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Assessment ability of Probiotic bacteria in human breast milk

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Abstract

The aim of this work is to study; Co-aggregation,and auto-aggregation of probiotic bacteria isolated from human breast milk.. Lactic acid bacteria are gram positive bacteria that are natural occupants of gastrointestinal tract of mammals including humans. Probiotics are made-up to those bacteria which have useful effects for the host . The isolated lactic acid bacteria were confirmed by gram staining, catalase test and and selected on the basis of safety properties-Arginine hydrolysis, blood haemolysis and Gelatin hydrolysis. Among the five isolates C2 strain shows potential probiotic properties. The probiotic properties of the isolated strain were confirmed by acid tolerance and bile tolerance test. The C2 strain showed acid tolerance and bile tolerance up to four hours. The auto-aggregation potential was studied and their co-aggregation potential was studied against nine different pathogenic bacteria. The percentage of absorption was calculated. The C2 strain showed 80% auto aggregation ability at fifth hour and showed maximum co aggregation ability with *Shigella flexineri* ,hence proved that the isolated lactic acid bacteria is an effective probiotic bacteria.

Keywords: 1.Auto aggregation ,2.Co-aggregation, 3.Lactic acid bacteria, 4.Probiotics, 5.Pathogenic bacteria.

Introduction

The health benefits of certain foods have been investigated for many years. Development of foods that support health and well-being is one of the key research concern of the food industry. This tendency has led to the increased production and consumption of foods enriched with dynamic components such as prebiotics, probiotics and symbiotics which are accepted as functional foods

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health (Salminen *et al*., 1999). The word 'probiotic' comes from the Greek language 'pro bios' which means 'for life' similar to antibiotics which means against life. Probiotic products include different enzymes, vitamins, capsules or tablets and some fermented foods that contain microorganisms which have beneficial effects on the health of host. They can contain one or several species of probiotic bacteria. They are just used as health supporting products. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora (Gismondo *et al*., 1999, Çakır 2003, Quwehand, 1999).

Probiotics have used for long time in food ingredients for human and to feed animals without side effects. The major criteria for being accepted as probiotics are resistance to low acidity, tolerance against bile salt.

Materials and methods

Enumeration of lactic acid bacteria from milk

The breast milk collected was serially diluted in sterile distilled water. Dilution of the first was prepared by transferring 1mlof milk to 9 ml of distilled water and mixed thoroughly. And the dilution were done up to 10^{-4} dilution. 0.1 ml of sample from each dilution (10^{-1}- $10^{-4)}$ were poured on to

separate petriplates containing MRS agar medium and spread plate has been done. The petriplates were incubated for growth at 37°C for 48 hours.

Catalase Test

Catalase test was performed to isolates in order to see their catalase reactions. A small amount of bacterial colony (LAB) was transferred to a surface of clean, dry glass slide using a sterilized loop. Placed a drop of 3% H_2O_2 on to the slide and mixed.

Gram staining

Cultures were grown in MRS mediums at 37°C for 24 h. After incubation,cells from fresh cultures were used for gram staining. Gram staining procedure was applied. Then, under light microscopy gram Positives and purified isolates were determined gelatin hydrolysis.
Further selection based on the isolated stain which shows better safety properties- gelatin hydrolysis blood haemolysis and arginine hydrolysis

Safety properties

Gelatinase Activity

Gelatinase activity of the isolates was investigated as described by Harrigan and Mac Cane (1990). In this method, inoculum of 18-24 hour old test bacterium is stab inoculated into tubes containing nutrient gelatine agar. The inoculated tubes were incubated at 25^0C for up to one week and checked every day. A strain of *Staphylococcus aureus* (MTCC 96) was used as positive control.

Hemolysis Activity

This test is performed to check whether the organism is hemolytic or not. The hemolytic activity was determined according to Guttmann and Ellar (2000). Isolates were screened on freshly prepared sterile blood agar plates containing 5% blood and plates were streaked with 16-18hours old culture and incubated at 37°C for 24-48hours. They were observed for clear zones surrounding the colonies (positive reaction for β-hemolysis).

Arginine Hydrolysis Test

Arginine MRS medium and Nessler's reagent were used in order to see ammonia production from arginine. MRS containing 0.3% L – arginine hydrochloride was transferred into tubes as 5mL and inoculated with 1% overnight cultures. Tubes were incubated at 37^0C for 24 hours. After incubation 100µL of cultures transferred on to a white background. The same amount of Nesseler's reagent was added to the cultures. The change in the colour was observed. Bright orange indicated a positive reaction while yellow indicated the negative reaction. A negative control, which does not contain arginine, was also used as negative control.

Assessment of Probiotic Potentiality

Resistance to Low pH

The ability to survive in low pH is considered as one of the important property of probiotic bacteria. Resistance to pH 3 is often used invitro assays to determine the resistance to stomach pH. Active cultures of isolated probiotic bacteria incubated for 16-18 hours were used. Cells were

harvested by centrifugation for 10 minute at 5000 rpm at 4°C. Pellets were washed out in phosphate – saline buffer (PBS) at pH 7.2. Then cell pellets were re-suspended in PBS (pH 3) and incubated at 37°C. Viable microorganisms were enumerated at the 0, 1, 2, 3 and 4 hours by pour plate techniques. Appropriate dilutions were done and plates were incubated at 37°C under aerobic conditions for 48 hours. Also growth was monitored by measuring absorbance at 620nm. The results obtained were expressed as Colony Forming Units (CFU).

Tolerance against Bile Salts

The organism was grown in MRS broth. The broth was centrifuged at 5000rpm for 10minutes. Supernatant was discarded; pellet was washed and re-suspended in PBS buffer (pH7.2). It was then again centrifuged at 5000rpm for 10minutes. After removing the supernatant, pellet was mixed with MRS broth supplemented with bile salt (0.3%). Viable bacterial count was enumerated using pour plate technique at 0,1,2,3 and 4 hours. Growth was monitored by measuring O.D at 620nm.

Auto-aggregation and Co-aggregation:

Auto-aggregation assay

Aggregation assays was performed according to the method of Kos *et al.,*2003 with certain modifications. Bacteria were grown for 18 hours at 30^0C in MRS broth. The cells were harvested by centrifugation at 5000 rpm for 15 minutes, washed twice and resuspended in phosphate buffered saline (PBS , pH 7.2). Cell suspensions (4ml) were mixed by vortexing for 10 seconds and auto-aggregation was determined during 5 hours of incubation at room temperature. At 1 hour intervals, 0.1 ml of the upper suspension as transferred to another tube with 3.9 ml of PBS and the absorbance was measured at 600 nm.

Result and Discussion

Isolation of Probiotic bacteria for screening

Five lactic acid bacteria were obtained from human breast milk. The individual bacterial isolates which show different morphology was sub cultured on to nutrient agar medium in order to obtain pure cultures. The pure isolates 5 colonies named as C1, C2, C3, C4 and C5 were maintained at 4°C in refrigerator for further studies. The cultures were subjected to routine morphological and biochemical tests using standard protocols and the results obtained were shown in Table 1.

Table 1: Morphological and biochemical results of bacterial isolates

Samples	Isolates	Gram Staining	Catalase Test	Colony Morphology
Milk	C1	+ve, rods	-ve	Cream colour small colonies
	C2	+ve,cocci in tetrads	-ve	Cream colour, pin point colonies
	C3	+ve, cocci	-ve	White colour, small colonies
	C4	+ve, cocci	-ve	Small, round, cream colonies
	C5	+ve, cocci	-ve	Cream colour, large round colonies

The results obtained showed that all the bacterial isolates, C1-C5 were gram positive. C1 was rod shaped whereas C2 to C5 isolates were cocci. The catalase test was negative for all the bacterial isolates. The colony morphology of C1 bacteria was cream colored with small colonies; C2 cream colored with pinpoint colonies; C3 white coloured with small colonies;C4 small, round, cream coloured colonies and C5 was cream coloured with large round colonies.

About 92 isolates of lactic acid bacteria were purified from frozen camels milk. Out of that 55.43% were identified as cocci and 44.56% as rods (Brasca *et al.,* 2008). In Sudan, 24 LAB were isolated from 12 samples of fermented camel's milk, in which 66.6% were rods and 33.3% were cocci (Ashmaig *et al.,* 2009). A total of 450 cultures were isolated from 25 samples of dromadedary milk collected from Layaounne, Morocco. From that 30 were identified as LAB (Khay *et al.*, 2011) .

Among the 5strains C2 showed maximum probiotic safety properties.so further studies with C2 strain and the results were shown in Table:2

Table:2 Probiotic safety properties of isolated strains

Isolated strains	Blood haemolysis	Arginine hydrolysis	Gelatin hydrolysis
C1	Positive	Negative	Negative
C2	Negative	Negative	Negative
C3	Positive	Negative	Negative
C4	Negative	Positive	Negative
C5	positive	Positive	Positive

Gram Staining

Gram staining of C2 strain observed under phase contrast microscope showed gram positive cocci in tetrads.

Catalase

C2 strain when analysed for catalase test do not showed the formation of gas bubbles, which showed that C2 strain is catalase negative.

Analysis of Probiotic properties of isolate

Resistance to Low pH

After incubation, optical density of the sample was measured at 620nm and viable cell count was also determined as colony forming unit. From which it is clear that the isolate was able to survive in pH 3 for 4 hours. A significant increase in O.D value was observed during the interval and the results obtained were shown in table 3. Hence it was concluded that the LAB isolates was tolerant to low pH.

Table 3: C2 strain showing resistance to p^H from 0 - 4 hours

HOURS	OD at 620nm	CFU per ml
0^{th} hour	0.532	TNTC
1^{st} hour	0.541	TNTC

2nd hour	0.567	TNTC
3rd hour	0.571	TNTC
4th hour	0.542	TNTC

One of the major selection criteria for probiotic strains is being resistant to low pH (Qwehand *et al.,* 1999; Cakir, 2003). Probiotic bacteria have to pass through different stressful conditions of stomach o reach the small intestine (Chou and Weimr, 1999; Cakir, 2003). Usually pH was as low as 1.0 in stomach, and in many of the invitro assays, pH 3 has been preferred. This is because of the fact that a major decrease in viability of strains was seen at pH 2 and below (Prasad *et al.,* 1998).

Tolerance against bile salts

The C2 strain, resistant to low pH, were screened for their ability to tolerate the bile salt. Strains that were grown in 0.3% bile salt for 0 to 4 hours (Figure3). The CFU values and OD at 620 nm were observed. According to the results the C2 strains were tolerant to 0.3% bile salt. . A significant increase in O.D value was observed during the interval and the results obtained were shown in table 3

Table. 3: C2 strain showing bile salt tolerance from 0 - 4 hours

Hours	**O.D at 620 nm**	**CFU per ml**
0th hour	0.025	TNTC
1st hour	0.075	TNTC
2nd hour	0.192	TNTC
3rd hour	0.487	TNTC
4th hour	1.001	TNTC

An important characteristic of LAB to survive in small intestine is bile tolerance. Bile resistance of many strains of bacteria are due to specific enzyme activity, bile salt hydrolase (BSH) which hydrolyse conjugated bile, thus its toxic effect will be reduced (Du Toit *et al.,* 1998). The enzyme hydrolases (BSHs) which causes hydrolyzation has been explained by Tanaka et al.,(2000) , that are seen in *Lactobacillus sp.* (De et al.,1995) and *Enterococcus sp.* (Augus, 2003). Even though the human gastro intestinal tract has varying bile concentration, the mean intestinal bile concentration is considered to be 0.3% w/v and the staying time is recommended to be 4 hours (Prasad *et al.*, 1998).

Auto aggregation assay

Auto aggregation ability of lactic acid bacteria is very important step necessary for new the selection of newly identified probiotic strains. Aggregation ability of a probiotic strain is more important for its colonization in the gastrointestinal tract. C2 strain was tested for auto aggregation ability which showed 80% aggregation after 5 hours (Figure 4). The results of auto aggregation at 1-5 hours were shown in Table.4

Table : 4 Auto aggregation ability of C2 strain

Time	1hr	2hr	3hr	4hr	5hr
Probiotic isolate	33.3%	40%	53%	66%	80%

One of the important properties of many bacterial strains is its aggregation ability. Aggregation plays a significant role in the formation of biofilms to protect the host from colonization by pathogens (An *et al., 2000*). Lactic acid bacteria are more competent for adhesion to intestinal cells because of their aggregation ability and hydrophobicity. Studies have shown that *Lactobacillus crispatusis* can stick on better to Caco-2 cells than its non-aggregation mutant (Oca and Macias, 2001).

Co-aggregation assay

Barrier effect is one of the prominent properties of probiotic bacteria which mean resistance to pathogens colonization. Interbacterial adherence of C2 strain with different food borne pathogens is shown in the table 5.

Table 5: Co-aggregation of C2 strain with different enteropathogens

Microorganism	Percentage of co-aggregation at 600 nm				
	1hour	2 hour	3 hour	4 hour	5 hour
Shigella flexineri	25	33	40	33	29
Pseudomonas aeruginosa	14	15	21	23	17
Staphylococcus aureus	29	26	24	20	17
Bacillus cereus	35	33	28	24	22
Vibrio cholerae	20	24	25	23	16
Salmonella typhimurium	22	23	16	13	8
Escherichia coli	8	13	13	9	6
Listeria	25	24	24	13	19
Vibrio para	18	25	25	21	17

C2 strain showed maximum co-aggregation with *Shigella flexineri* (29%) at 5 th hour followed by *Bacillus cereus*(22%) at 5 th hour. Minimum co-aggregation observed with *Salmonella typhimurium*(8%) and *E.coli* (6%) after 5 hours. All other pathogens tested against C2 strain showed 10 to 17% co- aggregation after 5 hours (Figure 5).

In some ecological niches like human gut, co-aggregation of bacterial strains plays a significant role. Studies have shown that co-aggregation abilities may allow lactic acid bacterial strains to inhibit the development of pathogenic strains in the gastrointestinal and urogenital tracts in a very close proximity (Botes *et al.,* 2008). Lactic acid bacteria is able to control a microenvironment around the pathogens and helps to increase the concentration of excreted antimicrobial substances in the process of coaggregation (Kaewnopparat *et al.,* 2013)

Conclusion

The study concluded that the isolated probiotic C2 strain from human milk meet several of the probiotic criteria. C2 strain was gram positive with cocci in tetrads, cream coloured with pinpoint colonies and showed catalase negative property. When analysed, C2 strain showed resistance to low pH and resistance against bile salts from 0-4 hours. Acid and bile tolerances result showed that this C2 strain is to be successful in survive in the intestine . Auto aggregation and co-aggregation results showed this strain is colonizing in compete with pathogens in gastrointestinal environment.Hence useful in prevention of enteric infections. Thus, these probiotic strains could be used for both preventive and therapeutic purpose in controlling intestinal infection.

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