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Isolation of Lactic Acid Bacteria with Probiotic Potential from Camel Milk

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Abstract: Three Lactic Acid Bacteria (LAB) namely *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactococcus lactis lactis* were isolated from un-pasteurized camel milk that was allowed to ferment spontaneously for one week. The probiotic potential of these isolates was investigated using *in vitro* antagonistic tests against *Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13076, *E. coli* 8739 strain, *Staphylococcus epidermis* and *Staphylococcus capitis*. The isolated LAB bacteria resisted the bactericidal effect of each other and did not produce antagonistic effect against *Staphylococcus* sp. However, *Lactobacillus plantarum* was able to inhibit the growth of the Gram negative tested bacteria with an average inhibition zone of 18 and 26 mm in diameter against *Salmonella* sp. and *E. coli* strains, respectively, while, *Lactobacillus pentosus* produced an average inhibition zone of 15 mm against *Salmonella* sp. and 25 mm against *E. coli* strains. *Lactococcus lactis lactis* antagonistic behavior was demonstrated by the production of 14 mm against *Salmonella* sp. and 20 mm inhibition zones against *E. coli* strains. In contrast to *Lactococcus lactis lactis* which was sensitive to low pH, both *Lactobacillus plantarum* and *Lactobacillus pentosus* demonstrated notable tolerance to acidic pH.

Key words: Probiotic, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactococcus lactis lactis*, *in vitro* antagonistic tests, camel milk

INTRODUCTION

Probiotics are live nonpathogenic microorganisms consist mainly of members of Lactic Acid Bacteria (LAB) such as *Lactobacillus* spp. and *Bifidobacterium* spp. and selected species of yeasts. These bacteria beneficially affect the host upon ingestion by improving the balance of the intestinal microflora and are important for the maturation of the immune system, development of normal intestinal morphology and maintaining a chronic and immunological balanced inflammatory response (Tannock, 2004). Probiotics have also been shown to possess inhibitory activities toward the growth of pathogenic bacteria. This inhibition could be due to the production of inhibitory compounds such as bacteriocins or reuterin, hydrogen peroxide, the alternation of pH values by the production of organic acids and competitive adhesion to the epithelium (Kolida *et al*., 2006).

In vitro methods are usually applied to measure the antagonistic action of probiotic microorganisms against pathogenic bacteria. These methods depend on bacterium-bacterium antagonism which regulates the proliferation and cell association of one bacterium by the metabolic products produced by the other (Jin *et al*., 1999). Different inhibitory compounds produced by LAB bacteria exhibit different effect on susceptible microorganisms. The bactericidal effect of bacteriocins is usually directed towards Gram positive species which are closely related to the producing bacterium (Jack *et al*., 1995). However, other antagonistic substances such as lactic acid and hydrogen peroxide can inhibit diverse types of microorganisms including food-born pathogens (Ito *et al*., 2003).

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Yogurt, cheese and fermented milk products are considered among the primary food sources of probiotics. However, there is some debate about whether dairy product starter cultures such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* should be considered probiotics. While traditional starter cultured dairy products are selected for their ability to rapidly produce desirable organoleptic qualities of cultured dairy products, the probiotic bacteria should be selected for the potential to provide specific health or nutritional benefits following consumption (Gilliland, 2001).

A research program was initiated at Kuwait Institute for Scientific Research (KISR) in 2005 to develop cost-effective probiotic additives that can improve health and does not pose any environmental health hazards. For this purpose, different susceptible samples were used to isolate potential probiotic including Arabian camel milk. The aim of this study is to assess the probiotic potential of lactic acid bacteria isolated from fresh and un-pasteurized Arabian camel milk.

MATERIALS AND METHODS

Sample Source and Enrichment Technique

Milk sample which was collected from a lactating Arabian camel (*Camelus dromedaries*) from a local camel breeding farm in the State of Kuwait was used for isolating LAB probiotic bacteria. The sample was allowed to ferment at room temperature for 1 week spontaneously without any additives through the raw milk endogenous microorganisms.

The enrichment process of the collected sample was carried out as follows: 10 mL volume of raw camel milk was added to 80 mL MRS (deMan, Rogosa and Sharpe) broth medium in 150 mL conical flasks. The enriched samples were incubated at 30°C for 1 week under static conditions. The high volume of the media provided suitable conditions for the facultative anaerobic microorganisms and made it unnecessary to incubate the samples anaerobically. The enrichment process was conducted in triplicate and repeated on weekly basis for one month period.

Isolation, Characterization and Identification of LAB Bacteria

The isolation process was carried out by streaking the enriched samples on MRS agar media and the isolated bacteria were incubated anaerobically at 37°C. The isolated bacterial cultures were characterized and identified using DNA sequencing technique which was carried out by MIDI labs laboratory (USA). The identification procedure is based on 16S rRNA gene sequencing similarity. The raw sequencing data files were compiled into sequences and sequence comparisons were obtained and analyzed using Applied Biosystems Microseq™ software or Genbank database libraries (Montgomery *et al*., 1999; Palys *et al*., 1997).

The identification process was also confirmed by fatty acid methyl ester analysis using the MIDI Sherlock Microbial Identification System. This identification process is based on similarity index which express how closely the fatty acid profile composition of an unknown sample compares with the mean fatty acid composition of the strain used to create the library entry listed as its match. The similarity index for these analysis ranged from 0.806 to 0.847.

Determining the Antagonistic Activity of Isolated LAB Using *in vitro* Tests

The antagonistic activity of the isolated LAB bacteria against *Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13076, *E. coli* 8739 strain, *Staphylococcus epidermis* and *Staphylococcus capitis* was determined using agar spot test (Jacobsen *et al*., 1999).

Prior to conducting the test, the potential probiotic LAB isolates were propagated in MRS broth medium and incubated anaerobically at 37°C for 48 h. For the agar spot test, 4 μL of propagated LAB bacterial isolates were spotted on the center of the surface of MRS agar medium containing only 0.2% glucose and 1.2% agar, in triplicate and incubated anaerobically for 24 h at 37°C to allow colonies to

develop. Approximately 10^7 cells of the test pathogen (i.e., heavy growth) in 15 mL of Nutrient agar were poured on the plate in which LAB were grown.

After incubation for 24 h at 37°C, the diameter of the inhibition zone around the LAB spot was measured. This clear zone was used as an indication of the ability of isolated LAB bacteria to antagonize the tested pathogen. The diameter of the clear zone (mm) was determined by measuring the diameter between LAB colonies and four different points of the clear zone surrounding the colonies and reporting the average. The antagonistic test was performed in triplicate. The data were statistically analyzed using one-way analysis of variance (ANOVA) test for significance at $p \leq 0.05$, using Analyze-it software version 1.73.

Tolerance of Isolated LAB to Acidic pH

The tolerance of the isolated LAB to acidic pH was performed as described by Gotcheva *et al.* (2002). Each bacterial isolate was grown in MRS (deMan Rogosa Sharpe) broth and incubated at 37°C overnight, then subcultured into fresh MRS broth and incubated for another 24 h. The bacterial cultures were then centrifuged at 5000 rpm for 10 min at 4°C and the pellets were washed twice in sterile phosphate-buffered saline (PBS, 0.1 M phosphate buffer, 0.8% NaCl, pH 7.2) and re-suspended in PBS. Each strain was diluted 1/100 in PBS at pH 1.0, 2.0 and 3.0 and incubated for 1, 2 and 3 h. Counts of surviving bacterial colonies were determined after plating the bacterial isolates on MRS agar with appropriate pH and incubating them anaerobically at 37°C overnight. Control samples without acidification were also prepared and similarly handled.

RESULTS

Characterization and Identification of LAB Bacteria

A number of bacterial species characterized as gram positive cocci or rods, catalase negative and non-spore forming were isolated from the tested camel milk sample and were identified as Lactic Acid Bacteria (LAB). The isolates were further identified using 16S rRNA gene sequencing from which three LAB species were distinguished namely: *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactococcus lactis lactis*.

***In vitro* Antagonistic Activity of Isolated LAB**

There was no significant difference ($p \leq 0.05$) in the antagonistic effect against the tested Gram negative pathogens between *Lactobacillus plantarum* and *Lactobacillus pentosus*. However, both cultures demonstrated significantly higher antagonistic activity when compared to *Lactococcus lactis lactis*. The highest antagonistic activity against Gram negative test pathogens, *E. coli* and *Salmonella* sp., was demonstrated by *Lactobacillus plantarum* with 26 mm in diameter inhibition zone against *E. coli* and 18 mm in diameter against *Salmonella enteritidis* (Table 1, Fig. 1, 2).

All the three isolated LAB species demonstrated significant inhibitory effect against *E. coli* compared to *Salmonella* sp. *Salmonella enteritidis* was found to be more susceptible to the inhibitory effect of the three isolated LAB species compared to *Salmonella typhimurium*. Additionally, the isolated LAB bacteria resisted the bactericidal effect of each other and did not produce antagonistic effect against Gram positive *Staphylococcus* sp.

Table 1: Antagonistic action of isolated LAB bacteria against *Escherichia coli* and *Salmonella* sp.

Bacteria ID	*S. typhimurium*	*S. enteritidis*	*E. coli*
Lactobacillus plantarum	17±0.8	18±0.7	26±0.5
Lactobacillus pentosus	14±0.6	17±0.8	25±0.4
Lactococcus lactis	12±0.3	15±0.5	20±0.4

The presented value is the average of three replica±SE. The clear zone measurement is in mm. The significant difference $p \leq 0.05$

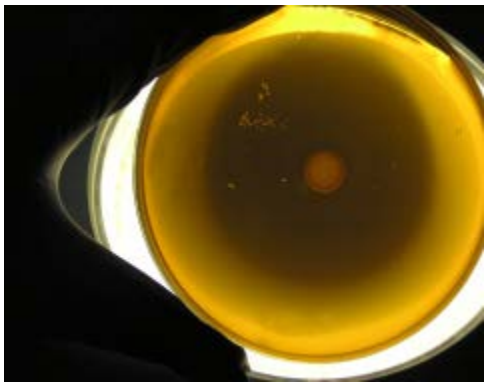

Fig. 1: Positive antagonistic effect of *Lactobacillus plantarum* against *Escherichia coli*

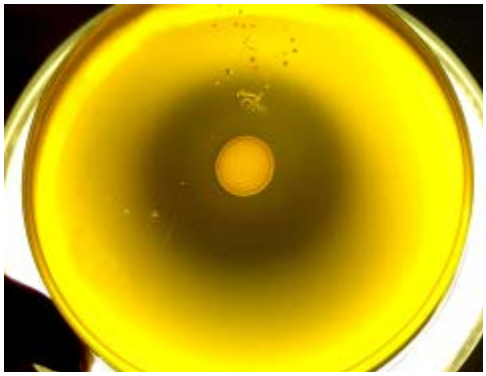

Fig. 2: Positive antagonistic effect of *Lactobacillus plantarum* against *Salmonella enteritidis*

Tolerance of Isolated LAB to Acidic pH

Both *Lactobacillus plantarum* and *Lactobacillus pentosus* survived an incubation period of 1 to 2 h at pH 3.0 with decrease in survival when the exposure time progressed for each strain. However, only *Lactobacillus pentosus* was able to survive 2 h at pH 2.0. *Lactococcus lactis lactis* did not demonstrate recordable survival rate at the tested pH values. No growth was observed in all isolated LAB strains at pH 1.0 for 1 h.

DISCUSSION

The inhibitory action of LAB bacteria is mainly due to the accumulation of main primary metabolites such as lactic and acetic acids, ethanol and carbon dioxide. Additionally, LAB are also capable of producing antimicrobial compounds such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins. The production levels and the proportions among those compounds depend on the strain, medium compounds and physical parameters (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). Still LAB were also able to control the growth of Gram negative pathogens including food born pathogens by the production of organic acids and hydrogen peroxide (Lu and Walker, 2001; Ito *et al*., 2003).

In this study, the probiotic potential of *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactococcus lactis lactis*, isolated from camel milk in controlling Gram negative and positive pathogens was investigated. The three isolated LAB species demonstrated a clear bacteriocidal effect against Gram negative pathogens *Salmonella* spp. and *E. coli*. However, no antagonistic effect against Gram positive *Staphylococcus* spp. or the closely related LAB species was detected. From this preliminary test it was concluded that the isolated LAB species controlled the tested pathogens using inhibitory activities other than bacteriocin production.

Probiotic microorganisms need to resist the adverse factors in the gastrointestinal tract as this will help them to reach the small intestine and colon of consumers and contribute in balancing the intestinal microbiota (El-Naggar, 2004). Most microorganisms are destroyed by low pH and hydrochloric acid in the stomach. In humans, the time from entrance to release from the stomach was reported to be 90 min (Berrada *et al*., 1991) and the bacteriocidal effect of the acid is evident at pH values below 2.5 (Maffei and Nobrega, 1975). In this study, both *Lactobacillus plantarum* and *Lactobacillus pentosus* demonstrated notable tolerance to acidic pH. However, the sensitivity of *Lactococcus lactis lactis* to acidic pH suggests its failure to survive the passage through a digestive system with typical conditions such as the low pH in the stomach.

Camel milk is gaining more popularity nowadays because of its high nutritional quality and therapeutic value (Sawaya *et al*., 1984; Strasser *et al*., 2006). In Kuwait, camel milk is mostly consumed fresh or when just soured (El-Amin and Wilcox, 1992). Fresh camel milk has a low lactic acid content of 0.03% and a pH of 6.5-6.7 (Shalash, 1979), however, when the milk is left to stand, the acidity and the lactic acid content rapidly increases (Ohris and Joshi, 1961). Although no study suggest that raw fresh milk should be used as probiotic (Blog, 2008), some researchers believe that a synergistic effect exists between components in dairy foods and probiotic cultures and that there are components in milk that turn on the beneficial genes in probiotic bacteria (Klaenhammer *et al*., 2007). Additionally, the presence of peptidoglycan recognition protein (PGRP), which is a protein not usually present in milk and was only detected in camel milk, has a role in passive immunity that is mostly related to its ability to avidly bound to lactic acid bacteria (Kappeler *et al*., 2004).

In conclusion, the results obtained from this study demonstrated the potential probiotic ability of the isolated LAB species from camel milk. In addition it is recommended that these species be further studied according to selection criteria like stimulation of immunological system and adhesion to epithelium tissue.

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