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Evaluation Activity of S-layer Proteins and Filtrate of *Lactobacillus spp.* Against Some Pathogenic Microorganisms and as Antitumor Agent *in vitro*

A Thesis

Submitted to the College of Science /Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

Raghad Abdullah Hassan

B.Sc. Biotechnology/ College of Science/ Al-Nahrain University, 2006

Supervised by

Dr. Maysaa Ch. AL-Yas — **Instructor**

Dr. Amina N. AL-Thwani — **Professor**

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Supervisors Certification

We, certify that this thesis entitled " Evaluation activity of S-layer proteins and filtrate of *Lactobacillus spp.* against some pathogenic microorganisms and as antitumor agent *in vitro*" was prepared by "Raghad Abdullah Hassan" under our supervision at the College of Science/Al-Nahrain University as a partial fulfillment of the requirements for the degree of Master of Science in Biotechnology.

Signature
Supervisor:Dr.Amina N.AL-Thwani
Scientific Degree: Professor
Date:

Signature
Supervisor: Dr. Maysaa Ch. AL-Yas
Scientific Degree: Instructor
Date:

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature
Name: Dr. Kadhim M. Ibrahim
Scientific Degree: Professor
Title: Head of Biotechnology Department
Date:

Committee Certification

We, the examining committee, certify that we have read this thesis entitled " Evaluation activity of S-layer proteins and filtrate of *Lactobacillus spp.* against some pathogenic microorganisms and as antitumor agent *in vitro*" and examined the student "Raghad Abdullah Hassan" in its contents and that in our opinion , it is accepted for the degree of Master of Science in Biotechnology.

(Chairman)
Signature
Name: Dr. Abdul W. Baqir
Scientific Degree: Professor
Date:

(Member)
Signature
Name: Dr.Ghazi M. Aziz
Scientific Degree: Assistant Professor
Date:

(Member)
Signature
Name: Dr.Shahlaa M. Salih
Scientific Degree: Instructor
Date:

(Member/Advisor)
Signature
Name: Dr. Amina N. AL-Thwani
Scientific Degree: Professor
Date:

(Member/Advisor)
Signature
Name: Dr. Maysaa Ch. AL-Yas
Scientific Degree: Instructor
Date:

I, hereby certify upon the decision of the examining comm

Signature
Name: Dr. Laith A. Z. Al-Ani
Scientific Degree: Assistant Professor
Title: Dean of College of Science
Date:

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Summary

This project was conducted to evaluate the activity of S-layer proteins isolated from *Lactobacillus* in comparison with the activity of concentrated filtrate of *Lactobacillus* against some pathogenic microorganisms and against tumor cell lines *in vitro*.

Twelve isolates of *Lactobacillus spp.* obtained from, vinegar, human milk, cow milk, yoghurt and vagina, were used to detect the S-layer protein (Slp) by Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) then extracted it by excised the Slp pand and treated with 6M guanidin hydrochloride (G-HCl) to eluted the protein from gel. The Molecular weights (MW) of Slps were estimated between (37-63 kDa) depending on the *Lactobacillus* species. The concentrations of Slp were estimated by using a Kit based on the Biuret method. One isolate of each of *Lactobacillus acidophilus* and *Lactobacillus casei*, were selected depending on the MW and concentrations of S-layer proteins.

The inhibitory effect of *Lb. acidophilus* and *Lb. casei* was determined against pathogenic microorganism; *Pseudomonas aeruginosa* , *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Candida albicans* on solid and liquid MRS media. Results revealed that less inhibitory activity against tested microorganisms was detected on the solid medium, compared to the liquid one. Greatest inhibitory effect of *L. acidophilus* and *L. casei* was appeared against *P. aeruginosa* when the zone of inhibition reached to 24 and 22 mm, respectively. It appeared that the inhibitory effect of *L. acidophilus* was more than that of *L. casei* against most of tested microorganisms, while S-layer proteins have no effect against pathogenic microorganisms.

Minimum inhibitory concentrations (MICs) of *Lb. spp.* concentrated filtrates were determined. Results showed 40% and 50% of the concentrated filtrate of both *Lactobacilli* were the MIC for *P. aeruginosa, E. coli*, respectively, where as MIC for *Sal. typhimurium* and *C. albicans* it was 60%, while 60% and 50% of *L. acidophilus* and *L. casei* respectively, were MIC against *Staph. aureus.* At such MIC,s of *Lb. spp.,* adhesion of *E. coli* and *Staph. aureus* to the uroepithelial cells was minimized when the average decreases recorded were (5-12) and (4-9) bacteria/cell after they were (50-60) and (29-35) bacteria/cell, respectively. Adhesion of *E. coli* and *Staph. aureus* to the uroepithelial cells was also decreased by S-layer proteins with average decreased (3-9) bacteria/cell for both tested bacteria.

When the cytotoxic activity of S-layer proteins and *Lactobacillus* concentrated filtrate by using different concentrations (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) was applied against two tumor cell lines (RD and L20B) and incubated for 48 hr., results confirmed that all extracts (S- layer proteins and filtrate) have cytotoxic effects. Moreover, *Lactobacillus* concentrated filtrate had the highest significant cytotoxic effect on growth of both tumor cell lines when compared with S-layer proteins.

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List of Abbreviations

Abbreviation	*Mean*
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CD	Cluster of differentiation
C-terminal	Carboxyterminus
DNA	Deoxyribonucleic acid
D.D.W	Deionized Distilled Water
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polysaccharides
GHCl	Guanidine hydrochloride
GI	Gastrointestinal
GIT	Gastrointestinal tract
GRAS	Generally Recognized As Safe
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton
L.	*Lactobacillus*
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
µg/ml	Microgram per milliliter

MIC	Minimun Inhibitory Concetration.
MNNG	*N*-methyl-*N*- nitro-*N*-nitrosoguanidine
MRS	de Manns Rogosa Sharp
MW	Molecular weight
N-terminal	Aminoterminal
P	Probability
PG	Peptidoglycan
pIs	Isoelectric point
ppm	Part per million
RPMI-1640	Rosswel Park Memorial Institute
SCWP	Secondary cell wall polymers
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFM	Serum free media
S-layer	Surface layer
SLH	S-layer homologous
Slp	Surface layer protein
spp.	Species
TEMED	Tetramethylethylenediamine
Th	T helper
TNF	Tumor necrosis factor
UEP	Uroepithelial

1.1 Introduction:

Probiotics lactic acid bacteria (LAB) have been expected to become a useful tool that could be used as a preventive substance instead of antibiotics. However, heavy use of antibiotic has become a major problem, since it results in drug-resistant bacteria, thus, alternative and non-pharmaceutical strategies for controlling enteropathogenic bacterial infection has been sought. Infection by oral challenge with such enteropathogens as *Salmonella typhimurium*, *Escherichia coli*, *Shigella sonnei* and *Listeria monocytogenes* has been efficiently suppressed by probiotic LAB feeding to rodents (Michail and Abernathy, 2002).

The administration of LAB contained in fermented foods, especially dairy products, has been found to exhibit a range of physiological and therapeutic effects, including enhancement of non-specific and specific immune responses, suppression of intestinal infection and alleviation of food allergies. However, the protective and immune-enhancing effects of probiotic LAB are known not as genus- or species-specific, but as strains. Accordingly, probiotic LAB strains have become very important in the fields of nutrition, health, and food for research and commercial development.

Probiotics LAB have mostly been found in animal sources, dairy products, human and animal intestines (Ishida-Fujii *et al*., 2007). From the past century, the beneficial role of probiotic bacterium in the intestinal lumen were described and many clinical benefits to these specific non pathogenic organism were studied like diarrhea treatment , antimicrobial activity , anticarcenogenic activity , immune modulation , reduction of cholesterol level and other (Oyetayo and Oyetayo , 2005).

The consumption of probiotic cultures may decrease cancer risk. The mechanisms by which lactic acid bacteria inhibit colon cancer may include alteration of the metabolic activities of intestinal microflora, alteration of

physicochemical conditions in the colon, binding and degradation of potential carcinogens, quantitative and qualitative alterations in the intestinal microflora incriminated in the production of carcinogens, production of antitumorigenic or antimutagenic compounds, enhancing the host's immune response and effects on the physiology of the host (Harish and Varghese, 2006).

An important property proposed for a probiotic bacterium is the ability to adhere and colonize host tissues, which enhances multiplication and survival of bacteria in the host and prevents colonization by pathogenic bacteria. Suppression of the growth of pathogens can also be achieved through competition for nutrients as well as by production of bactericidal components, such as bacteriocins, lactic acid or hydrogen peroxide (Reid and Burton, 2002).

Lactobacilli interact with the host via several distinct surface components. Adhesion to host tissues is considered to be the first step in bacterial colonization. The role of proteinaceous surface molecules in adhesion has been proposed in several studies (Lorca *et al.*, 2002). Like many other bacteria, several species of *Lactobacillus* have a surface (S-) layer as the outermost component of the cell (Åvall- Jääskeläinen and Palva, 2005). S-layers are periodic crystalline arrays that are composed of protein or glycoprotein subunits, which form a solid layer to cover the whole cell surface (Sára and Sleytr, 2000). The function of *Lactobacillus* S-layers characterized so far is involved in mediating adhesion to different host tissues. In addition to surface layer proteins (Slps) adhesive properties, the very large number of S-layer subunits present on the cell surface has prompted research aiming at the use of S-layers as a vehicle for the delivery of biologically active compounds, such as drug molecules, antibodies, enzymes and vaccine antigens (Sleytr *et al.*, 2007).

The members of the genus *Lactobacillus* are important residents of the gastrointestinal (GI) microbiota and have been subjects of increasing interest due to their possible role in the maintenance of GI health. Because of this putative health promoting properties, *Lactobacillus* species are widely used as probiotics.

Aims of the study:

- Extraction S-layer proteins from *Lactobacillus spp.* of different sources.
- Evaluating antimicrobial and antitumor activity of S-layer proteins in comparison with concentrated filtrate of *Lactobacillus in vitro*.
- Evaluating the activity of S-layer proteins and *Lactobacillus* concentrated filtrates to inhibit the adhesion of some pathogenic bacteria *in vitro*.

1.2 Literature Reviow

1.2.1 Probiotics:

A probiotic is defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/ WHO, 2002). Probiotics are selected; viable microbial dietary supplements that, when introduced in sufficient quantities, beneficially affect human organism through their effects in the intestinal tract (Holzapfel and Schillinger, 2002).

Probiotics have many attributes including the lack of adverse side effects associated with their use. The most frequently used genera fulfilling these criteria are *Lactobacilli* and *Bifidobacteria* (Harish and Varghese, 2006).

Overall, studies in *in vitro* systems and in a wide range of animal models provide considerable evidence that probiotics, and to a lesser extent prebiotics, have the potential to reduce colon cancer risk. The evidence from humans is less compelling, but nevertheless is suggestive of a cancer-preventing effect of fermented foods. The data from animal studies would suggest that using a combination of pro- and prebiotics may be the most effective strategy to maximize any anticarcinogenic effects **(**Burns and Rowland, 2000**)**. A prebiotic is defined as "nonabsorbable food components that beneficially stimulate one or more of the gut-beneficial microbe groups and thus have a positive effect on human health" (Gibson and Roberfroid, 1995). The concept of pre and probiotics is presented in fig (1-1).

There are a large number of probiotics currently used and available in dairy fermented foods, especially in yogurts. Lactic acid bacteria constitute a diverse group of organisms providing considerable benefits to humankind, some as natural inhabitants of the intestinal tract and others as fermentative lactic acid bacteria used in food industry, imparting flavor, texture and possessing preservative properties. Beyond these, some species are administered to humans as live microbial supplements, which positively

influence our health mainly by improving the composition of intestinal microbiota and relieve some abdominal pain. For this reason, they are called probiotics (Grajek *et al*., 2005).

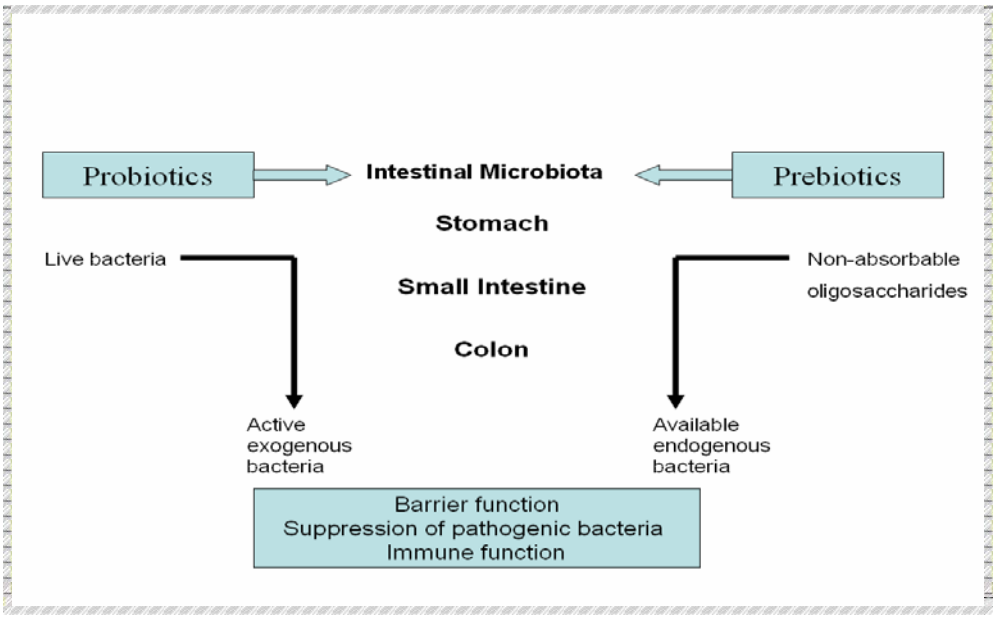


Fig (1-1): The probiotic and prebiotic concepts: altering the composition of intestinal microbiota by viable bacterial supplements versus non absorbable bacterial substrates. (Harish and Varghese, 2006).

Their beneficial effects may be mediated by direct antagonistic effect against specific groups of organisms, resulting in a decrease in numbers, or by an effect on their metabolism, or by stimulation of immunity. Probiotics antagonize pathogen through production of antimicrobial and antibacterial compounds and by compete with pathogens for available nutrients and for binding receptor sites that pathogens occupy (Kailasapathy and Chin, 2000 ; Rolfe, 2000). Probiotics reduce gut pH by stimulating the lactic acid producing microflora (Langhendries *et al*., 1995) improve immune function and stimulate immunomodulatory cells (Isolauri *et al*., 1995; Rolfe, 2000)

The resistance of people in developing countries to diseases can be improved upon by promoting the consumption of locally fermented foods that are rich in probiotic organisms. (Oyetayo and Oyetayo, 2005).

1.2.2 Probiotic microorganisms:

To achieve a probiotic status, microorganisms must fulfill a number of criteria related to safety, functional effects and technological properties (FAO/ WHO, 2001).

Ouwehand *et al*., in 1999 recorded that the probiotic microorganisms such as *Lactobacillus* and *Bifidobacteria* should not be pathogenic, have no connection with diarrhoeagenic bacteria and no ability to transfer antibiotic resistance genes, as well as be able to maintain genetic stability. To be recognized as functional food components, they should demonstrate the following properties: acid- and bile-stability, resistance to digestive enzymes, adhesion to intestine surface, antagonistic activity against human pathogens, anti-carcinogenic and anti-mutagenic activity, cholesterol-lowering effects, stimulation of the immune system without inflammatory effects, enhancement of bowel motility, maintenance of mucosal integrity, improvement of bioavailability of food compounds and production of vitamins and enzymes.

-The ideal probiotic microorganisms should have the following characteristics (Muhammed and Lakshmi, 2007):

- Non pathogenic to humans.
- High tolerance to bile and gastric acidity.
- Capability for easy proliferation in vivo.
- High survival rate through processing conditions (during harvesting, drying etc.)
- High stability at room temperature separately or when mixed with other ingredients.
- Lack of potential to develop virulence.

1.2.3 Lactic acid bacteria: classification and physiological characteristics:

Lactic acid bacteria (LAB) are a physiologically diverse group of organisms, which can be generally described as Gram-positive, non-sporing cocci or rods with lactic acid as the major product of carbohydrate fermentation. Traditionally, LAB comprises four genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. However, several new genera have been suggested for inclusion in the group of LAB due to taxonomic revision (Axelsson, 1998). The genus *Streptococcus* has been reorganized into *Enterococcus*, *Lactococcus*, *Streptococcus* and *Vagococcus*.

The "classical" classification schemes of LAB mainly relied on investigation of phenotypic characters. The introduction of modern molecular biology methods, in particular the comparison of ribosomal DNA sequences, has resulted in major revisions in LAB taxonomy and led to the introduction of several new genera into this group (Sillanpää, 2001).

Lactic acid bacteria are historically defined as a group of microaerophilic, Gram-positive organisms that ferment hexose sugars to produce primarily lactic acid. This functional classification includes a variety of industrially important genera, including *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus* species. The seemingly simplistic metabolism of LAB has been exploited throughout history for the preservation of foods and beverages in nearly all societies dating back to the origins of agriculture (Grajek *et al*., 2005). Based on morphological and biochemical characters, these presumptive lactic acid bacterium isolates were divided into 10 groups that included members of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. (Ennahar *et al*., 1998)

1.2.4 Antimicrobial compounds produced by lactic acid bacteria:

Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Oyetayo *et al*., 2003). The antimicrobial compounds produced by LAB can inhibit the growth of pathogenic bacteria of possible contaminants in the fermented products (Cintas *et al*., 1998).

1.2.4.1 Organic acids:

Fermentation by LAB is characterized by the accumulation of organic acids with accompanying reduction in pH. The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules (Podolak *et al*., 1996). Kashket in 1987 proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. Also, Earnshaw (1992) found that the undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems.

Lactic acid is the major metabolite of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts. However, different microorganisms vary considerably in their sensitivity to lactic acid. At pH 5.0 lactic acid was inhibitory toward spore-forming bacteria but was ineffective against yeasts and moulds (Woolford, 1975).

Acetic and propionic acids produced by LAB strains through heterofermentative pathways, may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang *et al*., 1986). They are more antimicrobially effective than lactic acid due to their higher percent of undissociated acids than lactic acid at a given pH (Earnshaw, 1992).

1.2.4.2 Hydrogen peroxide and carbon dioxide:

Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine hydroxy dinucleotide (NADH) peroxidase. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability. H_2O_2may also be as a precursor for the production of bactericidal free radicals such as superoxide (O2 -) and hydroxyl (OH**.**) radicals which can damage DNA (Yang, 2000). It has been reported that the production of H_2O_2 by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus*, *Pseudomonas* sp. and various psychotropic microorganisms in foods (Cords and Dychdala, 1993).

Carbon dioxide is mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, Eklund (1984) found that CO_2 may be play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability.

Carbon dioxide can effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotropic bacteria (Yang, 2000). The degree of inhibition by CO_2 varies considerably between the organisms. CO_2 at 10% could lower the total bacterial counts by 50%

(Wagner and Moberg, 1989) and at 20-50% it had a strong antifungal activity (Lindgren and Dobrogosz, 1990).

1.2.4.3 Aroma components:

Diacetyl is produced by strains within all genera of LAB by citrate fermentation. The antimicrobial effect of diacetyl has been known since the 1930s. It inhibits the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization (Yang, 2000). Jay (1982) showed that Gram-negative bacteria were more sensitive to diacetyl than Gram-positive bacteria; the former was inhibited by diacetyl at 200 μg/mL and the latter at 300 μg/mL.

Acetaldehyde is produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* by the action of a threonine aldolase, which cleaves threonine into acetaldehyde and glycine. Since *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in yoghurt cannot metabolize acetaldehyde, it accumulates in the product at a concentration of about 25 ppm. Acetaldehyde at 10-100 ppm inhibits the growth of *Staphylococcus aureus, Salmonella typhimurium* and *E. coli* in dairy products (Yang, 2000).

1.2.4.4 Fatty acids:

Under certain conditions, some *Lactobacilli* and *Lactococci* possessing lipolytic activities may produce significant amounts of fatty acids, e.g. in dry fermented sausage (Sanz *et al*., 1988) and fermented milk (Rao and Reddy, 1984). The antimicrobial activity of fatty acids has been recognized for many years. The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal activity of fatty acids is dependent on chain length, concentration, and pH of the medium (Gould, 1991). The antimicrobial action of fatty acids has been thought to be due to the undissociated molecule, not the anion, since pH had profound effects on their activity, with a more rapid killing effect at lower pH (Kabara, 1993).

1.2.4.5 Bacteriocins:

One class of *Lactobacillus* proteins important in survival within the host is the bacteriocins, which are produced by several *Lactobacillus* species and are antimicrobial against other microbes. These bacteriocins have a role in food industry, where they prevent spoilage, and promote quality of the products, but they are also proposed to suppress the growth of harmful bacteria species in the gastrointestinal tract and thus may have potential in clinical applications (Cotter *et al.*, 2005). Bacteriocins of *Lactobacillus* are inhibitory against several pathogens, such as *Helicobacter pylori* (Kim *et al.*, 2003) and *Listeria monocytogens* (Ghalfi *et al.*, 2006) also; it works against heterologous *Lactobacillus* species (Ouwehand, 1998).

Bacteriocins are proteinaceous compounds whose structures are diverse but classified into several classes with certain common structures, class I, antibiotics which are small peptides (e.g. nisin), class II, small heat-stable peptides, class III, large heat-labile proteins, and class IV, complex bacteriocins which are not well defined their mode of action generally targets cytoplasmic membrane and forms pore resulting in efflux of ions and sometime also ATP (Savadogo *et al*., 2006).

1.2.5 The genus *Lactobacillus:*

Members of the genus *Lactobacillus* are not only found in plants and in plant-derived materials, such as silage, grains and foods, but also in the gastrointestinal tract (GIT) of humans and animals (Stewart, 1997). Species of *Lactobacillus* form the most numerous genuses in the heterogeneous group of LAB. *Lactobacilli* are Gram-positive, facultative anaerobic, rods or coccobacilli, non-sporeforming, catalase negative, cytochrome absent, nutritionally demanding, acid-tolerant, and strictly fermentative bacteria producing lactic acid as the primary end product (Makarova *et al*., 2006).

Kingdom:	*Bacteria*
Division:	*Firmicutes*
Class:	*Bacilli*
Order:	*Lactobacillales*
Family:	*Lactobacillaceae*
Genus:	*Lactobacillus*

Dellaglio and Felis (2005) described approximately one hundred species belonged to the genus *Lactobacillus* these are subdivided by 16S rRNA analysis, DNA-DNA hybridization and other phylogenetic methods, into eight major groups; *Lactobacillus buchneri, delbrueckii, casei, plantarum, reuteri, sakei, salivarius,* and *brevis* group.

The *Lactobacillus delbrueckii* group was later renamed the *Lactobacillus acidophilus* group. Based on DNA-DNA-hybridization studies, the large *L. acidophilus* group was divided into six groups, A1-A4 and B1-B2, that corresponded to previously assigned species, *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovorus* (A3), *L. gallinarum* (A4), *L. gasseri* (B1) and *L. johnsonii* (B2) (Jakava-Viljanen, 2007).

Although six genome clusters of the *L. acidophilus* group have been designated as separated species with validly published names, they are difficult to distinguish solely on the basis of phenotypic characteristics. The taxonomy of the genus *Lactobacillus* has changed considerably as a consequence of the introduction of new genomic techniques for the identification of *Lactobacillus*.

The heterogeneity posses challenges and opportunities when characterizing or exploiting individual strains. At the time of writing (August 2007), 11 *Lactobacillus* genome sequences have been published, and at least 12 more sequencing projects are ongoing (Makarova and Koonin, 2007).

1.2.6 Therapeutic effect of LAB:

Lactic acid producing bacteria have many beneficial effects to promote human health. probiotics produce metabolites such as lactic acid and antibiotic-like substances called bacteriocins that suppress the growth of putrefactive microorganisms. Their metabolic activities also help in the pre-digestion of food components and the production of vitamins B, and improve the bioavailability of minerals and other nutrients, for example, isoflavones from soy milk (Pham and Shah, 2007). Additionally, Shahani in 1983 mentioned that cell wall components and metabolic products provided immune support and anti-inflammatory action. The major metabolic activities of probiotics include proteolysis (breakdown of food proteins), lipolysis (breakdown of food fat) and the conversion of lactose (milk sugar) to lactic acid. These changes are effected through the respective bacterial enzymes. Probiotics in the gastrointestinal tract could therefore help humans suffering from impaired digestion due to lack or dysfunction of the inherent digestive enzymes, by pre-digesting ingested food components (Alm, 1982).

Probiotics are now clinically proven to have a number of health benefits including:

- Usefulness in irritable bowel syndrome (Muhammed and Lakshmi, 2007).
- As anti-colon cancer effect (Mercenier *et al.*, 2003).
- Reduction of inflammatory or allergic reactions (Muhammed and Lakshmi, 2007).
- Skin health maintenance (Thestrup-Pedersen, 2003).
- Dental health maintenance (Meurman and Stamatova, 2007).
- In supporting healthy blood pressure levels (Muhammed and Lakshmi, 2007). As antihypertensive effect: Peptidase action on milk results in

antihypertensive tripeptides (angiotensin converting enzyme inhibitors), cell wall components act as angiotensin converting enzyme inhibitors.

- Immune functions and liver functions (Liong, 2007). Strengthening of non-specific defense against infection increased phagocytic activity of white blood cells, increased serum IgA after attenuated *Salmonella typhimurium* challenge, proliferation of intra-epithelial lymphocytes, adjuvant effect in antigen-specific immune responses, regulation of the Th1/Th2 balance and induction of cytokine synthesis (Mercenier *et al.*, 2003).
- In the management of vaginal infections, Inhibitor production (H_2O_2, biosurfactants (Muhammed and Lakshmi, 2007).
- In supporting cardiovascular health and wellness (Muhammed and Lakshmi, 2007) Some species of LAB, especially *Lactobacilli*, have the potential to assist in the reduction of elevated serum cholesterol levels, this beneficial action could involve their ability to assimilate cholesterol in to cellular membranes or it deconjugate bile acid (Pigeon *et al*., 2002).
- Decreased urease activity of *Helicobacter pylori* in humans after administration of a supernatant of a *Lactobacillus* culture. (Mercenier *et al.*, 2003; AL-Yas, 2006).
- Preventing and treating diarrhea, including infectious diarrhea, particularly from rotavirus (a virus that commonly causes diarrhea in children). (Pigeon *et al*., 2002).
- *Lactobacillus spp* have considerable effects against the urogenital pathogens (Mohamed, 2008).

Mechanisms for the benefits of probiotics are incompletely understood. However, as a general rule, include:

- Adherence and colonization of the gut (Adhesion of *Lactobacilli* to the host gastrointestinal tract is considered an important factor in health-promoting effects (Wang *et al*., 2008).
- Suppression of growth or epithelial binding/invasion by pathogenic bacteria and production of antimicrobial substances.
- Improvement of intestinal barrier function.
- Controlled transfer of dietary antigens.
- Stimulation of mucosal and systemic host immunity (Harish and Varghese, 2006).

1.2.7 *Lactobacillus* in the normal intestinal microbiota :

The normal intestinal microbiota works as a barrier against pathogens, contributes to degradation of some food components, stimulates the host immune system, and produces certain vitamins, enzymes and short-chain fatty acids (Holzapfel *et al*., 1998). The normal gut bacterial population of an adult human is estimated to comprise more than 400 species, with the predominance of obligate anaerobes (Rolfe, 1997). The presence and composition of *Lactobacilli* in the microbiota of the gastrointestinal tract of mammalian animals closely resemble those found in humans, although some variations at the species level occur depending on the host. Also, the anatomical differences of the alimentary canals influence the microbiota: e.g. the non-secreting stratified squamous epithelia in the fore-stomach of pigs are efficiently colonized by *Lactobacilli* (Tannock, 1999).

Several reports indicated that imbalanced normal microbiota (e.g. due to disease or use of broad spectrum antibiotics) is associated with an abnormally high presence of microbial species, such as *E. coli, Salmonella, Streptococci, Gardnerella* and yeasts, often associated with infective diseases, as well as with reduced levels of *Lactobacilli* (Coconnier *et al*., 1997; Mohamed, 2008).

Major natural habitats of LAB are the gastrointestinal and urogenital tracts of humans and animals, which provide stable conditions and a continuous supply of nutrients in the form of ingested food and secretions of the host. Commonly recovered *Lactobacillus* isolates from the human gastrointestinal tract include *L. acidophilus, L. salivarius, L. casei, L. plantarum, L. fermentum, L. brevis* and *L. reuteri* (Mikelsaar *et al.*, 1998).

In humans, the numbers of LAB vary greatly in different sections of the digestive tract and rise gradually when descending down the alimentary canal towards the colon. Important host-mediated factors that affect the growth of bacteria in the GI tract include acidicity, secretions such as bile, salts, immunoglobulins, enzymes, exfoliated cells, mucins and tissue exudate as well as the peristaltic movement (Tannock, 1999). To resist peristalsis, bacteria either have to adhere to intestinal surfaces or multiply at a fast rate. Since the peristaltic movement may be too rapid for significant bacterial multiplication (Fuller, 1989). Adherence to intestinal surfaces is probably an important bacterial factor contributing to succesful colonization at the upper intestinal regions (Tannock, 1992).

1.2.8 *Lactobacillus* as probiotics :

The ability to adhere to intestinal mucosa is considered an important requirement for microorganisms intended for probiotic use, allowing at least a temporary colonization of the human and animal intestinal tract. As microbial feed additives, they offer potential as an alternative to antimicrobials; both as a means of controlling pathogen carriage and improving growth rate and feed conversion. However, the mechanisms underlying the health effects and the host-probiotic communication in prophylactic and/or therapeutic treatments have remained poorly characterized. The probiotic strains are expected to fulfill several health-promoting characteristics and safety criteria (Mercenier *et al*., 2003):

- Non toxic and non pathogenic.
- Accurate taxonomic identification.
- Normal inhabitant of the targeted species.
- Capability to survive, proliferate and be metabolically active in the targeted site, which implies:

-resistance to gastric juice and bile.

-ability to persistent in the GIT.

-ability to adhere.

-ability to compete with the resident microbiota.

- Production of antimicrobial substances.
- Antagonism towards pathogenic bacteria.
- Ability to exert at least one clinically documented health benefit.
- Genetically stable.
- Amenability of the strain and stability of the desired characteristics during the processing, storage and delivery.
- Viability at high populations.
- Desirable organoleptic and technological properties when included in industrial prosesses.
- Isolation from suitable habitats.

The findings that colonization by *Lactobacilli* and other lactic acid bacteria, improves infection resistance of the host, have led to the production and consumption of probiotics. Several health-promoting effects of probiotics have been proposed e.g. prevention of the pathogen colonization in the GIT via competitive exclusion, and/or synthesis of inhibitory compounds (Isolauri *et al*., 2004).

1.2.9 Adhesive properties in *Lactobacillus*:

Adhesion to host tissues was considered to be the first step in bacterial colonization. The adhesion of *Lactobacilli* to the intestinal mucosal surface was a critical prerequisite for exerting beneficial effects to their host organisms and considered one of the main selection criteria for potential probiotics, as it prolongs their persistence in the intestine allowing them to exert their healthful effects longer (Wang *et al*., 2008).

Kos and his Colleque (2003) mentioned several factors contribute to the interaction of *Lactobacilli* with the host tissues, such as cell surface hydrophobicity and autoaggregation, lipoteichoic acids and cell surface proteins.

Granato *et al*. in 2004 showed that surface proteins of some *Lactobacilli* participate in adhesion to epithelial cell lines, GI mucins, or extracellular matrix proteins. *Lactobacilli* have been frequently observed to bind to epithelial cells and dissected tissue samples of the alimentary canal from human and animals, to intestinal mucus, to cultured human carcinomal intestinal cell lines and to the components of the extracellular matrix (ECM) (Jakava-Viljanen, 2007).

The proteinaceous nature of some surface components has been demonstrated and surface layer (S-layer) proteins detected in some *Lactobacillus* strains may be involved in adherence (Kos *et al*., 2003).

Greene and Klaenhammer (1994) hypothesized that proteinaceous molecules mediate the adhesion of *Lactobacilli* in the host intestine due to the reduced adhesiveness of *Lactobacilli* when treated with proteinases.

Rojas and Conway (1996) stated that bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesins (usually proteins) and complementary receptors.

Several *in vitro* models have been used to assess the effect of LAB on the adhesion and invasion of pathogenic bacteria. The epithelial cell line Caco-2 has often been used, and inhibitory effects against the adhesion and invasion of pathogens, such as *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, *Listeria monocytogenes* and *E. coli*, have been reported . However, some of the results were obtained by including the acidic growth medium of the LAB strains in the assays. It was later suggested that the low pH is deleterious to Caco-2 cells and leads to cell death (Sillanpää, 2001).

Rojas and Conway (1996) suggested that for successful colonization, of *Lactobacilli*, it should be resist the peristaltic movement by adhering to intestinal epithelia and/or mucus, particularly in the upper parts of the alimentary canal.

McGroarty, in 1993 found that adherence of LAB to intestinal epithelium could prevent pathogen colonization by steric hindrance or competition for epithelial receptors. As well as Lloyd and his Colleque (1977) stated that blocking the attachment of enteropathogens and undesirable microflora results from steric hindrance caused by the adhesion of lactobacilli or prevents colonization by exogenous bacteria, a phenomenon referred to as “colonization resistance” or “competitive exclusion”.

1.2.10 S-layer proteins (Slps):

S-layers are monomolecular crystalline arrays identified in hundreds of different species from the domains of bacteria and archaea as the outermost structure of the cell envelope. The S-layer consists of a single protein or glycoprotein subunit with molecular weights of 40 to 200 kDa that assembles into the two-dimensional S-layer sheet (Sára and Sleytr, 2000).

Many S-proteins are glycosylated or phosphorylated. Several S-layer proteins, extracellular enzymes and outer membrane proteins have an N- or C-terminal S-layer homology domain (SLH) (Sillanpää, 2001).

The S-layer sheet is attached to the underlying cell wall non-covalently and can usually be dissociated and solubilized into protein monomers by hydrogen bond-breaking agents (Pum and Sleytr, 1999).

Sleytr *et al*. (1996) developed different methods for the deattachment of S-layers and for their disintegration into protomeric units. Most S-layer proteins can be solubilized with high concentrations of agents that break hydrogen bonds (e.g., guanidine hydrochloride). Particularly S-layer proteins from gram-negative bacteria may be disintegrated by applying metal-chelating agents or cation substitution. These data have shown that the individual subunits of S-layers interact with each other and with the supporting cell envelope components through noncovalent forces. Isolated S-layer subunits frequently maintain the ability to recrystallize into regular arrays in suspension or on surfaces (including that cell envelope component they were originally associated with) upon removal of the agent used for their isolation.

S-layers are often lost during prolonged cultivation under laboratory conditions. Consequently, fresh isolates should be examined by electron microscopic techniques as soon as possible, preferably by freeze-etching of pellets of unwashed cells (Fig 1-2) in complete medium. High-resolution images of S-layers were also obtained by applying underwater atomic-force microscopy (Sleytr *et al*., 1999).

Fig (1-2): Electron micrograph of a freeze-etched preparation showing a whole cell with a hexagonally ordered S-layer lattice. Bar, 100 nm. (Sára and Sleytr, 2000)

S-layers are found in gram-positive and gram-negative bacteria and archaea, they can be associated with quite different supramolecular cell envelope structures. In gram-positive bacteria and archaea, the S-layer subunits are linked to the peptidoglycan-containing layer or to the pseudomurein. In gram-negative bacteria, attachment involves components of the outer membrane (e.g., lipopolysaccharides [LPS]). In archaea lacking a rigid wall layer, S-layers are the only wall component, being closely associated with the plasma membrane (Sára and Sleytr, 2000).

From a more general point of view, it is now evident that S-layers are dynamic closed surface crystals with the intrinsic ability to continuously assume a structure of low free energy during cell growth and division (Pum *et al*., 1991).

1.2.11 Expression of S-layer protein genes:

The S-layer proteins are among the most abundant bacterial proteins, representing 10 to 15% of the total cellular protein of the bacterial cell as indicated by Boot and Pouwels, in 1996 estimated that $5x10^5$ protein monomers are needed to cover the entire cell surface. This necessitates an efficient expression and secretion machinery for the S-protein, since approximately 500 copies of the monomer per second need to be synthesized in exponentially growing bacteria (Sleytr and Beveridge, 1999).

Boot and Pouwels, in 1996 analysed the amino acid composition of S-proteins and found they contain a relatively high number of threonine, serine and hydrophobic residues, but no or only a few cysteines or methionines. Most S-proteins are weakly acidic with isoelectric points (pIs) ranging from 3 to 6. An exception was found for the S-layer proteins of *Methanothermus fervidus* and for different *Lactobacilli*, which possess pIs of 8.4 and 9 to 11, respectively. Secondary structure measurements indicate that in most S-

layers 40% of the amino acids occur as β-sheets and 10-20% as α-helices (Sára and Sleytr, 2000).

Several researchers found the quantity of S-layer proteins detected in growth medium were miniscule, indicating a strict control of S-layer protein synthesis (Messner and Sleytr, 1992). Only a few organisms produce an excess of S-layer proteins shed into the growth medium, but in *Bacillus* spp. this seems to occur commonly. In *Bacillus thuringiensis*, free S-layer fragments have only been detected in cell cultures in late-exponential and stationary growth phases (Sidhu and Olsen, 1997).

1.2.12 S-layer proteins (Slps) of *Lactobacillus*:

S-layers are crystalline arrays of proteinaceous subunits located at the outermost part of the cell wall in several species of the genus *Lactobacillus*. *Lactobacillus* S-layers are relatively small, 25 kDa to 71 kDa in size (Åvall-Jääskeläinen and Palva, 2005). The molecular masses of *Lactobacillus* S-layer proteins vary from 43 kDa to 55 kDa (Åvall-Jääskeläinen, 2005). Based on electron microscopy, the S-layer subunits are composed of lattices with oblique, square or hexagonal symmetry (Sara and Sleytr, 2000). The oblique lattice type was identified in the S-layers of *L. acidophilus*, *L. brevis* and *L. helveticus* and the hexagonal lattice type in *L. casei* and *L. buchneri* (Jakava-Viljanen, 2007).

The S-layer subunits are non-covalently linked to each other and to the supporting cell envelope, and can be disintegrated into monomers by denaturing agents such as urea or Guanidin HCl, by metal-chelating agents or by cation substitution (Sara, 2001). Treatment of *L. brevis* ATCC 8287 cell with GHCl abolished binding of this strain to intestinal epithelial cell line and suggested the role of S-layer (Åvall-Jääskeläinen, 2005). Frece *et al.*, (2005) showed that treatment of *L. acidophilus* M92 cells with Lithium Chloride abolished the bacterial adhesiveness to mouse ileal epithelial cells.

In addition to peptidoglycan, the rigid cell envelope of *Lactobacilli* is composed of secondary cell wall polymers (SCWP) such as teichoic acid, lipoteichoic acids, lipoglycans or neutral or acidic glygans (Åvall-Jääskeläinen, 2005).

1.2.13 Functions of S-layer proteins:

The functions of Slps are not yet completely revealed but it has been proposed that these structures protect the microbe from hostile environmental agents and aid in maintaining cellular integrity (Åvall-Jääskeläinen and Palva, 2005). Several *Lactobacillar* S-layers have been identified as putative adhesins with an affinity for various tissue compartments or molecules. Adhesive S-layers have a role in inhibition of adhesiveness of pathogenic bacteria and thus can contribute to probiotic effects of *Lactobacilli*.

S-layers act as virulence factors in some pathogenic bacteria, which act as adhesins in several bacteria, mediating the adherence of these bacteria to epithelial cells and/or extracellular matrix (Hynönen *et al*., 2002).

The S-protein of *L. helveticus* CNRZ 892 functions as a receptor for a phage (Beveridge *et al.*, 1997). The S-layer of *G. stereothermophilus* functions as a molecular sieve by trapping high molecular weight solutes (Sára and Sleytr, 1987) and as an adhesion site for exoenzyme amylase (Jarosch *et al.*, 2001).

Surface layers have been proposed to have a role in cell shape determination and cell wall stabilization (Sleytr and Beveridge, 1999). Indeed, the extraction of S-layer protein reduced the viability of *L. acidophilus* at low pH, suggesting a protective role for the S-layer (Frece *et al.*, 2005). In addition, S-layer protein extracts from *L. helveticus* have been shown to inhibit enterohaemorrhagic *E. coli* adhesion to host epithelial cells (Johnson-Henry *et al*., 2007).

1.2.14 Attachment of the surface layer proteins to *Lactobacillar* cell wall:

The cell envelope of Gram-positive bacteria is composed of a cell membrane covered with a Peptidoglycan (PG) layer and secondary cell wall polymers. Peptidoglycan is comprised of glycan strands, these glycan strands are cross-linked by short cell-wall peptides, whose composition varies between bacterial species. Peptidoglycan network forms a huge macromolecular structure completely surrounding the cell (Ton-That *et al.*, 2004).

The cell wall has many critical functions, such as protection against the environment and cell lyses, but it also provides an attachment site for the surface proteins interacting with the host. Due to the different structural organization of the cell envelope in Gram-positive and Gram-negative bacteria, the cell envelope part to which the S-layer protein subunits can attach also differs according to species. However, no general mechanism of attachment of the S-layer subunit to the cell envelope that would depend on the classification of the bacterium by Gram staining has been found (Åvall-Jääskeläinen, 2005).

The rigid cell envelope of almost all Gram-positive bacteria is composed of secondary cell wall polymers (SCWP) such as teichoic acid, lipoteichoic acids, lipoglycans or teichuronic acids in addition to peptidoglycan (Neuhaus and Baddiley, 2003). When present in Gram-positive bacteria, the SCWP have been shown to be responsible for the anchoring of the S-layer protein to the cell envelope through different mechanisms as shown in fig (1-3). The S-layer homologous (SLH) motifs first identified by Lupas *et al.* (1994) are present at the N-terminal part of S-layer proteins in several Gram-positive bacteria and have been found to be responsible for the anchoring of S-layer protein to SCWP (Mader *et al*., 2004).

The S-layer protein from *L. acidophilus* has a two domain structure. A fragment containing the N-terminal two-thirds of the protein (SAN)

crystallized into a layer and was proposed to be composed of two subdomains with a surface exposed loop (Smit *et al*., 2002). The C-terminal part (SAC) was responsible for cell wall anchoring.

The C-terminal regions of the S-layer proteins of *L. acidophilus* ATCC 4356 and *L. crispatus* JCM 5810 are almost identical in sequence, and the cell wall binding domain in these bacteria has been shown to reside in the C-terminal region (Antikainen *et al*., 2002). Smit and Pouwels (2002) showed that in *L. acidophilus* an N-terminal repeat in the C-terminal SAC domain is most likely responsible for the anchoring of S-layer protein to cell wall fragments. For the S-layer proteins of *L. acidophilus* and *L. crispatus*, teichoic acids have been suggested to be involved in the binding (Antikainen *et al*., 2002; Smit and Pouwels, 2002).

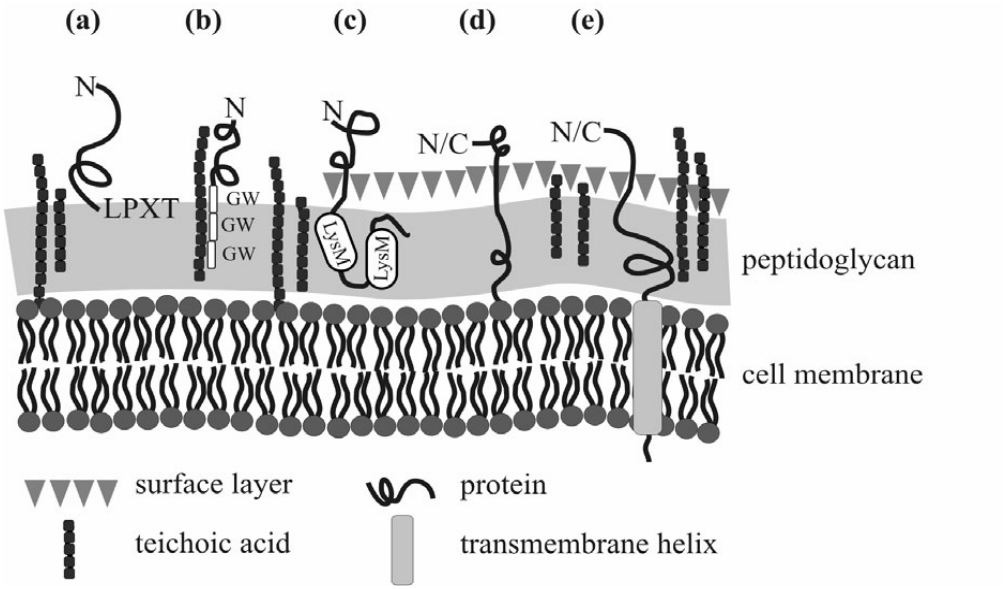


Fig (1-3): Mechanisms of protein anchoring in the Gram positive cell surface. a) LPXTG motif covalently anchors surface proteins to peptidoglycan b) Protein anchored to teichoic acids via GW motif c) LysM protein anchored to peptidoglycan d) Lipoprotein linked to cell membrane e) Transmembrane protein. N-and C-termini of proteins are indicated (N, C). GW, protein having GW motif; LysM, proteins with LysM domain (Antikainen *et al*., 2002).

1.2.15 *Lactobacillus* as live vaccine delivery vectors:

Vaccination represents one of the most efficient tools for the prevention and even eradication of infectious diseases. Since the majority of infections occur at or through the mucosal surfaces, the use of a mucosal route of vaccination instead of a parenteral route would be preferable. Compared with the parenteral route of vaccination, mucosal vaccination offers several advantages, such as prevention of the initial infection and replication of the pathogen at the site of entry, stimulation of both local and systemic immune responses, easy administration and low delivery costs (Åvall-Jääskeläinen, 2005).

Several benefits associated with LAB favour their use as vaccine delivery vehicles. These bacteria are considered to be safe organisms with a GRAS (generally regarded as safe) status. This is in contrast to other live vaccine carriers used (e.g., *Salmonella, E. coli*, vaccinia virus), which cannot be classified as safe (Pouwels *et al*., 2001). the long-term experience of their production in the food industry, the capacity of numerous strains to adhere and colonize to mucosal surfaces, beneficial health-effects for the hosts of several strains, intrinsic immunogenicity, resistance to bile acid, lack of lipopolysaccharides in their cell wall eliminating the risk of an endotoxic shock, and the capability to modulate the immune responses obtained by inducing cytokine production of the host (Seegers, 2002).When developing *Lactobacilli* as vaccine carriers, the choice of the appropriate *Lactobacillus* antigen carrier strain is of utmost importance. The factors that need to be considered include the capacity of the *Lactobacillus* strain to adhere to and colonize the relevant epithelial surfaces of the host, adjuvant and immune-stimulation properties of the strain, genetic amenability of the strain and the codon usage preferences of the strain. *Lactobacilli* have been shown to be capable of stimulating both non-specific host immune responses, including the enhancement of phagocytic activity of phagocytic cells, and specific host

immune responses, including the enhancement of humoral immune responses (Åvall-Jääskeläinen, 2005).

The intrinsic adjuvant activities of *Lactobacilli*, leading to the stimulation of immune responses to the co-administered antigen, are most likely based on the induction of cytokines by lactobacilli (Seegers, 2002). Due to importance of S-protein in the adhesion and their self-assembly properties, the possible therapeutic applications of Lactobacillar S-layers have gained increasing interest e.g. as targeted live antigen delivery vehicles to host tissues (Jakava-Viljanen, 2007).

S-layer could provide a superior expression level and surface density of the required antigen as compared to other bacterial antigen presentation systems. It has already been demonstrated that S-layer protein subunits can be modified to carry foreign epitopes as a uniform recombinant S-layer on the *Lactobacillus* cell surface (Smit *et al.*, 2002).

1.2.16 Antitumor activity of *Lactobacillus*:

The growth of the tumour cells can be inhibited by LAB. A preventive effect of LAB on malignant development could be mediated by production of antimutagenic substances, binding of mutagens, inhibition of procarcinogenic enzymes like nitroreductase and β-glucuronidase, increased production of β-glucosidase which release flavonoids and Deconjugation of bile salts (Lankaputhra and Shah, 1998).

Lactic acid bacteria may exert beneficial effects on tumor via different mechanisms: a) LAB induce an increase in the cytotoxic capacity of macrophages or CD8+ T cells, b) LAB are cytotoxic for tumor cells, c) LAB induce a non-specific local inflammatory reaction inducing a host-mediated immunological response against the tumor, d) LAB enhance the cytokines released which are involved with the cellular apoptosis induction, e) LAB give rise to specific immunity to the tumor (Perdigón *et al.*, 2001).

Goldin and Gorbach (1980) showed in mice that *Lactobacillus acidophilus* orally administered induced a decreased in the incidence of the colon cancer caused by 1-2 dimethylhydrazine dihydrochloride (DMH). The intratumour administration of *Lactobacillus casei* produced a total inhibition of the tumour, while simultaneous injection of *Lactobacillus casei* in different body sites had no effect on tumour growth and tumor inhibition by intravenous administration of *L. casei* in syngeneic mice and guinea pigs with carcinoma of the lung and liver, tumour respectively (Perdigón *et al.,* 2001).

Hosoda *et al*., (1992) proved in humans that oral supplements with *L. acidophilus* reduced acitivities of faecal bacterial enzymes such as β-glucuronidase, nitroreductase and azoreductase that are involved in pro-carcinogen activation.

2.1 Materials:

2.1.1 Equipment and Apparatus:

The following equipment and apparatus were used throughout the study:

Apparatus	Company	Origin
Anaerobic Jar	Rod well	England
Autoclave	Tomy	Japan
Centrifuge	Hermal	Germany
Compound Light Microscope	Olympus	Japan
Cooled centrifuge	Sanyo	———
Disk- tubes electrophoresis	Jokoh	———
Distillator	GallenKamp	England
ELISA reader	Organon Techniqa	Germany
Hot plate - magnetic stirrer	GallenKamp	England
Incubator	Sanyo	Japan
Laminar flow hood	Gelair class 100 Gelmaninstrument	England
Microtiter plate with 96 flat bottom well	Flow lab., Irvin	———
Millipore filter unit	Millipore and Whatman	———
Oven	Sanyo	Japan
PH- meter	WTW	Germany
Sensitive Balance	Mattler	Switzerland
Spectrophotometer	LKB Digital	Sweden
Vortex	Buchi	———
Water bath	GFH	England

2.1.2 Chemicals:

Material	Company	Origin
Agar-Agar	Difco	USA
Arabinose	BHD	England
Acrylamide	LKB	Sweden

Ammonium persulfate	BDH	England
Bromophenol blue	BDH	———
Calcium carbonate ($CaCO_3$)	BDH	———
Cellobios	BHD	———
Chlorophenol red	Fluka	Switzerland
Commassie brilliant blue R-250	LKB	Sweden
Cotton blue	Fluka	Switzerland
Crystal violet	Himedia	India
Esculine	BDH	England
Ethanol	Local made	Iraq
Ethylendiaminetetra acetic acid (EDTA)	Fluka	Switzerland
Fructose	BDH	England
Gelatine	Oxoid	———
Glacial acetic acid	BDH	———
Glucose	BDH	———
Glycerol	BDH	———
Glycine	Merck	Germany
Hydrochloric acid (HCl)	BDH	England
Iodine	Himedia	India
K_2HPO_4	BDH	England
Lactic acid	BDH	———
Lactose	BDH	———
Maltose	BDH	———
Mannitol	BDH	———
Mannose	BDH	———
Melibiose	BDH	———
Methanol	Merck	Germany
$MgSO_4.7H_2O$	Riedal-dehaeny	———
$MnSO_4.7H_2O$	BDH	England
N,N, methylene bis acrylamide	LKB	Sweden
N,N,N,N, tetramethylethylene diamide	Sigma	U.S.A
Neutral red	Sigma	———
Peptone	BDH	England
Phenol	Biomerieux, A.A.	France

Raffinose	BDH	England
Rhamnose	BDH	———
Ribose	BDH	———
Safranine	Himedia	India
Salicin	BDH	England
Sodium acetate hydrate	BDH	———
Sodiumdodecy sulfate (SDS)	BDH	———
Sucrose	BDH	———
Trehalose	BDH	———
Triammonium citrate	Fluka	Switzerland
Tris – base	Fluka	———
Trypsin	Oxoid	England
Tween 80	Oxoid	———
Yeast extract	Fluka	Switzerland

2.1.3 Culture Media:

2.1.3.1 Ready to use powdered media:

The following culture media were used for experimental work in this study:

Medium	**Company**	**Origin**
Litmus Milk Media	Biolife	Italy
Lactose broth	Oxoid	England
MacConkey Agar	Oxoid	———
Mannitol salt agar	Himedia	India
Manns-Regoza and Sharpe (MRS) Broth	Himedia	———
Nutrient agar	Himedia	———
Nutrient broth	Oxoid	England
Sabouraud dextrose broth	Difco	USA
Salmonella Shigella agar (SS agar)	Oxoid	England

2.1.3.2 Laboratory prepared media:

- MRS-$CaCO_3$ agar.
- Gelatin Medium.
- Carbohydrate fermentation broth.
- Peptone broth.

2.1.4 Ready to use reagents:

Reagent	Company	Origin
Oxidase	BioMerieux	France
Catalase	Al-mansor	Iraq
kovac´s	BioMerieux	France

2.1.5 Bacterial Isolates:

Bacterial isolates used in this study were obtained from different sources as indicated below:

Isolate	Source	Supplied by
Two isolates of *Lactobacillus acidophilus*	chicken intestine	College of Veterinary Medicine/ Baghdad University
Lactobacillus acidophilus	faeces of children	Biotechnology Research Centre /AL-Nahrain University
Lactobacillus casei		
Escherichia coli	Skin infection	Biotechnology Department/College of Science/Al-Nahrain University
Staphylococcus aureus		
Pseudomonas aeruginosa		
Salmonella typhimurium		
Candida albicans		

2.1.6 Cell lines:

Both cell lines were supplied by Tissue Culture Unit / Biotechnology Research Centre /AL-Nahrain University, Baghdad , Iraq.

- Rhabdomyosarcoma (RD) cell line(passage number 5):

  Human cell line was derived from biopsy specimen obtained from a pelvic Rhabdomyosarcoma of 7-years-old Caucasian girl (Mcallister *et al*., 1969). This cell line was adapted to grow on RPMI-1640 medium supplemented with 10% fetal calf serum in Biotechnology Research Centre /AL-Nahrain University instead of the original medium MEM which also supplemented with 10% fetal calf serum.

- L20B cell line (passage 19):

  L20B is a transgenic mouse cell line that expresses the gene for the human receptor for polio virus (CD155) on the cell surface (Pipkin *et al.*, 1993).

2.1.7 Protein Estimation Kit: (Randox / England)

Reagent composition:

- Reagent
  - Sodium hydroxide 200mmol/l
  - Potassium sodium taratrate 32mmol/l
  - Copper sulfate 18mmol/l
  - Potassium iodide 30mmol/l
- Protein standard 8g/dl

Reagent preparation:

The colour reagent and standard are ready for use.

2.2 Methods:

2.2.1 Stains:

❖ **Methylene blue stain (1%) (Atlas, 1995):**

It was prepared by dissolving 0.3 g methylene blue in 30ml of ethanol (95%v/v)

❖ **Lactophenol cotton blue stain solution (Atlas, 1995):**

It was prepared by dissolving the following ingrediants in 100 ml D.W: 20 g 0f phenol, 20 ml of Lactic acid, 40 ml of glycerol, then added 0.05 gm of cotton blue stain, that solution used to stain germ tube in *Candida*.

2.2.2 Reagent:

❖ **Chlorophenol red reagent:**

It was prepared by dissolving 0.2 chlorophenol red in 2 ml of ethanol 95% then the volume was completed to 100 ml with D.W (Cowan, 1974).

2.2.3 Media preparation:

2.2.3.1 Ready to use powdered media:

The media listed in (2.1.3.1) were prepared according to the instructions fixed on their containers as indicated by the manufacture. After adjustment of pH, they were sterilized by autoclaving at 121ºC for 15 min. Unless otherwise stated.

2.2.3.2 Laboratory prepared medium:

❖ **MRS-$CaCO_3$ Agar:**

This medium was prepared according to Harrigen and MacCane, (1976) by mixing 1.5 %(w/v) agar and (1 %w/v) $CaCO_3$ into MRS broth media then sterilized by autoclaving.

❖ **Gelatine Medium (Atlas *et al*., 1995):**

It was prepared by dissolving 12g of gelatine in 90 ml MRS broth medium, volume was completed with MRS broth to get a final concentration of 12% (w/v) then sterilized by autoclaving.

❖ **Carbohydrate fermentation broth:**

It was prepared according to Cowan (1974) by dissolving the following ingredients in 950 ml of distilled water.

Ingredients	Weight g/l
Peptone	10 g
yeast extract	5 g
Tween 80	1 ml
Sodium acetate hydrate	5 g
K_2HPO_4	2 g
Triammonium citrate	2 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.7H_2O$	0.05

Then 2% of Chlorophenol red was added as indicator and the pH was adjusted to 6.2-6.4. After that, the volume was completed to 1000 ml of D.W, then it was sterilized by autoclaving, 1% of each of the autoclaved sugar solutions (glucose, sucrose, fructose, mannitol, raffinose, lactose, trehalose and rhamnose) and membrane filtrated sugar solutions (arabinose, maltose, salicin, cellobios, ribose, melibiose, mannose and esculin) were added to sterilized medium. This medium was used for identification of *Lactobacillus spp*.

❖ **Peptone Broth Medium (Mackie and MacCartney, 1996):**

It was prepared by dissolving 5 gm of peptone in 100 ml of D.W., and sterilized by autoclaving. Then 5 ml aliquots were dispensed in sterile test tubes and stored at 4 °C until use.

2.2.4 Sterilization Methods (Baily *et al*., 1990)

❖ **Autoclaving:**

Bacterial cultural media, solutions, buffers and reagents were sterilized by autoclaving at 121°C and (15 lb/inch^2) for 15 min., while some sugar solutions and litmus media were sterilized by autoclaving for 5 min.

❖ **Membrane Filtration:**

Bacterial filtrates, some sugar solutions, dye solutions, antibiotic solutions and trypsine-versine solution as well as tissue culture medium (RPMI-1640) were sterilized throughout (0.22) µm in diameter Millipore filter papers.

❖ **Dry Heat Sterilization**

Electric oven was used to sterilize glasswares at 160-180°C for 3-2hr.

2.2.5 Sample collection:

2.2.5.1 Different samples:

Two samples of vinegar, five (3ml) of human milk (taken from healthy women), three of cow milk, and four of yoghurt were collected in sterile containers of Baghdad governorate from the period between 25/12/2007 and 31/3/2008 then samples were transferred to the laboratories for analysis within 2hr. after collection.

2.2.5.2 Vagina samples:

A total of 10 samples were obtained from the vagina of healthy premenopausal women by the gynecologist doctor in Kamal AL-Samarai hospital, Baghdad from 1/3 to 31/3/2008.

2.2.6 *Lactobacillus* isolation:

2.2.6.1 Isolation of *Lactobacillus* from different sources:

Lactobacillus isolates were isolated according to Buck and Gilliland (1995) as following:

One ml of each sample was transferred to a test tube containing 9 ml of sterilized MRS broth and incubated anaerobically overnight at 37℃, and this was repeated for three times to increase bacterial numbers, Serial dilutions were made from peptone water, and 1 ml of the last one was cultured on MRS-$CaCO_3$ agar and incubated for 24 hr. at 37℃ under anaerobic conditions using anaerobic jar. After incubation, colonies surrounded by clear zones (due to the production of acid hydrolyzing $CaCO_3$) were picked and grown on MRS broth. Morphological, microscopical examination and biochemical tests were performed.

2.2.6.2 Isolation of *Lac*tobacillus from vagina:

Vaginal swabs were cultured on chocolate agar then a loop full was taken and recultured in tubes contained sterile 10 ml of MRS broth, to isolate *Lactobacilli* as mentioned above.

2.2.7 Identification of *Lactobacillus spp.:*

2.2.7.1 Morphological Characteristics:

Local isolates of *Lactobacillus sp.* were first identified according to the morphological and cultural characteristics (shape, color, size, edges and hight) of colony on MRS agar plates (Harely and Prescott, 1996).

2.2.7.2 Microscopic Examination:

A Loop full of fresh culture of *Lactobacillus sp.* was fixed on a microscopic slide, fixed and stained with Gram stain, then examined under the light microscope to notice the cells shape, Gram reaction, grouping and spore forming (Harely and Prescott, 1996).

2.2.7.3 Biochemical Tests:

❖ **Catalase Test (Atlas *et al*., 1995):**

This test was performed by adding 2-3 drops of hydrogen peroxide (3%) to the mass of bacterial cells placed on the microscopic slide. Production of gaseous bubbles indicates a positive result.

❖ **Oxidase Test (Atlas *et al*., 1995):**

A clump of colonies from bacterial growth was picked with a sterile wooden stick and smeared on filter paper that moistened with a few drops of a freshly prepared oxidase reagent. Presence of deep purple color represents a positive result.

❖ **Gelatinase Test (Baron and Fingold , 1994):**

Gelatine liquefaction was detected by using gelatin medium inoculated with 1% of bacterial culture and incubated at 37˚C for 48hr. After that, it was put in the refrigerator (4˚C) for 30 minutes. The positive result was observed by gelatin liquefaction.

❖ **Acid and Curd Production in Litmus Media (Kandler and wises, 1986):**

Tubes containing litmus milk were inoculated with 1% bacterial culture and incubated at 37℃ for 24hr. anaerobically, changing in color, curd production and decreased in pH were observed as indicators of positive results.

❖ **Growth at 15 ºC and 45 ºC:**

Lactobacillus sp. isolates were used to inoculate test tubes containing MRS broth, and incubated at both 15 ℃ and 45 ℃ for 24 hrs. anaerobically, then, turbidity was measured and compared with control test tubes incubated at 37 ℃, turbidity appearance represents a positive result.

❖ **Carbohydrate Fermentation Test (Atlas *et al*., 1995):**

Tubes containing fermentation media were inoculated with 1% of *Lactobacillus spp.* isolates and incubated at 37 ºC for 5 days. Changing the medium colour from red to yellow indicates positive result.

2.2.8 Identification of Test Microorganisms:

2.2.8.1 Microscopic and Morphological examinations:

Loop full of fresh culture of test organisms was fixed on a microscopic slide then examined under the microscopic as mentioned in (2.2.7.2), also the isolates were cultured on different media (Nutrient agar, MacConkey agar, Mannitol salt agar, *Salmonella Shigella* agar and Sabouraud dextrose agar).

2.2.8.2 Biochemical Tests:

❖ **Catalase, oxidase, gelatinase** tests were done as mentioned above.

❖ **Coagulase test (Atlas *et al*., 1995):**

Several colonies of *Staphylococcus aureus* are transferred with a sterile loop to a tube containing 0.5 ml of human plasma. The tube was covered to prevent evaporation and incubated at 37^{o}C for 24 hr. Result was reading by tilting the tube and observing clot formation in the plasma, which indicates positive result. If plasma remains free-flowing with no evidence of a clot, the test is negative.

❖ **Indole Test (colle *et al*.,1996):**

Peptone broth containing test tubes were inoculated with fresh culture of isolates *and* incubated at 37 °C for 28 hr., then 0.05ml kovac´s reagent was added. Appearance of red ring formed at the top of the broth indicates a positive result.

❖ **Lactose fermentation test (Downes and Ito, 2001):**

Sterile tubes containing lactose broth was inoculated with test bacteria and incubated at 37 ºC for 24 hrs. After that, the positive result was recorded by production of CO_2 gas bubbles in Durham tube.

❖ **Surface growth (Evans and Richardson, 1989):**

The test was carried out by inoculating a small portion of the colony of *Candida albicans* into a tube containing Subourauds dextrose broth mixed well and incubated for 24-72 hrs at 28-30°C. Presence of growth layer at the surface of broth was indicated a positive result.

❖ **Production of germ tube (Evans and Richardson, 1989):**

A small portion of the isolated colony of *C. albicans* was emulsified in one ml of sterile human serum, then incubated for 2-3 hrs at 37°C, one drop of the suspension was placed on clean slide with drop of lactophenol cotton blue then examined microscopically for the production of germ tube. The result was observed by noticing short extension shape, budding.

2.2.9 Maintenance of bacterial Isolates:

Maintenance of bacterial isolates was performed according to Contreras *et al*. (1997) and as follow:

a. Daily working culture:

Broth media were inoculated with the isolates and then incubated at 37 ºC for 24 hr. Then, kept in the refrigerator at 4 ºC and reactivated weekly.

b. Stock culture:

Sterile glycerol (20%) was added to an exponential growth of bacterial isolates in screw-caped tubes and stored at -20ºC.

2.2.10 Protein electrophoresis:

2.2.10.1 Solutions used in electrophoresis:

The following buffers and solutions were used in protein electrophorasis (Lammili, 1970):

- Resolving gel buffer: (0.375M Tris-HCl at pH 8.8)

Tris	18.17 g
HCl	2.2 ml

The mixture was made to 100 ml with D.W.

- Stacking gel buffer: (0.125M Tris-Hcl at pH 6.8)

Tris	6.06 g
HCl	3 ml

The mixture was made to100 ml with D.W.

- Resorvoir buffer: (Tris-glycine at PH 8.3)

Tris	3 g
Glycine	14.4 g
SDS	1 g

The ingredients were dissolved in 1000 ml of D.W.

- Acryleamide solution :

Acryleamide	24 g
N,N,methylenebisacryleamide	1 g

These ingredients were dissolved in 100 ml Distilled water and the solution was filtered through watman No.1 filter paper. The solution was stored in dark bottle at 4℃ , and should be used within one month.

- Ammonium persulfate solution :

It was prepared by dissolving 250 mg of Ammonium persulfate in quantity of D.W and the volume was completed to 10 ml of D.W. and stored in dark bottle at 4℃ . The solution was used freshly.

❖ SDS – solution :

It was prepared by dissolving 10 g of SDS in 100 ml D.W.

❖ Bromophenol blue solution :

Dissolved 250 mg of Bromophenol blue in 100ml D.W.

❖ Lammili sample buffer:

It was prepared by mixing the following ingredients.

Staking buffer (0.5M Tris-Hcl PH6.8)	1 ml
Glycerol	0.8 ml
SDS 10%	1.6 ml
2-Mercaptoethanol	20µl
Bromophenol blue	50µl

❖ Staining solution :

It was prepared by mixing the following ingridients.

Coomassie brilliant blue R-250	0.25 g
Methanol	48 ml
Glacial acetic acid	4 ml
D.W.	48 ml

❖ Destaining solution:

Glycial acetic acid	7 ml
Methanol	5 ml
D.W	88 ml

The acid was added to mixture of methanol and D.W gradually.

❖ Fixing solution:

Trichloroacetic acid	57g
Sulphosalicylic acid	17g
Methanol	150ml
Distilled water	350ml

The acids were added to the mixture of methanol and D.W. gradually.

❖ EDTA solution (0.02 M)(pH 7.5)

Stock solution of EDTA was prepared by dissolving 0.744 g of EDTA in 100 ml D.W.

❖ Tris-hydrochloride Solution (0.5 M)

Stock solution of Tris-hydrochloride was prepared by dissolving 6 g of Tris-hydrochloride in 100 ml D.W.

❖ Guanidine hydrochloride (6 M):

It was prepared by dissolving 4.2 g of guanidine hydrochloride in 10 ml D.W.

2.2.10.2 Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS- PAGE):

- Gel preparation

Solution	Resolving gel (10%)	Stacking gel (3%)
Distilled water	9.32 ml	8.8 ml
Acrylamide	13.68 ml	3 ml
Stacking gel buffer	-	3.68 ml
Resolving gel buffer	8 ml	-
SDS 10%	0.64 ml	0.32 ml
TEMED	40 µl	20 µl
Ammonium per sulfate	0.36 ml	0.15 ml

- The resolving gel (10%) was poured in electrophoresis tubes. The tubes were left 30 minutes to complete solidification.
- Staking gel (3%) was added to resolving gel in the tubes. The tubes were left for 15 min to complete polymerization. After 24h they were placed in the electrophoresis apparatus.
- The electrophoresis system was connecting to power supply with current density 2mA/tube for 30min to remove positive ions to become the protein movement free.

- Take 80µl from each samples and stander proteins (γ-Globulin, Transferrin, Trypsine, Lysozyme) which dissolved in laemmili sample buffer previously and then added on the surface of the gels. Run the gel with current density 2mA/tube after an hour current density was increased to 3mA/tube until the dye band reaches the gel bottom. This usually takes 4-5 h.
- After the electrophoresis was completed the separation tubes were lifted and the gel was drawn carefully by injecting water between the gel and the tube wall with a syringe.
- After separation of the gel from the tube, it was placed in fixing solution for 30 min. then placed in staining solution for 30 min. Then the stained gels were placed in a distaining solution and from time to time the solution was replaced with a new one to obtain clear bands.

2.2.11 Detection of S-layer proteins

Lactobacillus cells grown in MRS broth were collected by centrifugation at 10,000 rpm for 10 min at 4ºC and washed once with 0.5M Tris-HCl, pH 7.5, then suspended 10 µl of the pellet in 200 µl of Laemmli sample buffer and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis 10% (SDS-PAGE) (Laemmli, 1970).

2.2.12 Extraction of the S-layer protein: (Vidgren *et al*., 1992)

The bands which located in the range between Transferrin and Trypsine was excised and cut into pieces. The protein was eluted from the gel pieces in 1.5 ml of 6 M guanidine hydrochloride-0.5 M Tris-HCl-2 mM EDTA, pH 7.5, by incubating in an end-over mixer at room temperature for 10 h. The eluate was dialyzed against 0.1M Tris-HCl, pH 8.5, at +4°C for 10 h. also analyzed by (SDS-PAGE), In order to ensure the purity of protein.

2.2.13 Determination of molecular weight:

The molecular weight of S-layer proteins determined by analyzed the pictures of column in photocapt analysis software.

2.2.14 Determination of Total Protein:

Protein concentration was estimated by using the specific kit which depended on Biuret method.

The principle of this assay was protein form a blue/violet complex when mixed with copper ions in alkaline solution (Biuret reagent) each copper ion binding with 5 or 6 peptide bonds. Tartarate was added as a stabilizer and iodide was used to prevent auto-reduction of the alkaline copper complex.

Procedure:

- Blank: by mixing 0.02 ml of D.W with 1ml of reagent.
- Standered: by mixing 0.02 ml of the standard with 1ml of reagent.
- Sample: by mixing 0.02 ml of sample with 1 ml of reagent.

The mixture was incubated at room temperature for 5 minute. Absorbance of sample (As) and standard (Astd) against reagent blank at wavelength of 546 nm, was measured.

Calculation:

$$\text{Total protein g/dl} = \frac{\text{As}}{\text{Astd}} \times \text{concentration of standard (Indicated in the kit).}$$

2.2.15 Testing the Inhibitory Activity of Lactic Acid Bacteria (LAB):

2.2.15.1 On Solid Medium (MRS Agar):

A culture of *Lactobacillus* was inoculated in MRS broth then incubated anaerobically at 37 ℃ for 24 hr., then culture was streaked on MRS agar plates and incubated at 37 ºC for 24, 48 and 72 hr.

After incubation, discs were made from the cultured agar using sterile cork borer (5mm). The discs were fixed on the surface of nutrient agar plates that were previously streaked with the tested pathogenic bacteria (*Pseudomonas aeruginosa, Esherichia coli, Staphylococcus aureus*, and *Salmonella typhimurium*) and Sabouraud dextrose agar plate that was previously streaked with *Candida albicans*, then streaked at 37 ºC for 24 hr.

Inhibition zones around the discs were measured by millimeter (Silva *et al*., 1987).

2.2.15.2 In Liquid Medium (MRS Broth)

Tube containing MRS broth medium was inoculated with 1% of fresh culture of each *Lactobacillus* isolates, then incubated anaerobically at 37 ºC for 24, 48 and 72 hr. After incubation, culture was centrifuged at 6000 rpm for 15 min., supernatant was taken and sterilized by filtration (Erdo rul and Erbilir, 2006). Inhibitory effect of *Lactobacillu*s crude filtrate was examined against tested microorganisms (*P. aeruginosa*, *E. coli*, *Sapht. aureus*, *Sal. typhimurium* and *C. albicans*) using well diffusion method by making wells on the surface of nutrient agar and Sabouraud dextrose agar plates that previously inoculated with test organisms, and 100 µl of crude filtrates of *Lactobacillus spp.* put in each well. Followed by incubation at 37℃ for 24 hr. Diameters of inhibition zones around wells were measured by millimeter (Ryan *et al*., 1996).

One hundred ml of filtrate was concentrated by oven at 40-45 ℃ to one-fold (50 ml), two -fold (25 ml) and three- fold (12.5 ml). Well diffusion method was used to detect the effect of each concentrated filterate against test organisms (*P. aeruginosa*, *E. coli*, *Staph. aureus*, *Sal. typhimurium* and *C. albicans*). Control containing concentrated MRS broth without *Lactobacillus spp.* was used.

2.2.16 Inhibitory effect of S-Layer proteins:

To evaluate the inhibitory effect of S-Layer protein extracted from *Lactobacillus spp.*, well diffusion method as in (2.2.15.2) was used but using S-layer protein instead of *Lactobacillu*s crude filtrate.

2.2.17 Determining Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *Lactobacillus spp.* Concentrated Filtrates:

Serial dilutions (10ml) of three fold concentrated filtrate of *Lactobacillus* were made in tubes containing sterile nutrient broth. The ratios were (10, 20, 30, 40, 50, 60, 70, 80 and 90%) giving final volume of 10 ml in each tube. After each concentration was inoculated by 0.1 ml of the test organisms (*P. aeruginosa*, *E. coli*, *Staph. aureus*, *Sal. typhimurium* and *C. albicans*), it was incubated at 37 ℃ for 24 hr. Growth intensity of each tube was observed by inoculation on nutrient agar and Sabouraud dextrose agar (for *Candida albicans*) then incubated overnight at (37°C) Results were recorded as growth (+), and no growth (-) (Atlas *et al*., 1995).

2.2.18 Bacterial Adhesion Test (Iwahi *et al*., 1982):-

2.2.18.1 Preparation of solution and buffer:

- **Phosphate Buffer Saline (PBS)**

It was prepared from the following ingredients:

Ingredient	Weight (g)
NaCl	8
KCl	0.2
Na_2HPO_4	0.15
KH_2PO_4	0.2

These ingredients were dissolved in 950 ml D.W. and pH was adjusted to 7.2, the volume was completed by D.W to 1000 ml sterilized by autoclaving, cooled and stored at 4°C (Freshney, 2000).

❖ **Fixative Solution:**

It was prepared by mixing (30ml) of methanol (99.8%) with (10ml) of acetic acid (99.8%). It was used for fixation of bacteria and uroepithelial cells during staining by methylene blue (Atlas *et al.*, 1995).

2.2.18.2 Preparation of *E. coli* and *Staph. aureus*:

Ten milliliter of nutrient broth medium was inoculated with bacterial growth culture, and incubated at 37°C for 24 hr. After that, the culture of bacteria was washed twice with PBS and concentrated by centrifugation at 1000 rpm for 20 min and resuspended in PBS.

2.2.18.3 Preparation of Epithelial Cells:

Uroepithelial cells were isolated from urine of some healthy females by centrifugation at 1000 rpm for 5 min then washed three times with PBS and recentrifuged at 1000 rpm for 10 min before resuspension in PBS.

2.2.18.4 Adhesion Test:

- A mixture of 0.2 ml of the bacterial suspension, 0.2 ml of the epithelial cells suspension and 0.1 ml of PBS were incubated at 37°C for 1 hr.
- Unattached bacterial cells to uroepithelial cells were removed by centrifugation in PBS at 1000 rpm for 10 min.
- Final pellet was resuspended in PBS. A drop of suspension was put on to a microscopic slide, air - dried, fixed with methanol: acetic acid (3:1) and stained with methylene blue.
- The adhered bacterial cells to epithelial cells were observed by the compound light microscope.
- The control contained only epithelial cells.

2.2.18.5 Effect of Concentrated Filtrates on Adhesion Property of Tested Organisms:

Minimum inhibitory concentration of the concentrated filtrates of *Lactobacillus spp*. isolate was used to investigate its effect on adhesion property of tested organisms on uroepithelial cells *in vitro* as following:

Nutrient broth medium containing minimum inhibitory effect of concentrated filtrates was dispensed in sterile tubes and incubated with a loopfull of each liquid culture of the tested bacteria at (37°C) for (24) hr.. Adhesion test as in (2.2.18.4) was reused to examine inhibitory effect of the concentrated filtrate after treatment.

2.2.18.6 Effect of S-layer proteins on Adhesion of Tested Organisms (Velraeds *et al*., 1998):

Mixtures consisted of 0.2 ml from each of the following, bacterial suspension, epithelial cells suspension and S-layer proteins isolated from *Lactobacillus spp*. were incubated at 37°C for 1 hr. Procedure was completed as in (2.2.18.4).

2.2.19 Cytotoxic Effect of *Lactobacillus* Concentrated Filterate and S-layer Proteins on Tumor Cell Lines:

Effect of the *Lactobacillus* concentrated filterates and S-layer protein isolated from *Lactobacillus* on two types of tumor cell lines (RD and L20B) were carried out in the Tissue Culture Unit of Biotechnology Research Centre /AL-Nahrain University, Baghdad, Iraq.

2.2.19.1 Tissue Culture Media

❖ **RPMI-1640 (Rosswell Park Memorial Institute) :**

It was prepared according to the method described by Freshney (2000) by dissolving the following ingredients in 950 of D.D.W.

RPMI-1640 with hepes buffer, L-glutamine	10.4g
Crystalline penicillin	0.5ml
Streptomycin	0.5ml
Fetal calf serum	10 %
Sodium bicarbonate	4.4 %

After adjusting the pH to 7.2, the volume was completed to 1000 ml and sterilized by filtration using 0.22 µm size filter paper. The medium was stored at (-20°C) until use.

2.2.19.2 Preparation of Solutions and Reagents:

The following solutions were prepared according to the method described by Freshney (2000).

❖ **Antibiotic Solutions:**

Benzyl Penicillin (1000000 i.u) was dissolved in 5 ml of D.W. and 1g streptomycin was dissolved in 5 ml D.W. Each solution was sterilized by filtration. From each one, 0.5 ml was added to 1L of RPMI-1640 then stored at -20ºC until use.

❖ **Sodium bicarbonate:**

It was prepared by dissolving 4.4 g of $NaHCO_3$ in100 ml of D.D.W, then sterilized by autoclaving and stored at 4°C.

❖ **Neutral red Solution (0.01%)**:

A portion of 0.01 g of the dye was dissolved in 100 ml of PBS.

❖ **Trypan blue Solution (1%):**

Stock solution was prepared by dissolving 1 g of the dye in 100 ml of PBS, then filtered by Wattman filter paper and stored at 4ºC. Working solution was made by diluting 1:10 in PBS before use.

❖ **Extraction dye solution:**

It was prepared by mixing PBS with absolute ethanol in the ratio of 1:1.

❖ **Fetal calf serum:**

It was supplied from Biotechnology Research Centre /AL- Nahrain University. The serum was already thermally inactivated, steriled and used directly for tissue culture media.

❖ **Trypsin-Versene Solution:**

Trypsin-versene	1.01 g
PBS	100 ml

Well mixed, sterilized with 0.22 µm filtering unit and stored at 4°C until use.

❖ **Preparation of S-layer protein dilutions:**

To prepare stock solution 1 ml of S-layer proteins of each *Lactobacillus spp*. were transferred to containers contained 1 ml of serum free medium, then sterilized by filtration using Millipore 0.22µm filter paper. From this stock solution two fold dilutions were made starting from concentration 1000µg/ml ending with the concentration 31.25 µg/ml. The dilutions were done in the wells of microtiter plate using serum free medium.

❖ **Preparation of *Lactobacillus* concentrated filtrate dilutions:**

A portion of 100mg from each of *Lactobacillus spp.* concentrated filtrate was dissolved in 10 ml to of serum free medium, then sterilized by Millipore 0.22µm filter. From these stock solutions two fold dilutions were made starting from concentration 1000µg/ml to 31.25 µg/ml. The dilutions were dumped in the wells of microtiter plate using serum free medium.

2.2.19.3 Cytotoxic Effect of *Lactobacillus* Concentrated Filterate and S-layer Proteins isolated from *Lactobacillus* on Tumor Cell Lines:

The growth inhibition was carried out according to a method that was adopted by Freshney (2000).

The method included the following steps:

- The cells (L20B and RD) were supplemented as a monolayer attached cells in falcon culture flasks (25) cm^2 containing RPMI-1640 medium. The cells were washed with sterile PBS under aseptic conditions after decanted off the old medium, and then a 2-3 ml of trypsin-versene solution was added with gentle shaking until the cells were detached from the flask surface. A quantity of 20 ml of the new growth medium was added to falcon containing a suspension of single cells with well stirring followed by transferring the contents of each falcon into another in a way that each falcon contained equal volume of both culture medium and cells (subculture). These falcons were incubated at 37°C for 24 hr. for L20B and RD cell lines.
- Counting of viable cells was carried out using trypan blue dye; dead cells usually take up the dye within a few seconds making them easily distinguished from viable cells. For this, one part of cell suspension was mixed with equal volume of diluted trypan blue stain (0.2 ml trypan blue in 1.6 ml PBS).
- Cell suspension was prepared by treating the container of (25) cm^2 with trypsin-versene solution followed by the addition of 20 ml of growth medium supplemented with 10% fetal calf serum. The cells were seeded in the wells of 96 well tissue culture plate, which carried out by transferring 200 µl of cell suspension in to each well, in somehow that each well had contained ($1x\ 10^5$) cell/well, and the plate was incubated overnight at 37°C for L20B and RD cell lines.

- The day after, the wells examined to inspect the formation of cell monolayer, and then 200 µl/well from each concentration (1000, 500, 250, 125, 62.5 and 31.5 µg/ml) that were previously prepared for each extract as much as three replicates, also 12 replicates were made for the control which contained only the cells with 200 µl/well of serum free medium (SFM), then the plates were wrapped with para-film and incubated at 37°C for 48 hr. in an incubator supplemented with (5%) CO_2.
- After elapsing the incubation period the media was decanted off, 50 µl/well of neutral red dye were added and incubated again for 2 h. after incubation, The contents of the plate were removed by washing the cells with PBS to remove the excess dye followed by the addition of 20 µl/well of extraction dye solution that draw out the dye from the viable cells that had stained. The results were read using ELISA reader at wave length 492 nm. The percentage of growth inhibition was calculated according to the following equation: (Gao *et al.*, 2003).

$$\text{Growth inhibition \%} = \frac{\text{Control} - \text{Treatment cell}}{\text{Control}} \times 100$$

2.2.20 Statistical Analysis:

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using SPSS computer program version 7.5.

Differences in results were considered significant at probability value equal or less than 0.05.

3. Results

3.1 *Lactobacillus* Isolates:

Lactic acid bacteria were primarily identified depending on the formation of clear zone around their growing colonies on MRS agar containing calcium carbonate (1%) as a result of acid production and $CaCO_3$ dissolving and that mentioned by many researchers (Buck and Gilliland, 1995; Kimoto *et al*., 2004).

3.1.1 Cultural and Morphological Characteristics:

All colonies of *Lactobacillus* isolates on MRS agar appeared; white to pale in color, round shape, convex, soft, mucoid and having smooth edges.

Microscopic examination showed that cells were gram positive, short or long bacilli, found mainly in chain containing 3-8 cells and non-spore forming. These results coincide with those mentioned by Jawetz *et al*. (1998).

Depending on the results of the cultural and microscopic examination, thirteen isolates may be belonging to the genus *Lactobacillus*

3.1.2 Biochemical Tests:

After lactic acid bacteria isolates were identified by biochemical tests, results in table (3-1) shown that they were negative to oxidase, catalase, and to the production of gelatinase after grow on gelatin medium, while they were positive to litmus milk test. When *Lactobacillus* isolates grew in MRS broth at 15 °C and 45 °C under anaerobic conditions, results showed that most of them grew at 45 °C but few of them could grew at 15 °C especially *Lactobacillus casei* and *L. plantarum* which are considered as mesophillic group of *Lactobacillus* bacteria. These results were agreed with those mentioned by Kandler and Weiss (1986).

Table (3-1) Biochemical tests of *Lactobacillus spp*.

Test	Lbvn1	Lbvn2	Lbh1	Lbh2	Lbh3	Lbc1	Lbc2	Lbc3	Lby1	Lby2	Lbvg1	Lbvg2	Lbvg3
Acid and curd production	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatinase test	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 15 C	+	+	-	-	-	+	-	-	+	-	-	-	-
Growth at 45 C	-	-	+	+	+	-	+	+	-	+	+	+	+
Species of isolates	***L.plantarum***	***L.plantarum***	***L.acidophilus***	***L.acidophilus***	***L.gasseri***	***L.plantarum***	***L.rhamenosu***	***L.curvatus***	***L.plantarum***	***L.acidophilus***	***L.acidophilus***	***L.acidophilus***	***L. fermentum***

Lactobacillus spp. were finally identified by carbohydrate fermentation tests. Results recorded as mentioned by Holt and Kriege (1986). Table (3-2) summarized the results of fermentation of 16 different sugars by *Lactobacillus* isolates. The following 6 species were identified; *L. acidophilus*, *L. plantarum*, *L. gasseri*, *L. rhamenosus*, *L. curvatus* and *L. fermentum*.

Table (3-2) Carbohydrate fermentation by *Lactobacillus* isolates:

NO. of isolates	Arabinose	Cellobiose	Esculine	Fructose	Glucose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Raffinose	Rhamnose	Ribose	Salicine	Sucrose	Trehalose	Species of isolates
Lbvn1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	*L.plantarum*
Lbvn2	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	*L.plantarum*
Lbh1	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	*L.acidophilus*
Lbh2	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	*L.acidophilus*
Lbh3	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	*L.gasseri*
Lbc1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	*L.plantarum*
Lbc2	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	*L.rhamenosus*
Lbc3	-	+	+	+	+	+	+	-	+	-	-	-	+	+	-	-	*L.curvatus*
Lby1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	*L.plantarum*
Lby2	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	*L.acidophilus*
Lbvg1	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	*L.acidophilus*
Lbvg2	-	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	*L.acidophilus*
Lbvg3	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	*L. fermentum*

Lbvn: *L. spp*. from vinegar, Lbh: *L. spp*. from human milk, Lbc: *L. spp*. from cow milk, Lby: *L. spp*. from yoghurt, Lbvg: *L. spp*. from vagina.

+ = positive result (fermenter), - = negative result (non fermenter)

3.2 Identification of test microorganisms:

Test microorganisms used in this study were obtained from Biotechnology Department/Al- Nahrain University. They were reidentified according to Cheryl *et al*. (2001) for *Candida albicans*, Jawetz *et al*. (1998) and De la Maza *et al*. (2004) for the test bacteria.

3.3 S-layer proteins and their extraction with Guanidine HCl:

Presence of crystalline arrays of protein (that so-called S-layer) covering the cell surface has been shown in several *Lactobacillus* species (Boot *et al.*, 1996).

Putative S-layer proteins on the bacterial cell surface can be deduced by the occurrence of a dominant protein band in the protein profile of non-lysed bacteria.

Twelve isolates of *Lactobacillus spp*. were analyzed by electrophoresis using 10% SDS-PAGE and the lane of proteins bands obtained were compared with four marker proteins (γ-globulin MW = 150 kDa, Transferrin MW = 80 kDa, Trypsine MW = 20 kDa, Lysozyme MW = 14 kDa).

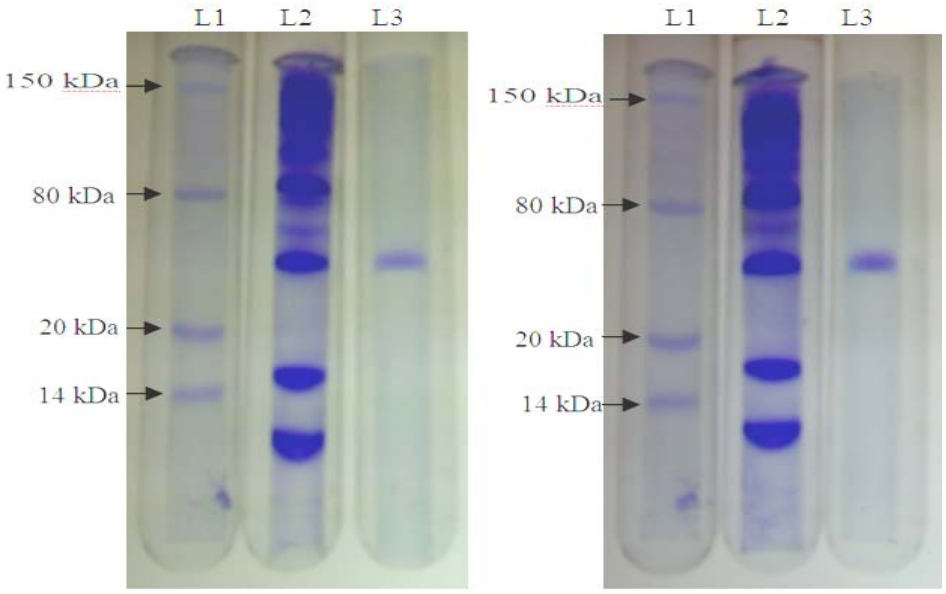
To extract S-layer protein, the band which located between Transferrin and Trypsine excised and treated with 6M G-HCl from crude column as mentioned in section (2.2.12).

Åvall-Jääskeläinen and Palva (2005) found that *Lactobacilli* surface layer proteins are among the smallest detected with molecular masses ranging from 25 to 71 kDa. The S-layer subunits are non-covalently linked to each other and to the supporting cell envelope, and can be disintegrated into monomers by denaturing agents such as urea or guanidin HCl, metal-chelating agents or by cation substitution (Sara, 2001).

Results of protein profile by SDS-PAGE revealed that seven bands with MW range between 10.26-108.71 kDa were obtained after analysis of *L. acidophilus* isolate (1) which isolated from chicken intestine. Then, detected band were excised and treated with 6M guanidine hydrochloride and analysed by SDS-PAGE. Results showed that only one band was obtained with MW 47.74 KDa. It was corresponded to the original band in crud column as shown in figure (3-

1) and (appendix 1-A). This came in accordance to Frece, *et al*. (2005) who mentioned that S-layer proteins of *lactobacilli* have molecular mass between 40 and 55 kDa.

Analysis of protein profile of *L. acidophilus* (2) isolate from chicken intestine gave eight bands with MW range between 11.50-177.74 kDa. Band with MW 50 kDa represented the S-layer protein, and treatment of this band with 6M guanidine hydrochloride gave one band with MW 48.37 kDa which corresponded to the original band in crud column (figure, 3-1) and (appendix1-A).



-A- -B-

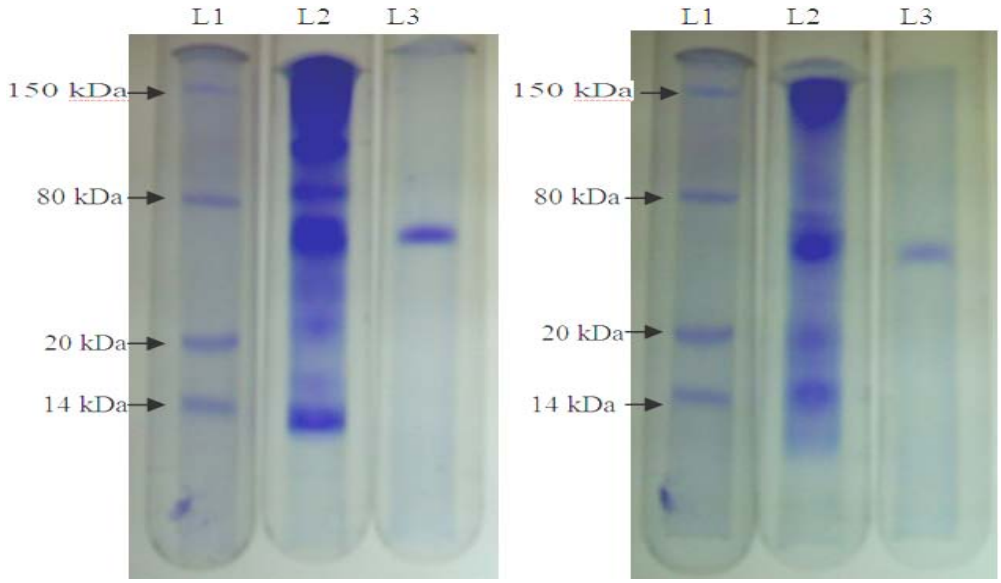
Figure (3-1): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (A) *L. acidophilus*1 and (B) *L. acidophilus*2 isolated from chicken intestine.

L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis

Results of protein profile analysis of *L. acidophilus* from feces of children showed seven bands with MW ranged between 13.10 -147.53 kDa. Band with MW 49.46 KDa represented the S-layer protein. On the other hand, five bands were obtained from *L.casei* of children feces with MW range

between 14.37- 292.50 KDa. Only band with MW 43.59 KDa represented S-layer protein.

Analysis S-layer from *L. acidophilus* and *L. casei* after treating with 6M guanidine hydrochloride gave two bands with MW 49.46 and 44.66 KDa, respectively as shown in figure (3-2) and (appendix 1-B).



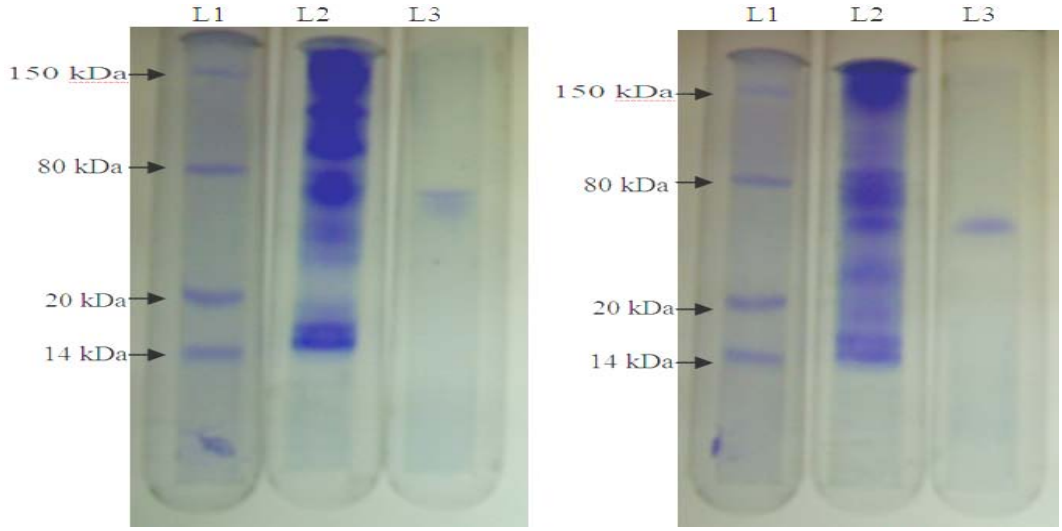
-A- -B-

Figure (3-2): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (A) *L. acidophilus* (B) *L. casei* isolated from faeces of children

L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis

Results in figure (3-3) and (appendix 1-C) indicated that bands with MW 46.52 and 44.25 KDa represented the S-layer protein of *L. plantarum* and *L. acidophilus* isolated from yoghourt, respectively. Treatment of these bands with 6M guanidine hydrochloride gave bands with MW 48.69 and 43.42 kDa, respectively, which were corresponded to the original band in crud column. Boot *et al* (1993) found that the molecular weight of surface protein was 43 kDa when extracted from *L. acidophilus* ATCC 4356 by treatment of whole cells with 4 M guanidine hydrochloride.

Analysis of protein profile of *L. gasseri* from human milk gave seven bands with MW range between 13.70 – 158.94 KDa. Band with 38.92 kDa represented S-protein; treatment of this band with 6M guanidine hydrochloride gave one band with MW 37.58 KDa was corresponded to the original band in crud column as shown in figure (3-4) (appendix 1-D).



-A- **-B-**

Fig (3-3): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (A) *L. plantarum* (B)*L.acidophilus* isolated from yoghurt

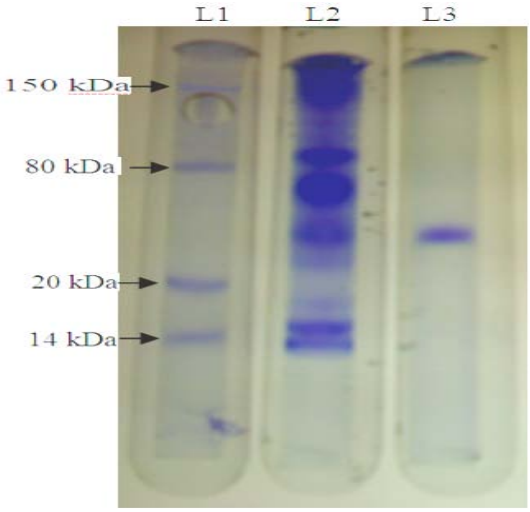


Fig (3-4): Protein profile analysis of *Lactobacillus gasseri* isolated from human milk by 10% SDS-PAGE

L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis

S-layer proteins did not appeared in protein profile analysis of *L. fermentum* isolated from vagina (figure, 3-5) and (appendix 1-D). This result was disagreed with that of Rojas *et al* (2002) who purified and characterized a 29-kDa cell surface protein from *L. fermentum*.

Kahala, *et al*. (1997) stated that among lactic acid bacteria, the S-layer seems to be a typical surface structure in several *Lactobacillus* species, e.g., in *L.acidophilus*, *L. helveticus*, *L. casei*, *L. brevis*, *L. buchneri*, *L.fermentum*, *L. bulgaricus* , and *L. plantarum* .

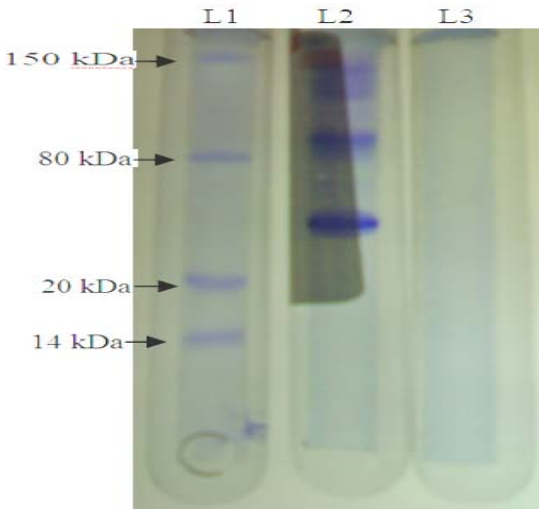
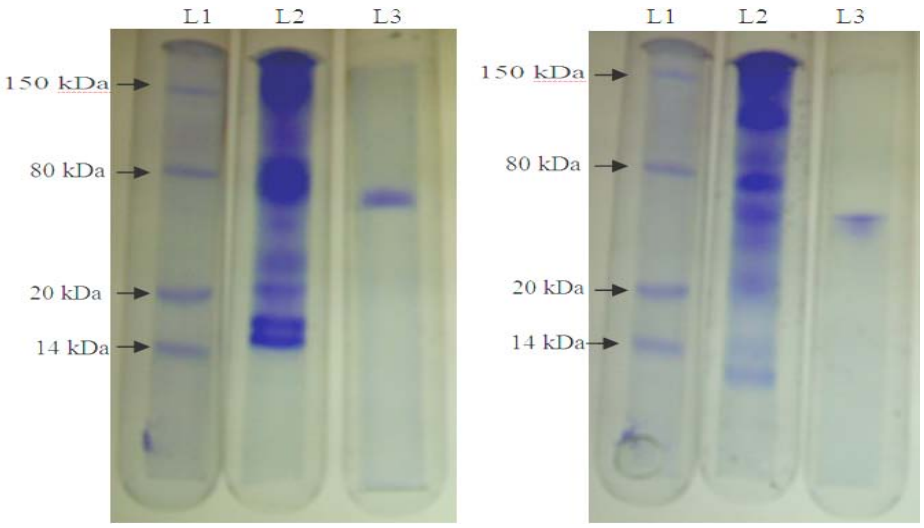


Figure (3-5): Protein profile analysis of *Lactobacillus fermentum* isolated from vagina by 10% SDS-PAGE.

L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis

Results of analysis of protein profile of *Lactobacillus plantarum* from vinegar, showed that only one band with MW of 63.06 kDa was visible, while *Lactobacillus plantarum* from cow milk gave S-layer band with MW 51.46 kDa, as indicated in figure (3-6) and (appendix 1-E).



-A- **-B-**

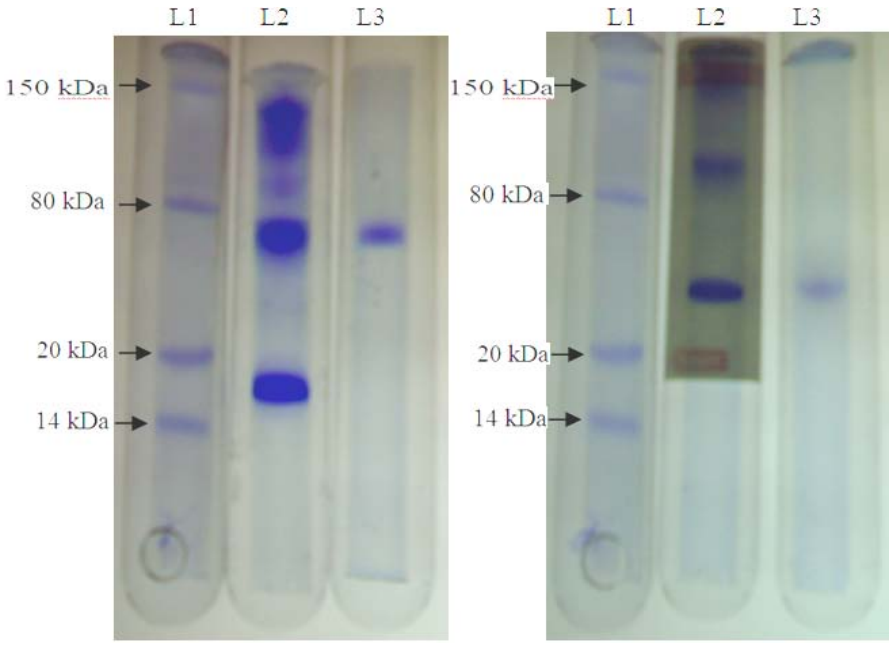
Figure (3-6): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE (A) *L.plantarum* from vinegar (B) *L.plantarum* from cow milk.

L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis

Protein profile analysis of *L. rhamenosus* and *L. curvatus* from cow milk showed that two bands were obtained with MWs 60.09 and 39.64 kDa, respectively, (figure, 3-7) and (appendix1-F).

Jakava-Viljanen and Palva (2007) found that the molecular masses of S-layer proteins of *Lactobacillus spp.* which isolated from pig intestine ranging between 45–62 kDa.

The molecular weight of S-layer protein is varied depending on species and sources of *Lactobacillus.* Most S-layers are composed of a single protein species which greatly varies in size related to different bacterial genera (Boot and Pouwels, 1996).



-A- **-B-**

Figure (3-7): protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (K) *L. rhamenosus* (L) *L. curvatus* isolated from cow milk.

L1: represents protein markers, L2: represents crude analysed cells of *Lactobacillus* and L3: is pure protein.

3.4 Concentrations of *Lactobacillus* S-layer proteins:

The concentrations of extracted S-proteins from *Lactobacillus* were determined by using Kit which depended on Biuret method.

Results of the concentrations of S-proteins showed that were ranged from 1.87 mg/ml for *L.acidophilus* (isolated from chicken intestine) to 0.13 mg/ml for *L. curvatus* (from cow milk) as shown in table (3-3). Under laboratory cultivation conditions, yield of the S-layer glycoprotein ranges between 0.5 and 2.0 g wet weight per litre of growth medium (Eshinimaev *et al*., 2002).

Table (3-3): Concentrations of S-layer proteins of *Lactobacillus* isolates

S-layer protein from		Concentration of protein (mg/ml)
Isolates	**sources**	
L. acidophilus1	From chicken intestine	1.87
L. acidophilus2		1.79
L. acidophilus	From feces	1.56
L. casei		1.39
L. plantarum	From yoghurt	0.83
L. acidophilus		0.32
L. gasseri	From human milk	0.55
L. plntarum	From vinegar	1.21
L. plantarum	From cow milk	0.57
L. rhamenosus		1.17
L. curvatus		0.13

Two S-layer proteins extracted from *L. acidophilus*1 and *L.casei* which their molecular weight were (47 and 44 kDa) and their concentrations were (1.87 and 1.39 mg/ml), respectively, were used in this study to evaluate the biological role of S-layer proteins.

3.5 Inhibitory effect of *Lb. acidophilus* and *Lb. casei* on pathogenic microorganisms:

Probiotic LAB has been known to show protective effects against pathogens (Ishida-Fujii *et al*., 2007). Numerous studies on human suggested that lactic acid bacteria at a dose of (10^9-10^{11} cfu/ml) per day can decrease the incident, duration and severity of some gastric and intestinal illness (Boonnaert and Rouxhet, 2000).

In the present study, different methods were used depending on medium used to estimate the effect of *L. acidophilus* and *L. casei* against some pathogenic

organisms, and the results obtained on both solid and liquid medium are as follows:

3.5.1 On Solid Medium (MRS agar):

Ability of *L. acidophilus* and *L.casei* to produce inhibition activity against pathogenic organism was tested by growing the isolates on MRS agar medium for different incubation periods (24, 48, 72 hr) at 37 ºC under anaerobic conditions. In this approach, AL-Kafaji and AL-Kasab (1992) used MRS agar medium to study ability of *Lactobacillus* isolates, to produce inhibition metabolites when grown under anaerobic condition, and obtained reasonable result. Anas *et al* (2008) found that several strains of *Lactobacillus* gave inhibition zones against *Staphylococcus aureus* on solid medium ranging from 3-8mm.

Table (3-4) shows the inhibitory effect of *L. acidophilus* and *L. casei* grown on MRS agar against the test organisms at three different incubation periods.

Both *Lactobacillus* isolates exhibited inhibitory effect against the pathogenic organisms, and highest effect was against *P. aeruginosa* after incubation for 24 hr. which reached 14 and 11 mm for *L. acidophilus* and *L. casei*, respectively, (appendix 2-A) this result agreed with Mobarez *et al* (2008) who mentioned that antibacterial activities of bacteriocines from *L. acidophilus* against *P. aeruginosa* was stronger than against *S. aureus*.

Composition of MRS media induce LAB to produce secondary metabolites with inhibitory effect against pathogenic bacteria and as it was mentioned by Garver and Muriana (1994) who found that the production of inhibitory materials by LAB is depended on the media used for growth and they found that Tween 80 induce the production of proteins (bacteriocine) by increasing activity of the bacteria, also Champangne *et al.* (1999) found that the growth

rate, maximum biomass levels and probiotic production were increased by raising yeast extract concentration from (0.5 g/l) to (5 g/l) which is the concentration that used in MRS media.

Table (3-4): Inhibitory effect of *Lb. acidophilus* and *Lb. casei* against the test pathogenic microorganisms on solid media estimated by diameter of inhibition zone (mm).

Microorganism	**Incubation time (hr)**	**Inhibition Zone Diameter of (mm)**	
		Lb.acidophilus	***Lb.casei***
Pseudomonas aeruginosa	**24**	**14**	**11**
	48	**10**	**8**
	72	**7**	**5**
Escherichia coli	**24**	**3**	**5**
	48	**9**	**11**
	72	**5**	**8**
Salmonella typhimurium	**24**	**10**	**9**
	48	**7**	**5**
	72	**4**	**-**
Staphylococcus aureus	**24**	**9.5**	**9**
	48	**8**	**8**
	72	**4**	**2**
Candida albicans	**24**	**9**	**8**
	48	**7**	**5**
	72	**4**	**-**

Incubation period of 24 hr. gave the highest inhibitory effect by *L.acidophilus* and *L.casei* against *P. aeruginosa*, *Staph. aureus*, *Sal. typhimurium* and *C. albicans* than other incubation periods of 48 and 72hrs, these results came in accordance with those obtained by AL-Marsoomy (2008) who found that *L. plantarum* and *L.casei* gave best inhibitory effect against *E. coli* after 24hr, also

Kontula (1999) found that *Lactobacillus spp.* needs 16 hr. after cultivation to reach the appropriate cell density for probiotic materials production, while these result disagreed with Al-Dulemy (2000) who found that inhibitory effect of LAB increased after 48 hr. of incubation but the inhibitory effect of these *Lactobacillus* against *E. coli* was better after 48 hr. of incubation this result agreed with AL-Yas (2006) who reported that inhibitory effect of LAB against *H. pylori* increased after 48 hr.

Differences in the above results of LAB against the pathogenic bacteria may be related to the type of bacteria, type of the inhibitory substance, its quantity and ability to distribute in the medium (Egorov, 1985). Aktypis *et al*. (1998) suggested that such differences in the inhibitory effect at different incubation periods may be related to the nature of LAB isolates used against the test bacteria itself, while Anas *et al*. (2008) referred the effect to the production of organic acids (such as lactic acid, acetic acid), hydrogen peroxide, diacetyl and production of bacteriocins, bacteriocin-like substances by the strains of *Lactobacillus*.

Present result confirmed that *L.acidophilus* showed better inhibitory effect against *P. aeruginosa*,*Staph. aureus*, *Sal. typhimurium* and *C. albicans* than *L. casei*. Other studies also clarified the inhibitory effect of *L. acidophilus* as the study of AL-Jeboury (2005) who found that the inhibitory effect of *L. acidophilus* was high against *Proteus mirabilis*, as well as Coollborn (2005) who recorded that *L.acidophilus* exhibit greatest inhibitory effect than other *Lactobacillus spp.* against pathogenic organism. *L. casei* gave more inhibitory effect than *L.acidophilus* against *E. coli* only after 48 hr. as shown in appendix (2-B). This result came in accordance with that obtained by Pishva *et al* (2009)

who found that *L. casei* possessed more inhibitory effect against *E. coli* than other *Lactobacillus spp.*

3.5.2 In liquid medium (MRS broth):

Well diffusion method was used to evaluate the inhibitory effect of *L. acidophilus* and *L. casei* filtrates, against pathogenic organisms.

Table (3-5) showed the highest effect of *L. acidophilus* and *L. casei* was against *P. aeruginosa* when the inhibition zones reached 24 and 22 mm, respectively. Maximum inhibition zone was 24 mm for *L.acidophilus* against *P. aeruginosa* followed by (*Staph. aureus, Sal. typhimurium* and *C. albicans*) which were (17 and 18 mm) respectively, after 24 hr. incubation period, which was higher than that recorded against same microorganisms on solid medium. This may be due to MRS broth exhibited wide spectrum inhibitory effect against Gram positive (*Staph. aureus*, *Baccillus subtilis*) and Gram negative bacteria (*E.coli, klebsiella spp., Proteus spp.*) (Gupta *et al.*, 1998), also Kubba (2006) who found that best inhibitory effect was gained when the MRS broth was used against pathogenic bacteria.

The filtrates of *L. acidophilus* and *L. casei* gave better inhibitory effect after 24 hr. of incubation, while increasing this period to 48 hr. showed lower inhibitory effect, while lowest effect appeared after 72 hr. against *P. aeruginosa*, *Staph. aureus, Sal. typhimurium* and *C. albicans*. these results was in agreement with those obtained by Aziz (2007) who found that best inhibitory effect of LAB against *Proteus mirabilis* was obtained after 24hr of incubation. The filtrates of *L. acidophilus* and *L. casei* gave more inhibitory effect against *E.coli* with inhibition zones of 18 mm and 19 mm, respectively, after 48 hr of incubation than other periods. Barfoot and Klaenhammer (1983) declared that death of test bacteria increased with increasing inhibitory substances like bacteriocin, acidophilin and plantaracin of LAB. Jin *et al*. (1996) recorded that

lactic acid was found to be the metabolic product responsible for inhibition of bacteria like *E. coli*.

Results in table (3-5) revealed that *L.acidophilus* gave inhibitory effect against *P. aeruginosa*, *Staph. aureus*, *Sal. typhimurium* and *C. albicans* better than that gave by *L. casei* this may due to acidophilin production from *L.acidophilius* as Lewus *et al*. (1991) reporteded. Appendix (3-A) and (4-C) showed that *L. casei* gave more inhibitory effect than *L.acidophilus* against *E. coli*, when inhibitory zone reached 15 mm after 24 hr. of incubation time, while *L.a* have 12 mm after the same of incubation time. Such results were similar with those obtained on the solid medium. On the other hand, Oyetayo (2004) mentioned that *Lactobacillus spp*. obtained from chicken caecum had been found to display antagonistic effect against other bacteria such as *E. coli* and *Salmonella spp*.

Production of directly inhibitory compounds (e.g., bacteriocin), reduction of luminal pH through short chain fatty acid production, competition for nutrients, adhesion sites on the gut wall, modulation of the immune response and regulating coloncyte gene expression are some of these mechanisms (Fooks and Gibson, 2002).

Table (3-5): Inhibitory Effect of *L.acidophilus* and *L.casei against* some pathogenic microorganisms in liquid media estimated by diameter of inhibition zone (mm).

Microorganism	**Incubation time (hr)**	**Inhibition Zone Diameter of (mm)**	
		L.acidophilus	***L.casei***
Pseudomonas aeruginosa	24	24	22
	48	21	20
	72	17	15
Escherichia coli	24	12	15
	48	18	19
	72	15	16
Salmonella typhimurium	24	17	16
	48	15	14
	72	13	11
Staphylococcus aureus	24	17	14
	48	15	13
	72	12	10
Candida albicans	24	18	17
	48	15	14
	72	13	11

Results of inhibitory effect for concentrated filtrate of *L.acidophilus* and *L. casei* against pathogenic microorganisms demonstrated in table (3-6). Filtrate of *L.acidophilus* and *L. casei* were concentrated to three folds by oven. The one - fold concentrated filtrate of LAB gave good inhibitory effect but the effect of two fold was better, while the three fold concentration showed highest inhibitory effect after 24hr. incubation because all the inhibitory substances were concentrated, zone diameter of *L.a* against *P. aeruginosa*, *E. coli*, *Sal. typhimurium*, *Staph. aureus*, and *C. albicans*, reached to 31, 17, 23, 24 and 27

mm, respectively, and *L.c* have zone diameter reached to 29, 21, 23, 21 and 25 mm, against them, respectively, as shown in appendix (5).these results agreed with Pfeiffer and Radler (1982) who found that there is a positive relationship between the diameter of inhibition zone and concentration of the inhibitory substance.

Table (3-6): Inhibitory Effect of *L.acidophilus* and *L.casei* concentrated filtrate against some pathogenic microorganisms estimated by diameter of inhibition Zone (mm).

Microorganism	**Concentrated**	**Inhibition Zone of Diameter (mm)**	
		Lb.a	***Lb.c***
Pseudomonas aeruginosa	**One-fold**	26	25
	Two-fold	29	27.5
	Three-fold	31	29
Escherichia coli	**One-fold**	13	17
	Two-fold	14	20
	Three-fold	17	21
Salmonella typhimurium	**One-fold**	20	18
	Two-fold	21	20
	Three-fold	23	23
Staphylococcus aureus	**One-fold**	18	16
	Two-fold	21	18
	Three-fold	24	21
Candida albicans	**One-fold**	20	20
	Two-fold	26	23
	Three-fold	27	25

3.6 Inhibitory effect of S-Layer proteins of *L. acidophilus* and *L. casei* on tested microorganisms:

When the inhibitory effect of S-layer proteins of *L.acidophilus* and *L.casei* by well diffusion method was evaluated. Results showed that S-layer protein had no inhibitory effect on growth of the pathogenic microorganisms. This result agreed with Johnson-Henry *et al*. (2007) who observed no any bactericidal activity of S-layer protein isolated from *Lactobacillus helveticus* against *E. coli*.

3.7 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of LAB Filtrates against pathogens:

Results (table 3-7) indicate that concentrations 10% and 20% of both *L.a* and *L.c* filtrates had no effect on the tested microorganism when clear growth of pathogenic microorganisms was observed after (24hr) of incubation. Adversely, 40% of both filtrates led to minimized growth (MIC) of *P. aeruginosa*, while concentration 50% of both LAB filtrates was needed to inhibit growth of this bacterium completely (MBC).

At the time that a sharp decrease in growth of *E. coli* was recorded by treatment with concentration 50% of both LAB filtrates, growth was completely inhibited by 60% concentration. The concentrations of 50 % and 60 % of *L. acidophilus* and *L. casei* filtrates respectively, were considered the MIC,s against *Staph.aureus*, where as 60 % and 70 % were the MBC,s. Concentration 60% of both *L.a* and *L.c* filtrates (MIC) were sharply reduced growth of *Sal. typhimurium* and *C. albicans*, while 70 % completely inhibited their growth. Mohamed (2008) found that concentration 50% of LAB was the MIC for *E. coli*, while 60% for *Staph.aureus* and *P. aeruginosa*. Al-Jeboury (2005) found

that the MIC of *L.acidophilus* and *L. plantarum* concentrated filtrates were 50% and 60% respectively, for *Proteus mirabilis* isolates.

Table (3-7): Minimum Inhibitory Concentrations (MIC,s) and Minimum Bactericidal Concentrations (MBC,s) of Concentrated Filtrates of *L. acidophilus* and *L. casei* against pathogens:

	Isolates	LAB filtrate concentration (%)								
		10	20	30	40	50	60	70	80	90
L. acidophilus	***Pseudomonas aeruginosa***	+	+	+	+	-	-	-	-	-
	Escherichia coli	+	+	+	+	+	-	-	-	-
	Salmonella typhimurium	+	+	+	+	+	+	-	-	-
	Staphylococcus aureus	+	+	+	+	+	-	-	-	-
	Cadida albicans	+	+	+	+	+	+	-	-	-
L. casei	***Pseudomonas aeruginosa***	+	+	+	+	-	-	-	-	-
	Escherichia coli	+	+	+	+	+	-	-	-	-
	Salmonella typhimurium	+	+	+	+	+	+	-	-	-
	Staphylococcus aureus	+	+	+	+	+	+	-	-	-
	Cadida albicans	+	+	+	+	+	+	-	-	-

Growth = + No Growth= -

3.8 Adhesion of *Escherichia coli* and *Staphylococcus aureus* :

Adherence of pathogenic bacteria to host epithelial cells is an important step in the initiation of the infectious process (Finlay and Caparon, 2005). Bacterial adhesion is initially based on non-specific physical interaction between two surface, which then enable specific interaction between adhesion

usually (proteins) and complementary receptors (Rojas and Conway, 1996). In the current study, adherence property of *E. coli* and *Staph . aureus* as well as how this property may be affected by LAB isolate and S-proteins, was investigated.

Adherence ability of *E.coli* and *Staph. aureus* to ureoepithelium (UEP) is shown in (Figure 3-8). Results clarified that the average number of *E. coli* adhering to UEP ranged from 50-60 bacteria/cell, whereas the number of *Staph. aureus* adhering to UEP ranged from 29-35 bacteria/cell.

Many researches confirmed that pili mediate attachment of uropathogenic *E. coli* to human urinary tract epithelium (Cook *et al*., 1995) while *Staph. aureus* and *streptococci pyogenes* adhere to host epithelial cells through the expression of surface proteins which bind to the host extracellular matrix proteins such as fibronectin and collagen (Jost and Billington, 2005).

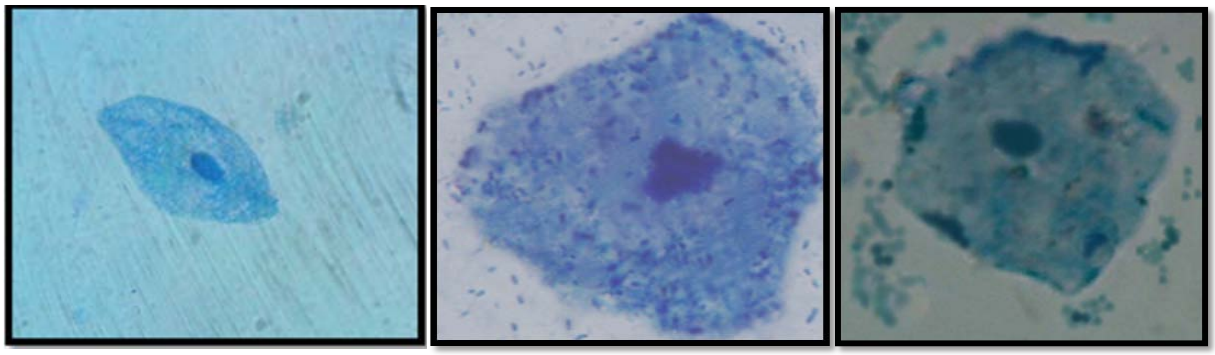


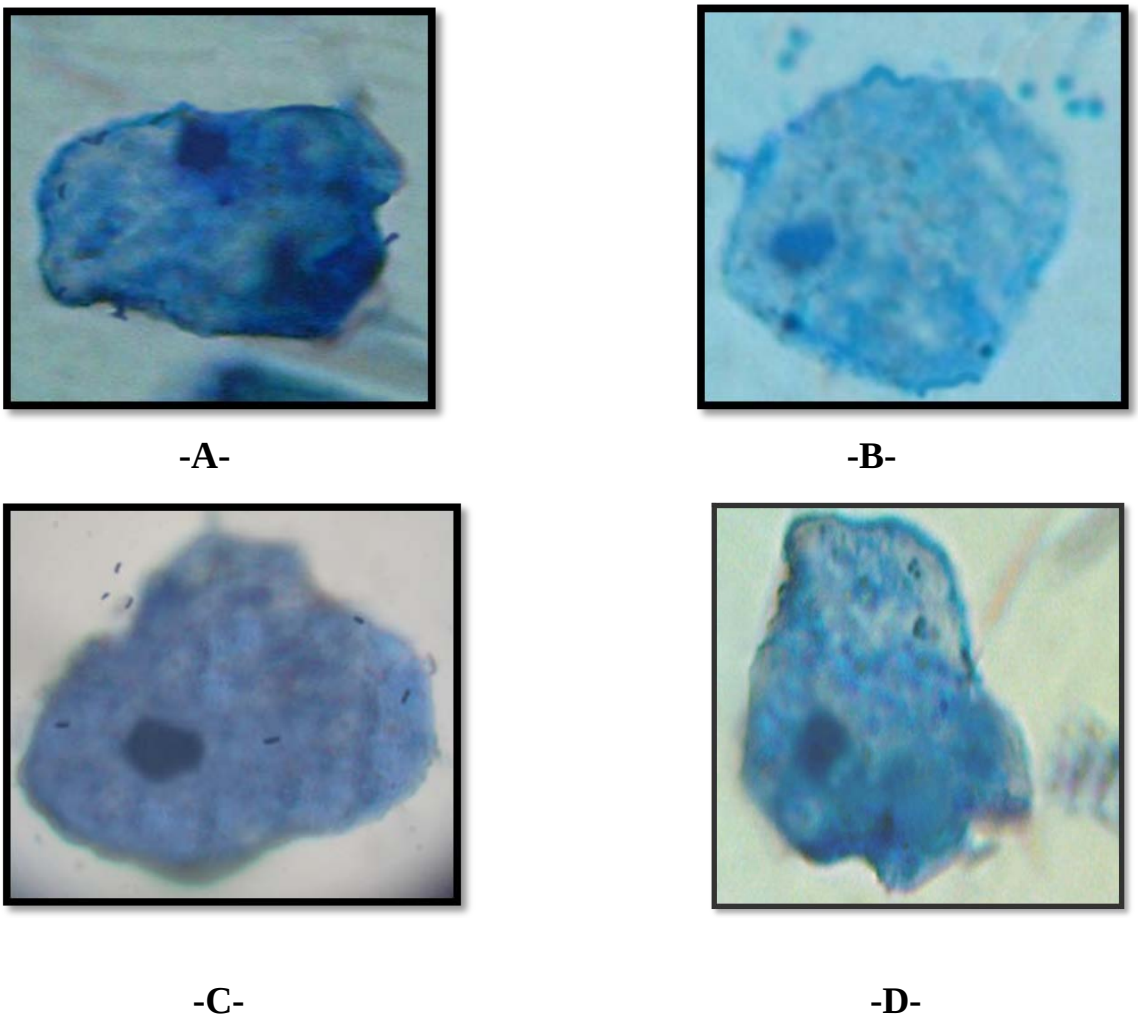
Figure (3-8): Microscopical Examination of Adhesion Property of *E. coli* and *S. aureus* Ureoepithelium cell under Oil - Immersion Objective (100xs). (A) Normal Uroepithelial cell (B) *E.coli* Adhered to Ureoepithelial Cell (C) *S. aureus* adhered to Ureoepithelial Cell.

3.9 Adhesion Inhibition by LAB Filtrates and S-layer protein:

The effect of concentrated filtrate of LAB and S-layer protein against adhesion property of *E. coli* and *Staph . aureus* were studied. Results showed that the three-fold concentrated filtrate of LAB (*L.acidophilus* and *L.casei*) minimized adhesion of *E. coli* to uroepethilial cell reaching an average of (5-12) bacteria / cells (fig. 3-9.A). In this aspect Blomberg *et al* (1993) found that *L. fermentum* produced a proteinaceous component detectable in spent culture fluid during growth in both complex and defined media; this component inhibited the adhesion of *E. coli* fimbriae to ileal mucus by interacting with mucus components.

The three-fold concentrated filtrates of both LAB minimized adhesion of *Staph. aureus* to the uroepethilial cell reached an average of (4-9) bacteria / cells (fig. 3-9.B). Study of Hawthorn and Reid (1990) found that precoating of LAB strains reduced the binding of uropathogenic (*Staphylococci* and *E. coli*) to 8 bacteria /cell.

Similar reduction also observed when the S-layer protein was used and, adhesion *E. coli* and *Staph. aureus* to the uroepethilial cells reached an average of (3-9) bacteria / cells. S-layer protein has the potential to play a role in the competitive exclusion of pathogens (Sára and Sleytr, 2000). Johnson-Henry *et al*. (2007) who found that S-layer protein extracted from *L. helveticus* had inhibition effect on enterohaemorrhagic *E. coli* adhesion to host epithelial cells.



-A- **-B-**

-C- **-D-**

Figure (3-9): Microscopical Examination of Adherence of *E.coli* and *Staph. aureus* to the Uroepithelium Cells after Treatment with the Concentrated Filtrate of LAB and S-layer protein (100 X).

-A- After treating *E. coli* with three-fold of LAB.

-B- After treating *Staph. aureus* with three-fold of LAB.

-C- After treating *E. coli* with Slp.

-D- After treating *Staph. aureus* with Slp.

3.10 Cytotoxicity effect of S-layer proteins of *L. acidophilus* and *L. casei* and *Lactobacillus* concentrated filtrates on Tumor Cell Lines:

Two tumor cell lines (RD and L20B) were used in this study for one time of exposure (48 hr). Cell lines were subjected to six concentrations of S-layer protein and of *Lactobacillus* filtrates (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) that were prepared.

Results obtained may be discussed as follows:

3.10.1 Effect of S-layer proteins and *Lactobacillus* filtrates on RD cell line:

After cancer cell line (RD) was treated with S-layer proteins and concentrated filtrates of *Lactobacillus acidophilus* and *Lactobacillus casei*, results showed significant cytotoxic effect started at the certain concentration and continued to the higher concentrations reaching the last concentration when compared with the control.

Surface protein from *Lactobacillus acidophilus* had significant cytotoxic effect ($P \leq 0.01$) on growth of RD cell line at the concentrations of 500 and 1000 µg/ml with growth inhibition percentage 26.61% and 49.81%, respectively, as shown in appendix (6-A) and figure (3-10).

S-layer protein from *Lactobacillus casei* showed significant toxicity ($P \leq 0.01$) on growth of RD cell line at the concentration 1000 µg/ml with growth inhibition percentage 47.19%, as shown in figure (3-10). As the concentration decreased, there was a decline in the inhibitory effect with no significant cytotoxic effect when compared with the control.

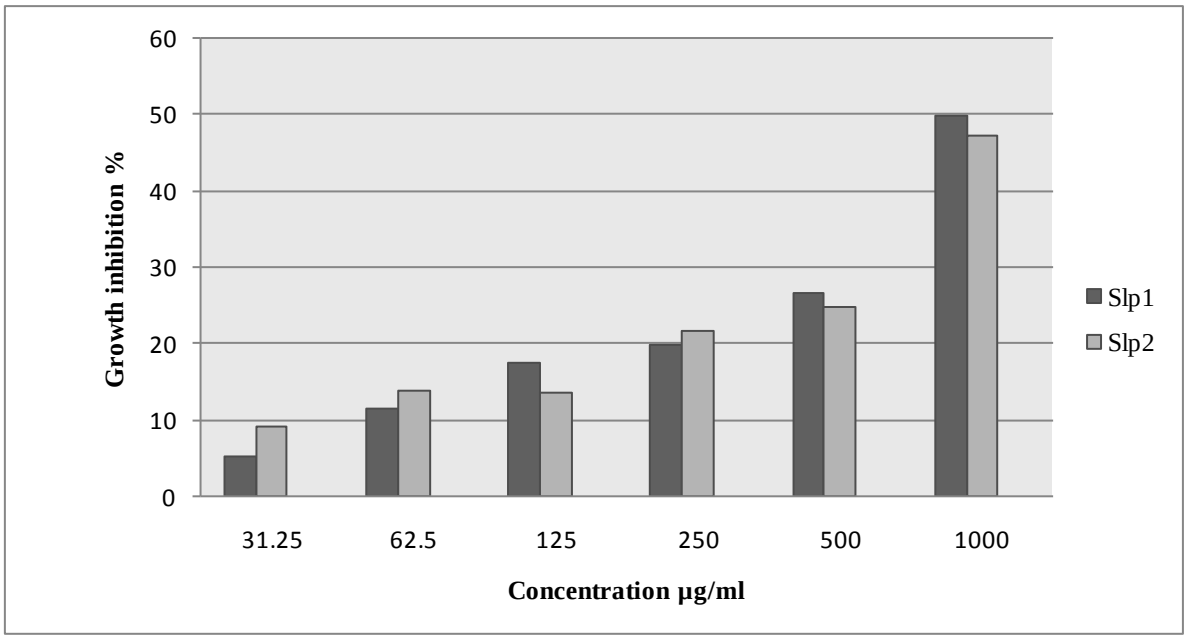


Fig (3-10): Histogram of growth inhibition percentages (GI %) for different concentrations of S-protein of *L.acidophilus* (Slp1) and S-protein of *L. casei* (Slp2) on RD cell line.

Appendix (6-B) revealed the effect of concentrated filtrates *Lactobacillus acidophilus* and *Lactobacillus casei* on growth of RD cell line and the percentage of growth inhibition represented in figure (3-11). There was a high significant cytotoxic effect ($P \leq 0.0001$) for the concentrated filtrate of *Lactobacillus acidophilus* which appeared at concentrations of 62.5 and 125 µg/ml with growth inhibition percentages 73.74% and 54.32%, respectively, but cytotoxic inhibition rate was decreased with increasing concentrations. The significant toxicity ($P \leq 0.001$) was appeared at the concentrations of 250, 500 and 1000 µg/ml to give growth inhibition percentages of 40.13%, 40.46% and 40.78%, respectively.

An explanation for this behavior, is that in the design of cell culture experiment it was important to be aware of the growth state of the culture, as well as the quantitative characteristics of cell strain or cell line . Cultures will vary significantly in many of their properties between exponential growth and stationary phase (Freshney, 1994).

The concentrated filtrate of *Lactobacillus casei* has significant effect ($P \leq 0.001$) when compared with the control at concentration of 1000 µg/ml with growth inhibition rate of 54.97%, the cytotoxic inhibition rate was increased with the concentration decreased. There was a high significant effect ($P \leq 0.0001$) at the concentrations of (125, 250, 500) µg/ml to give growth inhibition rates of (63.78%, 61.66% and 58.40%), respectively.

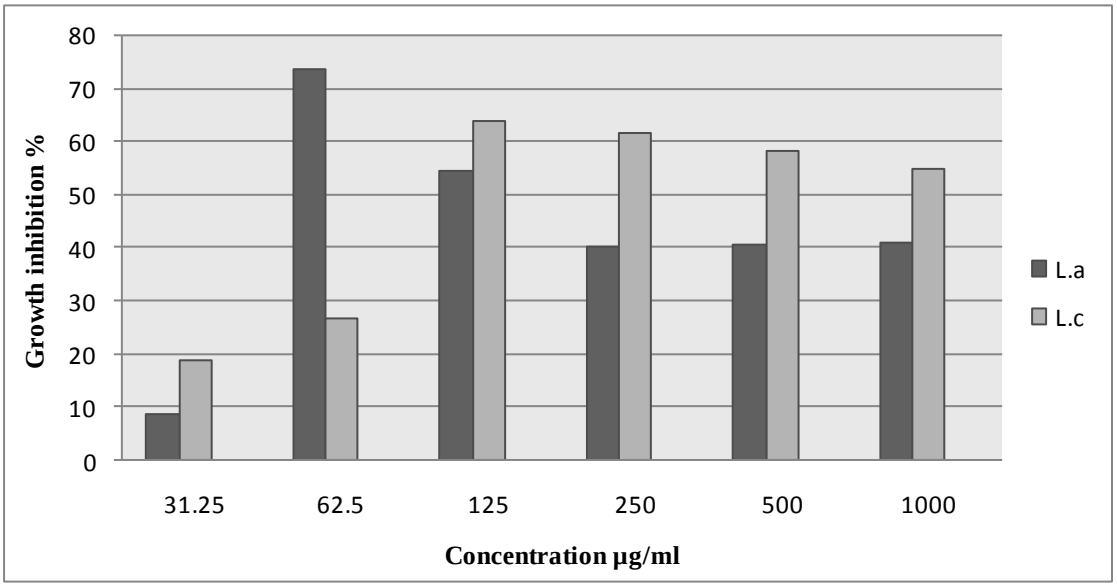


Fig (3-11): Histogram of growth inhibition percentage (GI %) for different concentrations of concentrated filtrate of *L.acidophilus* (*L.a*) and *L.casei* (*L.c*)on RD cell line.

3.10.2 Effect of S-layer proteins and *Lactobacillus* concentrated filtrate on L20B cell line:

Effect of S-layer proteins of *L.acidophilus* and *L.casei* on growth of L20B cell line at significant difference ($P \leq 0.05$) level, and growth inhibition percentages are shown in appendix (6-C) and figure (3-12). S-layer protein from *L.acidophilus* showed significant differences started from concentration 500 µg/ml ($P \leq 0.01$) to 1000 µg/ml when compared with the control. S-layer protein from *L.casei* had significant cytotoxic effect on growth of L20B cell line at the concentration 1000 µg/ml, when compared with control, while no significant cytotoxic effect was appeared when the concentration decreased below that.

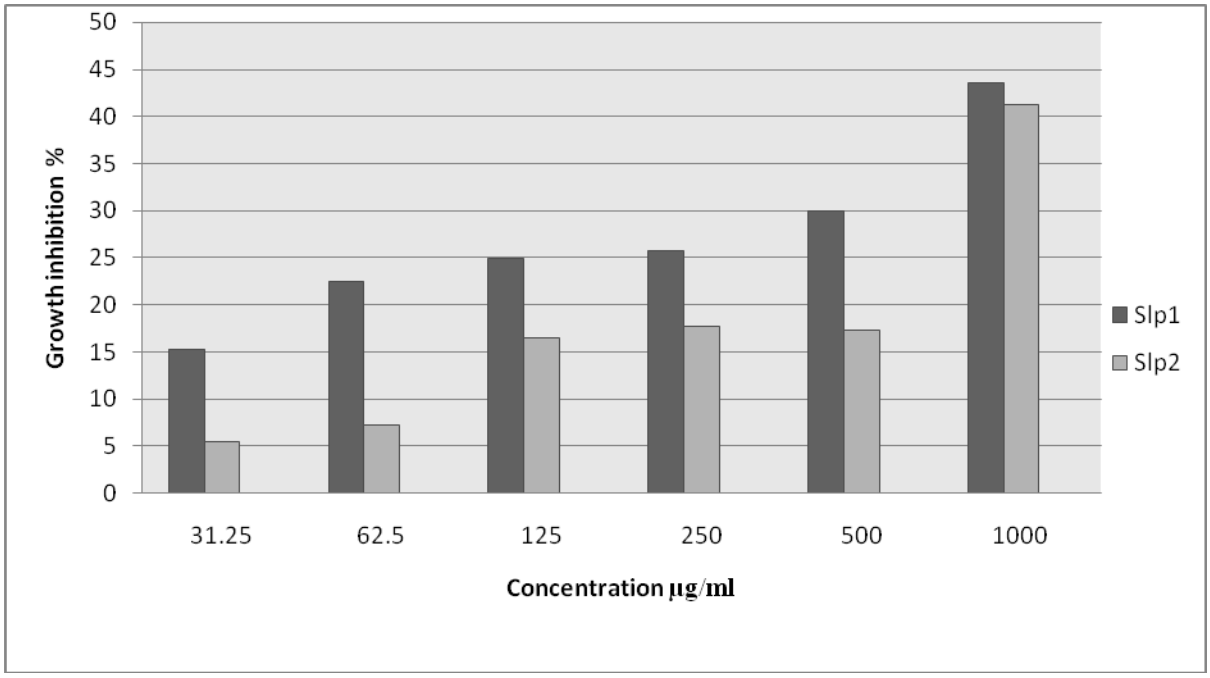


Fig (3-12): Histogram of growth inhibition (GI %) for different concentrations of S-layer protein of *L.acidophilus* (Slp1) and S-layer protein of *L. casei* (Slp2) on L20B cell line.

The significant cytotoxic effect ($P \leq 0.001$) of the concentrated filtrate of *Lactobacillus acidophilus* was started at concentration of 31.25 µg/ml with growth inhibition percentage of 40.36%. The significant cytotoxic effect was increased toward the higher concentrations ($P \leq 0.0001$)when compared with the control. Higher growth inhibition percentages 85.06%, 75.28% were observed at the concentrations of 125 and 250 µg/ml as shown in appendix (6-D) and figure (3-13).

Concentrated filtrate of *Lactobacillus casei* has high significant cytotoxic effect ($P \leq 0.001$) on growth of L20B started at concentration of 31.25 µg/ml with growth inhibition percentage of 33.67% when compared with the control. Significant effect was increased toward the higher concentrations, but concentrations of 125, 250 µg/ml have higher growth inhibition percentages (76.92%, 78.21%), respectively, compared to the concentrations of 500, 1000 µg/ml which gave growth inhibition percentages of 63.33%, 64.10%, respectively, as shown in appendix (6-D) and figure (3-13).

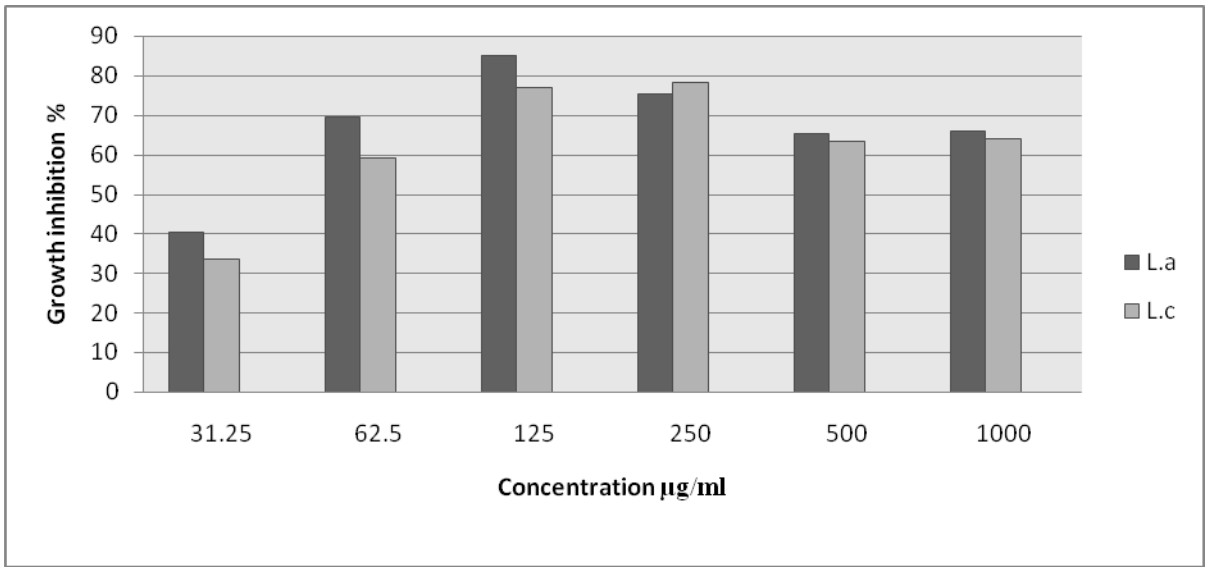


Fig (3-13): Histogram of Growth inhibition percentage (GI %) for different concentrations of concentrated filtrate of *L.acidophilus* (*L.a*) and *L.casei* (*L.c*) on L20B cell line.

Statistical analysis comparison between the effect of S-layer protein and concentrated filtrate of *L.acidophilus* and *L. casei* on RD cell line at the incubation period 48hr. clarified that S-layer protein of *L.acidophilus* had more effect than S-layer protein of *L.casei* because at the concentration 500 µg/ml it had significant difference of ($P \leq 0.01$) when compared with Slp of *L.c* which showed the same signifigant difference at the concentration 1000 µg/ml. On the other hand *L. acidophilus* filtrate had more effective than *L. casei* filtrate because at the concentration of 62.5 µg/ml had high significant difference ($P \leq 0.0001$), when compared with *L. casei* filtrate that showed high significant difference ($P \leq 0.0001$) at 125 µg/ml.

Lactic acid bacteria can inhibit genotoxicity of dietary carcinogens *in vitro* considering that the degree of inhibition was strongly species dependent (Burns and Rowland, 2000). El-Shafie *et al* (2008) when examined the cytotoxicity of four lactic acid producing bacteria (LAB) strains, (*Bifidobacterium animalis*, *Lactobacillus delbreukii*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*) on four human cancer cell-lines (HEPG-2 (Liver carcinoma); MCF7 (Breast carcinoma); HELA (Cervix carcinoma) and HCTI16 (colon carcinoma), they found that maximum inhibition was exerted by the *B.animalis* on the four cell lines which were totally unaffected by the *L. acidophilus*.

Statistical analysis comparison between the effect of S-layer protein and concentrated filtrate of *L.acidophilus* and *L. casei* on L20B cell line at incubation period of 48 hr. showed that the Slp of *L.acidophilus* more effect than Slp of *L.casei* because at the concentration 500 µg/ml it had significant difference ($P \leq 0.01$) when compared with Slp of *L.casei* that gave same significant difference at 1000 µg/ml when compared with control. In case of filtrates, the output revealed that *L. acidophilus* filtrate had more cytotoxic

effect than *L. casei* filtrate, because it had high significant effect ($P \leq 0.0001$) at concentration of 62.5 µg/ml, while *L. casei* filtrate had high significant difference ($P \leq 0.0001$) at concentration of 125 µg/ml. Depending on statistical analysis, the crude filtrate of LAB gave better cytotoxic effect on tumor cell line than S-layer protein, and concentrated filtrate of *L. acidophilus* was more effect than that of concentrated filtrate of *L. casei*.

Extracellular polysaccharides (EPS), which are metabolites of some LAB strains, were also reported to exhibit antitumor activity, macrophage activation, mitogenic activity, and induction of cytokines. However, most of these studies were performed in vitro and little information is available for in vivo experiments involving oral administration (Makino *et al*., 2006). The EPS are taken up by Peyer's patches in the intestine and stimulate antigenpresenting cells, such as dendritic cells, through tolllike receptors. This would result in selective enhancement of T-helper 1 (Th1) cell proliferation, and the subsequent production of IL-2 and IFN-*γ*, which are cytokines that are vital for cell mediated immune responses (Mossmann and Coffman, 1989).

Rhee and Park (2001) purified glycoproteins substances from a culture supernatant of *L. plantarum* KLAB21 cells, then they found that these substances possess antimutagenic activity against *N*-methyl-*N*- nitro-*N*-nitrosoguanidine (MNNG) on *Salmonella enterica* serovar *Sal*. *typhimurium* TA100 cells also Sekine *et al*. (1994) detected anti-tumor activity in peptidoglycans isolated from the *B. infantis* strain, ATCC 15697. From these researches, crude filtrate of *Lactobacillus* has many compounds which have cytotoxic effect against tumor cell lines and by different mechanisms.

Free fatty acids (FFA) represents one of some bacterial secondary metabolites, particularly short chain fatty acids such as butyrate, produced by bacterial

fermentation of dietary fibers, have been shown to exert inhibitory effects of the activation of NF kappa B, a transcription factor involved in the production of many proinflammatory cytokines or chemokines, especially in the presence of TNF-α, in an intestinal epithelial cell line and in macrophages (Inan *et al*., 2000). Several LAB strains have been shown to enhance cell-mediated immune responses, including T-lymphocyte proliferative ability, mononuclear cell phagocytic capacity, and natural killer (NK) cell tumoricidal activity (Cross *et al*., 2001).

One of the important secondary metabolites produced by LAB is hydrogen peroxide. Generate reactive oxygen species (ROS) such as H2O2 is capable of inducing apoptosis. Exposure to low doses of H2O2 induces apoptosis in a variety of cell types. Direct exposure of human hepatoma cell line (SMMC-7221) to hydrogen peroxide (H2O2) can induce apoptosis characterized by morphological evidence and fragmentation of DNA assayed by terminal deoxynucleotidyl transferase assay. Hydrogen peroxide can decrease the level of CD95, and it is confirmed that H2O2 can also activate the differential expression of some specific gene such as p53. The p53 tumour suppressor gene is an essential mediator of the mammalian cells and it required for apoptosis. Subsequently, p53 activates the transcription of several genes whose products are involved in DNA repair or apoptosis (Huang *et al*., 2000).

Supplementation of a high meat diet (72% beef) with *Lactobacillus acidophilus* (10^9- 10^{10} organisms/day) significantly decreased by 40 - 50% the activity of faecal β-glucuronidase, the enzyme, involved for release in the colon from their conjugated form, a number of dietary carcinogens, including polycyclic aromatic hydrocarbons. Similarly, bacterial ß-glycosidase hydrolyzes the plant glycoside cycasin to a carcinogen in the gut and

nitroreductase acting on N-nitroso compounds (which formed by the reaction of nitrite with secondary amines and amides of colonic microflora) many of which possess mutagenic and carcinogenic activity (Abdelali *et al*., 1995).

Interestingly the modulating effect of the *L.* strains was dependent on the type of basal diet fed on, Cole *et al*., (1989) demonstrated a significant reduction in ß-glucuronidase and ß-glucosidase activities when *Lactobacillus acidophilus* was fed for 3 days, with the effect persisting for 7 days after dosing ceased (Rowland and Tanaka, 1993).

Putative mechanisms of action of Lactic Acid Bacteria include, Induction of pro- and/or anti-inflammatory cytokines, Elevation of serum level of IL-10, Induction of maturation of dendritic cells (DC), Enhancement of serum antibody response to orally and systemically administered antigens, Enhanced immunoreactivity of spleen cells and phagocytes and Induction of oral tolerance to β-lactoglobulin (Perdigón *et al*., 2001).

Matsuguchi *et al.*, (2003) demonstrated that six heat-killed *Lactobacillus* strains examined induced the secretion of tumor necrosis factor alpha (TNF-α) from mouse splenic mononuclear cells, albeit to various degrees, when examined the stimulatory effects of lactobacilli on mouse immune cells. Several strains of mainly *lactobacilli* were found to induce IL-12 or IL-10, i.e. either a pro- or anti-inflammatory response (Th1 or Th2). *L. casei* Shirota induced production of proinflammatory cytokines IL-12 with subsequent production of IFN-γ in murine splenocytes (Kato *et al*., 1999).

More direct evidence for protective properties of probiotics against cancer has been obtained by assessing the ability of cultures to prevent DNA damage and mutations (which is considered to be an early event in the process of carcinogenesis) in cell cultures or in animals. It was indicating that the various

LAB can inhibit genotoxicity of dietary carcinogens *in vitro* considering that the degree of inhibition was strongly species dependent (Burns and Rowland, 2000).

Regarding the cytotoxicity of S-layer proteins on tumor cells, this effect may be because S-layer proteins are mostly composed of glycoproteins and involved in the adherence *Lactobacillus* to host tissues (Sara and Sleytr, 2000). Lee *et al* (2004) reported that binding of LAB may give a good effect on colon cancer by balancing the Th1/Th2 cytokine profile, but it is critically dependent on binding capacity of LAB to tumor/cancer cells and thus the pattern of cytokine production is that due to the LAB adhesion may be bacterial strain- specific.

Regarding susceptibility of the tumor cell lines to these extracts, it was noticed that L20B cell line was more sensitive than RD cell line, this may be due to the presence of several compounds that posses effective ability against this type of cell line, or due to the increased activity of Glutathione-S-transferase (GSTs) that acts as an anti-oxidation agent especially in cancer cells. The (GSTs) acted as an anti oxidant causing cellular detoxification by inhancing their combination with reduced glutathione leading the cancer cell toward programmed cell death , apoptosis (Medeiros *et al*., 2004).

RD resistance might be explained by “over expression” phenomena through genes responsible for binding - receptor blockage that prevents the cytotoxic effect of any treatment (Kim *et al*., 1998).

4.1 Conclusions:

- S-layer protein was detected in different species of *Lactobacillus* isolated from different sources; it had been appear that its molecular weight was different according to the species.
- *L. acidophilus* and *L. casei* had inhibitory effects against pathogenic microorganisms (*P. aeruginosa*, *E. coli*, *Staph. aureus*, *Sal. typhimurium* and *C. albicans*), but S-layer proteins had no such effect.
- Three-fold concentrated filtrates of LAB and S-layer protein had effect on the adhesion of *E.coli* and *Staph. aureus*.
- S-layer proteins and concentrated filtrate o*f L. acidophilus* and *L. casei* had cytotoxic effect on growth of both tumor cell lines RD and L20B depending on concentrations. The concentrated filtrate gave highest cytotoxic effect than S-layer protein.
- Cell line L20B was the most sensitive to the effect of three-fold concentrated filtrate of *Lactobacillus spp.* than the RD.

4.2 Recommendations:

- Studying the probiotic effect of mixed cultures of both *Lactobacillus acidophilus and Lactobacillus casei* against tumor cell lines and against pathogenic bacteria.
- Detection and purification the active compounds produced by probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* to evaluate their cytotoxic effect on tumor cell lines.
- Genetic study of S-layer gene which encoding to S-layer protein.
- Studying immunological effect of S-layer protein and LAB filtrate *in vivo*.

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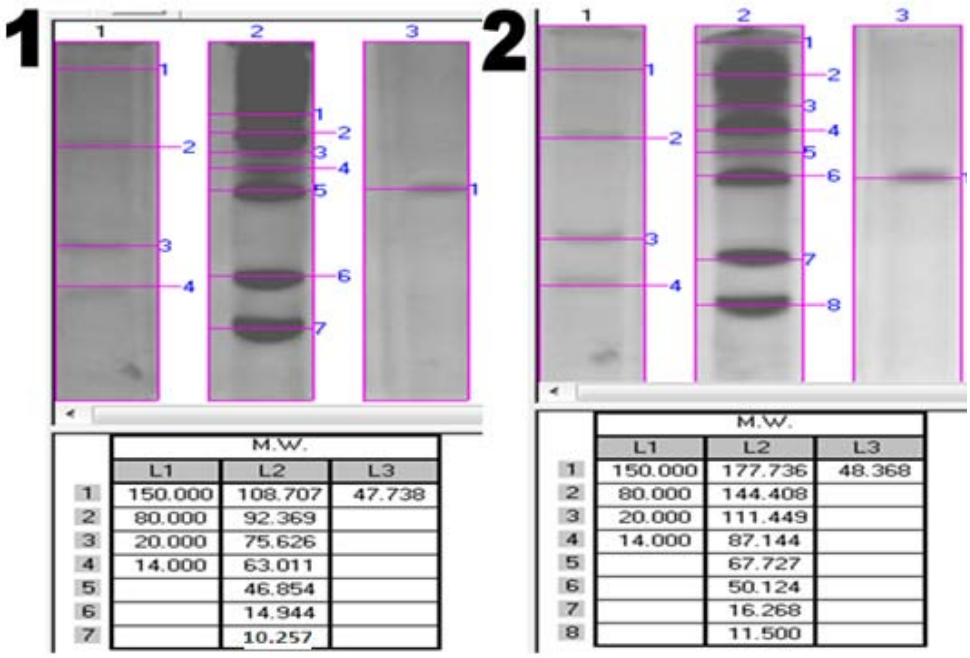
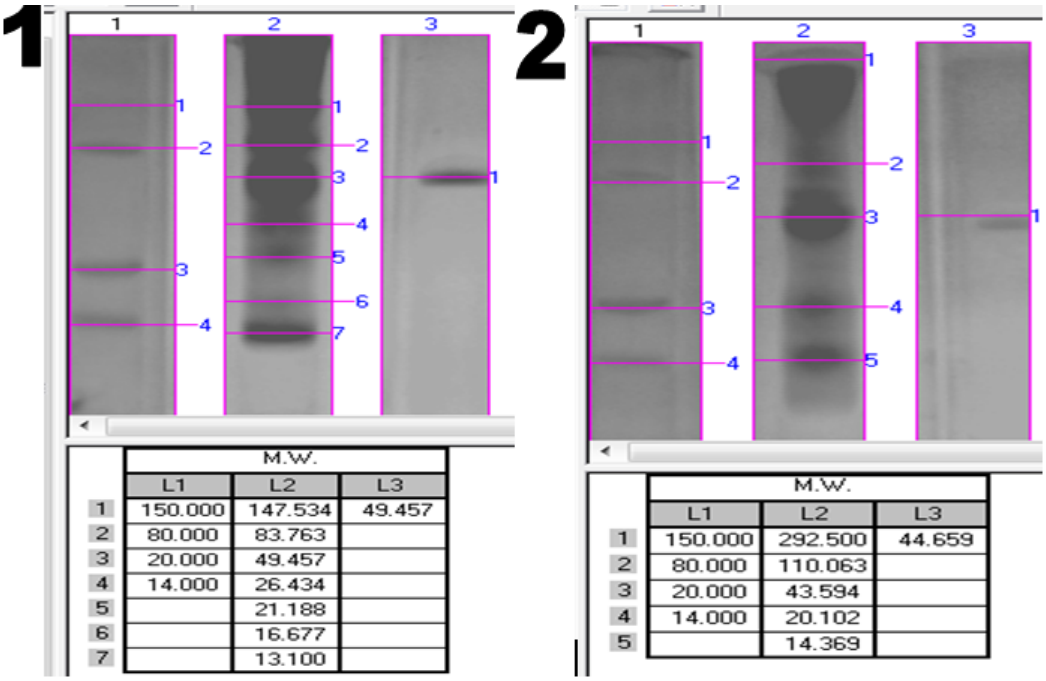


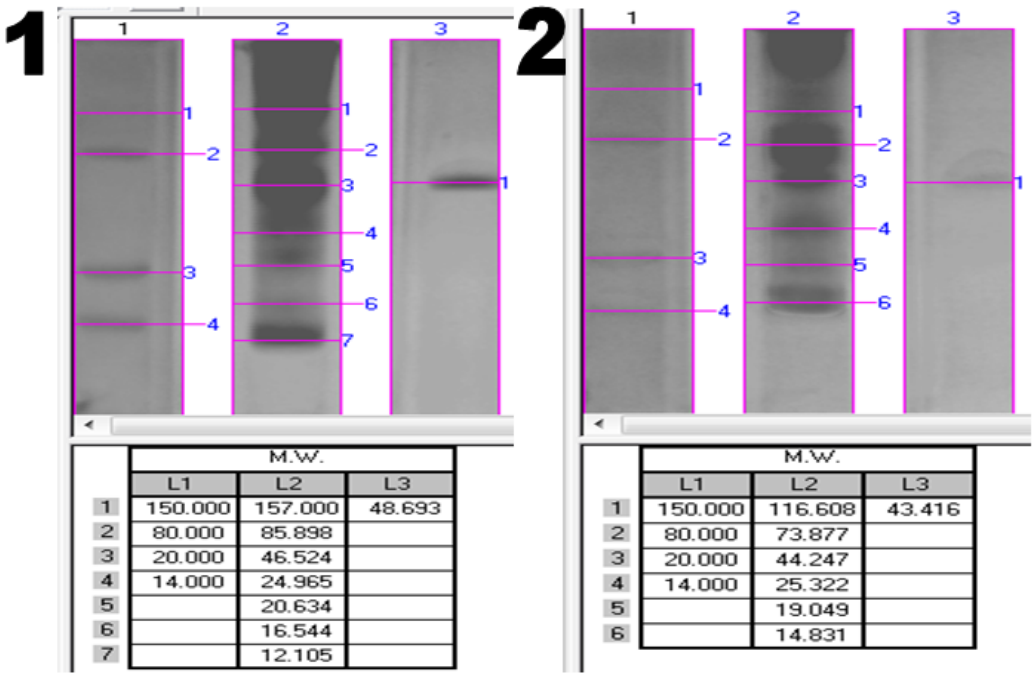
Appendix (1-A): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (1) *L. acidophilus*1 and (2) *L. acidophilus*2 isolated from chicken intestine.

L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis

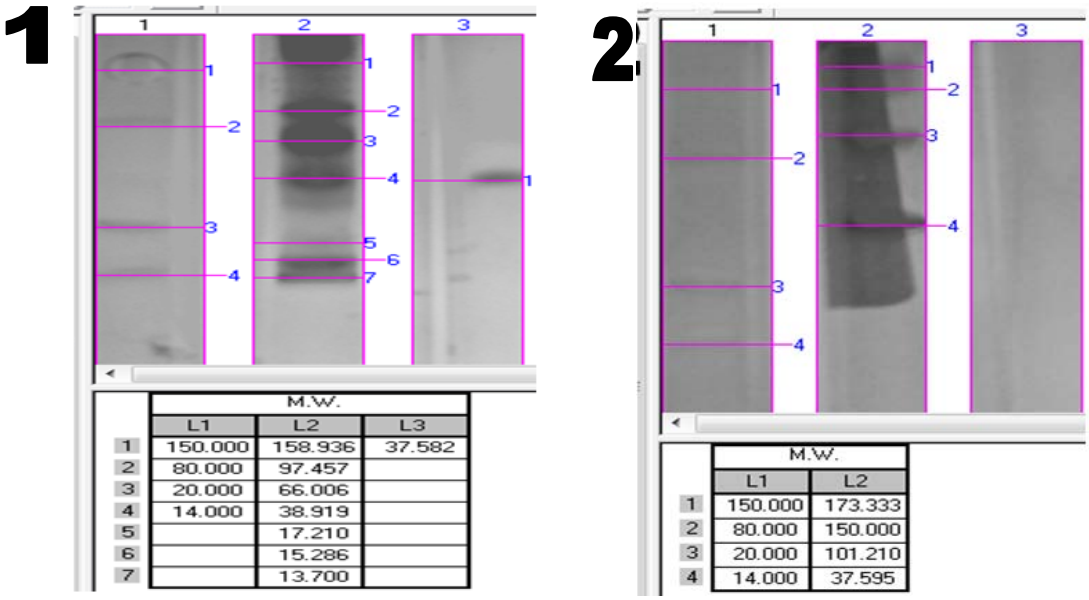


Appendix (1-B): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (1) *L. acidophilus* (2) *L. casei* isolated from faeces of children

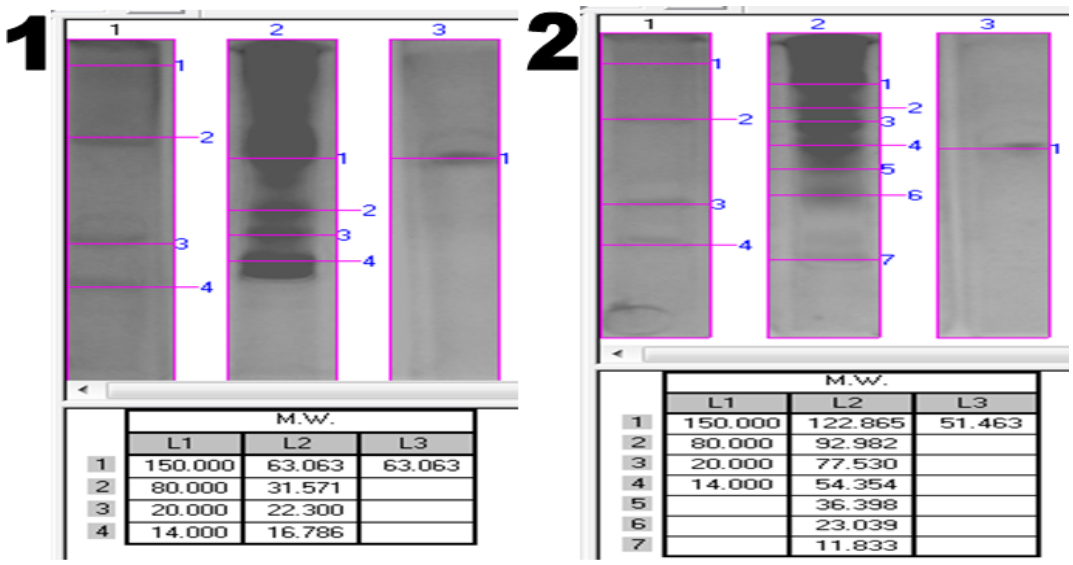
L1: Protein markers, L2: Crude analysis and L3: Pure S-protein analysis



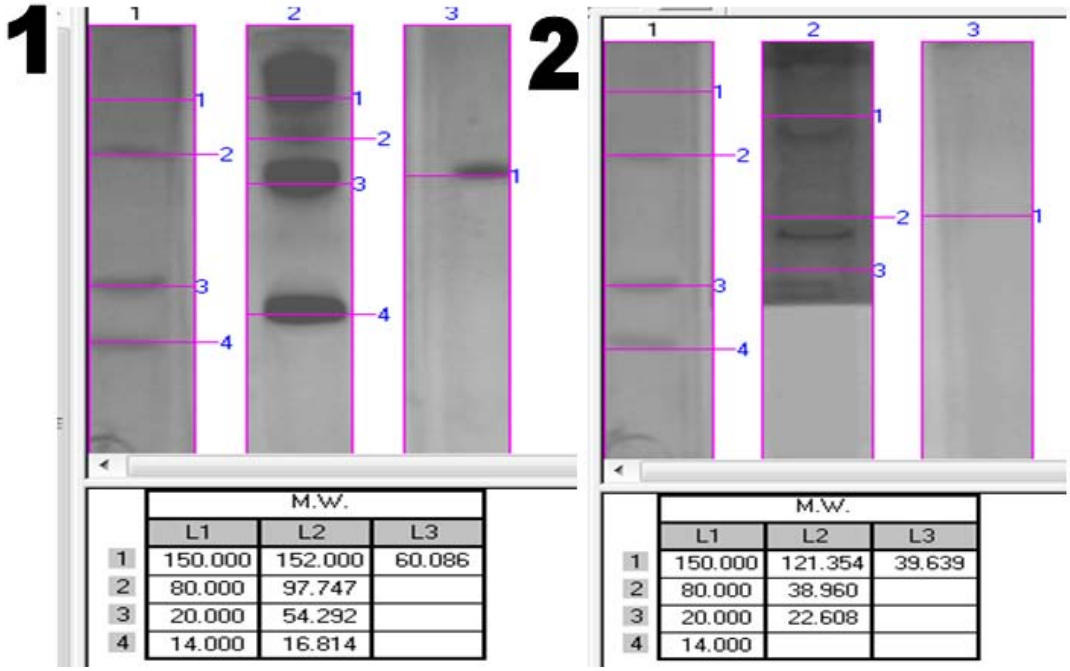
Appendix (1-C): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (1) *L. plantarum* (2)*L.acidophilus* isolated from yoghurt



Appendix (1-D): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE (1) *gasseri* isolated from human milk (2) *fermentum* isolated from vagina

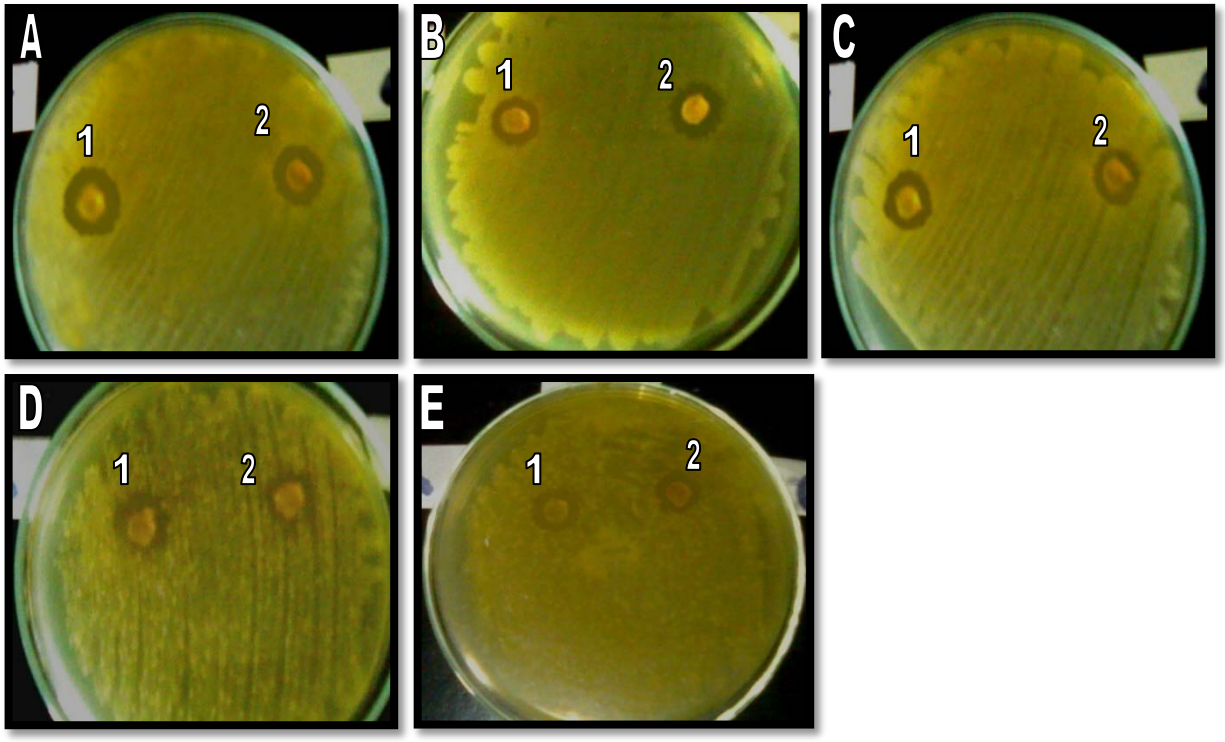


Appendix (1-E): Protein profile analysis of *Lactobacillus* by 10% SDS-PAGE (1) *L.plantarum* from vinegar (2) *L.plantarum* from cow milk.

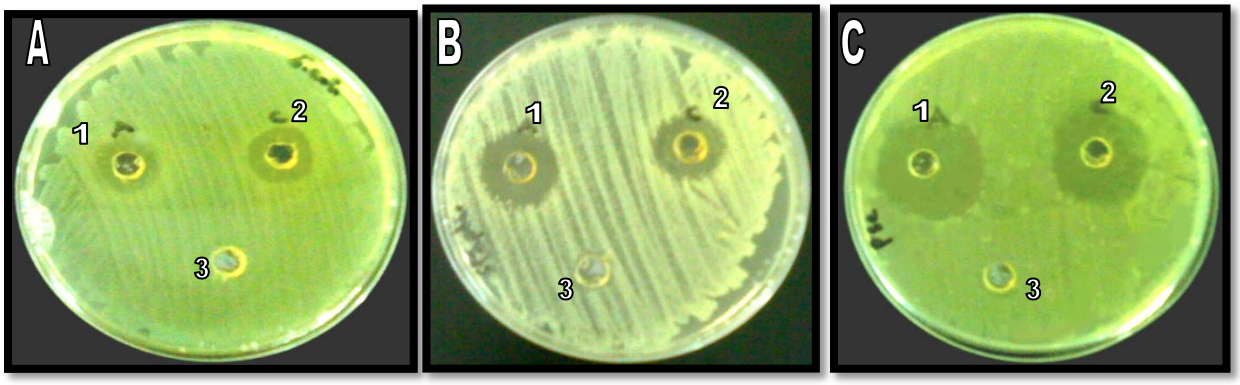


Appendix (1-F): protein profile analysis of *Lactobacillus* by 10% SDS-PAGE: (1) *L. rhamenosus* (2) *L. curvatus* isolated from cow milk. Photocapt analysis software was used to determine the molecular weight of proteins and tables designed by the program in which the molecular weight is calculated in kDa.

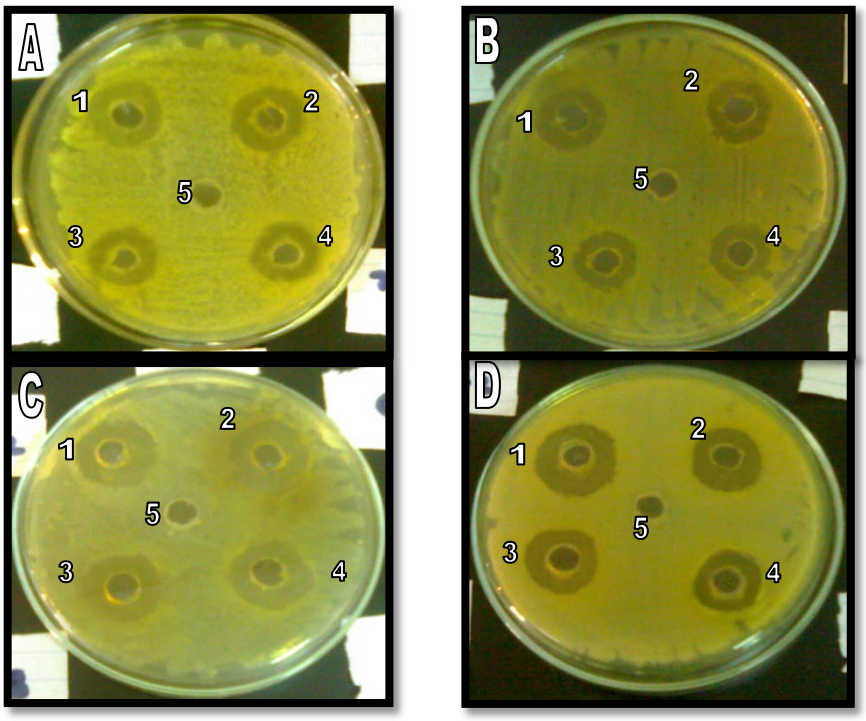
L1: represents protein markers, L2: represents crude analysed cells of *Lactobacillus* and L3: is pure protein.



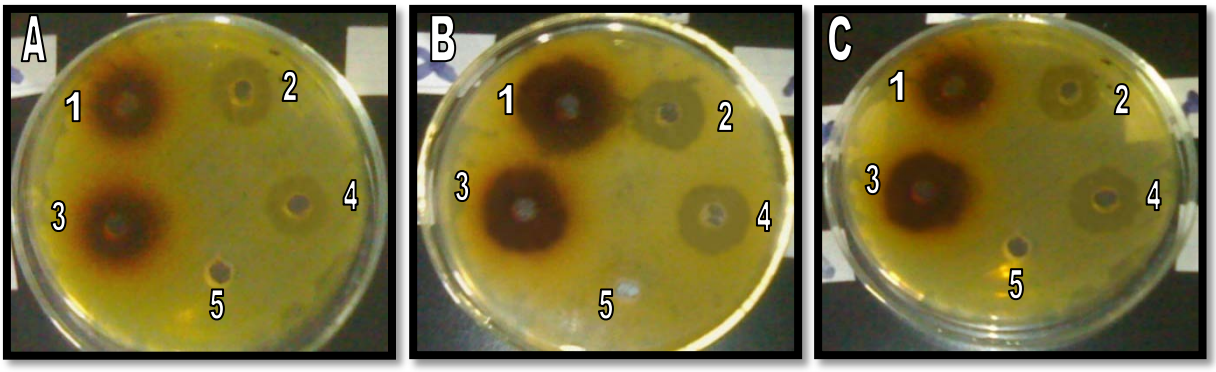
Appendix (2): inhibitory effect of *L. acidophilus* and *L. casei* on solid medium (MRS agar) against (A): *Pseudomonas aeruginosa* after 24 hr. (B): *E. coli* after 48 hr. (C): *C. albicans* after 24 hr. (D): *Sal. typhimurium* after 24 hr. (E): *Staphylococcus aureus* after 24 hr. (1): *L. acidophilus* (2): *L. casei*



Appendix (3): Inhibitory effect of LAB in liquid medium (MRS broth) after 24hr of incubation against (A): *E. coli* (B): *Staph. aureus* (C): *P. aeruginosa* (1) *L. acidophilus* (2): *L. casei* 3) control(MRS) without *Lactobacillus*.



Appendix (4): Inhibitory effect of LAB in liquid medium (MRS broth) after 48 and 72 hr of incubation against (A): *Sal. typhimurium* (B): *Staph. aureus* (C): *E. coli* (D): *P. aeruginosa* (1) *L. acidophilus* after 48hr (2) *L. casei* after 48 hr (3) *L.acidophilus* after 72hr (4) *L. casei* after 72 hr (5) control (MRS) without *Lactobacillus*.



Appendix (5): Inhibitory Effect of Concentrated Filtrate of LAB after grown in liquid medium (MRS broth) against
(A): *Sal.typhimurium* B): *C. albicans* (C): *E. coli* (1): three-fold of *L.a* (2): one-fold of *L.a* (3) three-fold of *L.c* (4) one-fold of *L.c* (5) control (contain concentrated MRS).

Appendix (6-A): Cytotoxic effect and growth inhibition percentages (GI %) of S-layer protein of *L.acidophilus* (Slp1) and S-layer protein of *L.casei* (Slp2) on RD cell line after 48 hr.

Extract Concentration(µg/ml)	O.D. mean ± S.E.*		Percentage of Growth Inhibition (GI %)	
	S-protein of *L.a*	S-protein of *L.c*	Slp1(*L.a*)	Slp2 (*L.c*)
31.25	0.751 ± 0.009 [a]	0.760 ± 0.00 [a]	5.30	9.20
62.5	0.703 ± 0.087 [a]	0.722 ± 0.02 [a]	11.35	13.73
125	0.654 ± 0.00 [a]	0.723 ± 0.00 [a]	17.53	13.62
250	0.636 ± 0.035 [a]	0.655 ± 0.00 [a]	19.80	21.74
500	0.582 ± 0.058 [ab]	0.630 ± 0.017 [a]	26.61	24.73
1000	0.398 ± 0.110 [b]	0.442 ± 0.00 [b]	49.81	47.19
Control	0.793 ± 0.057	0.837 ± 0.058		

*different letters= significant differences ($P \leq 0.05$) between the means.

Appendix (6-B): Cytotoxic effect and growth inhibition percentages (GI %) of concentrated filtrate of *L.acidophilus* (*L.a*) and concentrated filtrate of *L.casei* (*L.c*) on RD cell line after 48 hr.

Extract Concentration (µg/ml)	O.D. mean ± S.E.*		Percentage of Growth Inhibition (GI %)	
	filtrate of *L.acidophilus*	filtrate of *L.casei*	*L.acidophilus*	*L.casei*
31.25	0.559 ± 0.01 [a]	0.498 ± 0.07 [a]	8.81	18.76
62.5	0.161 ± 0.02 [c]	0.449 ± 0.07 [ab]	73.74	26.75
125	0.280 ± 0.01 [c]	0.222 ± 0.02 [c]	54.32	63.78
250	0.367 ± 0.01 [b]	0.235 ± 0.05 [c]	40.13	61.66
500	0.365 ± 0.005 [b]	0.255 ± 0.03 [c]	40.46	58.40
1000	0.363 ± 0.07 [b]	0.276 ± 0.04 [bc]	40.78	54.97
Control	0.613 ± 0.06	0.613 ± 0.06		

*different letters= significant differences ($P \leq 0.05$) between mean.

Appendix (6-C): Cytotoxic effect and growth inhibition percentages (GI %) of S-layer protein of *L.acidophilus* (Slp1) and S-layer protein of *L.casei* (Slp2) on L20B cell line after 48 hr.

Extract Concentration(µg/ml)	O.D. mean ± S.E.*		Percentage of Growth Inhibition (GI %)	
	S-protein of *L.a*	S-protein of *L.c*	Slp1	Slp2
31.25	1.400 ± 0.001 [a]	1.389 ± 0.04 [a]	15.31	5.51
62.5	1.290 ± 0.05 [a]	1.363 ± 0.09 [a]	22.52	7.28
125	1.249 ± 0.001 [a]	1.227 ± 0.002 [a]	24.95	16.53
250	1.237 ± 0.001 [a]	1.210 ± 0.004 [a]	25.71	17.69
500	1.165 ± 0.002 [b]	1.216 ± 0.001 [a]	30.03	17.28
1000	0.938 ± 0.234 [b]	0.863 ± 0.003 [b]	43.66	41.29
Control	1.665 ± 0.001	1.470 ± 0.001		

*different letters= significant differences ($P \leq 0.05$) between mean.

Appendix (6-D): Cytotoxic effect and growth inhibition (GI %) of concentrated filtrate of *L.acidophilus* (*L.a*) and concentrated filtrate of *L.casei* (*L.c*)on L20B cell line after 48 hr

Extract Concentration (µg/ml)	O.D. mean ± S.E.*		Percentage of Growth Inhibition (GI %)	
	filtrate of *L.acidophilus*	filtrate of *L.casei*	*L.acidophilus*	*L.casei*
31.25	0.854 ± 0.001 [b]	0.776 ± 0.003 [b]	40.36	33.67
62.5	0.434 ± 0.006 [c]	0.475 ± 0.04 [b]	69.69	59.40
125	0.214 ± 0.01 [c]	0.270 ± 0.05 [c]	85.06	76.92
250	0.354 ± 0.06 [c]	0.255 ± 0.04 [c]	75.28	78.21
500	0.496 ± 0.06 [c]	0.429 ± 0.02 [b]	65.36	63.33
1000	0.487 ± 0.09 [c]	0.420 ± 0.009 [b]	65.99	64.10
Control	1.432 ± 0.001	1.170 ± 5.00		

*different letters= significant differences (P≤0.05) between mean.

الخلاصة

أجريت هذه الدراسة لتقييم فعالية البروتينات السطحيه المعزوله من بكتريا حامض اللاكتيك العصويه بالمقارنة مع فعالية راشح البكتريا الخام المركز ضد بعض الاحياء المجهريه الممرضه وضد خطوط الخلايا السرطانيه داخل الزجاج.

أستخدمت (12) عزله من بكتريا حامض اللاكتيك المعزوله من الخل و حليب البشر و حليب البقر و اللبن و المهبل للكشف عن وجود البروتينات السطحيه بطريقة Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) ، ثم عزلت البروتينات بواسطة قطع حزمة البروتين السطحي ومعاملتها مع guanidin hydrochloride(G-HCl) تركيز 6 عياري لاسترجاع البروتين من الهلام. قدرت الاوزان الجزيئيه للبروتينا ت وكانت تتراوح ما بين (37- 63 kDa) حسب اختلاف انواع بكتريا حامض اللاكتيك، كذلك حسبت تراكيز البروتينات السطحيه بأستخدام عده تعتمد في آلية عملها على طريقة البيوريت . و تم أختيار العزلتين *Lactobacillus acidophilus* و *Lactobacillus casei* أعتمادا على الوزن الجزيئي و تركيز البروتين.

حددت الفعاليه التثبيطيه لبكتريا *L. acidophilus* و *L.casei* ضد الاحياء المجهريه الممرضه *Pseudomonas aeruginosa* و *Esherichia coli* و *Staphylococcus aureus* و *Salmonella typhimurium* و *Candida albicans* على وسط MRS الصلب والسائل، وسجلت اقل فعالية تثبيطيه على الوسط الصلب مقارنة بالوسط السائل الذي أعطى أفضل فعالية تثبيطية و أظهر تأثير تثبيطي أكثر ضد بكتريا *P. aeruginosa* أذ وصل القطر التثبيطي الى 24 و 22 ملم للعزلتين على التوالي. و كان التاثير التثبيطي لبكتريا *L.acidophilus* اقوى من التاثير التثبيطي لبكتريا *L. casei* ضد معظم الاحياء المجهريه المستخدمه . و عند دراسة الفعاليه التثبيطيه للبروتينات السطحيه لوحظ ان تلك البروتينات لا تمتلك تأثير تثبيطي ضد الاحياء المجهريه.

أظهرت نتائج التركيز المثبط الادنى MIC لرواشح مزروع بكتريا حامض اللاكتيك المركز لثلاث مرات ان نسبتي 40% و50% من كلا نوعي بكتريا حامض اللاكتيك العصويه ه ما التركيزين المثبطين الدنيا ضد بكتريا *P. aeruginosa* و *E. coli* على التوالي، و أما نسبة 60% فقد كانت التركيز المثبط الادنى ضد *Sal. typhimurium* و *C. albicans* بينما كانت نسبتي 50% و 60% هما التركيزان المثبطان الدنيا ضد بكتريا *Staph. aureus*.

أستخدم التركيز المثبط الادنى لدراسة ظاهرة الالتصاق لبكتريا *E. coli* و *Staph. aureus* على الخلايا الطلائيه ، وبينت النتائج فعالية الراشح المركز في التقليل من التصاق خلايا هاتين الممرضتين بالخلايا الطلائية بمعدل وصل الى 5-12 و 4-9 بكتريا /خلية بدلاً من 50- 60 و 29-35 بكتريا / خلية على التوالي كما وتناقص التصاق بكتريا *E. coli* و *Staph. aureus* بالخلايا الطلائيه قد تناقص ايضا بوساطة البروتينات السطحيه بمعدل (3-9) بكتريا /خلية لكلا النوعين من البكتريا.

درست الفعالية السميه للبروتينات السطحيه وراشح البكتريا باستخدام تراكيز مختلفه (1000 و500 و250 و 125 و 62.5 و 31.25 µg/ml) ضد نوعين من خطوط الخلايا السرطانيه (RD و L20B,) خلال فترة حضن 48 ساعه. أكدت النتائج أمتلاك جميع المستخلصات فعاليه سميه لكن تأثير رواشح بكتريا حامض اللاكتيك العصويه كان ذو قيمه معنويه عاليه على نمو هذين الخطين السرطانيين عند مقارنته بفعالية البروتينات السطحيه.

ألأهداء

أهدي هذه الرساله الى الذين أناروا لي دربي الطويل، و كانوا خير معين لي كلما وعرت المسالك.....

الى ملاذي الآمن أبي.....

هذه الرساله هي زرعك قد أثمر، وغرسك قد أزهر ، فمنك تعلمت كيف يكون الباحث صادقاً مع نفسه، مخلصا في عمله. فشكرا لك على المثال الذي قدمته لي بأعمالك.....

الى نور عيني أمي .....

ينبوع حنانك الذي لا ينضب امدني بالطاقة كلما أخذ مني الوهن مأخذه . فشكرا لكِ على صبركِ ومساندتكِ وشكراً لك على دعائكِ.....

الى خالتي أنتصار.....

لن أنسى دعمكِ و أمنياتكِ لأنجاز هذه الرساله، فشكرا لكِ لقد كنتِ مصدر ألهام لي .....

الى أخوي (ياسر وعمر).....

لن أنسى مساندتكما و صبركما معي خلال رحلة البحث. لقد كنتما خير عون لي فشكرا لكما.....

ألى أقربائي .....

لن أنسى دعواتكم ونصائحكم عند مطبات رحلة البحث، فشكرا لكم .....

الى أول من علمني حرفا و حتى آخر من تلقيتُ على يده علماً .....

أليكم جميعا أهدي هذا العمل المتواضع حباً و عرفاناً

رغـــــد

بِسْـــــمِ اللهِ الرَّحْمَنِ الرَّحِيْـــــمِ

إِنَّمَا إِلَـهُكُمُ اللَّهُ الَّذِي لَا إِلَهَ إِلَّا هُوَ وَسِعَ كُلَّ شَيْءٍ عِلماً

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صَـــدَقَ اللهُ العَظِيـــمُ

سورة طـــه (الآيه98)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم التقانه الاحيائيه

تقييم فعالية البروتينات السطحيه ورواشح بكتريا حامض اللاكتيك ضد بعض الأحياء المجهريه الممرضه و كعامل مضاد للسرطان داخل الزجاج

رساله

مقدمه الى كلية العلوم- جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانه الاحيائيه

من قبل

رغـــد عبـــدالله حسن

بكالوريوس تقانه احيائيه-جامعة النهرين- 2006

بأشراف

م.د. ميساء جاسب ألياس أ.د. آمنه نعمه الثويني

ذو الحجه 1430 تشرين الثاني 2009