# Investigation of probiotic properties of *Lactobacillus helveticus* 2/20 isolated from rose blossom of *Rosa damascena* Mill.

*Rositsa* Denkova-Kostova[1*], *Bogdan* Goranov[2], *Teodora* Tomova[2], *Velichka* Yanakieva[2], Denica Blazheva[2], *Zapryana* Denkova[1], and *Georgi* Kostov[3]

[1] Department of Biochemistry and Molecular Biology, Technological Faculty, University of Food Technologies, 4002, Plovdiv, Bulgaria
[2] Department of Microbiology, Technological Faculty, University of Food Technologies, 4002, Plovdiv, Bulgaria
[3] Department of Technology of Wine and Beer, Technological Faculty, University of Food Technologies, 4002, Plovdiv, Bulgaria

**Abstract.** A *Lactobacillus* strain was isolated from rose blossom of *Rosa damascena* Mill. and it was identified as belonging to the species *Lactobacillus helveticus* by the application of physiological-biochemical (API 50 CHL) and molecular-genetic methods (sequencing of the 16S rRNA gene). The presence of a number of probiotic properties of *L. helveticus* 2/20 was investigated. The strain exhibited high antimicrobial activity against pathogenic microorganisms that cause food toxicoinfections and intoxications. *L. helveticus* 2/20 survived in the simulated conditions of the gastrointestinal tract – pH = 2 and pepsin, pH = 4.5 and pancreatin and pH = 8 and pancreatin, as well as in the presence of up to 0.3% bile salts, retaining a significant concentration of viable cells. It has been shown that *L. helveticus* 2/20 cells begin multiplying after removing the extreme conditions. The strain allowed bioreactor cultivation and freeze-drying of the obtained concentrates, with the concentration of active cells in the lyophilic preparations exceeding $10^{12}$ cfu/g. The kinetic parameters of the batch cultivation process in a bioreactor with stirring and the maximum growth rate were determined, revealing the possibilities for scaling up of the fermentation process from laboratory to industrial conditions, as well as its management. After further research on the probiotic properties of *L. helveticus* 2/20, it can be included in the composition of probiotics and functional foods.

## 1 Introduction

The isolation of new strains with probiotic potential and the development of new foods for the needs of specific consumer groups is a major direction in nutrition science. Lactic acid bacteria and bifidobacteria are the main bacterial species in the production of probiotics and are part of the beneficial intestinal microflora. They are traditional cultures in the production of fermented foods [1 - 3]. Some strains of the genera *Lactobacillus*, *Bifidobacterium* and some representatives of *Propionibacterium* sp. are included in the composition of probiotics and foods due to their health beneficial effects [4 - 5]. Probiotic microorganisms contribute to the restoration of the intestinal balance, play an important role in maintaining health and improve the quality of some foods in which they are included, due to the organic acids (lactic acid, acetic acid, propionic acid), hydrogen peroxide and bacteriocins they produce [6 - 8]. The impact of probiotic bacteria is similar to that of normal intestinal microflora: insurance of colonization resistance; regulation of the gas composition and the redox potential of the intestines; regulation of the water-salt exchange; participation in detoxification processes; protection of the mucous membrane of the intestinal tract; strengthening the immune system - formation of a specific and non-specific immune response of the body. For this effect to occur, the ingested bacterial cells must be viable, and their number must be in the range of $10^6$ - $10^9$ cfu/g at the end of the product shelf life for a positive effect to be observed [9].

The strains of lactic acid bacteria belonging to the genera *Lactobacillus*, *Lactococcus* and *Bifidobacterium* have GRAS status (generally recognized as safe) and are safe for use [10]. EFSA has developed its own safety criteria with QPS status. According to FAO/WHO, newly isolated lactobacilli strains must be identified to species level by their phenotypic and genotypic characteristics, and a functional characterization of their probiotic properties must be made: to be harmless or apathogenic to the host, to be resistant to the simulative conditions of the gastrointestinal tract (low pH and bile juice), to adhere to the intestinal mucosa and colonize it, to produce organic acids and bacteriocins, to demonstrate antagonism to pathogenic and toxigenic microflora, to be suitable for clinical and nutritional use, to multiply rapidly and accumulate a high concentration of viable cells, to allow industrial fermentation and freeze-drying, to be able to be stored for a long time and to recover their activity quickly [11 - 19].

The widespread use of antibiotics and other antimicrobial agents has led to multiple resistance of pathogenic microorganisms [20]. This posed the problem

* Corresponding author: rositsa_denkova@uft-plovdiv.bg

of the rational therapy of infectious conditions and led to searching for new antimicrobial agents. One of the current trends is the selection of lactobacilli with probiotic potential [21 - 23].

It is known that as a result of the hydrolysis of milk protein under the action of the proteolytic systems of the probiotic strains, a wide range of bioactive peptides is formed [24]. Probiotic strains of *L. helveticus – L. helveticus* R 211 and *L. helveticus* R 389 that hydrolyze β and κ-casein to produce peptides that suppress ACE and lower blood pressure in humans, have been isolated [25]. Biopeptides produced by *L. helveticus* during the hydrolysis of milk protein exhibit immunostimulating, antimicrobial, mineral binding, antihypertensive, antimutagenic and anticarcinogenic activities [12, 25 - 34]. The uptake of *L. helveticus* increases the amount of secreted IgA in the bronchi and intestines [26, 28, 33, 35 - 36].

The aim of the present work was to examine the probiotic potential of a *Lactobacillus helveticus* strain isolated from the rose blossom of *Rosa damascena* Mill. for application in the production of probiotics and functional foods.

## 2 Materials and methods

### 2.1 Microorganisms

The research in the present work was carried out with *Lactobacillus helveticus* 2/20, isolated from rose blossom of *Rosa damascena* Mill. and identified to the species level by biochemical and molecular-genetic methods [54]. The pathogenic test-microorganisms used in the antimicrobial activity assay were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella abony* NTCC 6017, *Proteus vulgaris* J, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212.

### 2.2 Nutrient media

a) LAPTg10 - agar. Composition (g/dm$^3$): peptone – 15; yeast extract – 10; tryptone – 10; glucose – 10; agar - 15. pH was adjusted to 6.6 - 6.8 and Tween 80 - 1cm$^3$/dm$^3$ was added. Sterilization - 20 min at 121°C.

b) LBG agar. Composition (g/dm$^3$): tryptone – 10, yeast extract - 5, NaCl - 10, glucose – 10, agar - 20. pH is adjusted to 7.5. Sterilization - 20 min at 121°C.

c) MRS broth (Merck).

d) Sterile reconstituted skim milk powder with titratable acidity 16 ± 18°T. Composition (g/dm$^3$): skimmed milk powder (Scharlau). Sterilization - 15 min at 118°C.

### 2.3 Determination of the titratable acidity

The titratable acidity was determined by titration method according to standard protocol [37].

### 2.4 Determination of the number of viable cells in the freeze-dried preparation

The number of viable cells was determined by preparing appropriate ten-fold dilutions and spread plating on LAPTg10 agar. The inoculated Petri dishes were incubated in a thermostat for 48 h to 72 h at 37 ± 1°C until the appearance of countable single colonies.

### 2.5 Determination of biomass concentration

The biomass concentration in the experiments simulating the different departments of the gastro-intestinal tract (Determination of tolerance to bile salts; Determination of survival at low pH in the presence of pepsin and at alkaline pH in the presence of pancreatin) was measured on a micro-plate reader Spectrostar Nano (BMG LABTECH, Germany) against MRS broth as blank at λ = 600 nm. The sample volume was 200 µl with correction to 1 cm path length.

### 2.6 Determination of tolerance to bile salts

Fresh 24-h culture of *Lactobacillus helveticus* 2/20 was centrifuged for 15 min at 5000g. The obtained biomass pellet was washed twice with PBS-buffer and resuspended to the original volume in PBS-buffer. Samples of 750 µl MRS broth with 0.6% dry bile (Wako, Japan) were diluted in deep-well plates by making serial twofold dilutions until a concentration of 0.08% dry bile was reached. Each well was inoculated with 50 µl active bacterial culture and the trays were cultivated at 37°C. The optical density at λ = 600 nm was determined after 24 h.

### 2.7 Determination of survival at low pH in the presence of pepsin and at alkaline pH in the presence of pancreatin [38]

Fresh 24-h culture of *Lactobacillus helveticus* 2/20 was centrifuged for 15 min at 5000g. The resulting biomass precipitate was washed twice with PBS buffer and resuspended to the original volume in PBS buffer. 0.2 cm$^3$ of the cell suspension was incubated with 5 cm$^3$ of pH = 2 buffer solution containing 0.5% NaCl and pepsin (3.2 g/dm$^3$) (Sigma, 2,500 - 3,500 U/mg protein), buffer solution with pH = 4.5 and pancreatin; and buffer solution with pH = 7 and pancreatin at 37 ± 1°C for 24 h. Aliquots to determine the biomass concentration (optical density units) were taken at 0$^{th}$, 2$^{nd}$, 4$^{th}$ and 24$^{th}$ h.

### 2.8 Determination of the antibiotic susceptibility profile

Laboratory susceptibility testing was performed using the agar-diffusion method with paper discs by [39]. Fresh 24-h culture of *Lactobacillus helveticus* 2/20 was used to inoculate by spread plating plates with MRS-agar. Standard 6 mm paper disks soaked in different antibiotics were placed in the plates. The plates were incubated for 48 h at 37 ± 1°C. The diameter (in mm) of the inhibition zones formed around each of the antibiotic discs was recorded. The following designations were used for the

interpretation of the results: R - resistant (the inhibition zone being smaller than 8 mm), SR - intermediately sensitive (the inhibition zone being between 8 mm and 16 mm), S - sensitive (the inhibition zone being larger than 16 mm).

### 2.9 Determination of the antimicrobial activity – agar well diffusion method

Culture fluid (CF), biomass (B) and neutralized acellular supernatant (NASN) (pH 6.5) obtained from a 24-h culture of *Lactobacillus helveticus* 2/20 (concentration of viable cells – $10^{11}$ - $10^{12}$ cfu/cm$^3$) were used to determine the antimicrobial activity of *Lactobacillus helveticus* 2/20. The antimicrobial activity was tested against the following test microorganisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella abony* NTCC 6017, *Proteus vulgaris* J, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212.

Each of the test pathogenic microorganisms (suspension concentration of $10^7$ cfu/cm$^3$) was inoculated by spread plating into agar plates and wells (7 mm in diameter) were prepared after agar solidification. 0.1 cm$^3$ of CF, B and NASN were pipetted into the wells and allowed to diffuse into the agar for 1 hour at 4 ± 2°C.

The Petri dishes were then incubated at 37 ± 1°C for 24 h, after which the inhibition zones around the wells in mm were recorded.

### 2.10 Batch cultivation

Cultivation of *Lactobacillus helveticus* 2/20 was carried out in a laboratory bioreactor with a geometric volume of 2 dm$^3$ and a working volume of 1.5 dm$^3$. Management and monitoring of the main fermentation parameters was done by a Sartorius A2 controller, which included control loops for stirring rate, temperature, pH, etc.

Cultivation of *Lactobacillus helveticus* 2/20 was carried out in the following order: the apparatus was washed, then filled with 0.3% neomycin solution for cold sterilization for 24 h. After the sterilization time has ended, the device was washed several times with sterile physiological solution, after which it was ready to carry out the fermentation process.

The nutrient medium was loaded into the apparatus sterilely, using a peristaltic pump.

The process of batch cultivation was carried out in skimmed milk. The milk was sterilized at 121℃ for 20 min. After cooling to 39 - 40℃, the prepared medium in the bioreactor was inoculated with 5% (v/v) inoculum of a fresh 24-h culture of *Lactobacillus helveticus* 2/20 in MRS broth medium.

The cultivation process was carried out at 37℃, stirring rate of 150 rpm, without aeration.

The duration of the cultivation was 24 h, with periodic sampling of the culture fluid for analysis of total viable cell count of *Lactobacillus helveticus* 2/20 (cfu/cm$^3$) and titratable acidity.

### 2.11 Identification of the model parameters [40]

The kinetics of the process was described by the equation of the logistic curve:

$$\frac{dX}{d\tau} = \mu_m - \beta X \; X \qquad X = \frac{X_{in} e^{\mu_{max}(\tau - \tau_{in})}}{1 - \frac{X_{in}}{X_F}(1 - e^{\mu_{max}(\tau - \tau_{in})})} \qquad (1)$$

where:
$\mu_{max}$ - specific growth rate, h$^{-1}$;
X - concentration of biomass, cfu/cm$^3$;
$X_{in}$ - initial biomass concentration, cfu/cm$^3$;
$X_F$ - final concentration of biomass cfu/cm$^3$;
β - coefficient of internal population competition, cfu/cm$^3$h.

The kinetic parameters of the model were determined by analogy with [40, 41], after linearization of the equation under the condition that Δt = const:

$$\psi = 1 - \frac{X_t}{X_{\tau+\Delta\tau}} = 1 - \left(1 - \frac{X}{X_F}\right) \exp\ -\mu_m \Delta\tau \qquad (2)$$

where:
ψ - relative change of the microbial concentration;
Δt - time to change the microbial concentration from $X_t$ to $X_{t+\Delta t}$.

### 2.12 Freeze-drying

The freeze-dried concentrate of *Lactobacillus helveticus* 2/20 was obtained by a combined method for technological treatment - immobilization and freeze-drying, in the following stages of the process: primary processing, freezing and freeze-drying.

Primary processing involved dilution of the *Lactobacillus helveticus* 2/20 cultural suspension, obtained by batch cultivation, equilibration, dosing, and immobilization of the cell suspension by retention (inclusion) in the respective polymer matrix that served as a cryoprotective medium as well.

The immobilization matrix used was hydrocolloid and contained a 4% solution of highly esterified apple pectin and a 1.2% solution of sodium alginate in a ratio of 1:1. The cellular connection to the carrier was carried out by mixing the cell suspension of *Lactobacillus helveticus* 2/20 with the carrier (hydrocolloid matrix) at a temperature of 40 - 45℃ and homogenizing for 1 h and 30 min in a bioreactor with a stirring rate of 500 rpm.

The material thus prepared was subjected to freeze-drying, which combines two methods of preservation - freezing and drying under high vacuum. The samples were pre-frozen in chambers with forced air convection at a temperature of -30 to -35℃ for 12 - 15 h.

Freeze-drying was carried out under vacuum conditions in a sublimation installation "Hochvakuum-TG - 16.50" with contact heating of the plates in ICFT - Institute of Cryobiology and Food Technology – Sofia. The process parameters were as described in Table 1.

### 2.13 Processing of the results

Data from triplicate experiments were processed using Microsoft Office Excel 2013 software, using statistical functions to determine the standard deviation and maximum estimation error at significance levels of $p < 0.05$.

**Table 1.** Parameters of the freeze-drying process of the *Lactobacillus helveticus* 2/20 cell suspension

| Parameters | Unit | Parameter value |
|---|---|---|
| I. Object of research | - | Lactobacilli cellular suspensions with cryoprotective medium - pectin and sodium alginate |
| II. Layer thickness | Mm | 11 |
| III. Freezing | | |
| 1. Freezing temperature | °C | -30°C to -35°C |
| 2. Freezing rate | °C/sec | Slow method - 0.05 - 0.06 |
| 3. Eutectic temperature | °C | - 37 |
| 4. Curing temperature | °C | - 40 |
| IV. Freeze-drying | | |
| 1. Load on the bearing surface | kg/m$^2$ | 10.60 |
| 2. Drying temperature | °C | - 30 |
| 3. Temperature in the desublimator | °C | from - 55 to - 60 – before drying<br>from - 65 to - 70 – in final drying |
| 4. Partial pressure | Pa | from 30 to 34 |
| 5. Chamber pressure | Pa | from 20 to 27 |
| 6. Final drying temperature | °C | + 30 |
| 7. Maximum product temperature | °C | + 30 |
| 8. Residual moisture content | % | from 1.37 to 3.09 /according to BSS – no more than 6.0%/ |
| 9. Process duration | H | from 12 to 14 |
| 10. Storage conditions | | In envelopes of three-layer aluminum foil, closed under vacuum; stored at relative humidity no more than 35% at 20 - 22°C |

## 3 Results and discussion

### 3.1 Antimicrobial activity of *Lactobacillus helveticus* 2/20 against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella abony* NTCC 6017, *Proteus vulgaris* J, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212

In a series of experiments, the antimicrobial activity of *Lactobacillus helveticus* 2/20 against pathogenic microorganisms by the agar-diffusion method with wells was investigated (Table 2).

The biomass (B) was used to determine the inhibitory effect of the *Lactobacillus helveticus* 2/20 cells on the test microorganisms; acellular supernatant (ASN) without pH correction (with acidic pH) was used in order to determine the inhibitory effect of the lactic acid and other organic acids synthesized and accumulated in the medium by *Lactobacillus helveticus* 2/20 on the growth of the test-pathogenic microorganisms. In parallel, the activity of the acellular supernatant (NASN) neutralized to pH = 6.5 was recorded in order to eliminate the inhibitory effect of lactic acid and other organic acids produced by the *Lactobacillus helveticus* 2/20 and to qualitatively examine the ability of the studied lactobacilli strain to produce other substances with antimicrobial activity to suppress pathogenic microorganisms.

*Lactobacillus helveticus* 2/20 had pronounced antimicrobial activity against the pathogenic bacteria *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella abony* NTCC 6017, *Proteus vulgaris* J, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212. The observed high biomass activity indicated the presence of substances exhibiting inhibitory activity associated with the lactobacilli cell wall. The secretion of these substances with antimicrobial action probably occurred 24 hours after the start of the experiment. When neutralizing the organic acids synthesized by *Lactobacillus helveticus* 2/20 and accumulated in the medium, no antimicrobial activity against the growth of pathogenic bacteria that cause of food toxic infections and intoxications was found (Table 2). The observed dependencies are in compliance with the studies of Kaboosi et al. [42], Gerbaldo et al. [43].

**Table 2.** Antimicrobial activity of *Lactobacillus helveticus* 2/20 against pathogenic bacteria
$d_{well}$=7mm. "-" - no suppression of the growth of the pathogenic microorganisms by *Lactobacillus helveticus* 2/20 was found

| $d_{inhibition\ zones}$, mm / Sample | *Escherichia coli* ATCC 25922 | *Staphylococcus aureus* ATCC 25093 | *Salmonella abony* NTCC 6017 | *Proteus vulgaris* J | *Listeria monocytogenes* ATCC 19115 | *Enterococcus faecalis* ATCC 29212 |
|---|---|---|---|---|---|---|
| | $6 \times 10^9$ cfu/cm$^3$ | $1.7 \times 10^{12}$ cfu/cm$^3$ | $2 \times 10^{12}$ cfu/cm$^3$ | $1.5 \times 10^{12}$ cfu/cm$^3$ | $4 \times 10^{12}$ cfu/cm$^3$ | $4.2 \times 10^{12}$ cfu/cm$^3$ |
| CL | 13.33 ± 0.47 | 18.67 ± 0.47 | 13.17 ± 0.24 | 15.33 ± 0.47 | 19.50 ± 0.41 | 19.17 ± 0.24 |
| B | 9.33 ± 0.47 | 10.17 ± 0.24 | 8.33 ± 0.47 | 10.00 ± 0.00 | 12.17 ± 0.24 | 12.67 ± 0.47 |
| ASC | 10.17 ± 0.24 | - | 9.17 ± 0.24 | 9.17 ± 0.24 | 14.00 ± 0.82 | 11.33 ± 0.47 |
| NASN | - | - | - | - | - | - |

### 3.2 Antibiotic resistance of *Lactobacillus helveticus* 2/20

Knowledge of the antibiotic resistance of lactobacilli with probiotic potential is essential. On the one hand, this can be considered a significant criterion in the selection of probiotic cultures, due to the possibility of carrying out combined therapy with an antibiotic and a probiotic, with the aim of restoring the normal microflora of the gastrointestinal tract and/or the uro-genital tract [44]. On the other hand, a number of authors state the hypothesis that bacteria normally found in the body, including lactobacilli, can serve as a source of antibiotic resistance genes, transferring them to various pathogenic microorganisms [45]. Therefore, one of the conditions for the selection of potentially probiotic strains is knowledge of their antibiotic sensitivity.

Nineteen antibiotics with a different mechanism of action from the main antibiotic groups used in medical practice was selected and the sensitivity of *Lactobacillus helveticus* 2/20 to them was tested (Table 3). *Lactobacillus helveticus* 2/20 was resistant to the majority of antibiotics included in the present study, but it was sensitive to antibiotics affecting cell wall synthesis (penicillin, bacitracin and piperacillin). The antibiotic resistance spectrum data should be carefully analyzed when selecting probiotic strains due to the possibility of transfer of genetic elements conferring resistance [45]. The presence of acquired antibiotic resistance factors is considered highly undesirable [46].

In certain cases when using probiotics together with antibiotics, probiotic strains with a wider spectrum of resistance than their natural ones should not be selected [45]. In this regard, it is recommended that lactobacilli be screened for potentially transferable resistance to chloramphenicol, clindamycin, gentamicin, rifampin, tetracycline [46].

**Table 3.** Antibiotic resistance profile of *Lactobacillus helveticus* 2/20

| | Mechanism of action | Antibiotic | | Concentration | 2/20 |
|---|---|---|---|---|---|
| 1 | Inhibitors of the cell wall synthesis | Penicillin | P | 10 E/disc | S |
| 2 | | Bacitracin | Cm | 0.07 E/disc | S |
| 3 | | Piperacillin | P | 100 µg/disc | S |
| 4 | | Ampicillin | A | 10 µg/disc | R |
| 5 | | Oxacillin | O | 1 µg/disc | R |
| 6 | | Amoxicillin | Ax | 25 µg/disc | R |
| 7 | | Vancomycin | V | 30 µg/disc | S |
| 8 | Inhibitors of protein synthesis | Tetracycline | T | 30 µg/disc | R |
| 9 | | Doxycycline | D | 30 µg/disc | SR |
| 10 | | Gentamicin | G | 10 µg/disc | R |
| 11 | | Tobramycin | Tb | 10 µg/disc | SR |
| 12 | | Amikacin | Am | 30 µg/disc | R |
| 13 | | Lincomycin | L | 15 µg/disc | R |
| 14 | | Chloramphenicol | C | 30 µg/disc | R |
| 15 | | Erythromycin | E | 15 µg/disc | S |
| 16 | Inhibitors of the DNA synthesis and/or cell division | Novobiocin | Nb | 5 µg/disc | R |
| 17 | | Nalidixic acid | Nx | 30 µg/disc | R |
| 18 | | Rifampin | R | 5 µg/disc | R |
| 19 | | Ciprofloxacin | Cp | 5 µg/disc | R |
| d < 8 mm – R; < d < 16 mm – SR; d > 16 mm – S. | | | | | |

### 3.3 *In vitro* determination of the ability of *Lactobacillus helveticus* 2/20 to survive in conditions simulating the different departments of the gastrointestinal tract

#### *3.3.1 Resistance of Lactobacillus helveticus 2/20 cells to different pH values and enzymes*

One of the main criteria considered when selecting potential probiotic strains to ensure their viability and functionality when taken orally is to be tolerant to the acidic pH in the stomach, which can reach pH = 1.5 [4]. Pepsin has a pH optimum of action at pH = 1 - 2. It can attack peptide components in the cell wall. This affects cells in the logarithmic growth phase. Cells in the stationary growth phase are resistant to its action and retain their viability. At neutral pH values both in the buffer and in the nutrient medium, its action ceases. In the presence of nutrients the microbial cells grow and multiply and as a result their concentration in the medium increases.

*Lactobacillus helveticus* 2/20 was most sensitive to pH = 2 + pepsin. The concentration of active cells at pH = 2 + pepsin increased up to the $6^{th}$ h from the start of the process and was maintained until the $24^{th}$ h (Fig. 1). The optical density of the medium changed by 0.4 - 0.6 optical density units. At pH = 4.5 + pancreatin the growth inhibition of *Lactobacillus helveticus* 2/20 was observed by the $4^{th}$ h and a slight reduction by the $24^{th}$ h was determined. At pH = 8 + pancreatin, the same trend was observed, i.e. inhibition of the growth of *Lactobacillus helveticus* 2/20 by the $4^{th}$ h and a slight reduction by the $24^{th}$ h. The optical density of the medium changed by less than 0.1 optical density units (Fig. 1).

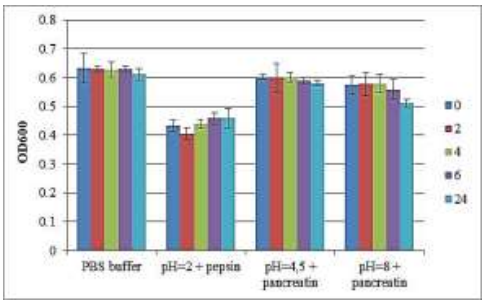


**Fig. 1.** Cell viability of *Lactobacillus helveticus* 2/20 in the conditions of acidic pH (pH = 2) + pepsin, pH = 4.5 + pancreatin and pH = 8 + pancreatin

After the 24th h, the survivors at the different pH values (2 + pepsin, at pH = 4.5 + pancreatin and at pH = 8 + pancreatin) were transferred to fresh MRS broth medium and cultured for 24 h at a temperature of 37°C. The results of these studies are presented in Fig. 2 and show that the surviving cells in the access of nutrients do grow and multiply. This is evidenced by the change in the optical density of the medium from 0.367 to 5.831 optical density units (Fig. 2).

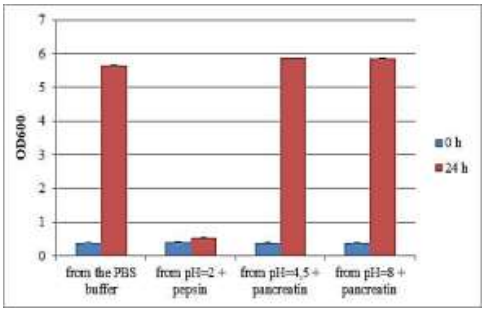


**Fig. 2.** Restoration of cell viability of *Lactobacillus helveticus* 2/20 cells after removal of the simulated gastrointestinal tract conditions

*Lactobacillus helveticus* 2/20 maintained high concentrations of viable cells in the artificially simulated gastrointestinal tract.

### *3.3.2 Survival of Lactobacillus helveticus 2/20 at different concentrations of bile salts*

Bile salts are composed of the salts of 4 types of bile acids (cholic, deoxycholic, chenodeoxycholic and lithocholic acids) and each of them can bind to glycine or form more complex salts. Bile salts or acids can be synthesized from cholesterol or extracted from the blood. After a meal, bile flows into the duodenum. It consists of 97% water, 0.7% bile salts, 0.2% bilirubin, 0.51% lipids (cholesterol, fatty acids and lecithin) and 200 meqv/l inorganic salts [34, 47]. Bile salts are a significant factor influencing probiotic strains in the intestinal tract. It is known that about three hours after taking the food, the concentration of bile salts in the small intestine reaches about 0.3%, and the retention of food in the small intestine is about 4 h [47]. This necessitated a study of the influence of different concentrations of bile salts on the growth of the studied strain in a liquid nutrient medium at different concentrations of bile salts.

The experimental data show that in the first hours of culturing of *Lactobacillus helveticus* 2/20 cells in the presence of 0%, 0.08%, 0.15%, 0.30% and 0.60% bile salts, the concentration of viable cells started increasing immediately after the introduction of the culture into the nutrient medium (Fig. 3). The growth continued until the 24th h, when the optical density of the medium was measured to be in the range of 0.021 to 5.765 optical density units (Fig. 3).

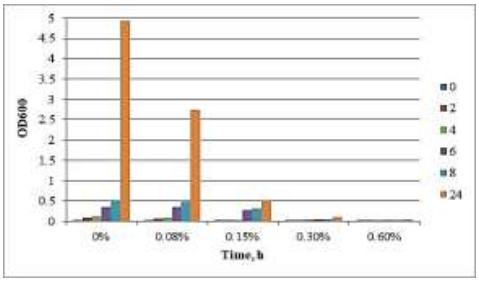


**Fig. 3.** Cell viability of *Lactobacillus helveticus* 2/20 at different concentrations of bile salts in the medium

*Lactobacillus helveticus* 2/20 survived in the presence of bile salts, retaining a significant amount of viable cells. In the human gastrointestinal tract, however, such extreme conditions are rarely created. In the presence of nutrients, the growth of the naturally resistant cells of *Lactobacillus helveticus* 2/20 will be observed.

The cell is an open dynamic system and conditions for bile salts to enter the cytoplasm are created. The cytoplasmic membrane is composed of phospholipids containing fatty acids, which upon interaction with bile salts form inclusion compounds (mostly with a crystal lattice), which only under certain conditions release the inclusion compound. This in turn disrupts the permeability of the cytoplasmic membrane and the transport of nutrients from the outside to the inside and from the inside to the environment. Young growing cells are especially sensitive. Cells in the stationary growth phase survive this stress and form colonies on the surface of the medium. Lower concentrations of bile salts in the medium allow the surviving cells to grow, and higher concentrations (0.6%) arrest its growth. The strain was affected by the presence of bile salts in the culture medium, and the degree of reduction in the concentration of viable cells depended on the concentration of bile salts in the medium.

### 3.4 Batch cultivation of *Lactobacillus helveticus* 2/20 in a laboratory bioreactor with mechanical stirring in skim milk medium. Determination of the process kinetics and identification of the model parameters

The possibility of obtaining a probiotic concentrate of active cells of *Lactobacillus helveticus* 2/20 by cultivation in reconstituted skim milk powder, was investigated. The

batch cultivation of the studied strain with correction and maintenance of pH = 5.7 was carried out in a laboratory bioreactor with mechanical stirring. The dynamics of biomass accumulation during the process were monitored (Fig. 4).

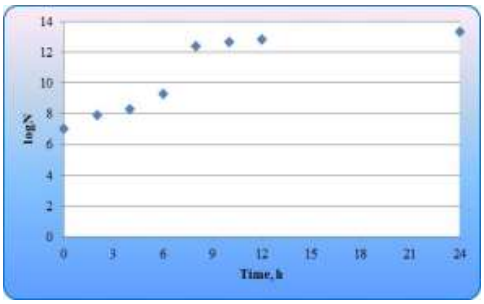


**Fig. 4.** Dynamics of biomass change during cultivation of *Lactobacillus helveticus* 2/20

The lag phase was short and difficult to determine. Therefore, according to eq. (2), the induction period (the time from the lag phase, during which the cells start to synthesize the necessary cellular structures and enzymes and pass from an unadopted state to an adapted state to the composition of the medium and the cultivation conditions), as well as the rate constant of adaptation $k_0$, have been determined. According to the model, the induction period ($\tau_0$) was 1.11 h, and the adaptation rate constant ($k_0$) was relatively high – 0.461 $h^{-1}$. The values of these parameters indicate that the strain quickly and easily adapted to milk, which is essential in the selection of strains included in the composition of probiotic starter cultures. From the 2nd to the 8th h, the culture was in exponential growth phase, and from the 8th to the 12th h, the strain entered the slow growth phase, in which there was a gradual increase in the concentration of active cells. The stationary growth phase occurred at the 12th h from the beginning of the process, when a high concentration of viable cells of $7.0.10^{12}$ cfu/$cm^3$ was achieved. From the 12th to the 24th h the concentration of viable cells increased slightly and was about $2.1.10^{13}$ cfu/$cm^3$.

Based on the conducted experiments, mathematical modeling of the growth kinetics was done and the maximum growth rate $\mu_m$ and the coefficient of intra-population competition, which are important bioprocess parameters of the culture, were determined. A comparison between the experimental data and the data obtained from the models was made. The results of these studies are presented in Fig. 5, and the values of the kinetic parameters are shown in Table 4.

**Table 4.** Kinetic parameters of the batch process of *Lactobacillus helveticus* 2/20

| μ | β | $X_{кр}$, | $R^2$ | e |
|---|---|---|---|---|
| $h^{-1}$ | $cm^3$/(cfu.h) | LogN | - | - |
| 0.103 | 0.016 | 14.02 | 0.9950 | 0.802 |

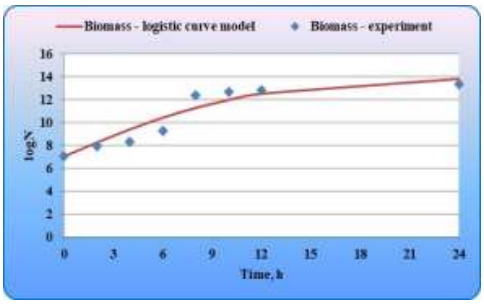


**Fig. 5.** Comparison of the experimental data with the model of the logistic curve for biomass accumulation during cultivation of *Lactobacillus helveticus* 2/20 at 40 ± 1°C in a bioreactor with mechanical stirring

The models agreed very well with the experimental results (Fig. 5). This was also confirmed by the high correlation coefficient and the low value of the identification error ($R^2$ and e) (Table 3). In addition, the model gave a theoretical value of the maximum biomass concentration of 14.03 LogN, which was close to the experimental one - 13.32 LogN. This shows that the derived mathematical model adequately described the process in the studied time interval and the identified kinetic parameters in it can be used for the scaling up of the fermentation process from laboratory to industrial conditions as well as for its management. *Lactobacillus helveticus* 2/20 was characterized by a relatively high maximum specific growth rate - 0.103 $h^{-1}$ and a low intra-population competition coefficient - 0.016 $cm^3$/(cfu.h). The values of these parameters in turn indicate that the conditions for the growth of this strain were optimal.

Fig. 6 shows the changes in the pH and the redox potential of the system. The pH value dropped rapidly from 6.38 at the beginning of the process to 5.7 at the 8th h, which once again testified to the rapid growth of the strain, even as a monoculture (Fig. 6).

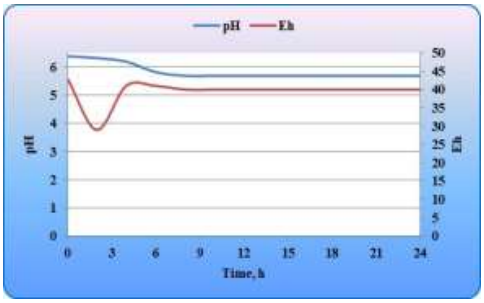


**Fig. 6.** Changes in the oxidation-reduction potential and the pH during cultivation of *Lactobacillus helveticus* 2/20 at 40 ± 1°C in a bioreactor with mechanical stirring

The pH value was then kept constant until the end of the fermentation process. The redox potential decreased from 43 mV at the 0th h to 29 mV at the 2nd h. Then there was an increase to 41 mV, which value was maintained from the 4th to the 6th h. At the start of the pH correction, the oxidation-reduction potential was 40 mV and it

remained constant until the end of the fermentation process.

### 3.5 Freeze-drying

The freeze-drying method is appropriate when the moisture content of the raw materials exceeds 30%. The most common food products contain significant amounts of free water (from 70% to 90%) for different types of products.

Like all preservation methods, freeze drying (lyophilization) requires the products to be processed to be of high quality. The pre-treatment of industrially valuable strains of lactic acid bacteria or mixed cultures is especially necessary. The pre-treatment aims to create conditions that guarantee maximum survival of bacterial cultures during freeze-drying. The vitality of the bacterial cultures in the freeze-dried preparation is determined by the ability of the bacterial cells to restore their original biochemical properties upon rehydration. For lactic acid bacteria, these properties include fermentative, proteolytic, aroma-forming, antimicrobial and other activities. When the microorganisms are stored in a dry state, they are in the so-called anabiosis. In this condition, it is assumed that their inherent biochemical processes are greatly slowed down, close to the cessation of all life processes. The survival of different types of microorganisms subjected to freeze-drying is different.

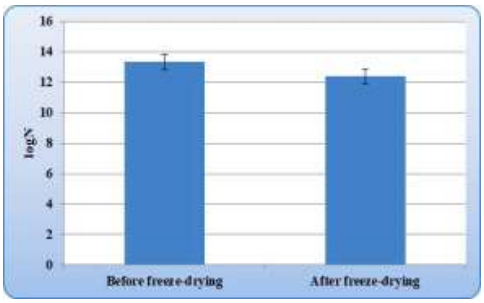


**Fig. 7.** Cell viability of *Lactobacillus helveticus* 2/20 in the freeze-drying process

The research done by Nikolov et al. [47] on the viability of lactic acid bacteria and in particular the microflora of traditional Bulgarian yogurt, shows that the survival of the studied and selected strains of *Lactobacillus* sp. during freeze-drying is also influenced by the growth stage at the time of drying. The survival of bacterial cells also depends on the environment in which they are located during the drying process. A number of substances contained in nutrient media for the cultivation of microorganisms have a protective effect and preserve the bacterial cells during drying. The concentration of bacterial cells also affects their survival in freeze-drying.

According to Nacheva et al. [48], the optimal concentration of the bacterial suspension for each specific microorganism is determined experimentally. According to Morichi [49], Zaŕate and Nader-Macias [50] at a concentration of lactic acid bacteria cultures of $10^{10}$ cfu/cm$^3$ and higher, greater survival was observed. Of essential importance for the survival of microorganisms is the way of applying heat to the cultures subjected to freeze-drying, with conductive heating, the survival of lactic acid streptococci is high, and of *L. bulgaricus*, which is very sensitive to freeze-drying, is low [48, 52-54]. After completion of the fermentation process, the biomass was immobilized in a hydrocolloid matrix. After freezing to a temperature of -40°C, it underwent freeze-drying. The microbiological status of the obtained lyophilizate was determined (Table 5 and Fig. 7).

**Table 5.** Microbiological status of the freeze-dried preparation of *Lactobacillus helveticus* 2/20

| Parameter | Norm according to BSS | Analysis result |
|---|---|---|
| 1. Total number of mesophilic aerobic and facultatively anaerobic bacteria | No more than 800 | < 10 |
| 2. *Escherichia coli* in 0.1 g of the product | Not to be found | Not found |
| 3. Sulphite-reducing clostridia in 0.1 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. Coagulase positive staphylococci in 1.0 g of the product | Not to be found | Not found |
| 6. Spores of microscopic fungi, CFU/g | No more than 100 | Not found |
| 7. Yeasts, CFU/g | No more than 100 | Not found |

The concentration of active cells in the dry product after freeze-drying was determined to be $2.5.10^{12}$ cfu/g and the survival rate was 93.02% (Fig. 7 and Table 5). The high survival rate indicated that the strain is xerotolerant and was little affected by the freeze-drying conditions. The resistance of the strain to the damaging factors of freeze-drying makes it valuable for production purposes, since not all lactobacilli show such high survival and resistance in production processes.

## 4 Conclusion

*Lactobacillus helveticus* 2/20 exhibited high antimicrobial activity against the pathogenic bacteria *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella abony* NTCC 6017, *Proteus vulgaris* J, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 29212. It was resistant to the majority of the most common antibiotics applied in clinical practice, which in turn opens up the possibility of its joint inclusion in complex therapy in the composition of probiotics. *Lactobacillus helveticus* 2/20 survived in the model conditions of the gastrointestinal tract, maintaining high concentration of viable cells (over $10^{13}$ cfu/g).

A batch fermentation process was carried out with *Lactobacillus helveticus* 2/20 and the kinetic parameters of the fermentation process were determined in order to obtain a concentrate with high content of viable cells ($10^{13}$ cfu/cm$^3$). These studies are necessary for the scaling-up and management of the fermentation process in industrial settings.

With the selected cryoprotective medium and the immobilization of the microbial cells, a freeze-dried preparation with high content of viable cells (over $10^{12}$ cfu/g) was obtained.

After examination of the other probiotic properties of *Lactobacillus helveticus* 2/20, it can be included in the composition of probiotics and probiotic foods.

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