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Original Article

اثر پری بیوتیک ها بر روی تولید باکتریوسین و اتصال به روده باکتری های *لیزینی باسیلوس اسفاریکوس* DY13 و *باسیلوس کلوسیی* DY14

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چکیده

سابقه و هدف: پروبیوتیک‌ها میکروارگانیسم‌های زنده‌ای هستند که درصورت مصرف شدن به میزان کافی سودمندند. به منظور تخمین قابلیت پروبیوتیکی این میکروارگایسم‌ها ضروری است که فواید بهداشتی، کارایی و ایمنی آنها مورد ارزیابی قرار گیرد. این مطالعه با هدف بررسی قابلیت اتصال به روده باکتری های های *لیزینی باسیلوس اسفاریکوس* DY13 و *باسیلوس کلوسیی* DY14 جدا شده از شیر انسان های سالم انجام شد. همچنین اثرات پری بیوتیک های طبیعی و تجاری بر روی تولید باکتریوسین با استفاده از این دو سویه نیز مورد ارزیابی قرار گرفت.

مواد و روش ها: سویه‌های های *لیزینی باسیلوس اسفاریکوس* سویه DY13 و *باسیلوس کلوسی* سویه DY14 از نظر توانایی تحمل شرایط مصنوعی معدی-روده‌ای مورد بررسی قرار گرفتند. علاوه بر آن، این جدایه‌ها از نظر ویژگی‌های چسبندگی به خود (auto-aggregation) و یکدیگر (co-aggregation) و همچنین ویژگی‌های آب گریزی سطح سلول مورد بررسی قرار گرفتند. اثر پری بیوتیکی ترکیبات (مانیتول، سوربیتول، سیر، پیاز و عسل) بر تحریک تولید باکتریوسین باکتری های *اشریشیا کلی، باسیلوس سرئوس، باسیلوس سوبتیلیس، سودوموناس ائروجینوسا و استافیلوکوکوس اورئوس* نیز مورد بررسی قرار گرفت.

یافته ها: در مقایسه با شاهد، توانایی چسبندگی به خود سویه های DY13 و DY14 به میزان ٤ درصد کاهش داشت. هر دو جدایه توانایی چسبندگی به یکدیگر قابل توجهی داشتند. درصد آبگریزی جدایه های DY13 و DY14 به ترتیب بین ۵۱ تا ۵۲/۷ درصد و ۵۹/۱ تا ٦٦/۱ درصد بود. بررسی مقایسه‌ای مربوط به اثر پری بیوتیک ها (مانیتول، سوربیتول، سیر، پیاز و عسل) بر روی رشد جدایه‌های باکتریایی و تولید باکتریوسین نشان داد که عسل بهترین منبع برای بهبود رشد سویه باکتری و همچنین تحریک تولید باکتریوسین است.

نتیجه‌گیری: با توجه به ویژگی های چسبندگی قابل توجه جدایه های مورد بررسی، این باکتری ها می توانند به عنوان پرو بیوتیک های مناسب و کارآمد همراه با عسل به عنوان پری بیوتیک مورد استفاده قرار گیرند.

واژگان کلیدی: باکتری های پروبیوتیک، سنجش چسبندگی، پری بیوتیک ها، فعالیت آنتاگونیستی، باکتریوسین.

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Original Article

The effect of prebiotics on bacteriocin production and gut adhesion potential of *Lysinibacillus sphaericus* DY13 and *Bacillus clausii* DY14

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Abstract

Background & Objectives: Probiotics are living microorganisms which when administrated adequately, confer benefits to hosts. In order to estimate the probiotic potential of these microorganisms, it is necessary to assess their health benefits, efficacy, and safety. This study was aimed to evaluate gut adhesion capacity of *Lysinibacillus sphaericus* DY13 and *Bacillus clausii* DY14 strains which were previously isolated from healthy human milk, and to assess the effects of natural and commercial prebiotics on bacteriocin production by these two strains.

Material & Methods: *L. sphaericus* DY13 and *B. clausii* DY14 isolates were evaluated for tolerating the artificial gastrointestinal conditions. Moreover, isolates were examined for auto-aggregation, co-aggregation, and cell surface hydrophobicity properties. The prebiotic effect of mannitol, sorbitol, garlic, onion and honey on the enhancement of bacteriocin production against *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was also evaluated.

Results: Compared to the control, auto-aggregation potential of DY13 and DY14 was found to be decreased by 4%. Both isolates have demonstrated preeminent auto-aggregation potential. The hydrophobicity percentage of DY13 and DY14 isolates typically ranged between 51-52.7% and 59.1-66.1%, respectively. Comparing the effect of selected prebiotics on bacterial isolates growth and bacteriocin production revealed that honey is the best source to improve the growth of bacterial strain, and also to stimulate bacteriocin production.

Conclusion: Due to considerable adhesion potential of both selected strains, they can be applied as suitable and efficient probiotics, along with honey as prebiotic.

Keywords: Probiotic bacteria, Adhesion assay, Prebiotics, Antagonistic activity, Bacteriocin.
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Introduction

The human intestinal environment is a good ecological niche for different kinds of commensal and symbiotic bacteria serving different functions and is a significant constituent of the gut mucosal barrier (1).

Bacteria that live in the human intestine and control the balance of intestinal microflora, and finally confer some physiological and beneficial effects on host health have been named as 'probiotics'(1). To be used as a probiotic organism, it should possess the properties such as acid resistance; bile tolerance; removal of pathogens or reduction in pathogenic adherence; production of acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth; safety; being non-pathogenic and non-carcinogenic; as well as adherence to host epithelial tissue (2).

The desired beneficial effects of probiotics can be enhanced by stimulating the growth of that organism by adding specific nutrients which are known as 'prebiotics'. Abundant environmental extremes like variations in pH, low oxygen levels, nutrient limitation, and higher osmolality are the potential impediments to the survival of microorganisms in the human gastrointestinal tract (3).

Lactic acid bacteria and other probiotic micro-organisms have numerous documented health effects (4). While choosing probiotic micro-organisms with beneficial health effects for the host, several criteria have to be met. Among these criteria, the most attractive one is the ability to adhere to the host tissue. Adhesion to the intestinal mucosa is considered to be a prerequisite for successful colonization and is important for immunomodulation by the probiotics (5).

Adhesion of micro-organisms is generally tested with mono-layers of intestinal tissue culture cells. Although this is a valuable method for assessing the adhesive abilities of bacteria to the intestinal mucosa, it does not take into interpretation, the possible adhesion to the mucus layer covering the epithelial cells of the intestine. The mucus layer has a dual role: It serves as a barrier for adhesion of certain micro-organisms to the underlying epithelium, while at the same time it can deliver a habitat for the adhesion of others (6).

Probiotics can be employed in the treatment and prevention of enteric infections and chronic inflammatory disorders of the gastrointestinal tract (GIT) (7). Some probiotic bacteria produce antimicrobial substances, including organic acids, hydrogen peroxide, and bacteriocins (8). Bacteriocins are proteinaceous antibacterial compounds that are bactericidal to several pathogens associated with mastitis, food spoilage and food-borne illnesses including *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus*, etc. (9, 10). They are typically degraded by the proteolytic action of GIT and seem to be non-toxic and non-antigenic to the animals. Therefore, bacteriocins could be used to improve the safety, and shelf life of many foods (11).

Human milk bacterial isolates providing health benefits such as protection of the host from pathogenic would be considered as attractive probiotic organisms (12). Human milk is a significant food for newborns to grow them up and protect the infants against some infections. High concentration of LAB in milk from the

healthy mother may play an important biological role during the first months of life. Studies on this biological fluid indicate that human milk is an exciting source for potential probiotic bacteria. In the present study, the gut adhesion ability of previously isolated bacteria (*Lysinibacillus sphaericus* DY13 and *Bacillus clausii* DY14) from healthy human milk has been described. Significant knowledge about the adhesive properties of probiotic organisms could, therefore, offer information about its possibility to colonize and modulate the immune system. The surface hydrophobicity of the tested microorganisms has been determined in order to test for a possible correlation between this physicochemical property and its ability to adhere to the intestinal mucus. This would in-turn offer an easy and swift method to screen for the potential adhesive probiotic strains.

Materials and Methods

Lysinibacillus sphaericus DY13 (GenBank accession number KP720575), and *Bacillus clausii* DY14 (GenBank accession number KP720576) strains have been isolated from healthy volunteer mother breast milk in the previous study (13). The pure cultures of the isolates were preserved in de Man, Rogosa and Sharpe (MRS) broth medium containing 20% (v/v) glycerol as frozen stocks at -80 °C. The cultures were revived prior to use by sub-culturing twice in MRS broth at 37 °C and 120 rpm for 24 hr (Hi-media, India).

Gut adhesion capacity

Preparation of gastric (G), intestinal (I), and gastric-intestinal (G+I) simulated juices

The simulated juices were prepared as described by Brinques et al (14) and Michida et al. (15). Simulated gastric juice was prepared by dissolving the pepsin (Sigma-Aldrich P7000) in sterile sodium chloride (NaCl) solution (0.5%, w/v) to an the absolute concentration of 3.0 g/L, and adjusting the pH to 2 with hydrochloric acid (1M HCl).

Simulated intestinal juice were prepared by adding pancreatic (Sigma-Aldrich P-1500) in sterile sodium chloride solution (0.5%, w/v) to a final concentration of 1 g/L, with 4.5% bile salts (Oxoid, Basingstoke, UK) and adjusting the pH to 8.0 with sterile NaOH (0.1 M). Total 1 ml of overnight grown bacterial culture containing approximately 5.8×109 cell/mL were incubated in 5ml artificial gastric juice for 3 hours at 37 °C, and in 5ml artificial intestinal juice for 4 hours at 37 °C in a separate sterile vial. After incubation, cells were recovered by centrifugation at 10,000rpm for 15 minutes. Cell pellets were washed twice with sterile normal saline (pH 7) and finally dissolved in 2ml normal saline (0.9% NaCl in sterile distilled water, pH 7). When bacteria travel in the gastrointestinal tract, they firstly encounter gastric juice for approximately 3 hr, and then with intestinal juice for 4 hr.

Thus for in-vitro, gastro-intestinal simulation, 1 ml of overnight grown bacterial culture (approximately 5.8×109 cells/mL) was incubated in artificial gastric juice for 3 hours at 37°C. Afterward, bacterial cells were recovered by centrifugation and washed with sterile normal saline (pH 7). This gastric simulated cell pellet was re-suspended in the artificial intestinal juice and incubated at 37 °C for 4 hours. Cell pellets were washed twice

with sterile normal saline (pH 7) and finally dissolved in 2ml N-saline (0.9% NaCl in sterile distilled water, pH 7). Bacterial cells collected after gastric simulation (G), intestinal simulation (I), and gastrointestinal simulated cell (G+I) has been employed for further process. As a persistence of cell viability study, cells were plated on MRS agar, and then incubated at 37 °C for overnight, and the viability was checked as a CFU (16).

Auto-aggregation and co-aggregation

Simulated bacterial cells (density of cell ~109 cells/mL) of L. sphaericus DY13 and B. clausii DY14 strain (control, gastric simulated, intestinal simulated and gastric-intestinal simulated) were studied for their capability to auto-aggregate and co-aggregate.

Co-aggregation was studied in the presence of Gram-positive bacteria including *Lactobacillus oris* DY17 (KP720579), *Lactococcus lactis* DY1 (KP720567), *Staphylococcus aureus* DY2 (KP720563), and *Micrococcus leriautusAPP16* (KF705255), as well as Gram-negative bacteria including *Pseudomonas aeruginosa* DY7 (KP720569), *Escherichia coli* MTCC 10312, *Serratia marcescens* DY3 (KP720564), and *Enterococcus faecalis* DY5 (KP720566), the bacterial strain which was previously isolated from the breast milk.

The cultures were grown in 10 ml MRS broth at 37 °C for 24 hours and then harvested (10,000 rpm, 20min and 4 °C). The cells were washed and re-suspended in sterile saline and adjusted to OD_{660nm}= 0.3. Auto-aggregation was determined spectrophotometrically as described by Todorov SD et al. (2008) (16) and by using the formula,

$$\%\text{Auto-aggregation} = [(A_i - A_f)/ A_i] \times 100$$

Where Ai is the initial OD at 620 nm, A_f refers to the optical density measured after the cells were incubated for 60 minutes and centrifuged for 2 minutes at 300×g. Co-aggregation was observed by determining the OD_{620nm} readings of paired cell suspensions after incubation for 60 minutes, and centrifugation for 2 minutes at 3000 rpm (17). The level of co-aggregation was calculated using the equation,

$$\%\text{ Co-aggregation} = 100 \times (A_i - A_f)/ A_i$$

Where A_i value refers to the initial OD at 620 nm which was taken immediately after adding the selected strains for co-aggregation, and A_f refers to the OD_{620nm} of the supernatant after the cells were centrifuged for 2 minutes at 3000 rpm (17). The experiment was conducted twice, with each time in triplicate.

Cell hydrophobicity

Cell surface hydrophobicity of isolates was estimated by Bacterial Adhesion to Hydrocarbons (BATH) as described by Geertsema-Doornbusch et al. (18) using xylene, chloroform, and ethyl acetate as the solvents. The simulated cell culture (~109 cells/mL) was employed to learn the cell hydrophobicity.

The simulated cells were washed twice in phosphate urea magnesium buffer (K_2HPO_4: 22.2 g/l; KH_2PO_4: 7.26 g/l; urea: 1.8 g/l; $MgSO_4$: 0.2g/l; pH: 7.1±0.2), and lastly suspended in the same buffer. The initial absorbance (Ai) of the suspension at 620 nm was taken immediately after being re-suspended in Phosphate Urea Magnesium (PUM) buffer. Two ml of cell suspension PUM

buffer was dispensed in clean and dry round bottom test tubes, followed by addition of one ml of xylene, ethyl acetate and chloroform in separate vessel. The contents were vortexed for 2 minutes. The tubes were left undisturbed for 1 hour at 37 ºC to allow the phase separation. The lower aqueous phase was cautiously removed with a sterile Pasteur pipette, and absorbance (Af) was recorded at 620 nm. Cell surface hydrophobicity in terms of percent (CSH %) was calculated using the following formula,

$$\%CSH = \left[(A_i - A_f) / A_i \right] \times 100$$

Effect of prebiotics on growth and bacteriocin production by the isolate

To study the effect of prebiotics on bacteriocin production by *B. clausii* and *L. sphaericus*, five prebiotics i.e. mannitol, sorbitol, garlic powder, onion powder, and honey were additionally added as 20.0 mg/ml (w/v) in MRS medium (Hi Media), and autoclaved. Media containing various prebiotic source was inoculated with *B. clausii* and *L. sphaericus* bacterial cultures, and the bacterial growth were measured by taking OD at 620 nm at different time intervals of 0, 24, 48, 72, 77, 94 hours. The supernatant is collected at same time intervals to study the bacteriocin activity, and the amount of crude bacteriocin was checked by taking OD at 280nm. The pH of the collected supernatant was merely adjusted to 7.0 using 1 M NaOH. Antimicrobial activity of the collected supernatant against indicator microbes like *E. coli* (MTCC 10312), *B. cereus* (MTCC 9762), *B. subtilis* (MTCC 1789), *P. aeruginosa* (MTCC 8076) and *S. aureus* (MTCC 9542) was studied by agar well diffusion method (19).

Statistical analysis

All the experiments were done in triplicate, and the results were conveyed as mean standard deviations. The values recorded in each experiment did not vary by more than 5%, and the single data points are presented in the figures with standard deviation bars. Data analysis was carried out with MS-Excel (Microsoft office 2013).

Results

Viability study of simulation bacterial strain:

To evaluate the survival rate in gastric environment, *B. clausii* DY14 and *L. sphaericus* DY13 strains were simulated in gastric juice (pH 2.0) for 3 hours. *B. clausii* DY14 had the highest survival rate over the 3 hours of exposure to simulated gastric juice (pH 2.0) as compared to the *L. sphaericus* DY13 (Figure1 and Table 1). After cell incubation in gastric juice, the viability of cells

Table 1. The effect of simulated gastric and small intestinal transit on the viability of selected *L. sphaericus* DY13 and *B. clausii* DY14 strains.

Sr. no.	Condition applied	Total bacterial count (CFU/ml)			
		L sphaericus DY13		*B. clausii* DY14	
		Control	Simulated	Control	Simulated
1	Gastric simulation	$10^9 \times 5.4$	$10^9 \times 3.1$	$10^9 \times 4.2$	$10^9 \times 2.6$
2	Intestinal simulation	$10^9 \times 5.8$	$10^9 \times 3.5$	$10^9 \times 4.7$	$10^9 \times 2.9$
3	Gastrointestinal simulation	$10^9 \times 4.6$	$10^9 \times 1.1$	$10^9 \times 5.3$	$10^9 \times 2.1$

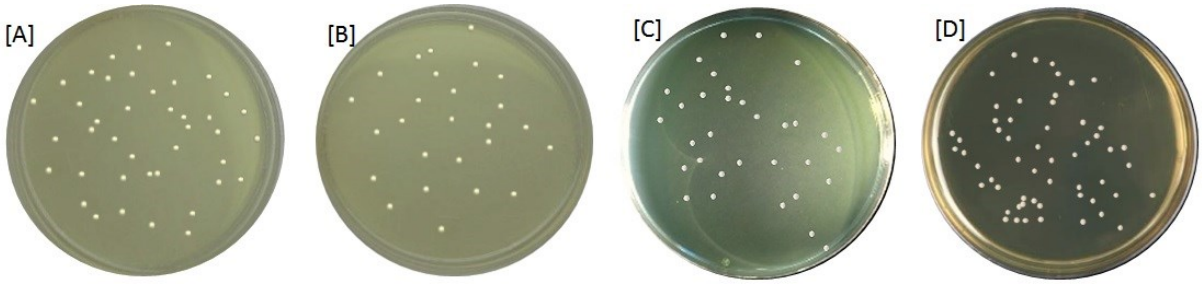

Figure 1. Cell viability after gastric simulation (G): [A] Control of *B. clausii* DY14, [B] Gastric simulated cell of *B. clausii* DY14, [C] Gastric simulated cell of *L sphaericus* DY13, [D] Control of *L sphaericus* DY13.

was decreased from 4.2×109 CFU/ml to 2.6×10^9 CFU/ml for *B. clausii* DY14 whereas for *L. sphaericus* DY13 it was decreased from 5.4×10^9 to 3.1×10^9 CFU/ml which obviously reveals that both strains belong to a moderately resistant group. Isolates were exposed to intestinal simulation in the presence of trypsin and bile salt (pH 8) which mimics the intestinal environment for 2 hours. Figure 2 depicts the survival of *B. clausii* DY14 and *L. sphaericus* DY13 in the presence of simulated intestinal juice. Viable cell counts of both strains were observed to be decreased from 4.7×10^9 CFU/ml to 2.9×10^9 CFU/ml after 4 hours incubation in simulated juices (Table 1). *B. clausii* DY14 and *L. sphaericus* DY13 were exposed to the

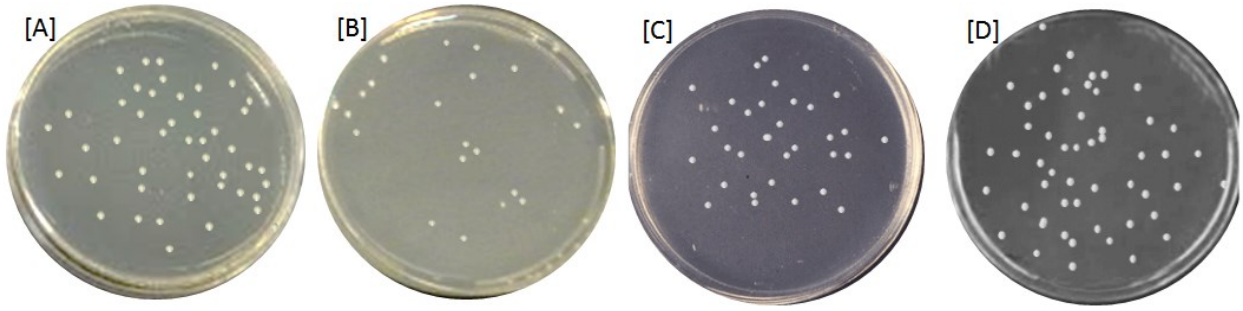

Figure 2. Bacterial cell viability after Intestinal simulation: [A] Control of *B. clausii* DY14, [B] Intestinal simulated cell of *B. clausii* DY14, [C] Intestinal simulated cell of *L sphaericus* DY13,[D] Control of *L sphaericus*

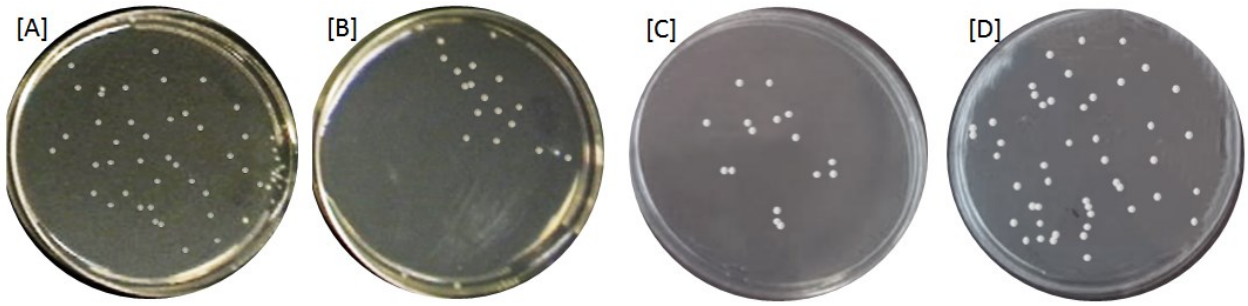

Figure 3: Cell viability count after gastrointestinal simulation (G): [A] Control of *B. clausii* DY14, [B] Gastrointestinal simulated cell of *B. clausii* DY14, [C] Gastrointestinal simulated cell of *L sphaericus* DY13, [D] Control of *L sphaericus* DY13.

combination of simulation (gastric+intestinal) i.e. gastric simulation for 3 hours, and intestinal simulation for 4 hours. Both isolates were found feasible after 24 hours incubation at 37 °C when spread on nutrient agar plates which clearly indicated the good tolerance of isolates against acidic and alkaline conditions prevalent in the gastrointestinal tract (Figure 3 and Table 1).

Gut adhesion capacity

Auto-aggregation and co-aggregation

Both strains showed high auto-aggregation abilities (Figure 4). Strain *B. clausii* DY14 and *L. sphaericus* DY13 control group have shown auto-aggregation in the range of 94-95% and 89-92%, respectively. But after gastric simulation, auto-aggregation potential decreased up to 4% which is very less. No significant influence on the auto-aggregation ability of bacterial cell was observed after gastric simulation, intestinal simulation, and gastrointestinal simulation.

The results reveal that both isolates have preeminent auto-aggregation potential. Different levels of co-aggregation were observed between various Gram-positive bacteria like *L. oris*, *L. lactis*, *S. aureus*, *M. leriautus*, and Gram-negative bacteria i.e. *P. aeruginosa*, *E. coli*, *S. marcescens*, and *E. faecalis* which were previously isolated from breast milk (Figure 5).

High levels of co-aggregation with *L. oris*, *E. faecalis*, and *L. lactis* were recorded (Figure 5). Both isolates used in this study have shown higher co-aggregation with Gram-positive microbes when compared to Gram-negative ones. Not much consequence of gastric simulation (G), and intestinal simulation (I) was observed on the co-aggregation ability of selected strain, however, the gastrointestinal simulated (G+I) bacterial cells have shown a diminutive decrease in co-aggregation percentage.

Cell hydrophobicity

Three different solvents were tested in this study: an apolar solvent, hexadecane, an acidic solvent, chloroform and a basic solvent, ethyl acetate. The results have clearly shown an

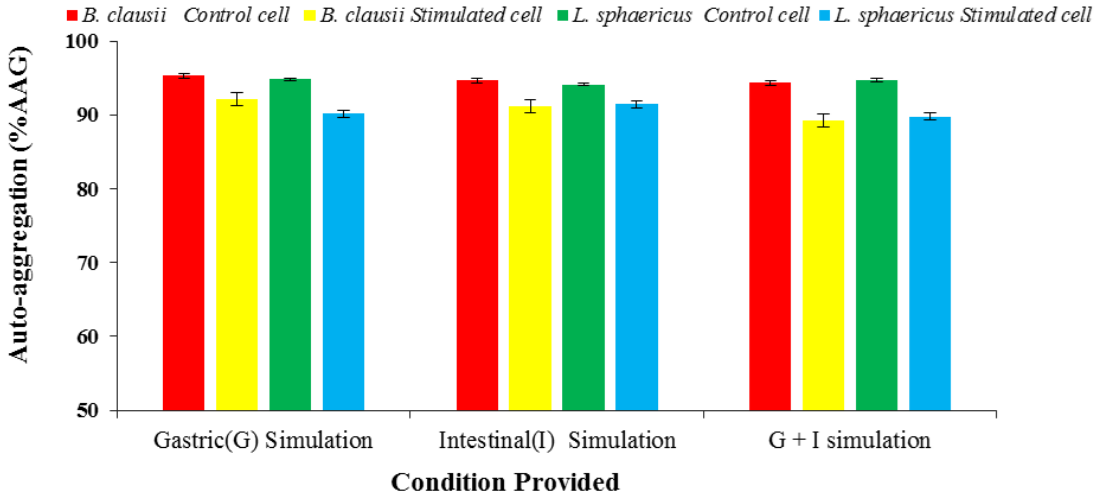

Figure 4. Auto-Aggregation potential of *B. clausii* DY14 and *L sphaericus* DY13 isolates.

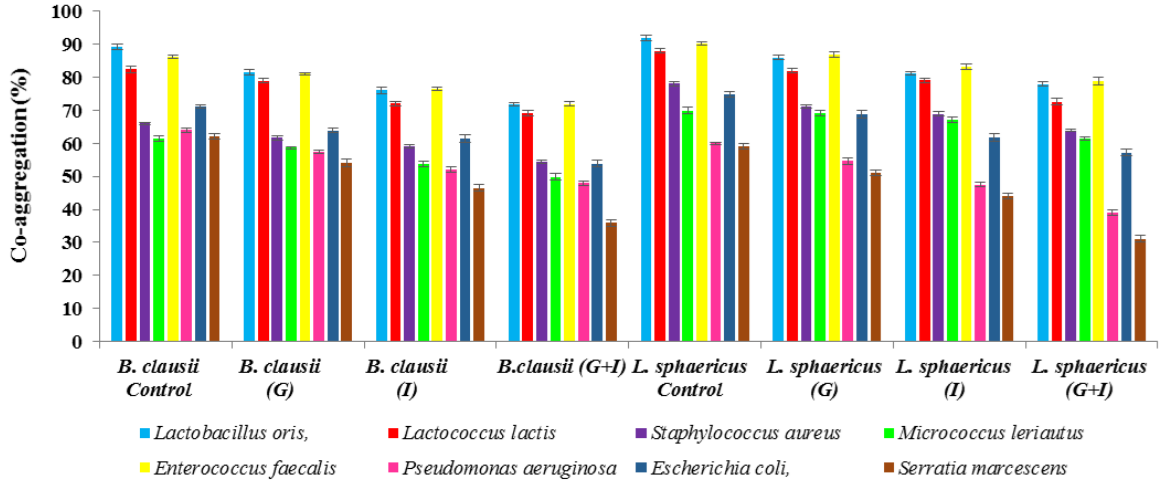

Figure 5. % Co-aggregation of *B. clausii* DY14 and *L. sphaericus* DY13 strains with Gram positive and Gram negative pathogens.

overall strong affinity of both *B. clausii* DY14 and *L. sphaericus* DY13 isolates to chloroform, the acidic solvent, and the electron acceptor. The hydrophobicity percentages for the tested *B. clausii* DY14 and *L. sphaericus* DY13 strains with chloroform ranged between 51-52.7% and 59.1-66.1%, respectively (Figure 6). Such greater adhesion values were compared with those obtained for hexadecane, as both solvents possess the same Vander Waals properties. The significant difference observed was due to the implication of Lewis acid-base interactions resulting from the electron donor and basic character of bacterial strains. The data obtained for ethyl acetate, a strongly basic solvent, and an electron donor, generated results contrary to those encountered with chloroform: the bacterial adhesion to this third solvent was low, ranging from 0.9 to 6.0%. It clearly confirmed the non-acidic cell surface character of the bacterial strains which were studied. Strains with hydrophobicity

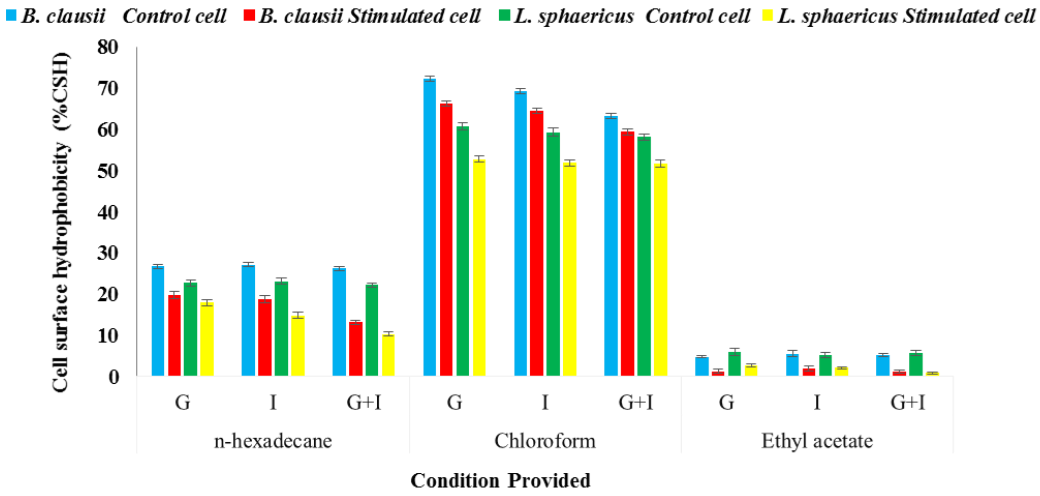

Figure 6. Cell surface hydrophobicity of *B. clausii* DY14 and *L sphaericus* DY13.

percentage greater than 40% were considered as hydrophobic.

The hydrophobicity of *B. clausii* DY14 strain has shown a greater affinity towards the solvents used for the study. Bacterial cells adherence is typically related to the cell surface characteristics. Cell surface hydrophobicity is a nonspecific interaction between microbial cells and the host. The results indicated that the cell surface of microorganisms studied was comparatively hydrophilic.

Effect of prebiotics on growth and bacteriocin production by the isolates

Prebiotics substances like mannitol, sorbitol, garlic, onion, and honey which were added to the media have shown a higher increase in bacteriocin production as an increase in bacterial growth rate subsequently results in more secondary metabolites extracellular secreted by bacteria.

In this study, major variations occurred in the bacteriocin production in different prebiotic

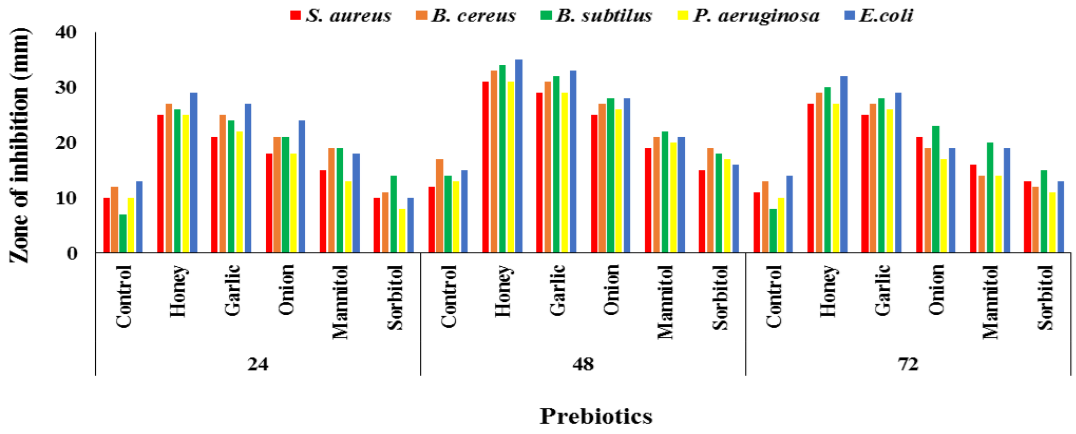

Figure 7. Antagonistic activity of crude bacteriocin of *L. sphaericus* DY13 against indictor microbes.

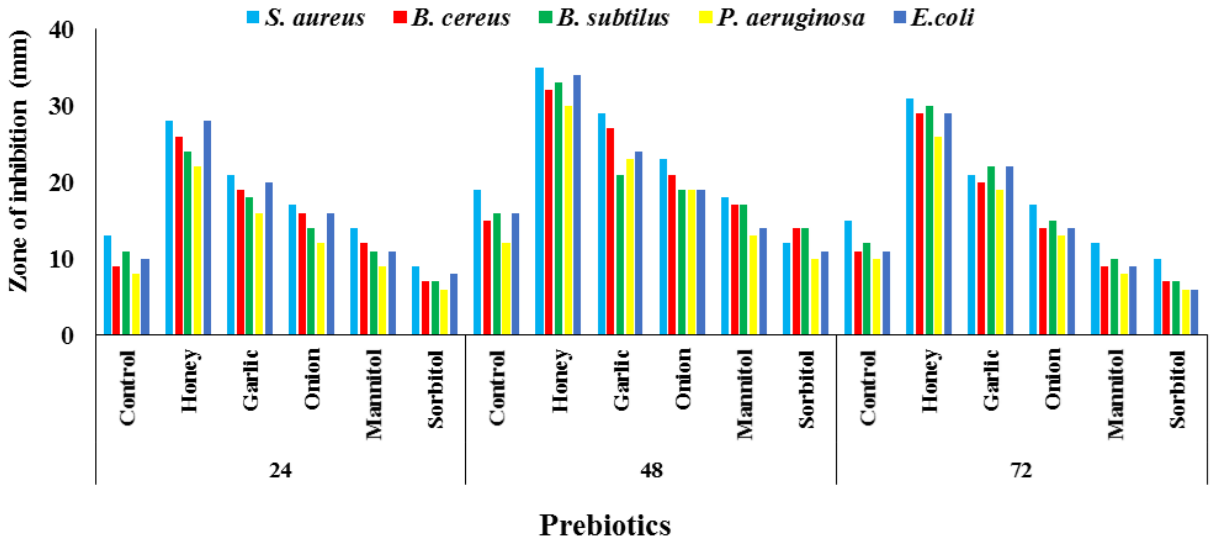

Figure 8. Antagonistic activity of crude bacteriocin of *B. clausii* DY14 against indictor microbes.

modified media. The enhanced bacteriocin production in medium containing prebiotics was due to the utilization of polyalcohol resulting in better cell growth, though there was no change in the final pH. In this study, the highest growth was observed in media containing honey, garlic, onion, mannitol, and sorbitol, respectively (Figures 7 and 8).

Discussion

Mother's milk constitutes an interesting source for delivering new and specific probiotic strains to neonates aiming at assisting a proper development of the gut microbiota, and the immune development in infants who for different reasons cannot be breastfed.

The results of this study indicate that mother's milk may be used as a potential natural source to isolate the effective strains of probiotics. The understanding of communal and potential probiotic bacteria that survive in the milk of healthy women is very restricted. It has been conveyed that breast milk bacterial isolates generally include Staphylococci, Streptococci, Micrococci, Enterococci, Bacilli, and Lactobacilli (20, 21).

The gastrointestinal transit commences by exposing the isolates to low pH and pepsin contained in simulated gastric juice. When the gastric juice is concealed, it has low pH of approximately 2.0. It has also been reported by Berrada et al., 1991 (22) that the time from entrance to release from the stomach is around 3 hours. Consequently, the survival of isolates in simulated gastric juice at pH 2.0 after 3 hours were examined and evaluated for viability. The survival of the isolates undergoing simulated gastric condition is absolutely necessary for its use as potential health benefits. Most of the studies ignore the effect of intestinal secretions on probiotic strains other than bile. Therefore, intestinal simulation adhesive properties of isolates were evaluated. The survival of isolates after a simulated intestinal condition is necessary for an exertion of potential health benefits. The capability of micro-organisms to adhere is often considered as one of the foremost selection criteria for potential probiotics.

Adhesion to the intestinal mucosa is thought to be an imperative property for colonization by preventing wash-out, especially in the small intestine where flow rates are comparatively high. Bacterial aggregation between microorganisms of the same strain (auto-aggregation) is of considerable importance of several ecological niches, especially in the human gut. Auto-aggregation may assist probiotic bacteria to adhere to mucus and epithelial cells, and survive harsh conditions in the gastrointestinal tract (23).

Furthermore, commercial probiotic strains were evaluated based on their auto-aggregation and co-aggregation abilities with potential Gram-positive and negative bacteria. For this purpose, the following strains were selected: Gram-positive bacteria like *L. oris*, *L. lactis*, *S. aureus*, *M. luteus*, and Gram-negative bacteria i.e. *P. aeruginosa*, *E. coli*, *S. marcescens*, and *E. faecalis*.

The results clearly indicated that *B. clausii* DY14 and *L. sphaericus* DY13 were the strains with greater co-aggregation percentage, and auto-aggregation abilities. Cell adhesion is a complex procedure involving contact between

the bacterial cell membrane and the interacting surfaces.

The capability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains that are used as probiotics. Several researchers have reported investigations on composition, structure, and interaction forces related to the bacterial adhesion to intestinal epithelial cells and mucus. In most cases, aggregation ability is related to cell adherence properties (24).

Bacterial aggregation between microorganisms of the same strain (auto-aggregation) or between genetically different strains (co-aggregation) is of substantial importance in several ecological niches, particularly in the human gut, where probiotics are to be active.

A relationship between auto-aggregation and adhesion ability has been reported for few bifidobacterial species, and also a correlation between adhesion ability and hydrophobicity, as measured by the microbial adhesion to hydrocarbons, has been observed in some lactobacilli, but these correlations have not been identified by other authors (25).

The bacterial adhesion to hydrocarbons test has been broadly employed for measuring cell surface hydrophobicity in lactic acid bacteria and bifidobacteria. Aggregation is a significant feature for biofilm formation. However, co-aggregation between the LAB and other bacterial cells may be considered a positive characteristic, as it is one of the steps required for the elimination of non-desirable strains from the GIT.

Auto-aggregation and co-aggregation are strain -specific, and most probably involve species-specific surface proteins. In this study, *B. clausii* and *L. sphaericus* have shown higher auto-aggregating abilities (>90%). Both isolates have a number of genes encoding for surface proteins that could function in recognition of or binding to components in the environment.

Some of these gene products are homologous to proteins with predicted functions such as mucus binding, aggregation promoting, and intracellular adhesion (26).

Furthermore, it has been suggested that the inhibitor- producing lactic acid bacteria which co-aggregate with pathogens may constitute an important host defense mechanism against infection in the urogenital tract (27). Also, a similar protective mechanism could operate in the GIT. Co-aggregation with potentially gut pathogens could, therefore, contribute to the probiotic properties attributed to the LAB. Adherence of bacterial cells is commonly related to cell surface characteristics.

Cell surface hydrophobicity is a non-specific the interaction between microbial cells and the host. The initial interaction may be weak, often reversible, and precedes subsequent adhesion processes mediated by more precise mechanisms involving cell surface proteins and lipoteichoic acids (28).

The determination of microbial adhesion to hexadecane, as a way to estimate the ability of a strain to adhere to epithelial cells, is a valid qualitative phenomenological approach. The results clearly indicate that the cell surface of microorganisms studied was relatively hydrophilic. In our present studies, *B. clausii* showed higher hydrophobicity than *L. sphaericus*. Similar results were recorded by Todorov and dicks; 2008 (16).

Microbial adhesion to two other solvents were

also examined. A selected bacterial strain which was tested here displayed a maximal affinity for an acidic solvent such as chloroform, and lower affinity for a basic solvent such as ethyl acetate.

These results demonstrated that selected bacterial strains are strong electron donors and weak electron acceptors, as confirmed by their hydrophilic cell surface properties. In other words, *B. clausii* and *L. sphaericus* have strong basic and weak acidic character. Microbial adhesion to hexadecane reflects cell surface hydrophobicity or hydrophilicity because electrostatic interactions are purely absent.

The values of hydrophobicity obtained with two other solvents, chloroform, and ethyl acetate, were viewed as a measure of electron donor/basic, and electron acceptor/ acidic characteristics of bacteria, respectively. Moreover, it should be noted that all these three solvents have similar Van der Waals properties. First, direct measurements of cell surface hydrophobicity and hydrophilicity were carried out by the partitioning of cells between aqueous and hexadecane partitions.

Very low percentages of studied bacteria (ranging from 10 to 26%) which adhered to this apolar solvent have demonstrated a hydrophilic surface. The determination of microbial adhesion to hexadecane as a means to estimate the capability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (29).

Because of the beneficial effects of prebiotics, there has been considerable interest in incorporating them into food products. The present studies have shown that prebiotics could stimulate probiotic growth and enzyme production (bacteriocin).

Honey and garlic have been estimated to be favorable carbon sources for the growth of probiotics. Similarly honey, garlic and onion have pretty high amounts of Fructooligosaccharide (FOS). However, there have been contradictory reports regarding the use of prebiotics to stimulate the growth of probiotic strains. Heubner et al. (30) reported that *L. paracasei* 1195 grow better in MRS medium containing 10 g/l FOS, and 10 g/l inulin than in glucose medium, however Kaplan and Hutkins (31) reported that three *Lactobacillus* strains, and one *Bifidobacterium* strain are unable to ferment FOS.

In an alternative study, Chou and Hou (32) investigated the effect of various prebiotics, including sorbitol, raffinose, and iso-malto-oligosaccharides towards the growth of *Bifidobacterium* in soymilk, and found that only iso-malto-oligosaccharides has shown a significant increase in growth after fermentation at 37°C for 48 hours.

In the present study, supplementation of synthetic prebiotic with mannitol has shown higher effect on the growth of B. clausii DY14 and *L. sphaericus* DY13 when compared to sorbitol. The use of mannitol as a prebiotic to promote the proliferation of probiotics has been studied to a small extent and its effect was found to be strain-dependent in the present study. Bacteriocin production has usually been studied in high-cost complex culture media.

Previous studies have clearly reported that multiple factors, including nutrients, temperature, and pH affect the production of bacteriocins (33). But the effect of prebiotic is still studied to a smaller extent, consequently,

the effect of prebiotics has been applied to effectively enhance bacteriocin production. The results obtained in this study evidently revealed that natural sources like honey, garlic, and onion could effectually enhance bacteriocin production of two selected strains than synthetic mannitol and sorbitol.

The investigation of other probiotic criteria for *L. sphaericus* DY13, and *B. clausii* DY14 strains, as well as characterization of the crude bacteriocin produced by these two strains and more investigation regarding the effect of prebiotics on enhancing specific crude bacteriocin are purposed for future studies.

Conclusion

Probiotics are micro-organisms that are commonly supposed to deliver health benefits when consumed. The term probiotic is presently used to name ingested microorganisms associated with benefits for humans and animals. The persistence of probiotics in food or in supplement form is to help regulate the ratio of beneficial-to-bad bacteria in the gut.

To assess the gut adhesion capacity of *L. sphaericus* DY13, and *B. clausii* DY14 strains which were previously isolated from mother's milk, and also to learn the effects of natural and commercial prebiotics on the production of bacteriocins is the focal objective of this study. Isolates were screened using properties like survival in simulated gastric and upper intestine contents, auto-aggregation, co-aggregation properties, and cell surface hydrophobicity.

In conclusion, isolated *L. sphaericus* DY13, and *B. clausii* DY14 strains from human milk possess potential probiotic properties as evidenced by their ability to survive under in-vitro gastrointestinal environment.

These characteristics may be advantageous for a probiotic culture to be successful in colonizing and competing with pathogens in the gastrointestinal environment. These indigenous probiotic isolates possess antagonistic activities against Gram-positive as well as Gram-negative food-borne and GIT pathogens which were enhanced by using honey as supplementary prebiotics and are beneficial both in food industries and in the medical sector.

Our results indicate that the ability to auto-aggregate, together with cell surface hydrophobicity and co-aggregation with pathogen strains can be used for preliminary screening in order to identify potentially probiotic bacteria suitable for human or animal use.

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