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## Cholesterol Reduction by Probiotic Bacteria *Lactobacillus Plantarum* From Cow's Milk

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### Abstract:

This study aims to study was to insulate and investigate the impact of some probiotic bacteria from different sources of milk and milk products in the reduction of cholesterol. Out of fourteen tested bacterial isolates, isolate no. (LAB 8) from raw cow's milk was the most potent isolate that reduced cholesterol by 33.07%. The cholesterol reduction potentiality by the isolate no (LAB 8) was optimized to 53.3% by studying and controlling the different cultural conditions as incubation temperatures, incubation periods, pH, cholesterol concentrations, carbon sources, nitrogen sources, phosphorus sources and UV radiation exposure. The highest ratio of cholesterol reduction by isolate no (LAB 8) was obtained after 6 days of incubation at 37°C in basal medium of cholesterol well-adjusted to pH 7, with addition of cholesterol (0.75 g/L) as a substrate, maltose (1.0 g/100 mL) and after exposure to UV radiation for 60 min. The selected bacterial isolate no. (LAB 8) was identified biochemically by VITEK®2 as *Lactobacillus plantarum*.

**Key words:** Cholesterol, *Lactobacillus plantarum*, VITEK®2, UV radiation

### Introduction:

Cholesterol is the most vital lipid present in all human cells,

responsible for the structure of cellular membranes and sterols (*Palani et al., 2021*). The most of

cholesterol mainly produces in liver (*Yehia et al., 2015*). Cholesterol is essential for sex hormones synthesis (*Young, 2001*), also it is been transformed into vitamin D in the kidney and skin and into bile acids in the liver that aid in fat digestion (*Russell, 2003*). A high level of cholesterol poses a risk for cardiovascular diseases, as the fat accumulation in the blood vessels impairs the blood supply to the organs. Several laboratories, clinical and epidemiological studies have found a correlation between hypercholesterolemia and an increased risk of cardiovascular disease, a notable cause of death in western countries (*Palani et al., 2021*). The World Health Organization (WHO) describes probiotics as living microorganisms that, when provided in appropriate quantities, contribute a health benefit to the host (*WHO, 2002*). In the 1970s, it was discovered that fermented milk containing *Lactobacillus* strain had a hypocholesterolemia effect in humans (*Mann and Spoerry, 1974*). *In vitro* or *in vivo* studies have been designed to examine the cholesterol-lowering. Since then, several effects of lactic acid bacteria (LAB), especially *Lactobacillus* and *Bifidobacterium* strains have been observed by *Pan et al. (2011)*; *Wang et al. (2012)*; and *Pereira*

and *Gibson (2002)* concluded a review from investigations on the hypocholesterolemic impact of probiotics in which, the fermentation of dairy products with the applicable bacteria strain can lower the concentrations of blood cholesterol and these strains do not usually exist in the intestine of human. Consumption of probiotic products on a daily basis can be a dietary resolution for achieving the long hypocholesterolemic effects (*Tsai et al, 2014*). Several studies have shown that different bacteria can lower cholesterol levels in aqueous systems like liquid media (*Saavedra et al., 2004*) and blood serum (*Hlivak et al., 2005*).

This current work was proposed to evaluate the dietary treatment role of some probiotic's bacteria in lowering cholesterol.

## **Materials and Methods:**

### **1. The isolation of cholesterol reducing probiotic bacteria:**

Fifteen samples of milk and milk product were collected from different domestic markets and farmers in Zagazig City, Egypt, through February to May 2019. The samples were suspended by shaking 10 g of each sample for 20 minutes in 90 ml of sterile saline solution (8.5 g NaCl/L), then 0.1 mL of each suspension was spread on the surface of plates containing MRS agar media

(de Man, Rogosa, Sharpe, Oxoid) for isolation of lactic acid bacteria under aseptic conditions and incubated for 2 days at 37°C.

## **2. Screening of bacterial isolates growth on cholesterol agar plate:**

The pure bacterial isolates were streaked on the surface of plates containing Minimal Salt Cholesterol (MSC) agar media, supplemented with cholesterol (0.2%) as a sole carbon source (*Nishiya et al., 1997*). The media including (g/L):  $\text{NH}_4\text{NO}_3$ , 17;  $\text{K}_2\text{HPO}_4$ , 0.25;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25;  $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ , 0.001; NaCl, 0.05; cholesterol, 1.5; agar 20 and Tween 80 (0.1 mL/L). To avoid the cholesterol coagulation, firstly it was suspended in 10 mL mixture (20% isopropanol plus 10% of Tween 80) then was added to the media. The pH was adapted to 7.0.

Utilization of cholesterol by bacterial isolates was evaluated *via* the growth of bacteria on these plates after 7 days of incubation at 37°C (*Wali et al., 2019*).

## **3. Screening of bacterial isolates for cholesterol reduction in liquid medium:**

The positively grown bacteria on (MSC) agar plates were incubated for 7 days in MSC broth media for 7 days at 37°C. The enzymatic colorimetric cholesterol oxidase-peroxidase (POD) method was used to assess the ability of

bacterial isolates to degrade cholesterol (*Kulkarni et al., 2013*). The assay was carried out by the estimation kit of cholesterol (Spinreact). The reagents were reconstructed according to the kit's instructions and the concentration of cholesterol standard was 200 mg/dL. All of the reagents were thoroughly mixed in accordance with the manufacturer's instructions. 10  $\mu\text{L}$  of cell free supernatant (CFS) was added to the reaction mixture, mixed by inversion, and incubated for 10 minutes at 37°C. At  $\lambda$  505 nm, the absorbance of the test and standard was compared to that of the blank. Uninoculated tube was served as control. The following formula was used to calculate cholesterol concentration:

$$\text{Cholesterol (mg/dL)} = \left\{ \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \right\} \times \text{Conc. of standard (mg/dL)}$$

Furthermore, the cholesterol reduction (mg/dL) and the percent of cholesterol reduction were assessed.

## **4. Biochemical identification and characterization of the most potent cholesterol reduction isolates:**

The most effective cholesterol reducer isolate was inoculated into the VITEK®2 Compact (bioMérieux) identification strip. Strains were cultured on MRS

agar for 18–24 hours at 37°C before analysis. The suspension of bacteria was adjusted to a McFarland standard of 0.50. The time it took to prepare the solution and fill the card was always less than 1 hour. Anaerobic and *Corynebacterial* identification cards (ANC) were used in the analysis. Every 15 minutes, the ANC Card was read automatically (Lee et al., 2011).

### **5. Optimization of cultural conditions for cholesterol reduction:**

The tested isolate was inoculated in liquid basal cholesterol medium, many growth conditions influencing cholesterol degradation were investigated, e.g. incubation temperatures (25–50°C) and incubation periods (2–10 days); pH values (4–9). Various concentrations of cholesterol (0.25–1.5 g/L) and carbon sources (sucrose, glucose, fructose, lactose and maltose) at a final carbon source concentration 1 w/v. Nitrogen sources including organic nitrogen sources (beef extract, meat extract, yeast extract and peptone); inorganic nitrogen sources (ammonium chloride, sodium nitrate and ammonium sulphate) were added to medium at concentration that was equimolecular to 17 g of  $\text{NH}_4\text{NO}_3$  and phosphorus sources ( $\text{KH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ ) also were investigated. The cholesterol

degradation assay was carried out, after the bacterial isolate was grown at under various conditions.

Bacterial suspension was exposed to UV light irradiation for different times (30, 40, 50 and 60 min.) at distance 20 cm from the UV source (254 nm). Then 100  $\mu\text{L}$  from bacterial suspension at each tested time was taken and spread on nutrient medium agar which incubated at 37°C for 24 hours. Then changes in count and morphogenesis in colony were determined and the suspected mutated bacteria were screened to determine their ability to reduce cholesterol (Jaivel and Marimuthu, 2010).

### **Results and Discussion:**

Fourteen isolates of bacteria were investigated for their growth on MSC agar media for 7 days at 37°C. All bacterial isolates utilized cholesterol and well-grown on MSC agar media (Figure 1) which agree with the results obtained by Wali et al (2019) for growth of *Bacillus pumilus* W1 and *Serratia marcescens* W8.

The purified 14 probiotic bacteria (Table 1) were screened to compare their effect in the reduction of cholesterol and the result showed that the bacterial isolate no. (LAB 8) isolated from cow milk was the most potent

cholesterol reducer by (33.07%) (Table 2). The obtained results agree with many authors who recorded the capability of various bacteria to lower the cholesterol in a liquid media such as *Kulkarni et al (2013)* who reported that the isolated bacteria from raw cow's milk revealed a cholesterol lowering activity and *Khiralla (2015)* who observed that the *Lactobacillus* isolates were the cholesterol reducer isolates. On other hand many studies which recorded the ability of *actinomyces* to reduce cholesterol level in liquid media as, *El-Naggar and El-Shweihy (2020)* who demonstrate that *Streptomyces anulatus* as cholesterol remover isolate.

The biochemical identification tests by VITEK<sup>®</sup>2 identified the isolate no. (LAB 8) as *Lactobacillus plantarum* (Table 3).

The optimum incubation period for the maximum growth and cholesterol degradation by *Lactobacillus plantarum* was achieved after 6 days of incubation (Figure 2). The obtained results agree with the results conducted by *ElBaz et al. (2017)* who isolated *Bacillus pumilus* and recorded high cholesterol degradation after 6 days of incubation. On the other hand, *Abou-Saty (2009)* recorded that the maximum cholesterol

degradation by *Streptomyces corchorusii* CX-3 was obtained after 10 days of incubation.

The optimal incubation temperature for the cholesterol degradation by *Lactobacillus plantarum* was 37°C (Figure 3). These results in agreement with the results obtained by *Aboseidah et al. (2017)* who observed that 37°C was the optimal incubation temperature for cholesterol reduction by *Enterococcus faecalis* W7 and *Enterococcus faecium* Y1. On other hand, many authors recorded that the optimal incubation temperature for cholesterol degradation by *Rhodococcus sp.* NCIM 2891 and *Streptomyces cavourensis* NEAE-42 was 30°C (*Ahmad and Goswami, 2013*) and (*El-Naggar et al., 2016*), 35°C for *Bacillus pumilus* W1 and *Serratia marcescens* W8 (*Wali et al., 2019*).

The obtained results indicated that the pH 7.0 was the optimum pH for *Lactobacillus plantarum* to achieve the maximal growth and cholesterol degradation by (Figure 4) that comes in agreement with results reported by *Saranya et al (2014)* who observed that the pH 7.0 was the optimal pH for *Bacillus* sp to lower the cholesterol and produce the cholesterol oxidase. These results contrasted with the results obtained by *Yazdi et al. (2001)*

who recorded that the optimal pH value for *Streptomyces fradiae* to decompose the cholesterol was 7.2.

The cholesterol in the present study was the sole source of carbon and its varying concentrations affect its decomposition. The maximum degradation was achieved at a concentration of 0.75 g/L (Figure 5). Even so, with increasing in cholesterol concentration increased, the hypocholestermic activity of *Lactobacillus plantarium* decreased significantly. This is consistent with the findings of Ouf et al. (2012), who found that the high concentrations of cholesterol were degraded more slowly than the low concentrations by *Streptomyces* cholesterol oxidase.

The results showed that the addition of maltose to the medium of growth elevated the cholesterol lowering activity of *Lactobacillus plantarium* compared with the case of maltose-free medium (Figure 6), while any increase in maltose concentration more than 1 g/100 mL inhibited the bacterial ability of cholesterol reduction.

In the present investigation the  $\text{NH}_4\text{NO}_3$  was the optimal nitrogen source for degradation cholesterol by *Lactobacillus plantarium* (Figure7) which agree with the results obtained by Ouf et al.

(2012) who noticed that the most appropriate nitrogen sources for the non-irradiated and radiated *Streptomyces fradiae* to reduce the cholesterol were  $\text{NH}_4\text{NO}_3$  and  $\text{Mg}(\text{NO}_3)_2$ . On the other hand, several studies such as Lee et al (1997) and Yazdi et al (2001) observed the yeast extract as the best nitrogen source for cholesterol degradation by *Rhodococcus equi* no. 23 and *Rhodococcus equi* 2C, respectively.

The results showed that  $\text{K}_2\text{HPO}_4$  was the optimal phosphorus source for cholesterol degradation by *Lactobacillus plantarium* (Figure 8) this agree with results obtained by Kim et al. (2002) who mentioned that the optimal phosphate source for cholesterol degradation by *Bacillus subtilis* was  $\text{K}_2\text{HPO}_4$  (0.025%).

Genetic improvement is one of the promising approaches for increased production of secondary metabolites by industrially important microorganisms. In the current study mutation was performed physically by using UV irradiation treatment led to change in count and morphology of the bacterial colonies (Figure 9) and by determination of reduction cholesterol percent by the mutant isolate it was found increasing in reduction of cholesterol (Figure 10) these results were agreed with Jaivel and Marimuthu (2010) who reported that the mutant JPM3-UV1 produced the maximum lovastatin comparing to the parent culture.

**Table (1)** Isolation of probiotic bacteria (lactic acid bacteria) from different milk sources.

Source	LAB growth
<b><u>Buffalo milk</u></b>	
Sample 1	+ve
Sample 2	+ve
Sample 3	+ve
Sample 4	+ve
Sample 5	+ve
Sample 6	+ve
<b><u>Chesse whey</u></b>	Sample 1
	+ve
<b><u>Cow milk</u></b>	Sample 1
	Sample 2
	+ve
<b><u>Goat milk</u></b>	Sample 1
	+ve
<b><u>Gohania milk</u></b>	Sample 1
	+ve
<b><u>Lactiuol milk</u></b>	Sample 1
	+ve
<b><u>Salt cheese whey</u></b>	Sample 1
	+ve
<b><u>Yogurt</u></b>	Sample 1
	Sample 2
	+ve
	+ve

*LAB growth: lactic acid bacteria growth; Positive growth: +ve; Negative growth: -ve.*

**Table (2):** Biochemical identification tests for identification of (LAB 8) by VITEK2.

<b>Identification information</b>													<b>Card:</b> ANC	<b>Lot Number</b> 24208740 3	<b>Expires:</b> Apr 20,2020 13:00
													<b>Completed:</b> Feb 23,2020 19:54 CST	<b>Status:</b> Final	<b>Analysis Time:</b> 6.98 hours
<b>Organism origin</b>													VITEK 2		
<b>Selected Organism</b>													99% probability <i>Lactobacillus plantarum</i> Bionumber:000010721773731		
<b>SRF Organism</b>															
<b>Analysis organisms and Tests to Separate:</b>															
<b>Analysis message:</b>															
<b>Contraindicating Typical Biopattern(s)</b> <i>Lactobacillus plantarum</i>															
<b>Biochemical details:</b> Installed VITEK 2 System Version: 05.02															
4	dGAL	-	5	LeuA	+	6	ELLM	-	7	PheA	+	8			
11	dCEL	+	13	TyrA	-	15	APPA	-	18	dGLU	+	20	c		
28	SAC	+	30	ARB	+	33	NAG	+	34	BGLUi	+	36			
39	GALi	-	41	AARA	-	42	AGALi	-	43	BMAN	-	44			
51	MTE	-	53	ESC	+	54	BdFUC	-	55	BNAGi	-	56	A		
59	PHOS	-	60	IARA	-	61	dRIB2	-	62	OPS	+	63	A		
MIC Interpretation guideline:															
Therapeutic Interpretation guideline:															
AES Parameter Set Name:															
AES Parameter Last Modified:															

**Table (3):** Screening of bacterial isolates for cholesterol reduction in cholesterol liquid media.

Isolates	Cholesterol residual (mg/dL)	% of Cholesterol reduction
LAB1	70.57	22.19
LAB 2	71.68	20.97
LAB 3	76.68	15.45
LAB 4	72.60	19.90
LAB 5	74.42	17.90
LAB 6	68.94	23.99
LAB 7	68.98	23.90
LAB 8	60.70	33.07
LAB 9	67.20	25.90
LAB10	61.5	32.19
LAB11	63.35	30.15
LAB12	63.40	30.00
LAB13	72.00	20.60
LAB14	71.50	21.16

LAB: Lactic acid bacteria, Control =90.7.



Figure (1): Positive growth of bacteria on cholesterol agar plate.

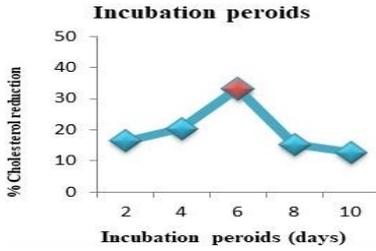


Figure (2): Effect of different incubation periods on cholesterol reduction by *Lactobacillus plantarum*

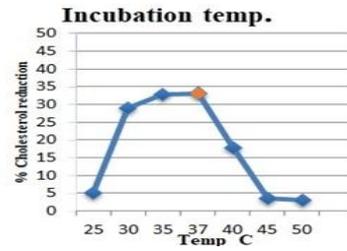


Figure (3): Effect of different incubation temperatures on cholesterol reduction by *Lactobacillus plantarum*

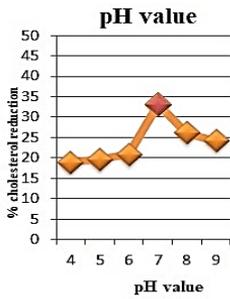


Figure (4): Effect of different pH values on cholesterol reduction by *Lactobacillus plantarum*

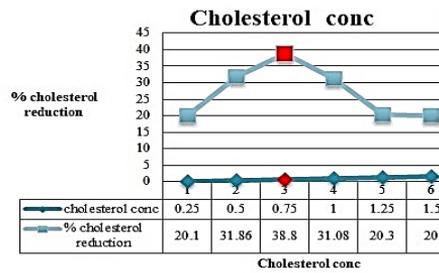


Figure (5): Effect of different cholesterol concentration on cholesterol reduction by *Lactobacillus plantarum*

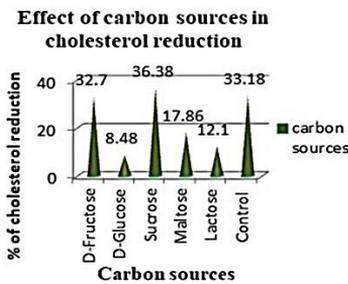


Figure (6): Effect of different carbon sources on cholesterol reduction by *Lactiplantibacillus plantarum*

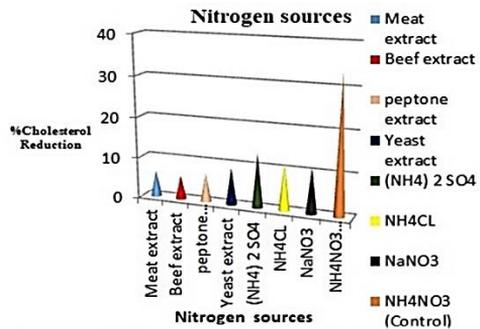


Fig. (7): Effect of different nitrogen sources on cholesterol reduction by *Lactiplantibacillus plantarum*

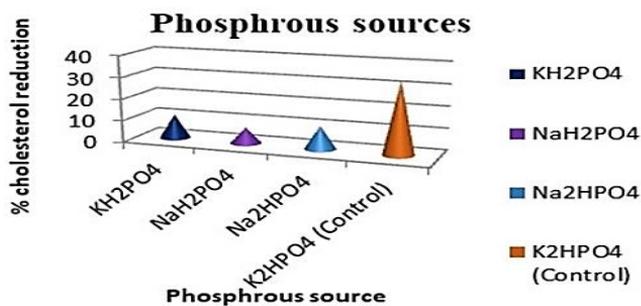


Fig.(8) Effect of different phosphorous on cholesterol reduction by *Lactiplantibacillus plantarum* □

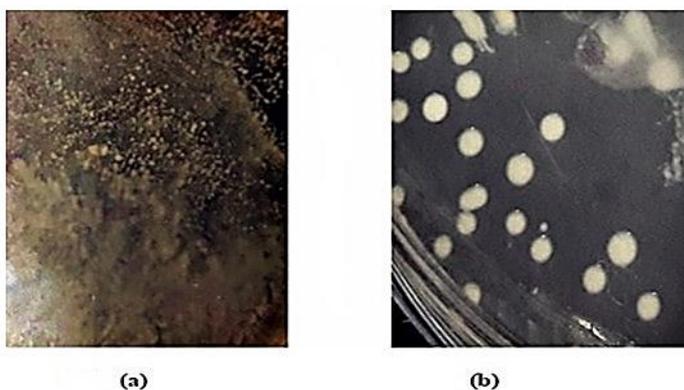


Figure (9): The effect of UV light on count and morphogenesis of *Lactobacillus plantarum* a, the bacterial growth at zero time; b, the bacterial growth after 60 min of UV exposure

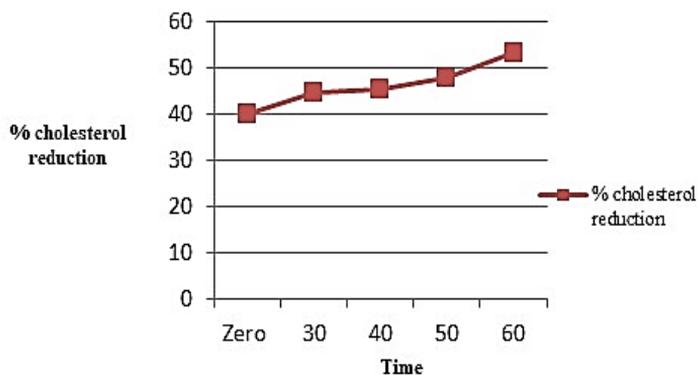


Figure (10): Effect of UV radiation exposure on cholesterol reduction.

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## الملخص العربى

## خفض الكوليسترول بواسطة بكتيريا اللاكتوباسيلس النافعة المعزولة من لبن البقر

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الهدف الرئيسى من هذه الدراسة هو عزل و دراسة تأثير بعض بكتيريا البروبيوتيك من مصادر مختلفة من الحليب ومنتجات الألبان في خفض نسبة الكوليسترول. تم فحص تأثير ١٤ عزله بكتيرية على خفض الكوليسترول و كانت العزلة (٨ LAB) المعزوله من حليب البقر الأكثر نشاطا في خفض الكوليسترول بنسبة ٣٣,07%. تم تحسين نشاط العزلة (٨ LAB) في تخفيض الكوليسترول ليصل إلى ٣,٥٣% و ذلك من خلال دراسة الظروف البيئية المختلفة مثل درجات حرارة الحضانة و فترات الحضانة و درجة الحموضة و تركيزات الكوليسترول و مصادر الكربون و مصادر النيتروجين و مصادر الفوسفور و التعرض للأشعة فوق البنفسجية. و أوضحت النتائج أن أعلى نسبة لخفض الكوليسترول بواسطة (٨ LAB) تم الحصول عليها بعد ٦ أيام من الحضانة عند ٣٧ درجة مئوية في الوسط القاعدي (الرقم الهيدروجيني ٧) مع إضافة ٧٥ جم/لتر كوليسترول كركيزة مع إضافة المالتوز بتركيز ١٠٠ جم/مل و بعد التعرض للأشعة فوق البنفسجية لمدة ٦٠ دقيقة. تم التعرف على (٨ LAB) كيميائياً بواسطة VITEK®2 على أنه *Lactobacillus plantarum*.