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ARTICLE

PROBIOTICS FROM HUMAN BREAST MILK

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Abstract

Probiotics , as defined by the Food and Agriculture Organization of United Nations (FAO) and World Health Organization (WHO) in 2001, comprise live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. The word 'probiotic' comes from the Greek language 'pro bios' which means 'for life' opposed 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. The present study involves the isolation of a probiotic bacteria from human breast milk. Among the five strains isolated from human breast milk, C2 strain showed maximum probiotic properties, so it was selected for the study. 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. C2 strain also showed antimicrobial property against several food borne pathogens. So the C2 strain which showed all the probiotic properties can be used for the preparation of probioticated foods.

Keywords: phytochemicals, germination, inhibition

Introduction

As today's consumers became aware of the link among lifestyle, diet and good health there is emerging demand for products that are able to enhance health beyond providing basic nutrition. The gastrointestinal tract of a healthy individual is colonized by a complex microflora containing many different species. A balance of these microorganisms in the gastrointestinal tract is important not only in promoting efficient digestion and maximum absorption of nutrients, but also in increasing the capacity of the host in excluding infectious microorganisms and hence preventing disease (Walter et al., 2003). The most commonly studied beneficial gut flora includes members of the genus Lactobacillus, especially, *L. acidophilus* and Bifidobacterium spp. (Tannock, 2002). To combat certain digestive disease and many other diseases like coleorectal cancer and cardiovascular disease ,the World Health Organization currently advocates the implementation of alternative disease control strategies, such as exploiting the prophylactic and therapeutic potential of these beneficial bacteria. These beneficial microflora were termed probiotics.

Probiotics , as defined by the Food and Agriculture Organization of United Nations (FAO) and World Health Organization (WHO) in 2001, comprise live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host. The word 'probiotic' comes from the Greek language 'pro bios' which means 'for life' opposed 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, Cakir 2003, Quwehand, 1999).

Probiotics have been used for long time in food ingredients for human and also to feed animals without any side effects. The excepted major criteria for being accepted as probiotics are resistance to low acidity and tolerance against bile salt. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much (Gismondo et al., 1999, Guarner et al., 2005). The concept of probiotics was first proposed by Elie Metchnikoff, a Nobel Laureate of the year 1908 . Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria (*Lactobacillus spp.*). Therefore, these bacteria affect the gut microflora positively and decrease the microbial

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toxic activity (Gismondo et al., 1999, Cakir 2003, Chuayana et al., 2003).

Materials and Methods

Isolation of lactic acid bacteria

Human breast milk was believed to contain many potential probiotic strains. Probiotic bacteria for the present work was isolated from human breast milk sample of a healthy mother. Breast milk sample was collected and stored at 4°C to protect from contamination and deterioration. Lactic acid bacteria (LAB) were isolated from human breast milk sample by using modified MRS (De Man, Rogosa and Sharpe) agar. The pH of the media was adjusted to 6.5. Isolation was carried out using spread plate technique at 10-1 and 10-2 dilutions. The plates were incubated at 37°C for 24-48hours. After incubation, individual colonies were selected and transferred into MRS agar slant. Selected colonies were inoculated by streak plate technique. The isolates were identified by their colony morphology, catalase reaction and Grams staining. A total of 22 isolates were isolated from human breast milk. From that five isolates were selected after original characterization.

Biochemical characterization

The distinct colonies obtained were further subcultured and gram staining was done to differentiate it as gram positive and gram negative bacteria.

Gram Staining

Gram staining is also known as differential staining technique. It is a procedure in which microbes are differentiated by their capacity to hold certain dyes. Staining depends on the ability of the microbial cell wall to resist decolourisation. Gram positive cells maintain the colour of the crystal violet even after decolourisation and become visible as pink colour but gram positive cells undergo decolourisation and absorb safranin and appear as purple colour.

Catalase Test

Catalase test was performed to isolates in order to see their catalase reactions. Overnight cultures of isolated bacteria were grown on MRS agar at suitable conditions. After 24 hours 3% hydrogen peroxide solution was dropped onto colonies into a glass slide. It was then examined for the presence or absence of bubbling or foam formation. Based on the observation recorded whether the organism has the catalase activity or not.

Analysis of Probiotic properties of isolate

The determination of probiotic properties of isolated bacteria can be confirmed by some selection criterias. They are resistance to low pH, tolerance against bile salts and antimicrobial activity.

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.

Antimicrobial activity

Modified agar well diffusion method was used to detect antimicrobial activities of cell free supernatant (CFS) produced from the isolates. Antibacterial activity was determined against: *Escherichia coli, Vibrio parahaemolytics, Listeria monocytogens, Vibrio cholera, Streptococcus fecalis, Pseudomonas aeruginosa, Bacillus cereus, Staphylocouus aureus* (MTCC 96), *Shigella flexineri, Salmonella typhimurium* and *Proteus mirabilis*. All of LAB isolates were incubated for 48 hour at 370C. After incubation cells were removed by centrifugation. The indicator organism is inoculated in nutrient broth and incubated at 37^0C for 5- 6 hours. The incubated organisms were swabbed on to the MHA (Muller – Hinton Agar) plates using swab and the CFS (Cell Free Supernatants) was used as antimicrobial agents. Using sterile tips the CFS was poured into the well of about 50µL and kept for incubation at 37^0C for 24 hours. Antimicrobial activity was evaluated by measuring zone of inhibition against the test organisms.

Results 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

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

Table 2. Absorbance of C2 strain from 0 to 4 hours

Hours	OD at 620nm	CFU per ml
0th hour	0.532	TNTC
1st hour	0.541	TNTC
2nd hour	0.567	TNTC
3rd hour	0.571	TNTC
4th hour	0.542	TNTC

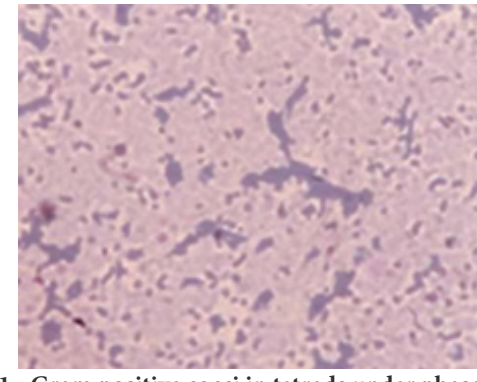

Figure 1 - Gram positive cocci in tetrads under phase contrast microscope

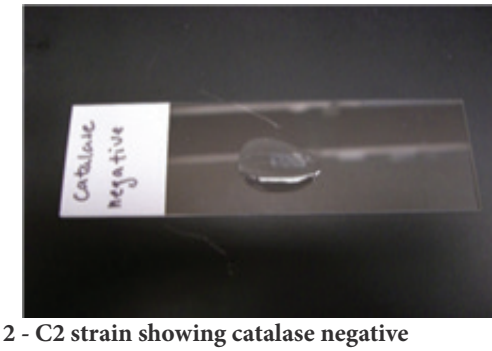

Figure 2 - C2 strain showing catalase negative

in Table 1.

The results obtained showed that all the bacterial isolates, C1-C5 were gram positive. C1 was rod shaped where as 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).

Biochemical characterization

Gram Staining

Gram staining of C2 strain observed under phase contrast microscope showed gram positive cocci in tetrads (Figure 1).

Catalase

C2 strain when analysed for catalase test do not showed the formation of gas bubbles, which showed that C2 strain is catalase negative (Figure 2).

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 (Figure 3). A significant increase in O.D value was observed during the interval and the results obtained were shown in table 2. Hence it was concluded that the LAB isolates was tolerant to low pH.

One of the major selection criteria for probiotic strains

Table 2. Absorbance of C2 strain from 0 to 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

Table 2. Absorbance of C2 strain from 0 to 4 hours

Organism	Zone of inhibition
Vibrio parahaemolytics	16mm
Listeria monocytogens	14mm
Vibrio cholerae	13mm
Streptococcus fecalis	10mm
Pseudomonas aeruginosa	10mm
Bacillus cereus	11mm
Shigella flexineri	9mm
Salmonella typhimurium	9mm
Escherchia coli	12mm
Staphylococcus aureus	11mm
Proteus mirabilus	12mm

is being resistant to low pH (Qwehand et al.,1999; Cakir, 2003). Probiotic bacteria have to pass through different stressful conditions of stomach to 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 (Figure 4). The CFU values and OD at 620 nm were observed. According to the results the C2 strains were tolerant to 0.3% bile salt.

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 L*actobacillus sp.* (De et al.,1995) and *Enterococcus sp.* (Augus, 2003). Even thoughthe 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).

Antimicrobial activity

Antimicrobial activity of C2 probiotic strain against different food borne pathogens such as *Escherichia coli, Vibrio parahaemolytics, Listeria monocytogens, Vibrio cholera, Streptococcus fecalis, Pseudomonas aeruginosa, Bacillus cereus, Staphylocouus aureus* (MTCC 96), *Shigella flexineri, Salmonella typhimurium* and *Proteus mirabilis* were tested and the result obtained were shown in Table 3.

The result obtained showed that *Vibrio parahaemolytics, Listeria monocytogens* and *Vibrio cholera* activity showed maximum antimicrobial activity with 16mm, 14 mm and 13 mm respectively. *Streptococcus fecalis, Pseudomonas*

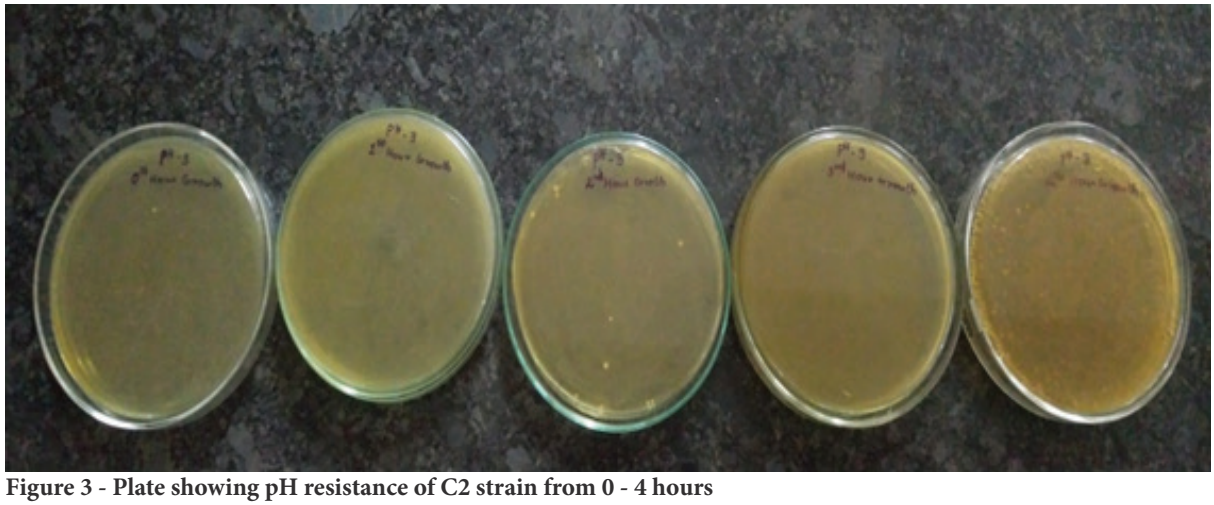

Figure 3 - Plate showing pH resistance of C2 strain from 0 - 4 hours

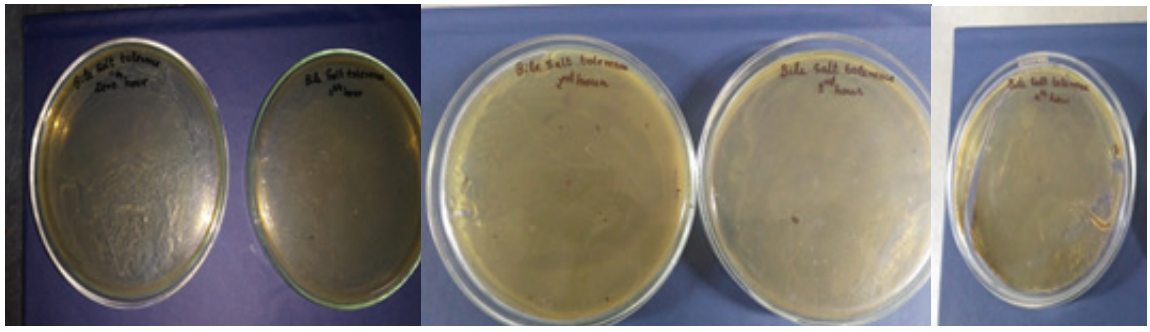

Figure 4 - Plates showing bile salt tolerance of C2 strain from 0 - 4 hours

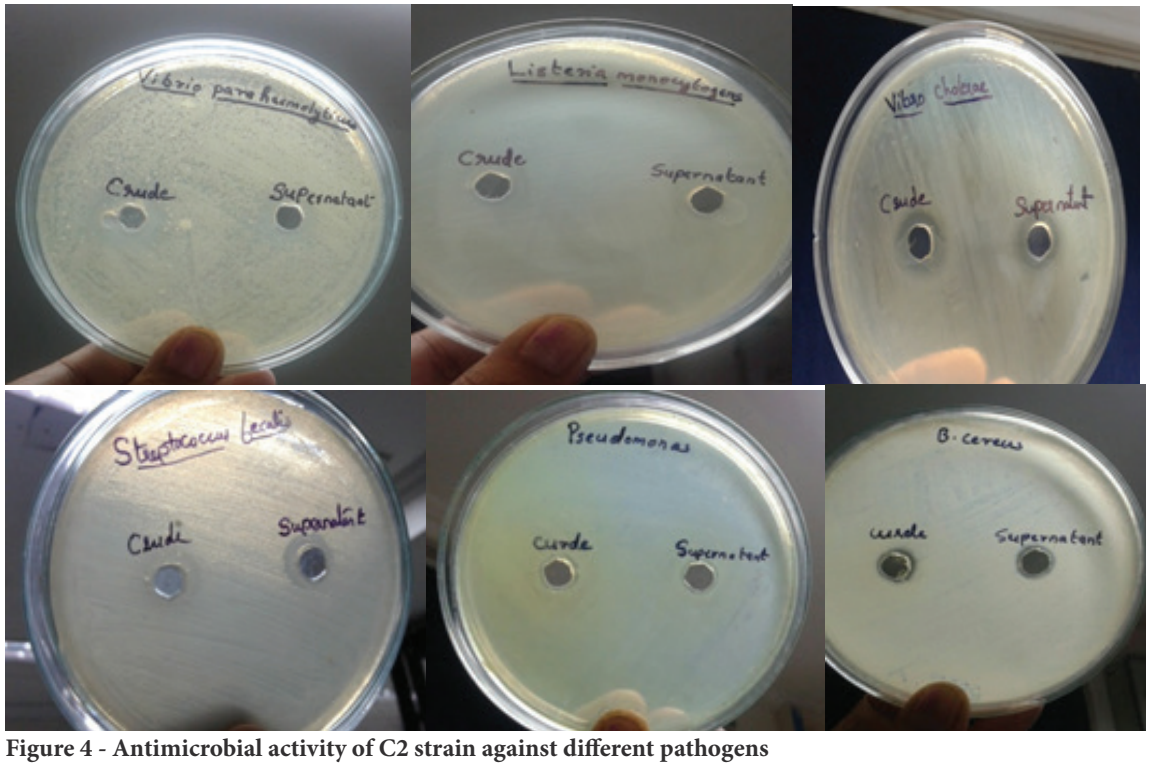

Figure 4 - Antimicrobial activity of C2 strain against different pathogens

aeruginosa, E.coli also showed significant inhibition. The minimum inhibition was showed by *Salmonella typhimurium* and *Shigella flexineri*.

Primary metabolites such as lactic acid, acetic acid, ethanol and carbon dioxide accumulated in LAB are mainly responsible for its inhibitory effect. In addition to this LAB also produces antimicrobial compounds such as formic acid, benzoic acid, hydrogen peroxide diacetyl, acetoin and bacteriocins. Depending on the type of strain, medium compounds and physical parameters the production levels and the proportion among these compounds differs (Tannock, 2004).

LAB has shown to possess inhibitory activities mostly towards Gram positive pathogens and closely related bacteria due to the bactericidal effect of protease sensitive bacteriocins (Jack et al., 1995). And some enterocins produced by *Enterococcus sp.* (Jennes et al., 1999) on Gram-negative bacteria through their synergetic effects with other antimicrobials has gained increased interest (Helander et al., 1997). LAB were also able to control the growth of Gram negative pathogens including food borne pathogens by the production of organic acids and hydrogen peroxide (Lu and Walker, 2001).

Conclusion

The present study involves the isolation of a probiotic bacteria from human breast milk. Among the five strains isolated from human breast milk, C2 strain showed maximum probiotic properties, so it was selected for the study. 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. C2 strain also showed antimicrobial property against several food borne pathogens. So the C2 strain which showed all the probiotic properties can be used for the preparation of probioticated foods.

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