**Original paper**

**Cholesterol Degradation by Some Bacteria Isolated from Food**

Ghada M. Khiralla*

Division of Medical Foods, National Organization for Drug Control and Research (NODCAR), 6-7, AboHazem St, Pyramids, Giza, Egypt

Received March 14, 2015 ; Accepted May 16, 2015

The study aimed to isolate and characterize probiotic strains with potential cholesterol degrading activity. Fourteen lactic acid bacteria isolated from lamb meat were screened on mineral salt agar supplemented with 0.2% cholesterol (MSC agar). Cell-free supernatants (CFSs) of these isolates were used as a crude source of extracellular cholesterol degrading enzymes. CFSs of GMK01, GMK02 and GMK03 isolates displayed high ability to degrading cholesterol (86.4, 86.1 and 84.6%, respectively). These isolates were identified as *Lactobacillus sakei* GMK01, *Lactobacillus rhamnosus* GMK02 and *Leuconostoc mesenteroides* GMK03. Strains were resistant to low acidity (pH 2.5) and bile salts (0.3%), able to adhesion to Caco-2 cells and have low rate of antibiotic resistance. Living cells of these strains were able to degrade cholesterol in MSC broth even after treatment with simulated gastrointestinal juice. The maximum cholesterol degradation (about 90%) was obtained on the third day. 4-cholesten-3-one was detected as a degradation product of cholesterol by *Leuconostoc mesenteroides* GMK03. The studied strains degrade cholesterol by different mechanisms and may suggest a new possibility for the mechanism underlying cholesterol degradation by LAB. In conclusion, the isolated strains could be suggested as potential pharmaceutical probiotic strains for food industry and human nutrition.

Keywords: lactic acid bacteria, probiotic, cholesterol, antibiotic resistance, gastrointestinal juice

**Introduction**

Worldwide, fermented foods processed using lactic acid bacteria (LAB) are known for their beneficial effects in human nutrition. Therefore, LAB are considered as promising probiotic candidates and being extensively studied to explore their desirable properties (Iranmanesh et al., 2014). One of the attractive properties is the ability of LAB to lowering cholesterol level in blood serum (Anderson and Gilliland, 1999; Ataie-Jafari et al., 2009). The species with cholesterol-lowering effects studied include genera as *Lactobacillus, Lactococcus, Enterococcus, Streptococcus* and *Bifidobacterium* (Pereira and Gibson, 2002).

Probiotics have been recommended to decrease cholesterol through different mechanisms including e.g. co-precipitation of cholesterol with deconjugated bile, cholesterol binding to cell walls, integration of cholesterol into the cellular membranes during growth, production of short-chain fatty acids during fermentation, and transformation into coprostanol (García et al., 2012; Lee and Salminen, 1995; Lye et al., 2010a,b; Pereira and Gibson, 2002). Cholesterol oxidase (cholesterol: oxygen oxidoreductase, EC 1.1.3.6) is known to be a key enzyme, which catalyses the oxidation of cholesterol to 4-cholesten-3-one and the reduction of oxygen to hydrogen peroxide (Sakodinskaya, 2000). This enzyme, especially deriving from bacterial cells, plays a significant role in the degradation of cholesterol in many fermented foods (Paniangvait et al., 1995).

Cholesterol-rich foods including chicken liver, turkey giblets, salmon, lamb, egg yolk, beef brain and shrimps are a rich source of new interesting cholesterol degrading bacteria (Ouf et al., 2012). The isolation of natural LAB from these foods to find new species and strains is of great interest. It is a group of industrially important...
microorganisms with generally regarded as safe (GRAS) status.

Therefore, the aim of the present study was to i) screening and identifying cholesterol degrading LAB from storage lamb meat as a cholesterol-rich food, ii) determine the important probiotic properties of the isolated strains, iii) studying the ability of the isolated LAB to degrade cholesterol after exposure to simulated gastrointestinal stress.

Materials and Methods
Isolation of lactic acid bacteria (LAB) Ten raw Lamb meat samples were collected from the local market of Giza City, Egypt. Samples were transferred to the laboratory and prepared as described in ISO/DIS 6887 part 1 and 2. From meat samples, 25 g were taken using to a depth of 2 – 3 mm, placed with 250 mL peptone water in a sterile plastic bag and homogenized. These initial suspensions were incubated at 37°C for 24 h in order to obtain enriched cultures. The enriched cultures were further streaked on MRS agar (de Man, Rogosa, Sharpe, Difco, USA) and incubated under anaerobic conditions (Anaerobic Jar, Sigma-Aldrich; with anaerobe atmosphere generation bag, product No 68061) at 37°C for 48 h. Single colonies picked off and sub-cultured in MRS agar at 37°C for 24 h. The cultures were examined microscopically and restreaked on the MRS agar medium for purification.

Screening of cholesterol degrading LAB The pure isolated bacteria were transferred to Minimal Salt Cholesterol (MSC) agar plates containing 0.2% cholesterol as a sole source of carbon and energy (Nishiya et al., 1997). MSC agar containing (g/L): NH₄NO₃, 17; K₂HPO₄, 0.25; MgSO₄.H₂O, 0.25; FeSO₄.H₂O, 0.001; NaCl, 0.05; cholesterol, 1.5; agar 20 and Tween 80 (0.1 mL/L). The pH was adjusted to 7.0. For suspending cholesterol and avoiding its coagulation, it was first dissolved in 10 mL solution of 20% isopropanol plus 10% of Tween 80 and then added to the medium. The ability of the isolated bacteria to decompose cholesterol was confirmed by measuring the zone of translucency around colonies on MSC agar after incubation at 37°C for 7 – 12 days (Yazdi et al., 2000). According to the clear zone obtained on MSC agar, cholesterol degrading strains were categorized to no (zone < 1 mm), weak (1 mm = zone < 3 mm), moderate (3 mm = zone < 6 mm) and strong (zone ≥ 6 mm) cholesterol degrading strain. To purify confluent colonies, they were picked up and subcultured by streaking on the same medium.

Cholesterol degradation by cell-free supernatant Fourteen cholesterol degrading strains (GMK01-GMK14) were subjected for preparation of cell-free supernatants (CFSs) as a crude source of extracellular cholesterol degrading enzymes. The developed colonies were further enriched in MSC broth and incubated at 37°C for 7 days. The enriched culture was centrifuged at 10000 xg for 5 min. CFS was then sterilized by using a 0.45-mm filter (Sartorius, Goettingen, Germany) and stored at –80°C until needed. The cholesterol degrading ability of CFS was measured by enzymatic colorimetric cholesterol oxidase - Peroxidase method (Kulkarni et al., 2013). The assay was performed using cholesterol determination kit (Kee Gad Biogen, India) according to the supplier instructions. The cholesterol standard had concentration of 200 mg/dL. All the reagents were mixed in the respective tubes labeled blank (buffer only), standard (buffer, standard cholesterol 200 mg/dL and cholesterol oxidase supplemented with the kits) and tested sample (buffer, standard cholesterol and 1 mL of CFS as a source of cholesterol degrading enzymes). The reaction mixture was thoroughly mixed and incubated for 10 min at 37°C. Absorbance of standard (A std) and tested sample (A sample) were read against the reagent blank at 505 nm. cholesterol reduction percentage was calculated from the following equation: % cholesterol reduction = [(A std – A sample)/ A std] x 100. This experiment was performed in triplicates, and % cholesterol reduction was averaged.

Identification of the cholesterol degrading bacteria According to the results of cholesterol degrading ability of CFSs, isolates that showed high ability to degrading cholesterol (GMK01, GMK02 and GMK03) were subjected for identification to species level. The carbohydrate fermentation profiles of the selected strains were determined using API 50 CHL medium (Bio-Merieux, Marcy l’Etoile, France) according to the manuacturer’s instruction (Iranmanesh et al., 2012). In addition, standard tests were performed for identification of the target strain in accordance with Bergey’s Manual of Determinative Bacteriology (Holt, 1994). These tests included cell form and size; Gram staining; spore formation; motility; colony pigmentation; production of UV-fluorescent pigments. Identification was confirmed by determine partial sequence of 16S rRNA gene sequences. Briefly, genomic DNA of studied bacteria was extracted (Govindarajan et al., 2007), and 16S rDNA was amplified in polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, 27F (5’-gagtttgatcactggctcag-3′) and 1492R (5’-tacggctacctgttacgactt-3′) (Byers et al., 1998). A 25 μL reaction mixture contained 1.25 U Taq polymerase, 0.2 mM dNTPs, 2.5 mM MgCl₂, 10 pmol of each primer, 2.5 μL of 10x reaction buffer, and 1 μg of template DNA. Aliquots of PCR reaction products were electrophoresed in 1% agarose, containing 10 μg/mL ethidium bromide. PCR product was purified using DNA-purification kits and send to Macrogen Co, Seoul, Korea for sequencing using 518F and 800R sequencing primers. The obtained sequences were compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI/BLAST available at http://www.ncbi.nlm.nih.gov/. The nucleotide sequence data have been deposited at the EMBL nucleotide sequence database under accession No. LN827925, LN827926 and LN827927 for the isolates GMK01, GMK02 and GMK03, respectively.

Antibiotic sensitivity The susceptibility to 15 antibiotics was determined using the disk diffusion method on Mueller Hinton agar (C.L.S.I., 2007). The antibiotics used in the present study were purchased from Bio-Rad, Laboratories, GmbH, Germany. These
Cholesterol Degradation by Some Bacteria Isolated from Food

Eighty-seven isolates collected from MRS agar were preliminary identified as LAB based on their Gram reaction, morphology and catalase test. All isolates were described as catalase-negative and Gram-positive bacilli in pairs or chains and cocobacilli (data not shown). Out of 87 isolates, only fourteen showed cholesterol degrading ability (determined as a clear zone) on mineral salt agar (Table 1). Out of these isolates only three isolates (GMK01, GMK02 and GMK03) showed strong ability (+++) to degrade cholesterol in the MSC agar. Six isolates (from GMK04 to GMK09) had moderate ability (+) to degrade cholesterol. The other isolates (GMK10 – GMK15) showed weak ability (+) to degrade cholesterol.

**Results and Discussion**

**Screening and identifying cholesterol degrading LAB**  
Lamb meat is well known as one of the most cholesterol-rich meat. Eighty-seven isolates collected from MRS agar were preliminary identified as LAB based on their Gram reaction, morphology and catalase test. All isolates were described as catalase-negative and Gram-positive bacilli in pairs or chains and cocobacilli (data not shown). Out of 87 isolates, only fourteen showed cholesterol degrading ability (determined as a clear zone) on mineral salt agar supplemented with 0.2% cholesterol (MSC agar). (Table 1). Out of these isolates only three isolates (GMK01, GMK02 and GMK03) showed strong ability (+++) to degrade cholesterol in the MSC agar. Six isolates (from GMK04 to GMK09) had moderate ability (+) to degrade cholesterol. The other isolates (GMK10 – GMK15) observed weak cholesterol degradation on MSC agar. The clear zones formed on MSC agar could be considered as a first indicator of at least 100 different cells from five separate fields. All tests were carried out in triplicate (n = 3), and the results were averaged.

**Quantifying the degradation of cholesterol by living bacterial cells**  
Strains GMK01, GMK02 and GMK03 were tested for their ability to degradation cholesterol before and after treatment with simulated gastrointestinal juices (SGIJ). Bacterial strains were activated in 10 mL of MRS broth at 37°C with reciprocal shaking (120 rpm) for 4 days. Every day, 2 mL were withdrawn for measuring growth of tested bacteria and for measuring the residual cholesterol. Growth was monitored by measuring the optical density at 600 nm (OD600) using a UV/Visible spectrophotometer (6105-Jenway, U.K.). All tests were performed in triplicates. Results are expressed as the mean of three replicates ± standard deviation. At each sampling period, the cells were removed by centrifugation (10000 xg for 5 min), the cholesterol was extracted from the supernatants and quantified by high-performance liquid chromatography (HPLC) as described later. The culture supernatant (1 mL) was extracted by 2 mL absolute methanol (HPLC-grade, Sigma, St Louis, MO, USA) and subjected to HPLC system (Shimadzu, LC10AT, Nakagyo-ku, Kyoto, Japan) equipped with a C18 column (Agilent Poroshell 120 EC-C18, 3.0 x 100 mm, 2.7 µm P/N 695975-302). Isocratic column elution was monitored by 1200 UV/visible detector at wavelength of 254 nm. The mobile phase was composed of acetonitrile/methanol (62: 38, v/v, HPLC-grade, Sigma, USA). The flow rate was 1 mL/min and the column temperature was 30°C. The standard cholesterol and 4-cholesten-3-one solutions (Sigma, USA) in methylene chloride were used for constructing the calibration curves (1.00 mg/mL). A linear dynamic range was established for cholesterol and 4-cholesten-3-one standard solutions of concentration ranging from 10 to 200 µg/mL.

**Results and Discussion**

**Quantifying the degradation of cholesterol by living bacterial cells**  
Strains GMK01, GMK02 and GMK03 were tested for their ability to degradation cholesterol before and after treatment with simulated gastrointestinal juices (SGIJ). Bacterial strains were activated in MRS broth at 37°C with reciprocal shaking (120 rpm) for 48 h. Cells were collected and washed twice with phosphate buffer (0.1 M, pH 6.8). Cells were resuspended in phosphate buffer to about 1x10⁶ CFU/mL. Resuspended cells (0.2 mL) were vortexed with gastric or small intestinal juice for 2 and 4 h, respectively, by pour plate method using MRS agar. Plates were incubated at 37°C for 48 h. Results of log (CFU/mL) are expressed as the mean of three replicates ± standard deviation.

**Bacterial adherence to Caco-2 cells**  
The adhesion ability of strains GMK01, GMK02 and GMK03 was assayed according to the method described by Jacobsen et al. (1999) with some modifications. Caco-2 cells (ATCC® HTB-37™; Manassas, USA) were subcultured according to manufacturer constructions. Briefly, washed Caco-2 cells were incubated with bacteria (1 x 10⁸ CFU/mL) or GIJ-treated cells were transferred to 245 mL of the MSC broth (containted 0.2% cholesterol, as a sole source of carbon and energy) and incubated at 37°C and 120 rpm for 4 days. Every day, 2 mL were withdrawn for measuring growth of tested bacteria and for measuring the residual cholesterol. Growth was monitored by measuring the optical density at 600 nm (OD600) using a UV/Visible spectrophotometer (6105-Jenway, U.K.). All tests were performed in triplicates. Results are expressed as the mean of three replicates ± standard deviation. At each sampling period, the cells were removed by centrifugation (10000 xg for 5 min), the cholesterol was extracted from the supernatants and quantified by high-performance liquid chromatography (HPLC) as described later. The culture supernatant (1 mL) was extracted by 2 mL absolute methanol (HPLC-grade, Sigma, St Louis, MO, USA) and subjected to HPLC system (Shimadzu, LC10AT, Nakagyo-ku, Kyoto, Japan) equipped with a C18 column (Agilent Poroshell 120 EC-C18, 3.0 x 100 mm, 2.7 µm P/N 695975-302). Isocratic column elution was monitored by 1200 UV/visible detector at wavelength of 254 nm. The mobile phase was composed of acetonitrile/methanol (62: 38, v/v, HPLC-grade, Sigma, USA). The flow rate was 1 mL/min and the column temperature was 30°C. The standard cholesterol and 4-cholesten-3-one solutions (Sigma, USA) in methylene chloride were used for constructing the calibration curves (1.00 mg/mL). A linear dynamic range was established for cholesterol and 4-cholesten-3-one standard solutions of concentration ranging from 10 to 200 µg/mL. Remaining cholesterol was calculated as a percentage from the cholesterol amount at the beginning of the experiment.

**Statistical analysis**  
The data obtained from three replicates were analyzed by a one-way ANOVA (SAS 8.2, Cary, NC, USA). Data of adhesion experiment was analyzed using two-way ANOVA to investigate the effect of SSIJ on adhesion of bacterial cells of each bacterial strain and across the studies strains. In all cases, these tests were followed by Duncan’s multiple range test at probability level = 0.05 to compare the significant differences between the mean numbers.
Table 1. Cholesterol degradation activity of cell-free supernatant* of lactic acid bacteria isolated from meat

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Cholesterol degradation in MSC agar</th>
<th>% cholesterol reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMK01</td>
<td>+++</td>
<td>86.4</td>
</tr>
<tr>
<td>GMK02</td>
<td>+++</td>
<td>86.1</td>
</tr>
<tr>
<td>GMK03</td>
<td>+++</td>
<td>84.6</td>
</tr>
<tr>
<td>GMK04</td>
<td>++</td>
<td>57.2</td>
</tr>
<tr>
<td>GMK05</td>
<td>++</td>
<td>52.2</td>
</tr>
<tr>
<td>GMK06</td>
<td>++</td>
<td>51.2</td>
</tr>
<tr>
<td>GMK07</td>
<td>++</td>
<td>41.2</td>
</tr>
<tr>
<td>GMK08</td>
<td>++</td>
<td>32.7</td>
</tr>
<tr>
<td>GMK09</td>
<td>++</td>
<td>32.7</td>
</tr>
<tr>
<td>GMK10</td>
<td>+</td>
<td>26.7</td>
</tr>
<tr>
<td>GMK11</td>
<td>+</td>
<td>26.4</td>
</tr>
<tr>
<td>GMK12</td>
<td>+</td>
<td>24.6</td>
</tr>
<tr>
<td>GMK13</td>
<td>+</td>
<td>24.3</td>
</tr>
<tr>
<td>GMK14</td>
<td>+</td>
<td>21.4</td>
</tr>
</tbody>
</table>

* cell-free supernatant was obtained after centrifugation at 10000 xg for 5 min and used as a crude source of cholesterol degrading enzymes.

MSC agar, mineral salt cholesterol agar supplemented with 0.15% cholesterol.

+ , weak (1 mm = zone < 3 mm); ++ , moderate (3 mm = zone < 6 mm); +++ strong (zone ≥ 6 mm) cholesterol degrading strain

for extracellular secretion of the enzymes involved in degradation of cholesterol. To confirm this hypothesis, cell-free supernatants (CFSs) were prepared and used as crude source of cholesterol degrading enzymes. CFS activities for degrading cholesterol were determined and the reduction percentage of cholesterol in a standard cholesterol solution (200 mg/dL) was calculated and presented in Table 1. The tested CFSs showed wide range of cholesterol degradation (from 20.3 – 86.4%). The CFS activity results were in harmony with that obtained on MSC agar, where the highest cholesterol degradation activities; 86.4, 86.1 and 84.6% were displayed by CFSs obtained from isolates GMK01, GMK02 and GMK03, respectively (Table 1). In a previous study, CFSs prepared from LAB isolated from raw cow milk have showed degradation activity ranged from 42.88 to 97.2% (Kulkarni et al., 2013). The highest cholesterol degrading isolates GMK01, GMK02 and GMK03 were subjected to biochemical and molecular identification. According to API 50 CHL results, the most potentiate isolates in degradation of cholesterol were defined as *Lactobacillus sakei* GMK01, *Lactobacillus rhamnosus* GMK02 and *Leuconostoc mesenteroides* GMK03 with identity percentage of 97.2, 95.6 and 85.7%, respectively (Table 2). From the carbohydrate utilization profile, all strains fermented lactose, D-mannose and ribose, however, *Lac. rhamnosus* was distinguished from *Lac. sakei* and *Leu. mesenteroides* by its ability to ferment more carbohydrates including adonitol, D-arabinose, gluconate, inositol, inulin, mannitol, melezitose, sorbitol and rhamnose (Table 2). The identification of these isolates was confirmed by the obtained partial sequences of 16S rRNA gene, which were deposited at the EMBL nucleotide sequence database under accession No. LN827925, LN827926 and LN827927 for the isolates GMK01, GMK02 and GMK03, respectively. These isolates displayed identity percentage of 99, 100 and 100% compared with the reference strains in NCBI database. Therefore, the highest cholesterol degrading isolates were considered as *Lactobacillus sakei* GMK01, *Lactobacillus rhamnosus* GMK02 and *Leuconostoc mesenteroides* GMK03 and subjected to the further experiments throughout the present work.

Antibiotic resistance of cholesterol degrading LAB The response to the different classes of antibiotics seems to depend on the species, where *Lac. sakei* GMK01, *Lac. rhamnosus* GMK02 and *Leu. mesenteroides* GMK03 showed resistance to 6, 3 and 5 antibiotics out of 15 studied antibiotics, respectively (Table 3). All studied strains are resistant to vancomycin and bacitracin. This result is in agree with those was previously evidenced (Coppola et al., 2005; Hamilton-Miller and Shah, 1998). Although vancomycin and bacitracin are both active against most Gram-positive bacteria, a number of *Lactobacillus* species are intrinsically resistant to theses glycopeptides (Felten et al., 1999; Swenson et al., 1990). This may be due to the hypothesis that vancomycin resistance in lactobacilli is intrinsic and chromosomally encoded (Çataloluk and Gogebakan, 2004). Most of protein synthesis inhibitors showed inhibitory effect against all tested strains (Table 3). These results are in agreement with those reported by Çataloluk and Gogebakan (2004). However, *Lac. sakei* GMK01 showed a resistance to oxacillic and and tetracycline. Similar result was obtained previously in another lactobacilli; *Lac. plantarum* (Cebeci and Gürakan, 2003). In general, it can be noticed that, strains GMK01, GMK02 and GMK03 did not show high rate of resistance to the studied antibiotics. This character is considered as an important criterion for selection of probiotic bacteria, where specific antibiotic resistance determinants carried on mobile genetic elements and constitute a reservoir of resistance for potential food or gut pathogens (Guimonde et al., 2013).

Resistance of LAB to simulated gastric and small intestinal juices Resistance of the studied strains GMK01, GMK02 and GMK03 toward simulated gastric and small intestinal juices (SGJ and SSJ) was studied (Fig. 1).

To survive passage through the stomach, probiotic bacteria must be tolerant of acidic environments. Stresses to ingested microorganisms begin in the stomach, which has a pH between 1.5 and 3, and continue in the upper intestine, which contains a 0.03 to 0.3% (w/v) concentration of bile salts. For probiotic strains, survival at pH 3 for 2 h and in a bile concentration of 0.3% (w/v) is considered optimal (Huang et al., 2013). Based on this statement, SGJ and SSJ were prepared using these ranges of pH, bile salts and enzymes as described in materials and methods. The studied strains showed relatively high resistance to treatment for 2 h with
Cholesterol Degradation by Some Bacteria Isolated from Food

5-keto-gluconate  _Lac. sakei_ GMK01 (97.2%)*  
Adonitol  _Lac. rhamnosus_ GMK02 (95.6%)*  
Amidon  _Leu. mesenteroides_ GMK03 (85.7%)*  
\(\beta\)-Arabinose  
\(\beta\)-Arabitol  
\(\beta\)-Lyxose  
\(\beta\)-Mannose  
\(\beta\)-Raffinose  
\(\beta\)-Tagatose  
\(\beta\)-Turanose  
Dulcitol  
\(\beta\)-Xylose  
Gluconate  
Glycerol  
Glycogen  
Inositol  
Inulin  
Lactose  
\(L\)-Arabinose  
\(L\)-Arabitol  
\(L\)-Fucose  
\(L\)-Sorbose  
Mannitol  
Melezitose  
Melibiose  
Rhamnose  
Ribose  
Saccharose  
Sorbitol  
\(\alpha\)-Methyl-\(\beta\)-glucoside  
\(\alpha\)-Methyl-\(\beta\)-mannoside  
\(\beta\)-Gentiobiose

+, positive reaction; - negative reaction
* identifying percentage according to the results obtained by API 50 CHL

SGJ at pH 3.0 and 2.5, however, growth of all strains was significantly \((p < 0.5)\) reduced due to exposure to SGJ at pH 2.0. No growth was recorded after treatment with SGJ at pH 1.5. These results demonstrated that bacterial cells can resist pH 2.5, however, some damages occurred due to treatment with pH 2.0. The reduction and total inhibition in bacterial growth after treatment with SSJ at pH 2.0 and 1.5, respectively, could be interpreted by the damage effect of low pH on the studied strains (Jacobsen et al., 1999). Bacterial counts obtained after treatment with SSJ at different bile salts concentration were recorded (Fig. 1). Although, reduction in bacterial counts was observed due to treatment with SSJ at the presence of 1.5, 2 and 3 mg/mL, statistically this reduction was insignificant \((p > 0.5)\). These results demonstrated the resistance of all tested strains to 0.3% bile salts (3 mg/mL). These results were in harmony with previous observations in other studies (Huang et al., 2013; Jacobsen et al., 1999; Vamanu et al., 2011). However, bacterial counts of all tested strains were significantly \((p < 0.5)\) reduced after treatment with SSJ contained 5 mg/mL bile salts. In harmony with this finding, high drop in viability of _Lac. rhamnosus_ has been noticed within four hours of exposure to 5 mg/mL bile salts (Vamanu et al., 2011). According to the obtained results, the isolated strains GMK01, GMK02 and GMK03 could be characterized by their tolerant to bile salts and low acidity. These characteristics are important in potential probiotics. Bile tolerance is necessary for bacterial growth and continued existence in the small intestine (Lee and Salminen, 1995) and acid tolerance is mandatory for survive passage of probiotics through the stomach (Henriksson et al., 1999), as well as to stay live in food (Lee and Salminen, 1995).

_Bacterial adherence to Caco-2 cells_ Adhesion ability of
690

Table 3. Antibiotic resistant pattern of three cholesterol degrading LAB isolated from raw meat

<table>
<thead>
<tr>
<th>Antibiotic (concentration) and mode of action</th>
<th>Lac. sakei GMK01</th>
<th>Lac. rhamnosus GMK02</th>
<th>Leu. mesenteroides GMK03</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of cell wall synthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oxacillin (1 µg)</td>
<td>R</td>
<td>-</td>
<td>R</td>
</tr>
<tr>
<td>Penicillin G (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacitracin (10 U)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Vancomycin (30 µg)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cefixime (5 µg)</td>
<td>R</td>
<td>-</td>
<td>R</td>
</tr>
<tr>
<td>Cloxacillin (5 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inhibition of protein synthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline (30 µg)</td>
<td>R</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>Clindamycin (2 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol (30 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gentamycin (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kanamycin (30 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin (10 µg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline (30 µg)</td>
<td>R</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

R, resistant; (-), sensitive

Fig. 1. Resistance of LAB strains to simulated gastric (A) and intestinal (B) juice after 2, and 4 h, respectively. Column with same letter within each group indicate insignificant change in bacterial cell counts (p > 0.05) due to change of pH in gastric juice (A) or change of bile salts concentration in intestinal juice (B). (n = 3)

Table 4. Adhesion ability of cholesterol degrading LAB cells to Caco-2 cells in absence (control) and presence of simulated small intestinal juice (SSIJ)

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of adhered bacteria per Caco-2 cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Lac. sakei GMK01</td>
<td>46 ± 7abc</td>
</tr>
<tr>
<td>Lac. rhamnosus GMK02</td>
<td>58 ± 10a,b</td>
</tr>
<tr>
<td>Leu. mesenteroides GMK03</td>
<td>63 ± 10a,b</td>
</tr>
</tbody>
</table>

*The mean number (n = 3) of bacteria ± SD per Caco-2 cell after examination of at least 100 different cells from five separate fields. Numbers with the same capital letter within row and number with the same small letter within column indicate insignificant difference p = 0.5

GMK01, GMK02 and GMK03 cells to Caco-2 cells in absence (control) and presence of simulated small intestinal juice (SSIJ) was studied (Table 4). The adherence of GMK01 was significantly lower than those recorded by GMK02 and GMK03 either in the absence or presence of SSIJ. In general, the isolated GMK01, GMK02 and GMK03 strains showed high ability to adherence to Caco-2 cells in the absence or presence of SSIJ. This finding indicated that the probiotic strains isolated in the present study can adhere to intestinal surfaces and consequently colonizes the gastrointestinal tract. This character is used to select potentially probiotic bacteria (Jacobsen et al., 1999), where the adherence to intestinal surface ensures the survival of probiotic cells in the digestive tract for a long time. Moreover, the high adhesion ability of probiotics provides them good chance to compete with the pathogenic bacteria to adhere to intestinal surface (Lee et al.,
Quantifying the degradation of cholesterol by living bacterial cells

Strains GMK01, GMK02 and GMK03 were selected in the present study for their ability to degrade cholesterol on MSC agar and their extracellular secretion of cholesterol oxidase. However, the cholesterol-lowering effects may also be attributed to the ability of probiotics to bind cholesterol, a process that is growth-dependent and strain-specific (García et al., 2012). Therefore, the ability of strains GMK01, GMK02 and GMK03 to degrade cholesterol before and after treatment with simulated gastrointestinal juices (SGIJ) was tested (Fig. 2). Exponential growth of the studied strains was recorded during the first 3 days when cholesterol was used as a sole carbon source in MSC broth. After 24 h, cholesterol consumption by GMK01, GMK02 and GMK03 was 22.5, 32 and 37%, respectively.
After 2 days, all strains observed about 70% cholesterol degrading ability. At the end of third day, *Lactobacillus rhamnosus* GMK02 showed the highest ability to degrading cholesterol (87.5%). After that, there is no significant $(p > 0.5)$ reduction in the cholesterol level by extension the incubation to 4 days. Same cholesterol degradation behavior was obtained by the strains treated by the SGII (Fig. 2). In agreement with the obtained results, many LAB including *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactococcus lactis* and *Pediococcus acidilactici* had ability to degrading cholesterol in MRS broth containing 0.3% bile slats (Iranmanesh et al., 2014). The resistance of the isolated strains to these stress conditions could be explained by those mentioned by Lye et al., (2010a). They demonstrated that, the occurrence of cholesterol during growth of LAB increases the level of saturated and unsaturated fatty acids. These fatty acids play an important role for improving the strength of bacterial cell membrane and consequently elevate cellular resistance against lysis (Lye et al., 2010a).

The mechanism of cholesterol reduction by LAB takes place by different bioactivities which include the inhibition of the hydroxymethylglutaryl coenzyme A reductase (Homma, 1988), uptake and/or co-precipitation of cholesterol (Tahri et al., 1996), cholesterol binding to cell walls (García et al., 2012), co-precipitation of cholesterol with deconjugated bile, production of short chain fatty acids during fermentation in the presence of prebiotics (Preter et al., 2007), enzymatic deconjugation of bile acids by bile-salt hydrolase (Lambert et al., 2008), incorporation of cholesterol into the cellular membranes during growth (Lye et al., 2010a) and conversion of cholesterol into coprostanol using bile salts and low acidity (pH 2.5). Moreover, these strains had high ability to adhere to Caco-2 cells. Therefore, the isolated *Lactobacillus sakei* GMK01, *Lactobacillus rhamnosus* GMK02 and *Leuconostoc mesenteroides* GMK03 could be considered as potential probiotic strains for food industry and human nutrition. Since cholest-4-en-3-one has positive uses against obesity, liver disease, and keratinization (Wu et al., 2015), *Leu. mesenteroides* GMK03 isolated in the present study could be considered as a promise probiotic strain. However, further studies are needed to understand the mechanism of cholesterol degradation using the extracellular cholesterol oxidase especially by *Leuconostoc mesenteroides*.

**References**


Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzano, G., and Sorrentino,
Cholesterol Degradation by Some Bacteria Isolated from Food


