

Full Length Research Paper

Cholesterol reduction *in vitro* by novel probiotic lactic acid bacterial strains of *Enterococcus* isolated from healthy infants' stool

Akram A. Aboseidah¹, Abdel-Hamied M. Rasmey^{1*}, Magdy M. Osman², Salha G. Desouky¹ and Nehal Kamal¹

¹Department of Botany and Microbiology, Faculty of Science, Suez University, Suez, Egypt.

²Department of Dairy, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt.

Received 4 May, 2017; Accepted 7 August, 2017

The present study aimed to isolate and evaluate the potentiality of some probiotic lactic acid bacteria from infants stool to degrade and reduce cholesterol level in fermentation medium. Out of 50 lactic acid bacterial isolates recovered from healthy infants (3 to 18 months old) stool, two bacterial isolates W7 and Y1 showed highest cholesterol reduction percentage (68.38 and 71.6%, respectively). The cholesterol reduction potentiality by the two isolates W7 and Y1 were optimized to 94 and 98%, respectively, by studying the different cultural conditions; inoculum size, inoculum age, pH, incubation temperature, incubation period and cholesterol concentration. The gas chromatography analysis of the fermentation extract revealed that the resulted end product of cholesterol was 5 α -cholestane-3 β ,25-diol (C₂₇H₄₈O₂) of molecular weight 404. The selected bacterial isolate Y1 was identified phenotypically and genotypically as *Enterococcus faecium* and deposited in GenBank under the accession number KY788356.

Key words: Cholesterol, *Enterococcus*, optimization, probiotic, gas chromatography.

INTRODUCTION

Cholesterol is a waxy steroid compound formed in all cells of the animal and human body and required for formation of steroid hormones (Young, 2001, Rigotti et al., 2003), a precursor for biosynthesis of bile acids in the liver that help the body to digest fat (Russell, 2003). Longstanding increased serum cholesterol levels may lead to atherosclerosis and as a result lead to development of cardiovascular diseases (CVDs) (Ngongang et al., 2016; Tsai et al., 2016). CVDs were

reported to be responsible for 30% of deaths worldwide and predicted to remain the leading causes of death (WHO, 2013). Both pharmacologic and non-pharmacologic approaches, including follow healthy diet, behavior alteration, and regular practice, are common strategies to lower serum cholesterol levels (Dunn-Emke et al., 2001), Although they record cholesterol-lowering ability, but in some cases unwanted side effects can be produced, such as gastrointestinal disorder (Davidson et

*Corresponding author. E-mail: am_rasmey@yahoo.com.

al., 1999).

There are some microorganisms that live as normal flora in animal and human gut, known as probiotics. The presence of these microorganisms in healthy form have different healthy benefits such as protection against gastrointestinal disorder (Vanderhoof and Young, 1998; Niv et al., 2005; Saxelin et al., 2005), reduction of cholesterol level in blood and lipids (Gilliland and Walker, 1990; Naidu et al., 1999), synthesizing extracellular bacteriocins as antimicrobial metabolites (Gill, 2003) and enhancing the immune system by production and circulation of serum antibodies (Maassen et al., 2000; Ezendam and van Loveren, 2006). An adequate quantity administration about 10^9 CFU per day of external probiotics especially lactic acid bacteria will be appropriate to maximize and balance the human gastrointestinal microbiota by replacing harmful pathogens and reinforcing the natural defence mechanisms (Casas and Dobrogosz, 2000; Ouwehand et al., 2002). Probiotics for human feeding are preferably isolated from the gastrointestinal (GI) tract of healthy human (Saarela et al., 2000).

Acid-producing probiotics have been extensively used for cholesterol reduction in human blood serum by applying these bacterial strains in various fermented foods, milk products and beverages (Pereira and Gibson, 2002; Ouwehand and Vesterlund, 2004) and several studies reported the hypocholesterol ability of lactic acid probiotics *in vitro* or *in vivo*, especially the strains of genera *Lactobacillus*, *Enterococcus* and *Bifidobacterium* (Pan et al., 2011; Wang et al., 2012; Huang et al., 2013; Hu et al., 2013; Tsai et al., 2016). Lactic acid bacteria of cholesterol reduction potentiality are now available as probiotics to consumers in forms of milk products such as yoghurts and cheese, formulated tablets and lyophilized powders. However, long-term hypocholesterolemic effects requiring continuous research for novel health enhancing probiotic strains from healthy individual origin is still attracting area.

Different mechanisms for cholesterol removal by probiotics have been reported, such as deconjugation of bile salts by bile-salt hydrolase (BSH) (Ahn et al., 2003), cholesterol absorption into bacterial cell membranes (Kimoto et al., 2002), production of short-chain fatty acids (SCFAs) during the probiotics growth (Trautwein et al., 1998), and cholesterol transformation into coprostanol (Lye et al., 2010). Additionally, several cultural conditions such as media composition, pH, temperature, and inoculum size may change the metabolic pathways by altering pyruvate metabolism and external electron acceptors resulted in different end-products (Axelsson, 1998; Annuk et al., 2003).

The main objective of this study was to isolate new lactic acid probiotic strains of human origin with potential of highly cholesterol reduction and optimize the cultural condition for the cholesterol reduction process in addition to follow the resulted end products formed by the selected

bacterial strain.

MATERIALS AND METHODS

Isolation of microorganisms

A total of 50 different healthy infants (3 to 18 months old) stool samples were obtained from different baby centers located at Ismailia and Suez Governorates of Egypt, transferred in sterilized plastic bottles to the laboratory and immediately used for isolation of lactic acid bacteria. Ten grams of each infant stool sample were aseptically homogenized in 90 ml of sterile saline solution and sequential decimal dilutions were done. One milliliter of each dilution was inoculated into sterilized plates and about 20 ml of melted de Man, Rogosa and Sharpe (MRS) agar medium of pH 6.2 were poured in each plate. The used MRS agar (De Man et al., 1960) medium contained (g/L distilled water): glucose 10, peptone 10, beef extract 10, yeast extract 5, K_2HPO_4 2, sodium acetate 5, tri-ammonium citrate 2, $MgSO_4 \cdot 7H_2O$ 0.2, $MnSO_4 \cdot 4H_2O$ 0.2 and Tween 80 (1 ml). The plates were incubated for 3 days at 37°C under anaerobic conditions (GasPak System - Oxoid, Basingstoke Hampshire, England). The separated colonies were picked, subcultured, maintained on MRS slants and stored at 4°C for further experiments.

Preparation of bacterial inoculum

A loopful of refreshed bacterial culture was inoculated and grown in bottles containing 20 ml of MRS broth medium and incubated for 24 h at 37°C under anaerobic conditions in CO_2 incubator.

Cholesterol degradation in fermentation medium by the isolated bacteria

Cholesterol stock solution was prepared by dissolving 10 mg of cholesterol in 1 ml of 96% ethyl alcohol and filter sterilized. For each culture to be tested, 70 μ l of cholesterol solution was added to 10 ml of MRS broth (final cholesterol concentration 70 μ g/ml) and 1% (v/v) of freshly grown culture was added and incubated anaerobically at 37°C for 24 h. After incubation, the cells were removed by centrifugation at 10,000 g for 10 min at 4°C.

Determination of cholesterol concentration in fermentation medium

The cholesterol concentration was determined in the supernatant using a colorimetric method described by Rudel and Morris (1973). Three milliliters of supernatant, 2 ml of 33% (w/v) KOH and 3 ml 96% ethanol were placed in a capped test tube, vortexed for 20 s and incubated for 15 min at 60°C in a water bath. After incubation, the mixture was removed and cooled under tap water, then 5 ml of hexane and 3 ml of water were added and vortexed for one min. One milliliter of the hexane layer was transferred into a dry clean test tube and evaporated under nitrogen gas. One milliliter of cholesterol liquicolor enzymatic kit (Spinreact Kit, Spain) was added (Guo et al., 2011). The solution was mixed and left for 5 to 10 min at 37°C and absorbance was measured at 500 nm with a spectrophotometer. The cholesterol reduction was calculated as percentage from the following equation: $A = 100 - (B/C) \times 100$, where A = % of cholesterol removed, B = absorbance of the sample containing the cells and C = absorbance of the sample without

cells.

Characterization and identification of the selected bacterial isolate Y1

Morphological characterization

The morphological growth characters of the selected isolate Y1 was conducted on MRS agar medium and the colony color, shape and texture were recorded. The cell shape and arrangement were determined by microscopic examination after Gram staining technique in accordance with Collins and Lyne (1985).

Physiological and biochemical characterization

The physiological and biochemical characteristics were estimated according to the standard methods. The selected bacterial isolate Y1 was examined for Catalase production (Wittenberg, 1964), carbohydrate utilization (Pridham and Gottlieb, 1948), growth at 6.5% NaCl, growth at different temperatures (15 and 45°C), production of CO₂ from glucose and production of NH₃ from arginine.

Genotypic characterization

DNA extraction: The extraction of bacterial DNA was performed according to the method of Kozaki et al. (1992) using DNA preparation kit (Jena Bioscience).

Polymerase chain reaction (PCR) amplification: The PCR amplification was performed using the 16S primers: 16S F: 5'-GAGTTTGATCCTGGCTTAG-3' and 16S R: 5'GGTTACCTTGTTACGACTT-3' and Qiagen Proof-Start Tag Polymerase Kit (Qiagen, Hilden, Germany) according to standard PCR protocol. The following substrates were combined in a total volume of 25 µl containing about 20 ng of template DNA, 12.5 µl PCR Master Mix, 20 pmol (2 µl) of both forward and reverse primers and the reaction was completed by 8.5 µl of deionized water. The reaction conditions were: an initial denaturation at 94°C for 5 min, 37 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s. A final extension was performed at 72°C for 5 min. PCR products were purified from gel with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Then analyzed by electrophoresis on 1.5% agarose gel in 1X TAE buffer and finally observed and pictured under UV light.

DNA Sequencing: Sequence similarity was recorded by searching the homology in the Genbank DNA database using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). CLUSTALW program (<http://clustalw.ddbj.nig.ac.jp/top-eh.html>) was used for evaluating multiple sequence alignment and molecular phylogeny. The tree view program was applied for phylogenetic tree illustration.

Optimization of cholesterol reduction by the selected bacterial isolates

Effects of different cultural conditions on cholesterol reduction by the selected bacterial isolates W7 and Y1 were studied in MRS broth medium as follows; cholesterol concentration (50, 70, 100, 125 and 150 µg/ml), incubation temperature (25, 30, 37 and 45°C), pH (3, 5, 6.5, 7 and 9), incubation time (14, 24, 48, 62 and 72 h), inoculum size (0.5, 1.5, 2.0, 2.5, 3.0 and 4.0%) and inoculum age (4, 8, 10, 18, 24, 42 and 48 h).

Extraction of the remaining cholesterol and its derivatives from the bacterial culture

The LAB isolate (Y1) was grown in MRS broth medium supplemented with cholesterol (70 µg/ml) under the optimized cultural conditions and the obtained bacterial cells with the remaining broth medium were homogenized in chloroform (1:1, v/v) by sonication. The solvent-supernatant mixture was agitated and separated by separating funnel and filtered on sodium sulphate anhydrous to remove any water from the extract. All obtained chloroform extracts were dried using rotary evaporator at 50°C under vacuum and the crude extracts were then subjected to analysis by gas chromatography-mass spectra to clarify the structure of the resulted metabolites.

Determination of cholesterol degradation products using gas chromatography-mass spectra (GC-MS)

The GC-MS analysis of the crude extracts was carried out at the Central Laboratory, National Research Center of Egypt with the following specifications: Instrument, a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-5MS column (30 m × 0.32 mm i.d., 0.25 µm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min. Temperature program: 50°C for 3 min; rising at 5°C/min to 300°C and held for 5 min. The injector and detector were held at 220 and 200°C, respectively. Diluted samples (1:10 diethyl ether, v/v) of 1 µl of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 50 to 500 (Said-AL Ahl and Omer, 2016).

Statistical analysis

Analysis of variance (ANOVA) was performed using CoStat V. 6.311 (CoHort software, Berkeley, CA94701). Cholesterol reduction mean values were compared at 5% significance level using Tukey's test. Least significant difference (LSD) test was used to test the significant differences between the whole means of different groups and compared with the critical difference at the 5% level.

RESULTS AND DISCUSSION

Fifty lactic acid bacterial isolates were recovered from the collected 50 infants stool samples based on criteria of lactic acid bacteria such as morphological shape, catalase negativity, Gram positive, and lactic acid formation. The selected bacterial isolates were tested for their potentiality to reduce or biodegrade cholesterol on basal cholesterol broth medium. A total of 10 out of 50 tested lactic acid bacterial isolates exhibited cholesterol reduction in high percentages (Table 1). Apparently, cholesterol reduction varied among the tested bacterial strains ranged from 48.22 to 71.6% reduction from the initial cholesterol concentration and the highest reduction mean values of 71.6 and 68.38% were obtained by the two isolates Y1 and W7, respectively. The results in the present study are in accordance with the experimental findings of Sieladie et al. (2011) who reported that about 11 lactobacilli strains isolated from raw cow milk exhibited cholesterol

Table 1. Mean values of cholesterol reduction percentage of the most active ten LAB isolates.

Isolate code	Cholesterol reduction* (%)
Y1	71.6 ± 0.05 ^a
H3	48.22 ± 0.02 ^e
W1	61.61 ± 0.04 ^{cd}
W2	64.6 ± 0.02 ^c
W4	65 ± 0.03 ^b ^c
W6	65 ± 0.04 ^{bc}
W7	68.38 ± 0.05 ^{ab}
Mix	61.61 ± 0.06 ^{cd}
YF- black	62.9 ± 0.09 ^c
YF- green	58.06 ± 0.08 ^d

*Values are means of three replicates ± standard deviation and values with the same letters are not significantly different.

degrading activity with cholesterol lowering potentials ranging from 42.88 to 97.20%. In the study conducted by Hassanein et al. (2013) the result revealed high cholesterol reduction (66.8%) by *Lactococcus lactis* KF 147. In this coincidence, Yehia et al. (2015) recorded maximum cholesterol reduction of 75.3% in liquid medium by *Enterococcus hirae* isolated from milk. Also, the highest cholesterol reduction by *L. lactis* subsp. *lactis* N7 was 97.0% as recorded by Kimoto et al. (2002). It has been reported that cholesterol removal by lactic acid bacteria appeared not to be the only strain specific but also growth dependent, as shown by studies that have evaluated cholesterol removal by probiotic cells during different growth conditions (Kimoto et al., 2002).

The two bacterial isolates W7 and Y1 were selected as highly cholesterol reducing lactic acid bacteria in the present study and subjected to identification and further experiments. The bacterial isolate W7 was identified as *Enterococcus faecalis* and published in another study for production of lactic acid by the same authors (Aboseidah et al., 2017), while the bacterial isolate Y1 was identified phenotypically and genotypically in the present study. The results of physiological and biochemical characterizations of the isolate Y1 are shown in Table 2. The obtained results revealed that the bacterial isolate was Gram positive and catalase negative. This isolate was able to tolerate NaCl (6.5%). Also, the bacterial isolate was able to grow at 15 and 45°C. The results also indicated that the isolate was unable to produce CO₂ from glucose and NH₃ from arginine. The tested isolate had the ability to ferment glucose, mannose, galactose, xylose, maltose, mannitol, lactose and arabinose but was unable to use sucrose and glycerol as a carbon source. Based on the taxonomic characteristics described earlier, the isolate Y1 was assigned to the genus *Enterococcus*.

The bacterial isolate Y1 was identified using phylogenetic analysis of 16S rRNA gene sequences. The partial 16S rRNA gene sequences of tested isolate was

matched with previously published bacterial 16S rRNA gene sequences available in National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Sequence analysis of the partial 16S rRNA gene sequence of 498 base pairs of the isolate Y1 revealed that this isolate had 96% similarity to *Enterococcus faecium*. A phylogenetic tree was constructed from a multiple sequences alignment of 16S rRNA gene sequences (Figure 1). The nucleotide sequences of the isolate Y1 was deposited in the GenBank nucleotide sequence database under new accession number KY788356.

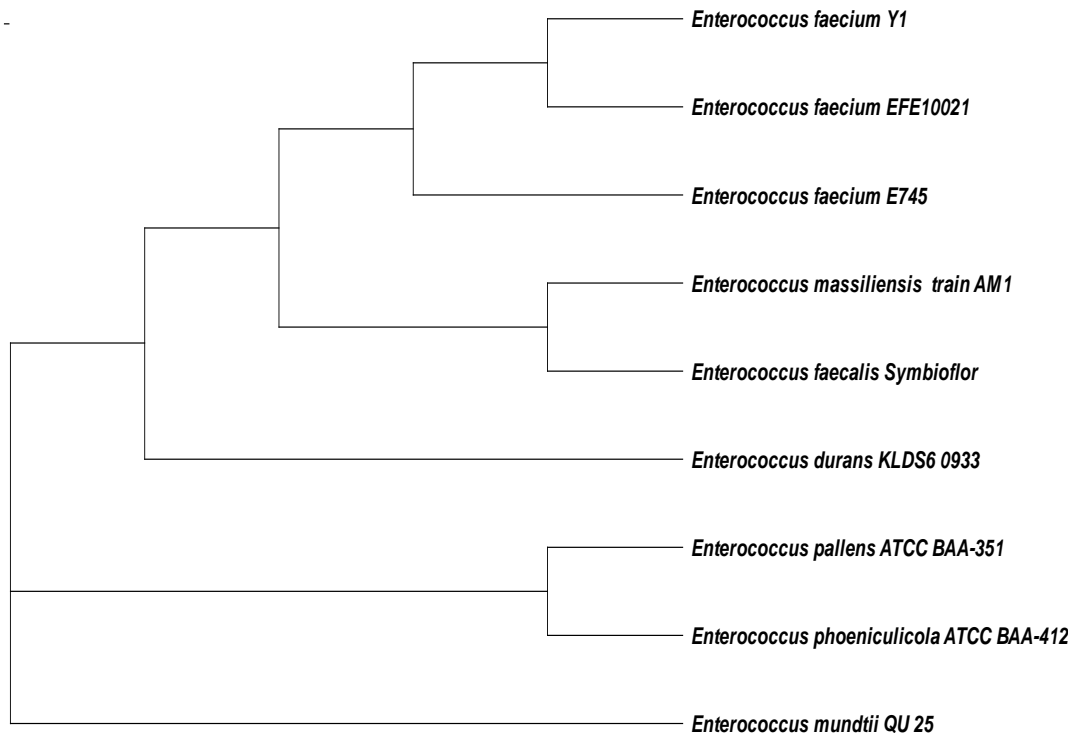
Figure 2 shows the effect of pH on cholesterol removal percentage by LAB isolates. The cholesterol reduction increased gradually with decreasing acidity of cultural medium until it reached maximum (65 and 78.5% for *E. faecalis* W7 and *E. faecium* Y1, respectively) at pH 6.5. At basic media the ability of bacteria to remove cholesterol decreased gradually with increasing alkalinity. Therefore, the optimum pH for maximum cholesterol reduction by the two selected isolates was pH 6.5. The study carried out by Kumar et al. (2013) suggested that a neutral pH is best for cholesterol reduction by *L. casei* LA-1. Also, Yazdi et al. (2001) reported that the optimal pH values for cholesterol decomposition are 7.2 for *Streptomyces fradiae* and 6.75 for *Rhodococcus erythropolis* ATCC 25544. The optimum production of enzymes required for metabolic process greatly affected by pH as enzymes, being proteins, contain ionizable groups; therefore, the pH of the culture medium affected their structure and function (Lekha and Lomane, 1997).

The effect of different incubation temperatures (25 to 45°C) on cholesterol reduction by the two tested bacterial isolates was studied and the obtained results are illustrated in Figure 3. The results revealed that the ability of bacterial isolates to remove cholesterol increased by increasing incubation temperature and reached to maximum values (80.7 and 85% for *E. faecalis* W7 and *E. faecium* Y1, respectively) at 37°C and then decreased by increasing temperature. Environmental and nutritional factors are known to influence the growth and decomposition of cholesterol by microorganisms. The optimum temperature for the decomposition of cholesterol in liquid medium by *E. hirae* was 37°C (Yehia et al., 2015). In contrast, several studies have reported other optimal incubation temperatures, such as 29°C for *R. erythropolis* ATCC 25544 (Sojo et al., 2002) and 30°C for *Bacillus subtilis* SFF34 (Lashkarian et al., 2010). The temperature range from 25 to 37°C is optimum for microbial growth and their enzymes activity and as a result maximum cholesterol removal was observed.

The effect of incubation time on cholesterol removal is observed in Figure 4. The maximum removal percentage was reported at 24 h of incubation. The bacterial isolate *E. faecium* Y1 removed 78.5% of cholesterol from the medium, while 65% of cholesterol was removed by isolate *E. faecalis* W7 at 24 h of growth. After that, the

Table 2. Morphological and biochemical characterizations of the selected bacterial isolate Y1.

Test	Observation
Colony morphology	White, circle, entire, convex
Gram stain	+
Cells shape	Cocci
Catalase production	-
Growth at 6.5% NaCl	+
Growth at 15°C	+
Growth at 45°C	+
Production of:	
CO ₂ from glucose	-
NH ₃ from arginine	-
Fermentation of:	
Xylose	+
Galactose	+
Arabinose	+
Maltose	+
Mannitol	+
Sucrose	-
Lactose	+
Glycerol	-
Mannose	+
Glucose	+

**Figure 1.** The neighbor-joining tree based on 16S rRNA gene sequences showing the position of *Enterococcus faecium* Y1 and related strains in GenBank.

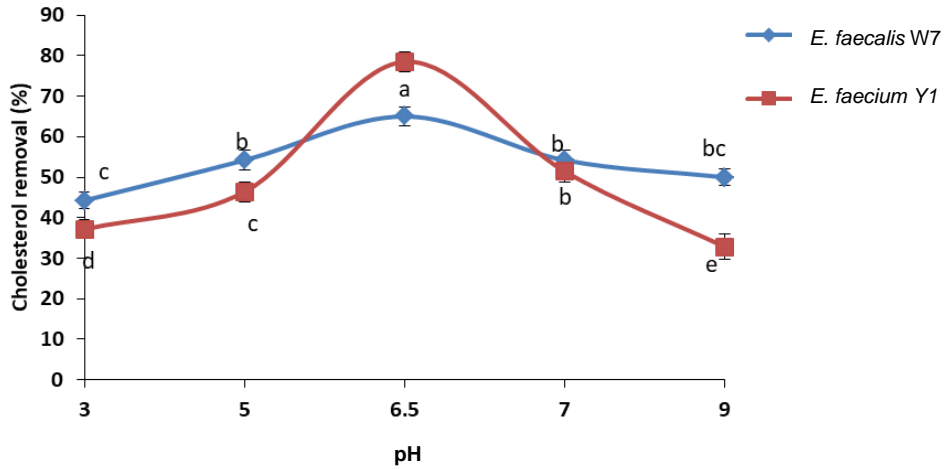


Figure 2. Effect of medium pH on cholesterol reduction percentage by *Enterococcus faecalis* W7 and *E. faecium* Y1.

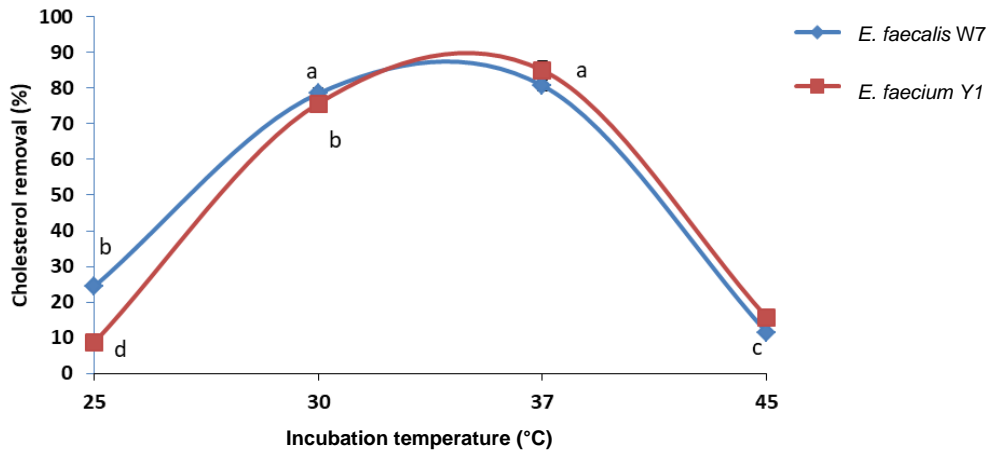


Figure 3. Effect of incubation temperature on cholesterol reduction percentage by *E. faecalis* W7 and *E. faecium* Y1.

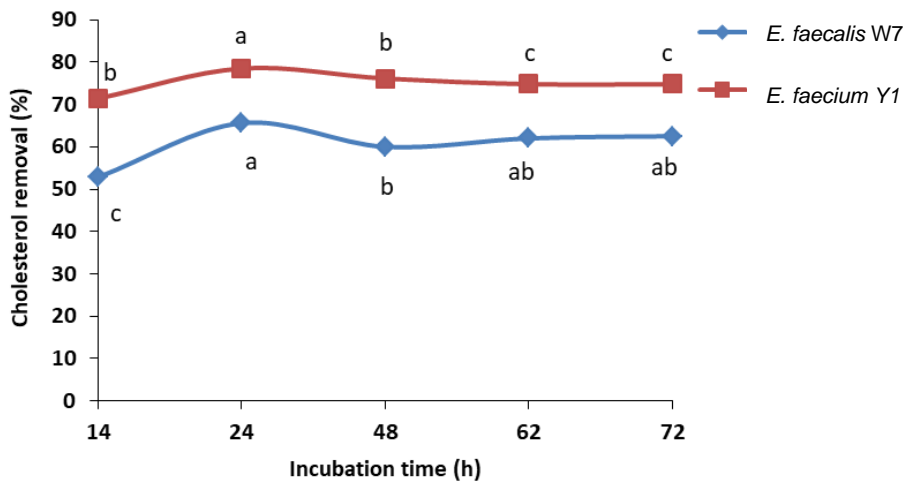


Figure 4. Effect of incubation time on cholesterol removal percentage by *E. faecalis* W7 and *E. faecium* Y1.

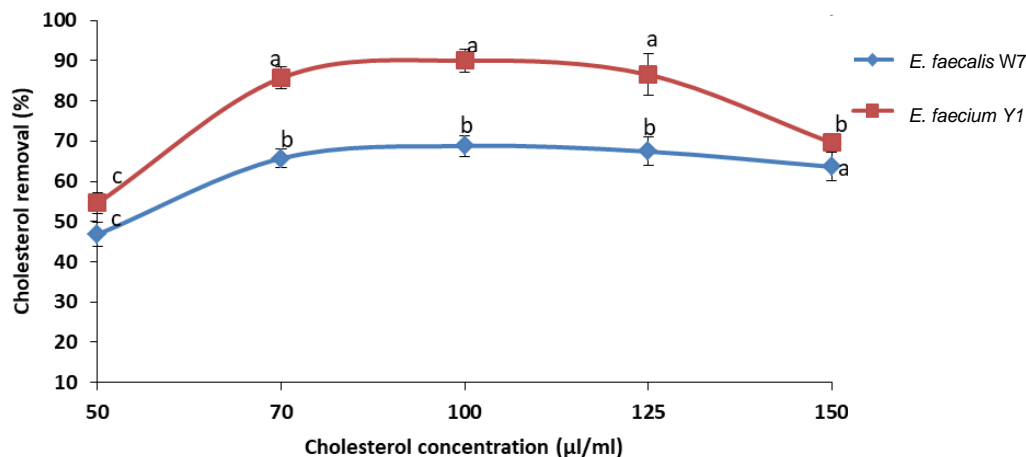


Figure 5. Effect of different cholesterol concentration on cholesterol removal percentage by *E. faecalis* W7 and *E. faecium* Y1.

ability of both isolates to remove cholesterol decrease gradually and finally reached stability at 48 h for W7 and 62 h for *E. faecium* Y1. Our findings is in accordance with the study of Kumar et al. (2013) who suggested that the ability of *L. casei* LA-1 to assimilate cholesterol was maximum after an incubation of 24 h. Hassanein et al. (2013) also found that the maximum percentage of cholesterol reduction by *L. lactis* KF147 was achieved at 24 h. Additionally, Yehia et al. (2015) observed that the optimal cholesterol reduction (75.1%) in liquid medium was achieved after one day. However, Mahrous (2011) recorded maximum cholesterol reduction from liquid medium by probiotics bacteria (24.32 to 45.3%) at 20 h.

The cholesterol reduction by the two highly degradable bacterial isolates *E. faecalis* W7 and *E. faecium* Y1 were studied at different concentrations from 50 to 150 µg/ml of cholesterol in culture broth medium (Figure 5) and the obtained results indicated that the highest reduction of cholesterol by both tested isolates was recorded at 100 µg/ml. The reduction of cholesterol by *E. faecalis* W7 in the cultural medium increased gradually with increasing cholesterol concentration until it reached the highest value of 68.8% at cholesterol concentration of 100 µg/ml. However, the maximum percentage of cholesterol removal by *E. faecium* Y1 was 90% at 100 µg/ml, then decreased at higher cholesterol concentrations. In a similar study, Mahrous (2011) found that maximum cholesterol reduction by *Lactobacillus acidophilus* P106 in liquid medium was obtained at 70 µg/ml cholesterol. While Hassanein et al. (2013) reported that *L. lactis* KF147 was able to remove 66.8% of cholesterol from MRS media supplemented with 100 µg/ml. Also, Pereira and Gibson (2002) reported high cholesterol removal ability (47%) by lactic acid bacteria and bifidobacteria isolated from human gut in broth media containing 100 µg/ml cholesterol. Guo et al. (2016) reported cholesterol reduction (41.29 to 56.61%) from liquid media containing

100 µg/ml of cholesterol by *Enterococcus* strains. Excessive cholesterol concentration may suppress bacterial ability of cholesterol reduction. Above a critical substrate concentration, a decreased water activity and onset of plasmolysis combine to cause a decrease in the rates of assimilation (Roukas, 1993).

The percentage of cholesterol removal at different growth stages of LAB were recorded in Figure 6. The maximum removal ability of *E. faecalis* W7 (93.5%) was observed at 42 h inoculum age, but in case of *E. faecium* Y1, the maximum removal value (94%) was reached at 18 h. In general, the results revealed that maximum cholesterol reduction was observed during the exponential growth phase and maximum biomass production when the cultures attained the stationary phase in which the medium nutrients is soon depleted and enriched with inhibitory products leading to decrease in bacterial biomass and rate of cholesterol removal (Kumar et al., 2013).

The effect of inoculum size on the cholesterol removal percentage is as shown in Figure 7. The highest value of cholesterol removal percentage by the two bacterial isolates *E. faecalis* W7 and *E. faecium* Y1 were 94 and 98%, respectively. In the case of isolate Y1, the ability of bacteria to remove cholesterol increased gradually with increasing inoculum size to reach maximum value of 98% at 3% inoculum size, while the maximum removal of cholesterol was reached at 2% inoculum size of isolate *E. faecalis* W7 then decreased slightly and became stable. The maximum removal percentage of cholesterol was recorded with inoculum size ranging from 2 to 3%. Our findings is in accordance with the study conducted by Kumar et al. (2013) who suggested that the ability of *L. casei* LA-1 to assimilate cholesterol is highly dependent on its growth, perhaps reflecting the growth of the inoculum used. Mahrous (2011) and Pereira and Gibson (2002) reported optimum cholesterol reduction with 1%

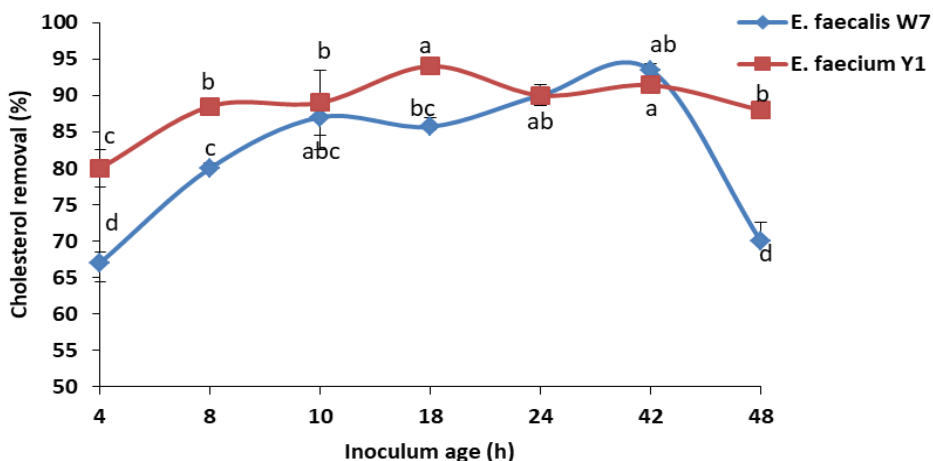


Figure 6. Effect of inoculum age on cholesterol removal percentage by *Enterococcus faecalis* W7 and *Enterococcus faecium* Y1.

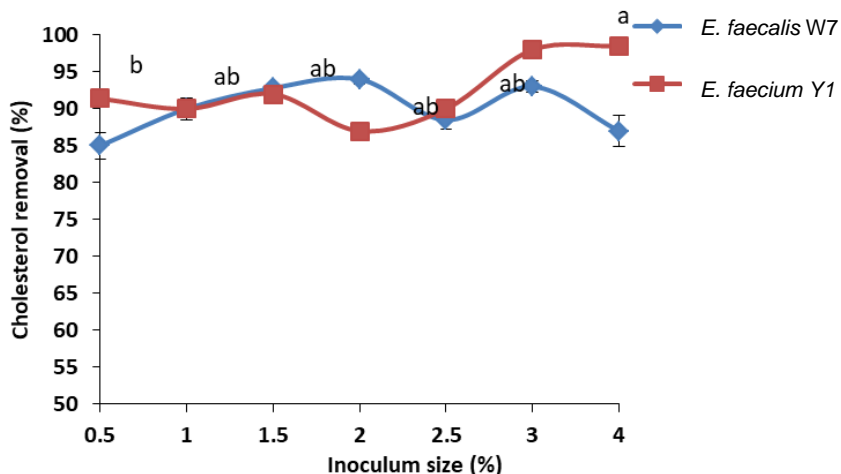


Figure 7. Effect of inoculum size on cholesterol removal percentage by *E. faecalis* W7 and *E. faecium* Y1.

Table 3. Gas chromatography – Mass spectra analysis of cholesterol degradation products by *Enterococcus faecium* Y1.

Test (h)	Retention time (RT)	Compounds	Area (%)	Molecular weight	Molecular formula
0	51.08	Cholesterol	5.39	386	C ₂₇ H ₄₆ O
4	51.09	Cholest-5-ene-16,22-dione,3á,26-dihydroxy-, 3-acetate	1.48	562	C ₄₀ H ₅₀ O ₂
8	51.08	5á-Cholestane-3à,25-diol	0.78	404	C ₂₇ H ₄₈ O ₂
24	51.07	5á-Cholestane-3à,25-diol	0.24	404	C ₂₇ H ₄₈ O ₂

inoculum size of probiotic bacteria.

The results of GC-mass spectra analysis of *E. faecium* Y1 extract at interval incubation time is shown in Table 3. At zero time, the prominent peak was cholesterol which was observed at 51.08 min of retention time, while at 4 h of incubation cholest-5-ene-16, 22-dione, 3á,26-dihydroxy-

, 3-acetate, was reported at 51.09 min of retention time and the peak of cholesterol became less predominant. At incubation time of 8 h, 5á-cholestane-3à,25-diol resulted in the peak at retention time 51.08 and the same result was observed at 24 h of incubation but by less amount (Figure 8). The results indicated that the bacterial isolate

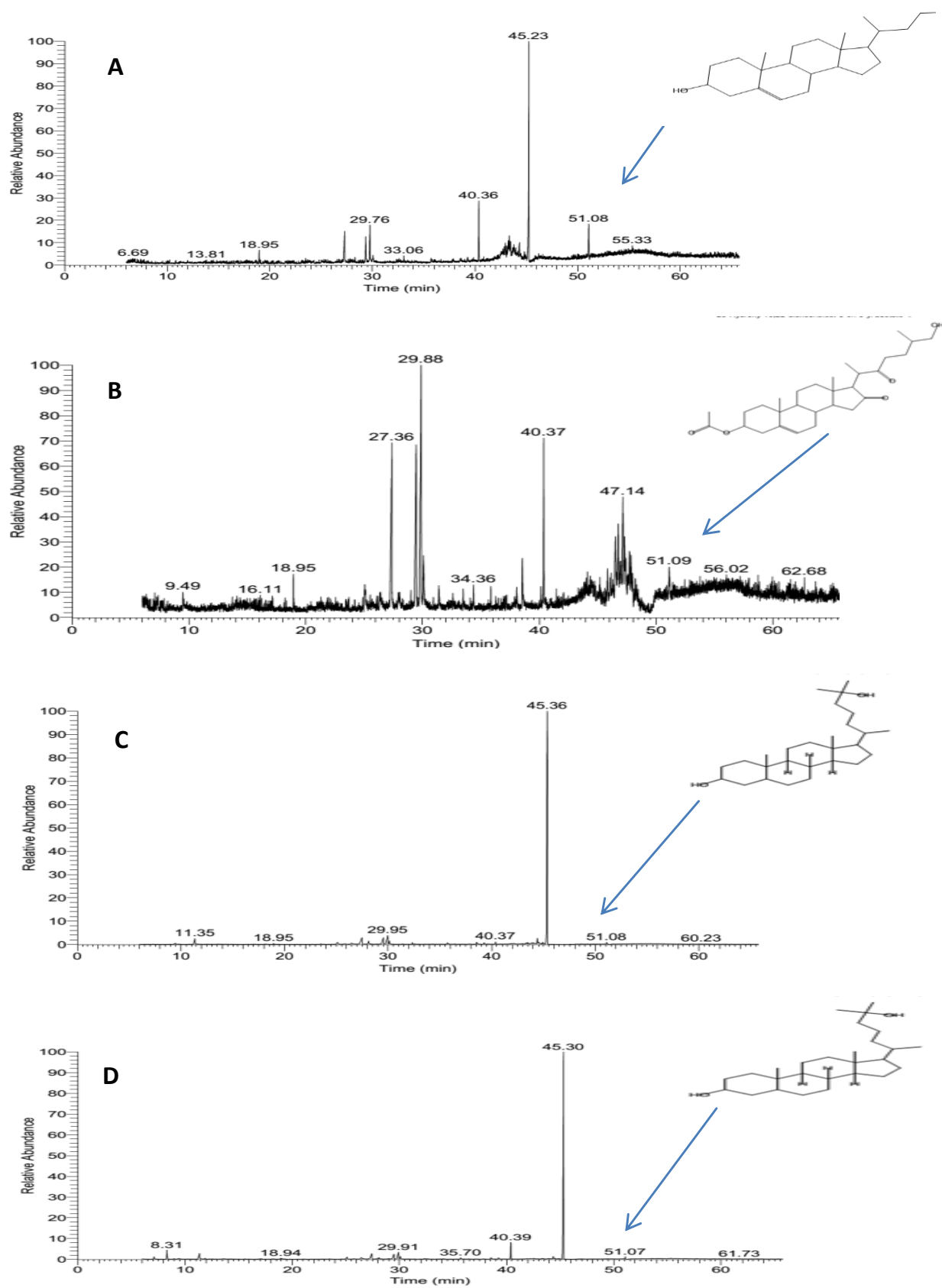


Figure 8. GC-mass analysis of cholesterol degradation by *Enterococcus faecium* Y1 at (A) zero time, (B) 4 h, (C) 8 h, and (D) 24 h of incubation period.

E. faecium Y1 transformed cholesterol to 5 α -cholestane-3 α ,25-diol through oxidation and other chemical reactions whereas the reduced amount of cholesterol was utilized by the bacterial cells for its assimilation metabolism. The produced metabolites were ketonic derivatives of cholesterol and this due to transformation of some functional groups of cholesterol giving rise to ketones (Saranya, 2014). These results are in accordance with Suzuki et al. (1986), who reported that some intestinal bacteria such as *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Enterobacteriaceae*, *Clostridium* and *Enterococcus* decompose cholesterol via the ChoX enzyme to cholest-4-en-3-one.

The reduction of cholesterol levels *in vitro* or *in vivo* by microorganisms may take place via enzymatic processes (Kovalenko et al., 2004) as the bacteria may decompose cholesterol due to their production of extracellular cholesterol oxidase (ChoX). ChoX attacks sterols at the 3 β -hydroxyl positions to form 4-cholestenone and H₂O₂. This is the first step of microbial degradation of cholesterol in the oxidation of the 3 β -hydroxyl group by ChoX (Sih et al., 1967) followed by the degradation of the side-chain (Shen et al., 1997; MacLachlan et al., 2000) by enzyme-complex possibly involving cytochrome P-450 and lyases leading to 4-androstene-3,17-dione, or 1,4-androstadiene-3 or 17-dione, and eventual breakdown of the steroid moiety with carbon dioxide and water being the final products. Also, the oxidation of cholesterol may result in the intermediate 3-ketosteroid as the final product (Motteran et al., 2001). Liu and Shan (2006) reported that cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one are produced from the oxidation of cholesterol degradation by bacteria. Doukyu (2009) reported that the ChoX enzyme from *Burkholderia cepacia* strain ST-200 produces 6-beta-hydroperoxycholest-4-en-3-one from cholesterol. Liu and Shan (2006) reported that cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one are produced through the oxidation of cholesterol. The end products resulted from cholesterol degradation by microorganisms and their quantity varies between the different microbial strains and depends on the incubation period of the tested strains.

Conclusion

The use of fermented dairy products containing probiotic lactic acid bacteria is considered as one of attractive and effective treatment for hypercholestermia. Therefore, obtaining a novel probiotic strains with cholesterol reducing abilities is required and will be safe alternative for clinical drugs. Consequently, in the present study a total of 50 different probiotic lactic acid bacterial strains were isolated from healthy infants' stool which showed various cholesterol lowering abilities in culture media. The most active isolates *E. faecalis* W7 and *E. faecium* Y1 were selected to optimize cholesterol removal process

under different condition. About 90% of cholesterol reduction was achieved by *E. faecium* Y1 under the optimized cultural conditions in the broth medium. In addition, the end products which resulted from the degradation process were also followed to study their impact when this probiotic strain was applied as cholesterol lowering agent in the dairy products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Aboseidah AA, Rasmey AM, Osman MM, Kamal N, Desouky SG (2017). Optimization of lactic acid production by a novel strain, *Enterococcus faecalis* KY072975 isolated from infants stool in Egypt. Eur. J. Biol. Res. 7:22-30.
- Ahn YT, Kim GB, Lim KS, Baek Y J, Kim HU (2003). Deconjugation of bile salts by *Lactobacillus acidophilus* isolates. Int. Dairy J. 13:303-311.
- Annuk H, Shchepetova J, Kullisaar T, Songisepp E, Zilmer M, Mikelsaar M (2003). Characterization of intestinal lactobacilli as putative probiotic candidates. J. Appl. Microbiol. 94:403-412.
- Axelsson L (1998). Lactic Acid Bacteria: Classification and Physiology, In: S. Salminen, Von Wright, A., Ouwehand, A. (Ed.), Lactic Acid Bacteria: Microbiological and Functional Aspects. New York: Marcel Dekker Inc. pp. 1-72.
- Casas IA, Dobrogosz WJ (2000). Validation of the Probiotic Concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. Microb. Ecol. Health. Dis. 12:247-285.
- Collins CH, Lyne PM (1985). Microbiological methods. 5th edn. Butterworths, London. <https://books.google.com.eg/books?isbn=0121361101>.
- Davidson MH, Dillon MA, Gordon B, Jones P, Samuels J, Weiss S, Isaacsohn J, Toth P, Burke SK (1999). Colesevelam hydrochloride (Cholestagel): a new, potent bile acid sequestrant associated with a low incidence of gastrointestinal side effects. Arch. Int. Med. 159:1893-1900.
- De Man J, Rogosa M, Sharpe ME (1960). A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130-135.
- Doukyu N (2009). Characteristics and biotechnological applications of microbial cholesterol oxidase. Appl. Microbiol. Biotechnol. 83:825-837.
- Dunn-Emke S, Weidner G, Ornish D (2001). Benefits of a low fat plant-based diet. Obesity 9:731.
- Ezendam J, Van Loveren H (2006). Probiotics: immunomodulation and evaluation of safety and efficacy. Nutr. Rev. 64:1-14.
- Gill HS (2003). Probiotics to enhance anti-infective defences in the gastrointestinal tract. Best Pract. Res. Clin. Gastroenterol. 17(5):755-773.
- Gilliland SE, Walker DK (1990). Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. J. Dairy Sci. 73:905-911.
- Guo L, Yang L, Huo G (2011). Cholesterol removal by *Lactobacillus plantarum* isolated from homemade fermented cream in inner Mongolia of China. Czech J. Food. Sci. 29:219-225.
- Guo L, Li T, Tang Y, Yang L, Huo G (2016). Probiotic properties of Enterococcus strains isolated from traditional naturally fermented cream in China. Microb. Biotechnol. 9: 737-745.
- Hu X, Wang T, Li W, Jin F, Wang L (2013). Effects of NS *Lactobacillus* strains on lipid metabolism of rats fed a high-cholesterol diet. Lipids Health Dis. 12:67.
- Huang Y, Wang X, Wang J, Wu F, Sui Y, Yang L, Wang Z (2013). *Lactobacillus plantarum* strains as potential probiotic cultures with cholesterol-lowering activity. J. Dairy Sci. 96:2746-2753.

- Kimoto H, Ohmomo S, Okamoto T (2002). Cholesterol removal from media by *Lactococci*. J. Dairy Sci. 85:3182-3188.
- Kovalenko NK, Kasumova SA, Muchnik FV (2004). Screening of the strains of lactic acid bacteria possessing hypocholesterinemic activity and their practical use. J. Mikrobiol. 66:33-42.
- Kozaki M, Uchimura T, Okada S (1992). Experimental manual for lactic acid bacteria. Asakurasyoten, Tokyo, Japan pp. 29-72.
- Kumar A, Kumar M, Ghosh M, Ganguli A (2013). Modeling in vitro cholesterol reduction in relation to growth of probiotic *Lactobacillus casei*. Microbiol. Immunol. 57:100-110.
- Lekha PK, Lonsane BK (1997). Production and application of tannin acyl hydrolase: State of the art. Adv. Appl. Microbiol. 44:215-260.
- Liu ZQ, Shan HY (2006). Cholesterol, not polyunsaturated fatty acids, is target molecule in oxidation induced by reactive oxygen species in membrane of human erythrocytes. J. Cell. Biochem. Biophys. 45:185-193.
- Lye HS, Rusul G, Liong MT (2010). Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol. J. Dairy Sci. 93:1383-1392.
- Maassen CB, van-Holten-Neelen C, Balk F, den Bak-Glashouwer MJ, Leer RJ, Laman JD, Boersma WJ, Claassen E (2000). Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains. Vaccine 18:2613-2623.
- MacLachlan J, Wotherspoon ATL, Ansell RO, Brooks CJW (2000). Cholesterol oxidase: sources, physical properties and analytical applications. J. Steroid Biochem. Mol. Biol. 72:169-195.
- Motteran L, Pilone MS, Molla G, Ghisla S, Pollegioni L (2001). Cholesterol oxidase from *Brevibacterium sterolicum* the Relationship between covalent flavinylation and redox properties. J. Biol. Chem. 276:18024-18030.
- Naidu AS, Bidlack WR, Clemens RA (1999). Probiotic spectra of lactic acid bacteria (LAB). Crit. Rev. Food. Sci. Nutr. 39:13-126.
- Ngongang EFT, Tiencheu B, Achidi AU, Fossi BT, Shinyuy DM, Womeni HM, François ZN (2016). Effects of Probiotic Bacteria from Yogurt on Enzyme and Serum Cholesterol Levels of Experimentally Induced Hyperlipidemic Wistar Albino Rats. Am. J. Biol. Life Sci. 4:48-55.
- Niv E, Naftali T, Hallak R, Vaisman N (2005). The efficacy of *Lactobacillus reuteri* ATCC 55730 in the treatment of patients with irritable bowel syndrome- a double blind, placebo-controlled, randomized study. Clin. Nutr. 24:925-931.
- Ouwehand AC, Salminen SJ, Isolauri E (2002). Probiotics: an overview of beneficial effects. Antonie. van Leeuwenhoek 82:279-289.
- Ouwehand AC, Vesterlund S (2004). Antimicrobial components from lactic acid bacteria. In: S. Salminen, von Wright A, Ouwehand A. (Ed.), Lactic Acid Bacteria: Microbiological and Functional Aspects. New York: Marcel Dekker, Inc. 139:375-396.
- Pan DD, Zeng XQ, Yan YT (2011). Characterisation of *Lactobacillus fermentum* SM-7 isolated from koumiss, a potential probiotic bacterium with cholesterol lowering effects. J. Sci. Food. Agric. 91:512-518.
- Pereira DI, Gibson GR (2002). Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. Crit. Rev. Biochem. Mol. Biol. 37:259-281.
- Pridham TG, Gottlieb D (1948). The utilization of carbon compounds by some actinomycetales as an aid for species determination. J. Bacteriol. 56:107-114.
- Rigotti A, Miettinen HE, Krieger M (2003). The role of the high-density lipoprotein receptor sr-bi in the lipid metabolism of endocrine and other tissues. Endocr. Rev. 24:357-387.
- Roukas T (1993). Ethanol production from carbopods by *Saccharomyces cerevisiae*. Food Biotechnol. 41:296-301.
- Rudel II, Morris Md (1973). Determination of cholesterol using ophthalmaldehyde. J. Lipid Res. 14:364-366.
- Russell DW (2003). The enzymes, regulation and genetics of bile acids synthesis. Annu. Rev. Biochem. 72:137-174.
- Saarela M, Mogensen G, Fonden R, Ma'tto J, Mattila-Sandholm T (2000). Probiotic bacteria: safety, functional and technological properties. J. Biotechnol. 84:197-215.
- Said-AL Ahi HAH, Omer EA (2016). Essential oil content and chemical composition of eight dill (*Anethum Geraveolens* L) cultivars cultivated under Egyptian conditions. Int. J. Pharm. Pharm. Sci. 8:227-231.
- Saranya S, Shekinah S, Rajagopal T, Vijayakumar J, Ponmanickam P (2014). Isolation and characterization of cholesterol degrading bacteria from soap and vegetable oil industrial waste. Indan. J. Biotechnol. 13:508-513.
- Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM (2005). Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol. 16:201-211.
- Shen Z, Corbin DR, Greenplate JT, Grebenok RJ, Galbraith DW, Purcell JP (1997). Studies on the mode of action of cholesterol oxidase on insect midgut membranes. Arch. Insect. Biochem. Physiol. 34:429-442.
- Sieladie DV, Zambou NF, Kaktcham PM, Cresci A, Fonteh F (2011). Probiotic properties of lactobacilli strains isolated from raw cow milk in the western highlands of Cameroon. Innov. Rom. Food. Biotechnol. 9:12-28.
- Sih CJ, Tai HH, Tsong Y (1967). The Mechanism of Microbial Conversion of cholesterol into 17-Keto Steroids. J. Am. Chem. Soc. 89:1957-1958.
- Sojo MM, Bru RR, Garcia FF (2002). *Rhodococcus erythropolis* ATCC 25544 as a suitable source of cholesterol oxidase: cell-linked and extracellular enzyme synthesis, purification and concentration. BMC Biotechnol. 2:1-9
- Suzuki K, Bruce WR, Baptista J, Furrer R, Vaughan DJ, Krepinsky JJ (1986). Formation of cholest-4-en-3-one by intestinal bacteria and its biological effect. Cancer Lett. 33:307-316.
- Trautwein EA, Rieckhoff D, Erbersdobler HF (1998). Dietary inulin lowers plasma cholesterol and triacylglycerol and alters biliary bile acid profile in hamsters. J. Nutr. 128:1937-1943.
- Tsai CC, Chou LC, Lai SE, Huang CC (2016). Effect of cholesterol lowering multiplex lactic acid bacteria on lipid metabolism in a hamster model. Afr. J. Microbiol. Res. 10(20):708-716.
- Vanderhoof JA, Young RJ (1998). Use of probiotics in childhood gastrointestinal disorders. J. Pediatr. Gastroenterol. Nutr. 27:323-332.
- Wang J, Zhang H, Chen X, Chen Y, Menghebilige Bao Q (2012). Selection of potential probiotic lactobacilli for cholesterol-lowering properties and their effect on cholesterol metabolism in rats fed a high-lipid diet. J. Dairy Sci. 95:1645-1654.
- WHO (2013). Cardiovascular Disease fact sheet No. 317, <http://www.who.int/mediacentre/factsheets/fs317/en/>.
- Wittenberg R (1964). Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. 35:13-14.
- Yazdi MT, Zahraei M, Aghaepour K, Kamranpour N (2001). Purification and partial characterization of a cholesterol oxidase from *Streptomyces fradiae*. Enzyme Microb. Technol. 28:410-414.
- Yehia HM, Hassanein WA, Ibraheim SM (2015). Purification and characterization of the extracellular cholesterol oxidase enzyme from *Enterococcus hirae*. BMC Microbiol. 15:178-190.
- Young DS (2001). Effects of diseases on clinical lab. Tests. 4th ed. Washington, D.C: AACC Press.