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The investigation of probiotic potential of lactic acid bacteria isolated from cow milk

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Abstract

Probiotic strains can be isolated from many sources, for human applications one of the main criteria is being mammal origin. Cow milk is an important nutrient source for human. Lot of studies showed that this fluid has beneficial effects on the health of human. One reason of being beneficial is explaining by the microflora of cow milk including beneficial lactic acid bacteria. In this research work, 12 isolate were identified as lactic acid bacteria by biochemical characterization and further studied for probiotic activity. Among them 9 lactic acid bacteria was posseses probiotic activity. Six *Lactobacilli* stains were identified as *Lactobacillus gasseri(1),Lactobacillus rhamnosus(1),Lactobacillus fermentum(2),Lactobacillus viridescens(3),Lactobacillus farciminis(1), Lactobacillus buchner(4).* All of the bacilli isolates were selected to observe potential probiotic activity. These isolates showed resistance to stomach pH (pH 3.0), tolerance against 0.3% bile salt concentration and antimicrobial activity against *Escherichia coli, Salmonella enteritids, Staphylococcus aureus, Listeria monocytogenes*. The isolates were used to investigate the tolerances of the isolates to other stress conditions, such as, growth at different NaCl solution, growth at different temperatures and in the presence of 0.5% $CaCO_3$. All of the isolate showed good tolerances in stress conditions. After investigation the probiotic properties of these isolates, they were identified by biochemical characterization techniques. It is observed that, cow milk is a source of potential probiotic strains.

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Introduction

The history of probiotics began with the history of man by consuming fermented foods that is well known that Greek and Romans are consuming very much (Gismondo MR *et al.,* 1999, Guarner F *et al.,* 2005). Russian researcher Ellie Metchnikoff, who got a nobel prize, firstly proposed the beneficial effects of probiotic microorganisms on human health.

More than 400 bacterial species exist in human intestinal tract. It is an enormously complex ecosystem that includes both facultatively anaerobic and anaerobic microorganisms (Naidu AS *et al.,* 1999). The numbers of genera is nearly steady, because they each have their own growth niches (Fooks LJ *et al.,* 1999). The composition of the gut microflora is constant but can be affected by some factors such as; age, diet, environment, stress and medication. Using of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and make the balance steady again (Fooks LJ *et al.,* 1999). Probiotics are suggested as food to provide for the balance of intestinal flora (Holzapfel WH *et al.,* 1998).

Milk and milk products are usually associated with probiotic bacteria, which provide supplements in maintaining beneficial intestinal balance (Isolauri E *et al.,* 2001). Probiotic bacteria may produce various compounds, which are inhibitory to the pathogen's growth, which include organic acids (lactic and acetic acids), bacteriocins, and reuterin. The organic acids not only lower the pH, thereby affecting the growth of the pathogen, but they can also be toxic to the microbes. *Lactobacilli* are known to produce many types of bacteriocins like acidophilin, acidolin, lactocidin, bulgarican, lactolin, lactobacillin and lactobrevin.

Enteric bacteria comprised of *Salmonella* species, *Shigella* species, Proteus species, *Klebsiella* species, *E. coli*, *Pseudomonas* species, *Vibrio cholerae* and *S. aureus* which are major etiologic agents of enteric infection (Ballal M *et al.,* 2002). There is increasing evidence that probiotics are beneficial in gastrointestinal disturbances, such as diarrhoea, dysentery, typhoid etc (Fooks LJ *et al.,* 1991). Also probiotics are acceptable because of being naturaly in intestinal tract of healthy human and in foods (Cakir I 2003).

However, the overall efficacy of probiotics from milk of domestic animals and the mechanisms by which probiotics ameliorate enteric infections are mostly unknown. Therefore an attempt was made to isolate *Lactobacillus* strains as probiotics from milk of domestic animals and compared its probiotics potential with commercial probiotic preparations and standard probiotic bacterial strains in prevention of enteric bacterial infections.

Materials and methods

Isolation of lactic acid bacteria from cow milk

The isolation material was cow milk obtained from 5 healthy cows from savar area. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were taken to the procedure for isolation. Pour plate technique was used to isolate the organisms. Samples were used directly and also diluted to 10^{-1} 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS (Man, Rogosa and Sharpe) agar (pH 6.2 and pH5.5), TPY (Trypticase Phytone Yeast) agar (pH 6.5) and MRS-cystein agar (pH 5.5). The plates were incubated at 30 °C for 3 days under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact).The isolates were examined according to their colony morphology, catalase reaction and gram reaction.

Gram staining

The gram reaction of the isolates was determined by light microscopy after gram staining. Lactic acid bacteria (LAB) are known to be gram positive. It means that they give blue-purple color by gram staining. Cultures were grown in appropriate mediums at 30 °C for 24 h under anaerobic

conditions. Cells from fresh cultures were used for gram staining. Then, under light microscopy gram positives and purified isolates were determined.

Catalase test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.

$2H_2O_2 \rightarrow 2\ H_2O + O_2$

Fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were choosed. Since, LAB are known as catalase negative.

Probiotic properties of isolates

For the determination of probiotic properties of isolates these major selection criteria were selected: resistance to low pH, tolerance against bile salt and the antimicrobial activity.

Resistance to low pH

Being resistant to low pH is one of the major selection criteria for probiotic strains (Quwehand AC *et al.,* 1999, Cakir I 2003). Since, to reach the small intestine they have to pass through from the stressful conditions of stomach (Chou LS *et al.,* 1999, Cakir I 2003). Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad RDD *et al.,* 1998). For selection the strains resistant to low pH, pH-adjusted to 3.0 was used. The time that takes during the digestion in the stomach is 3 hours. So, all the isolates were detected whether they were resistant to pH 3.0 or not.

Tolerance against bile

The strains, resistant to low pH, were screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 h (Prasad RDD *et al.,* 1998).

Antimicrobial activity

The selected strains were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator microorganisms Escherichia coli, S. enteritids, S. aureus, L. monocytogenes.

Physiological and biochemical characterization

Gas production from glucose

In order to determine the homonfermentative and heterofermentative characterization of isolates, CO_2 production from glucose test was applied. Citrate lacking MRS broths and inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. Then the test tubes were incubated at 30 °C for 5 days. Gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO_2 production from glucose.

Growth at different temperatures

Temperature test media, MRS containing bromecresol purple indicator, was prepared and transferred into tubes as 5 ml. Then fifty µl of overnight cultures inoculated to tubes and incubated for 7 days at 10 °C, 45 °C, and 65 ^{0} C. During these incubation time cells growth at any temperatures was observed by the change of the cultures, from purple to yellow.

Growth at different NaCl concentrations

Isolates were tested for their tolerance against different NaCl concentrations. For this purpose 4%, 6%, 8% and 10% NaCl concentrations were selected. Test mediums containing MRS broth were prepared according to the appropriate concentrations and transferred into tubes in 5 ml. these tubes were inoculated with overnight cultures and then incubated at 30 °C for 7 days. The change of the color from purple to yellow was proofed the cell growth.

Carbohydrate fermentation

Isolates were characterized according to their fermentation profiles of ability to ferment 8 different carbohydrates. All reactions were performed by pepton, NaCl, 1% sugar and .0025% phenol red as indicator. At first the media were prepared by adding the following ingredients mentioned above. After that the media were poured into the screw cap test tube each with 10ml. Then the tubes were autoclaved at 121 ^{0} C for 1 hour. Each of the tube was inoculated by 24 hour growth strain. All the reactions were performed twice. Also positive and negative controls were used to indicate any contamination. After overnight incubation at 30°C, the turbidity and the color change from red to yellow was recorded as positive fermentation results compared with the positive and negative control.

Growth in MRS+ 0.5% $CaCO_3$ Media

MRS media were prepared by adding 0.5% $CaCO_3$ with it. After that the media were strick with all the isolets. The plates were incubated over night at 30 ^{0} C in an anaerobic jar. All the isolates show good growth and create a clear zone around the colony. This helps to isolate single colony from the culture.

Results

Physiological and Biochemical Characterization

Bacteria isolated from different milk samples were identified as *Lactobacillus spp*. by observing their colony morphology, physiological and as well as some biochemical characteristics. Microscopically they were Gram-positive, rod shaped, catalase negative. All of the isolates were subjected to Gram staining and they were examined under light microscope. All the strains gave blue- purple color with staining; hence they all were Gram positive bacteria. All of the bacilli strains were selected with long and rounded end. Isolates were tested for catalase activity. They were all catalase negative. To test the gas production from glucose test tubes were observed for 5 days. But none of the strain showed any gas production. This indicates that all of the strains were homofermentative.

Another criterion for the identification the isolates was the ability of growth at different temperatures. From the results of 7 days observation, all of the strain show good growth rate at the temperature of 10°C, 45°C but they show very poor growth rate 65^0 C temperatures (Table1). Growth at different NaCl concentrations was observed. Most of the strain show very good growth rate at the concentration of 4%, 6%, 8% NaCl containing media but very poor growth rate at 10% NaCl concentration (Fig. 1).

Table 1. Growth at Different NaCl Concentrations.

Isolets	4% NaCl	6% NaCl	8% NaCl	10% NaCl
1	+	-	-	-
3	++	-	++	-
5	++	-	++	-
6	+	-	-	-
7	+++++	++++	+++	-
9	++++	++++	++	+
10	++++	-	++	+
11	++++	++++	++	+
12	+++	++++	-	-
13	++++	++	+	+
17	++++	++	++	+
18	++	+++	+	+

Legend: +, growth; -, no growth

The most useful test for the determination of strain differences is carbohydrate fermentation. Eight (with glucose) different carbohydrates were used for identification. They give different fermentation patterns when they are compared.

Differentiating characteristics of Lactobacillus species

The LAB were classified into the genera *Lactobacillus* based on their morphology and biochemical characters. The predominant *Lactobacillus sp.* was further classified to the species level. Each strain showed variation in their sugar fermentation pattern. Only tests that gave reproducible results were included in the classification scheme. The species identified *were L. gasseri (1), L. rhamnosus (1), L. fermentum (2), L. viridescens (3), L. farciminis (1), L. buchneri (4).*

Probiotic properties

Resistance to low ph & tolerance against bile

After the examination of all the isolates, the isolates that survived in pH 3.0 were taken to the next step. According to this experiment, among 12 isolates 9 of them were resistant to low pH. Experiments were run twice. Strains were detected in 0.3% bile concentration. According to the results all of the isolates are resistant to 0.3% bile salt except one isolate. All of the isolates are also able to grow in 0.3% bile salt as they survive (Fig. 2)

Table 2. Diameter of inhibition zones.

Isolates	Indicator Microorganisms			
	Diameter of inhibition zones (mm)			
No	*E. coli*	*S. enteritids*	*S. aureus*	*L. monocytogenes*
1	10	10	9	10
3	10	10	10	10
5	7	6	8	5
6	9	11	10	7
7	10	8	6	6
9	8	6	6	10
10	6	7	8	6
11	6	9	10	8
12	-	-	-	-
13	8	8	9	6
17	-	-	-	-
18	-	-	-	-

Antimicrobial activity

The selected strains were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator microorganisms *Escherichia coli, S. enteritids, S. aureus, L. monocytogenes*. The diameter of inhibition zones (Table2.) showed that all of the isolates have antibacterial effect on the indicator microorganisms.

Discussion

In this research work the isolated *lactobacilli*'s showed their inherent characters of probiotic potential and the following results were obtained finally. 1) Lactic Acid Bacteria were isolated from cow milk, 2) Probiotic properties of isolated bacteria were determined while they show the salt tolerance activity, different temperature tolerance tolerance activity and 3) Phenotyic and genotypic identifications were effectively diferentiate the isolates especially sugar fermentation patterns

support the genotypic characterization results. Two of them was determined that they could be potential probiotic strains even if some forward tests were applied.

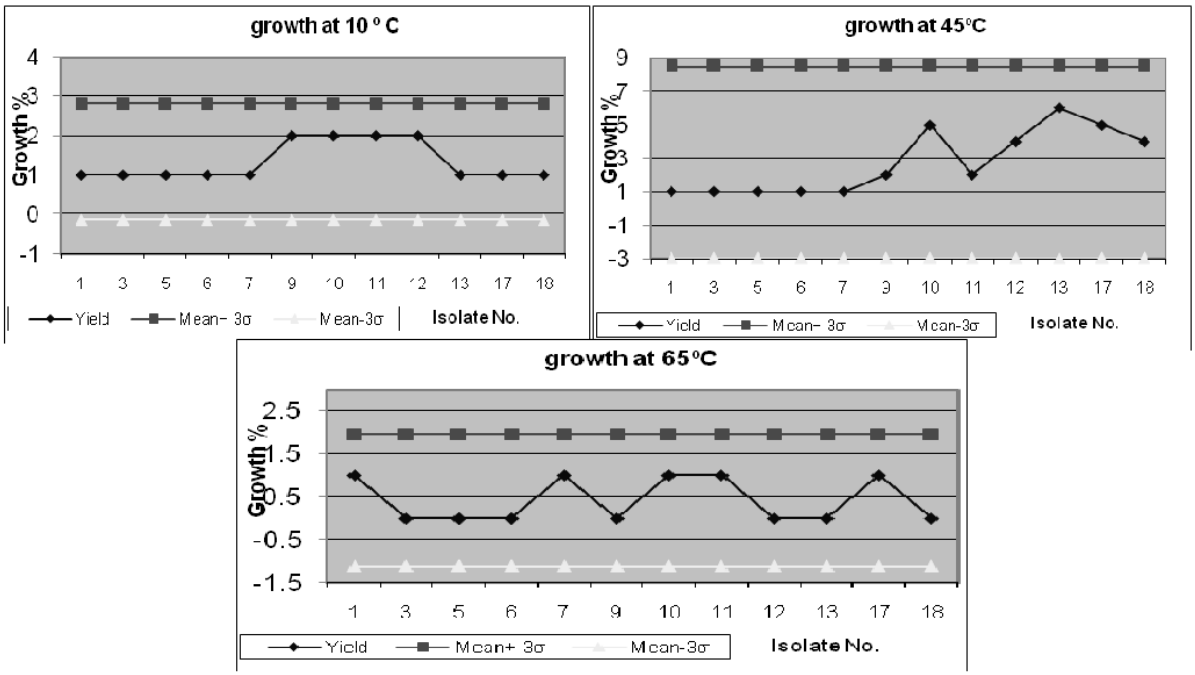

Fig. 1. Growth at different temperatures.

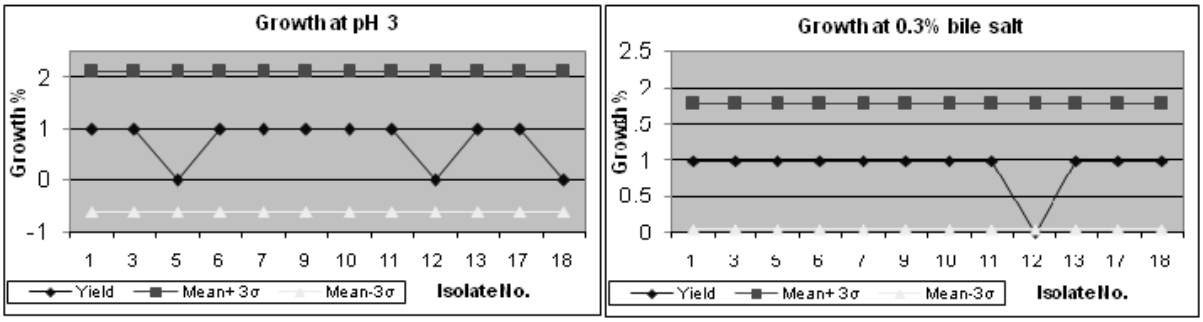

Fig. 2. Growth at low pH & bile salt.

In this study the first step was taken to use the isolates as cultures for probiotic products &12 isolate were identified as lactic acid bacteria by biochemical characterization and further studied for probiotic activity. Among them 9 lactic acid bacteria was posseses probiotic activity. Six *Lactobacilli* stains were identified as *Lactobacillus gasseri(1),Lactobacillus rhamnosus(1),Lactobacillus fermentum(2),Lactobacillus viridescens(3),Lactobacillus farciminis(1), Lactobacillus buchner(4).*

All of the bacilli isolates were selected to observe potential probiotic activity. The main criteria of being probiotic strains were determined which proved that cow milk is a source of potential probiotic strains. It will be beneficial to test the following characteristics;

1. Adhesion to mucosal surface.
2. Clinical studies for human health.
3. Technological properties (strain stability, viability in products, bacteriophage resistance).
4. Antibiotic resistance.
5. Growth at different stress condition.

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