Journal homepage: www.fia.usv.ro/fiajournal
Journal of Faculty of Food Engineering,
Ştefan cel Mare University of Suceava, Romania
Volume XII, Issue 3 – 2013, pag. 225 - 232

HIGHLY ACTIVE FREEZE-DRIED PROBIOTIC CONCENTRATES WITH LONG SHELF LIFE

***Rositsa DENKOVA[1], Zapryana DENKOVA[2], Velichka YANAKIEVA[2], Lyubka GEORGIEVA**

[1] *Department of "Biotechnology", Sofia University "St. Kliment Ohridski", Sofia, Bulgaria, rositsa_denkova@mail.bg*
[2]*Department of "Microbiology", University of food technologies, Plovdiv, Bulgaria, zdenkova@abv.bg*
* Corresponding author
Received August 21st 2013, accepted September 10th 2013

Abstract: *The application of probiotics in the composition of products intended to have long shelf life is limited due to their mandatory refrigeration storage. Increase in the resistance of probiotics to the production conditions and improvement of their viability throughout storage is achieved by microencapsulation. The preservation of probiotic strains for a long period of storage and low cost transportation and thus, resulting in ready-to-use cultures for dairy and other food-related industries including in the field of probiotics is facilitated by freeze-drying using appropriate cryoprotectant media. The used cryoprotectants are hydrocolloids which are natural, vegetable raw materials, with numerous beneficial physiological effects on the body. The aim of the present study is obtaining highly active probiotic freeze-dried bioproducts of selected probiotic strains by applying a combined modern biotechnology with the following main stages: selection, immobilization of the selected strains in the hydrocolloid matrix, cryoprotection, freezing and freeze-drying. The selected probiotic strains Lactobacillus plantarum X2 and Lactobacillus paracasei RN5 are immobilized and freeze-dried using combined hydrocolloid matrix - sodium alginate and high-ester pectin. The survival of the two lyophilized microorganisms after lyophilization and in the course of storage for nine months is monitored – both strains retain high concentrations of active cells. The used hydrocolloids are appropriate for the mechanical immobilization of cellular cultures and as cryoprotectants. The new combined biotechnology allows the preparation of probiotic bioproducts with long shelf life and intact probiotic potential for application as supplements for foods with dietary purpose and in the prophylaxis and treatment of gastrointestinal disorders.*

Keywords: *Probiotic, poly- and oligosaccharides, immobilization, cryoprotection, freezing, freeze-drying (lyophilization), Lactobacillus*

1. Introduction

When incorporating a probiotic strain into a food matrix there are two main problems to be addressed, namely the resistance of the strain to the technological conditions of food production [1] and the maintenance of cellular viability up to the expiring date of the food, since the manufacturer has to ensure an optimal administration of the probiotics throughout the whole shelf life of the product. That could lead to limitations of the use of probiotics in long-life products especially if they are not stored in a refrigerator. Freeze-drying is one of the most common methods used to

preserve probiotics. However, this method is not considered optimal, as it just protects probiotics from the humidity but does not confer protection from certain technological and environmental conditions such as varying temperatures, oxygen toxicity or passage through the intestinal tract. Decreased viability of the probiotics during or after freeze-drying is also a problem.

In this sense, microencapsulation, which is defined as a process in which the cells are retained within an encapsulating matrix or a membrane, may provide an approach for protecting probiotics. This technique would allow the isolation of probiotics from the environment thus increasing their resistance to the conditions of production and would also improve their viability throughout the storage. The protective effect of microencapsulation is due to the limited diffusion of inhibitory substances such as metabolic products from starter cultures, H_2O_2, lactic acid, and bacteriocins into the capsules [2, 3].

Freeze-drying of lactic acid bacteria (LAB) facilitates their preservation for a long period of storage, their low cost transportation resulting in ready-to-use cultures for dairy and other food-related industries, particularly in the emerging and continuously growing field of probiotics. In order to apply low rates of freezing, which are considered advantageous in industrial production of lactic acid starters because of their lower cost than high freezing rates (e.g. liquid nitrogen), suitable cryoprotective agents should be employed [4].

Various materials have been used for microencapsulation of probiotics, such as alginate [5], κ-carrageenan [6], cellulose acetate phthalate [7], gelatin [8] and pectin [2, 9].

The hydrocolloids used in the present study are sodium alginate and high-ester pectin. They act as matrices for the immobilization of the cell material and as cryoprotectants. They are natural, vegetable raw materials, with well known content of biologically active substances and beneficial physiological effects on the body [10, 11, 12].

Alginate is a linear heteropolysaccharide of D-mannuronic and L-guluronic acid extracted of various species of algae [13] which has interesting characteristics like its relative inexpensiveness, structural simplicity, and biocompatibility [3]. The use of alginate is limited due to its low physical stability in the presence of Ca^{+2}-chelating agents, monovalent ions and harsh environmental conditions [13]. Entrapment of probiotic bacteria in alginate matrices is among the most widely used immobisation techniques [14, 15]. This approach has been shown to enhance bacterial cell tolerance to alcohols, phenols, antibiotics or quaternary ammonium sanitizers [16] and resistance to adverse processing techniques such as freezing [17] and freeze-drying [18] and hostile environments such as simulated gastric environment [19]. Blending with other polymers such as starch [20] provides to some extent alginate gels with additional features and it has been reported as an effective method for the encapsulation of probiotics [3].

Pectins are high-molecular acid mucopolysaccharides composed of polygalacturonic acid in which some of the carboxyl groups are esterified with methyl alcohol residues. Several forms of pectin are produced – low-, medium- and high-ester pectin, depending on the methylation of the COOH-groups. When esterification affects less than 30% of the total number of carboxyl groups the pectin is called low-

ester pectin. When methylation concerns 30% to 60% of the -COOH-groups, the esterification is medium, and at esterification of 60% to 95.0% of the carboxyl groups the pectin is called high-ester pectin. Fruit pectins (citrus, apple) possess gelling ability, related to their high degree of esterification. The result of this activity is their sorption capacity, to which their hypolipidemic, detoxifying and radioprotective action are attributed. A number of dietetic foods like milk enriched with pectin, functional foods and herbal medicines, creams and others are produced in Bulgaria on the basis of pectin.

The main objective of the present study is to obtain highly active probiotic lyophilisates of selected strains of lactic acid bacteria by applying combined modern biotechnology.

2. Materials and Methods

Microorganisms:

Probiotic microorganisms: *Lactobacillus plantarum* X2 and *Lactobacillus paracasei* RN5, isolated from naturally fermented sourdough.

Media:

MRS-broth. Composition (g/dm^3): peptone from casein - 10, yeast extract - 4; meat extract - 8, glucose - 20; K_2HPO_4 - 2, sodium acetate - 5; diammonium citrate - 2; $MgSO_4$ - 0.2; $MnSO_4$ - 0.04; Tween 80 - 1 cm^3/dm^3; pH is adjusted to 6.5. Sterilization - 15 minutes at 118ºC.

MRS-agar. Composition (g/dm^3): MRS-broth + 2% agar. Sterilization - 15 minutes at 118ºC.

Saline solution. Composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

Hydrocolloid matrices:

pectin + sodium alginate / 1:1 / - 1.2% solution of sodium alginate, and 4% solution of pectin. The concentrations of the hydrocolloid solutions are determined on the basis of the physicochemical parameters of the hydrocolloids used, namely (Table 1):

Table 1
Physicochemical parameters of the hydrocolloids used

Total Moisture (%)	from 2.0 to 13.00%
Molecular weight – according to the certificate	from 5000 to 800 000
pH	from 3.0 to 8.0
Viscosity (mPa)	from 50.00 to 58.00
Dissolution temperature (°C)	Na – alginate - 18–25; Pectin /HEP/ – 20-22

The application of hydrocolloids of plant origin is in compliance with the requirements for physiological activity, safety and microbial stability [21].

Lyophilization

The new bioproducts are processed using a combined method of technological treatment that includes the following stages: primary processing of the cellular suspension, freezing and freeze-drying. In the primary processing the cellular suspensions of the selected *Lactobacillus* strains are diluted, equilibrated, dosed and immobilized by inclusion in the polymer matrix that acts as a cryoprotectant. These processes are performed before freezing.

A mechanical method for the immobilization of the microbial cells is applied. The immobilization includes the following processing steps: 1) Obtaining cellular suspensions by cultivation of the strains in MRS-broth for 24 hours at 37°C.

2) Mixing the obtained cellular suspensions with the prepared polymer solution at 40-45°C and homogenization for 1.5 hours in a bioreactor at 500 rpm.
Samples are pre-frozen in chambers with forced convection of the air environment at a temperature of -30°C to -35°C for 12-15 hours. The freeze-drying is performed in a vacuum sublimation installation "Hochvakuum-TG - 16.50" with contact heating of the plates in ICFT - Institute of Cryobiology and Food Technologies, Sofia, Bulgaria. The temperature regime for freeze-drying is programmed so as to obtain optimum drying speed that would ensure the good quality of the final products. The final drying off temperature ranges from +30°C to +35°C.
After lyophilization, the lyophilizates are digested in the granulator "Erveka". The digested lyophilized bioproducts are packed in three layer aluminum foil, sealed under vacuum.
A summary description of the results of the technological tests related to the whole cycle of freeze-drying of the specimens is given in Table 2.

Table 2

Parameters of freeze-drying

Parameters	**Unit**	**Value indicators**
I. Object of study	-	Cellular suspensions - lactobacilli and bifidobacteria in a cryoprotectant medium – high-ester apple pectin and sodium alginate
II. Layer thickness	mm	11
III. **Freezing**		
1.Temperature of freezing	°C	-30°C to – 35°C
2. Rate of freezing	°C/sec	Slow method - 0.05 - 0.06
3. Eutectic temperature	°C	– 37.00
4. Hardening temperature	°C	– 40.00
IV. Freeze-drying		
1. Load bearing surface	kg/m^2	10.60
2. Temperature of final drying off	°C	– 30.0
3. Temperature of the desublimer	°C	from - 55.0 to - 60.0 – before drying from - 65.0 to - 70.0 – in final drying off
4. Partial pressure	Pa	from 30.0 to 34.0
5. Chamber pressure	Pa	from 20.0 to 27.0
6. Temperature of final drying off	°C	+ 30
7. Maximum temperature of the product	°C	30
8. Residual moisture content of the product	%	from 1.37 to 3.09 /according to BS – no more than 6.0%/
9. Duration of the process	H	from 12 to 14
10. Storage conditions		In three layer bags of aluminum foil, sealed under vacuum, stored at a relative humidity less than 35% at 20°C – 22°C

Microbiology

1. The microbiological status of the native and the freeze-dried samples - acc. BS 1670-82 and Ordinance № 5 of the MH - SG 39/84, BS EN ISO 4833.

Indicators:

• lactic acid bacteria - CFU/g;
• total number of mesophilic aerobic and facultative anaerobic microorganisms - CFU/g;
• coliforms in 0.1 g of product;
• pathogens including *Salmonella* sp. in 25.0 g of product;
• pathogenic staphylococci in 1.0 g of product;

• sulfite reducing clostridia in 0.1 g of product;
• spores of microscopic molds, CFU/g;
• yeasts, CFU/g;

2. The number of viable cells is determined through appropriate serial dilutions of the samples and spread plating on coloured LAPTg10 – agar medium (to determine the number of viable lactobacilli cells) or on elective media (for the enumeration of the specific microorganisms).

3. The Petri dishes are incubated for 72 hours at 37±1°C until the appearance of countable single colonies. The count of the colonies is then used to estimate the number of bacteria in the original sample. Bacterial counts are transformed to log values.

4. The survival of lactobacilli in % is determined according to the following formula:

Survival, % = 100 - [(X_n / X_0) * 100], wherein:

X_n – the concentration of viable cells in the moment of reporting of the survival

X_0 – the concentration of viable cells before lyophilization.

3. Results and Discussion

The strains *Lactobacillus plantarum* X2 and *Lactobacillus paracasei* RN5, isolated from naturally fermented sourdough with proven probiotic properties [22, 23, 24, 25] are cultured in MRS-broth in a bioreactor with a volume of 2 dm^3 at constant stirring at 100 rpm at 37±1°C. Immobilization in a hydrocolloid matrix consisting of high-ester apple pectin and sodium alginate, followed by freeze-drying of the two strains is carried out. The resulting lyophilized bioproducts are stored at 20°C - 22°C and the changes in the concentration of viable cells of lactobacilli for nine months of storage is monitored (Fig. 1 and Fig. 2).

The examinations of the lyophilized bioproducts according to the standard methods show absence of insemination with pathogenic microflora (Table 3):

Table 3

Microbiological status of the probiotic lyophilisates

Type of pathogens	Norm according to BS	Number of tested microorganisms, CFU/g
1. General number of mesophilic aerobic microorganisms, CFU/g	No more than 800	310
2. Coliform bacteria in 0.l g of the product	Not to be found	Not found
3. Sulfite reducing clostridia in 0.l g of the product	Not to be found	Not found
4. *Salmonella* sp. in 25.0 g of the product	Not to be found	Not found
5. *Staphylococcus aureus* in 1.0 g of the product	Not to be found	Not found
6. Spores of microscopic molds, 100 CFU/g	No more than 100	35 – 40
7. Yeasts, CFU/g	No more than 100	32 – 48

No pathogenic microorganisms are found in the probiotic lyophilisates. The obtained lyophilizates meet the standard requirements for microbial purity of food. The absence of pathogenic insemination evidences that the overall process is carried out in compliance with the sanitary standards and requirements. The freeze-drying process has a bactericidal effect and does not lead to the development of conditions for insemination of the freeze-dried products. Moreover, the higher the degree of dehydration is, as in our bioproducts (residual moisture content -

below 5.0%), the lower the survival of the pathogenic microorganisms is.

Table 4

Survival of probiotic lactobacilli upon lyophilization (in %)

Strain	Before lyophilization	After lyophilization	Survival rate, %
Lactobacillus plantarum X2	$9.00x10^{13}$	$1.90x10^{13}$	95.26
Lactobacillus paracasei RN5	$6.00x10^{13}$	$1.60x10^{13}$	96.25

The results of the microbiological tests prove the high survival rates of the two strains after lyophilization - 95.26% for *Lactobacillus plantarum* X2 and 96.25% for *Lactobacillus paracasei* RN5 (Table 4). The results in Table 4 show a slight decrease of the number of viable cells in the process of freeze-drying, which is due to the optimally conducted process, including the use of a combined cryoprotectant hydrocolloid matrix. The observed decrease is by about 0.68 logN for *Lactobacillus plantarum* X2 and by 0.57 logN for *Lactobacillus paracasei* RN5.

The hydrocolloids used for the mechanical immobilisation of the probiotic strains of microorganisms, as well as as a cryoprotectant agent, sodium alginate and high-ester apple pectin, prove to be highly efficient, as evidenced by the high survival rate of the lyophilized microorganisms.

The probiotic lyophilized concentrates of viable cells of *Lactobacillus plantarum* X2 and *Lactobacillus paracasei* RN5, isolated from naturally fermented sourdough, are stored at 20°C - 22°C for 9 months, taking samples every three months and determining the number of viable cells. The results of this examination are shown on Fig. 1 for *Lactobacillus plantarum* X2 and Fig. 2 for *Lactobacillus paracasei* RN5.

Experimental data indicate that in the course of storage for nine months the concentrations of viable cells of the two probiotic concentrates are above 10^9CFU/g (Fig. 1 and Fig. 2).

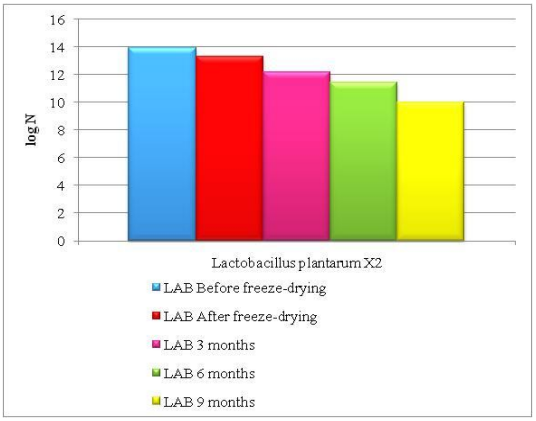

Figure 1. Survival of the cells of *Lactobacillus plantarum* X2 after lyophilization and during storage of the freeze-dried bioproduct for 9 months at 20°C - 22°C.

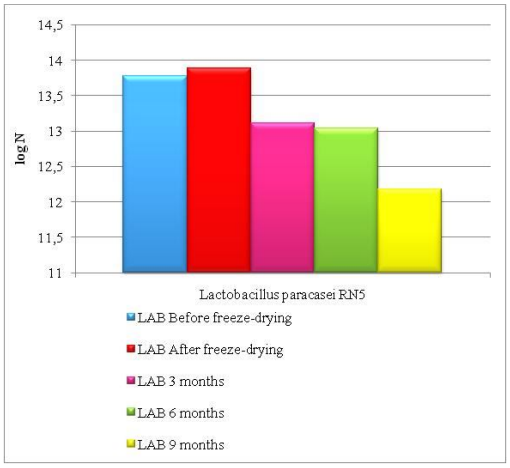

Figure 2. Survival of the cells of *Lactobacillus paracasei* RN5 after lyophilization and during storage of the freeze-dried bioproduct for 9 months at 20°C - 22°C.

According to Wolfson, 1999 [26] 10^9CFU/g is the required minimum concentration of cells of probiotic bacteria

in order for a concentrate to be defined as highly effective probiotic preparation.

The obtained results demonstrate that the main objective of our research is achieved - probiotic lyophilisates with high survival rate of the beneficial microflora after lyophilization are obtained. The number of viable cells before lyophilization is over 10^{13}CFU/cm^3 and after freeze-drying it remains over 10^{13}CFU/cm^3 i. e. the survival rate is high - 95.26% for *Lactobacillus plantarum* X2 and 96.25% for *Lactobacillus paracasei* RN5. This effect is due to the effectiveness of the hydrocolloids used as a cryoprotectant medium - a combined hydrocolloid matrix, consisting of sodium alginate and high-ester apple pectin.

A number of effects are attributed to the use of alginate and pectin as a hydrocolloid matrix for the immobilization of probiotic cultures: significant stabilization of the enzyme activities of the immobilized cells; stabilization and increase of the overall activity of the immobilized probiotic system, which is beneficial for their survival after lyophilization and during storage.

The high survival rate of the tested lactobacilli in the lyophilized biological products are due to the optimal conduction of the whole process - freezing conditions, applying suitable cryoprotectant medium and process parameters, proper determination of the duration of the cycle, which provides low residual moisture content in the final lyophilizates, resistance to the thermal processes and extension of their storage.

4. Conclusions

The survival and the viability of the probiotic lactobacilli bioconcentrates obtained through lyophilization are high – $1.60x10^{13}$ for *Lactobacillus paracasei* RN5 (survival percentage – 96.25%) and $1.90x10^{13}$ CFU/g for *Lactobacillus plantarum* X2 (survival percentage - 95.26%) - as a result of the applied technological approach. The combined hydrocolloid matrix of sodium alginate and high-ester apple pectin prove to be a highly effective cryoprotectant of endocellular type that increases the survival of lactobacilli during lyophilization. The absence of pathogenic microflora and the low residual moisture content of the new freeze-dried bioproducts determine the prolonged storage. During the monitored 9-month period no adverse changes in the microbiological safety and activity of the lyophilized probiotic strains are established.

5. References

[1] SAARELA M., MOGENSEN G., FONDÉN R., MÄTÖ J., MATTILA-SANDHOLM T., Probiotic bacteria: safety, functional and technological properties, *Journal of Biotechnology*, 84. 197 – 215, (2000).

[2] HEIDEBACH T., FÖRST P., KULOZIK U., Microencapsulation of probiotic cells for food applications, *Critical Reviews in Food Science and Nutrition*, 52. 291 – 311, (2012).

[3] MARTIN M. J., LARA-VILLOSLADA F., RUIZ M.A., MORALES M.E., Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT - Food Science and Technology*, 53. 480 – 486, (2013).

[4] CHAVARRI F. J., DEPAZ M., NUNEZ M., Cryoprotective agents for frozen concentrated starters from non-bitter *Streptococcus lactis* strains, *Biotechnology Letters*, 10(1). 11–16, (1988).

[5] MANDAL S., PUNIYA A. K., SINGH K., Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298, *International Dairy Journal*, 16. 1190–1195, (2006).

[6] ADHIKARI K., MUSTAPHA A., GRÜN I. U., Survival and metabolic activity of microencapsulated *Bifidobacterium longum* in

stirred yoghurt, *Journal of Food Science*, 68. 275–280, (2003).
[7] FÁVARO-TRINDADE C. S., GROSSO C. R. F., Microencapsulation of L. acidophilus (La-05) and B. lactis (Bb-12) and evaluation of their survival at the pH values of the stomach and in bile, Journal of Microencapsulation, 19. 485–494, (2002).
[8] ANNAN N. T., BORZA A. D., HANSEN L. T., Encapsulation in alginate-coated gelatin microspheres improves survival of the probiotic *Bifidobacterium adolescentis* 15703 T during exposure to simulated gastro-intestinal conditions, *Food Research International*, 41. 184–193, (2008).
[9] GEREZ C. L., FONT DE VALDEZ G., GIGANTE M. L., GROSSO C. R. F., Whey protein coating bead improves the survival of the probiotic *Lactobacillus rhammnous* CRL 1505 to low pH, *Letters in Applied Microbiology*, 54. 552–556, (2012).
[10] MURGOV I., DENKOVA Z., Probiotics on the threshold of the new millennium, *Journal "Milk"*, 27-29.35, (2001).
[11] IDRIS A., WAHIDIN S., Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized *Lactobacillus delbrueckii*, *Process Biochemistry*, 41(5): 1117-1123, (2006).
[12] GEORGIEVA L., Development of new cryobiotechnologies as a scientific basis for obtaining effective lyophilized foods for health, DSc Thesis, Institute of Cryobiology and Food Technologies, Sofia, Bulgaria, (2009).
[13] SMIDSRØD O., SKAJAK-BRAEK G., Alginate as immobilization matrix for cells, *Trends in Biotechnology*, 8. 71 – 78, (1990).
[14] CHAMPAGNE C.P., GAUDY C., PONCELET D., NEUFELD R.J., *Lactococcus lactis* release from calcium alginate beads, *Appl. Environ. Microbiol.*, 58. 1429–1434, (1992).
[15] KLINKENBERG G., LYSTAD K.Q., LEVINE D.W., DYRSET N., Cell release from alginate-immobilized *Lactococcus lactis* ssp. *lactis* in chitosan and alginate coated beads, *J. Dairy Sci.* 84. 1118–1127, (2001).
[16] LACROIX C., GRATTEPANCHE F., DOLEYRES Y., BERGMAIER D., Immobilised cell technologies for the dairy industry. In: NEDOVIC V., WILLAERT R. (Eds.), Applications of Cell Immobilisation Biotechnology, Springer, The Netherlands, pp. 295–321, (2005).
[17] SHEU T.Y., MARSHALL R.T., HEYMANN H., Improving survival of culture bacteria in frozen desserts by microentrapment, *J. Dairy Sci.* 76. 1902–1907, (1993).
[18] KEARNEY L., UPTON M., LOUGHLIN A.M.C., Enhancing the viability of *L.plantarum* inoculum by immobilizing the cells in calcium-alginate beads incorporating cryoprotectants, *Appl. Environ. Microbiol.*, 56. 3112–3116, (1990).
[19] CUI J.H., GOH J.S., KIM P.H., CHOI S.H., LEE B.J., Survival and stability of bifidobacteria loaded in alginate poly-l-lysine microparticles, *Int. J. Pharm.*, 210. 51–59, (2000).
[20] SULTANA K., GODWARD G., REYNOLDS N., ARUMUGASWAMY R., PEIRIS P., KAILASAPATHY, K., Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt, *International Journal of Food Microbiology*, 62. 47 – 55, (2000).
[21] WILLEMER H., Quality control during freeze-drying of biological products, 16th International Congress of refrigeration, Paris, Intrnational Institute of refrigeration, 78-83, (1983).
[22] DENKOVA R., GEORGIEVA L., DENKOVA Z., URSHEV Z., YANAKIEVA V., Biochemical and technological properties of *Lactobacillus plantarum* X2 from naturally fermented sourdough, *Journal of Food and Packaging Science Technique and Technologies*, Year I, №1, 2012, 59 – 64, (2012a).
[23] DENKOVA R., YANAKIEVA V., DENKOVA Z., ILIEVA S., PETKOVA A., Antimicrobial activity of *L.plantarum* strains isolated from different sources, Scientific conference with international participation "Food science, technique and technologies - 2012", 77 – 82, (2012b).
[24] DENKOVA R., ILIEVA S., NIKOLOVA D., EVSTATIEVA Y., DENKOVA Z., YORDANOVA M., YANAKIEVA V., Antimicrobial activity of *L.plantarum* X2 against pathogenic microorganisms, *Bulg. J. Agric. Sci.*, Supplement 2, 19. 108–111, (2013a).
[25] DENKOVA R., YANAKIEVA V., DENKOVA Z., GALENOVA E., Identification and survival in the model conditions of the gastrointestinal tract of the probiotic strain *L.casei* RN5, 23rd International Scientific Conference "Challenges for the scientists in relevance to the new program for science and innovations of EU "Horizon 2020" (in press), (2013b).
[26] WOLFSON, N. P., A probiotics primer, Nutrition Science News, 4(6). 276-280, (1999).