Bile Salts and Acid Tolerance and Cholesterol Removal from Media by some Lactic Acid Bacteria and Bifidobacteria

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ABSTRACT. In this study three strains of *Lactobacillus acidophilus* (DSM 9126, DSM 20079 and DSM 20242), two strains of Bifidobacteria *(infantis* DSM 20088 and *angulatum* DSM 20098) and *Streptococcus thermophilus* DSM 20617 were tested for acid tolerance, bile salt tolerance, capability to remove cholesterol and to deconjugate sodium taurocholate from the culture medium. Results showed that a considerable variation existed among cultures in their growth viability in the presence of bile salt, deconjugation of sodium taurocholate and assimilation of cholesterol from the medium. Moreover, the two cultures of bifidobacteria *(infantis* DSM 20088 and *angulatum* DSM 20098) were shown to be the most bile salts tolerant culture. All bacterial strains tested in this study exhibited sensitivity to acidity at pH 2. However, increasing the pH of the medium to 3 had improved the acid tolerance of all strains except *Streptococcus thermophilus*. Addition of 1% skim milk powder into medium at pH 2 increased the viability of all strains especially bifidobacteria strains. All tested strains removed less cholesterol from the broth (ranged from 3.08-29.68%) compared to those grown in broth supplemented with 0.2% bile salts (from 36.07-55.43%). Furthermore, considerable amount of cholesterol was precipitated with cells obtained from broth enriched with 0.2% bile salts. *Lactobacillus acidophilus* DSM 20079 appeared to be more active in deconjugation of sodium taurocholate (2.38 μmol/ ml) compared to the other strains, as well as being able to remove up to 66.61 mg of cholesterol (95.6%) from the culture medium and therefore, is regarded as a suitable candidate probiotic and adjunct culture.

INTRODUCTION

High serum cholesterol concentration is associated with the development of coronary heart disease (Usman and Hosono, 2000). Mann and Spoerry (1974) claimed that the consumption of fermented milk with *Lactobacillus acidophilus* reduced serum cholesterol level in Massai trip. Since then, the hypocholesterolemic effect of fermented dairy product has been observed in feeding studies either using humans (Harrison and Peat, 1975) or animals (Liong and Shah, 2006 and Daneilson et al., 1989). Many studies have reported the ability of *Lactobacillus acidophilus* (Gilliland et al., 1985 and Usman and Hosono, 1999) and bifidobacteria (Dambekodi and Gilliland, 1998) to assimilate cholesterol from laboratory media. Thus, both types of bacteria may have the potential to reduce serum cholesterol in humans. However, the ability to assimilate cholesterol from the media varied significantly amongst different bacterial strains. Many attempts have been made to elucidate the mechanism involved in the hypocholesterolemic action of lactic acid bacterial strains. One proposed mechanism is the assimilation of cholesterol by the cell wall during growth (Buck and Gilliland, 1994; Noh et al., 1997). Another mechanism is the deconjugation of bile salts by bacteria producing bile salt hydrolase. Most conjugated bile salts are recirculated through the enterohepatic circulation, while deconjugated bile salts are less soluble and excreted in the feces. The bile salts that are excreted must be replaced by new bile salts, which are formed from cholesterol in the body. Thus, the more bile salts

excreted, the more cholesterol is removed from the body. Furthermore, deconjugated bile salts do not stimulate the absorption of cholesterol and other lipids from the small intestine as well as do conjugated bile salts. Walker and Gilliland (1993) reported that some strains of *Lactobacillus* can deconjugate bile salts. While, Hill and Drasar (1968) suggested that *Lactobacillus* is incapable of deconjugated bile salts.

This study was conducted to compare bile salts and acid tolerance, assimilation of cholesterol and deconjugation of bile salts by different strain of lactic acid bacteria and bifidobacteria and to choose the most beneficial strain as adjunct culture in fermented dairy product.

MATERIALS AND METHODS

Bacteria

Three strains of *Lactobacillus acidophilus* (DSM 9126, DSM 20079 and DSM 20242), two strains of *Bifidobacterium (infantis* DSM 20088 and *angulatum* DSM 20098*)* and one strain of *Streptococcus thermophilus* DSM 20617 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ, Germany). Stock cultures were stored in 40% glycerol at –20°C. The organisms were subcultured 3 times before use in sterile de Man, Rogosa, Sharpe (MRS) broth using 1% inoculum and incubation for 20 h at 37°C.

Acid Tolerance

Acid tolerance of the cultures was studied by incubating the organisms in MRS broth (in some experiments 1% skim milk powder was added to MRS broth). The pH was adjusted to 2.0 with HCl 1N and the cultures were incubated at 37°C for 3 h. Each of the bacterial strains was subcultured at least 3 times before experimental use MRS broth was inoculated (10% vol/vol) with bacterial strain, and growth was monitored using the plate count method as described by Pereira and Gibson (2002). A 1-mL sample was taken at zero, 1.5 and 3 h, and serial dilutions were made using peptone water diluent. Samples were plated onto MRS agar, and the plates were incubated at 37°C for 48 h in an anaerobic jar (Becton Dickinson Microbiology Systems, Sparks, MD) with a Gas Generating Kit (Oxoid, Ltd., Mitsubishi Gas Chemical Company) except for *Streptococcus thermophilus,* which was incubated under aerobic condition. Acid tolerance was determined by comparing the plate count after 1.5 and 3h with the initial plate count at 0 h (results were expressed as percentage). The experiments were repeated twice.

Bile salts tolerance

Growth rate of bacterial cultures was determined in MRS broth containing different levels (0, 0.1, 0.3, 0.5 and 0.7%) of bile salts (oxgall). Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 h under anaerobic condition, except for *Streptococcus thermophilus,* which was incubated under aerobic condition. Optical densities were measured spectro-photometrically at 620 nm after 0, 3, 5 and 24 h.

Cholesterol removal

Cholesterol solution (10 mg/ml in 96% ethyl alcohol) was prepared and filtered sterilized. For each culture to be tested, 70 µl of cholesterol solution was added to 10 of

MRS broth (final cholesterol concentration 70 µg/ml) containing 0.2% bile salts (oxgall) or not containing. To the MRS broth, 1% of freshly grown culture was added and incubated anaerobically at 37°C for 20 h except *Streptococcus thermophilus* which was incubated under aerobic condition. An uninoculated sample was used as control. After incubation the cells were removed by centrifugation at 10,000 g for 10 min at 4°C and cholesterol was determined in the supernatant using modified Rudel and Morris (1973) method in which three ml of supernatant, 2 ml of 33% (wt/vol) KOH and 3 ml 96% ethanol were placed in a capped test tube, vortexed for 20 second 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 milliliters of cholesterol liquicolor enzymatic kit (Human-Gesellschaft fur Biochemica und Diagnostica mbh-Wiesbaden-Germany) was added. The solution was mixed and left for 5-10 min at 37°C and absorbance was measured at 500 nm with a spectrophotometer (LKB Biochrome ultrospec 11, Campridge, England). The ability of bacterial strain to remove cholesterol from media was calculated as percentage from the following equation:

$$A=100-(B/C)*100$$

Where A=% of cholesterol removed, B=absorbance of the sample containing the cells and C=absorbance of the sample without cells.

To measure the cholesterol removed with the cells, pellet cells obtained by centrifugation was resuspended in distilled water to the original volume of the culture and cholesterol was determined as mentioned above. Cholesterol remained with the pellet was calculated from the equation:

$$A= (B/C)*100$$

Where A= Cholesterol remained with the pellet (as percentage), B= absorbance of the sample containing the cells and C=absorbance of the sample without cells. It was observed that, sample containing no cells has no pellet and cholesterol was determined in the whole system.

Deconjugation of bile salts

Deconjugation of bile salts by bacterial strains was tested qualitatively through the plate assay as described by Ahn et al., (2003). To MRS agar containing 0.5 g/l cysteine, 1 mM of sodium taurocholate (Sigma Chemical Co., USA) was added. After autoclaving and solidifying, the plates were incubated anaerobically for 48 h before use. The plates were inoculated with active culture (20 µl) and incubated for 72 h at 37°C. Precipitated cholic acid around colonies were observed. Deconjugation of bile salts was also measured quantitavely by measuring released cholic acid as described by Walker and Gilliland (1993).

RESULTS AND DISCUSSION

Acid tolerance

The effect of pH on the viability of strains is presented in Fig. 1. Results showed that, the viability of all tested bacterial strains markedly decreased on incubation at pH 2 for 1.5 h. *Streptococcus thermophilus* DSM 20617 and *Bifidobacterium ifantis* DSM 20088 were the most acid sensitive of all tested strains. These strains completely lost their viability after 1.5 h at pH 2. *Bifidobacterium angulatum* DSM 20098 retained about 26% of initial viability after 1.5 h compared to 8% for *Lactobacillus acidophilus* DSM 9126, 20079 and 20242. Addition of 1% of skim milk powder into MRS broth greatly improved acid tolerance of all strains, particularly bifidobacteria, which retained about 98 and 89% for *B. infantis* DSM 20088 and 95 and 42% for *B. angulatum* DSM 20098 after 1.5 and 2 h, respectively (Fig. 2). There were major differences between the viability of bacterial strains at pH 3 compared with that at pH 2. *Bifidobacterium infantis* DSM 2288 and *B. angulatum* DSM 20098 retained about 100% viability after 1.5 h and sharply decreased after 3 h. While *L. acidophilus* DSM 9126, 20079 and 20242 retained about 50, 63 and 47% viability after 1.5h and 9, 30 and 3% after 3h, respectively. However, *S.thermophilus* was the most acid intolerant strain (Fig. 3).

Bile salts tolerance

In order to exert a beneficial effect in the digestive tract, probiotic culture must survive passage through the stomach and be tolerant to the bile salts concentrations in the small intestine (Sanders, 2000). Results from the comparison of different cultures for bile salts tolerance are shown in Figs (4-9). All strains exhibited considerable variations with regard to growth in control broth after 24h. The optical densities of *Lactobacillus* strains and *Streptococcus* reached to about 1.6 while it was about 1.1 for Bifidobacteria strains. With addition of bile salts to broth, the growth of strains also varied considerably. Bifidobacteria strains appeared to be the most resistant to bile salts while, the optical densities of treated samples increased than those of control after 24 h incubation. Moreover, the concentration of 0.5% bile salts had the highest enhancement for growth rate of bifidobacteria strains (Figs. 8 and 9). However, the growth rate of *L. acidophilus* DSM 9126 and DSM 20242 was greatly affected with addition of bile salts (higher than 0.1%), where *L. acidophilus* DSM 20079 showed an increase in optical densities up to 0.7% after 24 h incubation. *Streptococcus thermophilus* DSM 20617 also exhibited bile salts tolerance up to 0.5% bile salts. This finding is in good agreement with that observed by Pereria and Gipson (2002).

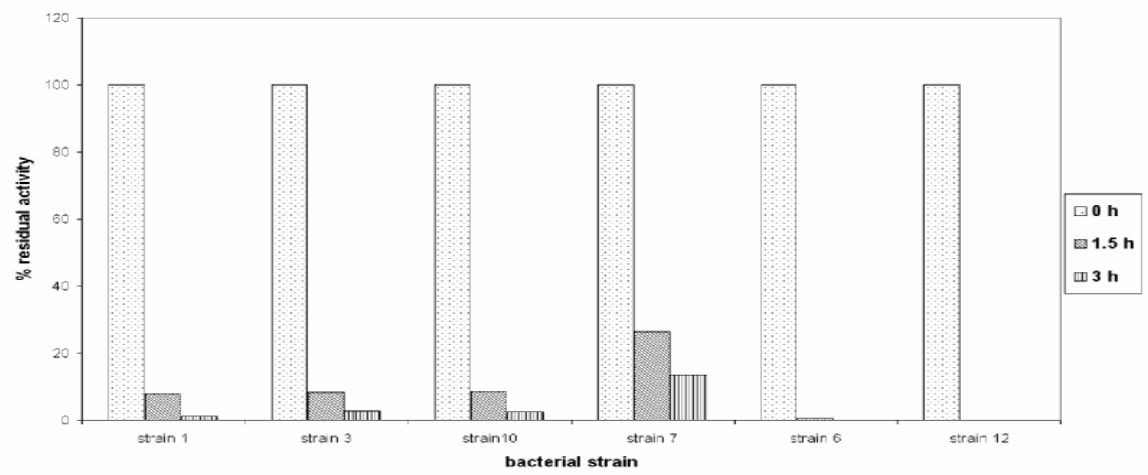

Fig. 1. Effect of pH on the viability of lactic acid bacteria grown in MRS broth (%residual activity at pH 2)

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098.

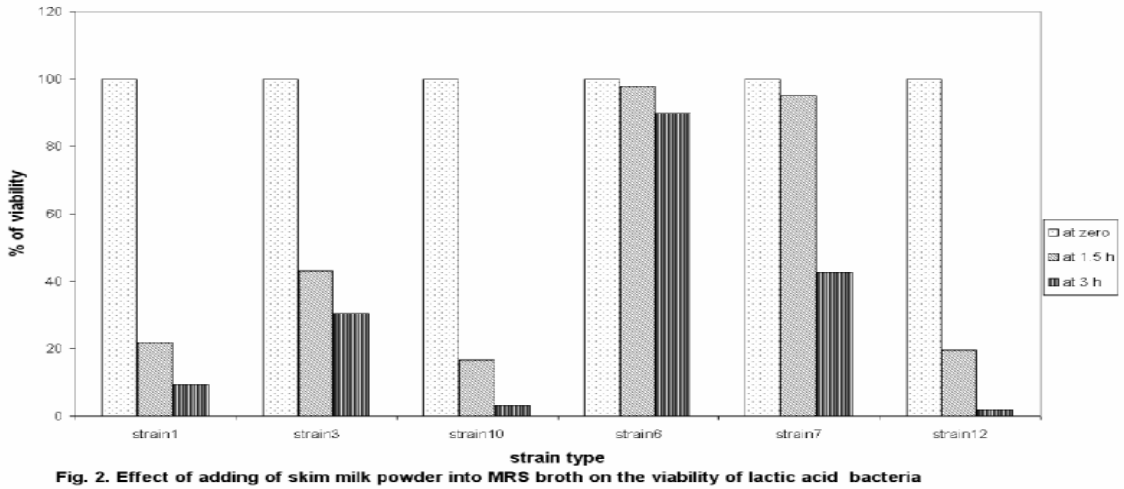

Fig. 2. Effect of adding of skim milk powder into MRS broth on the viability of lactic acid bacteria (%residual viability at pH 2)

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6:*Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. Angulatum* DSM 20098.

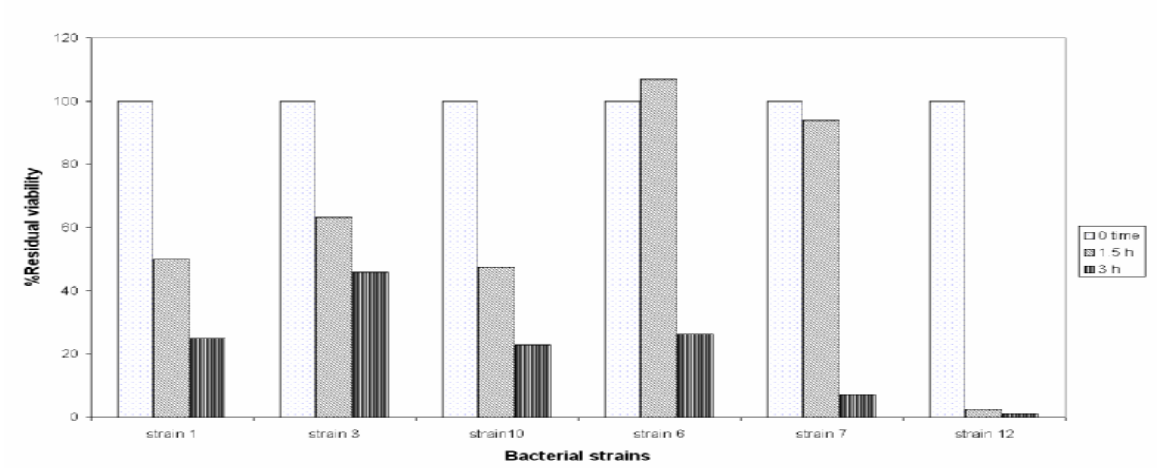

Fig. 3. Effect of pH on the viability of culture strains grown in MRS broth (residual viability at pH 3)

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098.

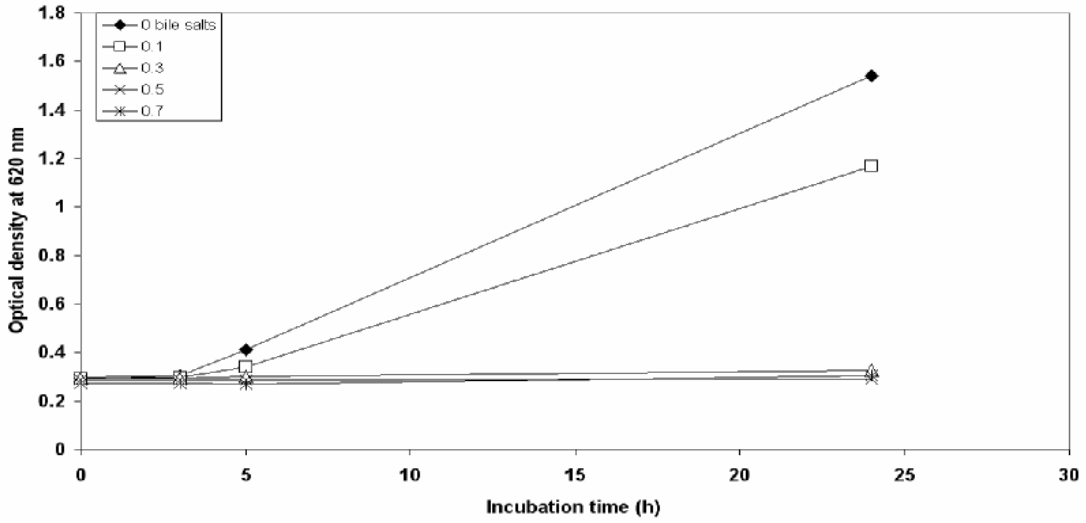

Fig. 4. Bile salt tolerance of *Lactobacillus acidophilus* DSM 9126 in MRS broth

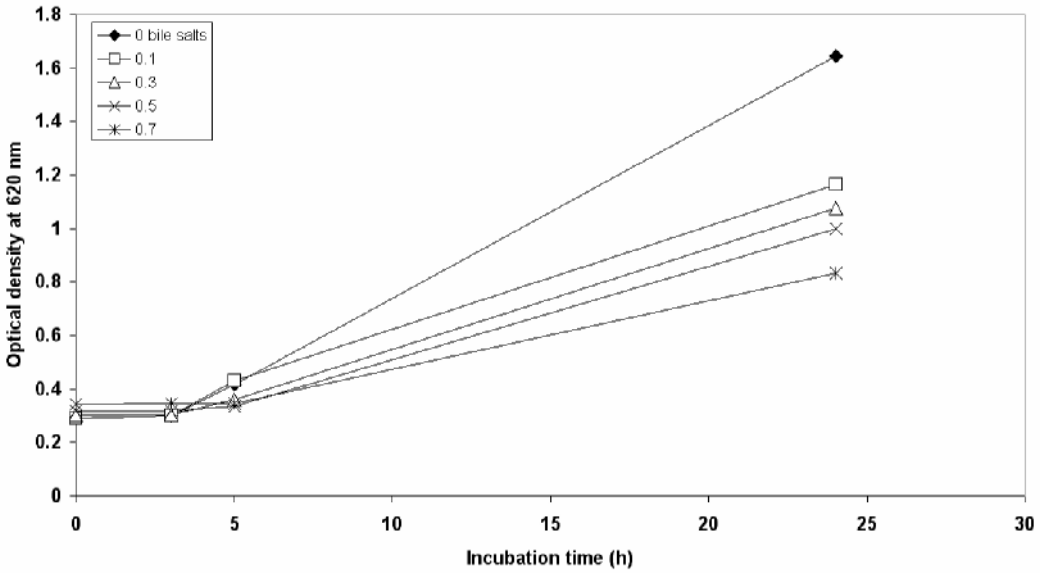

Fig. 5. Bile salt tolerance of *Lactobacillus acidophilus* DSM 20079 in MRS broth

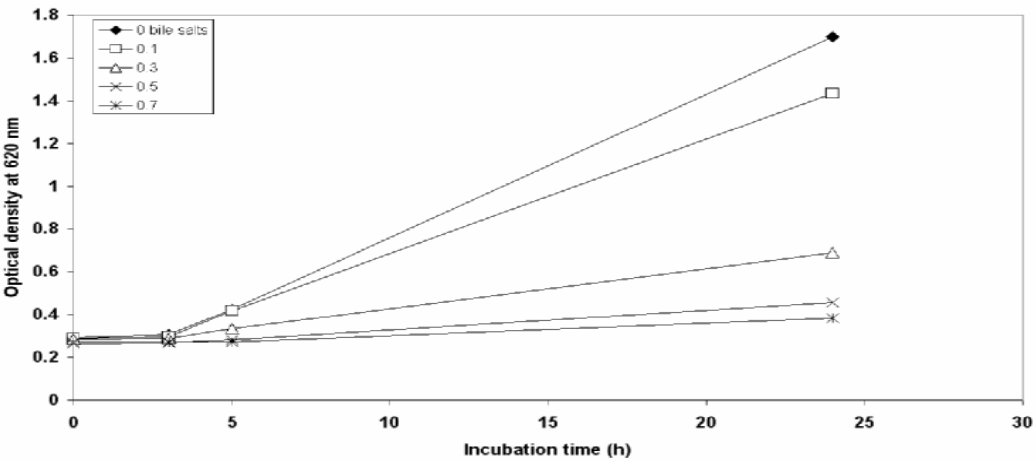

Fig. 6. Bile salt tolerance of *Lactobacillus acidophilus* DSM 20242 in MRS broth

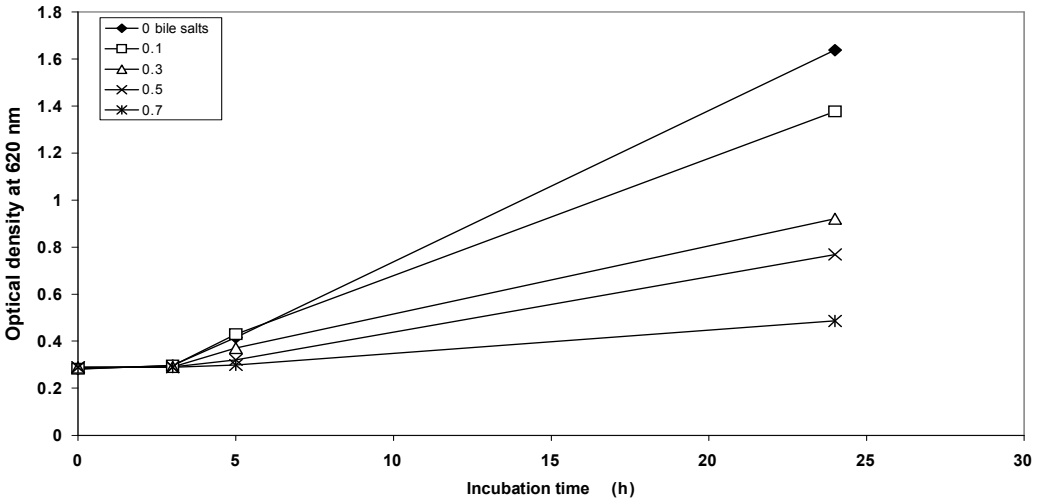

Fig . 7. Bile salt tolerance of *Streptococcus thermophilus* DSM 20617 in MRS broth

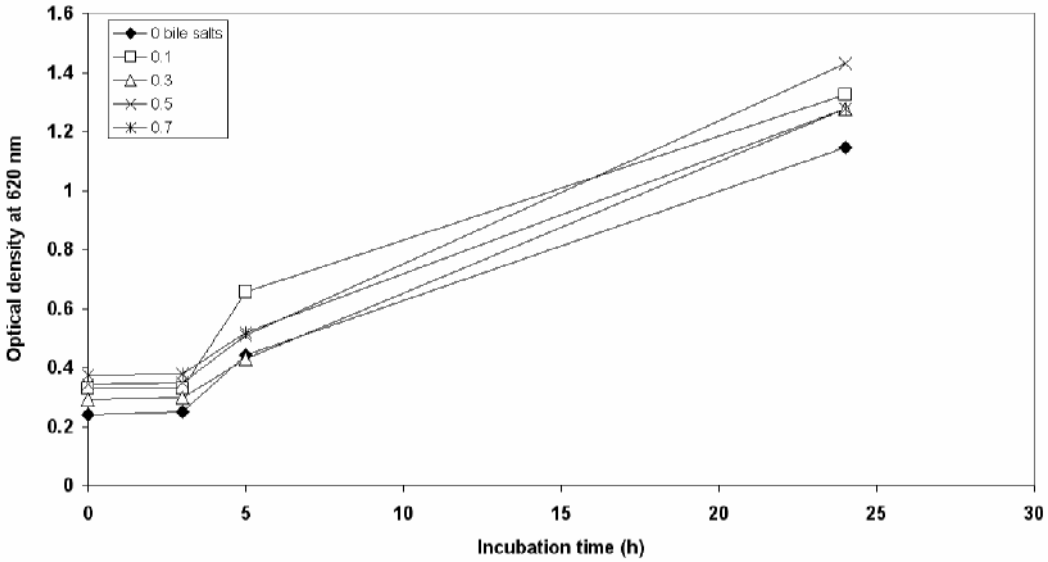

Fig. 8. Bile salt tolerance of *Bifidobacterium infantis* DSM 20088 in MRS broth

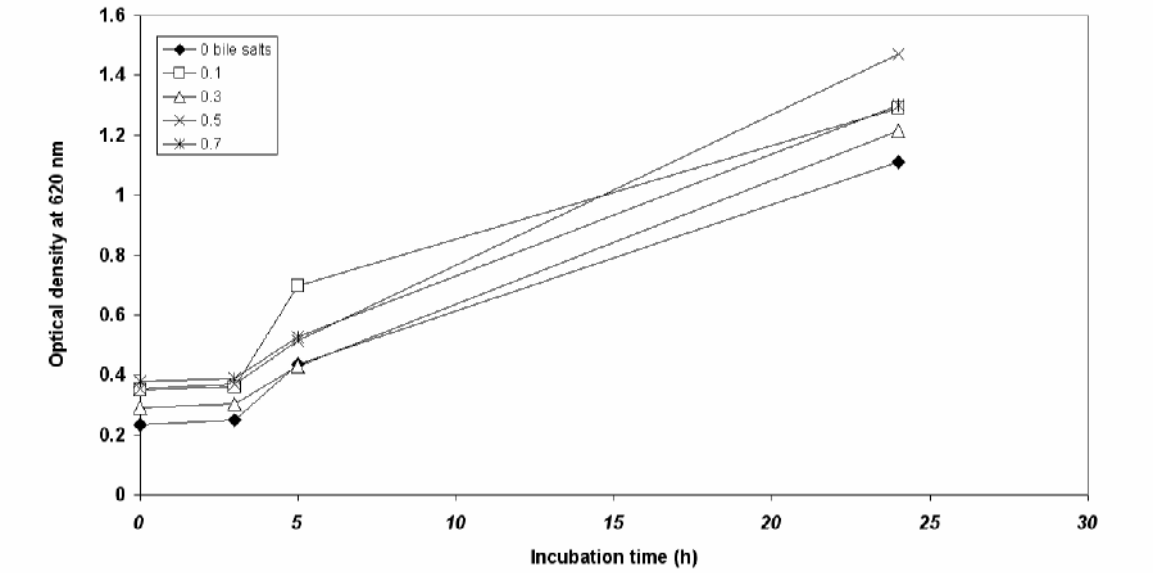

Fig. 9 Bile salt tolerance of *Bifidibacterium angulatum* DSM 20098 in MRS broth

Cholesterol assimilation

The percentage of cholesterol assimilated during 20 h of anaerobic growth at 37°C in MRS broth (Fig.10) revealed a wide variation among strains. All tested strains were able to assimilate cholesterol to some extent; the assimilation ranged from 3.08-29.68% presenting around 2.16-20.77 µg/ml. *Lactobacillus acidophilus* DSM 9126 exhibited high cholesterol assimilation (29.68%) compared to the other strains. Liong and Shah (2005); Lin and Chen (2000) and Dambekodi and Gilliland (1998) reported that *B. longum and L. acidophilus* are able to uptake cholesterol into their cellular membrane. Therefore, residual cholesterol was determined with the pellet obtained by centrifugation. As shown in Fig. 11, about 40% (27.59 µg/ml) of the cholesterol was precipitated with mass cells of *L. acidophilus* DSM 20242. Pereria and Gipson (2002) observed that the uptake of cholesterol by lactic acid bacteria and Bifidobacteria was higher in the medium containing 0.4% oxgall. This concentration is high enough to inhibit some strains in this studies (i. e. *Lactobacillus acidophilus* DSM 9126 and DSM 20242) (Figs. 4 and 6), and hence, MRS

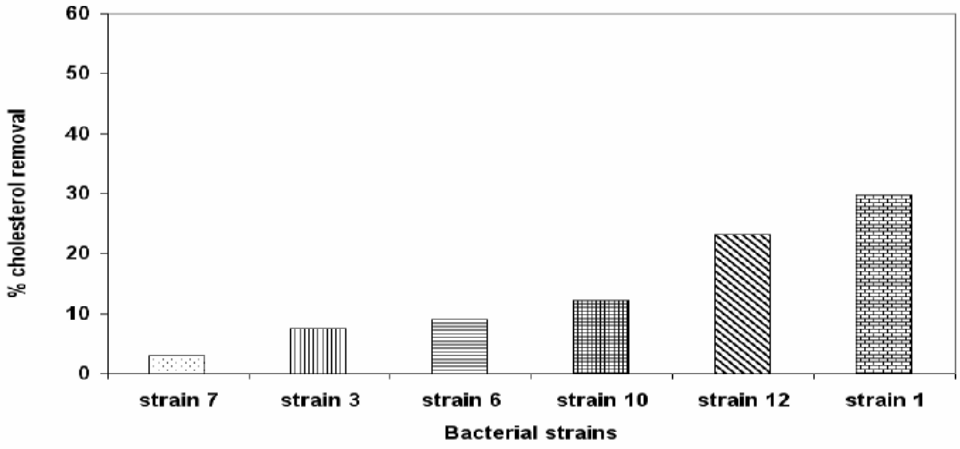

Fig. 10. Cholesterol removal from media by lactic acid bacteria and bifidobacteria

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098.

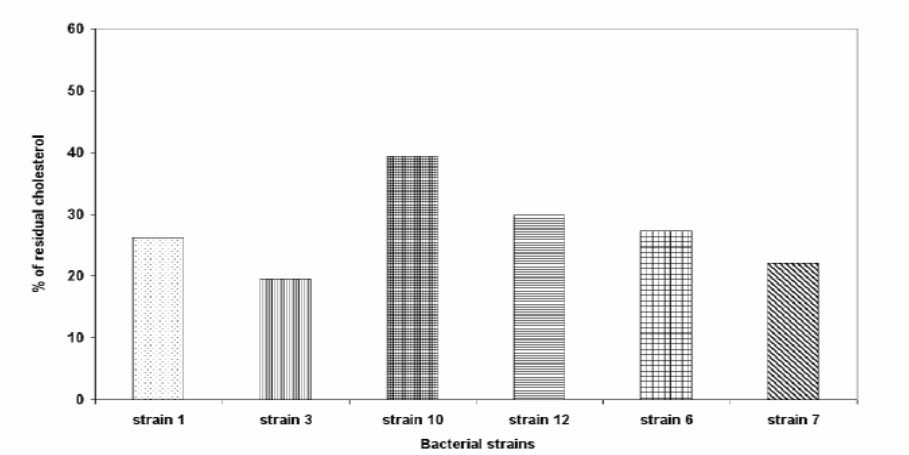

Fig. 11. Residual cholesterol with the pellet of bacterial strains after centrifugation

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098

supplemented with 0.2% oxgall was used. Results in Fig. 12 represent the effect of addition of bile salts on the assimilation of cholesterol from the media. Results revealed that addition of bile salts greatly improved the uptake of cholesterol from the media. *Lactobacillus acidophilus* strains showed the highest activity to assimilate cholesterol from the media and it ranged from 49.57-55.43% (34.70-38.8 µg/ml). Brashwars et al. (1998) reported that *L. casei* able to assimilate about 16.9-73.3 µg/ml cholesterol in MRS supplemented with 6 mM sodium taurocholate. However, removal by bifidobacteria strains ranged from 41.93-44.19% (29.35-30.93 µg/ml). Removed cholesterol by *Streptococcus thermophilus* was the lowest (about 36.07% equivalent to 25.25 µg/ml). Moreover, the precipitated cholesterol with the pellet cells was enhanced with addition of bile salts and *L. acidophilus* DSM 20079 increased the precipitated cholesterol up to 45.70% (31.99 µg/ml) (Fig. 13). Total assimilated and precipitated cholesterol was depicted in Fig. 14. It seems that all tested strains exhibited activity (ranged from 74.7-96.6%) towards assimilation and precipitation of cholesterol. *Lactobacillus acidophilus* strains DSM 20079 and DSM 9126 were among the most active in removing cholesterol from the growth medium.

Deconjugation of bile salts

Screening cultures for deconjugation of bile salts is shown in Fig.15. All cultures grown on sodium taurocholat-MRS agar plates formed fine precipitated white granules around and within the colonies to different extent. These white granules have been reported to be related to the solubility of bile salt at different pH (Dashkevicz and Feighner, 1989). The PK_a of taurin-conjugated and unconjugated bile salts are 1.9 and 5.0, respectively (Ahn et al., 2003). Thus, at acidic pH, unconjugated bile salts are protonated and precipitated.

The amount of released cholic acid in the broth containing sodium taurocholate was also determined. Data in Table 1 revealed that *L. acidophilus* DSM 20079 and *B. angulatum* DSM 20098 and *Bifidobacterium infantis* DSM 20088 librated more free cholic acid (2.38, 2.23 and 2.02 µmol/ml, respectivly) than did *L. acidophilus* DSM 9126 and *S. thermophilus* DSM 20617 (1.97 and 1.91 µmol/ml in order). Pereira et al. (2003) found that release of cholic acid from sodium taurocholate depends on the production of bile salt hydrolase by bacterial strains. Kim et al. (2004) also purified bile salt hydrolase from bifidobacteri strains.

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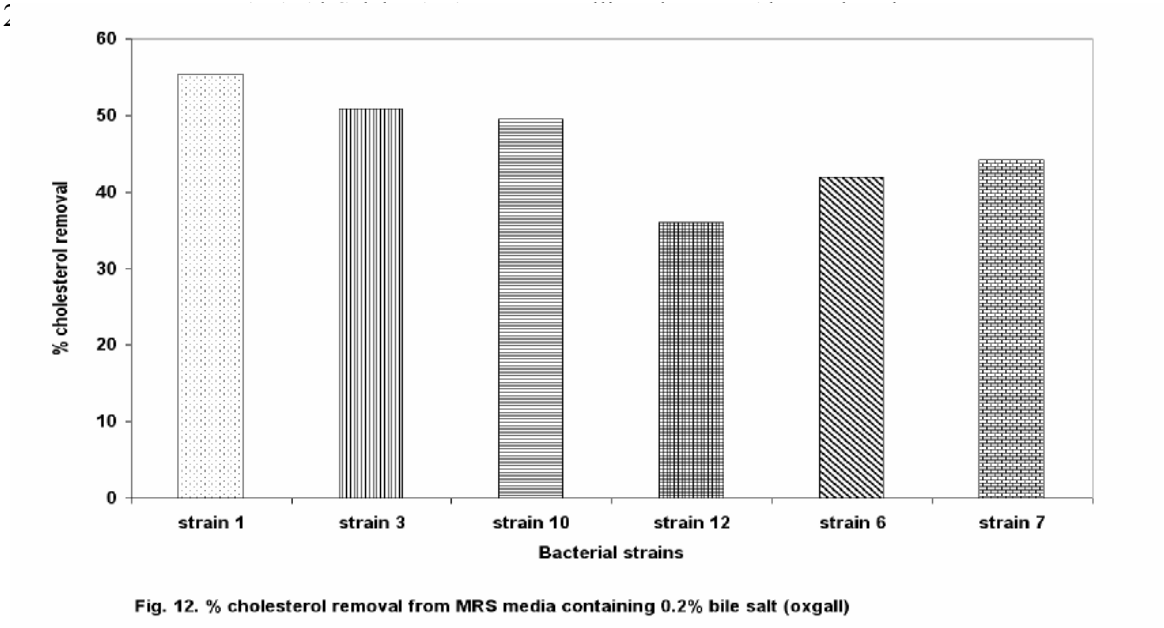


Fig. 12. % cholesterol removal from MRS media containing 0.2% bile salt (oxgall)

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulat*

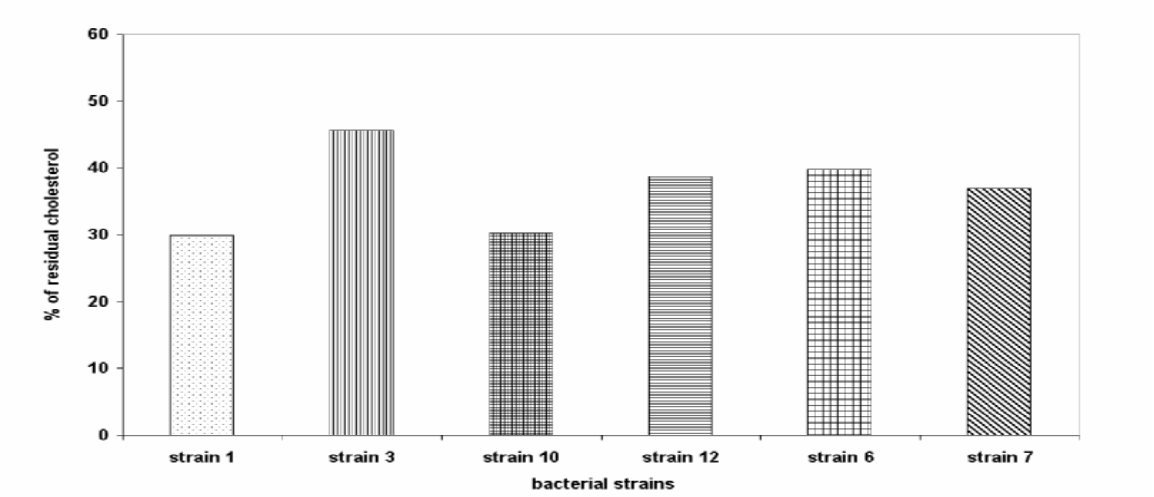


Fig. 13. % residual cholesterol with the pellet of cultures containing 0.2% bile salt (oxgall) after centrifugation

Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098.

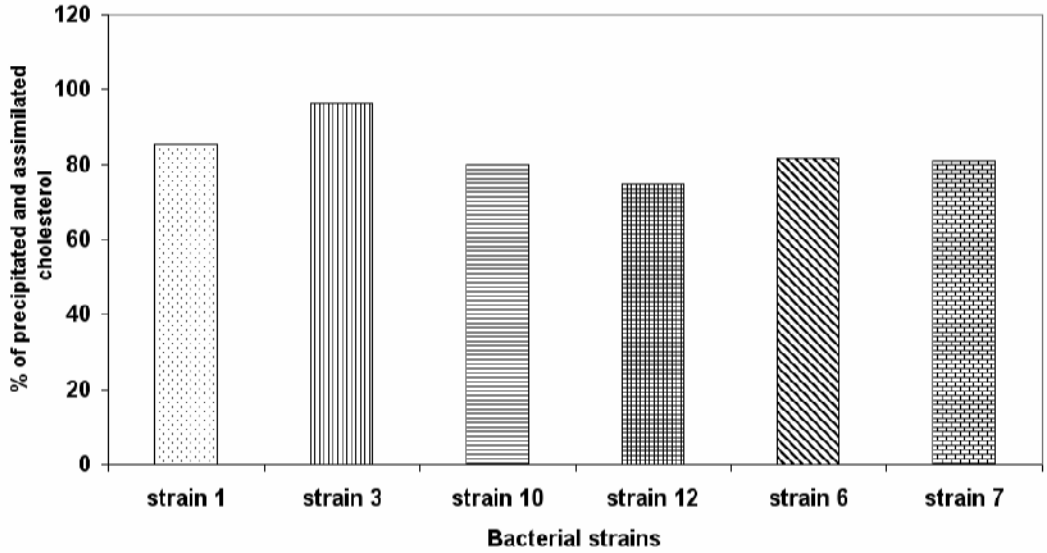
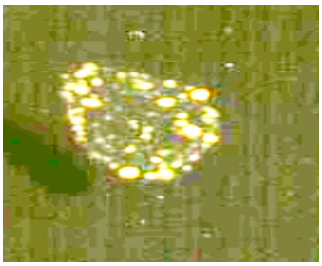


Fig. 14. Precipitated and assimilated cholesterol with different bacterial strains

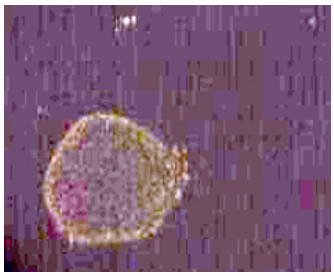
Strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lac. Acid*. DSM 20079, strain 10: *Lac. acid*. DSM 20242, strain 12: *Strept. thermophilus* 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098.

Fig. 15 Deconjugation of bile salt by bacterial strains grown on bile salt-MRS agar plate

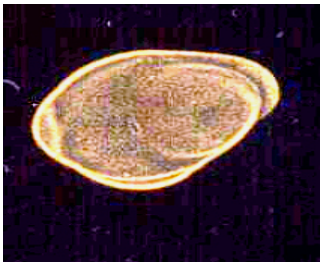


Strain 1

Strain 3



Strain 12



Strain 6

Strain 7

strain 1 is *Lactobacillus acidophilus* DSM 9126, strain 3: *Lacto. acid*. DSM 20079, strain 12:*Strept. thermophilus* DSM 20617, strain 6: *Bifidobacterium infantis* DSM 20088 and strain 7: *Bifido. angulatum* DSM 20098.

Table1.Deconjugation of sodium taurocholate (6 mM) by bacterial cultures in MRS broth

Bacterial strain	Free cholic acid (µmol/ ml)
Lactobacillus acidophilus DSM 9126	1.97
Lactobacillus acidophilus DSM 20079	2.38
Bifidobacterium infantis DSM 20088	2.02
Bifidobacterium angulatum DSM 20098	2.23
Streptococcus thermophilus DSM 20617	1.91

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تحمل الحموضة وأملاح الصفراء ونزع الكولستيرول من البيئات البكتيرية بواسطة بعض بكتريا حمض اللاكتيك وبكتريا البيفيدو

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الملخـــص: تم فى هذه الدراسة اختبار ثلاث سلالات من بكتريـــا *Lactobacillus acidophilus* (9126, 20079, 2042 DSM) وسلالتين من بكتريا الـــ Bifido (20088, 20098 DSM) وسلالة واحـــدة مـــن بكتريـــا *Streptococcus thermophilus* (20617 DSM) من حيث قدرتها على تحمل الحموضة وأملاح الصفراء وخفض الكولستيرول وفصل ملح توروكولات الصوديوم فى البيئات البكتيرية.

أوضحت النتائج أن هناك تباين بين هذه السلالات البكتيرية فى تحملها للحموضة وأملاح الصفراء وخفض الكولستيرول وفصل ملح توروكولات الصوديوم. إذ كانت بكتيريا الـــ Bifido أكثـــر الـــسلالات تحمـــلا لأمـــلاح الصفراء كما تأثرت جميع السلالات تحت الدراسة تأثرا كبيرا عند النمو فى بيئة ذات رقم حموضة (pH) ٢ ولكن عند زيادة رقم الـــ pH الى ٣ تحسنت قدرة جميع السلالات تحت الدراسة على تحمل الحموضـــة عـــدا بكتيريـــا *Streptococcus thermophilus*. زادت قدرة البكتيريا على تحمل الحموضة خاصة بكتيريا الـــ Bifido عند إضـــافة حليب فرز مجفف بنسبة ١% الى البيئات البكتيرية عند رقم pH ٢ وأظهرت النتائج ايضا قـــدرة منخفـــضة لهـــذه السلالات البكتيرية على خفض الكولستيرول من البيئات البكتيرية غير المحتوية على أمـــلاح الـــصفراء (٣,٠٨ – ٢٩,٦٨%) اذا ماقورنت بتلك المحتوية على ٠,٢ % املاح صفراء (٣٦,٠٧ – ٥٥,٤٣%). كما بينـــت النتـــائج أن هناك كميات اضافية من الكولستيرول ترسبت مع الخلايا البكتيرية المحتوية على ٠,٢% أملاح صـــفراء وكانـــت سلالة *Lactobacillus acidophilus* (20079 DSM) اكثر السلالات تحت الدراسة قدرة على فصل ملح توروكـــولات الصوديوم (٢,٣٨ ميكرومول/مل) وكذلك على خفض الكولستيرول سواء المترسب مع الخلايا البكتيرية أو المنزوع من البيئة بإجمالى ٩٥,٦% ولذا توصى الدراسة باستخدام هذه السلالة كمعاونات حيوية وكبـــادىء مـــساعد مـــع البادئات التقليدية فى إنتاج منتجات الحليب المتخمر.