

## Survivability of *Lb. Acidophilus* and *Bif. Bifidum* after Enzymatic Treatment and Lyophilization

R. NIKOLOVA<sup>1</sup>, Hr. CHOMAKOV<sup>2</sup> and Tz. TZVETKOV<sup>1</sup>

<sup>1</sup> Institute of Cryobiology and Food Technologies, BG – 1407 Sofia, Bulgaria

<sup>2</sup> University of Chemical Technology and Metallurgy, BG – 1000 Sofia, Bulgaria

### Abstract

NIKOLOVA, R., Hr. CHOMAKOV and Tz. TZVETKOV, 2007. Survivability of *Lb. acidophilus* and *Bif. bifidum* after enzymatic treatment and lyophilization. *Bulg. J. Agric. Sci.*, 13: 387-392

The objective of the present study is to trace the survivability of two strains of bacteria in a range of media, simulating the conditions of human gastrointestinal tract.

The strains used in this study are *Lactobacillus acidophilus* and *Bifidobacterium bifidum*.

Experiments have been conducted by treating the bacterial cells at pH 2 and pH 7 – 7.2.

Test *in-vitro* has been performed with strains treated with digestive enzymes such as pepsin and pancreatin. Cultivation has been carried out at temperature 37 °C for 90 minutes in thermostat.

Results of the test “in vitro” show 94 % resistance of cells as more resistant proved to be the strain of *Bifidobacterium bifidum*.

After lyophilization, both strains appeared to have high survivability that is measured to be 100% for *Bifidobacterium bifidum* after treatment.

**Key words:** *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, enzymatic treatment, lyophilization, probiotics

### Introduction

Probiotics, manufactured under industrial conditions and available on the market, have been selected on the basis of resistance to acids and gallbladder and survived after lyophilization. One of the preliminary requirements for probiotic ac-

tion is survivability in the intestinal tract.

A main indicator among the developed mass screening of all species within the *Lb. acidophilus* group is resistance to high acidity in stomach and bile acids in intestines (Saito, 2004).

Cells of *Lb. acidophilus* have on their surface S-layer proteins, the importance

has not been still clarified but it is considered that these cells have an essential role in their metabolism (Upreti et al., 2003).

Strains of *Lb. acidophilus*, producing bacteriocins as lactacin B (Barefoot et al., 1988), lactacin F (Muriana et al., 1991) and acidocin (Kanatan et al., 1992), have been isolated.

These characteristics of *Lb. acidophilus* make it especially suitable for the production of probiotic foods. Furthermore, it has high inhibitory activity against various pathogenic bacteria, present in food products such as *Listeria monocytogenes*, *Bac. cereus* and *Staph. aureus* (Itoh et al., 1995).

Recently a series of experiments have been conducted for the preparation of white brined cheese in Turkey and its transformation into a probiotic product (Kasimoglu et al., 2004).

In the process of investigating the survivability of *Lb. acidophilus* and *Lb. bulgaricus* in the gastrointestinal tract while incubating in the gastric juice, it has been confirmed that 50 % of the bacterial cells of *Lb. acidophilus* are able to survive at pH 3, 8 for 60 minutes (Floch et al., 1970).

Another group of microorganisms, actively taking part in the microbiota in the gastrointestinal tract, belong to the group of *Bifidobacterium*. Bifidobacteria inhabit the colon in man through the whole life cycle and are closely related to the overall healthy status. These bacteria are different from the lactic acid bacteria because of their metabolism and production not only of lactate but acetate as well as a main fermentable product (Stules et al., 1997). Bifidobacteria are associated with some probiotic effects, including antimicrobial action against pathogenic bacteria, reduce the risk of colon cancer, im-

mune modeling and maintain the balance in the intestinal microbiota. The investigations by adults (Mitsuoka et al., 1972; Duclouxcan, 1993) and children (Langhendries et al., 1995; Hudau et al., 1996) confirm that some strains of bifidobacteria are able to survive while passing through the gastrointestinal tract. The investigations in vitro have proved better survivability of bifidobacteria than the lactic acid bacteria (Havenar et al., 1994). Strain differences, related to the ability of bifidobacteria to survive by presence of acid and bile acids, have been also observed (Ihah et al., 1995). This makes the survivability very important indicator in the selection of probiotics (Kok et al., 1996; Kullen et al., 1997).

Based on the basic differences, regarding the ability of survivability of various strains, used as probiotics, we have decided to determine the survivability of a strain of *Lb. acidophilus* and one of *Bifidobacterium bifidum* in acid and alkaline media together with the presence of pepsin and pancreatin; such conditions are similar to these existing in the gastrointestinal tract in man. Another objective was to trace the survivability of the same strains after lyophilization.

## Materials and Methods

### Materials

- Strain *Lactobacillus acidophilus* NBIMCC 2448 = CNRZ 204.
- Strain *Bifidobacterium bifidum* – NBIMCC 1370 = CHR Hansen's Laboratorium A/S Denmark. The strain samples have been lyophilized, delivered by NBIMCC and maintained at the laboratory at ICFT.
- Sterile restored milk, obtained from 80 % skimmed dry milk, dissolved in 11

distilled cool water, sterilized at 121°C and pressure 1 atmosphere for 10 minutes.

Restored milk has been inoculated with both strains separated from each other. To 100 ml sterile fresh milk was added 4g of the strain sample in the lyophilized culture. It has been cultivated at 37 °C. After 6 hours the coagulated milk has to be cooled and kept in the fridge at 4 °C until the next day or about 16 hours.

#### **Methods and Equipment**

- A number of living cells – in a liquid growth medium (restored 80 % of the sterile skimmed milk) according to the method of limited dilutions. Their number is measured before and after lyophilization.

- Active acidity (pH – value) of pH parameter “Seibold G -103”.

- Microscopic picture – by means of coloured preparations in methylene blue, prepared from coagulated milks, obtained during the determination of the total number living cells.

- Calculating the survivability of the bacteria at different pH values after lyophilization.

#### **Experiments**

- Determination of pH- value and the number of living cells in the control sample of coagulated and cooled milk.

- Simulating conditions of gastrointestinal tract through pH – value change by adding sterile 1 N HCl to pH 2 and adding, while constantly mixing, pepsin suspension 7000 UI Sigma (dissolved 4 g dry pepsin in 100 ml distilled water). To 100 ml coagulated milk is added 50 ml pepsin suspension.

- Cultivation of the culture at 37 °C for 90 minutes. When the time is over, the number of the living cells is determined.

Simulating the conditions in duodenum through pH – value change by adding sterile 1 NaOH to pH 7 -7.2. By constantly

mixing is added pancreatin solution 1750 UI Sigma (dissolved 0.5 g dry pancreatin in 250 ml distilled water). To 100 ml coagulated milk is added 250 ml pancreatin solution.

- Cultivation at 37 °C for 90 minutes in thermostat, after that the number of all bacterial living cells is determined.

- The rest of the coagulated milk (after treating the samples with pH 7) has been sublimation dried by means of the installation Hochvakuum TG 16.50. The installation was prepared through cooling the plates minimum to -35 °C. Desublimator is cooled to minimum -60 °C that provides pressure sufficiently low partial pressure of water vapours over the product and in that way suitable conditions for mass delivery are provided. Correct determination of temperature and duration of drying provide the necessary residual humidity of the product.

#### **Results and Discussion**

During the cultivation of both strains, the inoculated sterile milk coagulates for 6 hours. On the following day, 16 hours after coagulation, pH value is 4, 5 for both strains.

Data for the total number of living cells during the treatment of *Lb. acidophilus* are presented on Table 1.

It is apparent from the Table 1 the initial number of living cells of *Lb. acidophilus* is lg 8, 40. Treatment conducted at pH 2 has negative influence on the survivability of cells. Data show a decrease in the number of cells with approximately one lg. When treated at pH 7, the number of living cells has been restored to its initial number that indicates destructive changes in the structure and enzymatic repertoire of the investigated strain of *Lb. acidophilus*.

**Table 1**  
**Number of living cells of *Lb. acidophilus* during treatment with different pH values**

Type of sample	Number of living cells lg CFU/ml		
	Before lyophilization	After lyophilization	Survivability during lyophilization
Untreated strain	8.4	7.88	94
After treatment at pH 2	7.18	ND	ND
After treatment at pH 7	8.65	7.98	92

“ND” – number of living cells not determined and not lyophilized

**Table 2**  
**Number of living cells of *Bifidobacterium bifidum* during treatment with different pH values**

Type of sample	Number of living cells lg CFU/ml		
	Before lyophilization	After lyophilization	Survivability during lyophilization
Untreated strain	7.4	6.98	94
After treatment at pH 2	6.65	ND	ND
After treatment at pH 7	6.4	6.4	100

“ND” – number of living cells not determined and not lyophilized

Data in Table 1 show that after lyophilization at pH 7 the survivability of the untreated strain is 94 % and 92 % of the treated one. The results obtained clearly indicate high survivability of the investigated strain during lyophilization. Similar results were established by other authors (Nacheva, 2003).

The microscopic picture of the un-

treated strain and treated one, as well as after lyophilization, is illustrated by thick well coloured rods, some of them are prolonged and twisted in shape.

Results about the total number of the living cells of the strain *Bifidobacterium bifidum* during treatment are presented in Table 2.

Data in Table 2 indicate the number of

**Table 3**  
**Results of the experiment *in vitro* for both strains**

Type of sample	Number of living cells lg CFU / ml	
	<i>Lb. Acidophilus</i>	<i>Bifidobacterim bifidum</i>
Untreated strain	8.4	8.65
After treatment at pH 2 and pepsin	7.65	8.17
After treatment at pH 7 and pancreatin	7.4	8.17

the living cells of the untreated strain is lg 7, 40. During treatment t pH 2 and pH 7 – 7.2 the viability of the cells has been decreasing with 10 %.

Data show the cells of the investigated strain of *Bif. bifidum* are more resistant at pH 2 compared to the cells of *Lb. acidophilus*. Higher survivability is also confirmed during lyophilization – for the treated strain at pH 7 - 100 %, as for the untreated one - 94 %. Results, referring to the high survivability of *Bifidobacterium bifidum* during lyophilization have been also proved by other authors (Havenar et al., 1994).

As a result of the investigation conducted, it has been established that the strain of *Bifidobacterium bifidum* has high survivability during treatment and lyophilization. This makes it especially a valuable probiotic strain.

In the microscopic picture it can be clearly observed that neither the untreated nor the treated and lyophilized strain show different morphological characteristics.

Probiotic characteristics of the strains are related to their ability to be developed at various pH values and their resistance to digestive enzymes that give them the

advantage while passing through the barriers of gastrointestinal tract in man.

With this purpose we conducted experiment “in vitro” – model system for investigating the resistance of probiotic strains in conditions, simulating these existing in gastrointestinal tract.

Results obtained are shown in Table 3.

Data in Table 3 show high survivability during the treatment of both strains. The number of living cells of *Lb. acidophilus* before treatment is lg 8, 40. During its cultivation at pH 2 with added pepsin, the number is reduced to lg 7, 65 with survivability of 91 %. Treatment with pH 7 and added pancreatin reduces the survivability with 12 % compared to the initial number.

The initial number of cells of *Bif. bifidum* is lg 8, 65. During treatment at pH 2 and added pepsin the strain shows better resistance – the number of living cells is lg 8, 17 with survivability of 94 %. It makes strong impression that the survivability of the same strain during cultivation at pH 7 and added pancreatin, where the number of the living cells and their survivability remain unchanged.

Data, obtained from the investigation

conducted, confirm the investigated strains *Lb. acidophilus* and *Bif. bifidum* are characterized with high survivability during treatment with digestive enzymes at pH 2 and pH 7. More resistant proved to be the strain of *Bif. bifidum*. These characteristics make the strains valuable for the preparation of probiotic foods.

On the basis of the investigation conducted the following conclusions might be outlined.

### Conclusions

During treatment of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* at pH 2 and pH 7 for 90 minutes, at 37 °C, the survivability of the cells is 90 %.

During lyophilization of the strain of *Lb. acidophilus* the untreated strains that survive are 94 %, and after treatment at pH 7 - 92%.

During lyophilization of the strain of *Bifidobacterium bifidum* the untreated strains that survive 94 %, and the treated strains at pH 7 - 100%.

The investigated strain of *Bifidobacterium bifidum* shows higher survivability at pH 7 compared to the strain of *Lactobacillus acidophilus*.

The investigated strains of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* show high resistance "in vitro" in the conditions of gastrointestinal tract.

High survivability of the investigated strains of *Lb. acidophilus* and *Bif. bifidum* at different pH values, during lyophilization as well as under the action of digestive enzymes, make these strains particularly valuable as probiotics.

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Received March, 1, 2007; accepted May, 10, 2007.