Evaluation of Probiotic Bacteria Exo-polysaccharides on Immune System.

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Abstract: Five Lactic acid bacteria (*Lactobacillus rhamnosus; Lactobacillus helveticus; Lactobacillus acidophilus; Lactococcus cremoris;* and *Lactococcus lactis)* were isolated from some Dairy products collected from Kingdom of Saudi Arabia (KSA) markets. Biomass and Exo-polysaccharides (EPS) production by Lactic acid bacteria (LAB) were studied under batch culture conditions using shake flasks. The effect of media type (M17 –MRS);Temperature (32,37& 42 °C); pH levels (5.5,6.5&7.0) and different carbon sources (fructose-sucrose) were investigated to maximize the biomass and Exo- polysaccharides production. The probiotic bacteria exo- polysaccharides obtained immunologically were evaluated using Ten Swiss albino strain mice's. The highest yield of biomass and EPS production were obtained with *Lactobacillus rhamnosus* grown on MRS medium with pH7.0, glucose as a sole carbon source at 37 ° C or 42 °C for 48 hours. The EPS produced by *Lactobacillus rhamnosus* were dosage orally with 10^8 UFC /ml / mice /day for 21 days to experimental group. The percentage of neutrophils (NU%), Lymphocytes (LY%), basophiles (BA%) and eosinophils (EO%) increased significantly ($P \leq 0.05$) in the experimental group (G1) compared to control group (G2). Immunoglobulin IgA showed a significant increase ($P \leq 0.05$) in the experimental group. However, no significant differences between G1 and G2 in immunoglobulin IgG. So. The present study concluded that *Lactobacillus* sp. had a power to activate the immune system.
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Key words: Probiotic bacteria, *Lactobacillus* sp., Exo-polysaccharides Fermentation, Immune system.

1. Introduction

The term " Probiotic " was first used in 1965, by Lilly and Stillwell, to describe substances secreted by one organism which stimulate the growth of another various bacterial genera most commonly used in Probiotic preparations are *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Enterococcus*, *Bacillus* and *Streptococcus*. The Probiotic effects ascribed to Lactic acid bacteria (LAB) and their fermented dairy products aris are not only from peptides and extracellular polysaccharides (Exo-polysaccharides) produced during the fermentation of milk which would allow a better understanding of the functional effects described to them. The mechanisms by which Probiotic bacteria affect the immune system are unknown yet, but many of them are attributed to an increase in the innate or in the acquired immune response.

Probiotics are available to consumers mainly in the form of dietary supplements and foods.They can be used as complementary and alternative medicine (CAM) A group of diverse medical and health care systems,practices,and products that are not presently considered to be part of conventional medicine. There are several reasons that people are interested in probiotics for health purposes. First, the world is full of microorganisms (including bacteria), and so are people's bodies, hand, skin, gut,and in other orifices. Another reason for the interest in probiotic stems from the fact there are cells in the digestive tract connected with the immune system. One theory is that if you alter the microorganisms in a person's intestinal tract (as by introducing probiotic bacteria), you can affect the immune system's defenses**. (Alvarez-Olmose and Oberhelman, 2001)**.The (LAB) may affect pathogens by means of competitive inhibition by competing for growth and there is evidence phagocytosis as well as increasing the proportion of T lymphocytes and Natural Killer cells **(Ouwehand *et al* 2000 and Reid *et al.,* 2003)**. There is a lack of knowledge concerning the immune mechanisms induced by Exo-polysaccharides produced by Lactic acid bacteria.One of the proposed mechanisms by which (LAB) mediate these health benefits is production of Exo-polysaccharides (capsule)**. (Salazer *et al.* 2009)** The (EPS) are associated with the external cell surface, which can be covalently bound forming loosely attached thereby developing a slime layer.**(Ruas and de los Reyes, 2005). (Liu *et al.,* 2011)** investigate that Exo-polysaccharides that stimulate macrophage production of cytokines and showed that certain LAB, such as *Lactobacillus casei, Lactobacillus rhamnosus*, and *Lactobacillus plantarum* enhance both systemic and mucosal immunity. Food containing probiotic bacteria are able to stimulate the immunoglobulin A (IgA) & (IgG) & (IgM) immune response **(Kaila *et al.,***

1992). *In vitro* studies have shown that several LAB strains promote the immunopotentiator capacity of cells of the innate immune system, including macrophages **(Kato *et al.*, 1983)**. It is known that the microflora of the gut stimulates the proliferation of epithelial cells **(Anderson, 2000)** and that colonization of the gut with commensally micro flora influences the development of the immune system **(Heyman *et al.*, 1986)**. Epithelial cells are important as the first line of defense because they are in constant contact with the bacteria and the bacterial products on their apical surface and because they are in close. The present study was carried out to elucidate the role of bacterial (EPS) in activating the immune system.

2-Material and Methods

2-1-Microorganisms

Probiotic bacteria were isolated from some Dairy products in KSA such as: Activia fermented laban & yoghurt full cream & Al Marai fresh laban & Al Marai fresh milk full cream and Vital fresh laban and yoghurt full cream. All strains were identified according to **Bergye's Manual of Systematic Bacteriology. (George *et al*. 2001).**

2-2- Media used.

MRS medium according to **De Mant *et al.* (1960)** and M17 medium according to **Terzoghi and Sandine, (1975)** were used.

2-3- Optimization of biomass and Exo polysaccharides production.

2-3-1-Effect of type of media.

Evaluation of different probiotic bacteria for biomass and EPS production. Isolates were inoculated with a ratio of 2.5% into 100 ml MRS broth or M17 broth in 250 ml capacity conical flasks. All flasks were incubated at 37 °C for 48 hrs.By the end of fermentation period, the cultures were evaluated for biomass net weight g/ l and, EPS concentration g/l.

2-3-2-Factors affecting on biomass and Exo-polysaccharides production.

The shake flasks system was used to study the effect of some factors on biomass and Exo-polysaccharides production by some Lactic acid bacteria isolates from dairy products. Modifications of standard medium (MRS medium) were carried out including the replacement of original carbon source (glucose) with different carbon sources (Fructose – Sucrose). The effect of different levels of pH (5.5, 6.5 & 7.0) and Temperatures (32, 37 & 42 ºC) were also studied to maximizing EPS production.

2-4-Microbiological evaluation.

2-4-1-Probiotic bacteria count.

Probiotic bacteria count (cfu/ml) were determined on MRS and M17 ager according to **De Man *et al.* (1960) and Terzaghi and Sandine, (1975).**

2-4-2-Growth curve:-

An inoculums of 20%(v/v) of isolates was inoculated into 250 ml conical flasks, containing 100ml of MRS or M17 for each media. All flasks were incubated at 37°C. After 48hrs, 1 ml was aseptically with drone from each flask under aseptic condition to determine the optical density (O.D) using Spectrophotometer (model v-200- RS, visible light, LW scientific).at 600 nm. **(Martins and Sa Correia,1994)**.

2-4-3-Biomass net weight:

An inoculums of 2.5%(v/v) of isolates was inoculated into 250 ml conical flasks, containing 100ml of MRS or M17 for each media.All flasks were incubated at 37 °C.After 48 hrs cells were harvested by centrifugation at 5000x for 5 min. The cells were dried at 70 °C for 48 hrs. Then, cells were weighted and calculated per 1 liter.

2-5- Recovery of Exo-polysaccharides (EPS):

The method of Pablo and**(Pablo an Analia, 2001)**was followed.100ml fermented medium was heated in boiling water bath for 15min in order to release the EPS attached to cells and to inactivate the enzymes. Cells were removed by centrifugation at 10000 g/15 min, at 20°C Polysaccharides were precipitated from the supernatant by the addition of two volumes of cold ethanol (96%), maintained at 4 °C for 24hrs and then centrifuged at 10000g.Pellets were suspended in 1L of hot water and dialyzed against distilled water for 24hrs at 4 °C.

2-5-1- Total sugars:

Total sugars were determined using the phenol sulfuric method as described by **Dubois, (1956).**

2-6- Calculation.

The specific growth rate (μ); Hourly growth rate were calculated from the exponential phase Yield factor; carbon utilization efficiency and conversion coefficient; yield and productivity were also calculated. according to **Painter& Marr, (1963).**

2-7- Experimental animals:

Ten adult males of Swiss albino strain mice weighing 25-30 grams and aged 2.5 to 3 months were obtained from animal house and divided into two groups. First group (G1) was the experimental group which dosage orally with 108 UFC/ml/mice/day **(Galdeano and Perdigon, 2006)** for along 21 days and the second group (G2) was control which dosage orally with saline. The animals were fed balanced rodents food and water *Ad libitum* At the end of experiment the heperinazed blood samples were collected by heart puncture to determined the differential counts of white blood cells (WBc). Blood plasma was then obtained by centrifugation at 5000 rpm for 15 minutes at 4ºC, and the plasma (supernatant) were transferred to clean plastic vials and stored at -20ºC until immunoglobulin's IgG and IgA analysis.

3.Results and Discussion:-

Selection of suitable medium for growth and EPS production:-

Growth parameters of Lactic acid bacteria grown on two types of media (MRS – M17) were recorded in Table (1), and (Fig1A& B). On the two types media used(MRS – M17) the growth parameters, { Specific growth rate (μ); hourly growth rate (HGR); doubling time (td); number of generation (N) and multiplication rate (MR) } of the five LAB used were ranged between 0.235 – 0.327; 0.94 – 1.33; 2.12 – 2.95; 2.71 – 3.77 & 0.34 – 0.47 and 0.295 – 0.809; 1.18 – 3.24; 0.86- 2.35; 3.4 – 7.69 & 0.57 – 1.16 respectively. This data sported by the results recorded by **(Degeest and De Vuyst, 2001).**

Table:(1).Growth parameters different of LAB growing on two types of media.

Lactic acid bacteria strains	Media used									
	MRS					M17				
	μ	HGR	td	N	MR	μ	HGR	td	N	MR
Lactobacillus rhamnosus	0.235	0.94	2.95	2.71	0.34	0.669	1.18	1.04	7.69	0.96
Lactobacillus. helveticus	0.327	1.31	2.12	3.77	0.47	0.809	2.43	0.86	9.3	1.16
Lactobacillus acidophilus	0.286	1.14	2.42	3.31	0.41	0.606	3.24	1.14	7.02	0.88
Lactococcus. cremoris	0.273	1.09	2.54	3.15	0.39	0.295	3.14	2.35	3.40	0.43
Lactococcus lactis	0.265	1.33	2.62	3.06	0.38	0.393	1.57	1.76	4.55	0.57

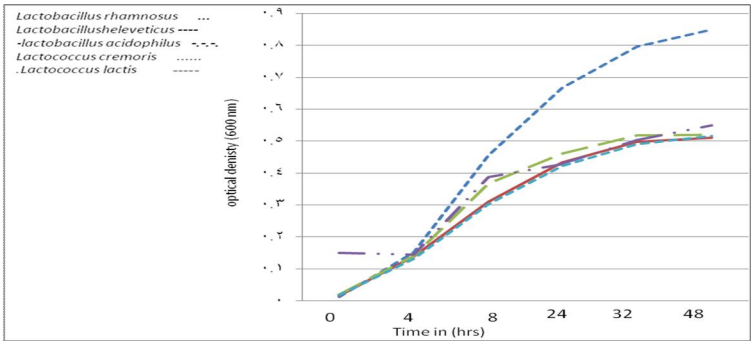

Fig.1 A: Growth curve of Lactic acid bacteria growing on MRS medium.

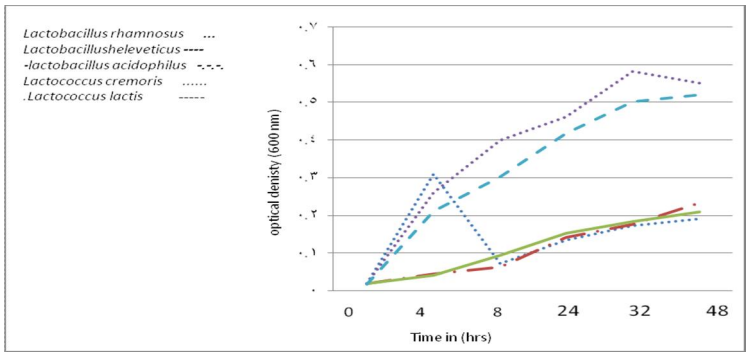

Fig.1 B: Growth curve of Lactic acid bacteria growing on M17 medium

The growth net weight and EPS production (g / L) were recorded in Table (2). In general, the biomass and EPS in MRS medium was superior than the biomass and EPS obtained on M17 medium except, *Lactococcus cremoris* and *Lactococcus lactis*. The highest results (biomass and EPS) were obtained *Lactobacillus rhamnosus* being, 6.3 g/ L and 0.86 g / L, respectively.

Table (2): Exo polysaccharides (EPS) production (g / L) and Biomass net weight (g / L) of different *Lactobacillus* strains growing in different media.

Lactic acid bacteria	Biomass net weight g / L		EPS production g / L	
	Media used		Media used	
	M17	MRS	M17	MRS
Lactobacillus rhamnosus.	0.72	6.3	0.145	0.86
Lactobacillus. helveticus	0.72	3.76	0.145	0.449
Lactobacillus acidophilus	0.69	3.56	0.144	0.431
Lactococcus. cremoris	5.73	4.35	0.698	0.547
Lactococcus lactis	4.94	3.45	0.591	0.411

Effect of different temperature on EPS production.

Effect of different temperature on Lactic acid bacteria growth and Exo-polysaccharides production are shown in Fig.(2 A &B). In general, there is a direct relation between biomass obtained, Exo-polysaccharides production and growth temperature with all Lactic acid bacteria used.

Temperature increased from 32 °C to 42 °C enhancing the biomass and Exo-polysaccharides production being 5.9 and 4.96 folds, respectively. The obtained results is parallel with the results recorded by **Gamar *et al.* (2004) and Belma Aslim, (2005).**

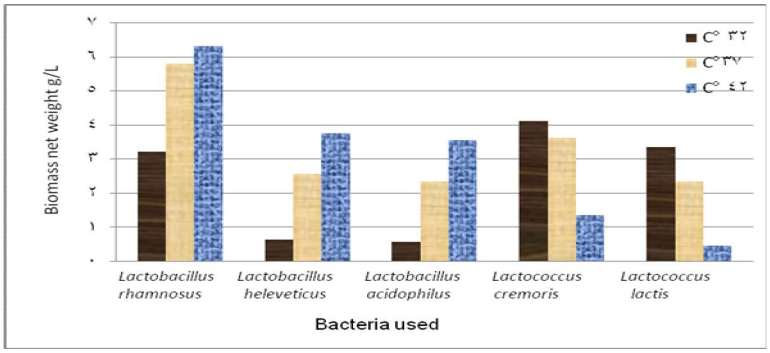

Fig. 2 A: Biomass net weight (g / L) of different Lactic acid bacteria growing on MRS medium at different temperature degree.

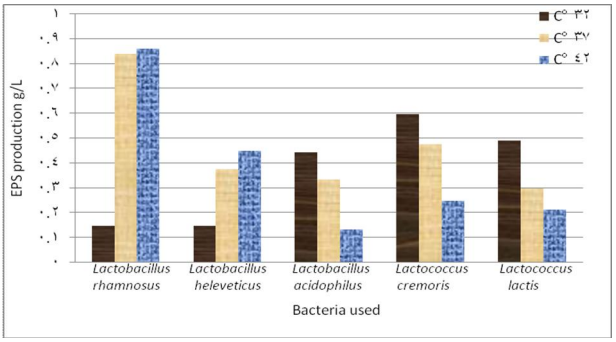

Fig. 2 B: EPS production (g / L) of different Lactic acid bacteria growing on MRS medium at different temperature degree.

Effect of initial pH on the biomass and EPS

Data illustrated in Fig (3 A & B) clearly show that, the maximum production with all Lactic acid bacteria used were obtained with pH 7 followed by pH6.5 and 5.5 being 6.30, 5.79, 3.21; 3.76, 2.57, 0.63; 3.56, 2.35, 0.57; 4.35, 3.62, 1.10; 3.45, 2.34, 0.45 and 0.860, 0.841, 0.145; 0.449, 0.374, 0.145; 0.431, 0.332, 0.144; 0.547, 0.574, 0.298; 0.411, 0.297, 0.291, respectively. **Gamar *et al.* (1997)** Reported that, the initial pH effect on the growth of microorganism and EPS Production,this results support the results obtained.

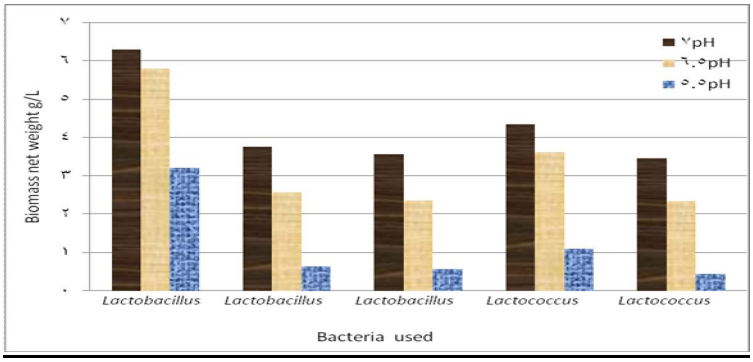

Fig.3 B: Biomass net weight (g /L) of different Lactic acid bacteria growing on MRS medium affected by initial pH

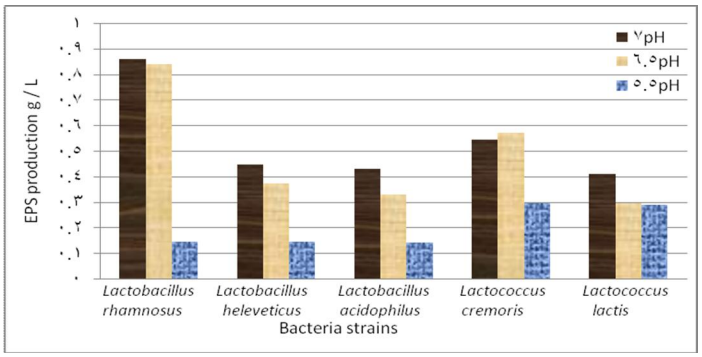

Fig.3 B: EPS production (g/L) of different Lactic acid bacteria growing on on MRS medium affected by initial pH.

Effect of different carbon sources on biomass and EPS production.

It's well known that different carbon compounds could be used as energy and carbon sources for growth of Lactic acid bacteria. Therefore, it was found of interest to study the influence of different carbon sources on growth and EPS production by different Lactic acid bacteria tested. Data presented in Fig (3A&B) show that, glucose gave the maximum yield of biomass and EPS production being, 7.89 g / L and 0.91 g / L, respectively with *Lactobacillus rhamnosus*.No grand difference of biomass and EPS production were found between the other carbon sources or Lactic acid bacteria tested. (see Fig 3A & B).

Data obtained noticed also that, Fructose gave a good results (biomass and EPS production) being,5.79 g / L and 0.87 g / L, respectively, with all Lactic acid bacteria used

We have shown that growth yield and EPS production are influenced by the carbon sources used. When *Lactobacillus rhamnosus* was grown in a chemically defined medium on mannose or glucose, lactose, it was found that EPS increased three or four time **(Zisu and Shah, 2000) &(Degeest and De Vuyst, 2000).** On the other hand**, Looijesteijn and Hugenholtz, (1999)** reported That, *Lctobacillus cremoris* NIZO B40 produced more EPS with glucose than the fructose.

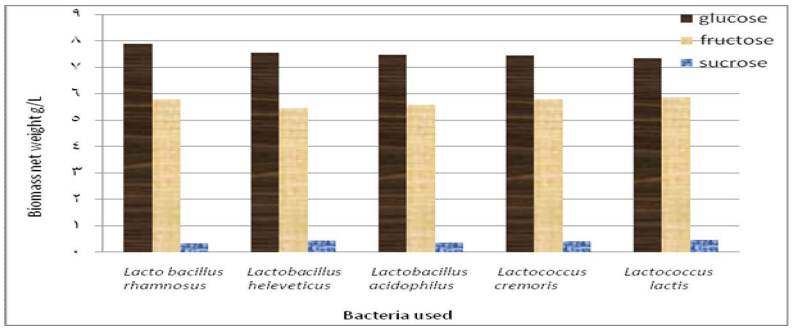

Fig. 3 A: Biomass production of different Lactic acid bacteria growing on MRS medium affected by different carbon sources.

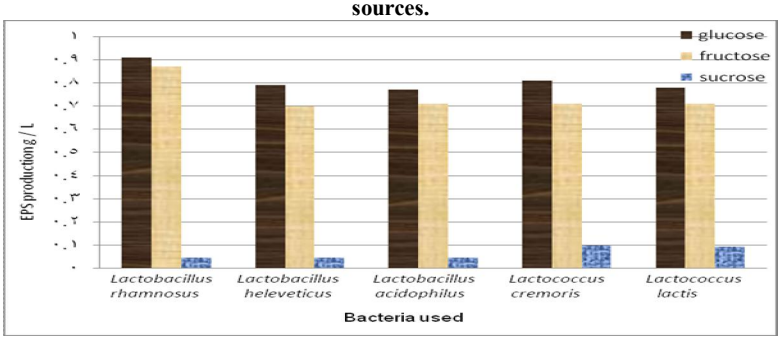

Fig. 3 B: EPS production of different Lactic acid bacteria growing on MRS medium affected by different carbon sources.

Effect of *Lactobacillus* sp. on immune system.

The present study shows that (Table: 3) the percentages of neutrophils (NU%), lymphocytes (LY%), basophiles (BA%) and eosinophils (EO%) increased significantly ($P \leq 0.05$) by treatment, while monocyte (MO %) was decreased in the second group G2 cells in the experimental group (G1). Immunoglobulin IgA showed a significant increased ($P \leq 0.05$) in the experimental group (G1). However, no significant differences between two groups in immunoglobulin IgG.

Table (3): Effect of probiotic bacterial polysaccharides on differential counts of blood and immunoglobulin's IgG. and IgA in Swiss albino mice.

Group	No. of Animals	NU (%)	LY (%)	MO (%)	EO (%)	BA (%)	IgG (mg/dl)	IgA (mg/dl)
G1 (Exp.)	5	70.12[a] ±.066	37.0[a] ±. 311	2.83 ±.028	4.57[a] ±.044	0.611[a] ±.022	1312.22 ±90.54	256.41[a] +15.6
G2 (control)	5	51.61[b] ±.061	29.30[b] ±.067	3.01 ±.024	1.67[b] ±.047	0.12[b] ±.082	1358.19 ±81.22	392.41[b] +17.2

Different letters means significant difference ($P<0.05$) (in column).

The abovementioned results were in agreement with **Fanning *et al.*(2011)** who found that This the serum antibody subtypes IgG3,IgG1, and IgG2a. mice treated with the × 109 EPS+ or EPS− *B. breve* strains on 3 consecutive days had no detectable serum IgG3 titers above preimmune levels. Although overall of IgA titers were a significantly higher ($P < 0.01$) level in mice treated with the EPS− *B. breve* strain of BALB/c mice.

Moreover**, Galdeano and Perdigon (2006)** studied that the influence of the probiotic bacterium *Lactobacillus casei* in the expression of receptors involved in the innate immune response, this bacterium was orally administered to BALB/c mice. They noticed that the main immune cells implicated are those involved in the innate immune response (macrophages) and that the T lymphocytes cell population would be involved in the immune activation observed by in vivo studies in conventional animals. that the main immune mechanism induced by the probiotic strain *L. casei* CRL 431 is the innate immunity with an influence in the clonal expansion of the IgA B-cell population. They also determined that histological slices of mononuclear cells from Peyer's patches and the number of IgA cells were increased with respect to the untreated control. So, The present study concluded that *Lactobacillus* sp. had a powerful to activate the immune system.

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