Technology for obtaining fermented products based on walnut milk

Kamelia Loginovska[1], *Alexandar* Valchkov[1 *], *Maria* Doneva[1], *Petya* Metodieva[1], *Svetla* Dyankova[1], *Daniela* Miteva[2], and *Iliana* Nacheva[1]

[1] Department of Cryobiology and Biologically Active Substances, Institute of Cryobiology and Food Technology, Agricultural Academy, Sofia, Bulgaria

[2] Department of Food Technology, Institute of Cryobiology and Food Technology, Agricultural Academy, Sofia, Bulgaria

Abstract. A technology has been developed for obtaining lyophilized fermented products based on walnut milk. Fermentation was carried out with two probiotic strains of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 and *Lactobacillus gasseri* NBIMCC 2450. In the capacity of prebiotic and cryoprotectant the composition of the products includes fructooligosaccharides (FOS) in three concentrations - 1, 2 and 4%. An increase in cell survival was found with an increase in the concentration of the fructo-oligosaccharides in the medium. After fermentation and lyophilization, *L. plantarum* showed better survival compared to *L. gasseri* in all samples examined. Probiotic cells fermented in medium with 4% FOS retain high viability after lyophilization - 93-96%, while those in medium with 2% FOS reach 85-93% survival. Fructooligosaccharides not absorbed by the cells at the end of the fermentation process render a cryoprotective effect and lead to a higher survival of the cells of both strains in the lyophilization process.

1 Introduction

Plant-based beverages are liquids obtained as a result of steeping, grinding and filtering processes, at which nutrients are extracted with water [1, 2, 3]. Fermentation of plant milks with selected probiotic bacteria significantly increases their health effect [4].

The growth and viability of probiotics in plant milk depends on its composition, fermentation process, type of probiotics, storage time, temperature, acidity, etc. [5, 6].

Lactiplantibacillus plantarum subsp. plantarum is one of the widely used probiotic microorganisms for fermentation and production of functional foods and beverages. The probiotic properties of *L. plantarum* may be a distinguishing feature for biocontrol against potentially harmful microorganisms during food processing and storage [7-11].

Lactobacillus gasseri is a homofermentative, thermophilic bacterium with probiotic properties that is found in the gastrointestinal tract, oral cavity and vaginal system. Studies related to determining the probiotic and technological properties of *L. gasseri* are still limited [12-14]. According to Gunyakti and Asan-Ozusaglam [12], *Lactobacillus gasseri* MA-4 has a high survival rate, owns good antimicrobial and antioxidant activity and aggregation ability.

Lactiplantibacillus plantarum subsp. plantarum and *Lactobacillus gasseri* are generally recognized as safe probiotic bacteria with GRAS status [7, 12]. Lin et al., [15] found that *L. plantarum* R9 and *L. gasseri* B1-27 strains could be isolated from human milk.

Fructooligosaccharides (FOS) are not hydrolyzed by the glycosidases of the small intestine and reach the cecum structurally unchanged. There they are metabolized by the intestinal microflora and form short-chain carboxylic acids, L-lactate, carbon dioxide, hydrogen and other metabolites. FOS are increasingly included in food products and formula milks because their prebiotic effect stimulates the growth of non-pathogenic intestinal microflora [16, 17]. There are data on the use of fructooligosaccharides in addition to the composition of fermented sour milk products and in the composition of fermented vegetable milks, most often soy-based [18-25].

Walnut milk represents an emulsified product in which the lipid content from the walnuts is dispersed in an aqueous phase, and the remaining components have a different effect on the stability of the product. Compared to cow's milk, which has about 3.2 g/100 mL of fat and 3.4 g/100 mL of protein, walnut milk is higher content in fat (18 g/100 mL) and lower content in protein (1.3 g/100 mL) [26-30].

Experiments have been conducted to obtain walnut milk-based probiotic drinks by fermentation of various types of lactic acid bacteria, including kefir with walnut milk [31-37].

* Corresponding author: aleksandar.valchkov@abv.bg

There is no scientific data to obtain lyophilized fermented walnut milk. Information on the use of *Lactobacillus gasseri* as a probiotic bacterium for walnut milk fermentation is also lacking.

The aim of the present study is to develop a technology for obtaining lyophilized fermented products based on walnut milk. Two fermentations will be carried out with *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 (ATCC 14917) and with *Lactobacillus gasseri* NBIMCC 2450 (ATCC 19992). As a prebiotic and cryoprotectant, fructooligosaccharides (FOS) will be included in the composition of the products in three concentrations - 1, 2 and 4%. The survival of the probiotic bacteria after fermentation and lyophilization of the two products, using different concentrations of FOS, will be tracked.

2 Materials and methods

2.1 Materials

- **Walnuts** - purchased from the commercial network in raw form.
- **Probiotic bacteria** – *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 strain [38, 39] and *Lactobacillus gasseri* NBIMCC 2450 [40, 41] strain provided by the National Bank for Industrial Microorganisms and Cell Cultures. *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 strain was isolated from sauerkraut (Denmark), and *Lactobacillus gasseri* NBIMCC 2450 strain was isolated from faeces, (Germany). Both strains were supplied in lyophilized form. MRS broth (De Man's medium, Rogosa & Sharpe, Oxoid), previously sterilized, was used to restore and activate the strains. The probiotic strains were cultured in a 37°C thermostat for 16-18 h. The technological suitability of the bacterial cultures after their incubation was determined by analyzing the morphology of the cells from the prepared microscopic preparations of the respective strains. To carry out the experimental part, the probiotic strains were reinoculated 3 times in MRS broth.
- **1, 2 and 4% fructooligosaccharides**.
- **2% fructose**.
- **4% glucose.**

2.2 Technological experiments

2.2.1 Pre-treatment of raw materials

The walnuts are peeled from their hard shells, then soaked in water at a ratio of 1:2 for a period of 12 h. The resulting mixture is mixed and ground to a uniform suspension, then filtered through a large filter - pore size of 160 μm. Natural walnut milk is obtained.

Figure 1 presents the technological scheme for the walnut milk, before fermentation. preparation of the starting raw materials for obtaining

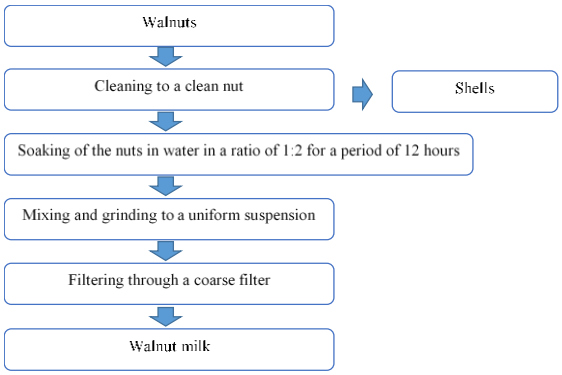

Fig. 1. Technological scheme for obtaining walnut milk, before fermentation

2.2.2 Preparation of inoculum for fermentation

Activated probiotic strains in an amount of 5-10% are cultivated in pre-sterilized MRS broth, statically at a temperature of 37°C for 16-18 h. After completion of the cultivation process, the technological status of the cultures was evaluated by determining the total number of viable cells (CFU/mL) and by microscopic analysis to study the morphology of the cells. The amount of inoculum added to the fermentation media was 1.6×10^6 CFU/mL.

2.2.3 Composition of fermentation media

Eight fermentation media were created with the following composition:

- **Fermentation medium 1 – control 1:** walnut milk, 2% fructose, 4% glucose, 10% inoculum of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447;
- **Fermentation medium 2:** walnut milk, 2% fructose, 4% glucose, 1% fructooligosaccharides, 10% inoculum of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447;
- **Fermentation medium 3:** walnut milk, 2% fructose, 4% glucose, 2% fructooligosaccharides, 10% inoculum of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447;
- **Fermentation medium 4:** walnut milk, 2% fructose, 4% glucose, 4% fructooligosaccharides, 10% inoculum of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447;
- **Fermentation medium 5 – control 2:** walnut milk, 2% fructose, 4% glucose, 10% inoculum of *Lactobacillus gasseri* NBIMCC 2450;
- **Fermentation medium 6:** walnut milk, 2% fructose, 4% glucose, 1% fructooligosaccharides, 10% inoculum of *Lactobacillus gasseri* NBIMCC 2450;
- **Fermentation medium 7:** walnut milk, 2% fructose, 4% glucose, 2% fructooligosaccharides, 10% inoculum of *Lactobacillus gasseri* NBIMCC 2450;

• **Fermentation medium 8:** walnut milk, 2% fructose, 4% glucose, 4% fructooligosaccharides, 10% inoculum of *Lactobacillus gasseri* NBIMCC 2450.

The active acidity (pH) of the media was adjusted with 20M NaOH to 6.8 - 6.9. Sterilization was performed in an autoclave at 121°C for 15 min, after which the media were cooled to 37°C and the inoculum was added to them. The fermentation process takes place at a temperature of 37°C for 16-18 h and until the pH of the culture media is not higher than 4.5.

2.2.4 Lyophilization of the obtained fermented walnut milks

Lyophilization takes place in three phases: **First phase** – freezing of fermentation media (1-8) with nut milk in a refrigeration plant to a temperature of -45°C; **Second phase** - sublimation (water removal at -22°/-25°C under deep vacuum); **Third phase** – desorption (further drying), by heating under deep vacuum, at positive temperatures - 25 - 28°C. The process lasted 48 h and was carried out in a laboratory sublimation installation of the company - "Hochvakuum-TG-16.50" with conductive heating of the plates and the residual pressure in the sublimator in the range of 1.33 - 13.33 Pa. The residual moisture content of lyophilized products reaches a maximum of 5%.

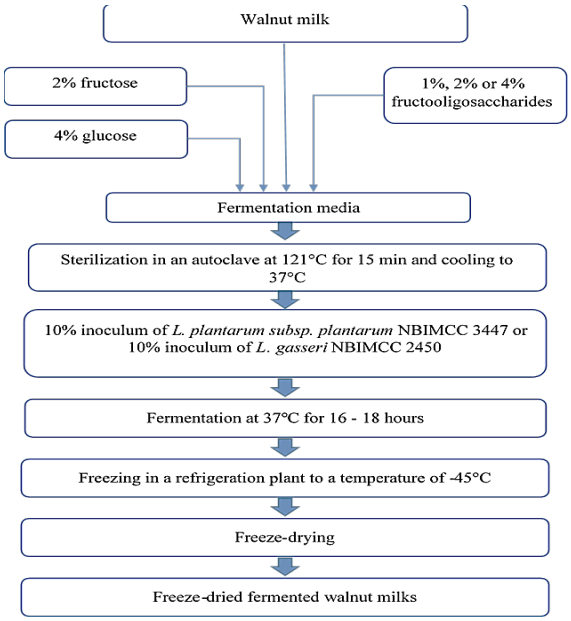

Fig. 2. Technological scheme for obtaining lyophilized fermented walnut milks with probiotic bacteria

As a result, three variants of lyophilized fermented walnut milk with *Lactobacillus gasseri* NBIMCC 2450 strain and 1, 2 and 4% FOS were obtained [42], three variants of lyophilized fermented walnut milk with *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 strain and 1, 2 and 4% FOS, one variant of lyophilized fermented walnut milk with *Lactobacillus gasseri* NBIMCC 2450 strain (control 1) and one variant of lyophilized fermented walnut milk with *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 strain (control 2).

Figure 2 presents the technological scheme for obtaining lyophilized fermented walnut milk.

2.3 Microbiological experiments

2.3.1 Determination of the total number of lactic acid bacteria (CFU/mL)

A determination of the total number of lactic acid bacteria in the eight fermentation media was performed after fermentation. Analysis was performed by counting colony-forming units per milliliter of strain-specific MRS agar medium. The method of falling decimal dilutions in saline was used. From them, a 1 mL sample was directly plated in MRS agar using the pour plating technique. Petri dishes were incubated for 72 h at 37°C. The number of lactic acid bacteria was calculated according to IDF standard 117 B:1991 by the formula:

$$N = \frac{\sum C}{V \times (n_1 + 0{,}1 \times n_2) \times d} \quad (1)$$

where: Σ C is the sum of all counted colonies in two successive dilutions, V – volume of inoculum, n1 is the number of petri dishes used for the first dilution, n2 is the number of petri dishes used for the second dilution and d is the dilution factor.

2.3.2 Survival of probiotic strains after lyophilization

The survival of the two probiotic strains after lyophilization in all investigated media was monitored. 1g of each lyophilized sample was rehydrated in 9 mL saline. The number of viable cells is accounted for through colony enumeration on MRS agar and recalculated to 1 mL of incubated material.

3 Results and discussion

Table 1 shows the number of viable cells of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 and *Lactobacillus gasseri* NBIMCC 2450 after fermentation in the culture media.

The data presented in Table 1 show that the addition of fructooligosaccharides to fermentation media stimulates the reproduction of probiotic bacteria. The viability of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 in the studied media was higher than that of *Lactobacillus gasseri* NBIMCC 2450. The highest number of viable cells was observed in the experimental samples with added 4% FOS for both strains, respectively for *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 - 9.5 logarithmic units, and for *Lactobacillus gasseri* NBIMCC 2450 - 9.0 LogN. The lowest survival of *L. plantarum* and *L. gasseri* was reported in the control media. A directly proportional dependency between the

amount of added fructooligosaccharides and the degree of cell growth of the two probiotic strains was observed.

Table 1. Survival of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 and *Lactobacillus gasseri* NBIMCC 2450 after fermentation (n = 24)

Variants of fermentation media	Survival after lyophilization			
	L. plantarum		*L. gasseri*	
	CFU/mL	LogN	CFU/mL	LogN
Fermentation media - controls	4×10^5	5.6	77	4.6×10^4
Fermentation media with 1% FOS	2.4×10^6	6.4	80	1×10^6
Fermentation media with 2% FOS	6.2×10^8	8.8	93	3.4×10^7
Fermentation media with 4% FOS	1.2×10^9	9.1	96	2.6×10^8

The difference in cell survival in control media and media with 1 and 2% FOS was from 0.7 to 2.2 logarithmic units, indicating that the selected concentrations of fructooligosaccharides had a beneficial and catalyzing effect on the reproductive process of both strains. In the media with 2 and 4% FOS, no significant difference in the number of viable cells was observed - for *L. plantarum* it was 0.1 LogN and for L. gasseri it was 0.2 LogN. When doubling the amount of FOS in the culture media (from 2 to 4%), no significant increase in the number of probiotic cells of the two bacteria was found.

Table 2 presents the survival data of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 and *Lactobacillus gasseri* NBIMCC 2450 after lyophilization of the studied samples.

Table 2. Survival of *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 and *Lactobacillus gasseri* NBIMCC 2450 after lyophilization (n=24)

Variants of fermentation media	Survival after fermentation			
	L. plantarum		*L. gasseri*	
	CFU/mL	LogN	CFU/mL	LogN
Fermentation media - controls	1.4×10^7	7.2	1.1×10^7	7.0
Fermentation media with 1% FOS	1×10^8	8.0	5×10^7	7.7
Fermentation media with 2% FOS	2.8×10^9	9.4	6.4×10^8	8.8
Fermentation media with 4% FOS	3.2×10^9	9.5	1×10^9	9.0

The lyophilization process for both strains in all fermentation media was successful. The difference in the percentage of surviving cells at *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 is the largest between the media with 1 and 2% FOS – 13%, and the lowest is between the experimental samples with 2 and 4% FOS, where it reaches 3%. For *Lactobacillus gasseri* NBIMCC 2450, with each increase in the amount of FOS, cell survival increased uniformly by 8-10%. The highest percentage of surviving cells after lyophilization in both strains was accounted in the culture media with 4% FOS. In all variants of fermentation media with added fructooligosaccharides, values of the number of surviving cells after lyophilization were found higher than 1 x 10^6 CFU/mL, which is a requirement of the World Health Organization for a probiotic product.

Both after fermentation and after lyophilization, *L. plantarum* showed better survival than *L. gasseri*. This is due to the fact that L. plantarum ferments to over 90% most of the carbohydrate sources, including mannitol and ribose. It is more resistant to pH changes and endures values between 4 and 9. The growth temperature range of this probiotic bacterium is also greater than that of *L. gasseri*, varying between 15 and 45°C. Thus, *L. plantarum* shows higher resistance than *L. gasseri*, both during and after fermentation, so and after lyophilization.

The input percentage of fructooligosaccharides in the fermentation media remains practically undigested by the cells in the fermentation process and during lyophilization the cryoprotective properties are stronger expressed, as a result of which we have a higher cell survival of both strains.

4 Conclusion

A technology has been developed for obtaining lyophilized products based on walnut milk fermented with two probiotic strains - *Lactiplantibacillus plantarum subsp. plantarum* NBIMCC 3447 and *Lactobacillus gasseri* NBIMCC 2450. As a prebiotic and cryoprotectant, fructooligosaccharides are included in three concentrations - 1, 2 and 4%. It was found that the importation of FOS to the culture media positively affected the fermentation of the probiotic strains. Probiotic cells that have undergone fermentation in medium with 4% FOS retain high viability after lyophilization - 93-96%, while those in medium with 2% FOS reach 85-93% survival. Fructo-oligosaccharides not digested by the bacteria at the end of the fermentation process have a cryoprotective effect and lead to a higher survival of cells from both strains in the lyophilization process.

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