plates were analysed and the following observations were made:

- all LAB developed both on MRS and Mueller-Hinton media;

- *Escherichia coli* culture has grown much more vigorously on Mueller-Hinton than on MRS medium;
- small areas of inhibition (approx. 1 mm) in the contact areas of *E. coli* with LAB in the wells were observed around most wells only on Mueller-Hinton medium, not on MRS medium;
- around the wells in Mueller-Hinton with *Lactobacillus pentosus* CMIT 1.60 (DSM 20314) and *Lactobacillus plantarum* CMIT 1.46 (ex. CMIT2, ex. 1188) large areas of inhibition of *E. coli* were observed (up to 8 mm for *Lb. plantarum* and up to 10 mm for *Lb. pentosus*);
- the same strains (*Lb. plantarum* and *Lb. pentosus*) did not produce areas of inhibition by cultivation on MRS medium with *E. coli* (Figures 3 and 4).

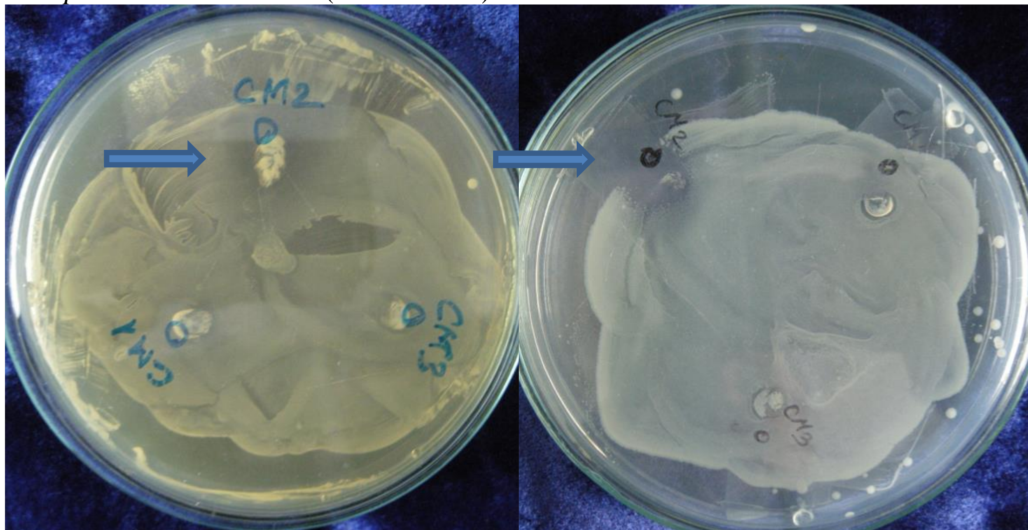

Figure 3. LAB and *E. coli***.** Left: on MRS *E. coli* grows up to the immediate vicinity of LAB. Right: on the Mueller-Hinton medium, small areas of inhibition of *E. coli* observed around wells with LAB and large area of inhibition by *Lb. plantarum* CMIT 1.46 (CMIT2) - in both plates marked with an arrow

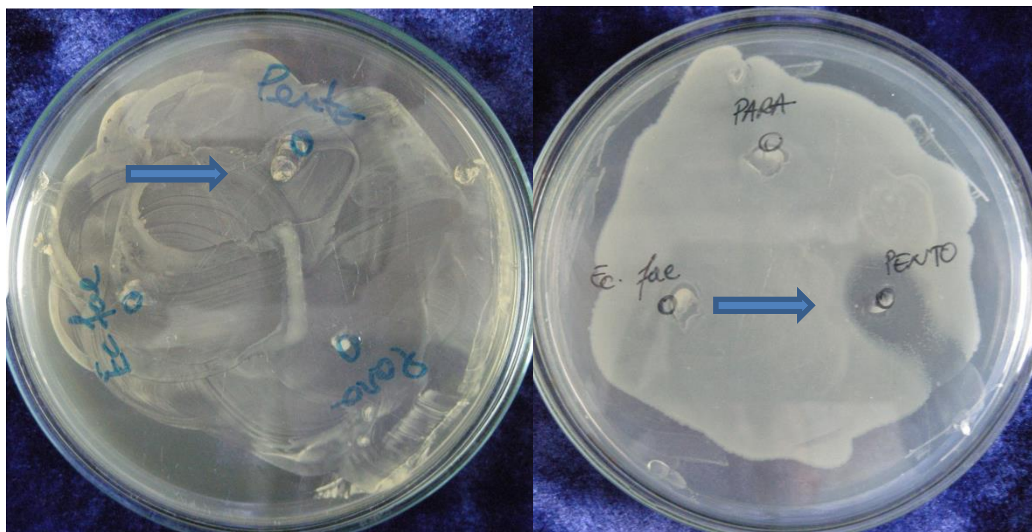

Figure 4. LAB and *E. coli*. Left: on MRS *E. coli* grows up to the immediate vicinity of LAB. Right: on Mueller-Hinton, small areas of inhibition of *E. coli* culture are observed around the wells with LAB and large area of inhibition by *Lb. pentosus* CMIT 1.60. (DSM 20314) - in both plates marked with arrow.

These observations demonstrate the ability of LAB to synthesize bacteriocins. Bacteriocins are antibacterial substances with an effect similar to that of antibiotics, synthesized by bacteria, but with an antimicrobial effect on a much smaller spectrum of bacteria than that of antibiotics. Regarding the synthesis of bacteriocins with anti-colibacillic effect by strains of *Lb. plantarum* and

Lb. pentosus only on the Mueller-Hinton medium, the following explanations can be issued.
One of them may be the effect of nutritional stress conditions to which LAB cultivated on Mueller-Hinton are subjected. While in the MRS formula the source of carbon and energy is provided by glucose – a carbon source readily to be metabolized - in Mueller-Hinton the source of carbon and energy is provided by starch, an organic polymer that must first be hydrolyzed with extracellular amylolytic enzymes and then the resulting glucose can be used by bacteria as an energy source in cellular metabolism. In general, stress conditions trigger synthesis of the secondary metabolism in microorganisms, through which they synthesize functional molecules that are released extracellular (hydrolytic enzymes, antimicrobial substances) involved in the competition for the nutrient substrate against other microorganisms. Several researchers [8,9] confirms the increased synthesis of bacteriocins on the Mueller-Hinton compared to cultures on MRS or other media.
Another explanation may be the following: *E. coli* - the bacterium against the antimicrobial activity of LAB is tested - is a microorganism capable of synthesizing and releasing proteolytic enzymes into the environment [10]. The Mueller-Hinton medium contains hydrolyzed casein as a source of nitrogen. In this case, *E. coli* has available amino acids ready for use directly in protein metabolism [11]. Therefore, as no complex protein substances are found in Mueller-Hinton formula (proteins are the necessary inducer for the synthesis of proteolytic enzymes), *E. coli* does not synthesize proteases in Mueller-Hinton. In contrast, in the MRS the nitrogen source is provided by a protein complex (peptone). Thus, *E. coli* is bound to trigger protease biosynthesis. These proteases also hydrolyse the bacteriocins (protein molecules) produced by the lactic bacteria in the respective plate.
These are the main reasons why Mueller-Hinton is used as a special medium for testing susceptibility to antibiotics and other antimicrobial substances [12].

Viability of lyophilized LAB

The two LAB strains producing bacteriocins in inhibitory concentration for colibacilli (*Lb. plantarum* and *Lb. pentosus*) were selected from the total of 17 strains tested. The two selected LAB strains were further tested for their ability to survive the preservation process by lyophilisation. In one of the lyophilisation methods (hereinafter referred to as ***Method 1***), the vials containing LAB in suspension with cryoprotectant, as explained in "Materials and methods", are frozen at -70ºC for 1 hour and subsequently lyophilized in the upper area of the lyophilizer (at about + 20ºC). In ***Method 2***, the vials containing LAB in suspension with cryoprotectant are frozen at -70ºC for 1 hour and lyophilized in the lower part of the lyophilizer, in the condenser area (around -30ºC).

Table 1 Viability of LAB preserved by the two lyophilisation methods

LAB strain	Number of viable cells before lyophilisation		Method 1			Method 2		
			Number of viable cells		Viability rate	Number of viable cells		Viability rate
	CFU/ml	Log_{10}/ml	CFU/ml	Log_{10}/ml	%	CFU/ml	Log_{10}/ml	%
Lb. plantarum	$3.6 \cdot 10^9$	9.55	$6.0 \cdot 10^7$	7.78	81.4	$1.1 \cdot 10^8$	8.04	84.2
Lb. pentosus	$1.2 \cdot 10^9$	9.08	$2.1 \cdot 10^7$	7.32	80.6	$1.6 \cdot 10^7$	7.20	79.3

The data in Table 1 indicate high viability percentages for both strains, with higher values for the strain of *Lb. plantarum*. Regarding the effect of the temperature in the lyophilizer (method 1 at + 20ºC and method 2 at -30ºC), the results indicate similar values for the *Lb. pentosus* strain, which is viable in proportion of 79-80% by applying both methods. Regarding the *Lb. plantarum* strain, the results show that lyophilisation at -30ºC ensures a slightly higher viability ratio (around 84%) compared to lyophilisation at + 20ºC.
We opted for the further application of method 2 (lyophilisation at -30ºC), because in terms of the technical conditions in which the two lyophilisation methods were performed, this method offers better working conditions. Applying method 1 (lyophilisation at + 20ºC), the bacterial suspension frozen at 70ºC is thawed during the lyophilisation process at high temperature, and the liquid in the lyophilisation

vial forms bubbles that rise to the cotton plug with which the vial is closed. This is a negative aspect, because the germs in the suspension disperse on the wall of the lyophilisation bottle, in some cases reaching the cotton plug. In the case of method 2, the suspension in the lyophilisation vial remains in a solid state, the water sublimating directly in the vapour state. Thus, at the end of lyophilisation by method 2 a dry product is obtained in the form of a pill on the bottom of the lyophilisation bottle (figure 5).

Figure 5. Lyophilized vials of *Lb. plantarum* (A) and *Lb. pentosus* (B) immediately after removal from the lyophilizer (left) and after stapling (right)

In figure 5 on the right, the two lyophilisation bottles on the front, marked A and B contains the lyophilisate obtained by method 2 on the bottom of the bottle in the form of a pill, and the two less visible on the back (A1 and B1) obtained by method 1 are dispersed on the wall of the vial.

Testing viability in time of lyophilized LAB

During this work stage we monitored the viability over time of lyophilized LAB by the two methods described above. The vials obtained with the two lyophilized LAB are stored in dark place, approximately + 4°C (in the refrigerator), and periodically one vial is opened and the number of viable bacteria is determined by the CFU (colony forming units) method. The period in which the viability of lyophilized LAB is monitored is three years.

Tables 2 and 3 show the viability values of the suspensions of LAB before lyophilisation and during storage in the refrigerator. Figure 6 shows graphically the evolution of the viability percentage in the tested period.

Table 2 Viability in time of lyophilized LAB by ”Method 1”

Moment of sampling	Viability					
	Lb. plantarum			*Lb. pentosus*		
	CFU/ml	Log_{10}/ml	Viability %	CFU/ml	Log_{10}/ml	Viability %
Before lyophilisation	$3.6 \cdot 10^9$	9.55	122	$1.2 \bullet 10^9$	9.08	124
After lyophilisation	$6.0 \bullet 10^7$	7.78	100	$2.1 \bullet 10^7$	7.32	100
2 months	$3.0 \bullet 10^7$	7.47	96.01	$2.0 \bullet 10^6$	6.30	86.06
4 months	$3.3 \bullet 10^7$	7.51	96.53	$2.1 \bullet 10^6$	6.32	86.33
3 years	$2 \bullet 10^7$	7.30	93.83	$2 \bullet 10^5$	5.30	72.40

The data on the viability of LAB presented in Tables 2, 3 and figure 6 indicate the following:

➢ Method 1 (lyophilisation at + 20°C), has the advantage of lower death rate during lyophilisation than in the case of method 2 (lyophilisation at -30°C). However, an improvement of the working technique is required because the bacterial suspension frozen at 70°C thaws during the lyophilization process at high temperature (+ 20°C), and the liquid in the lyophilization vials forms bubbles that reaches the cotton plugs.

Table 3 Viability in time of lyophilized LAB by "Method 2"

Moment of sampling	Viability					
	Lb. plantarum			*Lb. pentosus*		
	CFU/ml	Log_{10}/ml	Viability %	CFU/ml	Log_{10}/ml	Viability %
Before lyophilisation	$7.7 \cdot 10^9$	9.88	110.64	$8.0 \cdot 10^9$	9.90	121.47
After lyophilisation	$8.5 \cdot 10^8$	8.93	100	$1.4 \cdot 10^8$	8.15	100
2 months	$5.3 \cdot 10^7$	6.72	75.25	$3.2 \cdot 10^6$	6.50	79.75
4 months	$2.5 \cdot 10^6$	6.39	71.56	$3.7 \cdot 10^6$	6.57	80.61
3 years	$1.3 \cdot 10^6$	6.10	68.31	$1.2 \cdot 10^4$	4.10	50.30

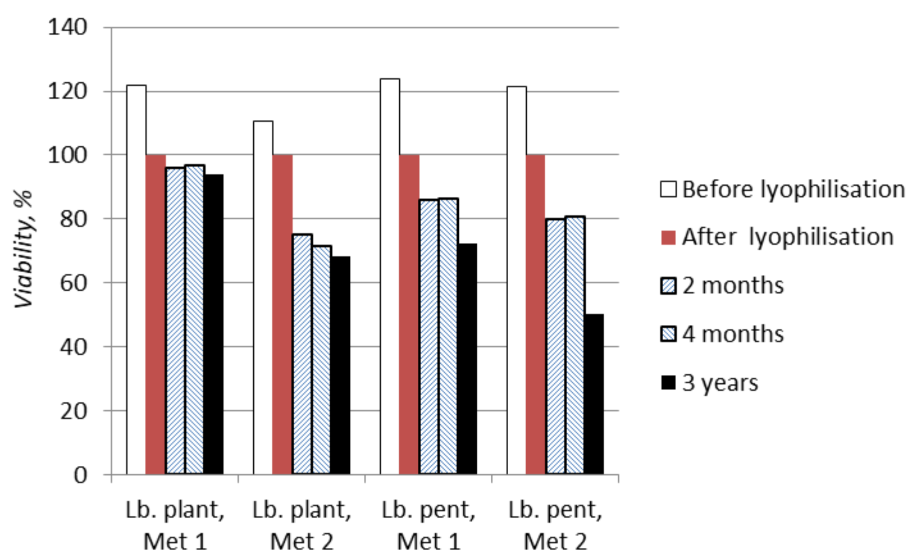

Figure 6. Evolution of the viability in the tested period

➢ (Lb. plant = *Lactobacillus plantarum*, Lb. pent = *Lactobacillus pentosus*, Met = lyophilisation method)

➢ In the case of method 2 (lyophilization at -30ºC), the working technique does not raise difficulties because the cell suspension, always remaining in the frozen state, does not foam under the action of vacuum and drying is much more uniform than in method 1. However, lower viability is obtained than in method 1, both during lyophilization and after storage of vials in the refrigerator for 3 years.

➢ The *Lactobacillus plantarum* strain used in this study is much better suited for preservation by lyophilization, 93% of the cells remaining viable when lyophilization is applied at -30ºC; unlike the *Lactobacillus pentosus* strain, in which the viability decreases to 72%, or even to 50% if lyophilization is applied at + 20ºC.

➢ In absolute terms, the most drastic reduction in viability resulted in the death of LAB cells from about 8 million living cells in one ml of rehydrated lyophilized suspension to 12000 living cells in one ml of lyophilized suspension of *Lactobacillus pentosus* rehydrated after three years of refrigerated storage. In contrast, the highest percentage of viability observed in this study caused the number of *Lactobacillus plantarum* bacteria to be reduced from 3600 million to 20 million in one ml of lyophilized and rehydrated suspension after three years of storage in the refrigerator.

➢ These data indicate that the starter cultures of lactic bacteria can be preserved in the lyophilized state for at least three years in the refrigerator. The inoculation percentage should be increased by 50% if the starter culture were prepared and preserved for three years by described techniques. The results obtained in this study sustain data obtained and published in previous research [13-15] regarding the viability of LAB and probiotic effect.

Lactobacillus plantarum has proven effects in other studies on the conservation of corn, alfalfa, sorghum silages, [16] so that lyophilised products containing this microorganism can be used as an inoculum in the biotechnology industry to obtain inoculants for crops preservation and improve quality of ensiled forages.

4. Conclusions

1. The ability of LAB to synthesize bacteriocins has been demonstrated. The synthesis of bacteriocins with anti-colibacilli effect by *Lb plantarum* and *Lb. pentosus* strains has been demonstrated on Mueller-Hinton culture medium.
2. Freeze-dried products containing viable lactic bacteria, stable for three years, in cold and dark storage conditions, were obtained.
3. These lyophilized products can be applied in:
- food industry as inoculants for the initiation of fermentations in the processes of preparation of fermented dairy products, with probiotic effect; the products obtained can be considered functional foods, with the effect of inhibiting colibacilli;
- feed additives industry for the production of probiotics applied to animal health;
- industrial biotechnology industry to obtain inoculants for preservation of fresh plant biomass.

References

1. Stoica I., Vassu Dimov T., Săsărman E., Biologia și taxonomia moleculară a microorganismelor - Colecția de culturi microbiene, Editura Arvin Press.
2. Săsărman Elena, Jurcoane Ștefania- Microbiologie industrială, Editura Mirton, Timișoara, 2000
3. Berg. R. D., The indigenous gastrointestinal microflora., Trends Microbiol, 2002, 4,430-435
4. Vintilă T., Dinu L. D., Tehnologia Produselor de Biosinteză, Manual de laborator, Editura Orizonturi Universitare, Timişoara, 2004. ISBN 973-638-156-0
5. Simione F.P., Brown E.M., ATCC Preservation Methods: Freezing and Freeze-Drying (2nd ed.), ATCC, Rockville, MD, 1991
6. Vintilă T., Microbiologie Aplicată: lucrări practice de laborator, Editura Agroprint, Timişoara, 2015.
7. Vintilă T., Săsărman E., Mic A., Studii privind conservarea în azot lichid a bacteriilor lactice, Sesiunea Ştiinţifică Anuală a FZB, Timişoara, 1999, 467-470
8. Bizani, D., Brandelli, A. Influence of media and temperature on bacteriocin production by *Bacillus cereus* 8A during batch cultivation. *Appl Microbiol Biotechnol,* 2004, 65, 158–162
9. Russell, J. B., and H. C. Mantovani, The bacteriocins of ruminal bacteria and their potential as an alternative to antibiotics, J Mol Microbiol Biotechnol, 2002, 4, 347-355
10. Shu Q., and H. S. Gill. Immune protection mediated by the probiotic Lactobacillus rhamnosus HN001 (DR20) against Escherichia coli O157:H7 infection in mice. FEMS Immunol Med Microbiol, 2002, 34, 59-64
11. Sakamoto, I., Igarashi, K. Kimura, A. Takagi, T. Miwa, and Y. Koga, Suppressive effect of Lactobacollus gasseri OLL 2716 (LG21) on Helocobacter pylori infection in humans. J Antimicrob Chemother, 2001, 47, 709-710
12. Corcionivoschi N., Drinceanu D., Probioticele la timpul prezent, Editura Mirton, 2009
13. Dupuis, C., C. Corre, and P. Boyaval, Proteinase Activity of Dairy Propionibacterium. Applied Microbiology and Biotechnology, 1995, 42, 750-755
14. Perdigon, G., S. Alvarez, M. Rachid, g. Aguero, and N. Gobbato, Immune system stimulation by probiotics, J Dairy Sci, 1995,78, 1597-1606
15. Salminen, S., and A. V. Wright, Lactic Acid Bacteria. Microbiology and Functional Aspects, New York, 2000.
16. Vintilă D., Vintilă, T., Dronca, D., Obtaining and conditioning lactic bacteria inoculants using bentonite, Bulletin of USAMV Cluj-Napoca, Animal Husbandry and Biotechnologies, 2006, 62, 238-242.