American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

American Journal of Food Technology 7 (11): 690-699, 2012
ISSN 1557-4571 / DOI: 10.3923/ajft.2012.690.699

Isolation of Lactic Acid Bacteria from Some Traditional Saudi Food

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ABSTRACT

Presently many dairy products are being used in Saudi Arabia. Monitoring and isolation of bacterial activity is of highly significance to sustainable health problems and issues. The objective of the study was to isolate bacteriocin-producing Lactic Acid Bacteria (LAB) from some traditional Saudi food. A total of 50 samples from dairy products, commonly consumed in Saudi Arabia, were screened for presence of natural LAB. The Cell-free Supernatants (CFS) of two LAB isolates exhibited antibacterial activities (inhibition zones >10 mm) against food-borne pathogens (*Lactococcus monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 29213 and *Salmonella enteritidis* ATCC13076). The growth inhibitory substances of CFS were sensitive to proteases (Protenase K and Pepsin) indicating the proteinaceous nature of inhibitors (bacteriocins) produced by the two LAB isolates. Their bacteriocins retained activity after thermal treatments (63°C for 30 min, 100°C for 10 min or 121°C for 15 min) or at pH ranging from 4.0 to less than 6.5. The two LAB isolates were phenotypically identified as *Lactococcus lactis* subsp. *lactis* (camel's milk) and *Lactobacillus paracasei* (goat's milk). The isolated LAB (*Lactococcus lactis* subsp. *lactis* and *Lactobacillus paracasei*) can be used as food preservatives and probiotics since they inhibited well-known food-borne pathogens such as *L. monocytogenes* and survived acidic conditions (pH 2.5) similar to those of the stomach.

Key words: Lactic acid bacteria (LAB), food borne pathogens, milk products, isolates, camel milk, goat milk, cow milk, inhibitors, antibacterial activity

INTRODUCTION

The Lactic-acid-bacteria (LAB) are gram-positive coccids or rods, catalase-negative, non-spore forming bacteria. They are fastidious, acid-tolerant and microaerophilic organisms. LAB are strictly fermentative and produce mainly lactic acid and other metabolites. *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus* and *Bifidobacterium* are important LAB genera (Robinson, 2002; Chen and Hoover, 2003). They are among the microflora of many food substances including raw milk. They also are present in the natural population of the gastrointestinal tract, GI (De Vuyst and Leroy, 2007; Rivera-Espinoza and Gallardo-Navarro, 2010). The LAB is important to food industry due to their ability to ferment sugar to lactic acid (homo-fermentative) and other metabolites such as alcohol and CO_2 (hetero-fermentative). Certain strains of LAB have technological properties indispensable in food production (starter cultures) especially the fermented dairy products. In general, starter cultures for fermented food are selected based on their technological potentials like acid and polysaccharide production, improving flavor and enhancing the nutritional value (e.g., reducing anti-nutrients) of foodstuff. Recently, the antimicrobial action of certain LAB against spoilage and pathogenic microorganisms is also taken as a desired characteristic for protecting food products (Robinson, 2002; De Vuyst and Leroy, 2007; Settani and

Corseti, 2008). The protective mechanism of LAB against pathogens in food is a multi-factorial i.e., pH reduction, production of antimicrobials (e.g., organic acids, hydrogen peroxide), competition for nutrients, displacing pathogens in the GI and production of bacteriocins. Bacteriocins are defined as peptides/proteins ribosomally synthesized and possessing antibacterial activity towards closely related bacteria. In fact, bacteriocin producers have the immunity to their own bacteriocins (Chen and Hoover, 2003; De Vuyst and Leroy, 2007; Settani and Corseti, 2008). They possessed inhibitory activity to some food-borne pathogens and spoilage bacteria such as *L. monocytogenes* and *Bacillus cereus* (Chen and Hoover, 2003; Cheikhyoussef *et al*., 2008). The LAB have a long history of safe consumption and thus are Generally Recognized as Safe (GRAS). Some bacteriocins are commercially produced in a partially-purified formulations (e.g., nisin as Nisaplin™) and have been used in food preservation (Chen and Hoover, 2003; Settani and Corseti, 2008).

Until to-date, screening studies on traditional fermented food products (dairy and non-dairy) have revealed a wide spectrum of LAB producing bacteriocins of different properties and specificity. In Europe, Africa, some of the Arab countries and other parts of the world, traditional cheeses (home-made types), raw milk, sourdough and vegetal products were explored as natural sources for bacteriocinogenic LAB (Nikolic *et al*., 2008; Casalta and Montel, 2008; Gerez *et al*., 2009). However, studies on isolating active LAB from camel's milk and other traditional Saudi food products are scarce. Therefore, the present study was conducted to isolate bacteriocinogenic LAB mainly from camel's milk and other Saudi food products available.

MATERIALS AND METHODS

Milk and other food samples: Fresh raw milk samples were randomly collected by hand milking from lactating Arabian camels (*Camelus dromedarius*), goats and cows of local farms in Al-Hasa, Saudi Arabia. The traditionally fermented milks from camel and cow milk were also included in the study. Dried goat's milk samples were purchased from traditional markets. Samples were immediately analyzed in the laboratory or, when necessary, they were stored overnight at 4°C prior to testing. Counts of endogenous LAB in tested food were carried out in MRS agars with an anaerobic incubation (Gas Pak) at 37°C for 48-72 h.

Isolation of LAB from samples: Ten milliliter or gram from experimental food products were aseptically blended for 2 min in a Laboratory Stomacher with 90 mL sterile peptone-NaCl water (Oxoid, UK). The samples were replicated three times. Serial dilutions ($1/10^2$-$1/10^7$) were further prepared using the same diluent. MRS or M-17 agar plates (Oxoid, UK) were spread with 0.1 mL diluted samples using a sterile glass rod. Plates were incubated anaerobically (Gas Pak) at 37°C for 48-72 h. Representative colonies were aseptically picked from plates containing 10-100 colonies. Isolates were further purified by streaking MRS/M17 agar plates with incubation as mentioned earlier. Gram-positive coccids/rods and catalase negative bacteria were considered as presumptive LAB. The isolates and indicators were propagated in the respective media (Table 1) and maintained in broths with 15% sterile glycerol at -20°C. Strains for routine use were maintained in agar deeps (LAB) or slants (non-LAB) and sub-cultured bimonthly.

Antibacterial activity of isolates

Agar spot assay (ASA): The deferred agar-spot test (Chen and Hoover, 2003) was used to reveal the antagonistic activity of isolated LAB against the indicators. Briefly, 5 μL of isolated LAB was spotted onto MRS agar and plates left to dry. Plates were inverted and incubated

Table 1: Indicator bacteria and growth conditions used in the study

Media and temperature	Indicators
MRS, 37°C (Gas Pak)	Lactic acid bacteria
	Lactobacillus bulgaricus DSM20081
	Lactococcus lactis sp. *lactis* ATCC11955
	Streptococcus thermophillus DSM20617
Nutrient broth (NB), 37°C	Food-borne pathogens
	Listeria monocytogenes ATCC7644
	Staphylococcus aureus ATCC29213
	Salmonella enteritidis ATCC13076
	Escherichia coli 7656

anaerobically (Gas Pak) at 37°C for 48-72 h. After incubation, 10 μL of each activated indicator was added to 10 mL sterile soft agar (MRS or Nutrient broths+0.75% agar) at 50°C. The inoculated soft agar (10^5 colony forming units, CFU mL^{-1}) was poured onto the above spotted agar. The plates were incubated at 37°C for 12-72 h. The antibacterial activity was indicated by a clear zone in the indicator lawn around the spot of the isolate.

Well diffusion assay (WDA): Cell-free Supernatants (CFS) of LAB isolates were screened for antagonistic activities against indicators by adopting the well assay described by Nikolic *et al.* (2008). The LAB isolates showing inhibition zones in the agar-spot method, as mentioned earlier, were only used in this test. Basically, CFS were obtained by centrifugation (9000 xg, 10 min. at 5°C) of isolates grown in broths (MRS or M17 at 37°C) for 48 h followed by 0.45 μm membrane filtration (Nalgene, USA). Eight milliliter of soft agar media (MRS for LAB or Nutrient broth plus 0.75% agar for pathogens) were seeded with 10^5 CFU mL^{-1} of the indicator. Each inoculated agar medium was overlaid onto MRS/Nutrient agar plates. Wells of 5 mm diameter and 100 μL capacity were made in media with a sterile stainless steel borer. Each well was filled with CFS and the plates were incubated at 37°C for 12-48 h. Clear inhibition zones around wells (including well diameter) in the lawn of indicators were measured in mm. The inhibition of indicators by hydrogen peroxide (H_2O_2) produced by isolates was ruled out by treating CFS with catalase (1 mg mL^{-1}) prior to the well assay.

Nature of inhibitory substances: CFS of LAB isolates (Lc and Lb) having antimicrobial activities in the well assay as described earlier were partially characterized using *L. monocytogenes* as an indicator since it was the most sensitive bacteria (wider inhibition zones) to their CFS.

Effect of enzymes: The CFS were tested for susceptibility to Proteinase K (Promega), Pepsin (Merck, Germany) or Lipase (Sigma, USA) using the well assay outlined by Nikolic *et al.* (2008). Twenty microliters of proteinase K (5 mg mL^{-1}), pepsin (5 mg mL^{-1} 0.002 M HCl) and lipase (5 mg mL^{-1}) were separately spotted adjacent to the edge of a well of CFS prior to incubation at 37°C for 18-72 h. Under the same conditions, untreated CFS, inactivated enzymes (boiled) in CFS and MRS broths containing only enzymes were the controls.

Thermal treatments and pH effects: CFS of active isolates were subjected to various treatments (63°C for 30 min, 100°C for 10 min or 121°C for 15 min). Meanwhile, other sets of CFS were adjusted to different pH (4.0, 4.6, 5.3 and 6.9) using sterile 2.5 N HCl or NaOH. Blanks

(MRS broths+2.5 N HCl or NaOH) were used under the same conditions. The residual antibacterial activities of treated CFS, controls and blanks were determined using the well assay of Nikolic *et al.* (2008).

Identification of active isolates

Phenotypic and biochemical tests: The isolates (Lc and Lb) which produced inhibitory substances sensitive to proteolytic enzymes (bacteriocins) were characterized using the phenotypical and biochemical tests as described by Nikolic *et al.* (2008). i.e., (a) gram stain and colony morphology, (b) growth at 15, 30 and 45°C in MRS broth for rods and in M17 broth for cocci, (c) salt tolerance (growth in MRS/M17 broths containing 4, 6.5 or 8% NaCl, (d) carbon dioxide (CO_2) production from glucose in MRS broth with Durham's tubes, (e) L-arginin hydrolysis, (f) esculin hydrolysis (for cocci), (g) citrate-utilization, (h) activity in milk and test in litmus milk and (i) diacetyl production-only for LAB which coagulated skimmed milk.

Carbohydrate fermentation of Lc and Lb were carried out by using the API 50 CHL strips and API CHL medium following the guidelines of manufacturer. Meanwhile, the identification of isolated LAB was done by a computerized database program provided by the manufacturer (BioMerieux, Marcy-l'Etoile, France).

Tolerance of isolates to acidic conditions: The two bacteriocinogenic isolates (Lc and Lb) were tested for their tolerance to acidic conditions similar to those of the stomach. The acid resistance was examined in MRS broth adjusted with HCl to a final pH of 2.5. Each isolate was separately inoculated (10^6 CFU mL^{-})[1] in MRS at pH 2.5. After exposures for 2 h, viable cells of bacteriocinogenic isolates were confirmed on MRS agar after anaerobic incubation for 48 h at 37°C (Klingberg *et al.*, 2005).

Statistical analysis: Data are means of three replications from each experiment. The results were statistically evaluated using the ANOVA-One Way (SPSS Version 10, SPSS Inc., Chicago) at 5% level of significance.

RESULTS AND DISCUSSION

Commonly consumed dairy products in Saudi Arabia and other arid and semi-arid regions of the Arabian and African countries were screened for their natural LAB in the present study. With the exception of dried goat's milk, the numbers of LAB ranged from 4.4-5.7 $\log_{10}$ CFU mL^{-1} (Table 2). Fermented samples from raw camel's or cow's milk were significantly ($p<0.05$) higher in LAB counts than the non-fermented samples. In fact, no previous investigations reported on LAB in raw and naturally-fermented dairy products in Saudi Arabia and other Gulf States. In other areas of the world, the LAB (7-9 $\log_{10}$ CFU mL^{-1}) were found in fermented camel's milk and raw

Table 2: Counts of LAB in traditional food screened in the study

Food	No. of samples	LAB ($\log_{10}$ CFU mL^{-1} or g)
Camel's milk (raw)	19	4.4±0.94[b]
Camel's milk (fermented)	12	5.3±1.10[a]
Cow's milk (fermented)	10	5.7±0.78[a]
Goat's milk (raw)	7	4.6±0.57[b]
Dried goat's milk	5	<1[c]

Values (Mean±SD) with different superscripts in a column are significantly different at $p<0.05$

cow's milk (Abdelgadir *et al*., 2008; Franciosi *et al*., 2009). It was clearly mentioned that raw milk is a better source of endogenous LAB than fermented products because some LAB strains were lost during preparations of fermented food, such as raw-milk cheeses (Franciosi *et al*., 2009).

Antibacterial activity of isolates

Agar spot assay: The agar spot method was used for preliminary screening of the antagonistic activity of LAB against some indicator bacteria. In this test, live LAB cells were in close contact with indicators. Out of the total twenty five isolates from test food, only seven isolates were non-beta hemolytic (blood agar) gram positive (coccids/rods) and catalase negative bacteria. Therefore, these seven LAB (persumptive) were screened for production of inhibitory substances in agar media. As shown in Table 3, clear inhibition zones around spotted LAB and in the lawn of indicators were exhibited by the seven isolates (CO, AL, SF, LO, GY, Lc and Lb) against at least one of the indicators. Yateem *et al*. (2008) and Nikolic *et al*. (2008) successfully isolated LAB (lactococci and lactobacilli) from camel's and goat's milk. Their isolates showed growth inhibitory activity against closely related LAB and some food-borne pathogens. Up till to-date, among the gram-positive bacteria only LAB was comprehensively exploited as a reservoir for antimicrobials with food applications (Cleveland *et al*., 2001).

Well diffusion assay: CFS of the seven LAB isolates giving positive results in the above agar spot assay were tested for antagonistic capabilities against indicators. Results confirmed that only CFS of two isolates (out of seven isolates) were found inhibitory to indicators with inhibition zones ranging from 14-20 mm (Table 4). However, turbid inhibition zones were resulted from CFS of only

Table 3: Inhibition activity of isolated LAB against indicators using the deferred spot assay

	LAB isolates codes						
Indicators	CO	AL	SF	LO	GY	Lc	Lb
Lactobacillus bulgaricus DSM20081	+	-	-	-	-	+	+
Lactococcus lactis sp. lactis ATCC11955	+	-	-	+	-	+	+
Streptococcus thermophillus DSM20617	+	-	-	-	-	+	+
Listeria monocytogenes ATCC7644	+	+	-	-	+	+	+
Staphylococcus aureus ATCC29213	+	-	-	-	-	+	+
Escherichia coli 7656	+	+	+	+	+	+	+
Salmonella enteritidis ATCC13076	+	+	-	+	-	+	+

+: Inhibited, -: No inhibition

Table 4: Antagonistic activity (well assay) of LAB isolates against indicator bacteria

	LAB isolates						
Indicators	CO	AL	SF	LO	GY	Lc	Lb
Lactobacillus bulgaricus DSM20081	10[b]	0	0	0	0	17[a]	15
Lactococcus lactis sp. *lactis* ATCC11955	-12	0	0	0	0	18	15
Streptococcus thermophillus DSM20617	-13	0	0	0	0	16	16
Listeria monocytogenes ATCC7644	-15	0	0	0	0	20	18
Staphylococcus aureus ATCC29213	-14	0	0	0	0	15	14
Escherichia coli 7656	-16	0	0	0	0	17	15
Salmonella enteritidis ATCC13076	-14	0	0	0	0	18	15

[a]Inhibition zones in mm, [b]Not clear inhibition (hazy) zones

one isolate (CO) against indicators (Table 4). Additionally, those turbid zones were overgrown by indicators upon extending incubation periods. In this regard, inhibitors (i.e., bacteriocins) produced by LAB are mainly active against closely related LAB and other food-borne pathogens such as *L. monocytogenes* and *Salmonella* spp. (Cleveland *et al*., 2001; Chen and Hoover, 2003; De Vuyst and Leroy, 2007; Nikolic *et al*., 2008). It is noteworthy that *L. monocytogenes, Staphylococcus aureus*, *Salmonella* spp. and *E. coli* O157:H7 are among the pathogens jeopardizing the food supply and accordingly human health.

Interestingly, there was no inhibition exhibited by CFS of the other four isolates (Table 4) which previously showed activity against some indicators in the agar spot assay (Table 3). Previously many researchers showed that less than 30% LAB isolates exhibiting inhibitory activities against indicators in the agar spot assay failed to produce the same activity in the well assay (Ammor *et al*., 2006; Alegria *et al*., 2010). In this regard, Alegria *et al*. (2010) pointed out that confirmation in liquid media (i.e., CFS) of the inhibition detected by the agar spot test is not always obtained. That was probably related to colony-associated antimicrobial compounds including fatty acids which have been considered to be responsible for the inhibitory effects observed in solid media (agar spot test).

Nature of inhibitory substances

Effect of proteolytic enzymes: From Table 5, CFS of both Lc and Lb lost activity against *L. monocytogenes* ATCC 7644 in the vicinity of spotted proteases (proteinase K and pepsin). On the other hand, controls and lipase-treated CFS had the same antagonistic activity against the same food-borne pathogen, *L. monocytogenes* ATCC 7644 (Table 5). Inactivation of the CFS for Lc and Lb clearly indicated the proteinaceous nature of inhibitors (bacteriocins). Bacteriocins of LAB are ribosomally synthesized, extracellularly released peptides which are active against closely related LAB and other non-LAB such as *L. monocytogenes*. In this regard, various LAB produced bacteriocins lost their antibacterial activity when treated with proteolytic enzymes such as Protenase K (Cleveland *et al*., 2001; Ammor *et al*., 2006; Nikolic *et al*., 2008; Alegria *et al*., 2010).

Sensitivity to thermal treatments and pH: Applications of heat treatments commonly used in food processing (pasteurization and sterilization) did not affect the antagonistic activity towards *L. monocytogenes* ATCC 7644. However, the Lc and Lb showed a substantial residual activity against the indicator (*L. monocytogenes* ATCC 7644) as determined by the well assay described above. The results in Table 6 showed that the pasteurization (63°C, 30 min) did not show any detrimental activity on the anti-listerial activity but the sterilization (121°C, 15 min) significantly ($p<0.05$) reduced the activity of bacteriocins for Lc and Lb by 92 and 83%, respectively. Meanwhile,

Table 5: Effects of enzymes on the inhibition activity of CFS for isolates against *L. monocytogenes* ATCC 7644 using the well assay

	LAB isolates	
Treatments	Lc	Lb
Control CFS	+	+
Treated CFS	-	-
Proteinase K		
Pepsin	-	-
Lipase	+	+

+: Inhibited, -: No inhibition

Table 6: Residual activity of CFS of LAB isolates after thermal treatments against *L. monocytogenes* ATCC 7644 using the well assay

	Residual activity (%) after treatments			
Isolates	Non-treated (control)	63°C, 30 min	100°C, 10 min	121°C, 15 min
Lc	100[a]	100	97	92*
Lb	100[b]	100	100	83*

[a]20 mm inhibition zones, [b]18 mm inhibition zones of controls, *Significant at $p<0.05$

Table 7: Effect of pH on inhibition (well assay) of *L. monocytogenes* ATCC 7644 by the CFS of LAB isolates

	Inhibition (diameter of zones in mm)					
Isolates	Control (pH 4.3)	pH 4.0	pH 4.5	pH 5.3	pH 6.0	pH 6.9
Lc	18	19	17	15	10*	0
Lb	12	14	11	10	8*	0

*Significant at $p<0.05$

boiling of bacteriocins for both Lc and Lb exhibited different trends on the inhibitory activity against the pathogen comparing to the corresponding controls (Table 6).

The antibacterial activity (well assay) towards *L. monocytogenes* ATCC 7644 was affected as the pH of CFS for both isolates was close to neutrality (pH>6.0). As depicted in Table 7, the inhibition activity (in mm) against the indicator bacteria was maintained when the pH of both CFS was between 4.3-5.3. However, there was a significant difference ($p<0.05$) on the antibacterial activity of isolates than controls at a pH value of 6.0 or above.

The results of the above treatments (Table 6, 7) were in agreements with the findings of Cleveland *et al.* (2001), Ammor *et al.* (2006), Nikolic *et al.* (2008) and Alegria *et al.* (2010). They stated that those bacteriocins (e.g., nisin) of LAB were thermostable and exerted their antibacterial activity when maintained in acidic conditions.

Identification of bacteriocin-producing LAB isolates

Phonotypical and biochemical tests: The two active isolates (Lc and Lb) were subjected to phonotypical and biochemical tests (Table 8). These were gram-positive, catalase negative non-spore forming bacteria. Morphologically, the Lc were cocci while the Lb were rods shape. The skim milk and litmus milk tests were positive (acid production and curdling at 37°C for 16 h) for both the isolates. There were also non-**β**-haemolytic strains in 5% blood agar media. The growth temperatures at 15 and 45°C varied between Lc and Lb. Meanwhile, salt tolerance at 6.5% of the two LAB isolates was also different. Other characteristics of both the isolates are listed in Table 8.

In addition to the phenotypical tests, carbohydrate assimilation for Lc and Lb was carried out using the API 50 CHL system. The fermentation profile of carbohydrates for Lc and Lb is summarized in Table 9. Based on the phenotypical tests and production of acids from carbohydrates, isolates were *Lactococcus lactis* subsp. *lactis* (Lc, raw camel's milk isolate) and *Lactobacillus paracasei* (Lb, goat's milk isolate).

Tolerance of isolates to acidic conditions: *Lactococcus lactis* subsp. *lactis* (Lc, camel's milk isolate) and *Lactobacillus paracasei* (Lb, goat's milk isolate) were able to survive under acidic conditions (pH 2.5) for 2 h. In order to survive and establish within the human GIT, some of the desirable properties of probiotics include their ability to inhibit pathogens and also to resist the

Table 8: Characteristics and preliminary identification of active LAB isolates

Test	Lc	Lb
Gram stain	+	+
Morphology	Coccids	Rods
Spores	-	-
Catalase test	-	-
Litmus milk (acid/reduction)	+	+
Gas in MRS	-	-
Growth at 15°C	w	-
30°C	+	+
45°C	-	+
Growth in NaCl (2 and 4%)	+	+
-6.50%	-	w
-8%	-	-
B-haemolysis	-	-
Arginin hydrolysis	+	-
Esculin hydrolysis	+	+
Citrate assimilation	w	-
Voges-Proskauer	-	-

+: Positive, -: Negative, w: Weak reactions

Table 9: Carbohydrate fermentation profile of LAB Isolates using the API 50 CHL system

	LAB isolate	
Carbohydrate	Lc	Lb
Glycerol	-	+
L-arabinose	+	+
D-ribose	+	+
D-galactose	+	+
D-glucose	+	+
D-fructose	+	+
D-mannose	+	+
D-mannitol	w	+
D-sorbitol	-	+
Methyl-ad-glucopyranoside	+	+
N-acetylglucosamine	+	+
Amygdalin	+	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
D-cellobiose	+	+
D-maltose	+	+
D-lactose	+	+
D-melibiose	+	+
D-saccharose	+	+
D-trehalose	+	+
Gentiobiose	+	+
D-tagatose	w	+
Gluconate	-	-

+: Positive, -: negative, w: Weak reactions

acidity (pH 2.5-3.5) of the stomach. The study results agree with those of Klingberg *et al.* (2005) who showed that exposure to pH 2.5 was a very discriminating factor. Similar research reported that *in vitro* methods are usually used to evaluate the antagonistic activity of probiotics against pathogenic microorganisms. Such methods depend on bacterium-bacterium antagonism which regulates proliferation and cell association of one bacterium by metabolites produced by the other (Yateem *et al.*, 2008).

CONCLUSIONS

The study showed that some traditional food items may be the natural sources for bacteriocin-producing LAB which can be used as food preservatives and probiotics.

ACKNOWLEDGMENTS

The author thanks the Deanship of Scientific Research of King Faisal University for partial support of the research project.

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