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IN VITRO ASSESSMENT OF PROBIOTIC SURVIVAL IN SIMULATED GASTROINTESTINAL CONDITIONS AND ADHESIVE PROPERTIES

The study focused on evaluating the functional properties of four lactic acid bacteria (LAB) strains – *Lactobacillus fermentum* 30, *Lactobacillus cellobiosus* 36, *Lacticaseibacillus paracasei* 36/1, and *Lacticaseibacillus paracasei* 30/1 – and their associations, aiming to assess acid resistance, bile tolerance, enzyme stability, and adhesion capabilities using *in vitro* methods. The survival rates of these LAB strains and their associations were investigated under simulated gastric juice (SGJ) and simulated intestinal juice (SIJ). In SGJ, the association *L. fermentum* 30 + *L. cellobiosus* 36 showed higher survival rates compared to individual strains, with approximately 75% viability after 2 hours incubation. Similarly, the association *L. paracasei* 30/1 + *L. paracasei* 36/1 exhibited enhanced survival in SGJ, maintaining about 80% viability. In SIJ, both associations demonstrated improved survival compared to individual strains, with *L. fermentum* 30 + *L. cellobiosus* 36 and *L. paracasei* 30/1 + *L. paracasei* 36/1 maintaining approximately 70% and 75% viability, respectively. The adhesion capabilities of these LAB strains and associations were evaluated using human erythrocytes. All strains displayed high adhesive activity, particularly notable in *L. paracasei* 36/1 + *L. paracasei* 30/1, which demonstrated a significantly higher adhesion index compared to other strains. These findings highlight the robust survival and adhesive properties of the LAB strains and their associations under simulated gastrointestinal conditions. Further research is warranted to explore their potential applications in promoting gut health and combating gastrointestinal disorders.

Key words: probiotics, tolerance, adhesion, simulated gastric juice, simulated intestinal juice.

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Пробиотиктердің жасанды асқазан-ішек жағдайларында тіршілігін және адгезивтік қасиеттерін *in vitro* бағалау

Бұл зерттеу сүт қышқылды бактериялардың (СКБ) төрт штаммы – *Lactobacillus fermentum* 30, *Lactobacillus cellobiosus* 36, *Lacticaseibacillus paracasei* 36/1 және *Lacticaseibacillus paracasei* 30/1 – және олардың ассоциацияларының функционалдық қасиеттерін бағалауға бағытталған. Қышқылға және өтке төзімділік, ферменттерге тұрақтылық және адгезивтік қабілеттері *in vitro* әдістерін қолдану арқылы зерттелді. Осы СКБ штаммдарының және олардың ассоциацияларының өміршеңдігі имитацияланған асқазан шырыны (ИАШ) және имитацияланған ішек шырыны (ИІШ) жағдайларында зерттелді. ИАШ-да *L. fermentum* 30 + *L. cellobiosus* 36 ассоциациясы жеке штаммдарға қарағанда жоғары өміршеңдік деңгейін көрсетіп, 2 сағаттық инкубациядан кейін шамамен 75% өміршеңдігін сақтады. *L. paracasei* 30/1 + *L. paracasei* 36/1 ассоциациясы да ИАШ-да өміршеңдіктің жақсартылған көрсеткіштерін көрсетіп, шамамен 80% өміршеңдігін сақтады. ИІШ-да екі ассоциация да жеке штаммдарға қарағанда жоғары өміршеңдік көрсетті: *L. fermentum* 30 + *L. cellobiosus* 36 және *L. paracasei* 30/1 + *L. paracasei* 36/1 тиісінше шамамен 70% және 75% өміршеңдігін сақтады. Осы СКБ штаммдарының және олардың ассоциацияларының адгезивтік қабілеттері адамның эритроциттерін қолдану арқылы бағаланды. Барлық штаммдар жоғары адгезивтік белсенділікті көрсетті, әсіресе *L. paracasei* 36/1 + *L. paracasei* 30/1 айқын көрінетін адгезия индексімен басқа нұсқалармен салыстырғанда айтарлықтай жоғары көрсеткіштер көрсетті. Бұл нәтижелер СКБ штаммдарының және олардың ассоциацияларының асқазан-ішек

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жолын имитациялау жағдайларында тұрақтылығы мен адгезивтік қасиеттерін атап көрсетеді. Олар ішек денсаулығын қолдау және асқазан-ішек ауруларымен күресуде, әлеуетті қолданылуын зерттеу үшін қосымша зерттеулер қажет.

Түйін сөздер: пробиотиктер, төзімділік, адгезия, жасанды асқазан шырыны, жасанды ішек шырыны.

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Оценка выживаемости пробиотиков в имитированных условиях желудочно-кишечного тракта и исследование их адгезивных свойств *in vitro*

Данное исследование сосредоточено на определении функциональных свойств четырех штаммов молочнокислых бактерий (МКБ) – *Lactobacillus fermentum* 30, *Lactobacillus cellobiosus* 36, *Lacticaseibacillus paracasei* 36/1 и *Lacticaseibacillus paracasei* 30/1 – и их ассоциаций с целью оценки устойчивости к кислоте, толерантности к желчи, стабильности к ферментам и адгезивных способностей с использованием методов *in vitro*. Выживаемость этих штаммов МКБ и их ассоциаций исследовалась в условиях имитированного желудочного сока (ИЖС) и кишечного сока (ИКС). В ИЖС ассоциация *L. fermentum* 30 + *L. cellobiosus* 36 имела более высокий уровни выживаемости по сравнению с отдельными штаммами, сохраняя около 75% жизнеспособности после 2 часов инкубации. Ассоциация *L. paracasei* 30/1 + *L. paracasei* 36/1 также проявила улучшенные показатели выживаемости в ИЖС, поддерживая примерно 80% жизнеспособности. В ИКС обе ассоциации также продемонстрировали повышенную жизнеспособность по сравнению с отдельными штаммами: *L. fermentum* 30 + *L. cellobiosus* 36 и *L. paracasei* 30/1 + *L. paracasei* 36/1 сохраняя около 70% и 75% жизнеспособности соответственно. Адгезивные способности этих штаммов МКБ и их ассоциаций оценивались с использованием эритроцитов человека. Все штаммы проявили высокую адгезивную активность, особенно заметную у *L. paracasei* 36/1 + *L. paracasei* 30/1, которая показала значительно более высокий индекс адгезии по сравнению с другими вариантами. Эти результаты демонстрируют устойчивость и адгезивные свойства штаммов МКБ и их ассоциаций в условиях имитации желудочно-кишечного тракта. Дальнейшие исследования необходимы для изучения их потенциального применения в поддержке здоровья кишечника и борьбе с заболеваниями желудочно-кишечного тракта.

Ключевые слова: пробиотики, толерантность, адгезия, искусственный желудочный сок, искусственный кишечный сок.

Introduction

Probiotics are living microorganisms that, when consumed in adequate amounts, are beneficial to the human organism. The idea behind their use is that taking probiotics can help restore and strengthen the local intestinal microbiota, which largely contributes to maintaining the health of the entire gastrointestinal tract (GIT) and its resistance to colonization by pathogens [1, 2]. Probiotics are usually used in the form of biologically-active food additives, or even therapeutic drugs in the form of tablets, capsules, powders, and sachets. All these forms are applied orally and therefore enter the GIT. In order for probiotics to flourish in the intestine and impart their beneficial effects, they have to be able to survive passage through the host's hostile digestive tract environment [3]. The main factors that are detrimental to the survival of probiotics in the stomach are the low gastric pH and the antimicrobial action of pepsin [4]. Probiotic bacteria also need to survive the environment in the small intestine where it is exposed to pancreatin and bile salts.

So, the first tool in the selection of a strain of probiotic interest is represented by *in vitro* methods aiming to ascertain the ability to survive passage through the upper GIT and arrive alive at its site of action [5].

Adhesiveness, attachment to epithelial cells of the GIT, is one of the important properties of probiotic strains, therefore the determination of adhesive properties is considered a necessary step for the study of probiotic microorganisms. An important biological property of bacteria of the genus *Lactobacillus* is their adhesive activity, which allows them to colonize the intestinal biotope and successfully implement antagonistic properties against pathogenic and opportunistic microorganisms [6]. The study of these property is necessary to predict the probiotic effect of lactobacilli on the intestinal microbioceno-

sis of a particular individual. It has been established that adhesive ability is a strain-specific feature, which should be taken into account when selecting probiotic cultures [6]. *In vitro* model systems have proved efficient for providing a good measure on the adhesive ability of a potential probiotic.

The present study was designed to assess *in vitro* the acid resistance, bile tolerance, enzyme stability, and adhesion capabilities of four lactic acid strains and their associations.

Materials and methods

Bacteria and growth conditions. The probiotic associations studied in this research include different microbial cultures. All strains were deposited in the Republic Collection of Microorganisms (Astana, Kazakhstan).

The first association comprises the following microbial cultures – *Lactobacillus fermentum* 30 and *Lactobacillus cellobiosus* 36. Strain *L. fermentum* 30 was isolated from a population of freeze-dried culture *L. fermentum* 29, extracted from the intestine of a healthy person. It is represented by gram-positive, asporogenic, immobile bacilli sized 0.5-0.7x1.0-3.0μm with blunt ends. The cells are often solitary, though short chains may occur. The strain is nonpathogenic and exhibits high antimicrobial activity with a broad spectrum and increased resistance to dehydration. Strain *L. cellobiosus* 36 was isolated from a population of freeze-dried culture *L. cellobiosus* 35. It is a rod with rounded ends of variable size: 0.5-0.7x1.5-5.5μm. The bacilli are immobile, asporogenic, and gram-positive, arranged singly, in short chains (3-5 cells each), and sometimes in longer chains. This strain is also nonpathogenic and shows high broad-spectrum antimicrobial activity.

The second association includes *Lacticaseibacillus paracasei* 36/1 and *Lacticaseibacillus paracasei* 30/1. Strain *L. paracasei* 36/1 was isolated from a population of freeze-dried culture *Lactobacillus cellobiosus* 36. It is represented by gram-positive, asporogenic, immobile bacilli sized 0.5-0.7x1.5-5.5μm with blunt ends, often solitary, though short chains may occur. Strain *L. paracasei* 30/1 was isolated from a population of freeze-dried culture *L. fermentum* 30. It is represented by gram-positive, asporogenic, immobile bacilli sized 0.5-0.7x1.0-3.0μm with blunt ends, often solitary, though short chains may occur.

The probiotics and their associations were cultured in a medium with the following composition (g/L): glucose – 15.0, yeast extract – 5.0, meat extract – 5.0, peptone – 10.0, ammonium citrate – 2.0, sodium acetate – 2.0, potassium phosphate monobasic – 2.0, sodium phosphate dibasic – 2.0, magnesium sulfate – 0.2, manganese sulfate – 0.05, cobalt chloride – 0.01, pH 6.5-7.0. The cultivation was carried out in an incubator at 35°C for 24 hours. After, the culture broth was centrifuged at 4000× g for 15 minutes (using a laboratory centrifuge RS-6MC, Dastan, Bishkek, Kyrgyzstan). The cells were separated from the supernatant and washed twice with physiological saline. A cell suspension with a concentration of 10^9 CFU/g was prepared.

Survival in Simulated Gastric Juice (SGJ) and Simulated Intestinal Juice (SIJ). SGJ was prepared by dissolving pepsin in sodium chloride solution (0.2%, w/v) to a final concentration of 3 g/L, and pH was adjusted to 2 with hydrochloric acid. SIJ was prepared with the following composition (g/L): sodium chloride – 6.8 g/L, potassium chloride – 0.4 g/L, calcium chloride dihydrate – 0.2 g/L, sodium bicarbonate – 1.5 g/L, bile salts – 5.0 g/L, and pancreatin – 1.0 g/L. The pH was adjusted to 6.8. Both solutions were filtered for sterilization through a 0.45 μm membrane.

One aliquot (1 mL) from each suspension was placed in 10 mL of SGJ. The tubes were incubated on an orbital shaker incubator ES-20 (Biosan, Riga, Latvia) (150 rpm) at 37°C for 2 hours. The samples were collected after 2 hours in SGJ, transferred into 10 mL of SIJ, and incubated as described above for SGJ.

Surviving bacteria were enumerated by pour plate counts in MRS agar incubated at 35°C for 48 hours. The survival of probiotics was presented as the number of viable cells (log CFU/g). The following equation was used to calculate the survival rate % of bacteria cells:

$$\text{Survival rate } \% = \frac{\log \text{CFU/g after treatment}}{\log \text{CFU/g after treatment}} \times 100 \quad (1)$$

Determination of adhesive activity. Adhesion was studied *in vitro* using human erythrocytes according to the method of Brilis et al. [7]. The cell substrate consisted of formalinized human erythrocytes of group 0 (1) Rh (+), pre-washed with a buffer solution by centrifugation (1000× g for 10 min). A suspension of erythrocytes with a concentration of 100 million/mL was prepared in the specified buffer.

For the experiment, a drop of buffer solution was applied to a microscope slide, into which suspensions of erythrocytes and microorganisms were suspended. The slide with erythrocytes and mi-

crobes was placed in a moist chamber for 30 min at 37°C, then the preparation was dried at the same temperature, fixed by heat, and stained according to Gram. Adhesive properties were assessed using the average adhesion index (AAI) − average number of microbes attached to 1 erythrocyte when counting at least 25 erythrocytes, considering no more than 5 erythrocytes in one field of view. To assess the adhesive properties of the microorganisms, criteria such as C and IMA were also used. C (erythrocyte participation coefficient in the adhesion process) is the percentage of erythrocytes with adhered microbes on their surface. IMA (index of microorganism adhesion) is the average number of microbial cells on one participating erythrocyte, calculated by the formula:

$$\text{IMA} = \frac{\text{AAI x 100}}{\text{C}} \quad (2)$$

A microorganism is considered non-adhesive when IMA ≤ 1.75, low-adhesive from 1.76 to 2.5, moderately adhesive from 2.51 to 4.0, and highly adhesive when IMA is above 4.0.

Statistical analysis. Unless otherwise stated, all experimental groups were analyzed in triplicate. Experimental measurements are presented as mean and standard deviation (mean ± SD). The difference between groups was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. All statistical analyzes were performed using SPSS software (version 28.0, IBM Corp., Armonk, NY, USA). A p value <0.05 was considered statistically significant.

Results and discussion

The most important characteristic of probiotic resistance is the preservation of cell viability in the aggressive conditions of the GIT. Although the ultimate model for determining the functional effectiveness of probiotics is a human organism, this model has ethical limitations. Therefore, in most studies, an «artificial GIT» system is used, simulating the physicochemical conditions of the main parts of the digestive system [8–11]. This is usually a buffer in which the pH value characteristic of a particular department is maintained and various digestive enzymes are added.

To determine the effect of gastric and intestinal conditions, studies were conducted on the comparative survival of four probiotic strains and their associations in SGJ and SIJ. To determine cell survival count after sequential incubation, the cells were enumerated by pour plate counts in a nutrient medium. The design of this series of experiments is shown in Figure 1.

In the GIT, the entry of bacteria is restricted due to the acidic pH of the stomach and the antibacterial action of pepsin. Probiotics need to bear the gastric juice discharge in the stomach where the pH can be as low as 2 to provide medical advantages [12]. Therefore, acid tolerance is frequently used as a significant paradigm for probiotic strain choice. The viability of the probiotic associations and individual strains in SGF after 2 hours of incubation is shown in Figure 2.

In SGJ, the probiotic association of *L. fermentum* 30 + *L. cellobiosus* 36 shows the highest survival rate, maintaining higher viability compared to the individual strains. By 2 hours in SGJ, the probiotic association retained about 75% viability, whereas the individual strains of *L. fermentum* 30 and *L. cellobiosus* 36 dropped to around 60% and 55%, respectively. This indicates a positive effect when these strains are combined.

Similarly, the probiotic association of *L. paracasei* 30/1 + *L. paracasei* 36/1 demonstrates a higher survival rate in SGJ for the first two hours, retaining about 80% viability. In contrast, the individual strains, *L. paracasei* 30/1 and *L. paracasei* 36/1, decreased to around 65% and 60%, respectively.

Overall, probiotic associations tend to have higher ($p < 0.05$) survival rates in SGJ compared to individual strains. The *L. paracasei* association (*L. paracasei* 30/1 + *L. paracasei* 36/1) shows the best survival rate in SGJ, outperforming the *L. fermentum* and *L. cellobiosus* combination.

Acidic pH environments may inhibit metabolism and reduce the viability of lactic acid bacteria. This study is consistent with other works that have shown that upon exposure to gastric acid with a pH of 2, a significant reduction in the number of bacteria could be recognized [13, 14].

After passing through the stomach, probiotics enter the intestine, where they face new challenges to their survival and activity. Intestinal juice contains several components that can significantly impact the viability of probiotic microorganisms. Among these, bile acids and pancreatin play crucial roles. This makes it critical to ensure the resilience of probiotics in such conditions. Alameri et al. [15] mentioned that probiotics should possess good resistance toward bile salts in order to survive in the human GIT. Therefore, high survival percentages indicate good bile salt tolerance. Bile plays an important role in the specific and nonspecific intestinal defense mechanism of the

gut, and the severity of its inhibitory action is primarily determined by bile salt concentrations [16]. Pancreatin is a mixture of digestive enzymes, including proteases, lipases, and amylases, which can disrupt bacterial cell membranes and alter their metabolic activity. Pancreatic enzymes are essential for the normal digestion of carbohydrates, fats, and proteins, respectively [17]. Therefore, the capability to endure these enzymes is a measure for the selection of probiotic bacteria [18].

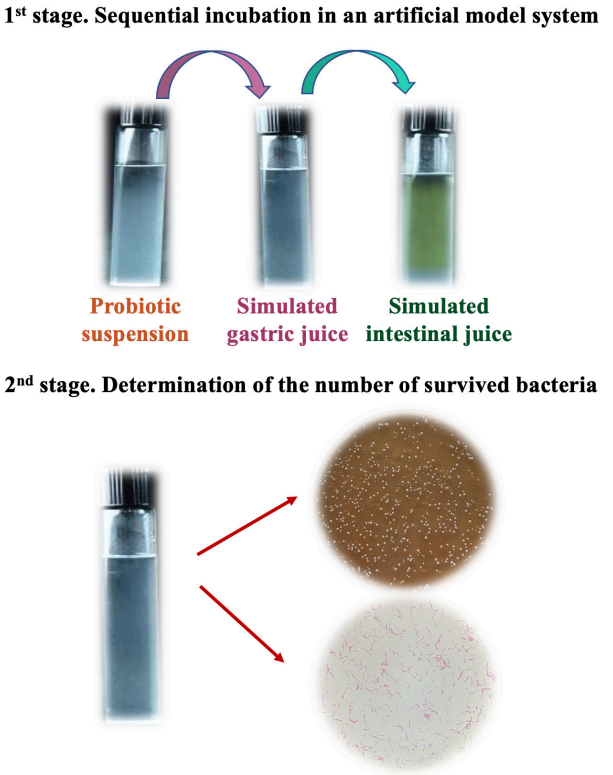

Figure 1 − Experimental design for the simulating upper GIT conditions

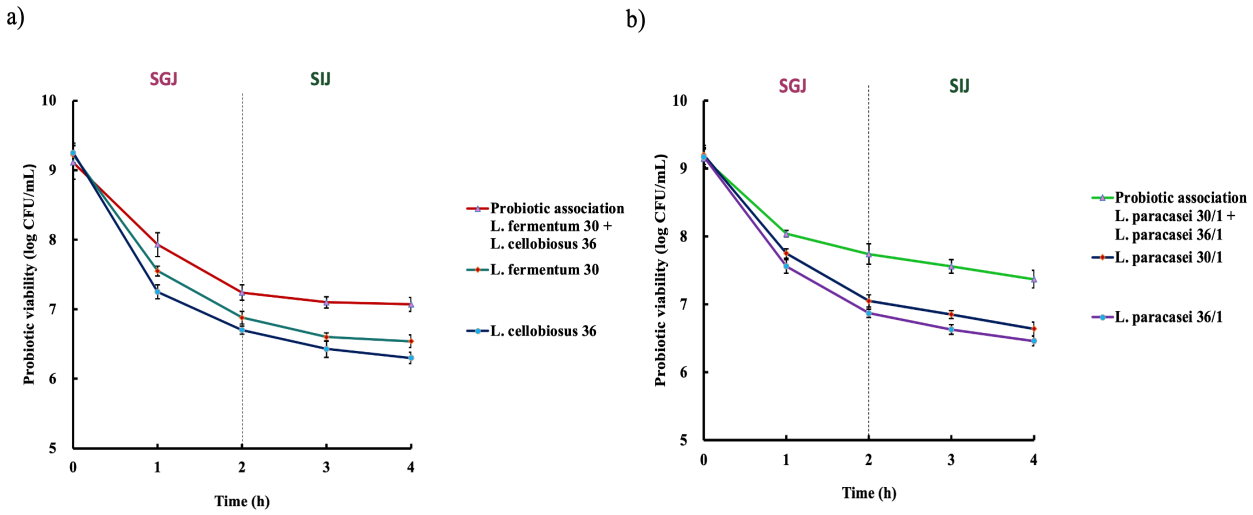

Figure 2 − Survival of probiotics in simulated gastric juice (SGJ) and simulated intestinal juice (SIJ):
a) probiotic association *L. fermentum* 30 + *L. cellobiosus* 36, *L. fermentum* 30, *L. cellobiosus* 36;
b) probiotic association *L. paracasei* 30/1 + *L. paracasei* 36/1, *L. paracasei* 30/1, *L. paracasei* 36/1

In SIJ, the probiotic association of *L. fermentum* 30 + *L. cellobiosus* 36 continued to show better survival compared to the individual strains, with a viability of approximately 70%. In contrast, the individual strains, *L. fermentum* 30 and *L. cellobiosus* 36, demonstrated about 60% and 55% viability, respectively.

The probiotic association of *L. paracasei* 30/1 + *L. paracasei* 36/1 maintained higher viability in SIJ as well, with about 75% survival. The individual strains, *L. paracasei* 30/1 and *L. paracasei* 36/1, had about 65% and 60% viability, respectively.

The effects of bile salts and pancreatin enzymes on the survival of probiotic cultures revealed that all have full tolerance to a concentration of 0.5% and 0.1% respectively. In general, the physiological concentration of human bile ranges from 0.3% to 0.5% [19]. Therefore, resistance to bile acid is an important characteristic that enables *Lactobacillus* to survive, grow, and remain active in the small intestine [20].

Both probiotic associations demonstrated enhanced survival in SIJ compared to the individual strains, but the improvement is more evident in SGJ. The *L. paracasei* association (*L. paracasei* 30/1 + *L. paracasei* 36/1) had the highest overall survival, indicating that combining probiotic strains can improve their resilience to gastric and intestinal conditions. The outcome of this work is consistent with the work done by Ribeiro et al. [21] which showed that the mixed probiotic culture had a high resistance after the passage through the gastrointestinal system.

Given that the most significant point of interaction between microorganisms and humans occurs on mucous membranes, understanding mucus adhesion is the primary target for controlling probiotic colonization. The most common method for quantitatively assessing mucus adhesion involves using a fluorescent indicator as a correlate of cell concentration. Surfaces are frequently modified by incubation with mucus or by culturing intestinal epithelial cells/tissue [22]. However, this method is expensive. Adhesion in *in vitro* conditions can also be studied by mixing human erythrocytes and microorganisms. This is a completely adequate experimental system, reflecting a positive correlation between the adhesiveness of lactobacilli and their colonizing ability [23, 24].

To quantify this activity, the microbial adhesion index was used, where low adhesion corresponds to an index value from 1.76 to 2.5, medium – from 2.51 to 4.00, and high – to an index value greater than 4.

The results obtained showed that all tested strains and probiotic associations demonstrated a high degree of adhesion with an index greater than 4 (Figure 3).

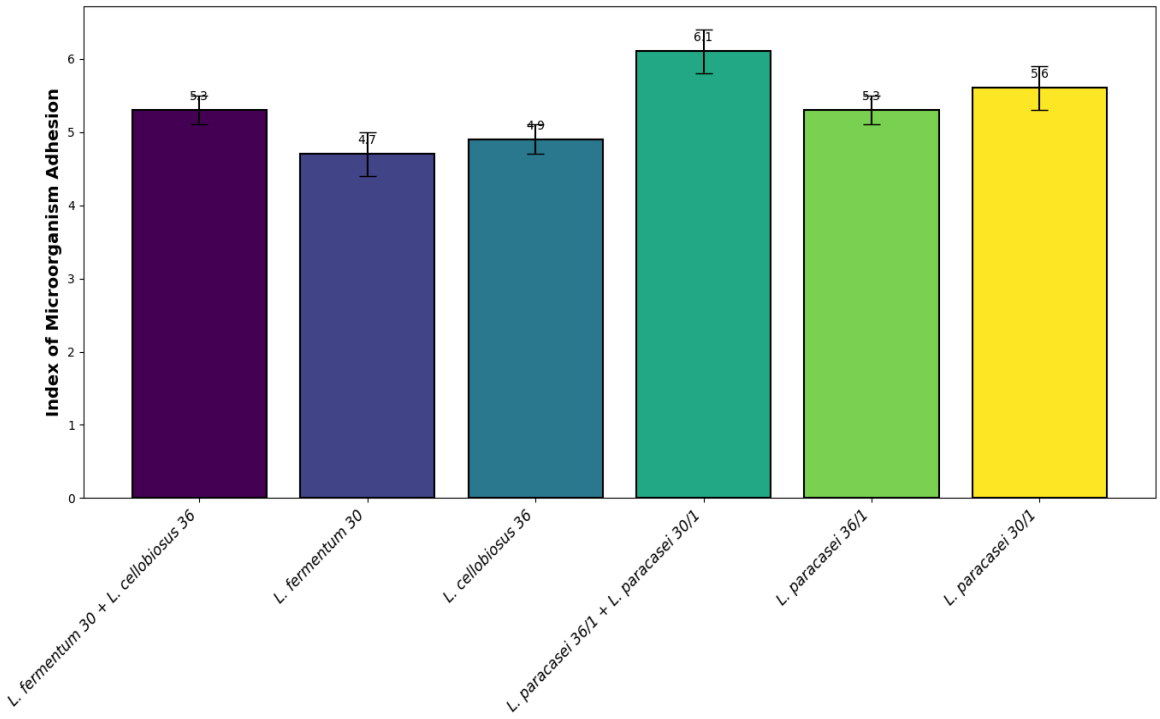

Figure 3 − The degree of adhesive activity of probiotic strains and their associations

The probiotic association *L. paracasei* 36/1 + *L. paracasei* 30/1 demonstrated the highest adhesive activity, which was statistically significant ($p < 0.05$) compared to the other strains and association. The higher adhesive activity of this probiotic association compared to the other one may be attributed to complementary surface proteins or the release of substances that enhance mutual adhesion, such as exopolysaccharides. Each strain may possess unique surface characteristics or adhesion mechanisms that, when combined, result in a synergistic effect [25], enhancing their ability to adhere to erythrocytes. This synergistic interaction could lead to a stronger and more stable adhesion, ultimately resulting in higher adhesive activity.

Conclusion

This study aimed to assess the survival under simulated gastrointestinal conditions and adhesive properties of selected LAB strains *in vitro*. The tested strains demonstrated robust survival rates in SGJ and SIJ, indicating their resilience to the harsh conditions of the GIT.

Specifically, the association of *Lacticaseibacillus paracasei* 36/1 + *Lacticaseibacillus paracasei* 30/1 showed superior survival rates in both SGJ and SIJ compared to individual strains, suggesting a synergistic protective effect when combined.

Moreover, the adhesive capabilities of *L. paracasei* 36/1 + *L. paracasei* 30/1 were noteworthy, indicating their potential to adhere effectively to gastrointestinal epithelial cells. Adhesion is critical for probiotics to colonize the gut mucosa and exert beneficial effects.

The findings highlight the promising probiotic potential of *L. paracasei* 36/1 + *L. paracasei* 30/1 due to their robust survival in simulated GIT conditions and strong adhesive properties. Further investigations, particularly *in vivo* studies, are warranted to explore their full potential as probiotics for promoting gut health and preventing gastrointestinal disorders.

Conflict of interest

All authors have read and are familiar with the content of the article and have no conflict of interest.

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