Development of bicomponent probiotic starter for dairy products

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Abstract.

Probiotics represent well-documented food supplements which benefit human health and appear frequently in dairy products. The beneficial health effects of *Limosilactobacillus reuteri* and *Lactobacillus acidophilus* make them crucial for developing functional and innovative food products. Standard microbiological techniques were employed to determine both the cellular density and antagonistic capabilities of lactobacillus cultures. The cultures were grown in different nutrient media under different environmental conditions. The research evaluated how these cultures affected growth inhibition and resistance to simulated gastrointestinal conditions and their physiological parameters were measured through titratable acidity tests against control samples.

The research focused on creating probiotic fermented milk drinks that fulfill technological requirements through optimization. The inoculum containing *Lm. reuteri, L. acidophilus,* and a control strain was added to pasteurized milk at 5% concentration. The titratable acidity measurements showed that the control sample and the formulations containing higher *L. acidophilus* concentrations produced more acid. The survival rates of different strains under simulated gastric juice (acidic pH) and mildly alkaline conditions (small intestine conditions) varied because some strains maintained viability through their adaptive mechanisms. The antagonistic tests evaluated both gram-positive and gram-negative bacterial strains. The probiotic samples displayed antibiotic-like properties with their highest activity occurring at the 24-hour incubation point. The results showed that a 1:1 ratio between *L. acidophilus* and *Lm. reuteri* produced efficient fermentation while also providing strong antagonistic effects against harmful microorganisms and high resistance to gastrointestinal stress. The research supports the creation of probiotic fermented milk beverages with improved health advantages through strategic probiotic strain selection and proportioning.

Keywords: probiotics, *Limosilactobacillus reuteri, Lactobacillus acidophilus,* fermentation, dairy products.

1. Introduction

The human skin together with oral cavities and the gastrointestinal tract maintains constant microbial contact with numerous microorganisms that spread across 400 square meters and number more than 500 bacterial species. These microbes help digestion while preventing pathogen entry and preserve immune system equilibrium. The goal of microbiota restoration stands as an essential therapeutic objective because antibiotics together with immunosuppressants and radiation disrupt the natural body balance of microorganisms.

The FAO/WHO defines probiotics as “live microorganisms which provide health benefits to hosts when consumed in proper amounts.” Medical applications focus on using these microorganisms to rebuild the body's natural gut microbial balance. *Lactobacillus* and *Bifidobacterium* together with *Escherichia*, *Enterococcus*, *Bacillus*, *Streptococcus* and *Saccharomyces* form the core probiotic genera. These microorganisms effectively treat pediatric

diarrhea and *Clostridium difficile* infections, and inflammatory bowel disease (IBD), and post-surgical complications through their role as immunomodulators by activating T cells and releasing cytokines and enhancing phagocytic activity and raising IgA levels (Przerwa et al., 2021).

Through the gut–brain axis probiotics produce neurotransmitters including GABA, serotonin, and acetylcholine to modify neural functions (Cryan et al., 2019; Socała et al., 2021). The development of "psychobiotics" for depression and anxiety, as well as autism spectrum disorder (ASD) and schizophrenia, is driven both by the observation of reduced microbial diversity in psychiatric disorders and by the fact that these substances avoid adverse side effects (Mörkl et al., 2020). ASD patients experience improvement in gastrointestinal and behavioral symptoms through probiotic intervention because of intestinal function improvement (Hurtado-Romero et al.).

The first probiotics, used in dairy products, consisted of lactic acid bacteria which continue to dominate research efforts (Chatterjee et al., 2021). The incorporation of fermented dairy and non-dairy products with lactic acid bacteria demands improved microbial cultivation methods together with regulatory approval and thorough quality monitoring practices (Aljadba et al.). *Limosilactobacillus reuteri* stands as a well-researched species which exists naturally within the human GIT as well as skin and urogenital tract and breast milk (Mu et al., 2018).

1. Antimicrobial activity.

Lm. reuteri is a bacterium that produces a range of antimicrobial products like reuterin, reutericillin, acetic and lactic acids, ethanol, and anti-inflammatory compound histamine (Amin et al., 2013). Due to these metabolites, the strain exhibits antimicrobial activity against a wide spectrum of pathogens like *E. coli, C. difficile* and *Salmonella* (Genís et al., 2017).

2. Reduction of lesions in gastric ulcer.

Helicobacter pylori infection serves as a primary cause of chronic gastritis and peptic ulcers and is a risk factor for gastric carcinoma. *Lm. reuteri* competes with *H. pylori* for glycolipid receptors on the gastric mucosa which leads to reduced bacterial growth and symptom relief for infected patients.

3. Control of body weight.

Lm. reuteri affects energy metabolism, increases the feeling of satiety, changes the composition of the microbiota, produces short-chain fatty acids, enhances intestinal barrier integrity and modulates bile acid metabolism (Foshati & Ekramzadeh, 2020). A close link between the microbiota and obesity has been proven (Daniali et al., 2020). In the study by Choi et al. (2021), *Lm. reuteri* strain MG5149 reduced body weight, adipose tissue, and adipocyte size by suppressing the expression of lipogenic proteins and regulating AMPK and acetyl-CoA carboxylase activity. At the same time, according to other reports, with excessive fructose consumption, *Lm. reuteri* can promote triglyceride accumulation (Huerta-Ávila et al., 2019).

4. Improved insulin sensitivity.

Consumption of *Lm. reuteri* SD5865 increases GLP-1 and GLP-2 levels, improves insulin sensitivity, stimulates insulin secretion, and inhibits GLP-1 degradation by DPP-IV enzyme. The Lr263 strain also reduces blood glucose, leptin, C-peptide and GLP-1 levels (Nadkarni et al., 2014).

5. Facilitating Liver Wellness

The early administration of *Lm. reuteri* DSM 17938 decreases liver enzyme levels (GGT, ALT, AST) and pro-inflammatory cytokines (IL-1, IL-2, IL-18) and pathogenic taxa and hepatic inflammatory gene expression according to Lo et al. (2014) and Jiang et al. (2021). The compound affects retinol metabolism and PPAR activation and oncogenesis-related pathways (Wong et al., 2017). The probiotics GMNL-89 and GMNL-263 reduce liver inflammation and apoptosis by modulating MAPK and NF-κB pathways (Gao et al., 2016). The maintenance of epithelial barrier integrity through short-chain fatty acid and polyamine and protein (p75, p40) prevents dysbiosis and liver disease (Matsumoto et al., 2011; Y. Wang et al., 2012). *Lm. reuteri* ZJ617 reduces intestinal permeability and hepatic cytokine release by blocking TLR4/MAPK/NF-κB and autophagy (Cui et al., 2019). The rat study by Hsu et al. (2017) demonstrated that Lr263 reduced ALT and AST levels while boosting antioxidant activity.

The IBD patient microbiome shows decreased diversity with elevated *Bacteroides* and *Enterobacteriaceae* and reduced *Firmicutes* with persistent inflammation (Wang et al., 2020). PAMPs detected by PRRs (TLR, NLR, CLR) activate proinflammatory signaling which serves as a primary mechanism in IBD (Sun et al., 2018). The prolonged activation of ERK1/2 causes *Lm. reuteri* to suppress IL-12 production (Özçam et al., 2019) while tryptophan catabolism produces indole-3-lactic acid to activate AhR and enhance IL-22 production which protects barrier integrity.

6. Use in Autism Spectrum Disorders (ASD)

There is no pharmacological intervention for primary symptoms of ASD, but *Lm. reuteri* has shown promise in relieving gastrointestinal and social dysfunction in ASD models (Santocchi et al., 2016). A pilot trial of Sgritta et al. (2019) demonstrated that three-month supplementation with *Lm. reuteri* (10^{10} CFU/day) improved behavioral scores

in children with ASD, likely by modulating the gut-brain axis. Higher levels of *Lm. reuteri* correlate with greater expression of the subunits of the GABA receptor and reduced antisocial behavior (Kong et al., 2020).

Interest in using *Lm. reuteri* in food matrix functionalization — such as in fermented, dairy, and non-dairy food products — continues to be high (Speranza et al., 2018). The strain possesses microbiota-modulating, immunomodulatory, neuroprotective, and metabolic activities that support its application in health-improving functional foods.

L. acidophilus remains the most commonly used probiotic species (Buck et al., 2005). It is highly adapted to harsh environments and performs homofermentation of sugars into DL-lactic acid. The *in vitro* characteristics of probiotics include acid and bile resistance, adhesion to colon cells, antimicrobial action, lactase production, and stability in food products (Khaleghi et al., 2010). *In vivo*, it enhances immunity, lowers serum cholesterol, enhances lactose utilization, and guards against infection (Bron et al., 2011). In short, *L. acidophilus* has a wide range of health benefits — from metabolic and gut-enhancing to immune and anticancer activities — making it an excellent ingredient in functional foods and drugs.

2. Methodology

The goal of the study was to develop a starter based on a combination of *Lm. reuteri and L. acidophilus*, which unites probiotic qualities with optimal technological characteristics, and to investigate the stability of products obtained in simulated conditions of the gastrointestinal tract.

The objectives of the study were:

1. To test the fermentation activity of milk with compositions with varying ratios based on *Lm. reuteri* and *L. acidophilus* in the dynamics for 48 hours.

2. To test the antagonistic activity of compositions of different composition based on *Lm. reuteri* and *L. acidophilus* in the dynamics for 48 hours against opportunistic species.

3. To test the resistance to the conditions of the gastrointestinal tract, namely to acidic conditions of the stomach and alkaline conditions of the small intestine of compositions of different composition based on *Lm. reuteri* and *L. acidophilus* in the dynamics for 48 hours.

For the preparation of seed material for *Lm. reuteri* and *L. acidophilus*, a bioreactor was utilised. Using smart sensors, pH was monitored at 6.5±0.1, temperature T=37±0.1°C. To prepare the inoculum, the overnight culture was suspended in sterile saline to a cell load of $1*10^9$ CFU/ml according to the density standard. The final control of the cell load of the studied cultures was performed according to the standard Koch method (Houchmandzadeh & Ballet, 2023) by culturing on MRS medium at T=37°C and t=24 h, followed by counting the resulting colonies.

To determine the antagonistic properties of the studied lactobacilli with the simultaneous introduction of test cultures, namely *E. coli*, *Sarcina lutea, Staphylococcus aureus* and *Proteus vulgaris*, the method for determining antibiotic activity by applying cross lines to the surface of meat-peptone agar was used. To do this, 0.1 ml of the suspension of the tested microorganisms was applied in a single line in the middle through the entire diameter of the Petri dish, and the test cultures were applied radially. The dishes were incubated in a thermostat at 37 °C for 48 hours.

The results were evaluated on a relative scale:

0 - no inhibition of test culture growth;

1 - test cultures grew well;

2 - test cultures grew partially;

3 - there was a significant inhibition of the growth of test cultures;

4 - test cultures were almost completely inhibited;

5 - test cultures were completely inhibited.

The cultivation of *Lm. reuteri* and *L. acidophilus* was carried out in pasteurized milk (2.5 % fat) at 37 °C for 48 hours in Erlenmeyer flasks with periodic shaking. Samples were taken after 8, 24 and 48 hours of cultivation.

The resistance of the tested samples to gastrointestinal conditions was determined according to the method developed by us, which maximally imitates the digestive process in the stomach and small intestine:

1. 1 ml of the sample was added to 9 ml of 0.1 N HCl and placed in a thermostat (T = 37 °C, incubation time 40±5 min).

2. 0.1 ml of the hydrochloric acid-incubated suspension was diluted 10^4-fold with saline. From the last test tube, 0.1 ml of the suspension was taken and applied in duplicate to Petri dishes.

3. 1 ml of the suspension incubated in acid was resuspended in 9 ml of sterile phosphate buffer with pH 7.4.

4. The suspension was incubated for 1 hour in a thermostat at T = 37 °C.

5. Incubated in phosphate buffer, the suspension was diluted 10^2-fold. The diluted suspension (0.1 ml) was applied to Petri dishes.

6. Cultivation of all Petri dishes was carried out for 48 hours at T = 37 °C.

7. After cultivation, the number of colonies was counted on a dark background of a counting board. The cell concentration was determined by the formula:

$$X=\frac{N*10^n}{V}, (1)$$

X - cell concentration, CFU/ml; N - average number of colonies in the inoculum of this dilution; 10 - dilution factor; n - serial number of the dilution; V - volume of the suspension taken for inoculation, ml.

The effective concentration ratio between the studied species was determined as follows:

1. Initial milk control test. The titratable acidity of the initial raw material was determined by the standard method according to DSTU 2661:2010 by titration with 0.1 N NaOH.

2. To compare certain physiological parameters of the studied lactobacilli, their cultivation was carried out in both pasteurized and sterilised milk. Sterilised milk was obtained by autoclaving at T = 121 °C for 30 min, p = 0.1 MPa.

3. 190 ml of milk was poured into the flasks and 10 ml of inoculum was added (Table 1):

Table 1. Ratio of the studied species in the inoculum

№ Sample	Species ratio (%)	
	Limosilactobacillus reuteri	*Lactobacillus acidophilus*
Sample 1	100	-
Sample 2	75	25
Sample 3	50	50
Sample 4	25	75
Sample 5	-	100

4. The starter culture Iprovit-SI2 (produced by the Institute of Food Resources of the National Academy of Agrarian Sciences of Ukraine), which includes *L. acidophilus* (sample No. 6), was used as a control sample.

Cell concentration of the inoculum:

- *Lm. reuteri* - $3.4*10^9$ CFU/ml.
- *L. acidophilus* - $4.1*10^9$ CFU/ml.
- Control - $1*10^{10}$ CFU/g.

The titratable acidity of milk was 18°T.

5. Fermentation was carried out in a thermostat at 37 °C, and the titratable acidity was determined at 8, 24 and 48 hours of the study in accordance with the method described in DSTU 2661:2010.

3. Results and Discussion

During the fermentation process, lactic acid bacteria alter the acidity of the raw material, which leads to a series of biochemical processes and, ultimately, to the formation of the final target product. Indicators of technological characteristics of industrial strains directly depend on their physiological and biochemical properties.

In previous studies (Chervetsova V. H. et al., 2022), the ability of *Lm. reuteri* and *L. acidophilus* strains to produce acid, as well as their potential combination in a two-component starter to produce a probiotic lactic acid beverage, was studied. As a control, aliquots of Galychyna yogurt, which is well established on the consumer market, were used. All samples were fermented with pasteurized milk, and titratable acidity was determined as the main indicator of the organoleptic properties of the resulting product after cultivation. The graph of changes in titratable acidity is shown in Figure 1.

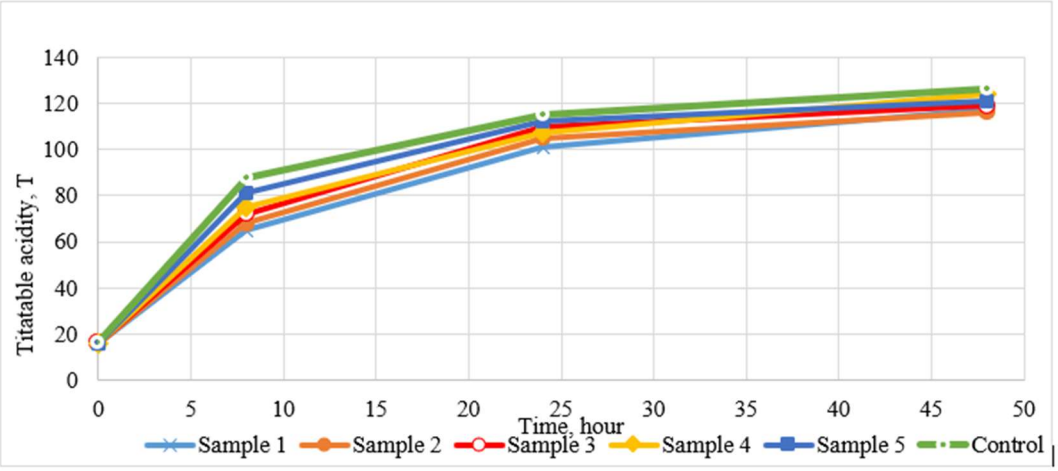

Fig. 1. Changes in titratable acidity

As can be seen from the data obtained, the best acid-forming capacity, i.e. the highest fermentation efficiency, was observed in the control sample. Samples № 4 and № 5 (with *L. acidophilus* concentrations of 75% and 100%, respectively) also had quite high values at the end of cultivation. Due to its physiological, biochemical and, above all, technological characteristics, this species proved to be a good acid-forming agent, which allows the creation of lactic acid beverages with optimal organoleptic properties.

Since probiotic fermented milk drinks pass through different parts of the human gastrointestinal tract, which differ in their physicochemical characteristics, model conditions of these parts were developed and their impact on the survival of lactobacilli in the final product was investigated. The incubation time of the samples corresponded to the generally accepted digestion time for humans.

Table 2. Resistance to gastric juice

Sample	8 hour		24 hour		48 hour	
	Initial bacterial titer	CFU/ml	Initial bacterial titer	CFU/ml	Initial bacterial titer	CFU/ml
1	$1,22*10^9$	$6,45*10^4$	$2,14*10^9$	$7,60*10^4$	$3,61*10^9$	$9,30*10^4$
2	$1,15*10^9$	$6,95*10^4$	$2,33*10^9$	$8,25*10^4$	$3,57*10^9$	$9,65*10^4$
3	$1,2*10^9$	$8,30*10^4$	$2,42*10^9$	$9,50*10^4$	$3,81*10^9$	$12,1*10^4$
4	$1,25*10^9$	$7,95*10^4$	$2,37*10^9$	$8,60*10^4$	$3,95*10^9$	$13,0*10^4$
5	$1,3*10^9$	$6,15*10^4$	$2,24*10^9$	$6,95*10^4$	$3,76*10^9$	$7,35*10^4$
6	$1,28*10^9$	$8,30*10^4$	$2,19*10^9$	$8,37*10^4$	$3,84*10^9$	$11,1*10^4$

The data obtained (Table 2) show that resistance to perchloric acid began to increase at the 24th hour of bacterial cultivation in all samples, and the maximum acid resistance was observed at the 48th hour of co-cultivation. Samples 3 and 4 (*Lm. reuteri/L. acidophilus* at ratios of 50/50 and 25/75, respectively) had the highest values.

The effect of further passage through various parts of the gastrointestinal tract, in particular the small intestine, was studied by transferring the samples to phosphate buffer with a pH 7.4. The viable lactic acid bacteria cell count (CFU/ml) showed a minor decrease, yet remained at a high level according to Table 3. Samples №1 (*Lm. reuteri/L. acidophilus* 100/0), № 2 (*Lm. reuteri/L. acidophilus* 75/25) and № 3 (Lm. reuteri/L. acidophilus 50/50) demonstrated the highest resistance to alkaline pH.

Table 3. Resistance to small intestine conditions

Sample	8 hour		24 hour		48 hour	
	Initial bacterial titer	CFU/ml	Initial bacterial titer	CFU/ml	Initial bacterial titer	CFU/ml
1	$1,22*10^9$	$4,10*10^4$	$2,14*10^9$	$6,31*10^4$	$3,61*10^9$	$6,83*10^4$
2	$1,15*10^9$	$2,53*10^4$	$2,33*10^9$	$3,58*10^4$	$3,57*10^9$	$5,17*10^4$
3	$1,2*10^9$	$1,50*10^4$	$2,42*10^9$	$4,29*10^4$	$3,81*10^9$	$10,5*10^4$
4	$1,25*10^9$	$5,83*10^3$	$2,37*10^9$	$2,46*10^3$	$3,95*10^9$	$1,80*10^4$
5	$1,3*10^9$	$2,19*10^3$	$2,24*10^9$	$8,34*10^3$	$3,76*10^9$	$4,62*10^4$
6	$1,28*10^9$	$1,34*10^4$	$2,19*10^9$	$1,55*10^4$	$3,84*10^9$	0

Antagonistic properties were determined for the following test microorganisms: *E. coli, S. lutea, St. aureus* and *Pr. vulgaris*. The results of the study are shown in Table 4.

Table 4. Determination of antagonistic activity (in relative units)

Sample	8 hour				24 hour				48 hour			
	E. coli	*S. lutea*	*St. aureus*	*Pr. vulgaris*	*E. coli*	*S. lutea*	*St. aureus*	*Pr. vulgaris*	*E. coli*	*S. lutea*	*St. aureus*	*Pr. vulgaris*
1	4±0,5	4±0,5	1±0,5	1,5±0,5	2,5±0,5	3±0,5	1,5±0,5	2,5±0,5	5±0,5	5±0,5	3,5±0,5	5±0,5
2	3±0,5	3,5±0,5	1±0,5	1,5±0,5	1,5±0,5	2±0,5	3,5±0,5	2±0,5	2±0,5	2±0,5	2±0,5	2±0,5
3	3±0,5	3±0,5	4±0,5	2±0,5	2,5±0,5	4±0,5	3,5±0,5	3±0,5	2,5±0,5	2,5±0,5	3,5±0,5	2,5±0,5
4	5±0,5	5±0,5	3±0,5	4±0,5	5±0,5	5±0,5	5±0,5	5±0,5	4,5±0,5	4±0,5	5±0,5	4,5±0,5
5	5±0,5	5±0,5	3,5±0,5	5±0,5	5±0,5	4,5±0,5	4,5±0,5	5±0,5	4,5±0,5	5±0,5	4,5±0,5	5±0,5
6	0±0,5	0±0,5	2±0,5	2,5±0,5	0±0,5	0±0,5	3,5±0,5	2,5±0,5	0±0,5	0±0,5	0,5±0,5	1,5±0,5

The results obtained indicate that samples 3, 4 and 5 (*Lm. reuteri/L. acidophilus* at ratios of 50/50, 25/75, and 0/100, respectively) have the best effect. We believe that the antagonistic activity against the test cultures is due to the synthesis of the antibiotic substance reuterin and other biologically active compounds produced by lactic acid bacteria.

Upon analysis of the processed data, it can be inferred that the optimal technological and probiotic attributes are evident in sample № 3, wherein the starter culture comprised *Lm. reuteri* and *L. acidophilus* in a ratio of 50/50.

4. Conclusion

1. The aim of the experiment was to determine the best ratio of *L. acidophilus* and *Lm. reuteri* to incorporate into starter cultures for fermented milk beverages. The hope was to create a formulation that would balance probiotic performance with desirable sensory properties. Different ratios were subjected to testing to determine how they would affect the fermentation process as well as any health benefits. Therefore, it was found that there is a combination that produces the most balanced performance of microbiological activity and quality of products.

2. The second part of the study involved assessing how the probiotic strains interacted with opportunistic microorganisms. The purpose of this assessment was to verify if the chosen combinations could stop the growth of potentially pathogenic bacteria. The tested cultures showed a detectable inhibitory effect, which proved their ability to enhance the microbial safety of fermented dairy products.

3. Among all the combinations tested, the best stable and effective results were in a 1:1 ratio of *L. acidophilus* with *Lm. reuteri*. This combination had the best fermentation kinetics, acidification capacity, and antimicrobial activity. Based on the above results, a culture of the balanced ratio can be considered as a promising candidate for a probiotic fermented milk drink with both technological efficacy and healthy properties.

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