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DETERMINING THE *IN-VITRO* CHOLESTEROL-REDUCING EFFICIENCY OF LACTOBACILLUS AND ENTEROCOCCUS STRAINS ISOLATEDFROM HUMAN BREAST MILK, FECES OF BREAST-FED INFANTS AND ANIMAL MILK (GOAT, COW AND BUFFALO)

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ABSTRACT

The present study was designed to evaluate the cholesterol-removal efficacy in-vitro of four Lactobacillus and four Enterococcus strains. All 8 isolates exhibited Bile Salt Hydrolase (BSH) activity (1.64 to 3.09 mm of precipitation zone) and cholesterol assimilation with and without bile salt (7.7% to 95.58%) but significantly higher-cholesterol assimilation was observed with bile salts (9.157% to 95.58%), Among these NB 16 and NB12 isolates manifested significantly higher cholesterol assimilation superior to other 6isolates with deoxycholic acid (95.58% and 94.85%) and cholic acid (92.17% and 88.9%) than ox bile and TDCA, and also exhibited a high cholesterol reduction ability in natural [egg yolk (NB7-34.46% to NB16-73.82%) and skimmed milk (NB7-28.22% to NB16-72.88%)] cholesterol media than synthetic cholesterol media without bile salt and cholesterol reduction potential by the 8 isolates were optimized by different cultural conditions, among these NB16 displayed an elevated cholesterol removal ability with 83.51% at1%inoculum size, 83.9% at 24 h inoculums age., 83% at pH-7, 83.17% at 37°C incubation temperature, 83.27% at 24 h incubation time and 83.51% at 70 µg/ ml cholesterol concentration these probiotic strain could be exploited as a potential biotherapeutic agent to reduce cholesterol levels and the risk of cardiovascular diseases.

KEY WORDS

Bile salt hydrolase, Cholesterol- lowering effect, Enterococcus faecium, Lactobacillus para casei.

I. INTRODUCTION

Cholesterol is a vital structural component of the animal cell membrane [1] and its imbalance in the blood is called hypercholesterolemia which is a leading risk aspect for cardiovascular diseases and also the main cause of death [2]. It may be averted by practicing drug therapies but usually, they possess undesirable side effects certain as gastrointestinal discomfort [3]. Hence, necessity is there for more natural approaches among which dietary intervention using probiotics have potent health-promoting benefits namely bio therapeutic agents [4, 5]. Previous studies have stated that total and low-density lipoprotein (LDL-C) cholesterol decreased by probiotics in which it was strongly related with elevated CVD risks. Due to it has led to an improved interest in probiotics as it was much less expensive then should be considered a "natural health remedy" [6].

In the 1970s experimental reports have stated that wild *Lactobacillus* strain have a cholesterol removal effect in human beings [7], after that many *in vitro* and *in vivo*

experiments have been performed to examine the cholesterol removal effect about lactic acid bacteria specifically in the strains of *Bifido bacterium* and *Lactobacillus* [8, 9,10]. The previous review on the cholesterol removal effect of probiotics concluded that the day by day consumption of products including lactic acid bacteria might also remain a dietary solution for long-term lowering effects of cardiovascular disease. [11, 12, 13].

Number of mechanisms for cholesterol reduction through probiotics have been recommended, such as like deconjugation about bile salts through bile-salt hydrolase (BSH) [14], Assimilation about cholesterol into cell membranes of the bacteria [15, 16], production about short-chain fat acids (SCFAs) for the duration of the growth about probiotics [17] then cholesterol transformation among coprostanol [18, 19] proposed as the BSH activity mechanism maximizes the amount of excretion. Such mechanism could be used in controlling serum cholesterol ranges by way of colonic microbes [20], due to this in past few decades the wide attention of the consumers, physicians and the researchers have attracted and used probiotic bacteria to decrease serum cholesterol levels [21]. It was once postulated that cholesterol assimilation was related with the availability of bile salts, it increased together with increasing concentration about bile salts within the medium [22] and numerous *in vitro* studies reported significantly higher cholesterol assimilation was observed in the existence of bile salt [23,24,25]. In accordance with the experimental findings [15, 26, 27, 28] cholesterol removal by probiotic appeared to not only strain specific but also growth dependent [15].

In the existent study of *Lactobacillus* and *Enterococcus* characterized as cholesterol-reducing probiotics by *in vitro* evidence.

II.MATERIALS AND METHODS

Bacterial strains: Strains were isolated from raw animal milk [Goat milk (*Enterococcus faecium NB44),* Cow milk (*Enterococcus faecalis NB7, Enterococcus faecium NB10)* and Buffalo milk (*Enterococcus faecium NB94)],* human milk *(Lactobacillus para casei NB12, Lactobacillus para casei NB14, Lactobacillus casei NB16)* and breastfeed Infant's fecal matter *(Lactobacillus para casei NB113*) by spread plate method [29,30] and maintained in MRS broth with 40 % sterile glycerol at –20°C [31] and evaluated for probiotics potential by using standard methods [32] and which exhibited acid tolerance (58-92%), bile tolerance (60-92%), synthetic gastric juice (60-90%), bacterial adhesion to hydrocarbons (93-96%), antimicrobial activity against food borne pathogens, antibiotic-resistant to 16 tested antibiotics out of 17, β-galactosidase activity upto1309±8mg/15 min, auto-aggregation (50-90%), fermented 17 sugars out 20, co- aggregation up to (62%), adhesion potential with 386 ± 15 bacterial cells (28.8%±1.5) per 100 cells of Caco-2 cell-line and identified as Lactobacillus para casei (NB12, 14 and 113), *Lactobacillus casei* (NB16), *Enterococcus faecium* (NB10, NB44 and NB94), and *Enterococcus faecalis* (NB7) by 16SrDNA sequencing method.

Chemicals: Cholesterol, Egg yolk, Skimmed milk, Bile salts, Calcium Chloride, De Man Rogosa and Sharpe broth/agar, were obtained from Hi-Media, Mumbai, India. Polyoxyethanyl cholesteryl sebacate, O-phthalaldehyde from Sigma, Saint Louis, USA. All the chemicals used were about an analytical grade.

Screening for bile salt hydrolase activity of *Lactobacillus* and *Enterococcus*.

Sterile filter discs placed on MRS agar plates with fresh culture, containing sodium salt of Tauro deoxycholic acid (0.5%) (w/v) and calcium chloride (0.037%) then the plates were incubated at 37°C for 72h an aerobically. Precipitation zone surrounding the filter disc around the colonies indicated the BSH activity of isolates, agar plates without supplementation were used as control [20, 33, 34].

***In vitro* cholesterol removal.**

MRS broth with 4% commercial hen egg yolk, 12% Skimmed milk cholesterol, Water-soluble cholesterol (polyoxyethanyl cholesteryl sebacate) and Synthetic cholesterol (70 µg/ml) as a sources through filter-sterilization with0.45 mm filter and inoculated with an 18 h culture (1% v/v) then incubated for 24 h at 37 °C. Then the cells were eliminated from culture broth by centrifugation for 15 min at 4°C and 6000 rpm [6, 15, 35]. Cholesterol remaining in the spent broth was determined by using the O-phthalaldehyde technique. One thousand micro liter of the supernatant was added to 1000 µl of potassium hydroxide (33% w/v) and 2000µl of ethanol (96%) was vortexed for 1 min, followed by heating for 15 min at 37 °C then 2000µl of the distilled water and 3000µl of the hexane were added after cooling, the tubes were then vortexed for 1 min then 1000µl of hexane layer was transferred to a glass tube

then evaporated on the water bath at 65°C. The residue was quickly dissolved into 2000µl of o-phthalaldehyde. After perfect mixing, 500µl concentrated sulphuric acid was added and the mixture was vortexed for 1 min. and allowed to stand for 10 min. Absorbance was read at 550 nm. [20, 34, 36, 37]. MRS broth including cholesterol without inoculation used as a control. All experiments have been done in triplicates. The proportion of cholesterol elimination was estimated the usage of the following formula:

$$= \frac{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in test sample}}{\text{Conc. of cholesterol in control.}} \times 100$$

***In-vitro* cholesterol removal with different bile-salts from media**

MRS broth was mixed with 0.30% of different bile salts including ox gall, CA, DCA, and TDCA and water-soluble cholesterol (70 µg/ml) through filter-sterilization (0.45 mm filter) and inoculated with (1% v/v) of an 18 h culture and incubated for 24 h at 37 °C [37,38,11], the cells were eliminated by centrifugation then the rest of the cholesterol concentration in the cell-free broth was estimated by using O-phthalaldehyde method [36].

***In-vitro* cholesterol removal by the dead, resting and growing cells with or without ox bile:**

MRS broth (15 ml) was inoculated with cultures (18 h) and incubated at 37°C for 24 h. The cells were recollected by using centrifugation for 15 min at 4°C for 6000 rpm, then washed and re suspended in distilled water (15 ml). Then the cell suspension was separated into three portions. First one was heat treated for 15 min at 121°C, centrifuged and recollected cells suspension was inoculated in cholesterol media (70 µg/ml) with and without ox bile (0.30%). The second one was centrifuged, and a recollected suspension was inoculated with 0.05 M phosphate buffer (pH 6.8) having cholesterol with and without ox bile. The third one was suspended in cholesterol media with and without ox bile and incubated at 37°C for 24 h. The cells were eliminated by centrifugation for 15 min at 4°C and 6000 rpm [15, 37, 39]. Then the spent stock was examined for cholesterol by the O-phthalaldehyde technique [36].

Optimization of cholesterol reduction by the isolates:

Effects of culture conditions on cholesterol reduction by isolates were studied in MRS broth with different concentrations of cholesterol (50, 70, 100, 150 and 200 µg/ml), incubation temperature (20, 30, 37 and 45°C), pH (3, 5, 6.5, 7 and 9), incubation time (12, 24, 48, 62 and 72 h), inoculum size (0.5, 1,1.5, 2, 3 and 4%) and inoculum age (8, 12, 18, 24,36 and 48 h)[40].

Statistical analysis

All assays were conducted in triplicates and the data were analyzed in Mean ± SEM (n=3) and standard deviations, by using Microsoft Excel (Version 7.0).

II. RESULTS AND DISCUSSION

Qualitative determination of bile salts hydrolase activity of LAB isolates:

Bile salt hydrolase activity was regarded as detoxifying activity wherein the conjugated bile salt is converted to free bile by producing bile salt hydrolase enzyme which catalyzes deconjugation activity [25, 41]. In the present study, the BSH activity was observed in 8 isolates. Wherein, the conjugated bile acid (Na-TDCA) taurine was deconjugated and produced ample amount of deoxycholic acid by BSH enzyme and diffused into the surrounding in the form of precipitation zone around the active colonies at different levels (1.64 to 3.09 mm). Among these, *Lactobacillus casei* NB16 displayed highest BSH activity (3.09mm) and *Enterococcus faecalis* NB7 displayed lowest BSH activity (1.67mm) (Fig. 1) and (Table.1). Our results were correlated with previous studies [10, 11, 34, 42, 43]. Since detoxified bile acids have a greater possibility of getting excreted through the intestinal tract because they are much less soluble and are much less possibility to be absorbed through the intestinal lumen than conjugated bile salts, deconjugated bile. This will increase the requirement of cholesterol because of de novo synthesis of bile acids to substitute their loss through feces [44].

Figure.1. BSH activity of Lactic acid bacteria on plate assay method

BSH activity of isolates manifested in the form of precipitation zone around the (disc contain) colonies at different levels (1.64 to 3.09 mm).

Table.1. Screening of the bile salt hydrolase by plate assay method

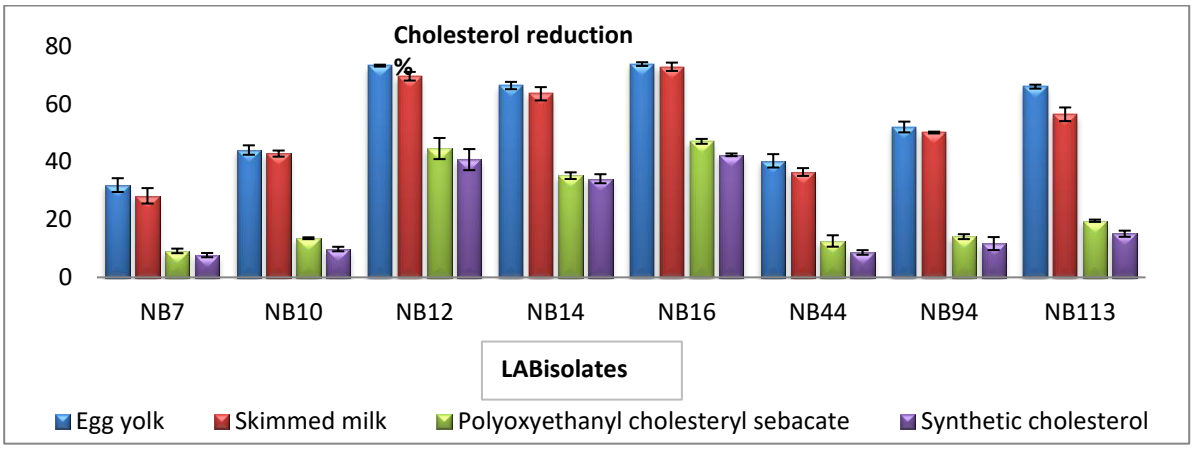
Strain	Precipitation zone diameter (mm)[a]	
	MRS agar	MRS agar (0.5%TDCA)
NB7	-[b]	1.64 ±0.28
NB10	-[b]	2.01 ±0.22
NB12	0.1±0	2.75 ±0. 2
NB14	-[b]	2.24± 0.1
NB16	0.3±0	3.09 ±0. 2
NB44	-[b]	1.74 ±0.28
NB94	-[b]	2.23 ±0.15
NB113	0.05±0	2.56 ± 0.2

a. Diameter of precipitation zone, b. Not detected, Values is expressed as mean ± SD in triplicates.

***In vitro* cholesterol removal from different Cholesterol media without bile salt:**

In the present study, ability of 8 LAB isolates to reduce biodegrade cholesterol from 4 different sources was investigated. All LAB isolates show the cholesterol assimilation ability and exhibited different levels of cholesterol reduction ranging from 7.7% to 73.82%. Egg yolk cholesterol-lowering ability ranged from 31.93% (NB7) to 73.82% (NB16), skimmed milk (NB7-28.22% to NB16-72.88%), water-soluble cholesterol (NB7-9.157% to NB16-47.07%) and synthetic cholesterol (NB7-7.7% to NB16-42.42%). All LAB Isolates exhibited a high cholesterol reduction ability in natural (egg yolk and skimmed milk) cholesterol media than water soluble and synthetic cholesterol media without bile salt. Among these NB16 and NB12 strains showed excellent cholesterol-lowering characteristics superior to other 6 isolates (Fig. 2). Our findings correlate with previous studies [38, 45, 46]. Previous experimental findings reported that about 11 lactobacilli strains isolated from raw cow milk exhibited cholesterol degrading activity ranging from 42.88 to 97.20%, *Enterococcus hirae* up to 75.3% and the extent of cholesterol reduction has been varying between the strains i.e *Lactococcus lactis KF 147* with 66.8% and *L. lactis subsp*. *Lactis N7* with 97.0% of cholesterol reduction [6, 15, 26, 28, 47, 48, 49]. Several reports have proven that *in vitro* model LAB can remove cholesterol including bile salt [10, 11, 15, 38, 42, 43]. This contradictory result suggests that cholesterol elimination seems to be strain specific [15, 26, 27, 28].

Figure.2. *In vitro* cholesterol removal from different Cholesterol media without bile salt



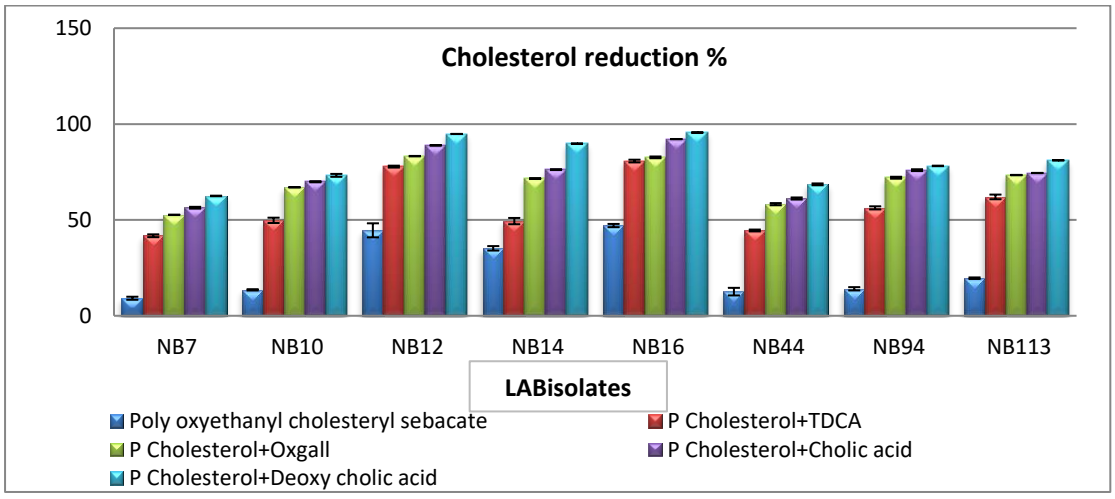
Egg yolk, Skimmed milk, Water-soluble (polyoxyethanyl cholesteryl sebacate) and Synthetic cholesterol removal ratio by Lactobacillus casei NB12, Values are expressed as mean ± SD in triplicates.

***In-vitro* cholesterol assimilation from media with different bile salts:**

In vitro cholesterol assimilation potential of LAB isolates including bile salts in media has been shown by numerous studies [11, 37].

In the present study *in vitro* cholesterol removing ability of 8 LAB isolates with 4different bile salts (ox bile [conjugated-97% + deconjugated- 3%) and TDCA (conjugated) CA and DCA (deconjugated)] was assessed. All the 8 LAB isolates showed significantly higher cholesterol removal ability in the presence of bile salts (41.74% to 95.58%) than without bile salt. Cholesterol removal ability of 8 LAB isolates with poly oxyethanyl cholesteryl sebacate without bile salt ranged from 9.157% (NB7) to 47.07% (NB16), with TDCA the capacity increased to 41.74% (NB7) and the highest was 80.72% (NB16), with ox bile NB7 showed 52.67% cholesterol removal capacity and NB16 showed 83.27%, while in presence of cholic acid NB7 showed 56.38 % capacity and NB16 showed 92.17% and with deoxycholic acid (NB7-62.52% to NB16-95.58%). Among these NB16 and NB12 isolates manifested significantly higher cholesterol assimilation superior to other 6 isolates with deoxycholic acid (95.58% and 94.85%) and cholic acid (92.17%and 88.9%) compared to the ox bile and TDCA (Fig.3). These results were in agreement with the previous reports [38, 50]. Which stated that higher cholesterol assimilation was observed in the presence of deconjugated bile acid than conjugated bile acids might be as a result of deconjugated bile acid high solubility, the detergent recreation of cholesterol then a high inhibitory effect of conjugated bile towards isolates may be one of the viable reasons [11, 24, 38].

Figure.3. *In-vitro* cholesterol assimilation from media with different bile salts



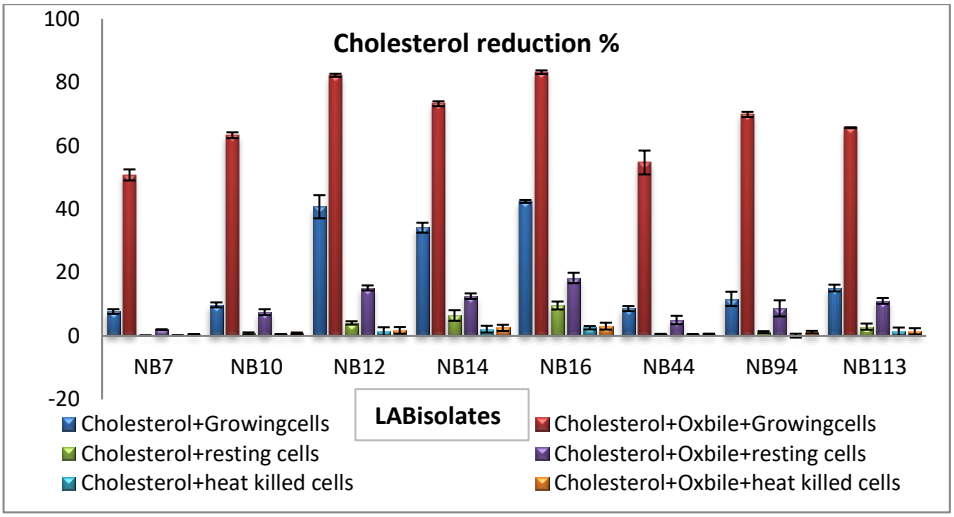
***In-vitro* cholesterol assimilation/ cholesterol removal percentage (%) from media with and without Oxbile by 8 isolates, Values are expressed as mean ± SD in triplicates.**

Cholesterol removal by the heat killed, resting and growing cells:

In vitro cholesterol reduction potential by the heat killed, resting and growing LAB strains from media containing ox bile has been shown by several studies [37, 38, 51].

In the present study, cholesterol removal ability of all 8 LAB isolates ranged from 0.1% to 83.16%. All 8 isolates growing on media containing ox bile showed significantly higher cholesterol reduction (NB7-50.77% toNB16- 83.16%) as compared to cells growing in the absence of ox bile (NB7-7.7% toNB16- 42.42%) and also as compared to the resting cells [(NB7-2% to NB16-18.29%) and without ox bile(NB7-0.11% toNB16- 9.6%)] and heat-killed cells [(NB7-0.5% toNB16- 3.09%) and without ox bile (NB7-0.13% toNB16- 2.58%)]. This could be as a result of the transiting microbes deconjugation activity about bile salts [52]. Among these NB 16 and NB12 isolates manifested significantly higher cholesterol assimilation compared to other 6 isolates (Figure.4). However, there was no considerable difference in the level of cholesterol removal between resting cells and heat-killed cells without ox bile. This indicated that heat treatment did not reduce the cholesterol removal potential however slightly higher cholesterol reduction potential was observed in resting cells including ox bile and many reviews have confirmed that availability of bile salts has a positive effect due to their high cell permeability [24, 45, 46]. However, heat-killed and resting with and without bile salt showed a small rate of cholesterol removal, indicating that cholesterol may also be removed via binding on to the cell surface [38, 53]. These results were correlated with the previous studies [11, 15, 37, 38].

Figure.4. Cholesterol removal by the heat killed, resting and growing cells with and without ox bile



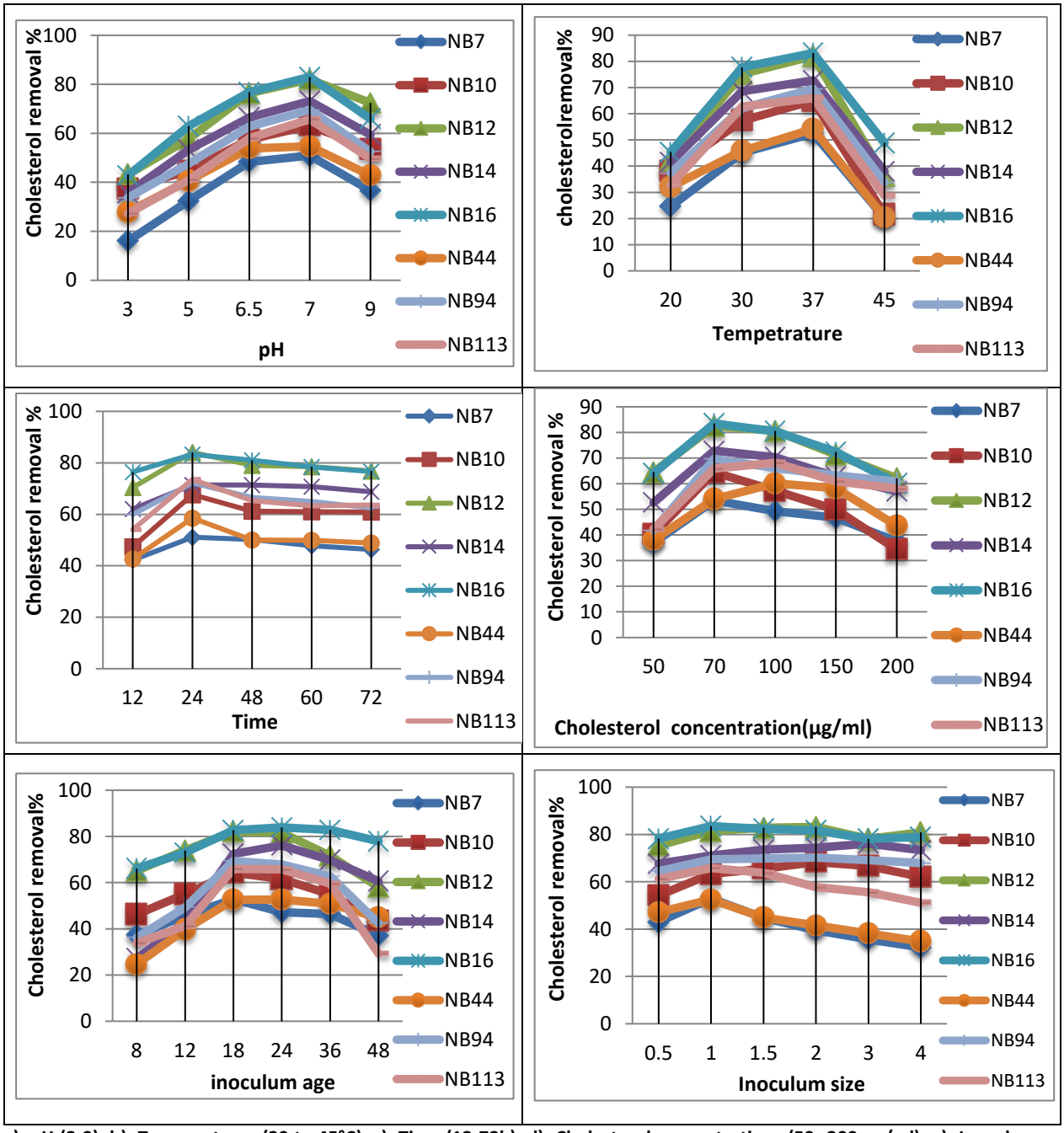
Cholesterol removal percentage (%) by the heat killed, resting and growing cells with and without ox bile by LAB isolates, Values are expressed as mean ± SD in triplicates.

Optimization of cholesterol reduction by the selected bacterial isolates:

The effect of pH (3-9) on cholesterol reduction ability by 8 LAB isolates was assessed. In all isolates it increased gradually with decreasing acidity and reached to maximum values (NB7- 50.94% to NB16-83%) and decreased gradually (NB7-36.61% to NB16-65.95%) with increasing alkalinity of culture medium, maximum cholesterol reduction by the 8 isolates was observed at pH 7(neutral) (Figure 5.a). These results are in agreement with the previous reports [40]. The previous experimental finding reported that for *L. casei LA-1, Streptomyces fradiae* show high cholesterol reduction ability at pH-7.2 and for *Rhodococcus erythropolis ATCC 25544* at pH-6.75 [54, 55]. The production of ideal enzymes is significantly altered by pH, via metabolic process therefore, the pH of the culture medium is of utmost importance for their structure and function [56]. The effect of temperatures (20 to 45°C) on cholesterol reduction ability by the 8 isolates was observed. It increased by increasing incubation temperature and reached to maximum values (NB7-52.47% to NB16-83.17%) at 37°C and then decreased when the temperature was increased (NB7-20.22% to NB16-48.92%) to 45°C (Figure.5. b.). The results are in accordance with the experimental findings reported earlier [40]. Previous experimental finding reported high cholesterol reduction ability for *E. hirae* shows at

37°C, *R. erythropolis ATCC 25544* at 29°C and *Bacillus subtilis SFF34* at 30°C [28,57,58,]. The optimal pH for decomposition of cholesterol is known to depend on the growth of the microorganisms which influences by environmental and nutritional factors [40].

Figure 5. Optimization of cholesterol reduction by the selected bacterial isolate



a). pH (3-9), b). Temperatures (20 to 45°C), c). Time (12-72h), d). Cholesterol concentrations (50- 200 µg/ml), e). Inoculums age (8-48h), f). inoculum size (0.5-4%), values are expressed as mean ± SD in triplicates

The effect of incubation time (12-72h) on cholesterol removal ability by the 8 isolates was observed. The maximum value was observed at 24 h of incubation ranging from (NB7-51.1% to NB12-84.06% and NB16-83.27). After that, cholesterol removal ability of 8 isolates decreases gradually and finally reached stability at 48 h with slight decreasing value (NB7-50.18% to NB12-79.01% and NB16-81%) (Figure 5c). Our results correlate with the previous reports [40], the previous experimental finding reported high cholesterol

reduction ability of L. *casei LA-1, L. lactis KF147, Enterococcus hirae* at 24 h and Lacto *bacillus acidophilus P10*6 at 20 h. [28, 49, 54, 59].

The effect of cholesterol concentrations from (50 to 200 µg/ml) on cholesterol removal ability was observed. The cholesterol removal ability of all the 8 isolates increased gradually with increasing cholesterol concentration upto70 and 100 µg/ml, in that 6 isolates (NB7-53.47%, NB10-64.48%, NB12-82.51%, NB14-72.89%, NB16-83.51% % and NB94-69.69%), showed maximum value at 70 µg/ml and remaining 2 isolates(NB44-60.15% and NB113-68.2%) reached the maximum value of at 100 µg/ml cholesterol, then cholesterol removal ability reduced with improved cholesterol concentrations (Figure 5.d). Our findings are in agreement with previous findings [40] which state that *Lactobacillus acidophilus P106* removed 90% at 70 µg/ml, *L. lactis KF147* removed 66.8%, *Enterococcus* strains removed (41.29%-56.61%), *lactic acid bacteria* and *bifidobacteria* removed (47%) at 100 µg/ml cholesterol [11, 49, 59, 60].

Excessive cholesterol concentration reduced water acti vity then the onset of plasmolysis may
suppress bacterial cholesterol assimilation ability [61].

The effect of age of the inoculate (8-48h) on cholesterol removal potential was studied. The maximum reduction potential of 5 isolates out of 8 isolates (NB7-52.8%, NB10-64.31%, NB12-82.18%, NB94-69.35% and NB113-65.93%) was observed at 18 h, remaining 3 isolates (NB14-76.11%, NB16-83.9% and NB44-52.67%) showed maximum reduction at 24 h inoculum age (Figure 5.e). High cholesterol assimilation was executed during the log phase and high biomass production executed when the cultures reached the stationary phase when the medium nutrients were depleted then enriched with inhibitory products leading to reduce in bacterial biomass and rate of cholesterol assimilation [40, 54].

The effect of inoculum size (0.5-4%) on the cholesterol removal ability was studied. Out of 8 isolates,4 isolates (NB7-52.97%, NB16-83.51%, NB44-52.52% yet NB113-65.81%) showed high cholesterol reduction capacity at 1% inoculum size and 3 isolates (NB10-68.34%, NB12-83.19% and NB94-70.26%) at 2% inoculum and NB14-76.11% at 3% inoculum size. NB16 showed high cholesterol reduction with 83.51% at 1% inoculum (Figure 5f). The high cholesterol assimilation was recorded with 1 to 2% inoculums size. Our results correlate with the previous findings [40, 54]. However, some experimental studies reported maximum cholesterol assimilation for *Lactobacillus fermentum* and *Lacto bacillus acido philus P106* at 1% inoculum size [11, 59].

IV.CONCLUSION

All l*actobacillus* and *enterococcus* strains demonstrated high bile salt hydrolase activity and cholesterol removal with and without bile salt in different cholesterol media. Among these, *Lactobacillus casei NB16* and *Lactobacillus para casei NB12* isolates executed significantly higher cholesterol assimilation with deoxycholic acid (95.58%and 94.85%). These results also indicate that milk is an excellent resource for cholesterol-reducing lactic acid bacteria. However, *in vivo* investigations are still required to confirm the hypo cholesterolemic effects and only after that one can recommend for the development of new functional food preparations for public health especially where cholesterol reduction in food is the main target.

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