

Full Length Research Paper

Cholesterol reduction by *Lactococcus lactis* KF147

Wesam A. Hassanein*, Nadia M. Awny and Shima M. Ibraheim

Department of Botany (Microbiology), Faculty of Science, Zagazig University, Zagazig, Egypt.

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Lactococcus lactis from yoghurt sample was confirmed based on alignment of 16S rRNA gene sequence in gene bank. The degree of acid and bile tolerance of strain *L. lactis* subsp. *lactis* KF147 was evaluated. The *L. lactis* KF147 was able to maintain viability for 2 h at pH 2 and growth in a medium with 0.4% (w/v) of bile acids. The maximum cholesterol reduction was 66.8% from the mMRS culture medium supplemented with 100 mg/L cholesterol. The tested strain was capable of binding cholesterol and removing 13% cholesterol from the medium. The growth of bacteria was affected by the presence of cholesterol in the medium. The growing bacterial cells had cholesterol reducing activity more than resting and dead cells. Transmission electron microscopy and IR analysis detected the cholesterol binding activity between *L. lactis* KF147 cells and the cholesterol. The fatty acid composition of the cells of *L. lactis* KF147 was altered by reducing cholesterol from the MRS medium that indicated incorporation of the cholesterol particles into cell membrane. According to these results, *L. lactis* KF147 revealed the probiotic potential in hypocholesterolemic effect on rats.

Key words: Cholesterol reduction, cholesterol binding, probiotic, hypocholesterolemic, *Lactococcus lactis* KF147.

INTRODUCTION

Cholesterol is a kind of steroid, found particularly in animal cell membranes (Paniangvait et al., 1995), and circulates in blood as a component of lipoproteins (Bansal et al., 2005). The majority of cholesterol synthesized from the liver (Kim et al., 2003), and the most intestinal cholesterol comes from diet, bile and intestinal secretions (Levy et al., 2007).

Cholesterol reduction by microorganisms takes place by different mechanical bioactivities which include cholesterol binding to cell walls of probiotics (Liong and Shah, 2005a), co-precipitation of cholesterol with deconjugated bile (Liong and Shah, 2006), production of short chain fatty acids upon fermentation by probiotics in the presence of prebiotics (De Preter et al., 2007), enzymatic deconjugation of bile acids by bile-salt hydrolase of probiotics (Lambert et al., 2008), incorporation of cholesterol into the cellular membranes of probiotics during growth (Lye et al., 2010a) and

conversion of cholesterol into coprostanol (Lye et al., 2010b).

Some microorganisms assimilated cholesterol and are able to grow on cholesterol as a sole carbon source. The non-pathogenic and pathogenic *Mycobacteria* (Gay and Sobouti, 2000) and different strains of lactic acid bacteria belonging to *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Leuconostoc* genera (Kovalenko et al. 2004) used cholesterol as carbon source.

Probiotics have been considered to have potential health-promoting benefits as biotherapeutic agents or a biological hypocholesterolemic agents (Park et al., 2007), and have cholesterol lowering effects in aqueous system as liquid media (Saavedra et al., 2004), blood of humans (Park et al., 2007) and rats (Nguyen et al., 2007).

Pereira and Gibson (2002b) found that, *Bifidobacterium infantis*, *Streptococcus bovis*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus faecalis*,

*Corresponding author. E-mail: wesamtarek2@yahoo.com.

Enterococcus faecium, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus pentosus* and *Lactobacillus acidophilus* N5, had *in vitro* cholesterol reduction abilities.

Prebiotics defined as non-digestible feed ingredients (Prado et al., 2008), are utilized by the intestinal microbial population to produce short-chain fatty acids which may lead to the reduced incidence of cardiovascular diseases (Dewailly et al., 2001) and improvement of lipid profiles (Wolever et al., 2002).

The objectives of this work were to study the ability of *L. lactis* KF147 for the bile and acid tolerance and their cholesterol reduction in the liquid media, study the fatty acid configuration, IR analysis and electron microscopy of *L. lactis* KF147 growing with and without cholesterol. Also, application of cholesterol reduction on rats was studied.

MATERIALS AND METHODS

Isolation and identification of cholesterol reducing bacteria

L. lactis KF147 was isolated from yoghurt sample on De Man, Rogosa and Sharpe (MRS) medium under anaerobic conditions. The composition of the MRS broth was (g/l): peptone, 10.0; yeast extract, 4.0; Tween 80, 1 ml; K₂HPO₄, 2.0; triammonium citrate, 2.0; sodium acetate, 3.0; MgSO₄-7H₂O, 0.2; MnSO₄-H₂O, 0.04; glucose, 10.0 (De Man et al., 1960). The isolate was confirmed for the identification based on alignment of 16S rRNA gene sequence available in gene bank.

Bile tolerance

L. lactis KF147 was evaluated for rapidity of growth in a MRS broth medium supplemented with and without bile acids according to Pereira and Gibson (2002a). Overnight cultures were inoculated 1% (v/v) into mMRS (modified MRS) broth containing 0.2 and 0.4% (w/v) oxgall and incubated anaerobically at 37°C for 12 h. Cultures were monitored each hour for growth using spectrophotometer at 650 nm. Comparison of cultures was based on their growth rates in each broth.

Acid tolerance

Overnight cultures of *L. lactis* KF147 were inoculated 1% (v/v) into MRS broth previously adjusted to pH 2.0 with HCl according to Pereira and Gibson (2002a). The mixtures were incubated anaerobically at 37°C for 2 h. One-milliliter samples were taken at various times (0, 15, 30, 45, 60, and 120 min), and added to 9 ml of half-strength peptone water plus 0.5 g of L-cysteine HCl /l, pH 7.0. 0.1 ml of each dilution was spread on the surface of plates containing MRS cholesterol agar medium. The plates were incubated at 37°C for 24 h under anaerobic conditions before enumeration.

The rate of bile and acid tolerance was calculated according to Xiao et al. (2003) as follows:

$$\text{Tolerance rate} = \frac{[(\text{CFU/ml}) / (\text{CFU/ml})_{\text{control}}] \times 100}{}$$

Cholesterol reduction

Bacterial liquid culture grown in mMRS with 100 mg/L cholesterol at

37°C for 24 h under anaerobic conditions was prepared. After centrifugation, quantitative determination of cholesterol was measured colourimetrically using Liebrman-Burchard reaction (Tietz, 1987).

The residual cholesterol was calculated according to the equation of Agerbaek and Richelsen (1995) as follows:

$$\text{Cholesterol (\%)/g (dry weight)} = \left[\frac{B - T}{B} \times 100 \right] / w$$

Where B is cholesterol content in the uninoculated control (mg/l), T is cholesterol content in the culture media (mg/l) and W is cells dry weight (grams) after 12 h of incubation.

Bacterial growth

This experiment was carried out to investigate the effect of cholesterol on the growth of *L. lactis* KF147 according to Liong and Shah (2005a). Aliquots (5 ml) were taken from bacterial cultures of 24 h age (cultivated in mMRS cholesterol liquid medium supplemented with 0.3% oxgall) every 2 h during culturing. Then, the bacterial growth was measured at 600 nm with a Spectronic 20 spectrophotometer and the percentage of cholesterol reduction was calculated as mentioned previously.

Cholesterol-binding activity

The cholesterol binding activity was estimated according to Pato et al. (2005). Sheep serum were added to MRS broth containing 2% sodium thioglycolate and 0.3% oxgall (MRSO) to obtain a concentration of 100 µg/ml cholesterol. Five milliliters of MRS broth was inoculated with 100 ml of active culture. The cholesterol binding ability was estimated by the following formula:

$$A = 100 - [(B/C) \times 100]$$

Where A: binding of cholesterol (%), B: cholesterol (mg/100 ml) in the supernatant of the inoculated MRSO broth, C: cholesterol (mg/100 ml) in the supernatant of MRSO broth without inoculation (control).

Electron microscopy

The bacteria cultivated in MRS broth with 100 mg/l cholesterol and without cholesterol were prepared for electron microscopy according to Lorian (1986). The bacterial cells were fixed (3% glutaldehyde), dehydrated (50% to absolute alcohol), embedded with epoxy, polymerized in oven at 60°C for 48 h and stained with 0.5% uranyl acetate followed by 0.4% lead acetate. Sections were examined and photographed using the transmitted electron microscope (Joel-JEM-100-CX).

IR spectroscopy analysis

This experiment was carried out to determine the functional groups of bacterial cell wall binding to cholesterol molecules. The bacteria cultivated in MRS broth with and without cholesterol were centrifuged, washed and prepared for IR (Infrared) analysis according to Kleiner et al. (2002).

Table 1. Experimental animal feeding design.

Group	Diet
ND	Normal diet (control).
HD	Hypercholesterol diet (control).
HDY	Hypercholesterol diet + yoghurt inoculated with <i>L. lactis</i> KF147.
HDMD	Hypercholesterol diet + milk + dead cells of <i>L. lactis</i> KF147.
HDYG	Hypercholesterol diet + yoghurt inoculated with <i>L. lactis</i> KF147 + glucose.

Effect of cholesterol on cellular fatty acid composition

This experiment was carried out to determine change in bacterial fatty acid composition as a result of cholesterol incorporation. Cellular lipids were extracted by a modified method of Liong and Shah (2005a). The fatty acids were estimated as methyl ester using the Hewlett-Packard of gas chromatograph (GC) (HP5890).

Cell state

The method used was modified from Marculescu et al. (2005). Cholesterol reduction by growing, resting and dead cells of *L. lactis* KF147 was expressed in dry weight to obtain uniformity in all treatments. The following equation was used:

$$\text{Cholesterol assimilation} = (C1 - C2) / (W2 - W1)$$

Where C1 and C2 were the amount of cholesterol present in the fermentation broths at time = 0 and 24 h, respectively, and W1 and W2 were the dry weight of the individual culture at time = 0 and 24 h, respectively.

Animal experiment

Thirty male albino rats (weight range was 102 to 110 g) were obtained from National Research Center, Giza, Egypt. The animals were divided into five homogeneous groups, each group contained six animals as Table 1 and housed individually in stainless steel cages fitted with a wire mesh bottoms and maintained on a 12 h light-dark cycle. Room temperature was controlled at 25 to 30°C with about 50% relative humidity. Cholesterol and oxgall was added at concentration of 5.0 and 0.5 g/kg diet according to Park et al. (2007).

During the feeding schedule, body weight gain, feed intake and feed efficiency ratio were determined weekly using an electrical digital balance. At the end of the experimental period, all rats were anesthetized with diethyl ether, after an overnight fasting, through inhalation (Portugal et al., 2006). Feed efficiency rate (FER) was calculated according to Kumar et al. (2010) the follows:

$$\text{FER} = \text{Weight gain (g)} / \text{feed intake (g)} \times 100$$

The suspension of bacterial culture was prepared (10^9 CFU/ml) daily for feeding the rats according to Pavan et al. (2003). Also, the yoghurt was prepared according to James et al. (1999) using *L. lactis* KF147 starter.

Blood samples

Blood samples were collected according to Oser (1965). Plasma total cholesterol (Young, 2001), plasma high density lipoprotein cholesterol (HDL) (Friedman and Young, 1997), plasma triglyceride

(Young, 2001), low density lipoprotein-cholesterol (LDL) (Bergmenyer, 1985) and total bile acid (Qureshi et al., 1984) were determined by using enzymatic colorimetric method kits.

Atherogenic index (AI) in serum was calculated according to Igarashi et al. (1997) by the following formula: AI = Total cholesterol - HDL/HDL.

Feecal samples

During the last week, faeces were collected from different experimental groups and dried to a constant weight at 55°C, weighted, then stored at refrigerator until analysis.

Feecal total bile acids were determined by the methods of Hishimoto et al. (1999) with some modification. Dried faeces (0.05 g) were extracted twice with 3.5 ml ethanol at 80°C for 1 h. After two extractions, the ethanol was evaporated under N₂ gas at 50°C, and the residue was dissolved in 2.5 ml ethanol. The amounts of total bile acids were analyzed with commercial test kit.

Statistical analysis

Recorded data were subjected to the statically analysis of variance according to Snedcor and Cochran (1980), and means separation was done according to Duncan (1955) at 5% level. Results were expressed as mean ± SEM and mean having the same alphabetical letter(s) within each column were not significantly different according to Duncan's multiple ranges test at 5% level.

RESULTS

The tested organism was confirmed for the identification based on alignment of 16S rRNA gene sequence available in gene bank. BLAST (Basic Local Alignment Search Tool) search indicated that, the tested *L. lactis* showed 99% identify to *L. lactis* subsp. *lactis* KF147 strain (accession number 013656.1).

Acid and bile tolerance

Table 2 represents the effect of bile salt concentrations on the viability of *L. lactis* KF147. The results indicated that, the bile tolerance rate at 0.2, 0.4 and 0.6% bile concentrations were 87.61, 94.56 and 74.7%, when respectively compared with the control sample (without bile salt). Also, Table 3 indicates that, the acid tolerance rate of *L. lactis* KF147 at pH 2.0 and 3.0, were 84.0 and 70.0%, respectively when compared with the control (6.8 pH).

Table 2. Effect of bile salt concentration on the viability of *L. lactis* KF147.

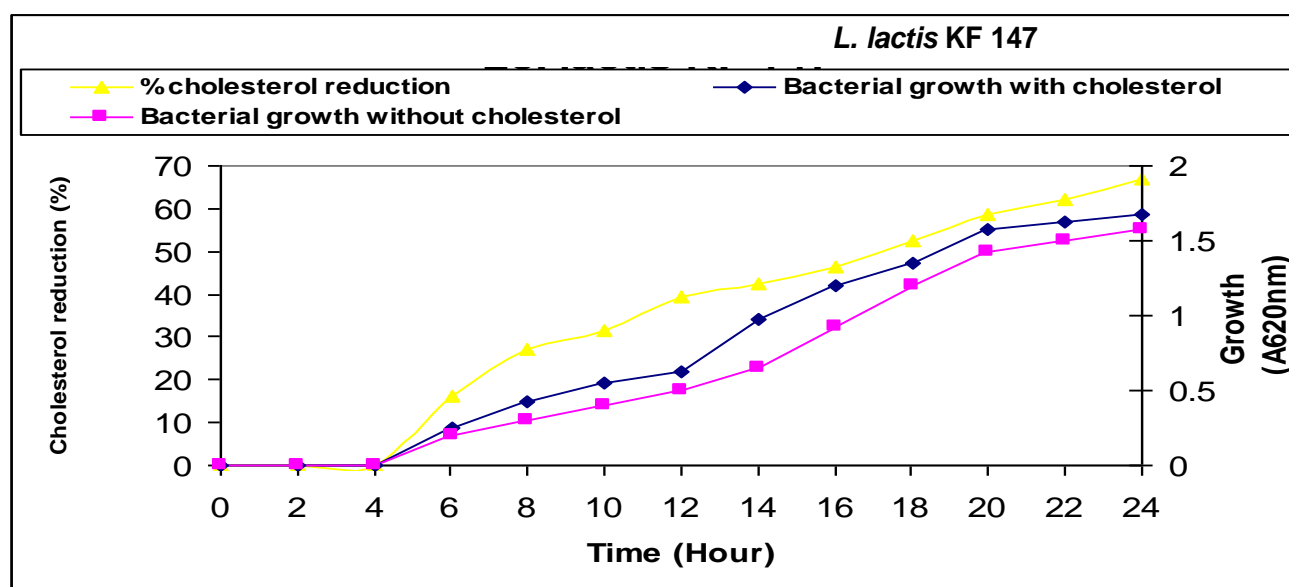
Bile concentration (%)	Bacterial counts expressed as Log ¹⁰ CFU/ml			BTR
	Incubation time (h)			
	0	4	8	
0.0	9.5 ± 0.50 ^{abcde}	10.2 ± 0.20 ^{abc}	10.5 ± 0.40 ^a	100
0.2	9.7 ± 0.70 ^{abcd}	9.5 ± 0.50 ^{abcde}	9.2 ± 0.40 ^{cdef}	87.61
0.4	9.4 ± 0.20 ^{bcde}	9.0 ± 1.00 ^{def}	8.7 ± 0.70 ^{def}	94.56
0.6	9.3 ± 0.30 ^{bcde}	8.2 ± 0.20 ^f	6.5 ± 0.50 ^g	74.7

BTR = Bile tolerance rate.

Table 3. Effect of acidity on the viability of *L. lactis* KF147.

pH value	Bacterial counts expressed as Log ¹⁰ CFU/ml					ATR
	Incubation time (h)					
	0	2	4	6	8	
2.0	7.2 ± 0.30 ^{gh}	7.0 ± 0.50 ^h	6.9 ± 0.60 ^{hi}	6.5 ± 0.40 ^{ij}	6.3 ± 0.20 ^j	84.0
3.0	8.5 ± 0.40 ^d	8.4 ± 0.10 ^d	8.1 ± 0.20 ^{de}	7.8 ± 0.50 ^{ef}	7.5 ± 0.40 ^{f^g}	70.09
6.8	9.4 ± 0.20 ^c	9.7 ± 0.30 ^c	10.2 ± 0.10 ^b	10.5 ± 0.30 ^{ab}	10.7 ± 0.60 ^a	100

ATR= Acid tolerance rate.

**Figure 1.** Effect of cholesterol on the growth of *L. lactis* KF147.

Cholesterol reduction

L. lactis KF147 was assayed for the reduction of cholesterol added in the liquid media. Residual and reduced cholesterol as well as percentage of cholesterol reduction were determined. The results indicated that, the cholesterol reduction was 66.8%.

Bacterial growth

The growth of *L. lactis* KF147 cultivated in MRS supplemented with 100 mg/l cholesterol and without cholesterol for different times is represented in Figure 1. The result indicated that, the O.D. of bacterial suspension at 620 nm after 24 h was 1.67 in the presence of cholesterol, while

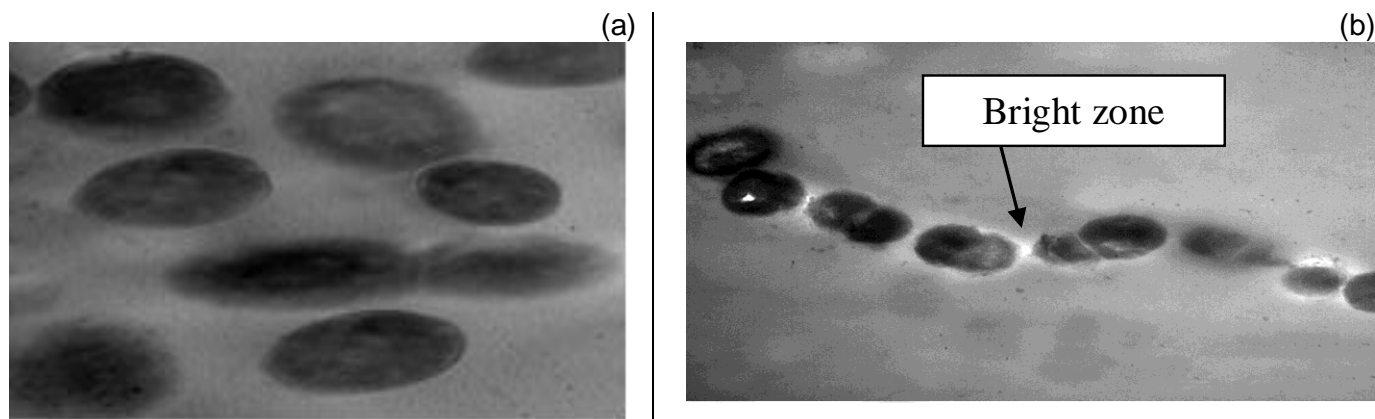


Figure 2. Transmission electron microscopy photo of *L. lactis* KF147 cells (a): Cells cultivated in the absence of cholesterol (20000 xs), (b) Cells cultivated in the presence of cholesterol (20000 xs).

the value decreased to 1.58 in the absence of cholesterol.

Cholesterol-binding activity

The result indicated the capability of *L. lactis* KF147 to bind to cholesterol and remove 13% cholesterol from the broth medium.

Electron microscopy

Electron microscopy study showed in Figure 2 indicated that, there was bright zone around the cells growing with cholesterol (Figure 2b) when compared with the cells cultivated without cholesterol (control) (Figure 2a). Also, non symmetrical cell division and reduction in cell size was observed in presence of cholesterol.

IR spectroscopy analysis

The mechanism of bonding functional groups between cholesterol and strain *L. lactis* KF147 was studied using IR analysis. The IR spectra in the range of 400 to 4000 cm^{-1} revealed characteristic binding peaks assigned to cholesterol and *L. lactis* KF147 biomass are presented in Figure 3a and b. It appeared that most obvious changes in the intensity for *L. lactis* KF147 biomass grown with cholesterol were detected in hydroxyl OH and amide stretching (N-H) groups and (P-H) stretching where intensity increased causing highly positive shift by 91.0 and 77.6%, respectively, as compared to *L. lactis* KF147 control. Also, there was moderate positive shift by 36.17 and 21.81% for (C=C) and (N-H) bonded to amide II, respectively. While slight positive shift 10.7 and 15.5% was for (COOR) ester group and (S=O) sulfamide bonds, respectively (Table 4).

Effect of cholesterol on cellular fatty acid composition

Fatty acid composition of *L. lactis* KF147 cells is shown in Table 5. It was found that, among unsaturated fatty acids, (C_{24:1}) represented the highest percentage in the presence or absence of cholesterol in culture medium followed by (C_{24:0}). In the presence of cholesterol, (C_{8:0}), (C_{14:1}), (C_{15:1}), (C_{16:1}) and (C_{18:2}) were lost after the cell analysis. While, the lower percentage of fatty acids were for (C_{20:2}) and (C₁₈). Total saturated (SFA) and unsaturated (UFA) fatty acids of the cells grown without cholesterol were 51.18 and 43.82%, respectively. Their percentages were increased to 53.2 and 60.87%, respectively after adding cholesterol to the medium.

Cell state

The result in Figure 4 shows that, the highest microbial mass and the percentage of cholesterol reduction were achieved when *L. lactis* KF147 active growing cells (control) were cultivated followed by resting cells (inactive cells) then dead cells (heat-killed cells). The percentages of cholesterol reduction were 65.3, 32.9 and 29.7%, respectively.

Rats body weight and feed efficiency

Concerning the animal experiments after 6 week of treatment, final body weight (160.7 g) of the normal diet (ND) was lower in comparison with other groups as shown in Table 6. The hypercholesterolaemic diet (HD) group animals, which were fed on cholesterol enriched diet, showed a relatively higher body weight (163.8 g) in comparison with other experimental groups. The highest body weight gain was recorded in the HDYG group

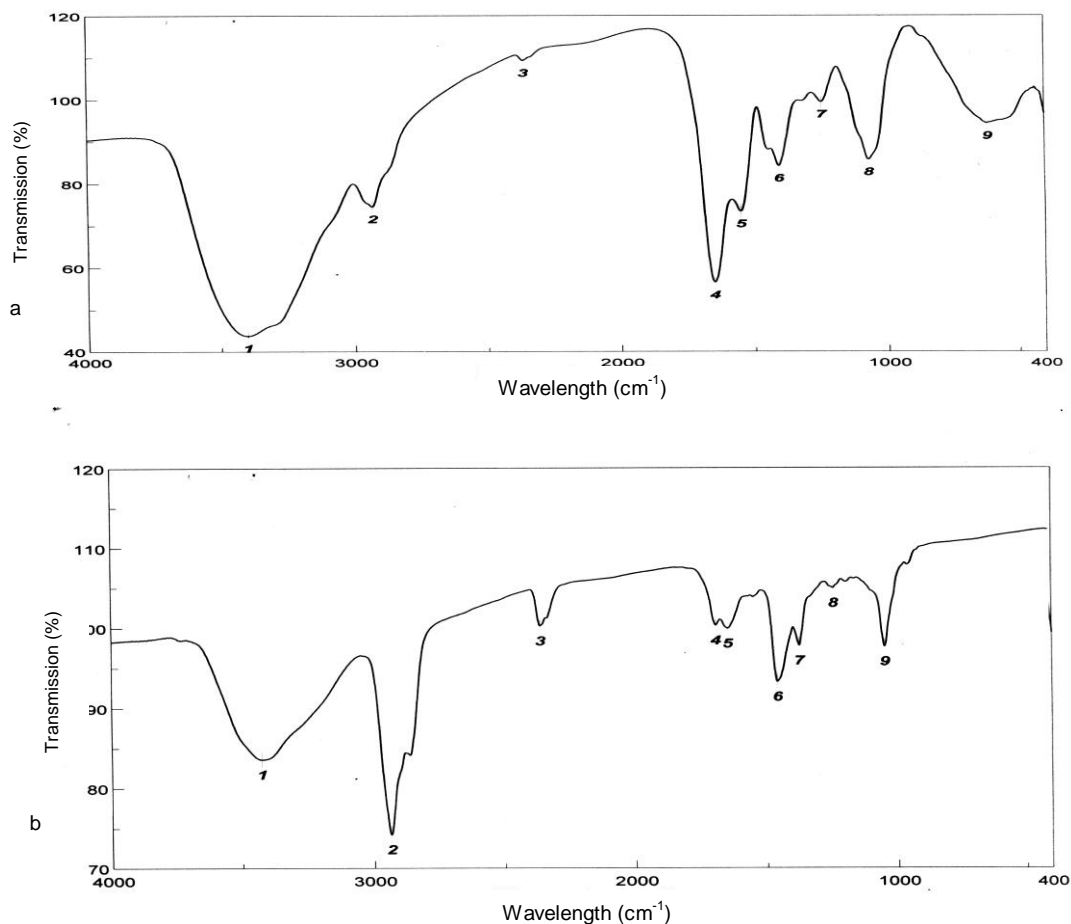


Figure 3. IR analysis of (a) the cells of *L.lactis* KF147 and (b) the cells of *L.lactis* KF147 grown with cholesterol, respectively.

Table 4. Functional groups of *L. lactis* KF147 non-loaded and loaded with cholesterol and the corresponding infrared absorption wave lengths.

IR label	Non-loaded biomass		Loaded biomass		Assignment
	Wave length (cm ⁻¹)	Intensity	Wave length (cm ⁻¹)	Intensity	
1	3401.82	43.74	3423.99	83.56	Bounded hydroxyl groups (OH ⁻) and primary and secondary amines and amides stretching (N-H) group
2	2933.2	74.47	2936.09	74.19	v (C-H) asymmetric stretching
3	2360.44	109.23	2360.44	100.23	v (C-H) symmetric stretching
4	1650.77	56.47	1691.27	100.29	v (P-H) stretching
5	1551.43	73.37	1645.95	99.91	v (C≡C) groups
6	1407.78	84.23	1457.92	93.26	v (COOR) Ester group
7	1247.72	99.43	1375	97.77	v (C=O) bonded to amide I
8	1072.23	85.74	1244.83	104.44	v (N-H) bonded to amide II
9	621.19	94.31	600.71	108.95	v (S=O) sulfamide bonds

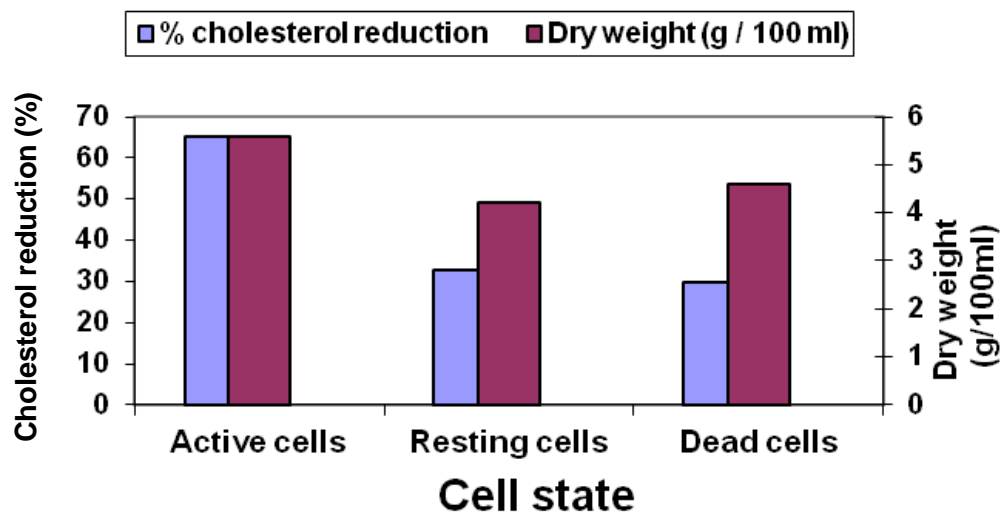
(196.7 g), while the body weight gain of HDMD group (166.7 g) was slightly lower than that of other groups. Similarly, feed intake was higher in HD group (465.8 g)

than in other groups and the ND group (410.3 g). The feed efficiency in rats fed HDYG group (23.37) was greater than those of the HDY (19.6) and HDMD (139) groups.

Table 5. Effect of cholesterol on fatty acids composition.

Fatty acid	Control without cholesterol	Treated with cholesterol
Caprylic C ₈	9.34 ± 1.30 ^a	ND
Capric C ₁₀	3.18 ± 0.10 ^a	2.87 ± 0.30 ^a
Lauric C ₁₂	4.8 ± 0.40 ^a	3.90 ± 0.20 ^b
Tridecylic C ₁₃	2.15 ± 0.10 ^a	3.12 ± 0.12 ^b
Myristic C ₁₄	3.26 ± 0.06 ^a	2.30 ± 0.10 ^b
Myristoleic C _{14:1}	1.42 ± 0.02 ^a	ND
Pentadecanoic C ₁₅	3.32 ± 0.30 ^a	2.37 ± 0.07 ^b
Cis-10-Pentadecanoic C _{15:1}	1.87 ± 0.17 ^a	ND
Palmitic C ₁₆	1.77 ± 0.27 ^a	5.04 ± 1.00 ^b
Palmitoleic C _{16:1}	2.98 ± 0.08 ^a	ND
Heptadecanoic C ₁₇	1.73 ± 0.13 ^a	1.95 ± 0.15 ^b
Cis-10- Heptadecanoic C _{17:1}	2.62 ± 0.02 ^a	3.98 ± 0.06 ^b
Stearic C ₁₈	4.93 ± 0.50 ^a	2.73 ± 0.10 ^b
Elaidic C _{18:1}	2.25 ± 0.20 ^a	17.7 ± 2.00 ^b
Linoleic C _{18:2}	2.88 ± 0.40 ^a	ND
Gamma-linolenic C _{18:3}	1.76 ± 0.20 ^a	1.98 ± 0.13 ^b
Arachidic C ₂₀	1.88 ± 0.07 ^a	1.76 ± 0.06 ^b
% saturated fatty acids (SFA)	51.18	43.82
% unsaturated fatty acids (UFA)	53.2	60.87

ND = Not detected.

**Figure 4.** Cholesterol reduction by active, resting and dead cells of *L.lactis* KF147.

Plasma lipid profile

The effect of dietary treatments on plasma lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) is recorded in Figure 5. The reduction of total cholesterol levels for HDY, HDMD and HDYG groups were 11.55, 7.82 and 10.58%, respectively as compared to the ND and HD which were 85.7 and 99.2 mg/dl, respectively. Also, the reduction in LDL-cholesterol

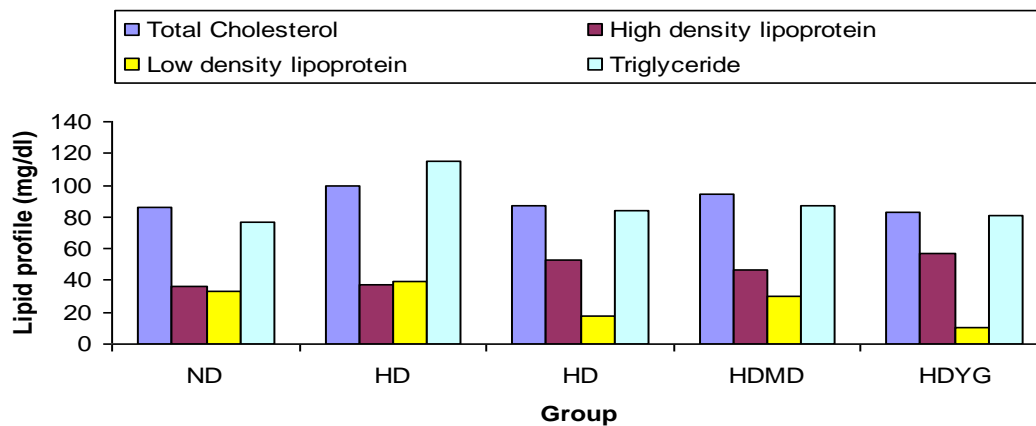
was 56.8, 40.0 and 63.18%, respectively when compared with HD (39.1 mg/dl) and ND (33.66 mg/dl) group. While, the increase in HDL-cholesterol values was 22.95, 30.81 and 11.83%, respectively when compared with the HD (37.2 mg/dl) and ND (36.7 mg/dl) group. While, the reduction in triglyceride values was 8.7, 5.81 and 8.02%, respectively when compared with the HD (114.8 mg/dl) and ND (76.7 mg/dl).

The reduction of serum total bile acid concentrations

Table 6. Effect of different feeding treatment on the body weight gain of rats.

Experimental group	Initial weight (g)	Final weight (g)	Body weight gain(g/6 weeks)	Feed intake (g/6 weeks)	FER
ND	110.4 ± 5.00 ^a	160.7 ± 5.70 ^c	50.3 ± 0.30 ^g	410.3 ± 10.00 ^c	12.26 ± 0.26 ^d
HD	108.2 ± 4.00 ^a	163.8 ± 1.40 ^c	55.6 ± 0.20 ^f	465.8 ± 4.00 ^a	11.94 ± 0.40 ^d
HDY	107.6 ± 3.00 ^a	190.0 ± 2.0 ^a	82.4 ± 0.30 ^b	420.5 ± 5.50 ^c	19.6 ± 0.60 ^b
HDMD	102.9 ± 1.45 ^a	166.7 ± 2.70 ^c	63.8 ± 0.80 ^d	456.2 ± 6.00 ^{ab}	13.9 ± 1.40 ^{cd}
HDYG	106.3 ± 3.00 ^a	196.7 ± 4.08 ^a	90.4 ± 0.40 ^a	386.8 ± 6.80 ^d	23.37 ± 1.00 ^a

Feed efficiency rate (FER) = weight gain (g)/feed intake (g) × 100.

**Figure 5.** Effect of different feeding treatment on serum lipid of rats.

were 28.1, 27.2 and 25.0% for HDY, HDMD and HDYG groups, respectively when compared with the HD and ND which were 4.3 and 3.5 ($\mu\text{mol/l}$) groups, respectively. On the contrast, the increase concentrations of fecal total bile acid were 2.8, 2.1 and 3.5 (mg/g of feces), respectively, when compared with ND and HD which were 1.4 and 1.2 (mg/g of feces) (Table 7). The AI of treatment groups decreased after 4 week feeding when compared with the control HD group (1.6). The recorded AI were 0.6, 1.0 and 1.24 for HDY, HDMD and HDYG groups, respectively.

DISCUSSION

Hypercholesterolemia is considered as a major risk factor for the development of coronary heart disease and stroke (Aloglu and Oner, 2006). Probiotics are viable microorganisms that establish beneficial effects on the health of the host when they are ingested (Kimoto et al., 2004). One of the beneficial health effects related to probiotics is their ability to reduce serum cholesterol levels.

L. lactis KF147 under investigation isolated from yoghurt sample had a potential to survive under bile and acid environment up to 0.4% bile and pH 2 for 8 and 2 h,

respectively. This is in harmony with the report of Kumer et al. (2010). They recorded that, different strains of *L. plantarum* tolerated 2% bile up to 76-23 for 3 h. In this relation, Morelli (2007) reported that, in the laboratory, acid and bile are added to the media in order to mimic conditions encountered in human gastrointestinal tract. *Lactobacillus paraplantarum* II 32 showed acid resistance and bile salt tolerance. This strain survived at pH 2 after 2 h culture and the living bacterial number could reach 10^4 CFU/ml. In addition, the growth of this strain delayed for less than 0.5 h in MRS broth with 0.3 to 0.4% bile salt, when the absorbance values both increased to 0.6 units (Liu et al., 2009).

The time from entrance to release from the stomach has been estimated to be approximately 90 min, with further digestive processes requiring longer residence time. Stresses for organisms begin in the stomach, with pH between 1.5 and 3.0, and in the upper intestine that contains bile (Corzo and Gilliland, 1999). Survival at pH 3.0 for 2 h and at a bile concentration of 1000 mg/l is considered optimal acid and bile tolerance for probiotic strains (Usman and Hosono, 1999).

Results of the present study indicated that the highest cholesterol reduction was 66.8% by *L. lactis* KF147. In this coincidence, the highest cholesterol reduction by *L. lactis* subsp. *lactis* bv. *diacetylactis* N7 and *L. plantarum*

Table 7. Effect of different tested feeding treatments on total bile acid in plasma ($\mu\text{mol/l}$) and in faeces (mg/g of faeces) of experimental rats.

Feeding treatment	Plasma total bile acid ($\mu\text{mol/l}$)	Total bile acid in faeces (mg/g of faeces)
ND	3.5 \pm 0.62 ^c	1.4 \pm 0.02 ^{ef}
HD	4.3 \pm 0.72 ^a	1.2 \pm 0.37 ^f
HDY	2.3 \pm 0.32 ^{de}	2.8 \pm 0.12 ^{bc}
HDYE	2.6 \pm 0.39 ^{cd}	2.6 \pm 0.22 ^c
HDMD	3.2 \pm 0.55 ^{bc}	2.1 \pm 0.01 ^d
HDYG	1.5 \pm 0.62 ^f	3.5 \pm 0.11 ^a

were 97.0 and 68-97% as recorded by Kimoto et al. (2002) and Kumer et al. (2010), respectively. Also *L. lactis* subsp. *lactis* bv. *diacetylactis* N7 achieved the highest cholesterol removal by recovery with the resuspended cells. Thus, cholesterol removed was not metabolically degraded (Kimoto et al., 2002).

Concerning the relationship between cholesterol reduction and bacterial growth, the result showed that the maximum percentage of cholesterol reduction by *L. lactis* KF147 was achieved at 24 h and the growth in the presence of cholesterol was higher than that without cholesterol. This may be due to, utilization of cholesterol by many lactic acid bacteria as carbon source stimulated their growth (Kovalenko et al., 2004). In this relation, Kimoto et al. (2002) found that, in the presence of cholesterol, Lactococci N7 reached a higher cell density than in cholesterol absence. The dry weight of the cells growing in the absence and presence of cholesterol was 219 and 544 mg/l, respectively. Also, Liang and Shah (2005b) observed that, cholesterol assimilation may be influenced by biomass levels.

The result of the present study represents the capability of *L. lactis* KF147 to bind cholesterol and remove 13% cholesterol from broth. This explained that, for high removal of cholesterol, cholesterol could be strongly attached to the cell surface (Tahri et al. 1995). In this relation, Pato et al. (2005) showed that, *L. lactis* subsp. *lactis* B-4 exhibited the highest cholesterol-binding ability by removing 15% cholesterol from media broth, while *L. lactis* subsp. *lactis* I-2775 showed the lowest activity (5%).

Moreover, electron microscopy and IR analysis were studied to increase the understanding about cholesterol binding activity of *L. lactis* KF147. According to the electron microscopy, there was bright zone around the cells of *L. lactis* KF147 grown with cholesterol, reduction in cell size, and non-symmetrical cell division when compared with the cells grown without cholesterol. The bright zone around the cells may be referred to the secretion of exopolysaccharides and it may have a role in cholesterol binding as reported by Tahri et al. (1995). They stated that some probiotics could produce exopolysaccharides which adhere to the cell surface and could absorb cholesterol. Role of extracted *L. lactis*

KF147 exopolysaccharides in cholesterol reduction will be studied in the future work.

Usman and Hosono (1999) reported that the differences between groups in their hypocholesterolemic effect are related to the mechanism of cholesterol binding to bacterial cell walls which varies between bacterial species. The binding differences of the different bacterial species are due to chemical and structural properties of their cell wall peptidoglycans and even dead cells may have the ability to bind cholesterol in the intestine.

With regards to infrared analysis, there was no previous literature to explain microbial cell surface groups by which cholesterol bind to them but IR analysis was used by Kirschner et al. (2001) in identification and classification of enterococci. In the present study, IR analysis indicated that, there was affinity between cholesterol and *L. lactis* KF147 biomass through OH, (P-H), (C \equiv C), (N-H) bonded to amide II, (S=O) sulfamide bonds and (COOR) ester groups which had positive shift by 91.0, 77.6, 36.17, 21.81, 15.52 and 10.72, respectively as compared to the control. This indicated an arrangement of functional groups on the surface of *L. lactis* KF147 cells.

Also, this may explain that the groups facilitated the binding between cholesterol molecules and cell surface. Tahri et al. (1995) reported that, cholesterol could be attached strongly to the cellular surface, where more than 40% of cholesterol was extracted from cells of *Bifidobacterium breve* ATCC 15700 only after several washings and sonication. On the other hand, Kimoto-Nira et al. (2007) suggested cholesterol was bound to bacterial cells and this was a result of the chemical and structural properties of their cell wall peptidoglycans, which contain various amino acid compositions that facilitate the attachment of cholesterol to cellular surfaces.

Regarding GC analysis, there was a difference in the fatty acid distribution pattern for *L. lactis* KF147 cells grown with or without cholesterol. Also, in the presence of cholesterol, total SFA and UFA were increased, suggesting that the fatty acids composition of the cells was altered because cholesterol was incorporated into the cellular membrane after its removal from the media (Kimoto et al., 2002).

The changes in the fatty acid composition may in turn cause a change in membrane fluidity (Fukushima and Nakano, 1996). The incorporation of cholesterol into the cellular membrane increased the concentration of saturated and unsaturated fatty acids, leading to increased membrane strength and subsequently higher cellular resistance toward lysis. This mechanism determines the possible locations of the incorporated cholesterol within the membrane phospholipid bilayer of probiotic cells. The authors incorporated fluorescence probes into the membrane bilayer of probiotic cells that were grown in the absence and presence of cholesterol. Enrichment of cholesterol was found in the regions of the phospholipid tails, upper phospholipids and polar heads of the cellular membrane phospholipid bilayer in cells that were grown in the presence of cholesterol as compared to the control cells, indicating incorporation of cholesterol in those regions (Lye et al., 2010a).

Moreover, ability of active, resting and dead cells of *L. lactis* KF147 to reduce cholesterol was studied. The result showed that, the microbial mass and the percentage of cholesterol reduction during growth (growing cells) was higher than that by dead cells (heat-killed cells) suggesting that the difference in the amount of cholesterol reduced between the heat killed cells and growing cells was due to the uptake of cholesterol by *L. lactis* KF147. Thus, it seems that *L. lactis* KF147 can remove cholesterol from media both by binding of cholesterol to dead cells and by the uptake of cholesterol into living cells during growth. This is in agreement with report of Liu et al. (2009). They showed that, cholesterol removed by dead and resting cells of *L. paraplantarum* II 32 were 5.64 and 5.90 (mg/g of dry weight), respectively when compared with growing cells, which was 16.98 mg/g of dry weight.

With regards to the animal experiments, the results indicated the effect of dietary cholesterol after 4 weeks on body weight and serum profile for HDY, HDMD and HDYG groups when compared with the HD and ND groups. It was found that there was increase in body weight of rats in all experimental groups. This in similarity to that of Chang et al. (2001) who observed an increase in daily weight gain and an improvement in feed conversion in pigs using a *Lactobacillus* supplement. The increase in the body weight in rats fed with diet containing the cultured dairy product may be due to the increase in the efficiency in nutrient utilization associated with the availability of more digestible protein. Also, feeding the rats with either of the yoghurt or probiotic yoghurt resulted in rapid decline of pathogenic coliforms, and significant increase in body weight gain (Christopher et al., 2006).

In the present study, total cholesterol, triglyceride, LDL and serum total bile level decreased and HDL cholesterol and fecal total bile increased in all the experimental groups of rats under test after the period of feeding when compared with the control groups (HD and ND). Also,

Kumer et al. (2010) indicated that, supplementation of the diet with *L. plantarum* Lp91 resulted in a significant reduction in plasma total cholesterol, LDL-cholesterol and TAG by 23.26, 38.13, and 21.09 %, respectively. Also, a significant enhancement of 18.94% in HDL-cholesterol in hypercholesterolaemic rats was observed.

L. acidophilus had the potential of reducing risk of coronary heart disease by 6 to 10% by reducing the serum cholesterol (James et al., 1999). Pereira and Gibson (2002b) found that, the hypercholesterolemia effect of probiotic bacteria are due to their ability to ferment food-derived indigestible carbohydrates to produce short-chain fatty acids in the gut, which can then cause a decrease in the systemic levels of blood lipids by inhibiting hepatic cholesterol synthesis and/or redistributing cholesterol from plasma to the liver.

Prebiotics are decreasing cholesterol absorption accompanied by enhancing cholesterol excretion via feces, and the production of short-chain fatty acids upon selective fermentation by intestinal bacterial microflora (Arjmandi et al., 1992), increase the viscosity of the digestive tract and increase the thickness of the unstirred layer in the small intestine, and thus inhibit the uptake of cholesterol (Dikeman et al., 2006). This may lead to a higher cholesterol catabolism in the liver that contributed to a hypocholesterolemic effect.

Also, the result of the present investigation show that the concentration of serum bile acid decreased. This is in agreement with the report of De Rodas et al. (1996). They found that, the serum total bile acid was decreased for pigs fed the diet with *L. acidophilus*. Also, the increase of fecal bile acid when compared with the control was detected previously with Xiao et al. (2003). They found that, the fecal total bile acid for all animal experiment groups was increased for the groups fed mixed culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and bifidobacterium milk.

Moreover, AI of treatment groups increased sharply after four weeks feeding on the hypercholesterolaemic diet in comparison with that recorded with the ND group. However, the AI of the probiotic treatment groups was found to decrease when compared with the HD group. This is similar to the report of Kumer et al. (2010) for strain *L. plantarum*. The AI reflects the balance between risk and protective lipoprotein forces and indicates its protective effects against cardiovascular diseases (Natarajan et al., 2003).

Conclusion

Since, reduction in serum cholesterol is associated with a 2 to 3% reduction in estimated risk for coronary heart disease; the reduction is associated with intake of fermented milk containing certain probiotic bacteria. *L. lactis* KF147 isolated from yoghurt was capable of surviving at pH 2 and bile (0.4%) mimic the stomach and the

environment of the intestine. The ability to reduce cholesterol from broth culture during growth was because of independence on whether cells were viable, dead or in resting state.

Transmission electron microscopy and the shift of functional groups on cell surface detected by IR analysis indicated the cholesterol binding activity between the tested *L. lactis* KF 147 and cholesterol. Change in fatty acid composition by reducing cholesterol from the medium indicated incorporation of the cholesterol particles into cell membrane of cells. Moreover, *L. lactis* KF147 had hypocholesterolemic effect on serum plasma profile of rat.

This research gave awareness about the link between diet and health and the fact that probiotic containing foods are generally perceived as "safe and natural". Thus, establishing the effective probiotic properties of Lactococcus bacteria which could be incorporated into dairy products or other functional foods, alternatively for high cost pharmaco-logical therapy.

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