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***In vitro* Treatment with Intact Cells or Cell Lysates of *Lactobacillus* and *Spirulina* Induced Lowering Effects on Induced Hypercholesteremia**

¹Shymaa Kamal, ¹Ragaa A. Hamouda, ¹Hoda Mahrous, ²Mohammed Labib Salem, ¹Hanafy A. Hamza and ³Ekbal Abd Elhafez

¹Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat, Sadat City, Egypt

²Department of Zoology, Faculty of Science, Division of Immunology and Biotechnology, Tanta University, Egypt

³National Organization of Drug Control and Research, Egypt

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Corresponding Author:

Shymaa Kamal

Genetic Engineering and Biotechnology
Research Institute (GEBRI),
University of Sadat, Egypt

ABSTRACT

Increase in the levels of cholesterol is harmful, it is a risk factor for cardiovascular disease. This study was carried out to compare the *in vitro* anti-hypercholesteremia effects of different concentrations of probiotic bacteria (*Lactobacillus casei* and *Lactobacillus plantarum*) and cyanobacteria (*Spirulina platensis*) isolated as intact cells or cell lysates. The results showed that *S. platensis* is more effective. The mixture of cell lysates from *L. casei*, *L. plantarum* and *Spirulina platensis* lowered the level of cholesterol by 65% as compared to the inhibitory effect of *L. casei* (32%), *L. plantarum* (33%) and *S. platensis* (47%). Their significant differences by using the mixture of testing bacteria as intact cells and cell lysate in the percentage of cholesterol reduction.

Key words: Cholesterol, *in vitro*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Spirulina platensis*

INTRODUCTION

Coronary Heart Disease (CHD) is currently a leading cause of death worldwide, this disease is still increasing and has become a true pandemic that respects no borders (WHO., 2009). Elevated blood cholesterol (hypercholesterolemia) is an important risk factor associated with atherosclerosis and coronary heart diseases. Although, there are multiple risk factors for CHD, hypercholesterolemia remains a major determining factor for this pathology (Castelli *et al.*, 1986). To decrease the incidence of CHD, it is therefore necessary to reduce the level of serum cholesterol in hypercholesterolemic subjects (Murray and Lopez, 1997). The WHO has predicted that, by 2030, cardiovascular diseases will remain the leading causes of death, affecting approximately 23.6 million people around the world, the risk of heart attack is three times higher in those with hypercholesterolemia, compared to those who have normal blood lipid profiles (WHO., 2009).

Recent modalities for lowering blood cholesterol levels involve dietary management, behavior modification, regular exercise and drug therapy (Dunn Emke *et al.*, 2001). Pharmacological agents that effectively reduce cholesterol levels are available. But they are expensive and known to have severe side effects (Bliznakov, 2002). Probiotics are defined as living microorganisms, which upon ingestion in certain numbers; exert health effects beyond inherent basic nutrition (Guarner and Schaafsma, 1998). There are many reports describing probiotic health-promoting effects on gastrointestinal infections, anti-microbial and anti-viral activity, improvement in lactose metabolism, reduction in serum cholesterol level and blood pressure, improvement in mineral absorption, stabilization of the gut mucosal barrier, anti-mutagenic and anti-carcinogenic properties, immune system stimulation, anti-diarrheal and anti-constipation properties, urogenital infections and improvement in inflammatory bowel disease (Sanders, 1999; Saarela *et al.*, 2000).

Lactic acid bacteria, predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, constitute a significant proportion of probiotic cultures in nutritional supplements, pharmaceuticals and functional foods (Del Piano *et al.*, 2006). Lactic Acid Bacteria (LAB) with active Bile Salt Hydrolase (BSH) or products containing them have been suggested to lower cholesterol levels through interaction with host bile salt metabolism (De Smet *et al.*, 1998). Klein *et al.* (2008) proposed the mechanism based on the ability of certain probiotic lactobacilli and bifidobacteria to de-conjugate bile acids enzymatically, increasing their rates of excretion.

Cholesterol, being a precursor of bile acids, converts its molecules to bile acids replacing those lost during excretion leading to a reduction in serum cholesterol. This mechanism could be operated in the control of serum cholesterol levels by conversion of deconjugated bile acids into secondary bile acids by colonic microbes. The use of such orally applied microorganisms (probiotics) is a major aim of the concept of functional food. Recently, there has been much interest in LAB, especially lactobacilli, due to their beneficial effects on health including anti-cholesterol, anti-diabetic, anti-pathogenic, anti-carcinogenic properties and stimulation of the immune system (Nagpal *et al.*, 2007). Bengmark *et al.* (1998) reported that, both of *Lactobacillus plantarum* and *Lactobacillus casei* have bile salt hydrolase enzyme, *Lactobacillus plantarum* the predominating *Lactobacillus* species on oral and intestinal human mucosa, has shown the ability to survive the passage through the human gastrointestinal tract and to establish itself for at least a shorter time in the intestine after consumption.

Recently, a trend has been started to add microalgae (cyanobacterial biomasses) into fermented milks in order to increase the functional product characteristics via promoting viability of probiotics as well as to enhance the nutritional attributes (Varga *et al.*, 2002). *Spirulina* is blue-green microalgae, which contain high-anti-oxidant components, abundant amino acids, high-quality proteins, Fe and Ca, unsaturated fatty acids and many types of vitamins, including A, B₂, B₆, B₈, B₁₂, E and K. *Spirulina* have anti-viral, anti-inflammatory and anti-tumor effects and reduce blood lipid profile, blood sugar, body weight and wound healing time. Therefore, they are known as therapeutic and functional food (Dillon and Phan, 1993). It seems that the co-addition of microalgae and probiotics stimulates growth and increases viability and acid production of the probiotic bacteria (Webb, 1982).

The Aim of the present study is to investigate the potential effect of different concentrations of probiotic bacteria (*Lactobacillus casei*, *Lactobacillus plantarum*) in comparison with *Spirulina platensis* (cyanobacteria) and mixtures of them in the reduction of cholesterol *in vitro*.

MATERIALS AND METHODS

Bacterial strains: *Lactobacillus plantarum* p9 and *Lactobacillus casei* ATCC 7469 isolated and identified by

Hoda *et al.* (2010). *Lactobacillus plantarum* p9 was cultivated in nutrient broth (Lab M, IDG, UK) and incubated at 37°C for 24 h and preserved in reconstituted skim milk in Eppendorf tube. *Lactobacillus casei* ATCC 7469 was cultivated in MRS agar medium and incubated at 37°C for 24 h and preserved in reconstituted skim milk in Eppendorf tube. The culture was preserved in reconstituted skim milk in Eppendorf tubes, stored at -80°C with glycerol (20%, v/v). Prior to use, strain was sub cultured (1%, v/v) twice in MRS broth and adjusted at 1×10^7 CFU mL⁻¹.

Isolation and identification of cyanobacteria: Isolation and purification of cyanobacteria were made according to the methods described by Rippka (1988) Briefly, *S. platensis* was isolated after repeated light migrations on solid medium (Zarrouk, 1966).

S. platensis was identified according to Vonshak (1997).

Alga cultivation: The cyanobacterium *S. platensis*, was cultivated in Zarrouk medium (Zarrouk, 1966) at 25±2°C, pH 10 with continuous illumination using cool white fluorescent tubes (2500 Lux) and twice daily shaking by hand for 15 days. Cells were collected by filtration using filter paper 8 mm pore size (Screen printing paper) and was washed with buffer solution (pH 7), diluted to be known volume and processed for further inoculation.

Dry weight measurement: For dry weight measurement homogenous suspension of known quantities of *Spirulina* sample was filtered through screen-printing paper and oven dried at 75°C for 2-6 h.

Cell lysate and intact cells of microbial strain

Preparation of cell lysate: Cells were harvested by centrifugation at 4°C for 30 min (5,000 rpm) after overnight incubation at 37°C and the pellet was washed twice with 20 mM sodium phosphate buffer (SPB, pH 7.4), then re-suspended in SPB. Washed cell suspension was disrupted with an ultrasonic cell disrupter (Brands on 4°C) and filtered (0.45 µm, Millipore). Cell debris was removed by centrifugation (10,000×g for 10 min and concentrations were measured by the Bradford method (Bio- Rad Laboratories) and adjusted to 10 mg mL⁻¹ (Kim *et al.*, 2006).

Preparation of the intact cells: Cells were washed twice with SPB and resuspended in SPB. The total cell number was adjusted to 10⁷ CFU mL⁻¹ (Kim *et al.*, 2006).

Treatments: 1-Standard, 2- *Lactobacillus plantarum* p9 3- *Lactobacillus casei* ATCC 7469, 4- *Spirulina platensis*, 5-*L. plantarum* p9+*L. casei* ATCC 7469, 6-*Spirulina platensis*+*L. casei* ATCC 7469, 7-*Spirulina platensis* +*L. plantarum* p9, 8- *Spirulina platensis*+*L. casei* ATCC 7469+*L. plantarum* p9. All treatments tested as intact cells and cell lysate.

Cholesterol binding assay: Different concentration (200, 400, 600, 800 and 1000 $\mu\text{g mL}^{-1}$) of cell lysates and intact cells for *L. plantarum* p9, *L. casei* ATCC 7469 and *Spirulina* sp. were prepared from stock of ten milligrams of lyophilized cells per mL suspended in 1 mL of cholesterol-ethanol solution (100 μg of cholesterol dissolved in 1 mL of 60% ethanol), vortexed and incubated at 37°C for 1 h in a shaking water bath. The mixture was then centrifuged at 1118 \times g for 10 min and unbound cholesterol in the supernatant was determined by enzymatic analysis and the tests were carried out in triplicate. An enzymatic colorimetric kit used for the determination of cholesterol was obtained from Biodiagnostic Company Dokki, Giza, Egypt (Richmond, 1973). The absorbance of the sample and standard against blank was measured at 517 nm. The percentage of cholesterol lowering effect was determined by this equation:

$$\text{Cholesterol reduction (\%)} = \frac{A_{517 \text{ standard}} - A_{517 \text{ sample}}}{A_{517 \text{ standard}}} \times 100$$

Statistical analysis: Data obtained was subjected to analysis of variance and the means were compared using the Least Significant Differences (LSD) tested for the 0.05 levels, as recommended by Snedcor and Cochran (1982).

RESULTS AND DISCUSSION

Consumption of fermented milk containing bacterial strains provides beneficial effects, such as the reduction of serum cholesterol levels of human (Gilliland *et al.*, 1985; Mann and Spoerry, 1974). The relationship between Lactic Acid Bacteria (LAB) and the serum cholesterol has become important subject for study, studies evaluating this relationship have found that lactobacilli or bifidobacteria can exhibit hypocholesterolaemic properties in animal models (Gilliland *et al.*, 1985; Nguyen *et al.*, 2007) and in humans (Agerbaek *et al.*, 1995; Anderson and Gilliland, 1999).

Several hypotheses have been proposed to explain the mechanism of action of (LAB) lower cholesterol as: Consumption of cholesterol by intestinal bacteria, thus reducing the amount of cholesterol available for absorption (Pigeon *et al.*, 2002; Pereira and Gibson, 2002). Cholesterol may be bound to the bacterial cellular surface (Liong and Shah, 2005) or incorporated into the bacterial cellular membranes (Lye *et al.*, 2010a) or converted into coprostanol by cholesterol reductase, which is produced by strains of lactobacilli (Lye *et al.*, 2010b). Some bacterial species excrete bile salt hydrolase, leading to increased bile excretion in feces (Begley *et al.*, 2006).

Result in Fig. 1a, shows the percentage reduction of cholesterol when incubated with different concentrations of *L. plantarum* intact cells and cell lysate. The reduction of cholesterol increased with increasing concentrations of *L. plantarum* intact cell and cell lysate. *L. plantarum* cell lysate has the highest effect in cholesterol reduction than intact cell. Naruszewicz *et al.* (2002) reported that the supplementation of diet with *Lactobacillus plantarum* 299v

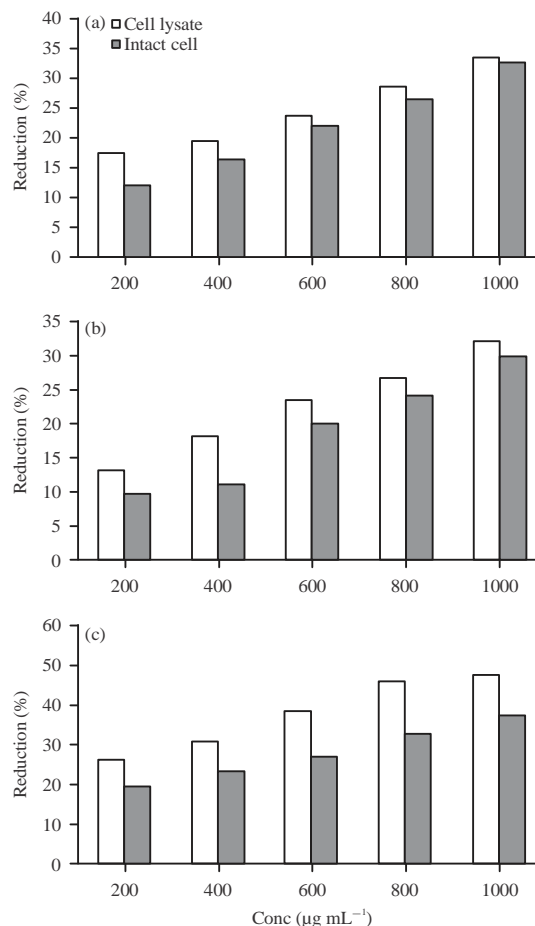


Fig. 1(a-c): Effect of different concentrations of (a) *Lactobacillus plantarum*, (b) *Lactobacillus casei* and (c) *Spirulina platensis* intact cells and cell lysate on reduction of cholesterol

significantly lowered LDL cholesterol concentration. Viljoen and Wierzbicki (2008) suggested that the probiotics reduce serum cholesterol levels due to their capability to compete with cholesterol for intestinal absorption.

Result in Fig. 1b, show the effect of different concentrations of *L. casei* intact cell and cell lysate on lowering cholesterol. The results revealed that the cell lysate was more effective than intact cells. The highest concentrations were caused highest percentage reduction of cholesterol by 32.2% *L. casei* cell lysate.

Result in Fig. 1c, shows the effect of different concentration of *Spirulina platensis* intact cells and cell lysate on reduction of cholesterol *in vitro*. These results indicate that all treatments showed reduction of cholesterol *in vitro*. The highest concentration of *Spirulina platensis* cell lysate (1000 $\mu\text{g mL}^{-1}$) caused the maximum lowering of cholesterol (47.37%).

Figure 2a illustrate the mean percentage of reduction of cholesterol by different concentrations of mixture of *L. plantarum* and *L. casei* intact cells and cell lysate. The obtained results indicate that all concentrations caused

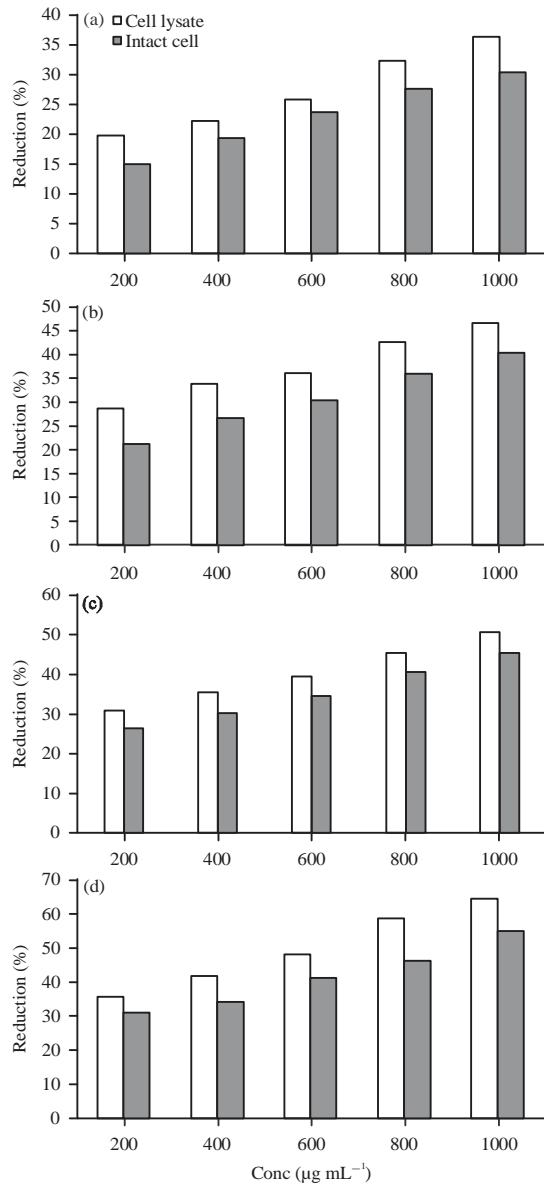


Fig. 2(a-d): Effect of different concentrations of mixture of (a) *Lactobacillus plantarum* and *Lactobacillus casei*, (b) *Spirulina platensis* and *Lactobacillus casei*, (c) *Spirulina platensis* and *Lactobacillus plantarum* and (d) *Lactobacillus plantarum*, *Lactobacillus casei* and *Spirulina platensis* intact cell and cell lysate against cholesterol

reduction of cholesterol and the percentage of reduction increase by increasing the concentrations.

The lowering effects of cholesterol were observed in (Fig. 2b) by effects mixture of *Spirulina platensis* and *L. casei* intact cell and cell lysate on cholesterol *in vitro*. The concentration 1000 µg mL⁻¹ of *Spirulina platensis* + *L. casei* cell lysate and intact cell caused the percentage reduction of cholesterol *in vitro* 46.6 and 40.8 and 40.6, respectively.

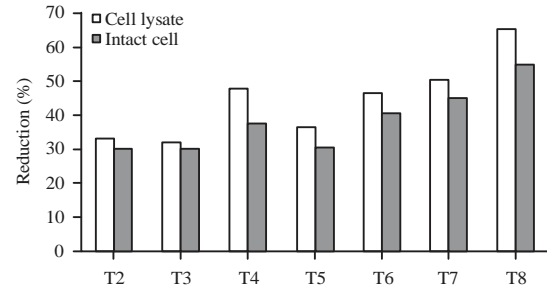


Fig. 3: Comparison between different treatments *Lactobacillus plantarum*, *Lactobacillus casei* and *Spirulina platensis* each and mixture on cholesterol reduction at 1000 g mL⁻¹ of intact cells and cell lysate, T2: *Lactobacillus plantarum* p9, T3: *Lactobacillus casei* ATCC 7469, T4: *Spirulina platensis*, T5: *Lactobacillus plantarum* p9+*Lactobacillus casei* ATCC 7469, T 6: *Spirulina platensis*+ *Lactobacillus casei* ATCC 7469, T7: -*Spirulina platensis*+ *Lactobacillus plantarum* p9, T 8: *Spirulina platensis*+ *Lactobacillus casei* ATCC 7469+*Lactobacillus plantarum* p9

The results in Fig. 2c informed clearly the effect of different concentrations of mixture from *Spirulina platensis*+ *L. plantarum*, intact cell and cell lysate against cholesterol. Results indicate that different concentrations of the mixtures of *Spirulina platensis* + *L. plantarum* (200, 400, 600, 800 and 1000) demonstrate the lowering of cholesterol level *in vitro* for intact cells (26.21, 30.58, 34.46, 40.29 and 44.85%, respectively) and cell lysate (31.06, 35.43, 39.32, 45.14 and 50.48%, respectively).

The data obtained from Fig. 2d show that the mixture of *L. plantarum*, *L. casei* and *Spirulina platensis* cell lysate and intact cells were more effective on cholesterol reduction and increase with concentration from 200-1000 µg mL⁻¹. The results revealed that the mixture was more effective in lowering cholesterol as cell lysate (65%) than intact cells (55%) with concentration of 1000 µg mL⁻¹.

The percentage reduction of cholesterol by *Spirulina platensis* is more effective than *L. plantarum* and *L. casei*, meanwhile the mixture of *L. plantarum* and *L. casei* and *Spirulina platensis* was given the maximum cholesterol reduction. Prakash and Kumari (2011) studied the preparation of low-fat and high-protein frozen yogurt enriched with papaya pulp and *Spirulina* with the objective to find out the optimum level of *Spirulina* that could be incorporated to obtain a better-quality frozen yogurt.

Results of Fig. 3, informed that the percentage reduction of cholesterol by *Spirulina platensis* is more effective than *L. plantarum* and *L. casei*, mean while the mixture of *L. plantarum* and *L. casei* and *Spirulina platensis* was given the maximum cholesterol reduction. Prakash and Kumari

(2011) studied the preparation of low-fat and high-protein frozen yogurt enriched with papaya pulp and *Spirulina* with the objective to find out the optimum level of *Spirulina* that could be incorporated to obtain a better-quality frozen yogurt.

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