**Original Article**

Co-Aggregative Effect of Probiotics Bacteria against Diarrheal Causative Bacteria

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Abstract

Probiotics have been used for over a century to prevent and treat diseases. They can reduce the effects of gastroenteritis and are now used to treat acute diarrhea. This study aimed to evaluate the co-aggregative effects of probiotics bacteria against diarrheal causative bacteria. For this purpose, 11 isolates of probiotic bacteria were used in the current study, including three _Lactobacillus plantarum_, one _Lactobacillus gasseri_, two _Lactobacillus fermentum_, three _Lactobacillus acidophilus_, and two _Lactococcus garvieae_ isolates. All isolates were tested for antibiotic susceptibility, autoaggregation ability, adhesion ability, antibacterial activity, acid tolerance, and bile salts tolerance. The results showed that most of them had the ability to autoaggregate after 4 h, with the highest percentage of 57.14% for _L. fermentum_. For the antibiotic susceptibility test, all the isolates showed resistance against trimethoprim/sulfamethoxazole, except one isolate. Moreover, all the isolates, except one, were susceptible to both vancomycin and tetracycline. All tested isolates had adhesion ability with different survival rates, which reached 34.57% for _L. plantarum_ in acidic conditions. Besides, the highest survival rate was 85.17%, which belonged to _L. garvieae_, for bile salt tolerance. Probiotic isolates had an antibacterial effect against diarrhea-causative bacteria with an inhibition diameter of 17-49 mm for different _Lactobacillus_ spp. and _Lactococcus_ spp. isolates. Furthermore, the co-aggregation ability of probiotic isolates against diarrhea-causative bacteria was studied, and results showed that probiotic isolates had a co-aggregative effect against diarrhea-causative bacteria, _Escherichia coli_, _Shigella sonnei_, and _Providencia alcalifaciens_, after 24 h of incubation. The highest co-aggregative effect of probiotics isolates belonged to _L. fermentum_ and _L. acidophilus_ against _P. alcalifaciens_ with a co-aggregation percentage of 100%, while the lowest co-aggregation rate was 14.29% against _E. coli_. The findings revealed the probiotic properties and co-aggregative effects of probiotic bacteria against diarrhea-causative bacteria.

Keywords: Auto aggregation, Diarrhea, _Lactobacillus spp., Lactococcus spp.,_ Probiotics bacteria

1. Introduction

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (1). The history of probiotics dates back to the first use of cheese and fermented products, which were well known to the Greeks and Romans who recommended their consumption (2). Probiotics have been used to prevent and treat diseases for more than a century, and their use has lately increased (3). Probiotic medication may mitigate the effects of gastroenteritis in resource-constrained environments (4). Probiotics treat acute diarrhea as they activate immunological signaling pathways, produce anti-pathogenic factors, and cause the host to secrete them to combat enteric infections. Before they can be used as probiotics to regulate intestinal flora, the safety and digestive system stability must be ensured (3, 5).

Gram-positive probiotic bacteria, like _Lactobacillus_ spp., have been widely used to treat and prevent diarrhea caused by gastrointestinal infections (6).

Lactobacillus spp., *Lactococcus* spp., and *Leuconostoc* spp. have effectively prevented and treated various diarrheal diseases. Anti-infective mechanisms of probiotics include regulation of the gut microbiota, promotion of intestinal barrier function, competition for adhesion and nutrition, modulation of the host immune system, and synthesis of antimicrobial compounds (7).

In general, probiotic bacteria, which are beneficial to health, are best obtained from a human host and should have resistance to gastrointestinal acids and bile. They were classified as Generally Recognized as Safe (GRAS). These bacteria are characterized by their ability to produce antimicrobial substances and compete with pathogens to prevent their colonization of the intestine, and humans are the preferable source for them (8).

Most diarrheal infections are caused by pathogenic bacteria, such as *Escherichia coli*, which cause gastrointestinal tract infections in developed countries, with symptoms, including stomach pain, bloating, and moderate diarrhea (9). In addition, it can cause illness in humans and animals and contain pathogenic genes. Moreover, it can cause an infection after intestinal adherence by fimbriae (10). An estimated 160,000 deaths per year across all age groups are attributed to *Shigella* spp., a major cause of diarrhea among young children in developing nations. It should also be mentioned that the strain *Shigella sonnei* causes shigellosis disease in developed nations (8). In this regard, the current study aimed to detect probiotic properties and the co-aggregation effects of probiotic bacteria against diarrhea-causative bacteria.

2. Materials and Methods

2.1. Bacterial Isolates
2.1.1. Probiotic Bacteria

This study included 11 isolates of probiotics bacteria, namely *Lactobacillus gasseri* (Lb1), *Lactobacillus acidophilus* (Lb2, Lb3, and Lb4), *Lactobacillus plantarum* (Lb5, Lb6, and Lb7), *Lactobacillus fermentum* (Lb8 and Lb9), and *Lactococcus garvieae* (Lc1 and Lc2). These isolates were identified by the

Vitek 2 system (bioMérieux, France) and obtained from the Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.

2.1.2. Diarrhea Causative Bacteria

Five diarrhea causative isolates, including *E. coli* (11,38,41), *S. sonnei* (1), and *Providencia alcalifaciens* (1), were isolated from children with diarrhea aged between 3 days and 11 years old who visited hospitals in Baghdad, Iraq. These isolates were identified by cultural, microscopical, and biochemical tests as well as a Vitek 2 system.

2.2. Detection of Probiotic Properties
2.2.1. Acid tolerance

For this experiment, 10 ml of De Man, Rogosa, and Sharpe (MRS) broth was inoculated with 0.1 ml of an overnight culture of *Lactobacillus* spp. and *Lactococcus* spp. isolated (9×10^8 CFU/ml, O.D600 0.134), which had adjusted to a pH of 2.5 with 1N HCl. Subsequently, the mixture was put into a CO_2 incubator at 37 °C for 3 h. Afterward, the number of viable bacteria was determined by plating serial dilutions on the MRS agar. Colony numbers of isolates on MRS agar were compared and the results were used to evaluate their acid tolerance. The percentage of bacteria that survived after being incubated for 0 and 3 h on MRS agar was calculated using the plate count method. The survival rate (%) was defined as the comparison of the number of viable colonies after incubation (N) with the initial number of viable colonies (N_0) (11):

Survival rate (%) = $[N/N_0] \times 100\%$
(N: the number of colonies after 3h), (N_0: the number of viable colonies at zero time)

2.2.2. Bile Salts Tolerance

At this stage, 100 µl (9×10^8 CFU/ml, O. D_{600} 0.134) of *Lactobacillus* spp. and *Lactococcus* spp. were inoculated in 10 ml of MRS broth and MRS-bile salt broth with and without 0.3% (w/v) bile salt and incubated at 37 °C for 3 h. After incubation, the number of viable bacteria was determined by plating successive dilutions on MRS agar. The survival rate

was determined using the plate count method at 0 and 3 h post-inoculation. The survival rate (%) was calculated by comparing the number of surviving colonies (N) after incubation with the original number of surviving colonies in the control group (N_c) (12):

Survival Rate (%) = [N/N_C]×100%

(N: the number of colonies after 3 h in bile salt availability), (N_C: the number of colonies without bile salt for control)

2.2.3. Adhesion Ability (Congo Red Binding Assay)

The *Lactobacillus* spp. and *Lactococcus* spp. isolates were cultivated by streaking on the prepared MRS-congo red agar and incubated in a CO_2 incubator at 37 °C for 48-72 h. The red colonies indicate positive results for congo red-bound cells (13).

2.3. Antibiotic Susceptibility Test

Antibiotic susceptibility tests for 10 different antibiotics, including ampicillin, ciprofloxacin, cefotaxime, cefoxitin, gentamicin, imipenem, meropenem, trimethoprim/sulfamethoxazole, and vancomycin, were tested using the Kirby-Bauer method, as described by WHO, and according to the guidelines established by the Clinical Laboratory Standards Institute (2021) (14). Moreover, the susceptibility or resistance of an isolate to a given antibiotic was determined by measuring the diameter of the inhibition zone that formed around the discs in millimeters.

2.3.1. Antibacterial Activity (Well-Diffusion Method)

Antibacterial activity of *Lactobacillus* spp. and *Lactococcus* spp. isolates were studied using the well-diffusion method. The MRS broth was inoculated with 2% of bacterial isolates ($9×10^8$ CFU/ml, absorbance: 0.134 OD_{600}) for 48 h in a CO_2 incubator at 37 °C. After the incubation period, these cultures were centrifuged in a cooling centrifuge at 4,000 rpm for 30 min to obtain the supernatant, then sterilized with Millipore filter paper (0.22 µm). Mueller-Hinton agar was prepared and cultivated with 100 µl of diarrhea-

causative bacteria using a glass rod spreader. Subsequently, the wells (6 mm in diameter) were formed after that, and all of them were filled with sterilized supernatants of probiotics isolates, while the control well was filled with only a sterilized MRS broth. The plates were incubated for 24 h at 37 °C, and the inhibition zones around the wells were measured in millimeters using a metric ruler (15, 16).

2.4. Auto-Aggregation Assay

2.4.1. Visual Assay

Overnight growth of probiotics bacteria ($9×10^8$ CFU/ml, absorbance: 0.134 OD_{600}) was inoculated in MRS broth and incubated aerobically for 24 h at 37 °C. After the incubation period, the tubes were vortexed for 10 s and left statically for 4, 9, and 24 h at 37 °C, and the results were observed. Positive results show sedimentation at the bottom of the tubes, while negative results show turbidity (17).

2.4.2. Spectrophotometry Assay

The bacterial growth culture was prepared as mentioned in the visual assay section. After that, 0.1 ml of the upper portion was transferred to a tube containing 3.9 ml of phosphate buffer saline (PBS) solution and shaken; afterward, the OD_{600} nm was recorded. The other part was left statically for 4 h at 37 °C. After 4 h, 0.1 ml of the transparent upper part was transferred to other tubes that contained 3.9 ml PBS and the OD 600 was recorded after shaking each tube (18). The auto-aggregation percentage was recorded according to Jánošíková, Pálková (19):

(Auto-aggregation) % = [A_0 - A/A_0] × 100%

(A_0): Initial OD of each isolate, recorded before stagnation.

(A): Final OD of each isolate, recorded after 4, 9, and 24 h.

2.5. Co-Aggregative Effect of Probiotics Bacteria against Diarrhea Causative Bacteria

The bacterial inoculum of *Lactobacillus* spp. and *Lactococcus* spp. isolates and pathogenic bacteria were

prepared in an auto-aggregation assay. Moreover, 2 ml of the pathogenic bacteria inoculum was separately mixed and vortexed with *Lactobacillus* spp. and *Lactococcus* spp. inoculum in a tube for 10 s. The control tubes contained 4 ml of probiotic and 4 ml of pathogenic bacteria separately, each vortexing for 10 s. All the tubes were left statically for 4 and 24 h at 37 °C. After 4 and 24 h, the OD_{600} was recorded for 0.1 ml from the clear upper portion, which was mixed with 3.9 ml of PBS (17). The percentage of co-aggregation was calculated according to the equation described by Motey, Owusu-Kwarteng (16).

$$(Co-aggregation)\% = \frac{(Ax + Ay/2) - A(x+y)}{(Ax + Ay/2)} \times 100\%$$

Ax: OD for probiotic bacteria.

Ay: OD for diarrhea causative bacteria.

A(x+y): OD for probiotic bacteria and diarrhea-causative bacteria mixture.

3. Results and Discussion

3.1. Detection of Probiotic Properties

3.1.1. Acid Tolerance

The acid tolerance of 11 probiotic bacteria isolates was evaluated on MRS agar with a pH of 2.5, as shown in figure 1. The acid survival rates of Lb1, Lb2, Lb3, Lb4, Lb5, Lb6, Lb7, Lb8, and Lb9 were 4.32%, 5.97%, 14.54%, 3.58%, 29.29%, 34.57%, 2.58%, 2.9%, and 4.16%, respectively (Figure 1). Moreover, the acid survival rates of *Lactococcus garvieae* (Lc1 and Lc2) were 1.72%, and 9.65%, respectively, as illustrated in figure 1. The best tolerance rates belonged to the Lb5 and Lb6 isolates (29.29% and 34.57%, respectively), which were higher than those of the other studied probiotic isolates (20). According to Sadeghi, Panahi (21), Lb5 and Lb6 were mildly resistant (10-16%) and sensitive (>10%), respectively. Moreover, four groups have been mentioned for the survival rate for acid tolerance. It should be noted that the tolerance of *L. plantarum* for acidic environments varies by species and strain.
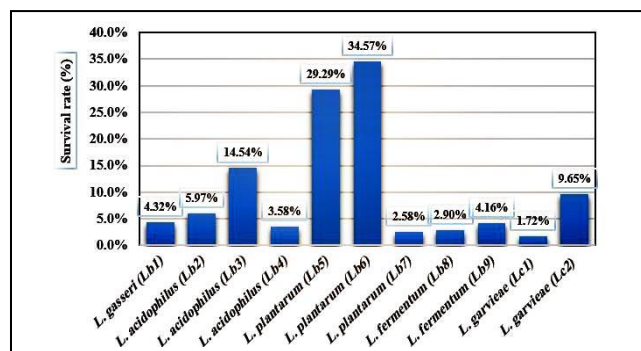

Figure 1. AcidTolerance and survival rate of probiotics bacteria

Liu, Xu (22) mentioned that the probiotic bacteria should resist and adapt to the intestinal acids for colonization of the cellular layer at low pH values (pH=2.5). This condition may affect the cellular layer (dynamical proton pump, reactions of tolerating acids, and repairing) as well as the mechanisms of acid tolerance generation and preservation to the macro-molecules.

In their study, Ayyash, Abdalla (20) mentioned the proton flow mechanism for the protection against acidic conditions, in which the proton flows in large amounts and the homeostasis maintenance becomes low. This disturbance in the homeostasis inside the cells causes energy draining. The acids cause denaturation of protein, DNA, and physiological and biochemical processes, damage to the membrane, and cellular death.

3.1.2. Bile Salts Tolerance

The results showed that the probiotic isolates were grown in the MRS broth with 0.3% of bile salt with different survival rates. Accordingly, the survival rates of Lb1, Lb2, Lb3, Lb4, Lb5, Lb6, Lb7, Lb8, and Lb9 were 45.19%, 17.87%, 12.79%, 29.65%, 11.46%, 3.03%, 48.29%, 36.09%, and 53.8%, respectively. Moreover, Lc1 and Lc2 showed high survival rates (51.87% and 85.17%, respectively), compared to the other probiotic isolates, all of which showed mild resistant rates against the bile salts, except Lb6, which was sensitive to the bile salts and had a survival rate of 3.03%, as shown in figure 2.
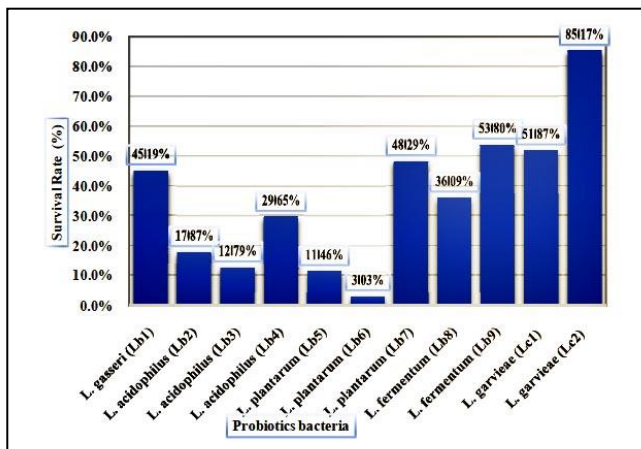
Figure 2. Bile salt tolerance of probiotics bacteria

Khromova, Epishkina (23) reported that all the growing colonies of probiotic bacteria on the agar with bile salt were less than those on the control plates (without bile salt). The probiotic bacteria isolates have many ways to resist the bile salts, such as alterations in the structural components of lipids, pumps regulating the flowing of these salts, and preservation enzymes, such as hydrolase. The affinity of bile salts to lipids (high hydrophobic) makes it target the probiotic bacteria cellular structural lipids and cause changes in it. This affects their cellular shapes and changes their external membrane which is associated with cell protection and resistance (5).

3.1.3. Adhesion Ability (Congo Red Binding Assay)

The results revealed that all the 11 studied probiotic isolates were grown on the congo red MRS agar surface with red color colonies within 48-72 h, as shown in figure 3. The appearance of the red colonies indicated the adhesion ability of probiotics bacteria. Ambalam, Kondepudi (13) mentioned that protein structures on the outer layer of bacterial cells play a role in adhesion and biofilm development. Accordingly, the bright red-colored appearance of colonies is due to the S-layer, which plays a role in the adhesion to the cells of the intestine.

Alp, Kuleaşan (24) reported that the S-layer, fimbriae, lipoteichoic acid, saccharides, and proteins for mucoid binding are found on the outer membrane of the cell used for adhesion. This layer is related to the characteristics of probiotics (adhesiveness, aggregation ability, and microorganism suppression) and does not affect the adhesive characteristics of all the bacteria. This has led to the belief that there are other adhesion compositions. Affhan, Dachang (25) reported that CR plays a role in bacterial adhesiveness and biofilms, which bind with the outer saccharides of bacterial cells.
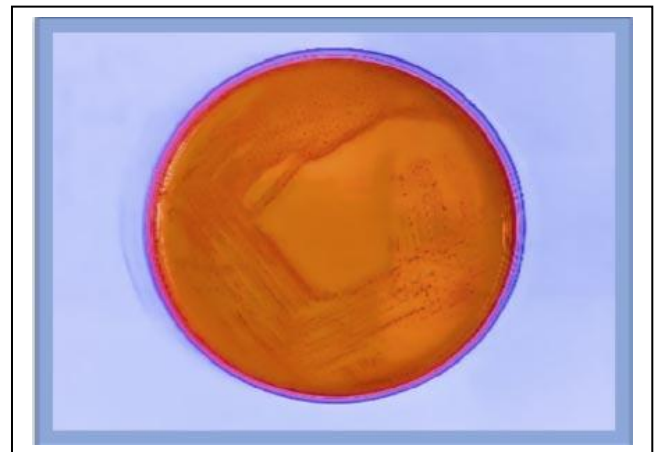
Figure 3. The appearance of probiotics bacterial colonies on Congo Red-MRS agar after (48-72) h at 37 °C

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3.2. Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed on 11 probiotics isolates, one *L. gasseri* (Lb1), three *L.*

acidophilus (Lb2, Lb3, Lb4), three *L. plantarum* (Lb5, Lb6, Lb7), two *L. fermentum* (Lb8, Lb9), and two *L. garvieae* (Lc1, Lc2), by using disc diffusion test, for 10 antibiotics from different classes. These antibiotics included carbapenem class (imipenem and meropenem), cephalosporin (cefotaxime and cefoxitin), folate pathway (trimethoprim-sulfamethoxazole), penicillins (ampicillin), tetracyclines (tetracycline), fluoroquinolones (ciprofloxacin), cephamycins (cefoxitin), glycopeptide (vancomycin), and quinolones (gentamicin). The results showed that the isolates had a variable resistance to the tested antibiotics (Table 1).

Most of the studied probiotic bacteria showed resistance to antibiotics (cefotaxime, gentamicin, and trimethoprim-sulfamethoxazole). Furthermore, most of them were sensitive to ampicillin, vancomycin, imipenem, and tetracycline. Dobreva, Koprinarova (26) showed that the resistant genetic material against beta-lactam antibiotics is transferable. Moreover, four, seven, six, one, and six isolates were resistant to ampicillin, cefotaxime, cefoxitin, vancomycin, and ciprofloxacin, respectively. This resistance is due to a lack of permeability or disturbance in the cellular wall and multi-drug carriers. Furthermore, the resistance against the quinolones group is due to the cellular wall construction, permeability, and influx mechanisms (26).

Based on the findings, most of the studied isolates showed resistance against gentamicin. This is consistent with the results of a study performed by Kim, Lee (5). Dobreva, Koprinarova (26) and Thumu and Halami (27) in their studies found two resistant genes against tetracycline in probiotic bacteria *tet*(M) and *tet* (S). The probiotic bacteria could be used as a treatment for intestine disease due to their resistance to antibiotics (28). The ability to resist antibiotics has many benefits since diseases affect the antibiotic by changes in the microflora and their imbalance in the gut.

3.2.1. Antibacterial Activity (Well-Diffusion Method)

The antibacterial activity of probiotic bacteria was detected using the suitable diffusion method. The results showed that the probiotic isolates had an antibacterial effect against diarrheal causative bacteria with an inhibition diameter of 17-49 mm for different *Lactobacillus* spp. and *Lactococcus* spp. isolates (Table 2).

Table 1. Antibiotic susceptibility test for probiotics bacteria. R: Resistant. S: Sensitive. I: Intermediate

Antibiotic Bacterialisolates	Meropenem	Vancomycin	Imipenem	Cefoxitin	Gentamicin	Cefotaxime	Ciprofloxacin	Tetracycline	Ampicillin	Trimethoprim /Sulfamethoxazole
L.gasseri (Lb1)	S	S	S	S	R	R	S	S	S	R
L. acidophilus (Lb2)	S	S	S	S	I	R	S	S	S	R
L. acidophilus (Lb3)	S	S	S	S	I	S	S	S	S	R
L. acidophilus (Lb4)	I	S	I	I	R	R	I	S	I	R
L. plantarum (Lb5)	S	S	S	S	R	R	I	S	S	R
L. plantarum (Lb6)	I	S	S	I	R	S	I	S	I	R
L. plantarum (Lb7)	S	S	S	S	R	S	S	S	S	R
L. fermentum (Lb8)	S	I	S	R	R	I	I	I	R	R
L. fermentum (Lb9)	S	S	S	S	I	S	S	S	S	R
L. garvieae (Lc1)	I	S	S	I	I	R	I	S	S	R
L. garvieae (Lc2)	I	S	I	R	S	S	I	S	S	S

Table 2. The inhibition diameter of the probiotics bacteria (CSF) against diarrheal causative bacteria

Probiotic Bacteria Pathogenic Isolates	*L. gasseri* (Lb1)	*L. acidophilus* (Lb2)	*L. acidophilus* (Lb3)	*L. acidophilus* (Lb4)	*L. plantarum* (Lb5)	*L. plantarum* (Lb6)	*L. plantarum* (Lb7)	*L. fermentum* (Lb8)	*L. fermentum* (Lb9)	*L. garvieae* (Lc1)	*L. garvieae* (Lc2)
	Inhibition diameter (mm)										
E. coli (3)	32	33	35	32	31	36	34	37	34	36	39
E. coli (7)	28	19	31	24	27	33	21	16	35	26	26
E. coli (11)	36	38	17	36	26	21	17	37	26	18	29
E. coli (14)	35	24	35	34	33	36	36	32	33	31	36
E. coli (35)	31	36	49	38	35	48	41	35	36	38	39
E. coli (41)	26	26	21	25	21	26	41	28	24	36	22
S. sonnei (3)	36	36	36	39	38	37	35	38	42	34	36
S. sonnei (4)	18	23	23	41	21	33	31	19	33	33	41
P. alcalifaciens (1)	35	26	22	27	38	21	23	34	30	24	26

Lin, Hsieh (29) mentioned that the diameter size for the inhibition zone was divided into three categories, namely small (11-16 mm), moderate (17-22 mm), and large (>23 mm). Kaewchomphunuch, Charoenpichitnunt (30) reported that the probiotic isolates could inhibit the growth of diarrhea-causative bacteria and that the cell-free supernatant has more antimicrobial activity than the filtrated elements due to the presence of various materials. Affhan, Dachang (25) indicated that the cell-free supernatant obtained from probiotic bacteria has a protective effect against diarrhea-causative bacteria, preventing the adherence ability of the host cells and forming a biofilm. Many methods and products have shown antibacterial effects, such as H_2O_2, protein complexes (fatty and lactic acids), phenol, and competition for nutrients (31).

The inhibitory mechanism of lactic acid may lead to the solubility of non-dissociated lactic acid inside the cytoplasm membrane and the low solubility of dissociating lactate, which causes acidic cytoplasm and proton motive force failure. It affects the transmembrane pH gradient and cell growth energy. The antimicrobial activity was observed in the acetic and lactic acid, especially in *L. plantarum*, as reported by Wang, Ma (32).

3.3. Auto-Aggregation Assay

The results of the visual assay of aggregation probiotic bacteria were observed after 4, 9, and 24 h for 11 isolates at 37 °C in the aerobic condition. When the bacteria were suspended in constant broth culture and left in a stand for 4, 9, and 24 h, the bacterial isolates aggregated with each other and settled down at the bottom of the broth tube. Nine isolates gave positive results after 4 h of incubation, including Lb7, Lb8, Lb9, Lb2, Lb3, Lb4, and Lc1, Lc2. However, three isolates (Lb1, Lb5, and Lb6) revealed negative results after 4 h but positive results after 24 h (Table 3).

Table 3. Auto aggregation ability of probiotics bacteria (Visual Assay). (-): Negative result; (+): Positive result

Probiotics isolates	Auto-aggregation		
	4h	9h	24h
L.gasseri (Lb1)	-	-	+
L.acidophilus (Lb2)	+	+	+
L.acidophilus (Lb3)	+	+	+
L.acidophilus (Lb4)	+	+	+
L.plantarum (Lb5)	-	-	+
L.plantarum (Lb6)	-	-	+
L.plantarum (Lb7)	+	+	+
L.fermentum (Lb8)	+	+	+
L.fermentum (Lb9)	+	+	+
L.garvieae (Lc1)	+	+	+
L.garvieae (Lc2)	+	+	+

The spectrophotometry assay results showed different rates of autoaggregation; accordingly, the autoaggregation rates of Lb8, Lb9, Lc1, and Lc2 were 57.14%, 14.89%, 21.87%, and 8.33%, respectively. However, Lb7 had a lower autoaggregation rate (1.85%), while Lb5 and Lb6 showed negative results after 4 h. Moreover, the autoaggregation rates of Lb2, Lb3, and Lb4 isolates were 15.25%, 8.92%, and 11.11%, respectively. In the present research, the highest aggregation rate was 57.14% which belonged to Lb8, as shown in figure 4.
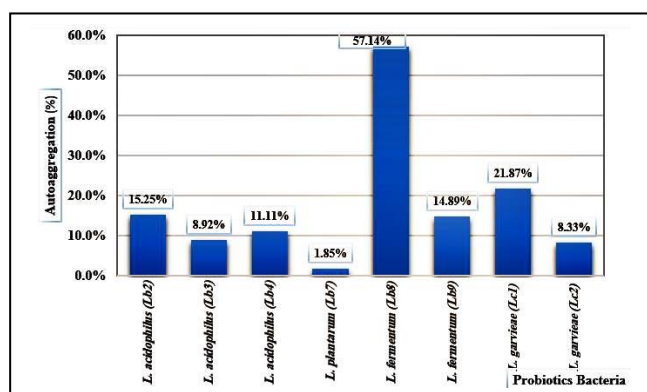

Figure 4. Auto-aggregation percentage of probiotics bacteria after 4h of incubation

Khojah, Gomaa (33) stated that the tested strains showed high autoaggregation rates after 24 h, and the probiotic bacterial strains used this feature to colonize the intestine. This ability may prevent pathogenic bacteria, which have the ability of adhesion to the intestine cells, from colonization in the intestine. In a study performed by Sadeghi, Panahi (21), the highest aggregation rate was approximately 29%, which is regarded as a strong aggregation ability.

3.4. Co-Aggregative Effect of Probiotics Bacteria against Diarrheal Causative Bacteria

Five diarrhea causative isolates (*E. coli*(11,38,41), *S. sonnei*(1), and *P. alcalifaciens*(1)) and three isolates from probiotic bacteria (Lb2, Lb8, and Lc1) were selected for this experiment. The co-aggregation rates of *L. acidophilus* against the pathogenic isolates of *E. coli*(38), *S. sonnei*(1), and *P. alcalifaciens*(1) after 4 h of incubation were 12.2%, 14.29%, and 6.85%,

respectively. Two of the isolates did not show co-aggregation ability against *E. coli*(11, 41) after 4 h.

After 24 h of incubation, the co-aggregation rates against *E. coli*(11), *E. coli*(38), *E. coli*(41), *S. sonnei*(1), and *P. alcalifaciens*(1) were 89.74%, 60%, 56.52%, 84.21%, and 100%, as summarized in table 4. The results for Lb8 revealed no co-aggregative effect after 4 h; however, after 24 h, its co-aggregation rates against *E. coli*(11), *E. coli*(38), *E. coli*(41), *S. sonnei*(1), and *P. alcalifaciens*(1) were 83.78%, 92.86%, 14.29%, 94.44%, and 100%, respectively (Table 5).

Table 4. Co-aggregative effect of Lactobacillus acidophilus (Lb2) against diarrheal causative bacteria.(-): Negative result of co-aggregation

Pathogenic isolates	Co-aggregation(%)	
	4h	**24h**
E. coli (11)	-13.16	89.74
E. coli (38)	12.20	60.00
E. coli (41)	-2.38	56.52
S. sonnei (1)	14.29	84.21
P. alcalifaciens (1)	6.85	100.00

Table 5. Co-aggregative effect of Lactobacillus fermentum (Lb8) against diarrheal causative bacteria.(-): Negative result of co-aggregation

Pathogenic isolates	Coaggregation (%)	
	4h	**24h**
E. coli (11)	-78.72	83.78
E. coli (38)	-16.98	92.86
E. coli (41)	-49.09	14.29
S. sonnei (1)	-51.22	94.44
P. alcalifaciens (1)	-59.09	100.00

Furthermore, the results after 4 h revealed that the percentages against *E. coli*(11), *E. coli*(38), *S. sonnei*(1), and *P. alcalifaciens*(1) were 5.26%, 21.95%, 11.43%, and 9.59%, respectively. However, after 4 h, Lc1 showed negative results for co-aggregation against *E. coli*(41). After 24 h, the co-aggregation rates of Lc1 against *E. coli*(11), *E. coli*(38), *E. coli*(41), *S. sonnei*(1), and *P. alcalifaciens*(1) were 90.48%, 96.30%, 70.21%, 67.74%, and 75%, respectively. All the results showed that the co-aggregation rates were higher after 24 h, compared to 4 h of incubation (Table 6).

Table 6. Co-aggregative effect of Lactococcus garvieae (Lc1) against diarrheal causative bacteria, (-): Negative result of co-aggregation

Pathogenic isolates	Co-aggregation (%)	
	4h	24h
E. coli (11)	5.26	90.48
E. coli (38)	21.95	96.30
E. coli (41)	-11.90	70.21
S. sonnei (1)	11.43	67.74
P. alcalifaciens (1)	9.59	75.00

In their study, Alp, Kuleaşan (24) mentioned that the intestine secretes mucus continuously to remove the pathogen from its tissue. The binding of probiotic bacteria with the pathogen happened through co-aggregation. The presence of mucus that is secreted in limited amounts in the intestine keeps the beneficial bacteria, such as the probiotic bacteria, by adherence to the intestinal mucus glycoproteins by mucus-binding proteins. These bacteria protect the intestine from pathogenic bacterial colonization, decreasing the ability of the pathogen to connect to the tissue of the intestine (34).

The S-layer in probiotic bacteria was efficient in co-aggregation. When this layer was removed from the probiotic bacterial strains, they lost their co-aggregation abilities (24). This protein layer reacts with a particular surface cellular position of the pathogen that leads to modifications on it, making the pathogenic bacterial cells agglomerate with the probiotic bacteria, which prevents tissue infestation. The probiotic bacteria have several methods to aggregate with the pathogenic bacteria in addition to the S-layer; for instance, the lectin-like proteins located at the surfaces of many bacterial cells aid in cellular interaction. Moreover, protein 32 kDa, which is secreted by probiotic bacteria, as well as a specialized peptide (sex pheromones), and the exo-polysaccharide play roles in the co-aggregation (24).

Probiotic bacteria have many characteristics and mechanisms to prevent pathogenic bacteria from colonization in the intestine and infection. One of them is the co-aggregation with pathogenic bacteria, as mentioned in a study conducted by Sadeghi, Panahi

(21). Bacteriocins, peptides, organic acids, and volatile compounds generated by LAB isolates are antimicrobial. Antibacterial activity of dead cells shows that cell membrane and cytoplasm are antimicrobial against food-borne pathogens (35).

Based on the results, *Lactobacillus* spp. and *Lactococcus* spp. local isolates had probiotics properties and co-aggregative effects against diarrheal causative bacteria after 24 h of incubation.

Authors' Contribution

Study concept and design: J. A. S. S.

Acquisition of data: S. R. H.

Analysis and interpretation of data: S. R. H.

Drafting of the manuscript: S. R. H.

Critical revision of the manuscript for important intellectual content: J. A. S. S.

Statistical analysis: J. A. S. S.

Administrative, technical, and material support: S. R. H.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

References

1.   Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-14.

2.   Gogineni VK, Morrow LE, Gregory PJ, Malesker MA. Probiotics: history and evolution. J Anc Dis Prev Rem. 2013;1(2):1-7.

3.   Mosaddek ASM. Effect of Probiotics in the Treatment of Acute Watery Diarrhoea in Children Admitted to a Tertiary Care Hospital in Bangladesh: A

Non-Randomized Prospective Clinical Trial. IJCRIMPH. 2022;14(2):1-7.

4.   Pernica JM, Arscott-Mills T, Steenhoff AP, Mokomane M, Moorad B, Bapabi M, et al. Optimising the management of childhood acute diarrhoeal disease using a rapid test-and-treat strategy and/or Lactobacillus reuteri DSM 17938: a multicentre, randomised, controlled, factorial trial in Botswana. BMJ Glob Health. 2022;7(4):e007826.

5.   Kim H, Lee Y-S, Yu H-Y, Kwon M, Kim K-K, In G, et al. Anti-Inflammatory Effects of Limosilactobacillus fermentum KGC1601 Isolated from Panax ginseng and Its Probiotic Characteristics. Foods. 2022;11(12):1707.

6.   Ariyoshi T, Hagihara M, Takahashi M, Mikamo H. Effect of Clostridium butyricum on gastrointestinal infections. Biomedicines. 2022;10(2):483.

7.   Kora AJ. Probiotics in the prevention and treatment of diarrheal disease. Probiotics in the Prevention and Management of Human Diseases. 2022:107-15.

8.   Pakbin B, Didban A, Brück WM, Alizadeh M. Phylogenetic analysis and antibiotic resistance of Shigella sonnei isolates. FEMS Microbiol Lett. 2022;369(1):fnac042.

9.   Ulfman LH, Schloesser JE, Kortman GA, van den Belt M, Lucas-van De Bos E, Roggekamp J, et al. A Double-Blind, Randomized Intervention Study on the Effect of a Whey Protein Concentrate on E. coli-Induced Diarrhea in a Human Infection Model. Nutrients. 2022;14(6):1204.

10.   Sekhi RJ. Diarrhea in children an infants caused by e. Coli: a review article. 2022.

11.   Hassanzadazar H, Ehsani A, Mardani K, Hesari J, editors. Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Veterinary Research Forum; 2012: Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

12.   Kaboré D, Sawadogo-Lingani H, Dicko MH, Diawara B, Jakobsen M. Acid resistance, bile tolerance and antimicrobial properties of dominant lactic acid bacteria isolated from traditional "maari" baobab seeds fermented condiment. Afr J Biotechnol. 2012;11(5):1197-206.

13.   Ambalam P, Kondepudi KK, Nilsson I, Wadström T, Ljungh Å. Bile stimulates cell surface hydrophobicity, Congo red binding and biofilm formation of Lactobacillus strains. FEMS Microbiol Lett. 2012;333(1):10-9.

14.   Weinstein M, Lewis J, Bobenchick A, Campeau S, Cullen S, Galas M, et al. CLSI M100 ED31: 2021

Performance Standards for Antimicrobial Susceptibility Testing. Accessed; 2021.

15.   Koohestani M, Moradi M, Tajik H, Badali A, editors. Effects of cell-free supernatant of Lactobacillus acidophilus LA5 and Lactobacillus casei 431 against planktonic form and biofilm of Staphylococcus aureus. Veterinary Research Forum; 2018: Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

16.   Motey GA, Owusu-Kwarteng J, Obiri-Danso K, Ofori LA, Ellis WO, Jespersen L. In vitro properties of potential probiotic lactic acid bacteria originating from Ghanaian indigenous fermented milk products. World J Microbiol Biotechnol. 2021;37(3):1-13.

17.   Melgar-Lalanne G, Rivera-Espinoza Y, Téllez-Medina DI, Hernández-Sánchez H. Cell surface properties of halotolerant probiotic lactobacilli. J Adv Biotechnol. 2015;4(3):404-13.

18.   Kos B, Šušković J, Vuković S, Šimpraga M, Frece J, Matošić S. Adhesion and aggregation ability of probiotic strain Lactobacillus acidophilus M92. J Appl Microbiol. 2003;94(6):981-7.

19.   Jánošíková L, Pálková L, Šalát D, Klepanec A, Soltys K. Response of Escherichia coli minimal ter operon to UVC and auto-aggregation: pilot study. PeerJ. 2021;9:e11197.

20.   Ayyash MM, Abdalla AK, AlKalbani NS, Baig MA, Turner MS, Liu S-Q, et al. Invited review: Characterization of new probiotics from dairy and nondairy products—Insights into acid tolerance, bile metabolism and tolerance, and adhesion capability. J Dairy Sci. 2021;104(8):8363-79.

21.   Sadeghi M, Panahi B, Mazlumi A, Hejazi MA, Komi DEA, Nami Y. Screening of potential probiotic lactic acid bacteria with antimicrobial properties and selection of superior bacteria for application as biocontrol using machine learning models. LWT. 2022;162:113471.

22.   Liu Z, Xu C, Tian R, Wang W, Ma J, Gu L, et al. Screening beneficial bacteriostatic lactic acid bacteria in the intestine and studies of bacteriostatic substances. J Zhejiang Univ Sci B. 2021;22(7):533-47.

23.   Khromova NY, Epishkina JM, Karetkin BA, Khabibulina NV, Beloded AV, Shakir IV, et al. The Combination of In Vitro Assessment of Stress Tolerance Ability, Autoaggregation, and Vitamin B-Producing Ability for New Probiotic Strain Introduction. Microorganisms. 2022;10(2):470.

24.   Alp D, Kuleaşan H, Korkut Altıntaş A. The

importance of the S-layer on the adhesion and aggregation ability of lactic acid bacteria. Mol Biol Rep. 2020;47(5):3449-57.

25. Affhan S, Dachang W, Xin Y, Shang D. Lactic acid bacteria protect human intestinal epithelial cells from Staphylococcus aureus and Pseudomonas aeruginosa infections. Genet Mol Res. 2015;14:17044-58.

26. Dobreva L, Koprinarova M, Bratchkova A, DANOVA S. Antibiotic susceptibility of lactobacillus plantarum strains, isolated from katak. Bulg J Vet Med. 2022;25(1).

27. Thumu SCR, Halami PM. Acquired resistance to macrolide–lincosamide–streptogramin antibiotics in lactic acid bacteria of food origin. Indian J Microbiol. 2012;52(4):530-7.

28. Hamed E. Isolation, characterization and identification of lactic acid bacteria as probiotic. Ann Agric Sci Moshtohor. 2021;59(2):311-22.

29. Lin P-P, Hsieh Y-M, Tsai C-C. Antagonistic activity of Lactobacillus acidophilus RY2 isolated from healthy infancy feces on the growth and adhesion characteristics of enteroaggregative Escherichia coli. Anaerobe. 2009;15(4):122-6.

30. Kaewchomphunuch T, Charoenpichitnunt T, Thongbaiyai V, Ngamwongsatit N, Kaeoket K. Cell-free culture supernatants of Lactobacillus spp. and Pediococcus spp. inhibit growth of pathogenic Escherichia coli isolated from pigs in Thailand. BMC Vet Res. 2022;18(1):1-13.

31. Iorizzo M, Ganassi S, Albanese G, Letizia F, Testa B, Tedino C, et al. Antimicrobial Activity from Putative Probiotic Lactic Acid Bacteria for the Biological Control of American and European Foulbrood Diseases. Vet Sci. 2022;9(5):236.

32. Wang W, Ma H, Yu H, Qin G, Tan Z, Wang Y, et al. Screening of Lactobacillus plantarum subsp. plantarum with potential probiotic activities for inhibiting ETEC K88 in weaned piglets. Molecules. 2020;25(19):4481.

33. Khojah E, Gomaa MS, Elsherbiny EG, Elawady A, Darwish MS. The In Vitro Analysis of Postbiotics in Functional Labneh to Be Used as Powerful Tool to Improve Cell Surfaces Properties and Adherence Potential of Probiotic Strains. Fermentation. 2022;8(3):122.

34. Sengupta R, Altermann E, Anderson RC, McNabb WC, Moughan PJ, Roy NC. The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. Mediators Inflamm. 2013;2013.

35. Alameri F, Tarique M, Osaili T, Obaid R, Abdalla A, Masad R, et al. Lactic acid bacteria isolated from fresh vegetable products: Potential probiotic and postbiotic characteristics Including immunomodulatory effects. Microorganisms. 2022;10(2):389.