The Combination of In Vitro Assessment of Stress Tolerance Ability, Autoaggregation, and Vitamin B-Producing Ability for New Probiotic Strain Introduction

Natalya Yu. Khromova, Julia M. Epishkina, Boris A. Karetkin *, Natalia V. Khabibulina, Andrey V. Beloded ©, Irina V. Shakir and Victor I. Panfilov

Abstract: The health benefits of probiotics are beyond doubt. The positive effects of lactobacilli and bifidobacteria on the function of many body systems have been repeatedly proven by various studies. To completely realize the potential of probiotic microorganisms, the strains should be tested by the greatest combination of characteristics that contribute to the wellness of the host. In this work, for the first time, a combined assessment of the probiotic properties and vitamin B-producing potential of various species and strains of bifidobacteria and lactobacilli was carried out. The presence of an additional advantage, such as vitamin-producing ability, can prevent vitamin deficiency both at the level of the consumption of fermented foods, when the enrichment will occur naturally on the spot, and during colonization by these intestinal strains, when synthesis will occur in vivo. To select potential probiotics, the stress tolerance ability of 16 lactic acid bacteria and bifidobacteria strains to low pH values, bile, and proteolytic enzymes, as well as their ability to autoaggregate, were studied under conditions of modeling the gastrointestinal tract in vitro. The ability of the strains to extracellularly accumulate water-soluble B vitamins was evaluated by capillary electrophoresis. Among the tested strains of bifidobacteria, B. adolescentis VKPM AC-1662 is of interest; it was characterized by the greatest stress tolerance ability and the ability to autoaggregate, in addition to the extracellular synthesis of riboflavin and pyridoxine. Among lactic acid bacteria, L. sakei VKPM B-8936 demonstrated the greatest tolerance to low pH, L. plantarum VKPM B–11007 to duodenal conditions, L. acidophilus VKPM B-2213 to pepsin, and L. salivarius VKPM B–2214 to pancreatin. The highest percentage of autoaggregation was observed in L. salivarius VKPM B–2214, which also accumulated the largest amount of pantothenic acid, but it was sensitive to stress conditions. The obtained results could be used to create new products enriched with probiotics and B vitamins.

Keywords: probiotics; Bifidobacterium; Lactobacillus; probiotic properties; water-soluble B vitamins; biofortification; stress tolerance ability; aggregation ability

1. Introduction

Fortification of food products with chemically synthesized vitamins is a traditional way of increasing their biological value in order to prevent vitamin deficiency, which can have negative effects on the development and functioning of the body. A lack of B vitamins in humans can lead to various diseases. Folic acid deficiency can lead to osteoporosis, coronary heart disease, decreased cognitive function and Alzheimer’s disease, hearing loss [1], and an increased risk of breast cancer [2]. Riboflavin deficiency can lead to skin and liver damages, as well as changes in glucose metabolism in the brain [3]. Thiamine deficiency causes beriberi disease, which can have consequences for both the cardiovascular and the nervous system. Niacin deficiency can lead to pellagra, lack of pantothenic acid to acne, pyridoxine deficiency to dermatitis and epileptic seizures, and a
lack of biotin can cause growth retardation in children [4]. Recently, the efficacy of synthetic vitamins has been in doubt and so they need to be analyzed. It is reported that their bioavailability is worse than that of natural forms of vitamins, which means they might not be as effective [5]. Also, some studies show that chemically synthesized vitamins can even have a negative effect on human health. For example, high consumption of foods enriched with chemically synthesized folic acid, not the natural form, in some people can lead to masking vitamin B12 deficiency, leukemia, arthritis, and an increased risk of cancer [6]. At the same time, synthetic vitamins are quite expensive due to the large number of stages of production. Finally, consumer requirements for food quality are increasing and preference is given to a healthy diet, so much attention is paid to the absence of synthetic additives in the composition.

Biofortification by fermentation of lactic acid bacteria and bifidobacteria with vitamin-producing ability is a promising approach for obtaining foods with increased concentrations of natural forms of B vitamins. Most members of the Lactobacillus and Bifidobacterium are normal representatives of the human intestinal microbiota and are generally recognized as safe (GRAS), which makes them the most suitable candidates for these purposes. Some authors insist that careful selection of starter cultures with increased vitamin B-synthesizing ability can enhance the concentration of folic acid in yogurts over 200 µg L$^{-1}$ [7–9] or beverages, and thereby lead to the development of innovative functional products [10]. Such foods will not only be healthy, but also economically beneficial. The strains of lactic acid bacteria (LAB) selected according to the specified criteria have already been used as starters for bread and pasta production enriched with riboflavin, which leads to an increase in this vitamin concentration of about 2–3-fold in the final product [11]. It was shown in model rats with folic acid deficiency that yogurt fermented with the vitamin-producing strain L. plantarum GSLP-7 V restores the disturbed microbiota and increases levels of serum folate and homocysteine, while folic acid aggravated intestinal dysbiosis [12]. Moreover, vitamin production is considered a criterion in the functionality assessment in some studies—for example, on isolation of new LAB from various sources such as vegetable raw materials [13].

Vitamin-producing lactic acid bacteria and bifidobacteria strains may become potential candidates for their synthesis in vivo in the intestine after colonization [14]. It is reported that LAB synthesizing folate and riboflavin can be used as an additional treatment in people suffering from inflammatory bowel diseases [15]. To ensure the effects, LAB have to reach the colon in a viable state and be characterized by high stress tolerance when passing through the digestive tract (tolerance to acidic pH, bile, and proteolytic enzymes). These abilities refer to probiotic properties [16]. In addition to stress tolerance, the strains should have a high aggregation ability, which is often associated with adhesion to the intestinal epithelium and colonization properties [17]. They will have more advantages over nonaggregated strains that can be easily removed from the intestinal environment [18].

In this study, for the first time, the probiotic properties and B-vitamin-producing potential of various bifidobacteria and lactobacilli strains are evaluated in order to select the most promising candidates. Their stability under conditions that model the human gastrointestinal tract passage, autoaggregation ability, and ability to synthesize and extracellularly accumulate water-soluble B vitamins are studied. The obtained results can be used in medicine, healthcare, the food industry, and farming in order to expand the applications of LAB and bifidobacteria.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The bifidobacteria strains B. adolescentis AC-1662, B. pseudolongum subsp. pseudolongum AC-1785, B. longum subsp. infantis AC-1912, B. breve AC-1911, B. longum subsp. longum AC-1665, and B. bifidum AC-1779 and lactic acid bacteria L. acidophilus B-2105, L. plantarum B-11007, L. acidophilus B-6551, L. acidophilus B-2213, L. salivarius B-2214, L. casei B-2873, L. acidophilus B-6553, L. rhamnosus B-8238, L. sakei B-8936, and L. paracasei subsp. paracasei
B-4079 were used in this study. Freeze-dried samples were purchased from the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) in sealed glass vials. The sources of origin of microorganisms, according to VKPM, are presented in Table 1.

Table 1. The origins of the organisms used in the study.

| Species                               | Strains            | Origin                  |
|----------------------------------------|--------------------|-------------------------|
| Bifidobacterium adolescentis           | VKPM AC-1662       | Human                   |
| Bifidobacterium pseudolongum subsp. pseudolongum | VKPM AC-1785   | Pig                     |
| Bifidobacterium longum subsp. infantis | VKPM AC-1912       | Human                   |
| Bifidobacterium breve                  | VKPM AC-1911       | Human                   |
| Bifidobacterium longum subsp. longum   | VKPM AC-1665       | Human                   |
| Lactobacillus bifidum                  | VKPM AC-1779       | Human                   |
| Lactobacillus acidophilis              | VKPM B-2105        | Fermented dairy product |
| Lactobacillus plantarum                | VKPM B-11007       | Lactobacterin (medication) |
| Lactobacillus acidophilis              | VKPM B-6551        | Human                   |
| Lactobacillus acidophilis              | VKPM B-2213        | Human                   |
| Lactobacillus salivarius               | VKPM B-2214        | Human                   |
| Lactobacillus casei                    | VKPM B-2873        | Human                   |
| Lactobacillus acidophilis              | VKPM B-6553        | Human                   |
| Lactobacillus rhamnosus                | VKPM B-8238        | Human                   |
| Lactobacillus sakei                    | VKPM B-8936        | Italian dry-cured sausage |
| Lactobacillus paracasei subsp. paracasei | VKPM B-4079   | Fermented sugar beet tops |

To obtain the inocula, bifidobacteria were inoculated in a Bifidum medium [19] and incubated at 37 °C for 24 h. The bifidum medium has the following composition (Formula Per Liter): 30 g casein tryptone (211610; Difco Laboratories, Detroit, MI, USA); 5.0 g yeast extract (Springer, Maisons-Alfort, France); 10.0 g D-glucose (361105; Dextrose, Roquette, Lestrem, France); 0.5 g cysteine hydrochloride (C7477; Sigma-Aldrich, Munich, Germany); 2.5 g sodium chloride (S9888; Sigma-Aldrich, Munich, Germany); 0.5 g magnesium sulfate (212486; PanReac Aplichem, Barcelona, Spain); 0.5 g ascorbic acid (A4034; Dia-m, Moscow, Russia); and 0.3 g sodium acetic acid (S2889; Sigma-Aldrich, Munich, Germany).

To obtain the inoculate of lactobacilli, the strains were cultured in a de Man–Rogosa–Sharpe (MRS) broth [20]. To determine the ability of bifidobacteria and lactobacilli to produce B vitamins, a folic acid assay medium was used according to the Difco™ formula with some modifications in the composition (approximate formula per liter): 12.0 g casamino acids (tryptone) (T2559; Sigma-Aldrich, Munich, Germany); 40.0 g dextrose (361105; Roquette, Lestrem, France); 20.0 g sodium citrate (141655; PanReac Aplichem, Barcelona, Spain); 0.2 g L-cystine (C8755; Sigma-Aldrich, Munich, Germany); 0.2 g DL-tryptophan (T3300; Sigma-Aldrich, Munich, Germany); 20.0 mg adenine sulfate (145815; Sigma-Aldrich, Munich, Germany); 2.0 mg guanine (G11950; Sigma-Aldrich, Munich, Germany); 20.0 mg uridine (U3003; Sigma-Aldrich, Munich, Germany); 2.0 mg thiamine hydrochloride (W332208; Sigma-Aldrich, Munich, Germany); 4.0 mg pyridoxine hydrochloride (P6280; Sigma-Aldrich, Munich, Germany); 2.0 mg riboflavin (R9504; Sigma-Aldrich, Munich, Germany); 2.0 mg niacin (N4126; Sigma-Aldrich, Munich, Germany); 1.0 g dipotassium phosphate (141512; PanReac Aplichem, Barcelona, Spain); 1.0 g monopotassium phosphate (141509; PanReac Aplichem, Barcelona, Spain); 0.4 g magnesium sulfate (142486; PanReac Aplichem, Barcelona, Spain); 20.0 mg sodium chloride (S9888; Sigma-Aldrich, Munich, Germany); 20.0 mg ferrous sulfate (1413624 PanReac Aplichem, Barcelona, Spain); and 20.0 mg manganese sulfate (131413; PanReac Aplichem, Barcelona, Spain) (pH adjusted to 7.0).

2.2. Tolerance to Simulated Human GI Tract

The cultures were centrifuged (Eppendorf, Hamburg, Germany Centrifuge 5417C, 7000 rpm, 15 min) after 24 h of growth (daily culture) and washed once in a 1:1 v/v ratio
with a Ringer solution of the following composition (g L$^{-1}$): 8.69 sodium chloride (S9888; Sigma-Aldrich, Munich, Germany); 0.30 potassium chloride (191494; PanReac AppliChem, Barcelona, Spain); 0.48 calcium chloride (131232; PanReac AppliChem, Barcelona, Spain), pH 6.4 [21]. The precipitate was resuspended in simulated gastric juices (see below) in a 1:1.33 ratio [22] and incubated at 37 °C. Sampling was carried out for 20, 40, 60, and 90 min of the experiment for bifidobacteria and for 30, 60, and 90 min for lactobacilli and the viable cells was counted. After 90 min, the samples were centrifuged, the supernatant was decanted, and a medium simulating the conditions of the duodenum (see below) was added in a 1:3 v/v ratio [22]. If the strain was found to completely lose viability in gastric juices, the simulated intestinal conditions test was repeated with fresh daily culture. Samples were taken at 60, 120, and 180 min.

The effect of the bile medium on the viability of bifidobacteria and lactobacilli was also studied [22]. The precipitates were obtained as described above and resuspended in a bile medium (1:1). The incubation was carried out at 37 °C with sampling at 60, 120, and 180 min.

2.2.1. Preparation of Simulated Gastric Juices

During the experiment, simulated gastric juices of the following composition (g L$^{-1}$) were used: 3.50 dextrose (361105; Roquette, Lestrem, France); 2.05 sodium chloride (S9888; Sigma-Aldrich, Munich, Germany); 0.60 potassium dihydrogen phosphate (A1043; PanReac AppliChem, Barcelona, Spain); 0.11 calcium chloride (131232; PanReac AppliChem, Barcelona, Spain); and 0.37 potassium chloride (191494; PanReac AppliChem, Barcelona, Spain), pH 2.0. The solution was autoclaved at 115 °C for 20 min. The following components were sterilized by filtration (0.2 µm, Millipore, St. Louis, MO, USA) and added to the solution after cooling: 0.05 g L$^{-1}$ pork bile (48305; Sigma, St. Louis, MO, USA); 0.10 g L$^{-1}$ lysozyme (CAS Number 9066-59-5; Cagliificio Clerici S.p.A., Cadorago, Italy), and 13.30 mg L$^{-1}$ pepsin (12762; JSC “Plant of Endocrine Enzymes”, Moscow, Russia) [23].

2.2.2. Preparation of Simulated Duodenal Environment

During the experiment, a medium [24,25] of the following composition (g L$^{-1}$) was used: 6.43 sodium chloride (S9888; Sigma-Aldrich, Munich, Germany); 0.50 potassium chloride (191494; PanReac AppliChem, Barcelona, Spain); 0.21 calcium chloride (131232; PanReac AppliChem, Barcelona, Spain); 0.05 potassium dihydrogen phosphate (A1043; PanReac AppliChem, Barcelona, Spain); 0.05 magnesium chloride (131396; PanReac AppliChem, Barcelona, Spain); 5.67 sodium hydrogen carbonate (131638; PanReac AppliChem, Barcelona, Spain); 0.15 urea (U5378; Sigma-Aldrich, Munich, Germany); albumin (CAS Number 9048-46-8; “Sonac Loenen BV”, GD Loenen, Netherlands); 10.00 pork bile (48305; Sigma, St. Louis, MO, USA); 1.00 lipase (CAS Number 9001-62-1; Cagliificio Clerici S.p.A., Cadorago, Italy); and 6.00 pancreatin (A0585; Panreac, Barcelona Spain), pH 6.0.

2.2.3. Preparation of Bile Medium

During the experiment, a medium [26] of the following composition was used: 30 mL of sodium chloride (S9888; Sigma-Aldrich, Munich, Germany), 175.3 g L$^{-1}$ solution; 68.3 mL of sodium hydrogen carbonate (131638; PanReac AppliChem, Barcelona, Spain), 84.7 g L$^{-1}$ solution; 4.2 mL of potassium chloride (191494; PanReac AppliChem, Barcelona, Spain), 89.6 g L$^{-1}$ solution; 200 mL of hydrochloric acid (170266; LenReaktiv, Moscow, Russia), 37% w/v solution; 10 mL of urea (U5378; Sigma-Aldrich, Munich, Germany), 25 g L$^{-1}$ solution; 10 mL of calcium chloride dihydrate (121214; PanReac AppliChem, Barcelona, Spain), 22.2 g L$^{-1}$ solution; albumin (CAS Number 9048-46-8; “Sonac Loenen BV,” Netherlands), 1.8 g L$^{-1}$; and pork bile (48305; Sigma, St. Louis, MO, USA), 6.0 g L$^{-1}$, pH 8.0.

2.2.4. Tolerance to Enzyme Preparations

The tolerance of microorganisms to pepsin and pancreatin was evaluated separately. The bacterial cell precipitate was resuspended in the buffered enzyme (pancreatin or
pepsin) solution in a 1:1.33 ratio. The enzyme solutions were previously sterilized by filtration (0.22 µm, Pall Corporation, Port Washington, NY, USA) [22,27].

To obtain the pancreatin (A0585; Panreac Applichem, Barcelona, Spain) solution (6 g L⁻¹), a phosphate buffer of the following composition (g L⁻¹) was used: 8.00 sodium chloride (S9888; Sigma-Aldrich, Munich, Germany); 0.24 sodium hydrogen phosphate (131679; PanReac Applichem, Barcelona, Spain); 0.20 potassium chloride (191494; PanReac Applichem, Barcelona, Spain); 0.24 potassium dihydrogen phosphate (A1043; PanReac Applichem, Barcelona, Spain) [28], pH 7.0. Pepsin (12762; JSC “Plant of Endocrine enzymes”, Moscow, Russia) was dissolved in the citrate–HCl buffer (pH 3.0) at 13.5 g L⁻¹ [23,25]. To prepare the citrate–HCl buffer at pH 3.0, 21.0 g of citric acid (C2404; Sigma-Aldrich, Munich, Germany) was dissolved in 200 mL of 1 M NaOH, brought up to 1 L with distilled water, and 40.3 mL of this solution was diluted with 0.1 M HCl to 100.0 mL [29].

2.3. Determination of Total Viable Count and Survival Percentage

Enumeration of Lactobacillus strains’ viable cells (CFU mL⁻¹) was carried out by plating 10-fold dilutions of the samples in sterile PBS on MRS agar. Plates were incubated at 37 ± 1 °C for 48 h. Enumeration of Bifidobacterium strains’ viable cells (CFU mL⁻¹) was carried out by plating 10-fold dilutions of the samples in sterile PBS on Bifidum agar. Plates were incubated under anaerobic conditions provided by the BD GasPak™ (BD Biosciences, Franklin Lakes, NJ, USA) anaerobic container system at 37 ± 1 °C for 48 h. Counting was performed in triplicate and the results are expressed as log₁₀ (CFU mL⁻¹).

The survival percentage was calculated as the percentage of LAB or Bifidobacterium colonies grown on MRS or Bifidum agar compared to the initial bacterial concentration:

\[
\% \text{ survival percentage} = \frac{\log \text{ CFU of viable cells survived}}{\log \text{ CFU of initial viable cells inoculated}} \times 100. \quad (1)
\]

2.4. Spectrophotometry Autoaggregation Assays

The ability to autoaggregate was tested as described by Collado, Meriluoto, and Salminen [18] according to the autoaggregate percentage. The cells were harvested after 24 h of incubation by centrifugation (3200 × g, 4 °C, 20 min), washed twice with a phosphate–salt buffer (PBS: 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), resuspended in the same buffer under sterile conditions, adjusted to OD₆₀₀ = 0.25 ± 0.05, and incubated at 37 °C. OD was controlled at different time intervals (0, 3, 6, 12, or 24 h). The percentage of autoaggregation was expressed according to:

\[
A\% = \frac{(A₀ - Aₜ)}{A₀} \times 100, \quad (2)
\]

where \(A₀\) is the absorption (A₆₀₀ nm) at 0 h and \(Aₜ\) is the absorption (A₆₀₀ nm) at different time intervals (0, 3, 6, 12, or 24 h).

2.5. Determination of Water-Soluble B Vitamins

The analysis of water-soluble B vitamins free forms and their concentrations in the fermentation broth of probiotic bacteria was carried out with a capillary electrophoresis system “Kapel-105M” (Lumex Ltd., St. Petersburg, Russia) with positive polarity, equipped with a special geometry quartz capillary similar to amino acid (pore inner diameter 50 microns, total/effective length 75/65 cm) and the specialized software Elforun® (S-Pb, St. Petersburg, Russia). The preparation of samples for research consisted of the microbial cells’ precipitation using an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) at 7500 rpm for 10 min. After that, the supernatants were placed in Amicon Ultra-15 centrifuge concentrators (Merck, Darmstadt, Germany) with a 3 kDa pore diameter cellulose membrane and centrifuged for 15 min at 7500 rpm for complete purification. Separation, identification, and determination of the concentrations of water-soluble vitamins thiamine (B1), riboflavin (B2), pantothenic acid (B3), pyridoxine (B6), and folic acid (Bc) were carried out by capillary zone electrophoresis (CSE), and that of the vitamin nicotinamide (B5) by
micellar electrokinetic chromatography (MEKC) according to the practical guidelines for the use of “Kapel” capillary electrophoresis systems [30]. Detection conditions for vitamins B1, B2, B3, B6, and Bc: capillary: quartz, 50 microns, 75 cm; voltage +25 kV; wavelength at the beginning of the analysis 200 nm, after the detection peak B1 267 nm, after the peak B2 and before the end of the analysis 200 nm; temperature 30 °C; pressure at the beginning of the analysis 0 mbar, after the peak B6-30 mbar until the end of the analysis. Detection conditions for vitamin B5: capillary: quartz, 50 microns, 75 cm; voltage +25 kV; wavelength 200 nm; temperature 40 °C; pressure 0 mbar. The standards (HPLC grade) of all B vitamins for calibration were purchased from Lumex, Ltd. All samples were tested in triplicate.

2.6. Statistical Analysis

All the measurements were performed in triplicate, and the results were expressed as the mean with standard deviation (±SD). The data were analyzed by an ANOVA test with Tukey’s multiple comparison using MathLab (MathWorks, Natick, MA, USA) software. Statistical significance was determined at \( p = 0.05 \).

3. Results

3.1. Stress Tolerance Ability

3.1.1. Tolerance of \textit{Bifidobacterium} and \textit{Lactobacillus} Strains to Simulated Gastric Juices

The standard conditions for incubation in real or simulated gastric juices have not been fully developed yet [31]. However, it is known that, depending on the consumed food and beverages, incubation time in the stomach can vary from 15 to 194 min [32]. Therefore, to simulate the presence of lactobacilli and bifidobacteria in the stomach, an incubation period of 90 min was determined. As the death rate of bifidobacteria will be greater than that of lactobacilli, the measurements for the strains of \textit{Bifidobacterium} genus were carried out more frequently. In this case, only the final results of the test (after 90 min) are applicable for intergenera comparison.

The results of the research on the survival of six bifidobacteria strains and 10 lactobacilli strains during the incubation in simulated gastric juices at pH 2.0 for 1.5 h are shown in Tables 2 and 3, respectively. Each tested strain of bifidobacteria demonstrated a considerable decrease in the viable cell count during the first 30 min of incubation. The strains of \textit{B. adolescentis} VKPM AC-1662, \textit{B. pseudolongum} subsp. \textit{pseudolongum} VKPM AC-1785, and \textit{B. breve} VKPM AC-1911 showed the highest tolerance to the effects of simulated gastric juices. The marked decrease in the population was observed in the \textit{B. longum} subsp. \textit{infantis} VKPM A C-1912, \textit{B. longum} subsp. \textit{longum} VKPM AC-1665, and \textit{B. bifidum} VKPM AC-1779 strains. The strain \textit{B. bifidum} VKPM AC-1779 was characterized by the decay of viable cells after 40 min of exposure.

Table 2. Tolerance of \textit{Bifidobacterium} strains to simulated gastric juices (pH 2.0).

| Strain | Survival Percentage (% ± SD) |
|--------|-------------------------------|
|        | 20 | 40 | 60 | 90 |
| \textit{B. adolescentis} VKPM AC-1662 | 95 ± 0.4 \(^a\) | 81 ± 0.9 \(^a\) | 71 ± 0.2 \(^a\) | 67 ± 0.7 \(^a\) |
| \textit{B. pseudolongum} subsp. \textit{pseudolongum} VKPM AC-1785 | 88 ± 0.9 \(^b\) | 86 ± 1.1 \(^b\) | 63 ± 0.4 \(^b\) | 65 ± 0.8 \(^b\) |
| \textit{B. longum} subsp. \textit{infantis} VKPM AC-1912 | 75 ± 0.2 \(^c\) | 54 ± 0.9 \(^c\) | 0 \(^c\) | 0 \(^c\) |
| \textit{B. breve} VKPM AC-1911 | 81 ± 1.1 \(^d\) | 60 ± 0.6 \(^d\) | 60 ± 0.2 \(^d\) | 60 ± 0.8 \(^d\) |
| \textit{B. longum} subsp. \textit{longum} VKPM AC-1665 | 80 ± 1.2 \(^d\) | 50 ± 1.2 \(^c\) | 0 \(^c\) | 0 \(^c\) |
| \textit{B. bifidum} VKPM AC-1779 | 63 ± 0.5 \(^e\) | 0 \(^f\) | 0 \(^c\) | 0 \(^c\) |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (\( p < 0.05 \)) within each time comparison.
Table 3. Tolerance of *Lactobacillus* strains to simulated gastric juices (pH 2.0).

| Strain                | Survival Percentage (% ± SD) | Time (min) |
|-----------------------|------------------------------|------------|
|                       | 30              | 60          | 90          |
| *L. acidophilus* VKPM B-2105 | 97 ± 0.7 a | 84 ± 1.2 a | 81 ± 0.7 a |
| *L. plantarum* VKPM B-11007 | 99 ± 0.3 b | 84 ± 0.5 a | 71 ± 0.8 b |
| *L. acidophilus* VKPM B-6551 | 96 ± 1.4 a | 84 ± 0.6 b | 82 ± 0.6 c |
| *L. acidophilus* VKPM B-2213 | 99 ± 0.5 b | 86 ± 0.1 c | 74 ± 2.0 d |
| *L. salivarius* VKPM B-2214 | 89 ± 0.8 c | 89 ± 0.5 d | 72 ± 0.4 e |
| *L. acidophilus* VKPM B-6553 | 90 ± 0.8 c | 88 ± 1.4 e | 87 ± 0.3 e |
| *L. salivarius* VKPM B-22873 | 97 ± 1.1 a,b | 93 ± 0.9 f | 81 ± 1.3 a,c |
| *L. sakei* VKPM B-8936 | 98 ± 0.7 a,b | 94 ± 0.4 f | 93 ± 0.3 h |
| *L. paracasei* subsp. *paracasei* VKPM B-4079 | 85 ± 1.6 d | 81 ± 1.0 f | 78 ± 0.6 i |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (*p* < 0.05) within each time comparison.

All the studied lactobacillus strains also demonstrated a decrease in the viable cell count under the model conditions of simulated gastric juices (Table 3). The strain *L. sakei* VKPM B-8936 demonstrated the greatest resistance to low pH values, while *L. salivarius* VKPM B-2214 had the lowest. Total inactivation of the lactobacillus population was not observed for any strain after 1.5 h of incubation.

3.1.2. Tolerance of *Bifidobacterium* and *Lactobacillus* Strains to the Simulated Duodenal Environment

The time of food transit via the small intestine is usually 1 to 4 h, which gives a total transit time in the upper gastrointestinal tract of 3 to 8 h [33]. Therefore, in order to simulate the passing of lactobacilli and bifidobacteria in the intestine, the incubation period of 3 h was established. The viability of lactobacilli after 3 h in the duodenum-simulating conditions was comparatively higher than in bifidobacteria (Tables 4 and 5). At the same time, the survival rate of both lactobacilli and bifidobacteria was strain-specific and differed between the cultures.

Table 4. Tolerance of *Bifidobacterium* strains to simulated duodenal environment (pH 6.0).

| Strain                     | Survival Percentage (% ± SD) | Time (min) |
|----------------------------|------------------------------|------------|
|                            | 60              | 120         | 180         |
| *B. adolescentis* VKPM AC-1662 | 100 ± 0.02 a | 100 ± 0.03 a | 98 ± 0.7 a |
| *B. pseudolongum* subsp. *pseudolongum* VKPM AC-1785 | 100 ± 0.03 a | 92 ± 1.6 b | 80 ± 2.1 b |
| *B. longum* subsp. *infantis* VKPM AC-1912 | 96 ± 1.7 b | 86 ± 0.4 c | 86 ± 1.5 c |
| *B. breve* VKPM AC-1911 | 89 ± 1.0 c | 92 ± 0.5 b | 80 ± 0.6 b |
| *B. longum* subsp. *longum* VKPM AC-1665 | 87 ± 0.8 d | 87 ± 0.5 c | 87 ± 0.2 c |
| *B. bifidum* VKPM AC-1779 | 89 ± 1.1 c | 87 ± 0.9 c | 87 ± 1.3 c |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (*p* < 0.05) within each time comparison.

The strain *B. adolescentis* VKPM AC-1662 had the highest survival rate, almost equal to 100%. The greatest sensitivity to the conditions of the intestinal environment was demonstrated by the bifidobacteria *B. pseudolongum* subsp. *pseudolongum* VKPM AC-1785 and *B. breve* VKPM AC-1911 strains, which had the survival percentage of 80% after 3 h of incubation.

Among lactobacilli, the lowest decrease in population was observed in the *L. plantarum* VKPM B-11007 and *L. acidophilus* VKPM B-2213 strains. The survival rate of the *L. salivarius* VKPM B-2214 strain was the lowest.
Table 5. Tolerance of *Lactobacillus* strains to simulated duodenal environment (pH 6.0).

| Strain                  | Survival Percentage (%± SD) | Time (min) |
|-------------------------|----------------------------|------------|
|                         | 60 | 120 | 180   |
| *L. acidophilus* VKPM B-2105 | 91 ± 1.7 a | 90 ± 0.9 a | 89 ± 0.6 a |
| *L. plantarum* VKPM B-11007 | 100 ± 0.04 b | 99 ± 0.4 b | 99 ± 0.6 b |
| *L. acidophilus* VKPM B-6551 | 95 ± 0.4 c | 84 ± 1.6 d | 84 ± 1.9 c |
| *L. acidophilus* VKPM B-2213 | 98 ± 0.6 d | 94 ± 0.8 e | 89 ± 1.3 a |
| *L. salicarius* VKPM B-2214 | 96 ± 0.9 e | 92 ± 1.2 f | 86 ± 0.7 c |
| *L. casei* VKPM B-2873 | 99 ± 0.3 bd | 96 ± 1.4 e | 92 ± 0.8 d |
| *L. acidophilus* VKPM B-6553 | 99 ± 0.8 bd | 97 ± 0.2 e | 89 ± 0.3 a |
| *L. rhamnosus* VKPM B-8238 | 99 ± 0.6 bd | 97 ± 0.9 e | 95 ± 1.1 e |
| *L. sakei* VKPM B-8936 | 99 ± 0.6 bd | 97 ± 0.9 e | 95 ± 1.1 e |
| *L. paracasei* subsp. *paracasei* VKPM B-4079 | 99 ± 0.6 bd | 97 ± 0.9 e | 95 ± 1.1 e |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.

3.1.3. Tolerance of *Bifidobacterium* and *Lactobacillus* Strains to Digestive Enzymes

To clarify the effect of enzymes as principal components of digestive juices, the strains were tested separately for pepsin and pancreatin action. A study of the effect of the proteolytic enzymes, such as pepsin and pancreatin, on the survival of bifidobacteria showed that they had an equally strong effect on every considered strain. Total death of microorganisms occurred after 1 h of incubation. The results of the research on the sensitivity of lactobacilli to pepsin and pancreatin are shown in Tables 6 and 7.

Table 6. Tolerance of *Lactobacillus* strains to pepsin.

| Strain                  | Survival Percentage (% ± SD) | Time (min) |
|-------------------------|----------------------------|------------|
|                         | 60 | 120 | 180   |
| *L. acidophilus* VKPM B-2105 | 96 ± 1.5 a | 80 ± 1.3 a | 80 ± 0.6 a |
| *L. plantarum* VKPM B-11007 | 98 ± 0.4 b | 83 ± 1.2 b | 72 ± 0.9 b |
| *L. acidophilus* VKPM B-6551 | 92 ± 0.5 c | 85 ± 0.8 c | 85 ± 0.2 c |
| *L. acidophilus* VKPM B-2213 | 98 ± 0.3 ab | 92 ± 0.6 d | 91 ± 0.7 d |
| *L. salicarius* VKPM B-2214 | 94 ± 1.9 d | 88 ± 1.0 e | 84 ± 0.6 e |
| *L. casei* VKPM B-2873 | 92 ± 0.5 c,d | 84 ± 0.8 b,ce | 81 ± 1.1 a |
| *L. acidophilus* VKPM B-6553 | 96 ± 0.9 a | 91 ± 0.6 d | 83 ± 1.3 a |
| *L. rhamnosus* VKPM B-8238 | 87 ± 0.7 a | 74 ± 1.5 f | 71 ± 0.8 b |
| *L. sakei* VKPM B-8936 | 90 ± 0.7 c | 84 ± 0.7 c,e | 73 ± 0.1 b |
| *L. paracasei* subsp. *paracasei* VKPM B-4079 | 73 ± 0.2 e | 67 ± 1.6 e | 58 ± 1.0 f |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.

Table 7. Tolerance of *Lactobacillus* strains to pancreatin.

| Strain                  | Survival Percentage (%±SD) | Time (min) |
|-------------------------|----------------------------|------------|
|                         | 60 | 120 | 180   |
| *L. acidophilus* VKPM B-2105 | 100 ± 0.03 a | 92 ± 1.0 a | 91 ± 0.8 a |
| *L. plantarum* VKPM B-11007 | 97 ± 0.7 b | 92 ± 0.9 a | 91 ± 0.2 a |
| *L. acidophilus* VKPM B-6551 | 100 ± 0.1 a | 95 ± 0.5 bc | 94 ± 0.6 b |
| *L. acidophilus* VKPM B-2213 | 98 ± 0.2 c | 95 ± 0.9 b,ce | 93 ± 0.5 b |
| *L. salicarius* VKPM B-2214 | 99 ± 0.1 a | 98 ± 0.4 d | 98 ± 0.4 c |
| *L. casei* VKPM B-2873 | 98 ± 0.6 c | 96 ± 0.3 c | 93 ± 0.9 b |
| *L. acidophilus* VKPM B-6553 | 98 ± 0.3 bc | 98 ± 0.5 d | 97 ± 1.2 c,d |
| *L. rhamnosus* VKPM B-8238 | 100 ± 0.2 a | 96 ± 1.4 b,c | 96 ± 0.9 d |
| *L. sakei* VKPM B-8936 | 95 ± 1.0 d | 94 ± 0.6 b | 88 ± 1.4 e |
| *L. paracasei* subsp. *paracasei* VKPM B-4079 | 96 ± 0.6 e | 92 ± 0.9 a | 90 ± 1.5 f |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.
The inactivation effect of pepsin on the lactobacilli viability was higher than that of pancreatic. If the survival rate of lactobacilli after 3 h of incubation with pancreatin was about 90% for all the cultures, the sensitivity to pepsin greatly varied between the strains, reaching 60% in some cases. Thus, the strain L. paracasei subsp. paracasei VKPM B-4079 demonstrated the lowest tolerance to pepsin, and L. acidophilus VKPM B-2213 the greatest.

3.1.4. Tolerance of Bifidobacterium and Lactobacillus Strains to Bile

The results of the study on bile’s effect on the viability of bifidobacteria and lactic acid bacteria strains are shown in Tables 8 and 9. Almost every strain considered showed a decrease in the population of viable cells. The most sensitive to the presence of bile in the medium was the B. breve VKPM AC-1911 strain (the survival rate was 70%). The B. bifidum VKPM AC-1779 strain was characterized by the greatest resistance to the effects of bile; the survival rate hardly changed (about 99%).

### Table 8. Tolerance of Bifidobacterium strains to bile.

| Strain                        | Survival Percentage (% ± SD) | Time (min) |
|-------------------------------|-----------------------------|------------|
|                              |                            | 60         | 120        | 180         |
| B. adolescentis VKPM AC-1662 | 89 ± 0.6 ± 1.3 ± 1.5        | 89 ± 0.6   | 89 ± 0.6   | 89 ± 0.6   |
| B. pseudolongum subsp. pseudolongum VKPM AC-1785 | 99 ± 0.1 ± 0.8 ± 1.8 | 89 ± 0.8   | 88 ± 1.8   |
| B. longum subsp. infantis VKPM AC-1912 | 94 ± 0.4 ± 1.0 ± 1.1 | 88 ± 1.0   | 87 ± 1.1   |
| B. breve VKPM AC-1911         | 89 ± 0.8 ± 1.8 ± 0.5        | 79 ± 1.8   | 70 ± 0.5   |
| B. longum subsp. longum VKPM AC-1665 | 98 ± 0.4 ± 0.1 ± 0.8 | 89 ± 0.1   | 80 ± 0.7   |
| B. bifidum VKPM AC-1779       | 99 ± 0.2 ± 0.04 ± 0.04      | 99 ± 0.2   | 99 ± 0.04  |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.

### Table 9. Tolerance of Lactobacillus strains to bile.

| Strain                        | Survival Percentage (% ± SD) | Time (min) |
|-------------------------------|-----------------------------|------------|
|                              |                            | 60         | 120        | 180         |
| L. acidophilus VKPM B-2105    | 98 ± 0.2 ± 0.6 ± 0.7        | 98 ± 0.6   | 98 ± 0.6   |
| L. plantarum VKPM B-11007     | 90 ± 2.0 ± 1.2 ± 1.0        | 88 ± 1.2   | 86 ± 0.6   |
| L. acidophilus VKPM B-6551    | 88 ± 1.6 ± 0.7 ± 1.9        | 81 ± 0.7   | 79 ± 1.9   |
| L. acidophilus VKPM B-2213    | 88 ± 0.6 ± 0.1 ± 0.8        | 74 ± 0.4   | 70 ± 1.0   |
| L. salivarius VKPM B-2214     | 97 ± 0.5 ± 0.8 ± 0.6        | 90 ± 0.8   | 76 ± 0.5   |
| L. casei VKPM B-2873          | 95 ± 0.1 ± 0.9 ± 1.2        | 85 ± 0.9   | 83 ± 1.2   |
| L. acidophilus VKPM B-6533    | 88 ± 1.3 ± 0.3 ± 0.7        | 81 ± 0.3   | 78 ± 1.5   |
| L. rhamnosus VKPM B-8238      | 86 ± 0.8 ± 0.3 ± 0.2        | 76 ± 0.3   | 72 ± 0.4   |
| L. sakei VKPM B-8936          | 95 ± 0.2 ± 0.0 ± 1.0        | 85 ± 0.9   | 77 ± 1.0   |
| L. paracasei subsp. paracasei VKPM B-4079 | 88 ± 1.7 ± 0.4 ± 0.6 | 82 ± 0.4   | 80 ± 0.6   |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.

Among the Lactobacillus strains considered, the L. acidophilus VKPM B-2213 strain demonstrated the greatest sensitivity to bile, and L. acidophilus VKPM B-2105 the lowest.

3.2. Autoaggregation Assays of Bifidobacterium and Lactobacillus Strains

The results of the study on the autoaggregation of bifidobacteria strains are shown in Table 10. The highest percentage of autoaggregation was demonstrated by the strain B. adolescentis VKPM AC-1662: 69% after 24 h of incubation. B. longum subsp. longum VKPM AC-1665 aggregation was a little lower. The other strains demonstrated low autoaggregation ability.
which the maximum amount was detected for the last one. Thiamine, pantothenic acid, AC-1665, L. salivarius VKPM B-6551, L. acidophilus VKPM B-2213 (its concentration was higher in the medium before fermentation). The riboflavin that was found in B. adolescentis in concentration was observed. The ability to accumulate pyridoxine extracellularly was detected in the nutrient medium was consumed by all cultures except B. adolescentis VKPM AC-1662, B. pseudolongum 15 ± 0.2, L. paracasei VKPM B-4079 have a high potential ability to attach to epithelial cells and mucosal surfaces.

### Table 10. Autoaggregation percentage of Bifidobacterium strains.

| Strains                  | 3 h ± | 6 h ± | 12 h ± | 24 h ± |
|--------------------------|-------|-------|--------|--------|
| B. adolescentis VKPM AC-1662 | 15 ± 0.4 | 48 ± 0.8 | 53 ± 0.8 | 69 ± 0.8 |
| B. pseudolongum subsp. pseudolongum VKPM AC-1785 | 9 ± 0.8 | 12 ± 0.4 | 14 ± 0.3 | 13 ± 0.3 |
| B. longum subsp. infantis VKPM AC-1912 | 12 ± 0.4 | 15 ± 0.5 | 13 ± 0.2 | 11 ± 0.2 |
| B. breve VKPM AC-1911 | 12 ± 0.4 | 32 ± 0.6 | 30 ± 0.5 | 31 ± 0.2 |
| B. longum subsp. longum VKPM AC-1665 | 20 ± 0.2 | 56 ± 0.2 | 59 ± 0.6 | 67 ± 1.0 |
| B. bifidum VKPM AC-1779 | 34 ± 0.9 | 47 ± 0.6 | 55 ± 0.8 | 57 ± 0.9 |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.

The results of the Lactobacillus strains autoaggregation test are shown in Table 11. The greatest autoaggregation was observed in the L. salivarius VKPM B-2214 strain: 56% after 24 h of incubation. Also, a high autoaggregation ability was observed in the L. acidophilus VKPM B-6551, L. acidophilus VKPM B-2213, and L. rhamnosus VKPM B-8238 strains. The aggregation percentages of the other lactobacilli strains considered were lower.

### Table 11. Autoaggregation percentage of Lactobacillus strains.

| Strains                  | 3 h ± | 6 h ± | 12 h ± | 24 h ± |
|--------------------------|-------|-------|--------|--------|
| L. acidophilus VKPM B-2105 | 10 ± 0.4 | 28 ± 0.5 | 40 ± 0.9 | 47 ± 1.0 |
| L. plantarum VKPM B-11007 | 9 ± 0.2 | 9 ± 0.2 | 7 ± 0.6 | 8 ± 0.5 |
| L. acidophilus VKPM B-6551 | 25 ± 0.3 | 50 ± 0.8 | 52 ± 0.7 | 55 ± 0.8 |
| L. acidophilus VKPM B-2213 | 12 ± 0.3 | 49 ± 0.2 | 50 ± 0.6 | 53 ± 0.6 |
| L. salivarius VKPM B-2214 | 14 ± 0.2 | 18 ± 0.1 | 26 ± 0.7 | 56 ± 1.1 |
| L. casei VKPM B-2873 | 6 ± 0.1 | 13 ± 0.3 | 12 ± 0.3 | 32 ± 0.7 |
| L. acidophilus VKPM B-6553 | 5 ± 0.2 | 8 ± 0.1 | 8 ± 0.3 | 25 ± 0.3 |
| L. rhamnosus VKPM B-8238 | 16 ± 0.3 | 44 ± 0.8 | 46 ± 0.8 | 50 ± 0.7 |
| L. sakei VKPM B-8936 | 11 ± 0.1 | 10 ± 0.2 | 10 ± 0.2 | 12 ± 0.5 |
| L. paracasei subsp. paracasei VKPM B-4079 | 5 ± 0.2 | 8 ± 0.1 | 5 ± 0.2 | 7 ± 0.2 |

Values are the mean ± SD of three determinations of three replicates. The superscripts indicate statistically significant differences (p < 0.05) within each time comparison.

Thus, the strains B. adolescentis VKPM AC-1662, B. longum subsp. longum BKMIM AC-1665, L. salivarius VKPM B-2214, L. acidophilus VKPM B-6551, L. acidophilus VKPM B-2213, and L. rhamnosus VKPM B-8238 have a high potential ability to attach to epithelial cells and mucosal surfaces.

### 3.3. The Study of Lactic Acid Bacteria’s and Bifidobacteria’s Ability to Accumulate B Vitamins Extracellularly

While studying the ability of lactic acid bacteria and bifidobacteria to produce B vitamins, it was assumed that, if a specific vitamin was found in the supernatant, the considered strain was able not only to synthesize, but also to excrete vitamins simultaneously with their accumulation in the medium. The results of the studies are presented in Tables 12 and 13.

Based on the results, each bifidobacteria culture considered consumed nicotinamide (its concentration was higher in the medium before fermentation). The riboflavin that was detected in the nutrient medium was consumed by all cultures except B. adolescentis AC1662 and B. longum subsp. infantis AC-1912, which excrete it in the medium, such that an increase in concentration was observed. The ability to accumulate pyridoxine extracellularly was detected in B. adolescentis VKPM AC-1662, B. pseudolongum subsp. pseudolongum VKPM AC-1785, B. longum subsp. infantis VKPM AC-1912, and B. longum VKPM AC-1665, among which the maximum amount was detected for the last one. Thiamine, pantothenic acid, and folic acid were not detected in the supernatant of the fermentation broths.
Table 12. Concentration of B vitamins in bifidobacteria fermentation broths.

| Strain                        | Thiamine (B1) | Riboflavin (B2) | Pantothenic Acid (B3) | Nicotinamide (B5) | Pyridoxine (B6) | Folic Acid (Bc) |
|-------------------------------|---------------|-----------------|-----------------------|-------------------|-----------------|-----------------|
| B. adolescentis VKPM AC-1662 | —             | 2.180 ± 0.021   | —                     | 1.462 ± 0.040     | 7.974 ± 0.040   | —               |
| B. pseudolongum VKPM AC-1785 | —             | —               | —                     | 1.462 ± 0.040     | 7.974 ± 0.040   | —               |
| B. longum subsp. infantis VKPM AC-1912 | —         | 2.009 ± 0.011   | —                     | 1.554 ± 0.020     | 20.680 ± 0.080  | —               |
| B. breve VKPM AC-1911        | 0.503 ± 0.012 | —               | —                     | —                 | 2.010 ± 0.021   | —               |
| B. longum VKPM AC-1665       | —             | 0.613 ± 0.031   | —                     | 0.304 ± 0.060     | —               | —               |
| B. bifidum VKPM AC-1779      | —             | —               | —                     | —                 | —               | —               |
| medium before fermentation   | 1.191 ± 0.011 | —               | —                     | 52.970 ± 0.091    | —               | —               |

Values are the mean ± SD of three determinations of three replicates. The concentrations of each B vitamin differed significantly between the strains (p < 0.05).

Table 13. Concentration of B vitamins in lactic acid bacteria fermentation broths.

| Strain                        | Thiamine (B1) | Riboflavin (B2) | Pantothenic Acid (B3) | Nicotinamide (B5) | Pyridoxine (B6) | Folic Acid (Bc) |
|-------------------------------|---------------|-----------------|-----------------------|-------------------|-----------------|-----------------|
| L. acidophilus VKPM B-2105    | —             | 0.917 ± 0.010   | —                     | 10.220 ± 0.032    | 43.640 ± 0.050  | 4.096 ± 0.020   |
| L. plantarum VKPM B-11007     | —             | —               | —                     | 46.650 ± 0.020    | 3.453 ± 0.010   | —               |
| L. acidophilus VKPM B-6551    | —             | —               | —                     | 0.671 ± 0.010     | 1.100 ± 0.010   | —               |
| L. acidophilus VKPM B-2213    | 0.171 ± 0.010 | —               | —                     | 49.920 ± 0.011    | —               | —               |
| L. salivarius VKPM B-2214     | —             | —               | 138.600 ± 0.021       | 38.130 ± 0.030    | 1.500 ± 0.010   | —               |
| L. casei VKPM B-2873          | 19.540 ± 0.023| —               | 3.966 ± 0.020         | —                 | 11.400 ± 0.030  | —               |
| L. acidophilus VKPM B-6553    | 0.472 ± 0.020 | —               | —                     | 43.030 ± 0.030    | 2.944 ± 0.020   | —               |
| L. rhamnosus VKPM B-8238      | 14.72 ± 0.010 | —               | 52.990 ± 0.100        | 27.690 ± 0.010    | 6.824 ± 0.010   | —               |
| L. sakei VKPM B-8936          | —             | —               | —                     | 5.111 ± 0.010     | 13.880 ± 0.012  | —               |
| L. paracasei subsp. paracasei VKPM B-4079 | —         | —               | —                     | —                 | 61.370 ± 0.010  | —               |

Values are the mean ± SD of three determinations of three replicates. The concentrations of each B vitamin differed significantly between the strains (p < 0.05).

Each Lactobacillus strain also consumed nicotinamide. The ability to produce thiamine in the medium was found in L. casei VKPM B-2873 and L. rhamnosus VKPM B-8238; pantothenic acid in L. acidophilus VKPM B-2105, L. salivarius VKPM B-2214, L. casei VKPM B-2873, and L. rhamnosus VKPM B-8238; and pyridoxine in L. acidophilus VKPM B-2105, L. plantarum VKPM B-11007, L. acidophilus VKPM B-6551, L. casei VKPM B-2873, L. rhamnosus VKPM B-8238, and L. sakei VKPM B-8936. Folic acid was detected in the fermentation broths of L. salivarius VKPM B-2214 and L. acidophilus VKPM B-6553.

Among the Lactobacillus strains considered, L. salivarius VKPM B-2214 is of the greatest interest since the pantothenic acid concentration reached 138.600 mg L$^{-1}$ in the fermentation broth.

4. Discussion

Carrying out proper in vitro studies to screen potential probiotic strains is a necessary first step before in vivo experiments to establish the possible health benefits of probiotics [34]. An important evaluation criterion during the selection is stress tolerance to passing through the digestive tract, which characterizes the resistance of the probiotics to acid and bile, as well as the production of antimicrobial compounds and the ability to aggregate with intestinal cells [34,35]. The acidic gastric juices and bile salts are the main obstacle to the survival of probiotic bacteria and reaching the distal part of the intestine [36].

The results obtained in our research show that the stress tolerance of bifidobacteria and lactobacilli to the effects of low pH, proteolytic enzymes, and bile varies greatly, both between species and between strains of the given species. The effect of artificial gastric
juices on bifidobacteria strains led to the death of B. longum subsp. infantis VKPM AC-1912, B. longum subsp. longum VKPM AC-1665, and B. bifidum VKPM AC-1779. Similar results were reported by Charteris et al. [37], who showed a lack of resistance to simulated gastric conditions (pH 2.0 for 90 min) in the B. bifidum, B. animalis, B. infantis, B. breve, and B. adolescentis strains. Strong differences in resistance to low pH between strains within the species B. longum were observed by Izquierdo et al. [38]. In our study, the B. adolescentis VKPM AC-1662 strain, which was isolated from the human intestine, was characterized by the greatest acid resistance. The effect of the model duodenum conditions on the bifidobacteria survival was less harmful, and the survival rate for all strains was above 80%. The strain B. adolescentis VKPM AC-1662 also showed the highest stability.

When comparing the tolerance of the tested lactobacilli and bifidobacteria to the gastrointestinal tract model conditions, the higher resistance of the former was anticipated. They were resistant to both simulated gastric juices and bile. Acid resistance is a characteristic of most lactobacillus species due to their ability to control intracellular pH [39]. Some of them (e.g., Lactobacillus casei, Lactobacillus reuteri, Lactobacillus vaginalis, Lactobacillus fermentum, and Lactobacillus casei) may be members of the gastric microbial community [40]. In our research, the strain L. sakei VKPM B-8936 showed the greatest resistance to low pH. Similar results were reported by Song et al. [41], who showed the acid resistance of the Lactobacillus sp. NN 8829, L. casei MB3, L. sakei MA9, and L. sakei CH8 strains under incubation conditions at pH 2.5. Thus, the obtained data are generally consistent with the results presented earlier. On the other hand, taking into account the confirmed strain-specific tolerance, the accumulation of information on new strains is theoretically and practically valuable. The experiment dedicated to the treatment of bifidobacteria populations separately with pepsin and pancreatin led to the death of all tested strains. Lactobacilli were more resistant to the effects of proteolytic enzymes. The greater sensitivity of lactobacilli was marked in the experiments with pepsin.

The bacterial cell aggregation of probiotic strains is often associated with potential ability to adhere to the intestinal epithelium and mucous membranes [17]. In most cases, bacterial autoaggregation is mediated by homotypic interactions between surface proteins [42]. The tested cultures of lactic acid bacteria and bifidobacteria demonstrated some degree of autoaggregation, which differed greatly between the strains. The strains B. adolescentis VKPM AC-1662, B. longum subsp. longum VKPM AC-1665, and B. bifidum VKPM AC-1779, isolated from the intestines of adults, showed strong autoaggregation ability. Among lactobacilli, the highest percentage of autoaggregation was observed in L. salivarius VKPM B-2214, isolated from human saliva. It was also characterized by the strong dependence of bacterial cells’ aggregation on exposure time, which was greatest after 24 h. It was reported previously that L. acidophilus is characterized by high values of autoaggregation [43]. In our study, high autoaggregation ability was also observed in L. acidophilus VKPM B-6551, L. acidophilus VKPM B-2213, and L. rhamnosus VKPM B-8238.

The reference probiotic bacteria, e.g., L. rhamnosus GG or L. casei Shirota, can be applied in research on probiotic properties to improve the reliability of the data comparison [44–46]. The lack of a reference probiotic can be considered a limitation of our study. However, it should be noted that the standards were not used in the numerous works mentioned above. The FAO/WHO guidelines [47] also do not establish strict requirements. Moreover, discrepancies in the data on the stress tolerance of the same reference strain and in the same tests were noted between studies.

The analysis of the obtained results on lactic acid bacteria’s and bifidobacteria’s ability to extracellularly production of water-soluble B vitamins showed that the consumption of some vitamins detected in the nutrient medium before fermentation and the excretion of others into fermentation broths occurred. So, riboflavin was not consumed by B. adolescentis VKPM AC-1662 and B. longum subsp. infantis VKPM AC-1912. Hou et al. [48] showed that fermentation of soy milk with Bifidobacterium longum B6 and B. infantis CCRC 14633 cultures increases the content of riboflavin. Pyridoxine accumulation was observed in the strains of B. adolescentis VKPM AC-1662, B. pseudolongum subsp. pseudolongum VKPM AC-1785, B.
longum subsp. infantis VKPM AC-1912, and B. longum VKPM AC-1665. The obtained results are consistent with the data of Deguchi, Morishita and Mutai [49], who linked the ability to synthesize certain vitamins with the species and strain. The extracellular synthesis of folic acid was demonstrated in the studies of Deguchi, Morishita and Mutai [50] and Pompei et al. [50], but it was not detected for the considered strains. Although *Bifidobacterium bifidum* and *Bifidobacterium longum* subsp. *infantis* are classified as species/strains with a high level of biosynthetic properties of folic acid [50].

The B vitamins’ extracellular synthesis by lactobacilli was poor, especially when modern techniques such as capillary electrophoresis were applied. In our study, the ability to excrete thiamine, folic acid, and pantothenic acid was considered for some strains of lactobacilli. *L. salivarius* VKPM B-2214 is of interest due to its ability to produce up to 138.600 mg L$^{-1}$ of pantothenic acid.

Thus, *B. adolescentis* VKPM AC-1662 can be considered the best candidate for probiotics, since it is characterized by the greatest stress tolerance and a high autoaggregation percentage, as well as the ability to accumulate riboflavin and pyridoxine extracellularly. The application of folate-producing bifidobacteria has already been shown in rats [51] and humans [52]. On the other hand, data on the correlation of the synthesis of B vitamins in vitro and in the intestine are poor; additional research is required, which may involve significant methodological difficulties. Among the lactic acid bacteria, the greatest tolerance to low pH values was demonstrated by *L. sakei* VKPM B-8936; *L. plantarum* VKPM B-11007 showed tolerance to duodenal conditions, *L. acidophilus* VKPM B-2213 to pepsin, and *L. salivarius* VKPM B-2214 to pancreatin. The highest autoaggregation percentage was observed in *L. salivarius* VKPM B-2214, which also accumulated the most pantothenic acid of the studied strains. *L. casei* VKPM B-2873 and *L. rhamnosus* VKPM B-8238 was also characterized by the accumulation of thiamine and pyridoxine in addition to pantothenic acid. These strains of lactobacilli have the potential to be used as starter cultures in functional product manufacturing.

5. Conclusions

In this research, for the first time, the combined assessment of the probiotic and vitamin-producing potential of various bifidobacteria and lactobacilli species and strains was carried out in order to select potential probiotics with high stress tolerance and the ability to synthesize B vitamins. The selected strains with the best characteristics, such as *L. salivarius* VKPM B-2214, *L. casei* VKPM B-2873, and *L. rhamnosus* VKPM B-8238, can be used as potential starter cultures to increase B vitamins in a number of probiotic foods. *B. adolescentis* VKPM AC-1662 can be introduced in the diet as a probiotic, potentially capable to synthetize B vitamins in vivo in the intestine. The conducted studies of lactobacilli and bifidobacteria strains, devoted to the ability to extracellularly produce B vitamins in combination with the assessment of probiotic properties, can be considered the first stage in their introduction for commercial use. It should be pointed out that studies on B vitamin accumulation in the bacterial biomass, as well as their production in other nutrient media, have not been carried out in this work. Therefore, research into both the effect of various cultivation conditions on the accumulation of B vitamins (in order to increase their concentrations), and the fermentation of the strains in other nutrient media (including milk or plant substrates) should be undertaken in the future.

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