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Effect of Probiotics on The Relative Activity of Hyaluronidase and Streptokinase from Locally Isolated *Streptococcus pyogenes*

A Thesis

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By

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Dedication

To the women who raised me as a seed in life and irrigated me by her kindness … to her dear and noble soul…….. my Mother

To those whom I live for their sake, and I owe them my happiness……. my Sisters and Brother

To every loving and loyal heart … I dedicate my work.

Yasameen

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Yasameen

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Summary

This study aimed to investigate the inhibitory effect of probiotic microorganisms against *Streptococcus pyogenes.* A total of 85 pharyngeal swaps were collected from children infected with pharyngitis. Four isolates *S. pyogenes* were obtained and then evaluated for the activity of its spreading factors for infection streptokinase and hyaluronidase.

As a probiotic microorganism, *Lactobacillus acidophilius* after propagating in MRS medium, results revealed that's both unconcentrated and concentrated filtrate possessed inhibitory activity against three (S16, S20 and S28) of the four isolates of *S. pyogenes*, while the fourth isolate S30 had no such effect.

When *Streptococcus salivarius* was also used as a probiotic, it was able to exerts inhibitory activity against all four isolates of *Streptococcus pyogenes* by using the deferred antagonism test

Adversely, concentrated filtrate of the probiotic yeast, *Saccharomyces boulardii*, had no inhibitory effect against any of the four isolates of pathogenic *S. pyogenes* used in the study.

All *Streptococcus pyogenes* isolates were resistant to erythromycin, azithromycin, chloramphenicol, clindamycin, but sensitive to amoxicillin, tetracycline, and vancomycin, and have variant result with penicillin.

When activity of the two enzymes (streptokinase and hyaluronidase) produced by *S. pyogenes* was tested, results showed that all its four isolates possessed the ability to produce streptokinase by using both the casinolytic assay and radial caseinolysis assay.

Regarding hyaluronidase, results showed that only three of the *S. pyogenes* isolates (S16, S20 and S28) were able to produce the enzyme when detected by the two methods; plate assay and turbidity reduction assay, while isolate S30 was unable to do so by any of the two methods used.

After subjection of the two crude enzymes (streptokinase and hyaluronidase) to the treatment with each of the; crude extraction of bacteriocin from *S. salivarius*, concentrated filtrates of *Lb. acidophilus*, concentrated filtrates of *S. boulardii* and of the six antibiotics(erythromycin, azithromycin, chloramphenicol, clindamycin, amoxicillin, tetracycline, and vancomycin) results obtained could be summarized as follows:

- Streptokinase activity was completely inhibited by the bacteriocin while it was partially inhibited by *S. boulardii.*
- Streptokinase activity was not affected by the filtrate of *Lb. acidophilus*.
- Only amoxillin, tetracycline and chloramphenicol from those antibiotics used in this study caused partial inhibition in the activity of streptokinase.
- Hyaluronidase activity was completely inhibited by the bacteriocin that produced by *S. salivarius.*
- Partial inhibition of hyaluronidase was occurred by treating with each of the of *S. boulardii* and *Lb. acidophilus* filtrates.
- Any of the six antibiotics used in this study had no effect on the hyaluronidase activity.

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Abbreviation	Meaning
GAS	Group A streptococcus
SOF	The surface-anchored serum opacity factor
HDL	high-density lipoprotein
SLS	Streptolysin S
SLO	streptolysin O
SPEs	Streptococcal pyrogenic exotoxins
MRS	de Man, Rogosa and Sharpe

1. Introduction and Literature Review

1.1 Introduction:

Streptococcus pyogenes (group A streptococcus) is an important species of gram-positive extracellular bacterial pathogens. Group A streptococci colonize the throat or skin and are responsible for a number of suppurative infections and non- suppurative sequelae. As pathogens they have developed complex virulence mechanisms to avoid host defenses (Bisno, 1995).

They are the most common cause of bacterial pharyngitis and are the cause of scarlet fever and impetigo. The concept of distinct throat and skin strains arose from decades of epidemiological studies, in which it became evident that there are serotypes of group A streptococci with a strong tendency to cause throat infection, and similarly, there are other serotypes often associated with impetigo (Wannamaker, 1972).

The streptokinases are a family of secreted streptococcal proteins with the common function of converting plasminogen to plasmin, human plasminogen is often used by invasive bacteria as a virulence factor and this process has been recognized as a critical step in *S. pyogenes* invasion (Walker *et al.,* 2005).

Plow *et al.* (1995) explained that healthy host, active plasmin dissolves intravascular fibrin clots and participates in the repair and remodeling of tissue.

Cole *et al.* (2006) mentioned that these same mechanisms are exploited by *S. pyogenes* to promote systemic spread. Plasminogen bound to the bacterial cell surface may also be converted into plasmin through the action by the activity of the GAS plasminogen activator streptokinase (SK). SK is a highly efficient plasminogen activator that

plays a critical role in the invasive pathogenesis of GAS (Coleman *et al.*, 1999).

Production of hyaluronidase by group A streptococci had been suggested to aid the organism in its spread through the connective tissue hence, hyaluronidase had been designated as one of the spreading factors of microbial origin, because of its ability to attack the hyaluronic acid present in the cement substance of host tissues (Wessels *et al.,* 1991).

Probiotics used as a term to describe the use of live microorganisms as food supplements improving the intestinal microbial balance of the host (Salminen *et al.,* 1999). There strategies devised the use of probiotics as an alternative therapy for treatment and prevention of bacterial infections (Fooks and Gibson, 2002; Bomba *et al.,* 2006). There is a growing interest in probiotics as a safe therapeutic agent through their ability to alleviate food allergies (Gorbach, 2000), enhance non-specific and specific immune responses, suppress intestinal infections, and anticarcinogenic activity (Grajek *et al.,* 2005).

Doron and Gorbach, (2006) found that probiotics have several mechanisms to exert their beneficial effects; they prevent colonization, cellular adhesion, invasion by pathogenic organisms, they have antimicrobial activity, and they modulate the host immune response.

For the importance of *S. pyogenes* infection and the use of probiotics mentioned above, this study was aimed to:

Investigate inhibiting effect of probiotics on the virulence factors streptokinase and hyaluronidase that produce from local *Streptococcus pyogenes* isolate. To achieve these goals had been performing the following steps:

- Isolation and Identification of *S. pyogenes* from local health center.
- Investigating the effect of probiotics on *S. pyogenes*.
- Detection of streptokinase and hyaluronidase production by *S. pyogenes.*
- Studying the effect of probiotics on the activity of both enzyme.

1.2 Literature Review:

1.2.1 *Streptococcus pyogenes*:

Streptococcus pyogenes (group A streptococcus) is a gram-positive coccoid-shaped bacterium that grows in chains and produces small white to grey colonies with a clear zone of β-hemolysis on blood agar (James and McFarland, 1971).

Beall *et al.*(1996) reported that group A streptococcus (GAS) is distinguished from other groups of β-hemolytic streptococci by a group-specific polysaccharide located in the cell wall. Serologic grouping by the Lancefield method is precise, but group A organisms can be identified more readily by any one of a number of latex agglutination, co-agglutination, or enzyme immunoassay procedures.

The GAS cell is a complex structure; it is covered with a hyaluronic acid capsule that gives the colonies a mucoid or water drop appearance. Protruding from the cell surface and into the hyaluronic capsular layer are microscopic hair-like fimbriae, which promote adherence of GAS to epithelial cells and extracellular matrix proteins (Fischetti, 1989). Beachey, *et al.* (1983) pointed out that the fimbriae are composed of a surface-anchored M protein adopting a coiled-coil structure, closely associated with lipoteichoic acid polymers.

Mora *et al.,* (2005) declared that GAS recently has been recognized to express surface pili, which correspond to the classical "T antigen" in earlier serologic typing schemes. Roughly, half of GAS strains display the capacity to opacity mammalian serum, through the activity of the surface-anchored serum opacity factor (SOF) protein, which binds to apolipoproteins to displace high-density lipoprotein (HDL) to form lipid droplets (Courtney *et al*, 2006).

Serologic diversity among various pilus T antigens and SOF proteins can be helpful supplemental tools to aim typing in epidemiologic characterization of GAS strains (Johnson *et al.*, 2006).

Nizet (2002) explained that GAS produce and release into the surrounding medium a large number of biologically active extracellular products. Some of these are toxic for human and other mammalian cells. Streptolysin S (SLS) is a small oxygen-stabile toxin responsible for β-hemolysis of GAS on blood agar, while streptolysin O (SLO) is an oxygen-labile, cholesterol-dependent toxin related to staphylococcal α-hemolysin. Both SLS and SLO injure cell membranes, not only lysing red blood cells, but also damaging other eukaryotic cells and membranous subcellular organelles.

McIver (2009) stated that streptolysin O is antigenic while streptolysin S is not. Streptococcal pyrogenic exotoxins (SPEs) are secreted factors with the capacity to act as super-antigens and trigger T-cell proliferation and cytokine release. GAS elaborates a broad-spectrum cysteine protease with multiple host targets, a variety of specific peptidases that cleave host chemokine and complement factors, and several nucleases (Llewelyn and Cohen, 2002).

The expression of GAS virulence genes is controlled by the interplay of several transcriptional regulatory systems (Kreikemeyer *et al.*, 2003). Genome sequencing has shown that bacteriophages have played an important role in the evolutionary genetics of GAS, including the transfer of genes encoding antibiotic resistance, SPEs and other virulence determinants (Banks *et al.*, 2004).

1.2.2 Virulence factors of GAS:

Group A streptococcus (*Streptococcus pyogenes*) is a serious human pathogen, due to its prevalence and the severe (even fatal) diseases it causes. While uncomplicated pharyngitis is the disease syndrome most commonly associated with this organism, the GAS is also capable of causing a variety of invasive diseases,

including necrotizing fasciitis, myositis, bacteremia, streptococcal toxic shock syndrome, and pneumonia. In addition, the delayed of rheumatic fever and acute glomerulonephritis may follow some types of GAS infection in some people (Hook *et al.*,1960).

Streptococcus pyogenes has several virulence factors that enable it to attach to host tissues, evade the immune response, and spread by penetrating host tissue layers (Patterson, 1996). A carbohydrate-based bacterial capsule composed of hyaluronic acid surrounds the bacterium, protecting it from phagocytosis by neutrophils (Ryan and Ray, 2004). In addition, the capsule and several factors embedded in the cell wall, including M protein, lipoteichoic acid, and protein F (SfbI) facilitate attachment to various host cells (Bisno *et al*, 2003). Ryan and Ray (2004) stated that M protein also inhibits opsonization by the alternative complement pathway through binding to the host complement regulators. The M protein found on some serotypes is also able to prevent opsonization by binding to fibrinogen.

1.2.2.1 Streptodornase (DNase enzyme):

Most strains of *S. pyogenes* secrete up to four different DNases, which are sometimes called streptodornase. The DNases protect the bacteria from being trapped in neutrophil extracellular traps (NETs) by digesting the NET's

web of DNA, to which are bound neutrophil serine proteases that can kill the bacteria.(Buchanan *et al,* 2006).

1.2.2.2 Streptococcal chemokine protease:

Streptococcal chemokine protease is responsible for preventing the migration of neutrophils to the spreading in the infection area (Hidalgo *et al.,* 2006). The serine protease Scp C, which is released by *S. pyogenes*, degrades the chemokine IL-8, which would otherwise attract neutrophils to the site of infection. C5a peptidase, although required to degrade the neutrophil chemotaxin C5a in the early stages of infection, is not required for *S. pyogenes* to prevent the influx of neutrophils as the bacteria spread through the fascia (Hidalgo *et al,* 2004).

1.2.2.3 Superantigens:

Superantigens secreted by many strains of *S. pyogenes.* This pyrogenic exotoxin is responsible for the rash of scarlet fever and many of the symptoms of streptococcal toxic shock syndrome (Patterson,1996).

1.2.2.4 Streptokinase

Streptokinase is an extracellular enzyme produced by various strains of β-hemolytic Streptococci. The enzyme is a single polypeptide that exerts its fibrinolytic action indirectly by activating the circulatory plasminogen.

The complete amino acid sequence of streptokinase was first established by Jackson and Tang (1982). Streptokinase has a molar mass of 47 kDa and is made up of 414 amino acid residues (Malke and Ferretti, 1984).

As a protein, it exhibits its maximum activity at a pH of approximately 7.5 and its isoelectric point is 4.7 (Castellino, *et al.,*1976). The protein contains

nocystine, cysteine, phosphorous, conjugated carbohydrates and lipids.. Streptokinase produced by different groups of streptococci differs considerably in structure (Malke, 1993).

Streptokinase enzymes convert inactive plasminogen to plasmin which digests fibrin and prevents clotting of the blood. The relative absence of fibrin in spreading bacterial lesions allows more rapid diffusion of the infectious bacteria. Plasminogen contains several structural domains, consisting of the amino- terminal peptide, followed by five kringle domains (K1–K5) and the carboxy-terminal serine protease catalytic domain (Ponting, *et al.,* 1992).

Christensen (1984) explained that Lysine-binding sites present in K1, K4, and K5 are responsible for the interaction of plasminogen with a number of other molecules such as fibrin, plasminogen receptors and other cellular surfaces . The circulating, soluble form of plasminogen (Glu-plasminogen) is maintained in a compact, closed conformation through lysine-dependent interactions between the amino-terminal peptide (Mangel *et al*, 1990).

On binding to mammalian or bacterial receptors, a conformational change is induced in Glu-plasminogen, producing an extended, activation-susceptible form (Parry *et al*, 2000). Cleavage of the Lys77- Lys78 peptide bond in Glu-plasminogen by plasmin removes the amino-terminal peptide domain producing Lys-plasminogen which is open conformation that is more readily activated (Ramakrishnan *et al*, 1991).

The pathogenic streptococci produce of extracellular products is streptokinase. The primary biological activity known to be associated with streptokinase is the specific activation of human plasminogen (Wulf and Mertz, 1968). Castellino and Bajaj (1977) reported that streptokinase binds to plasminogen stoichiometric ally and triggers a conformational change and self-

cleavage that results in the formation of the serine protease plasmin which can enzymatically cleaves fibrin and fibrinogen. Ginsberg (1981) explained that streptokinase has been referred to as a streptococcal spreading factor due to its ability to effect the lysis of clots and fibrin deposits which limit areas of infection.

1.2.2.5 Hyaluronidase:

Hyaluronidase is an enzyme capable of degrading hyaluronic acid, a major component of the extracellular matrix of body tissues, as well as being the major or sole component of the capsular material of certain bacteria (Laurent, and Fraser, 1992). Various Gram-positive microorganisms including species of *Streptococcus*, *Staphylococcus*, *Clostridium*, *Propionibacterium*, *Peptostreptococcus* and *Streptomyces* produce hyaluronatelyase. Hyaluronidase activity has also been associated with some Gram-negative organisms (Hynes and Walton, 2000).

Laurent and Fraser (1992) reported that hyaluronate is a linear unsulfated glycosaminoglycan polymer, with an average molecular mass greater than 10000. The polymer is made up of alternating N-acetyl- glucosamine and glucuronic acid residues linked by glycosidic bonds (Fig.1-1).

Hyaluronate is found in many body tissues and fluids of higher organisms such as umbilical cord, synovial fluid, cartilage, brain, muscle, and is a major component of the extracellular matrix especially in soft connective tissue. Half of the hyaluronate in the body is found in the skin. It is also present in high concentrations in rooster combs and is produced by certain bacteria such as the streptococci. The hyaluronate isolated from all these various sources has an identical chemical structure (Mark *et al.,* 2009).

Many pathogenic bacteria able to establish infections at the mucosal or skin surface produce the enzyme hyaluronidase. Since hyaluronate is a major constituent of the ground substance of most connective tissues, skin hyaluronidase may be an essential component in enabling the spread of the pathogens from an initial site of infection (Cheng *et al.,* 1995)

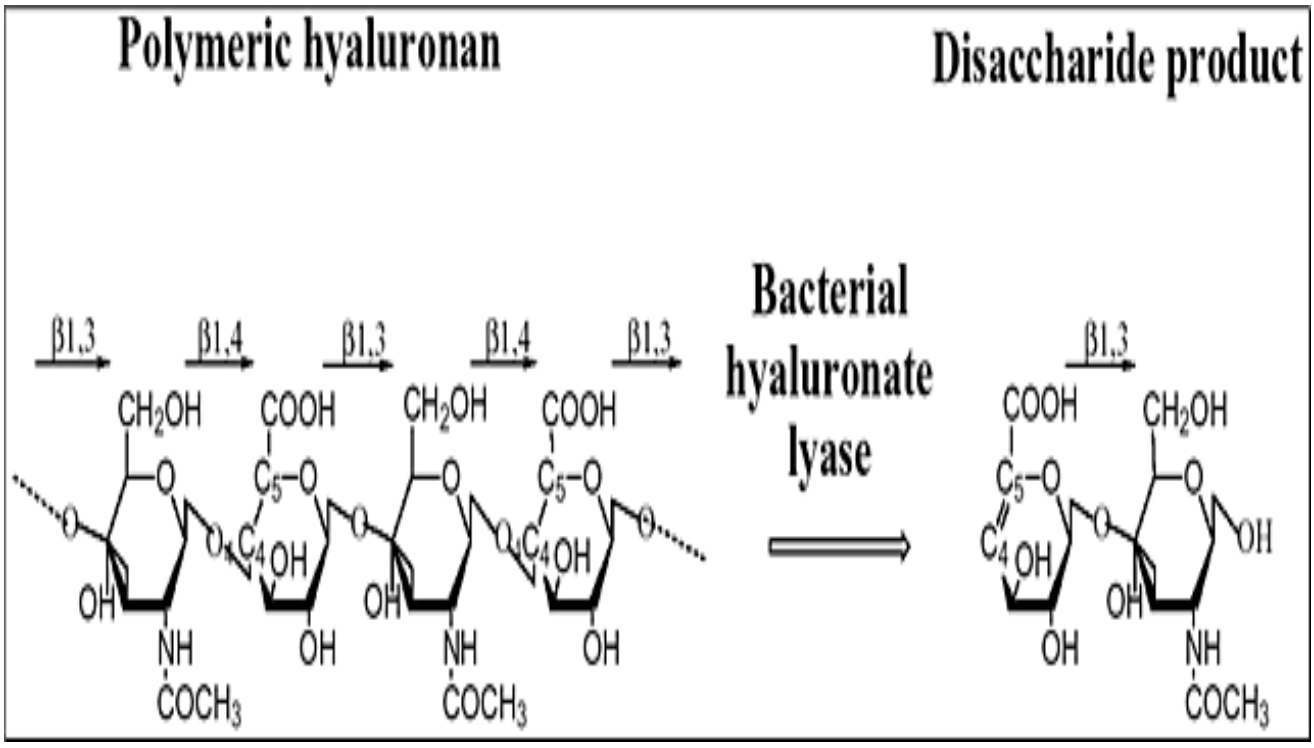
Seddon *et al*., (1990) declared that hyaluronidase degradation of hyaluronate are disaccharides (Fig.1-1) which can be transported and metabolized intracellularly to supply needed nutrients for a pathogen as it replicates and spreads. The hyaluronidase, or proteins associated with the enzyme, are induced in the presence of substrate. Addition of hyaluronate to growth media also results in increased levels of hyaluronidase in group A streptococci (Homer *et al*, 1997).

Tam and Chan (1985) reported that hyaluronidase production by oral pathogens belonging to *Peptostreptococcus* sp. may play a role in the pathogenesis of periodontal disease . The ability to hydrolyze the ground substance of gingival tissue may lead to periodontal destruction by enabling the bacteria to spread and increased permeability of the gingiva connective tissue may lead to increase spread of bacterial toxins.

Streptococcus pyogenes have been shown the ability to produce hyaluronidase (Schaufuss *et al.,* 1989). This enzyme has been suggested to play a role in the spread of infection causing more damage (Calvinho *et al*., 1998).

Streptococci produce an antiphagocytic capsule of which the sole component is hyaluronate. therefore, seems reasonable to hypothesize that the hyaluronidase produced is going to deplete this protective capsule leaving the organisms potentially susceptible to phagocytosis, as the capsule is susceptible

to degradation by the hyaluronidase. However, continued production of hyaluronidase establishes a mean for the organism to degrade host connective tissues allowing for bacterial spread. Perhaps other anti-phagocytic factors such as M-protein provide protection for *S. pyogenes* during hyaluronidase production (Oliver *et al*., 1998).



Fig(1-1). Structure of hyaluronate and the products after eliminative cleavage by a bacterial hyaluronatelyase. The boxed region shows the repeating unit of N-acetylglucosamine and glucuronic acid linked L-1-4 that constitutes hyaluronate. The repeating disaccharide is linked L-1-3 to the adjoining disaccharide (Homer *et al*., 1997)

1.2.3 Pathogenicity of *Streptococcus pyogenes*:

1.2.3.1 Adherence and colonization

Host-pathogen interactions occur due to binding of surface streptococcal ligands to specific receptors on host cells. Attachment of group A

streptococci to pharyngeal or dermal epithelial cells is the most important initial step in colonizing the host (Baddour *et al.*, 1990). Without strong adherence mechanisms, group A streptococci could not attach to host tissues and would be removed by mucous and salivary fluid flow mechanisms and exfoliation of the epithelium (Courtney *et al.*, 1999).

Hasty and Courtney (1996) pointed out that in skin attachment and colonization by group A streptococci, a site of previous damage may be important in overcoming the dermal barrier. Specific adhesion allows competition between normal flora and group A streptococci for tissue sites where normal flora reside. The investigation of adherence determinants of both streptococcal and host cells is vital to the understanding of pathogenic mechanisms in disease and in the development of anti-adhesive or vaccines to prevent colonization (Hasty *et al.*, 1992).

Adherence has been outlined in these reviews as an initial weak interaction with the mucosa which is followed by a second adherence event which confers tissue specificity and high-avidity adherence (Hasty *et al.*, 1992). In addition, one could speculate that the presence of multiple adhesions in strains could give them the advantage of more avid adherence and potentially enhanced virulence. Although not yet well understood, environmental factors expressed in a particular body site may be important cues for expression of adhesions important for colonization of a tissue-specific site (Courtney *et al.*, 1997). It is possible that movement of streptococci from the mucosa or skin into deeper tissues may be facilitated by specialized adhesion mechanisms. Recent studies indicate that adherence to particular types of host cells may induce localized cytokine production and inflammatory responses (Wang *et al*, 1997).

1.2.3.2 Intracellular invasion:

New evidence suggested that group A streptococci not only adhere to epithelial cells but also invade them (LaPenta *et al*, 1994). In another research, LaPenta *et al* (1996) reported that group A streptococci have the potential to invade human epithelial cells at frequencies equal to or greater than classical intracellular bacterial pathogens, such as *Listeria* and *Salmonella* spp. This initial report generated considerable interest and was confirmed by several laboratories (Jadoun *et al*, 1998). Fluckiger *et al* (1998) also ensured the invasion of epithelial cells by group A streptococci. High-frequency invasion requires expression of M protein (Cue *et al.*, 1998) and/or fibronectin-binding proteins such as SfbI (Molinari *et. al.*, 1997). Both the M1 protein and SFbI are considered invasions because latex beads coated with either protein are efficiently internalized by epithelial cells.

Two theories have been proposed for the role of internalization of group A streptococci in disease pathogenesis. It has been suggested to potentially play a role in the carriage and persistence of streptococci, as stated above. Second theory suggests that internalization may lead to invasion of deeper tissues(LaPenta, *et al.*, 1994), A study by found that low virulence was associated with internalization.

Perhaps both theories are correct depending on the virulence and properties of the invading bacterium and whether the invasion is of the throat or skin epithelium. It is also possible that internalization of group A streptococci by host epithelial cells represents successful containment of the pathogen by the host. This hypothesis is supported by the observation that poorly encapsulated strains are internalized most efficiently but are relatively virulent in infection models (Schrager *et al*,1996).

1.2.4 Pharyngitis

Pharyngitis is an inflammation of the mucous membranes and submucosal structures of the pharynx. There are many causes of pharyngitis. *Streptococcus pyogenes* accounts for approximately 30% of cases with the remainder being mostly viral (Wannamaker,1972). Hing *et al.* (2006) found that Group A streptococcus was responsible for 5 to 15% of cases of pharyngitis in adults and 20 to 30% of cases in children.

Ebell *et al* (2000) demonstrated that Streptococcal pharyngitis occurs most commonly among children between 5 and 15 years of age. In temperate climates, the incidence is highest in winter and early spring. The onset of symptoms in patients with streptococcal pharyngitis is throat pain, fever, chills, malaise, headache, and particularly in younger children abdominal pain, nausea, and vomiting (Wannamaker, 1972).

Brink *et al*. (1951) explained that streptococcal pharyngitis is accompanied by scarlet fever, which is manifested as a finely popular erythematous rash that spares the face, may be accentuated in skin folds, and may desquamate during convalescence. Among children younger than 3 years of age, exudative pharyngitis due to streptococcal infection is rare. In this age group, streptococcal infection may be manifested as coryza, excoriated nares, and generalized adenopathy. In most persons, fever resolves within 3 to 5 days, and throat pain resolves within 1 week, even without specific treatment (Denny *et al*, 1953).

1.2.5 Antibiotic resistance problem in *S. pyogenes* :

pharyngitis is one of the most common infections encountered in children. Most bacterial infections are caused by *S. pyogenes* (Bisno, *et al.,* 1997). In addition, resurgence of severe forms of disease caused by *S. pyogenes*

has been detected in various parts of the world (Givner, *et al.,* 1991 and Stevens, *et al.,* 1994). Although all *S. pyogenes* strains remain exquisitely sensitive to penicillin, erythromycin has been the drug of choice for individuals who cannot take penicillin.

However, there is a concern that a significant incidence of erythromycin resistance of *S. pyogenes* has been reported from the various regions of the world (Maruyama, *et al.,* 1979 and Cornaglia, *et al.,* 1998). Because the nonsuppurative sequels of *S. pyogenes* infections are still an important problem and invasive infections due to *S. pyogenes* have been increase recently.

Neeman, *et al.,* in 1998 reported that antibiotic treatment has been demonstrated to be unable to eradicate group-A Streptococci in up to 30% of patients with pharyngotonsillitis . thus an antibiotic, targeted against the strains of group-A Streptococci that have entered into the respiratory epithelial cells, which is unable to penetrate the cell membrane (such as penicillins), would fail to eradicate the bacteria.

Several explanations have been advanced, such as coexistence of oropharyngeal beta-lactamase- producing bacteria, interference by aerobic and anaerobic commensals, penicillin tolerance, reinfection. at present, many studies support the hypothesis that the intracellular niche may protect group a Streptococcus from penicillin, which does not reach high intracellular concentration (Sela and Barzilai, 1999).

thus, internalization and intracellular survival represent a novel explanation for penicillin eradication failure. in fact, the ability of group a Streptococci to persist in the throat following antibiotic therapy corresponds with their capacity to adhere to and be internalised by epithelial cells.

Jefferson in 2004 had been suggested that biofilm may also have a role in *S. pyogenes* infections have been reported. Hidalgo- Grass , *et al.*,(2004) had observed that structured communities appear to be present in necrotizing fasciitis lesions, and Neely *et al.* (2002)found similar characteristics in amodel of S. pyogenes myositis in zebrafish.

Akiyama *et al.* (2003) reported that *S. pyogenes* from a murine model of impetigo was embedded in glycocalyx, a feature also observed with human lesions. While still uniformly susceptible to penicillin, *S. pyogenes* strains may be resistant to macrolides, with resistance rates which vary considerably in different countries (Cornaglia and Bryskier, 2004).

1.2.6. Probiotics

Probiotics is a term derived from the Greek, meaning "for life". Nobel laureate Elie Metchnikoff formulated the probiotic concept approximately 100 years ago. He proposed that consumption of certain 'lactic bacilli' would be beneficial to humans by maximizing health-promoting activities of the gastrointestinal microbiota and minimizing their potentially harmful effects (Metchnikoff, 1907; Casas and Dobrogosz, 2000). From then on, probiotics have been scientifically studied and its definition has evolved and emerged from the gradual progress of understanding.

Lilley and Stillwell (1965) described probiotics as substances secreted by one microorganism to stimulate the growth of another, as opposite to an antibiotic. Parker (1974),who defined probiotics as organisms and substances which contribute to intestinal microbial balance, was the first to include microorganisms into the definition which is in the sense that it is used today. Later, Fuller (1989) recognized probiotics as "a live microbial feed supplement

which beneficially affects the host (humans or animals) by improving its intestinal microbial balance".

Furthermore, the probiotics concept was broadened, as "a viable mono- or mixed- culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (Havenaar and Huisin't Veld, 1992). The definition of probiotics continues to be improved, and was made official by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host" (Araya *et al*, 2001; 2002). Scientific findings are continuing, and the new challenge on the probiotics definition is that the beneficial effects do not have to come from viable probiotic cells; non-viable probiotics can also have health effects such as epithelial competitive exclusion, immune modulation and carcinogen binding in the host (Ouwehand and Salminen, 1998). World-wide interest in probiotics is increasing, and with continuous accumulation of knowledge the probiotics concept will surely enter a new era (Salminen *et al*, 1999).

1.2.7 Mechanism of action of probiotics:

1.2.7.1 Adherence and colonization to gut:

Probiotic microorganisms are able to adhere to the epithelial cells due to their anti – adhesive effects which block adherence of the pathogens (Lin *et al*, 2008). Anti adhesive effects might be due to competitive exclusion for the same receptor by the probiotics and the pathogens, secretion of proteins that destroy the receptor, induction of bio-surfactants, establishing a biofilm, and production of receptor analogues (Mack,*et al.,* 2003 and Oelschlaeger, 2010).

1.2.7.2 Competition of probiotics for limiting resources:

Almost all bacteria need iron as an essential element with the exception of *Lactobacillus* which didn‟t need iron in their natural habitat (Weinberg, 1997). *Lactobacillius acidophilus* and *Lb. delbrueckii* were found to be able to bind ferric hydroxide at their surface making it unavailable to pathogenic microorganisms. This mechanism is of crucial advantage in competition with other microorganisms which depend on iron (Elli *et al.,* 2000)

1.2.7.3 Anti–invasive effects of probiotics:

Not only adhesion but also invasion of epithelial cells is an important property for full pathogenicity of many gut pathogens; the ability to inhibit bacterial invasion of gut epithelial cells by pathogens is rather wide spread among probiotics (Hess *et al*., 2004). Number of researchers confirmed that some probiotics (like *Lactobacillus* and *Bifidobacterium* strain Bb12) had the ability to secrete factors which interfere with the invasion of host epithelial cells by *Salmonella typhimurium* (Ingrassia *et al*., 2005; Botes *et al.,* 2008).

1.2.7.4 Production of antimicrobial substances:

Wohlgemuth *et al* (2009) reported that lactic acid bacteria including *Lb. plantarum* and *Lb. acidophilus* had the ability to inhibit growth of Gram positive and Gram negative bacteria. This is due to their ability to produce organic acids (lactic acid and acetic acid), hydrogen peroxide, bacteriocin-like substances, and possibly bio-surfactants (Bierbaum and Sahl, 2009 ; Oelschlaeger, 2010).

1.2.7.5 Immunomodulatory effects of probiotics:

Isolauri *et al*. (1995) mentioned that many probiotic strains were able to stimulate production of immunoglobulin A (IgA) that helps in maintaining

humoral immunity of the intestine by binding to the antigen and limiting their access to the epithelium. Children at age of (2-5) years who were vaccinated with rotavirus and received *Lb. rhamnosus* GG showed an increase in the production of IgA producing cells. Probiotics, such as *Salmonalla, Shigella, Streptococcus*, are usually absorbed orally. They stimulate the gastro intestinal immunity by enhancing the specific and non specific immune response, inhibiting of pathogen growth, and translocation which will reduce the chance of infection from common pathogens (Delcenserie *et al*, 2008).

1.2.8 *Lactobacillus* as probiotics:

Lactobacilli are often considered to be commensal or beneficial participants in human microbial ecology, and considerable researchesare being carried out on the effects for the use of lactobacilli as additives in both human and animal diets (Hummel *et. al.* 2007). The probiotic activity of lactic acid bacteria (Lactobacilli Streptococci and Bifidobacteria) has been emphasized.

A number of health benefits have been claimed for probiotic bacteria and are also being recommended as a preventive approach to maintaintheir beneficial effects on humans including:

- The balance of intestinal microflora (Shah, 2007).
- Stabilization of intestinal microflora,excluding colonization of entero-pathogenic bacteria byadhesion to the intestinal wall and competition for nutrients, (Denev., 2006).
- Reduction of lactose intolerance (de Vrese *et al.*, 2001).
- Prevention of antibiotic-induced diarrhoea (Pochapin., 2000).
- Prevention of colon cancer (Wollowski *et al.*, 2001).
- Stimulation of the immune system (Isolauri *et al.*,2001).

In order to survive and colonise in the gastro-intestinal tract (GIT), probiotic microorganisms should express high tolerance to acid and bile and ability to adhere to intestinal surfaces. However, in vivo testingis expensive, time consuming and requires approval by ethical committees. Hence, reliable on vitromethods for selection of promising strains have been used by researchers (Jacobsen *et al.*,1999).

One of the unique features of probiotics is their antibiotic resistance expression and transferability as there is great concern over possible spread of resistance determinants to human pathogenic and opportunistic bacteria(Ammor *et al.* 2007).

Lactobacilli are highly competitive largely due to their applications in the production of fermented food. They can also produce antimicrobial substances including bacteriocins that have ability to inhibit pathogenic and food spoilage bacteria. These compounds have shown to exert specific antagonistic properties against Gram-negative and Gram-positive pathogens(Servin, 2004).

Adhesion of lactic acid bacteria (LAB) to mucosal surfaces has been studied *in vitro* using Caco-2 cells (Duary *et al.*,2011).The a forementioned points indicate that screening and selection of novel probiotic strains iscritical, thereby depending on definite criteria. Most efficient strains will be the strains that are robustenough to survive the harsh physicochemical conditions present in GIT.

Lactobacillus acidophilus is a well known and well studied probiotic microorganism. However, it is nowclear that different strains undoubtedly vary in their efficiency and probiotic potentialsm. (Ng *et al.*,2009).

1.2.8.1 Inhibitory compounds produced by *Lactobacillus acidophilus:*

- Carbon dioxide (CO_2): Carbon dioxide is produced by *Lactobacillus acidophilus* by fermenting sugars that provide anaerobic conditions which will in turn inhibit growth of the obligate aerobic microorganisms, lowering the pH, interfering with the cellular enzymes reactions, and destroying the cellular membrane (Adams and Nicolaides, 1997).
- Organic acids: Organic acids, such as pyruvate, helps lowering the pH and inhibits growth of many pathogenic Gram negative and positive bacteria. However lactic acid and acetic acid have a synergistic inhibitory effect against *Salmonella and E. coli* (Servin, 2004).
- Hydrogen Peroxide (H_2O_2): Hydrogen peroxide is produced in presence of oxygen and accumulates due to the inability of *Lactobacillus acidophilus* to produce the catalase enzyme. Hydrogen peroxide has an inhibitory effect against many Gram negative and Gram positive bacteria because of its oxidative effects; it also breaks cellular nucleic acids and proteins (Naidu *et al*., 1999; Kullisaar *et al*., 2002).
- Bacteriocins: Bacteriocins produced by *Lactobacillus* spp. are extracellular ribosomally synthesized peptides, usually ranging in size from 20 to 60 amino acids with bactericidal activity, and are immune to their own action including acidocin and lacticin produced from *Lb. acidophilus*, plantaricin C which is abroad-spectrum bacteriocins from Lb. plantarum, and Gassericin from Lb. gasseri (Tamime, 2005). Coconnier *et al*., (2000) stated that bacteriocins produced by *Lb. acidophilus* inhibited several enteropathogens *in vivo* and *in vitro*, including *H. pylori and Salmonella enterica var. typhimurium*. Cleusix *et al.*, (2008) reported that *Lb. reuteri* produces a bacteriocin (reuterin)

which has a broad-spectrum activity against Gram positive and Gram negative bacteria, also against yeast, fungi, protozoa, and viruses.

1.2.9 *Streptococcus salivarius* as probiotics

Streptococcus salivarius K12, is also known as BLIS (bacteriocin-like inhibitory substance) K12 (Tagg, 2004). It releases two lantibiotic bacteriocins named salivaricin A2 and salivaricin B, with high efficiency. Via these two lantibiotics, encoded by a 190 kb megaplasmid, BLIS K12 can effectively counteract the growth of β-hemolytic (group A) *Streptococcus pyogenes,* a common cause of pharyngitis, tonsillitis, and acute otitis media (Hyink *et al.,* 2007).

Wescombe *et al.,* (2006) reported that this inhibitory action is strongly linked to the release of lantibiotics because BLIS K12 P(-), the same strain without the 190 kb plasmid, does not show any antagonism of growth of *Streptococcus pyogenes*. In addition to its action against *S. pyogenes*, BLIS K12 can also inhibit growth of *Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Micrococcus luteus, Streptococcus anginosus, Eubacterium saburreum, and Micromonas micros* (Burton *et al.*, 2005).Many of these are potential pathogens in the ear and oral cavity, causing acute otitis media and halitosis.

Burton *et al.* (2006) found that BLIS K12 colonizes the upper respiratory tract of infants (oral cavity, nasopharyngeal and adenoid tissues) and with good persistence, given that after only 3 days of administration, it can still be detected 32 days later. Therefore, because of its good colonization capability and very high safety profile, combined with its reputed ability to counteract oral pathology, Tagg *et al.* (2006) decided to evaluate the preventive role of BLIS K12 when administered to children having a history of recurrent streptococcal pharyngitis and/or tonsil

1.2.10 Yeasts as probiotics:

Yeasts are group of unicellular microorganisms most of which belong to the fungi division of Ascomycota. They had been known for thousands years and considered as safe in food industry for various types of fermentations (Olver *et al*., 2002; Muňoz *et al*., 2005), like the production of alcohols (wine, beer), whey, bread, food additives, enzymes, vitamins, flavoring agents, production of microbiologically media and extracts, as alternative source of high nutritional value proteins, and single cell protein (Bekatorou *et al.,* 2006).

1.2.10.1: *Saccharomyces boulardii:*

Several *Saccharomyces boulardii* strains are commercially available now and had been used for over a decade in animal production industry as probiotic due to improving growth and reproduction, and reduction of morbidity and mortality of young animals (Zhang *et al*, 2000). The recorded poor survival of the probiotic bacteria in yogurt, led to the incorporation of probiotic yeast *Saccharomyces boulardii* due to its ability to survive in bio-yogurt reaching 10^7cfu/g. Despite inability of yeast to utilize lactose, it is able to utilize available organic acids, as well as galactose and glucose derived from bacterial metabolism of milk sugar (lactose) in dairy products (Lourens-Hattingh and Viljoen, 2001). *S. boulardii* resists the gastric acidity, proteases, and antibiotics. It is used usually in a lyophilizes form (Kühle *et al*, 2005).

Czerucka and Rampal (2002) reported some probiotic properties of *Saccharomyces* such as its ability to survive through the gastrointestinal tract (GIT) and the antagonist interaction with GI pathogens of *E. coli*, *Shigella*, and *Salmonella*. Some strains of *S. cerevisiae* had been used in humans as probiotics for many years, because they affect the intestinal microflora with

clinical efficiency for prevention and treatment of antimicrobial-associated diarrhea (Elmer, 2001).

Pérez-Sotelo *et al*., (2005) explained some of the mechanisms helping in understanding ability ofyeasts to protect the hosts against pathogens. These mechanisms include: stimulation of the immune system, degradation of bacterial toxin (by the yeasts proteolytic enzymes), inhibition of bacterial adherence to gastrointestinal epithelial cells (by releasing a protease able to digests the bacterial receptors of certain pathogens such as *Clostridium difficile*), and formation of yeast-bacterial conglomerates (by bacterial adhesion to yeast cell wall).

Bekatorou *et al* (2006) found that *S. boulardii* was first isolated from lici fruit in Indonesia. This species is a thermophyle, non pathogenic yeast, used for more than 50 years as a feed probiotic supplement and a therapeutic agent to treat many gut disorders like diarrhoea. Moreover, it is safe, resists antibiotics, achieves high cell number in the intestine in a short time, does not permanently colonize the intestine, and quickly cleared after the cease of administration. Edwards-Ingram *et al.* (2007) declared that *S. boulardii* is considered as a conspecific with *S. cerevisiae*, based on comparative genomic hybridization, genetic finger printing, and gene sequencing. Zanello *et al*. (2009) pointed out that the two yeast speciesare differed genetically, metabolically, and physiologically. Also, the alternation in the copy number of *S. boulardii* genes may contribute to increase growth rate, and better survival in acidic environment, in addition to the ability of *S. boulardii* to grow faster than *S. cerevisiae* at 37°C.

1.2.10.2 Anti-microbial activity of *Saccharomyces boulardii*

Within the intestinal luminal, *S. boulardii* exerts several anti-microbial activities that could be divided in two groups:

Group one: Direct anti-toxin effects.

The anti-toxin action elicited by *S.boulardii* is mainly due to small peptides produced by the yeast. A 54 kDa serine protease is able to inhibit enterotoxin and cytotoxic activities of *Cl. difficile* by degradation of toxin A and B and receptors sites of toxin A on the enterocyte cell surface. Others *Saccharomyces* strains fail to show such activities (Castagliuolo *et al.*,1999). *S.boulardii* produces a phosphatase enzymes ables to dephosphorylate endotoxins (such as lipopolysaccharide of *E. coli* 055B5) and inactivates its cytotoxic effects (Buts and De Keyser, 2006). This mechanism may account for the protection afforded in cases of sepsis.

Group two: Inhibition of growth and invasion of pathogens:

Mumy *et al.* (2008) reported in an *in vitro* study that *S. boulardii* directly inhibited the growth of several pathogens (*Candida albicans*, *E. coli*, *Shigella*s p, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Entamoeba hystolitica*), and cell invasion *by Salmonella typhimurium* and *Yersinia enterocolitica*. .A study by Murzyn *et al.* (2010) suggested that caprice acid, a factor secreted by *S.boulardii*,is responsible for inhibition of *C. albicans* filamentation.

2.Materials and Methods:

2.1 Materials:

2.1.1 Apparatus and equipment:

The following apparatus and equipment were used to perform this study:

Apparatus or equipment	Company	Origin
Autoclave	Express	Germany
Compound light microscope	Feica	Germany
Cooled centrifuge	Harrier	UK
Durham tubes	Marienfeld	Germany
Incubator	Memmert	Germany
Laminar air flow hood	Esco	Germany
Micropipette	Gilson	France
pH- meter	Radiometer	Denmark
UV/VIS-Spectrophotometry	BUCK	USA
Vaccum oven	MTI	USA
Vetic2	Biomereiux	France
Water bath	Gallen Kamp	England
Water distiller	GLF	Germany

2.1.2 Chemicals and biological materials:

Material	Company	Origin
Crystal violet	Syrbio	Switzerland
Iodine		
Safranine		
Acetone		
Glacial acetic acid	Scharlau	Spain
Ethanol		
Peptone water	Himedia	India
Methyl red		
Peptone		
Beef extract		
Esculin		
Bile salt		
Agar		
Skim milk		
Casein		
Yeast extract		
Hyaluronic acid	Sigma	USA
Ribose	BDH	England
Sorbitol		
NaCl salt		
Phenol red		
Ferric citrate		
Mannitol		
Lactose		
Catalase		
Oxidase		
Tris		

HCl	BDH	England
NaOH		
TCA		
l-Cysteine , Glucose		
Bovine serum albomin		
Sodium acetate		
Dextrose		
Gelatin		
Fructose		
Maltose		
Sucrose		
l-cysteine HCl		
Iodine		
Calcium Carbonate		
Bromocresol purple		
Lactophenol-cotton blue stain		
Dipotassium hydrogen phosphate		
KOH, α-naphthol		

2.1.3 Antibiotic discs: (Bioanalyse / Turkey):

Antibiotic	Symbol	Concentration (µg/disc)
Amoxicillin	AX	10
Azithromycin	AZM	15
Bacitracin	B	0.04
Clindamycin	CL	30
Chloramphenicol	C	30
Erythromycin	E	15
Penicillin	P	10
Tetracycline	TE	30
Vancomycin	VA	30

2.1.4 Ready-to-use media:

All media listed below were prepared according to the instructions on containers by their manufacturing company:

Medium	Company	Origin
Blood agar base	Himedia	India
Brain heart infusion agar		
Brain heart infusion broth		
MRS broth		
MRS agar		
MRVP broth		
Potato dextrose agar (PDA)		
Simon citrate agar		
Sabouraud dextrose broth(SDA)		
Sabouraud dextrose agar		
Tryptic Soy Broth		

2.1.5 Laboratory-prepared media:

2.1.5.1 Blood agar: (Collee *et. al,* 1996)

It was prepared by addition of 5% human blood to warm autoclaved blood agar base, mixed and poured into plate and kept at 4 C until use for identification of *Streptococcus* spp.

2.1.5.2 Bile Esculin Agar : (Downes and Ito, 2001)

Peptone (5 g), Beef extract (3 g), Esculin (1g), Bile salts (40 g), Ferric citrate (0.5 g) and Agar (15 g) were dissolved in1000 ml distilled water with

the aid of heat, then it was dispensed into tubes (5 ml each) before sterlized by autoclaving and allowed to solidify as slants. It was used for identification of *Streptococcus* spp.

2.1.5.3 Purple Broth with Carbohydrates: (Forbes *et al.,* 2007)

Peptone (10 g), Sodium Chloride (5.0 g) and promcresol Purple (0.02 g) were dissolved in 1000 ml distilled water with the aid of heat, then it was dispensed into tubes (5 ml each) before sterilized by autoclaving. When preparing dissolve 10 g of the desired carbohydrate in the basal aseptically added ,separately. Used for identification of *Streptococcus pyogenes*.

2.1.5.4 Skim milk agar : (Baron and Finegold, 1994)

Skim milk (100 g) and agar (15 g) were dissolved in 1000 ml D.W., then sterilized by the autoclave at 121°C for 5 min. This medium were used for detection of streptokinase enzyme by the plate method.

2.1.5.5 Brain heart serum albumin medium (BHSA):(Samantha *et al.*, 2004)

It was prepared by addition of hyaluronic acid (previously sterilized by filtration through millipore filter 0.2µm) to 100 ml of the autoclaved and cooled to 50°C medium (which contained 52 g/l of brian heart infusion agar), then mixed well to give a final concentration of 0.04% of hyaluronic acid. After that, sterilized bovine serum albumin was add to it (with constant stirring) to give a final concentration of 1%. The final pH of was adjusted to 6.8 before distributing into Petri dishes. After solidification, plates were kept at 4°C to provide a firm surface. This medium used for detection of hyaluronidase enzyme.

2.1.5.6 Yeast fermentation medium:

It was used for identification of *Saccharomyces* spp and prepared according to Atlas, (1995) from the following ingredients: peptone (7.5 g), yeast extract(4.5 g) and Bromothymol blue (0.6 g/10 ml) (1 ml) which were dissolved in 1000 ml D.W., before sterilized by the autoclave. then the carbohydrate source (0.6/10 ml) solution prepared in (2.1.8.1) (which was previously sterilized by membrane filtration, (1 ml) was added.

2.1.5.7 Sugar fermentation medium:

This medium was used for identification of *Lactobacillus* spp. and prepared according to Harrigan and MacCance, (1976) by using MRS broth containing 0.005% (v/v) bromocresol purple. After pH was adjusted to 6.2, the medium was distributed into test tubes, then sterilized by the autoclave. Later on, each sugar (mannitol, xylose, lactose, sucrose, esculin, fructose, maltose, , ribose, , sorbitol) was aseptically added ,separately, to the medium to obtain a final concentration of 2%.

2.1.6 Samples collection:

Samples were collected from the Central Hospital of the child in Baghdad by taking pharyngeal swaps from children infected with pharyngitis for the peroid from (6-10-2013) to(8-12-2013)..

2.1.7 Probiotic microorganisms:

2.1.7.1 *Lactobacillus acidophilus*:

It was obtained from the Department of biology at College of Science, Al-Mustansiriya University, which was orignally isolated from vagina.

2.1.7.2 *Streptococcus salivarius*

It was obtained as a compacted capsule from Amzone Web Site supplied by Now Foods (USA)

2.1.7.3 *Saccharomyces boulardii*

It was obtained as a compacted capsule from Amzone Web Sitesupplied by Pure Encapsulations (USA).

2.1.8 Preparation of solutions, buffers and reagents:

2.1.8.1 Solutions

- **Sugar solutions:**

Sugars used in yeast fermentation tests were prepared according to Atlas, (1995) by dissolving 0.6 g of each of glucose, fructose, lactose, maltose and sucrose in 10 ml of D.W. All sugar solutions were sterilized by membrane filtration throughout 0.22 µm millipore filter.

- **Trichloroacetic acid(TCA)5%: (Sutar *et al*., (1986)**

It was prepared by dissolving 5 g of TCA in 100 ml D.W.

- **Hyaluronic acid solution 1%: (Tam and Chan, 1983)**

It was prepared according to manufacturing company by dissolving 0.25 g of hyaluronic acid in 25 ml of D.W. then sterilized by filtration through milipore filter 0.22 mm. It was used as a substrate for hyluronidase enzyme.

- **HCl solution (3.3M): (Sutar *et. al*. (1986)**

It was prepared by adding 2.75 ml of stock HCl (12M) to 7.25 ml D.W.

- **Acetic acid (2N): (Tam and Chan, 1983)**

It was prepared by adding 17.6 ml of stock acetic acid (17M) to 132.4 ml D.W.

2.1.8.2 Buffers:

✓ **Tris-HCl buffer pH: 8: (Ronald *et al,* 1995).**

This buffer was prepared from following solutions:

- Solution A:HCl 0.2M

  It was prepared by addition of 0.8 ml of stock HCl 12M to 49.2 ml D.W.
- Solution B: Tris(hydroxymethylaminomethane) 0.2M

  It was prepared by dissolving 1.2 g of Tris in 50ml D.W.
- Solution C: Tris-HCl buffer

  It was prepared by mixing 26.8 ml of solution A with 50 ml of solution B and 50 ml of D.W.

✓ **Acetate buffer pH 4.2: (Ronald *et al.,* 1995).**

This buffer was prepared from following solutions:

- Solution A: acetic acid 0.2M

  It was prepared by adding 1.17 ml of stock acetic acid (17M) to 98.8 ml D.W.
- Solution B:sodium acetate 0.2M

  It was prepared by dissolving1.64 g of sodium acetate in 100 ml D.W.
- Solution C: Acetate buffer

Itwas prepared by mixing 36.8 ml of solution A with 13.2 ml of solution B and 50 ml of D.W.

2.1.8.3 Reagents:

- **Albumin reagent: (Tam and Chan, 1983)**

  It was prepared by dissolving 2.5 g of bovin serum albumin in 250 ml of acetate buffer, pH was adjusted to3.0 by HCl (2N), the solution was heated to 93 for 30 min. then the volume completed to 1000 ml by the same buffer.

- **Voges–Proskaure reagent (VP): (Collee *et al.*, 1996)**

It was used with acetoin formation test which consisted of the following :
VP1 reagent : 40% KOH , it was prepared by dissolving 40 gm of KOH in a little of D.W. then completed the volume to 100 ml of D.W.
VP2 reagent : It was prepared by dissolving 5 g of α-naphthol in a little of absolute ethanol then completed the volume to 100 ml of absolute ethanol.
Both reagents were kept in cooled dark place until use.

- **Methyl red reagent: (Collee *et. al.*, 1996)**

  It was prepared by dissolve 0.01g of methyl red in 30 ml of ethanol, then add 20 ml of D. W.

- **Oxidase reagent:** (Collee *et al.*, 1996)

  This reagent was prepared by dissolving 1 g of tetramethyl-p-phenylene diamine dihydrochloride powder in 100 ml D.W.

- **Catalase reagent:**

  This reagent was prepared according to Collee *et al.*, (1996) by mixing 3 ml of hydrogen peroxide solution (22%) with 97 ml D.W.

- **Kovac's reagent** (Atlas *et al.,* 1995)

  It was prepared by dissolved 10 g ρ-Dimethyl-aminobenzaldehyde in 150 ml of isoamyl alcohol with heating in a water bath at 50oC and concentrated HCl 50 ml was added slowly . The reagent was prepared in small quantities and stored in refrigerator.

2.2 Methods:

2.2.1 Sterilizing methods: (Baily *et al.*, 1990).

- **Wet-heat sterilization:**

Microbial culture media, solutions, buffers, and reagents were sterilized by the autoclave at 121°C (15 Ib/inch^2) for 15 min unless otherwise stated.

- **Dry-heat sterilization:**

Electric oven was used to sterilize glassware at 180 °C for 3 hrs.

- **Membrane Filtration:**

Microbial filtrates, sugar solutions, and dye solutions were sterilized throughout (0.45) and (0.22) µm in diameter millipore filters, and Whatman No.1 filter papers.

2.2.2 Sampling:

For isolation of *Streptococcus pyogens*,a total of 85 samples were collected from the Child Central Hospital in Baghdad, by taking pharyngeal swabs from children (2-14 years) infected with pharyngitis during the period from 6/10 to 8/12/2013. Swab samples were aseptically transferred under cooling conditions to the laboratory for analysis.

2.2.3 Isolation of *Streptococcus pyogens* :

According to Rijal *et al*. (2009), the throat swab was rubbed, while being rotated over the area of the surface on a blood agar plate. The plate was, then, incubated at 37°C for 24 hrs in a candle jar.

2.2.4 Identification of *Streptococcus pyogenes*:

2.2.4.1 Microscopic examination:

All specimens were subjected to the microscopic examination by staining with Gram stain (Atlas *et al*. , 1995).

2.2.4.2. Biochemical tests:

Suspected *Streptococcus pyogens* isolates were subjected to the biochemical tests mentioned by Murray *et. al.* , (2003) as follow:

2.2.4.2.1 Haemolysin production:

A loopfull from each suspected isolate culture was inoculated on human blood agar and incubated at 37°C for 24 hrs in a candle jar to examine its ability to produce haemolysin. Presence of clear zones around the colonies represents β- haemolysis and considered as a positive result .

2.2.4.2.2 Catalase test

Bacterial growth on a blood agar plate was flooded with 1.0 ml of 3% hydrogen peroxide (catalase reagent in 2.1.8.3) and observed for effervescence (bubbling) which indicates a positive test.

2.2.4.2.3 Bacitracin Test:

A bacitracin disk was fixed in the middle of plate, that already was streaked with the suspected isolate.. The plate was incubated for overnight in the candle jar at 37°C. Formation of a zone of inhibition is considered as a positive test.

2.2.4.2.4 Indole test:

A β-haemolytic colony was inoculated into peptone water broth and incubated at 37°C for 24 hrs in a candle jar. After incubation,few drops of Kovac's reagent (2.1.8.3) were added. A positive result was detected by formation of a red Indole ring at top of the test tube.

2.2.4.2.5 Methyl-red test:

A β-haemolytic colony was inoculated in MR-VP broth and incubated at 37°C for 24 hrs in a candle jar. After incubation, 3-4 drops of methyl red reagent (2.1.8.3) were added. Converting media color to red is a positive result.

2.2.4.2.6. Citrate test (Simmon's Citrate slant):

A loopful of β haemolytic colony was streaked onto a simmon citrate agar slant, then incubated for 24 to 48 hrs at 37°C in a candle jar. A positive reaction was detected by a formation of blue color.

2.2.4.2.7 Vogas-Proskauer test:

One β haemolytic colony was inoculated in MRVP broth and incubated at 37°C for 24 hrs in a candle jar. Then two drops of VP1 and four of VP2

(as mention in 2.1.8.3) were added. Appearance of red color after 30 min indicates a positive result.

2.2.4.2.8 Bile esculin test

Test tubes containing 5 ml each of bile esculin agar slant as prepared in (2.1.5.2) were inoculated with fresh culture of each β haemolytic suspected isolate and incubated at 37°C for 24-48 hrs in a candle jar. Appearance of blackening indicates a positive result that mean bacteria had ability to hydrolysis esculin and tolerance to bile.

2.2.4.2.9 Acid formation in carbohydrate broths:

A single colony of each bacterial isolate was inoculated into the carbohydrate broth tube (Item 2.1.5.3), then incubated at 37°C for 24-48 hrs in a candle jar. Appearance of yellow color indicates a positive result.

2.2.4.3 Identification of bacteria by VITEK 2 system:(David, 2004)

The VITEK 2 is an automated microbiology system utilizing growth-based technology. Used for bacterial identification.

2.2.4.4 Maintenance of *Streptococcus pyogenes* isolates:

Bacterial isolates were maintained according to Johanson *et al*., (1988) as follows:

- **Short – term storage (few weeks):**

Bacterial isolates were maintained for few weeks by culturing on plates of brain heart agar, and incubated at 37°C for overnight. The plates were then tightly wrapped with parafilm and stored at 4 °C.

- **Medium – term storage (1 – 3 months):**

  Bacterial isolates were maintained as stab culture for few months by inoculated in small screw capped bottles containing (5 – 8) ml of sterile brain heart agar (as slants), then incubated at 37°C for 24 hrs in a candle jar. The bottles were tightly wrapped with parafilm and stored at 4°C.

- **Long time storage: (Boonaert and Rouxhet, 2000)**

  Test tube containing 10 ml sterile brain heart broth was inoculated with the bacterial isolate, and incubated at 37°C for 24 hrs in a candle jar. After incubation, sterile glycerol (20%) was added and mixed by vortex before freezing at (– 20)°C.

2.2.5 Probiotic microorganisms:

2.2.5.1 *Lactobacillus acidophilus*:

2.2.5.1.1 Propagating *Lactobacillus acidophilus*:

According to Buck and Gilliland (1995), *L. acidophilus* isolates were propagated by transferring 1 ml of their cultures to a test tube containing 9 ml of sterile MRS broth, then incubated anaerobically for overnight at 37°C. This process was repeated three times to increase intensity of the bacteria cells.

2.2.5.1.2 Identification of *Lactobacillus acidophilus*.:

A) Microscopic examination:

A loopfull from the fresh culture of each *Lactobacillus* isolate was fixed on a glass slide, then stained by Gram's staining and examined under the oil – immersion lens of the compound light microscope to examine cells Gram reaction shape, grouping, and spore forming (Harely and Prescot, 1996).

B) Biochemical tests:

a) Catalase test: As in item (2.2.4.2.2):

b) Oxidase test: (Atlas *et al.*, 1995)

By a sterile wooden stick, a single colony was put onto a filter paper, then a drop of the oxidase reagent (2.1.8.3) was added to it. Changing colony color to dark purple indicates a positive result.

c) Sugar fermentation test: (Collins and Lyne, 1985)

Tubes containing sugar fermentation medium (as prepared in 2.1.5.7) were inoculated with culture of the *Lactobacillus* isolate, and incubated anaerobically at 37°C for 7 days. Changing bromocresol purple color from purple to yellow indicates a positive result

2.2.5.2 *Streptococcus salivarius* (Oral probiotic) :

2.2.5.2.1 Activation:

Contents of an oral probiotic (*S. salivarius*) capsule was dissolved in 10 ml of normal saline, then 1ml of this solution was added to 100 ml of brain heart infusion broth before incubated anaerobically at 37C^{o} for 24 hrs.

2.2.5.2.2 Identification of *S.salivarius*:

A) Microscopic examination: as in item (2.2.4.1)

B) Biochemical tests:

a) Catalase test: as in item (2.2.4.2.2)

b) Oxidase test: as in item (2.2.5.1.2)

c) Haemolysin production: (2.2.4.2.1)

d) Vogas-Proskauertest: as in item (2.2.4.2.7)

e) Bile esculine test: as in item (2.2.4.2.8)

2.2.5.3 *Saccharomyces boulardii:*

2.2.5.3.1 Activation:

From a compact capsule containing dried yeast *Saccharomyces boulardii* was obtaind. The yeast was activited by culturing in Sabouraud dextrose broth (SDB) at 25°C for 48 hr. After incubation, serial dilutions were made in SDB, and a loopfull from the last dilution was streaked on PDA (Barnett *et al.*,1985).

2.2.5.3.2 Identification of *Saccharomyces boulardii* .:

A) Microscopic examination:

A loopfull from the suspected *S. boulardii* isolate was put on a glass slide, then drops of lactophenol – cotton blue were added before examining under the 40× objective lens of the compound light microscope.

B) Carbon sources fermentation test (Atlas *et al* , 1995):

Yeast fermentation medium (Item 2.1.5.6) was prepared by dissolving all ingredients (except carbohydrate solution) into the major portion of D.W., and completing the volume to 1L with D.W.. The medium then was distributed into test tubes (5 ml each) previously contained inverted Durham tubes, before sterilizing in the autoclave. After cooling, 1 ml of each carbohydrate solution was added to each tube and mixed thoroughly. Positive fermentation turns the medium into yellow color with the formation of gas bubbles in the Durham tubes.

2.2.6 Antibiotic susceptibility test for *S. pyogenes* : (Atlas, *et al.*, 1995)

Single colonies grown of *S. pyogenes* isolate on blood agar for (24) hrs. By using sterile cotton swab, a touch of bacterial culture was transferred to blood agar and streaked three times to ensure even distribution of the inoculum, the inoculated plates were placed at room temperature for 10 min to allow absorption of excess moisture, then the discs of antibiotic (amoxicillin, azithromycin, chloramphenicol , clindamycin, tetracycline, penicillin and vancomycin) were fixed by sterile forceps on the surface of plates and gently pressed down on the surface of agar.

Discs were arranged so as to avoid the development of overlapping of inhibition zones and the plates were incubated at 37 ºC for 24 hours. After incubation , measured the diameters of inhibition zones (clear area around discs) were measured by ruler .

2.2.7 Detecting antimicrobial activity of probiotics against *S. pyogens*:

2.2.7.1 *Saccharomyces boulardii*:

Yeast isolates possessing antimicrobial activity were determined by growing the isolates in SDP then the medium was distributed in 100 ml conical flasks (each one contained 50 ml) before sterilized by the autoclave. The flasks were inoculated with 1 ml of each yeast isolate suspension that already grown for 48 hrs, and incubated at 28°C for 24 hrs.

After incubation yeast suspension was centrifuged at 3000 rpm for 15 min to obtain cells – free culture suspension, then the suspension was filtrated using millipore filters (0.45) , this unconcentrated filtrate.

Saccharomyces boulardii filtrates were concentrated as follows: One hundred mls of the unconcentrated filtrate were concentrated to (50 ml) by putting in the vacuum oven at (40-45) °C to make a the one-fold concentrated filtrate. The experiment was repeated on the one-fold concentrated filtrate to obtain the two-fold concentrate filtrate (25 ml), and same thing was done for the three-fold concentrate filtrate (12.5 ml).

By using Izgü and Altinbay (1997) method, 5 mm diameter wells were made by a cork borer in solidified brain heart agar in plates that were already inoculated with 0.1 ml of *S. pyogens* culture by spreading. The wells were filled with the unconcentrated and concentrated yeast filtrates, then plates were incubated at 37˚C for 24 hrs. After incubation, inhibition zones were measured by a ruler (in mm).

2.2.7.2 *Lactobacillus acidophilus:*

The filtrate was obtained from growing each Lactobacillus isolates in MRS broth with pH 6 and 2% inoculum, then incubated anaerobically at 37°C for 24 hrs (Lewus et al., 1991). After that the isolates were centrifuged at (6000 rpm) for 10 min to obtain cell – culture – free liquid, which was sterilized by filtration (millipore filter 0.45) this unconcentrated suspension (Piard *et al.*, 1990 and Martinez-Gonzalez *et al.*, 2004).

One-fold, two-fold and three-fold concentrated filtrates were made from the unconentrated *L. acidophilus* filtrate as in item 2.2.8.1.

5 mm diameter wells were made by a cork borer and placed on the surface of brain heart agar plates that was already spread with 0.1 ml of *S. pyogens* culture, The wells were filled with the unconcentrated and concentrated *L. acidophilus*, then incubated at 37°C for 24 hrs. Inhibition zone diameters (in mm) were measured (Al-Kassab and Al- Khafaji, 1992**).**

2.2.7.3 *Streptococcus salivarius*:

The inhibitory activity of (bacteriocin-like inhibitor substance / BLIS) produced by *S. salivarius* was evaluated by using deferred antagonism test, essentially that described by Tagg and Bannister (1979) as follow:. A 1-cm wide diametric streak culture of *S. salivarius* inoculated onto trypticase soy broth with the addition of 2% yeast extract,1.5%, $CaCO_3$, 5% of human blood and incubated in a candle jar for 24 hrs at 37°C. After removing the macroscopic cell growth with a glass slide, residual cells on the agar surface were killed by exposure to chloroform vapour for 30 min. The agar surface was then aired for 30 min and the isolated *S. pyogens* was streaked across the line of the original streak culture with cotton swabs. After incubation for 18 hr in a candle jar at 37°C, the extent of inhibition of each *S. pyogenes* isolate was recorded.

2.2.7.3.1 Preparations of crude BLIS:

Preparation of Crude BLISs that produced by *S. salivarius* were obtained by freeze-thaw extraction of cultures of *S. salivarius* that had been grown for 18 hr at 37◦C in a candle jar on tryptic soy broth supplemented with 2% yeast extract, 1% $CaCO_3$, and 0.7% agar, adjusted to pH 6.5 before autoclaving. The freeze thaw extraction process consisted of the agar cultures (entire agar plate including bacterial lawn) being frozen at -20 °C and then subsequently thawed.

It was essentially that agar cultures were thawed at room temperature for 3 to 4 hr. After the freeze-thaw cycle broke the gel structure, cells were removed by centrifugation of the filtrates at 10,000 x g for 10 min at 4°C and then filtered by Millipore filter (**0.45mm)** the supernatant referred to as the crude bacitracin (Jack *et al.*, 1994).

2.2.8. Detection of streptokinase production:

2.2.8.1 Radial caseinolysis assay:

Streptococcus pyogens was grown in 25 ml of brain heart broth at 37 °C untill the turbidity reached the level of 0.6 OD at 600 nm, The culture was centrifuged for 25 minutes at 10,000 g. After the supernatant was filtered through a 0.45 µm millipore filter, it was collected and labelled as crude enzyme. (Mohammad *et. al.*, 2009 and Saksela, 1981).

(Wu *et al*., 1998) the skim milk agar medium was prepared and wells were punctured in agar plate. 25 µl of streptokinase crude enzyme was loaded into the wells and kept for 12 hrs incubation at 37 °C. Positive result recorded by create clear zone around well in skim milk agar plate.

2.2.8.2 Casein digestion method

Streptokinase activity was determined indirectly by the casein digestion method, which is based on determination of the liberated tyrosine from digested casein after plasminogen activation (Mounter and Shipley 1957)

The activity was determined according to a modified method of Sutar *et. al*. (1986). Reaction mixture (2 ml) containing 10 mg casein, 50 mM Tris-HCl, pH 8.0 prepared in (2.1.8.2), The absorbance at 600nm of each cultures of

bacterial isolates were adjusted to 0.5nm by dilution with sterile medium. The suspension was centrifuged to pellet the cells, and the supernatant fluid was removed and filtered by a 0.45-mm-pore-size filter unit, add 0.1 ml of filtered streptokinase crude enzyme. The reaction was carried out at 37°C for 20 minutes before terminated by the addition of 2.6 ml 5% (w/v) of trichloroacetic acid (TCA) (in 2.1.8.1) and 0.4 ml 3.3 M HCl (2.1.8.1) . Reactions were then kept on ice for 30 minutes after which they were filtered through Whatman paper No.1. Absorbance of the TCA soluble fractions was measured at 280 nm.

2.2.9 Detection of hyaluronidase production:

2.2.9.1 Plate method:

Brian heart serum albumin (BHSA) agar prepared in (2.2.3.5) was poured in the plates to a depth of 3 to 4 mm, After solidification, plates were kept at 4°C to provide firm surface for wells making which were filled with 25 µl of the clear supernatant of overnight *Streptococcus pyogenes* broth culture, the plate were then incubated at 37°C for 24 hrs. After incubation, the plate were flooded with 2 N acetic acid prepared in (2.2.2.1) for 10 min. Diameters of the hydrolysed or clear zone (production of hyaluronidase) by the isolates were measured in mm (King *et al.*, 2004).

2.2.9.2 Turbidity reduction assay:

The reaction mixture containing 0.25 ml (0.04 %) hyaluronic acid (HA), 0.5 ml D.W. and 0.25 ml of albumin reagent (Item 2.1.2.3), The absorbance at 600nm of each cultures of bacterial isolates were adjusted to 0.5nm by dilution with sterile medium. The suspension was centrifuged to pellet the cells, and the supernatant fluid was removed and filtered by a 0.45-

mm-pore-size filter unit .and 0.5 ml of the supernatant were add, mixed and incubated at 37 °C for 30 min. At the end of incubation time, the tubes were cooled in ice bath. To the above mixture, 0.1 ml of acetic acid (2 N) (2.1.8.1) was added to precipitate the remaining HA . Tubes containing sterile broth or broth from inactive cultures became turbid while those containing broth from hyaluronidase producing organism remained clear on addition of the acid (Tam and Chan, 1983).

2.2.10 Effect of probiotics on streptokinase and hayluronidase activity:

To measured the effect of each probiotics, 1 ml of each of the followings:

- concentrated *Lactobacillius acidophilus* filtrates (prepared in item 2.2.3.6)
- concentrated yeast filtrates (prepared in item 2.2.3.7)
- crude BLIS(prepared in item 2.2.3.8.1)

was added, separately, to the reaction mixture, then the activity was measured by using turbidity reduction assay for hyaluronidase and casein digestion method for streptokinase

2.2.11 Effect of antibiotics on streptokinase and hayluronidase activity:

To measured the effect of the antibiotics, 1 ml of each of :

- Tetracyclin 30 µg/ml
- Chloramphenicol 30 µg/ml
- Vancomycin 30 µg/ml

- Azithromycin 15 µg/ml
- Clindamycin 2 µg/ml
- Amoxillin 25 µg/ml

was added, separately, to the reaction mixture, then the activity was measure by using the turbidity reduction assay for hyaluronidase and casein digestion method for streptokinase.

3. Results and Discussion

3.1 Isolation of *Streptococcus* isolates:

After culturing the 85 pharyngeal swap samples of children on blood agar, results showed that 135 bacterial isolates were obtained, but only 30 of them were able to produce type β-hemolysis around their colonies. Formation of such type of complete hemolysis zone is usually a characteristic of *S. pyogenes* (Nizet, 2002).

3.2 Identification of bacterial isolates:

3.2.1 Cultural characteristics:

The 30 suspected bacterial isolates were replated on blood agar and incubated for 24 hrs at 37^{o} C in a candle jar. After incubation, their colonies were small glossy, grayish-white, translucent, and surrounded by distinctive β hemolysis zones. These characteristics come in accordance with the corresponding cultural characteristics of *S. pyogenes* that mentioned by Ananthanarayan and Paniker (2005).

3.2.2 Microscopic characteristics:

When suspected isolates were stained by Gram staining method, their cells appeared under the microscopic as G-positive, spherical, cocci arranged mainly in chains, as mentioned by Ananthanarayan and Paniker (2005).

3.2.3 Biochemical characteristics:

The suspected *Streptococcus pyogenes* 30 isolates and were then subjected to the related biochemical tests. Results showed that four of the 30 β-hemolysis producing isolates were the most sensitive ones to bacitracin as shown in table (3-1). Upon such finding, only these four isolates were subjected to the remaining biochemical tests, because of in contrast to most other streptococci, *S. pyogenes* is uniformly sensitive and large inhibition zones are

formed round bacitracin discs on blood agar (Cunningham, 2000) as shown in Figar (3-1) .

Table (3-1) Bacterial isolates that gave bacitracin sensitive.

Isolate symbol	Susceptibility	Bacitracin inhibition diameter(mm)*	Isolate symbol	Susceptibility	Bacitracin inhibition diameter(mm)*
S1	R	-	S16	S	13mm
S2	R	-	S17	R	-
S3	R	-	S18	R	-
S4	R	-	S19	R	-
S5	R	-	S20	S	14mm
S6	R	-	S21	R	-
S7	R	-	S22	R	-
S8	R	-	S23	R	-
S9	R	-	S24	R	-
S10	R	-	S25	R	-
S11	R	-	S26	R	-
S12	R	-	S27	R	-
S13	R	-	S28	S	14mm
S14	R	-	S29	R	-
S15	R	-	S30	S	13mm

*** (S) sensitive to bacitracin, (R) resistance to bacitracin.**

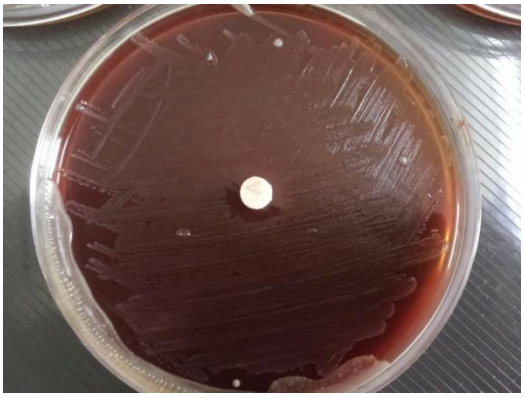


Figure (3-1) Bacitracin sensitivity test

Table (3-2) Biochemical tests for *Streptococcus pyogenes.*

Biochemical test	Isolate			
	S16	S20	S28	S30
Growth under anaerobic condition	+	+	+	+
β-haemolysis	+	+	+	+
Catalase	-	-	-	-
Oxidase	-	-	-	-
Indole	-	-	-	-
Methyl red	-	-	-	-
Voges Proskauer	-	-	-	-
Citrate	-	-	-	-
Growth in 6.5% NaCl (bile salt)	-	-	-	-
Esculin Hydrolysis	-	-	-	-
Gelatin hydrolysis	-	-	-	-
Glucose fermentation	+	+	+	+
Lactose fermentation	+	+	+	+
Maltose fermentation	+	+	+	+
Mannitols fermentation	+	+	+	+
Sorbitol fermentation	-	-	-	-
Ribose fermentation	-	-	-	-

(+) positive result, (-) negative result.

Results illustrated in table (3-2) showed that all the four isolates were unable to hydrolyse gelatine and esculin, not producing catalase, oxidase, and

gelatinase enzymes, as well as negative for methyl red, citrate, Voges-Proskauer and Indole tests. They were also unable to tolerate bile salts when can't grow in presence of 6.5% sodium chloride. Adversely, they were able to ferment four of the used carbon sources (glucose, lactose, maltose and mannitol), but not ribose and sorbitol. Holt (2000) in Bergey's Manual of Determinative Bacteriology declared that such characteristics usually are coming in accordance with those belonging to *Streptococcus pyogenes*.

3.2.4 Identification of bacterial isolates by VITEK system:

Identification of bacterial isolates was also done by the VITEK system, recently installed at the Central Health Laboratory/Ministry of Health, by using the GP (Gram positive) card.

Table (3-3) showed the performance of the VITEK 2 system for the identification of bacterial isolate that gave all of isolates were *S. pyogenes*. In general, the VITEK 2 system is an easy-to-handle system that provides a rapid (4 to 15 h) and reasonably accurate means for the identification of most commonly isolated species of *Enterococcus* and accurately detects. However, the system needs further improvement in its accuracy of identification, interpretation of results, and database. One of the most important advantages of the VITEK 2 system is the significant reduction in handling time, which will have a positive impact on the work flow of the clinical microbiology laboratory

Table (3-3) Result of VITEK2 system .

Bacterial isolates	Probability	confidence
S16	97%	Excellent identification
S20	85%	Acceptable identification
S28	95%	Very good identification
S30	88%	Acceptable identification

Table(3-4) : Vitek test result for S16 isolate

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
AMY	-	CDEX	-	dSOR	-	dMNE	-	AGLU	+
APPA	-	ProA	-	LAC	-	SAC	+	PHOS	+
LeuA	+	TyrA	+	dMAN	-	BGAL	-	BGUR	-
AlaA	+	ILATk	-	SAL	-	AMAN	-	dGAL	-
dRIB	-	NC6.5	-	ADH1	+	PyrA	+	BACI	-
NOVO	-	O129R	-	BGAR	-	POLYB	-	PUL	-
dRAF	-	dXYL	-	AGAL	-	dMAL	+	ADH2s	-
OPTO	+	AspA	-	URE	-	MBdG	-		
PIPLC	+	BGURr	-	NAG	-	dTRE	+		

Table(3-5) : Vitek test result for S28 isolate

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
AMY	-	CDEX	-	dSOR	-	dMNE	+	AGLU	+
APPA	+	ProA	-	LAC	+	SAC	+	PHOS	+
LeuA	+	TyrA	+	dMAN	-	BGAL	-	BGUR	-
AlaA	+	ILATk	-	SAL	-	AMAN	-	dGAL	+
dRIB	-	NC6.5	-	ADH1	+	PyrA	+	BACI	-
NOVO	+	O129R	-	BGAR	-	POLYB	+	PUL	-
dRAF	-	dXYL	-	AGAL	-	dMAL	+	ADH2s	+
OPTO	+	AspA	-	URE	+	MBdG	-		
PIPLC	-	BGURr	-	NAG	+	dTRE	+		

Table(3-6) : Vitek test result for S30 isolate

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
AMY	-	CDEX	+	dSOR	-	dMNE	-	AGLU	+
APPA	+	ProA	+	LAC	+	SAC	+	PHOS	+
LeuA	+	TyrA	+	dMAN	-	BGAL	-	BGUR	-
AlaA	+	ILATk	-	SAL	-	AMAN	-	dGAL	-
dRIB	-	NC6.5	-	ADH1	+	PyrA	+	BACI	-
NOVO	+	O129R	-	BGAR	-	POLYB	-	PUL	+
dRAF	-	dXYL	-	AGAL	-	dMAL	+	ADH2s	+
OPTO	+	AspA	-	URE	-	MBdG	-		
PIPLC	-	BGURr	-	NAG	-	dTRE	+		

Table(3-7) : Vitek test result for S20 isolate

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
AMY	-	CDEX	+	dSOR	-	dMNE	+	AGLU	+
APPA	+	ProA	+	LAC	-	SAC	+	PHOS	+
LeuA	+	TyrA	+	dMAN	-	BGAL	-	BGUR	-
AlaA	+	ILATk	-	SAL	-	AMAN	-	dGAL	+
dRIB	-	NC6.5	-	ADH1	+	PyrA	+	BACI	-
NOVO	+	O129R	-	BGAR	-	POLYB	+	PUL	+
dRAF	-	dXYL	-	AGAL	-	dMAL	+	ADH2s	+
OPTO	+	AspA	-	URE	-	MBdG	-		
PIPLC	-	BGURr	-	NAG	+	dTRE	+		

3.3 Identification of *Lactobacillus acidophilus* isolate:

Lactobacillus acidophilus used in this study was supplied Despite that it was already identified by the source previously, but it was reidentified, and the following description of cultural and biochemical were obtained.

3.3.1 Cultural characteristics:

After propagating *L. acidophilus* isolate on the MRS agar medium, their colonies appeared white to pale color, round shaped, soft, mucoid, convex, with smooth edges. (Jawetz *et al.*, 2010).

3.3.2 Microscopic characteristics:

After *Lb. acidophilus* isolate colonies were Gram stained and examined, results revealed that they were gram positive, short to long bacilli, clustered in long and short chains, and non-spore forming. This description is also mentioned by Hammes and Vogal, (1995).

3.4.3: Biochemical characteristics:

Result showed that *Lb.acidophilus* isolate were negative to catalase, oxidase. Regarding sugars, they were able to ferment glucose, fructose,

galactose, sucrose, lactose and maltose, but unable to ferment mannitol, ribose, and xylose as reported by Hammes and Vogal (1995) and Carr *et al.* (2002).

3.4 Identification of *Streptococcus salivarius*:

A standard strain of *S. salivarius* was identified depending on the cultural, microscopic, and biochemical examination. Results obtained are expressed as follows:

3.4.1 Cultural characteristics:

Appearance of *S. salivarius* colonies on blood agar was characterized as small, glossy, grayish-white, translucent, without hemolysis (gamma hemolysis). These results come in accordance with the corresponding cultural characteristics mentioned by Hardie and Bowden (1976).

3.4.2 Microscopic characteristics:

Cells of the bacterial isolate were also identified according to the Gram reaction and other microscopic characteristics. Result showed that they were Gram positive-cocci arranged mainly in chains. These results agreed with the microscopic characteristics that mentioned by Hardie and Bowden (1976).

3.4.3 Biochemical characteristics:

Results showed that the *S. salivarius* isolate gave negative result for catalase and oxidase and bile-esculin tests, but positive result for Vogas-proskuar (VP). It was unable to produce hemolytic zone (gamma hemolysis) as reported by Murray *et al.* (2003).

3.5 Identification of *Saccharomyces boulardii*:

A standard strain of *S. boulardii* were reidentified depending on the cultural, microscopic, and biochemical characteristics. Results are expressed as follows:

3.5.1 Cultural characteristics:

The strain colonies produced after incubation on SDA (Sabouraud dextrose agar) were characterized as rounded, convex, soft, white to cream, with regular edges. These results come in accordance with the corresponding cultural characteristics that mentioned by Barnett *et al.* (1990).

3.5.2 Microscopic characteristics:

Cells of the standard yeast strain were examined under the microscope after staining by lactophenol-cotton blue. Results illustrate that the cells were mainly single ovoid and also grouping as clusters like a beehive. Barnett *et al.* (1990) ensured that *Saccharomyces boulardii* has such characterization.

3.5.3 Biochemical characteristics:

After performing the cultural and microscopic examinations, the yeast strain was subjected to number of biochemical tests. Results showed that these cells had the ability to ferment all sugars tested (glucose, fructose, galactose, sucrose and maltose) except lactose. Such findings come in accordance with what are described by Lodder, (1974) and Barnett *et al.* (1985).

3. 6 Antibiotics susceptibility of *S. pyogenes*

Results showed that all *S. pyogenes* isolates were resistant to erythromycin, azithromycin, clindamycin chloramphenicol but sensitive to amoxicillin, tetracycline and vancomycin , on other hand they variant in result with penicillin as shown in table (3-4)

Table (3-8) Result of antibiotics susceptibility test

S. pyogenes isolates	AX	AZM	CL	TE	VA	E	P	C
S16	S	R	R	S	S	R	S	R
S20	S	R	R	S	S	R	R	R
S28	S	R	R	S	S	R	R	R
S30	S	R	R	S	S	R	S	R

***(S) sensitive (R) resistant, (AX) amoxicillin, (AZM) azithromycin, (CL) clindamycin, (TE) tetracycline, (VA) vancomycin, (E) erythromycin, (P) penicillin , (C) chloramphenicol.**

Antimicrobial resistance is an important problem in the management of patients with infectious diseases. Wittler *et al.,* (1990) declared that *Streptococcus pyogenes* remains susceptible to penicillin during the past 70 or 80 years but the reason for this unique lack of development of resistance to penicillin is unknown On the other hand, numerous reports have demonstrated significant prevalence of erythromycin resistant *S. pyogenes* around the world during the past three decades (Cornaglia *et al.,* 1998). This resistance has been temporally related to increased or excessive use of macrolide antibiotics. Because of this relation, it is important to determine the geographic prevalence of resistant *S. pyogenes* to facilitate clinical care and to address public health concerns.

Furthermore, several reports demonstrated that the increase in the incidence of erythromycin resistant *S. pyogenes* strains is related to increased macrolide treatment (Kürekçi *et al.,* 1996). Macrolide antibiotics, especially new ones such as clarithromycin and azithromycin, are widely selected for the treatment of upper respiratory tract infections such as sore throat. In a study from Ankara /Turkey , erythromycin resistant *S. pyogenes* strains were increased from 3.29% to 15.74% in a 7-year period (Kürekçi *et al.,* 1996). A substantial increase in

erythromycin resistance was associated with the increase in the consumption of macrolide antibiotics.

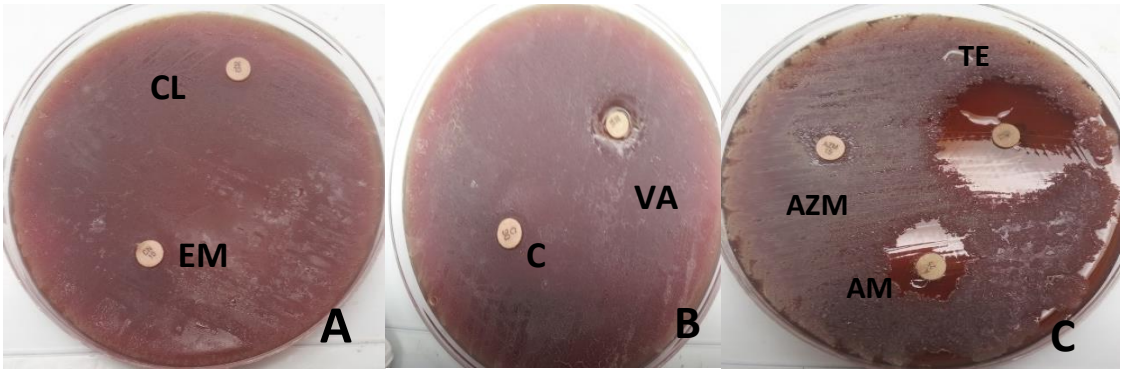


Figure. (3-2) antibiotic susceptibility test A) resistant to clindamycin and erythromycin B) resistant to chloramphenicol and sensitive to vancomycin C) sensitive to tetracycline and amoxicillin resistant to azithromycin.

*** (AX) amoxicillin, (AZM) azithromycin, (CL) clindamycin, (TE) tetracycline, (VA) vancomycin, (E) erythromycin, (P) penicillin , (C) chloramphenicol.**

3.7Antimicrobial activity of filtrates of *Lb. acidophilus*, *Streptococcus salivarius* and *Saccharomyces boulardii* against *S. pyogenes*:

3.7.1 Inhibitory effect of *Lb. acidophilus* against *S. pyogenes*:

As a probiotic microorganism, results revealed that *Lb. acidophilius* excreted inhibitory activity against three (S16, S20 and S28) of *S. pyogenes*, while had no effect against the fourth isolate S30 (Table 3-5). The most effective inhibitory activity was achieved by the three-fold concentrated and unconcentrated filtrates against isolate S20 with the highest recorded inhibition zones of 22 and 20 mm, respectively (Fig.3-3).

Against all *S. pyogenes* affected isolates, the three-fold concentrated filtrate exhibited more effective inhibitory activity than the unconcentrated filtrate, especially against isolate S28 when the inhibition zone reached 20 mm comparing to 15 mm for the unconcentrated filtrate.

These results agreed with Jacobsen *et al.* (1999) who found that the antagonist activity of lactic acid bacteria might be referred to its ability in producing organic acids (which lowers the pH) and bacteriocins, in addition to competition on the nutrients with the pathogenic bacteria.

Jin *et al.* (1996) found that a strain of *Lactobacillus* isolated from chickens was able to inhibit the growth of *Streptococcus*, *Salmonella* spp. and *E. coli*.

Result obtained by Barefoot and Klanhammer (1983) ensured that death of the tested bacteria was increased by increasing the concentration of *Lb. acidophilus* filtrate due to increasing the concentration of the inhibitory compounds especially the bacteriocins.

Table 3-9: Inhibitory effect concentrated and unconcentrated filtrates of *Lb. acidophilus* against *S. pyogenes.*

Streptococcus pyogenss **isolate**	**Inhibition zone (mm)***	
	unconcentrated filtrates	**concentrated filtrates**
S16	16	18
S20	20	22
S28	15	20
S30	0	0

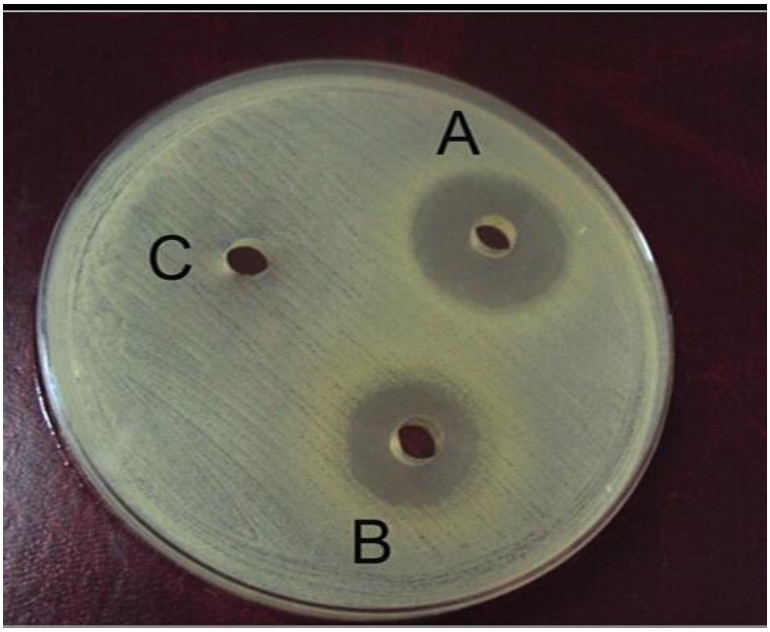


Figure. (3-3) Inhibition zones of (A) concentrated and (B) unconcentrated filtrates of *Lb. acidophilus* against *S. pyogenes* S20 isolate (C) control MRS broth.

Mishra and Lambert (1996) founded that the inhibitory effect of *Lb. acidophilus* is resulting from killing action of the bacitracins as they bind with the cytoplasmic membrane, which affects its permeability, and cause death of the sensitive cell.

Acetic acid and lactic acid are mainly produced by lactic acid bacteria; they affect the cytoplasmic membrane and diffuse in the cytoplasm quickly and cause bacterial death (Adams and Nicolaides, 1997; Ogawa *et al.*, 2001).

3.7.2 Inhibitory effect of *S. salivarius* against *S. pyogenes*:

In this study, *S. salivarius* was also used as a probiotic against *S. pyogenes*. Results in table (3-6) show that the probiotic bacteria exhibited effective inhibitory activity against all four isolates of the pathogenic bacteria by using the deferred antagonism test (Fig. 3-4). Highest effect was reported against isolate S20 when the antagonism inhibitory zone reached 5.3 mm, while the lowest (3 mm) was against S30 isolate.

Table (3-10) Inhibitory effect of *S. salivarius* against *S. pyogenes* by deferred antagonism test after incubation for24 hrs at 37 °C.

***S. pyogenes* isolate**	**Inhibition zone (mm)***
S16	4 mm
S20	5.3 mm
S28	5 mm
S30	3 mm

***the antagonism inhibition zone was measured (in mm) from the distance between the original producer line (*S. salivarius*) and the inhibition line of *S. pyogenes*.**

Tagg (2004) in this regard, reported that *S. salivarius* is one of the predominant commensal bacteria of oral cavity which known to produce bacteriocins and bacteriocin-like inhibitory substance, making the *S. salivarius* strains, in his study promising candidates for development of oral probiotics against oral infection disease .

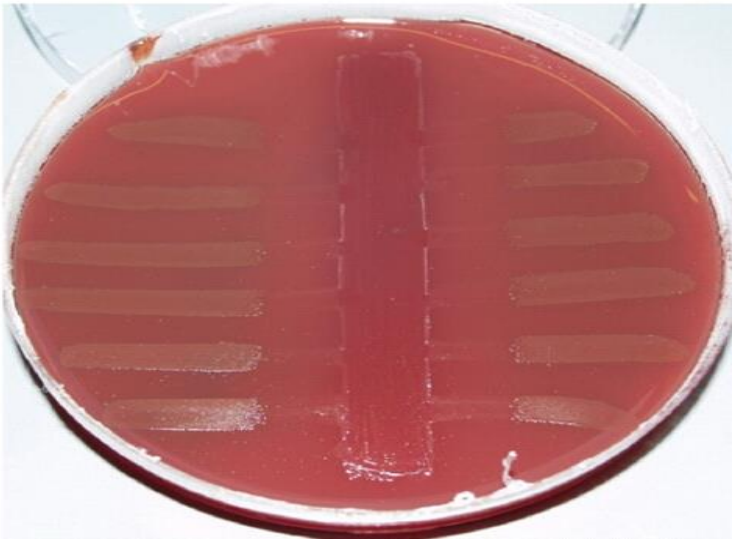


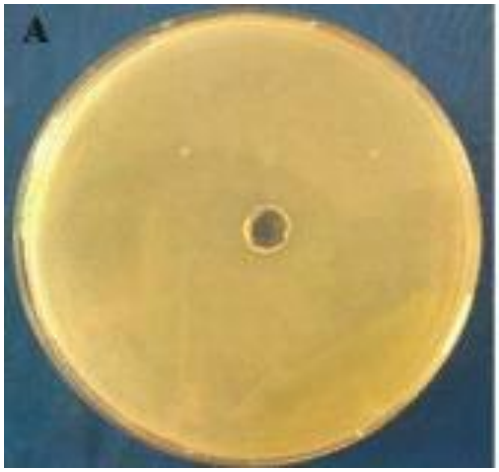
Figure (3-4) Inhibitory effect of *S. salivarius* against *S.* pyogenes by using the antagonism test procedure.

It has already been shown that *S. salivarius* can antagonises action of the main etiological agent of bacterial pharyngitis in children, *S, pyogenes.* Indeed, lozenges containing *S. Salivarius* are sold in some countries as an oral probiotics to maintain throat health as reported by Wescombe *et al*. (2011).

Moreover, Reid *et al*. (2003) found that the use of *S. salivarius* as probiotics in clinical practice has been tested by using the deferred antagonism test. Burton *et al.* (2006) reported strong inhibition by *S. salivarius* against Gram positive bacteria.

3.7.3 Inhibitory effect of *Saccharomyces boulardii*:

Results showed that *S. boulardii* isolates had no inhibitory effect on the pathogenic bacteria *S. pyogenes* as in figure (3-5). Despite that Buts and De Keyser (2006) mentioned this probiotics yeast as possessing beneficial properties, improving the gut immune response and intestinal barrier, no such beneficial property was detected by *S. boulardii* in the recent study from the standpoint of inhibitory activity. However, Maioli *et al.* (2014) reported that only few studies have investigated the effect of this probiotics yeast on pharyngitis and mucositis, and in some cases, the results are contradictory.



Figure(3-5) Antimicrobial activity of *Saccharomyces boulardii* filtrate against *Streptococcus pyogene*s

3.8 Streptokinase production by *S. pyogenes*:

By using two different methods Radial caseinolytic assay and Casein digestion method, it was found that all four isolates of *S. pyogenes*, were able to produce streptokinase enzyme by creating clear zones around the wells in skim milk agar plate. As shown in fig.(3-6).

Fig. (3-7) shows that isolate S20 gave the highest optical density (relative activity) of streptokinase among the four isolates of *S. pyogenes*, when the O.D. reached 1.14.

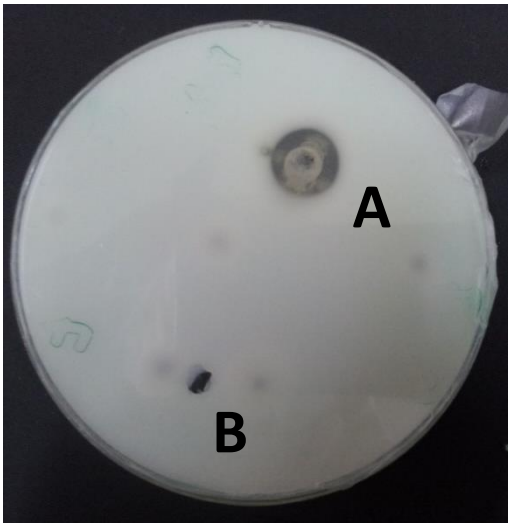


Figure. (3-6) Biological activity of streptokinase estimated by the Radial Caseinolysis assay. A) supernatant of *S. pyogenes* (S16) broth containing streptokinase B) control (brain heart broth).

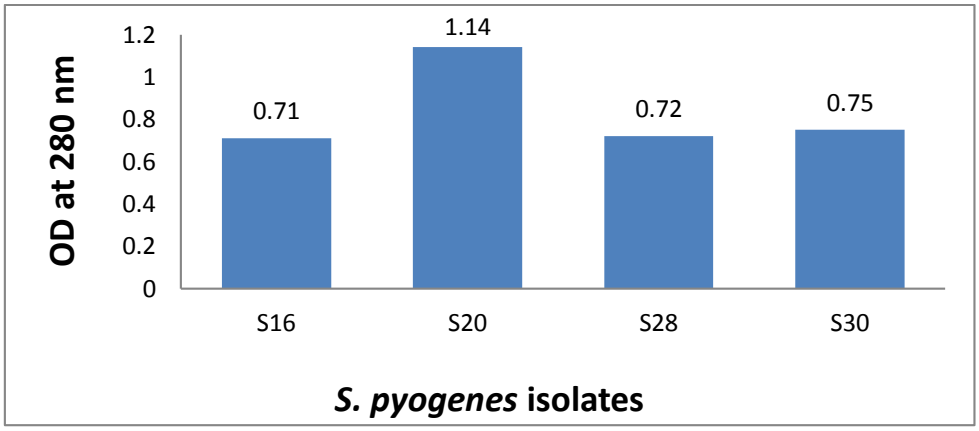


Figure. (3-7) Relative activity of Streptokinase produced by the four isolates of *S. pyogenes* estimated by Casein digestion method

This result was in agree with that of Vandamme *et al.* (1996) who reported that most of the streptokinase are obtained from β-haemolytic streptococci of human and animal origin which belong to the Lancefield groups C, G and *S. pyogenes*.

Streptokinase assay with radial caseinolytic activity is used mainly for the qualitative determination of the enzyme which depends on direct measurement of the area of the transparent lysis zone in a skim milk agar (Saksela, 1981)

The second enzyme method was assayed upon growing bacterial strains on different media by the common casein digestion method (Muller *et al.*, 1989), the absorbance at 280 nm for the supernatant was measured and converted to the amount of tyrosine equivalent.

3.9 Production of hyaluronidase by *S. pyogenes*:

Results showed that all isolates of *S. pyogenes*, except S30, were able to produce hyaluronidase enzyme by using both plate method and turbidity reduction assay.

In the first method (plate method), wells were made and filled with the supernatant of overnight *S. pyogenes* isloates, and upon addition of acetic acid on the plated surface the hydrolyzed zones around of the wells were appeared (Smith and Willett , 1968; King *et al.*, 2004). As shown in Table (3-7), highest diameter zone (12 mm) was produced by isolate S16, and upon this finding, it was used for the qualitative determination of the enzyme.

The principle of using turbidity reduction assay for detection of hyaluronidase depended on the reduction in turbidity of the broth medium when inoculated with hyaluronidase producing *S. pyogenes* isolates. After 30 min of incubation, the broth appeared clear, which meaning a positive result, while the non-hyaluronidase producing isolate broth remained turbid broth (negative result) (Sahooa *et al.*, 2009)

Table(3-11) Screening of *S. pyogenes* isolates for production of hyaluronidase.

***S. pyogenes* isolate**	**ΔT* (A600 nm)**	**Hydrolyzed zone (mm) ****
S16	0.27	12 mm
S20	0.28	7 mm
S28	0.28	10 mm
S30	0.074	-

ΔT*-Reduction in turbidity of broth cultures after 30 min incubation at 37 °C (A600nm) (Uninoculated culture broth as blank) - Increase in turbidity (A600 nm) after 30 min. at 37 °C.(Sahooa, et al., 2009)

**** Diameter was calculated after subtracting the diameter of the well (5 mm).**

Upon addition of the acid, tubes containing sterile broth or broth from inactive cultures became turbid, while those containing broth from hyaluronidase producing organism remained clear. The isolates exhibiting reduction in turbidity as given in Table (3-7).

3.10 Effect of probiotics and antibiotics on streptokinase activity:

When the crude enzyme was incubated at room temperature for 10 min with the crude extraction of bacteriocin from *S. salivarius* and with the probiotics (concentrated filtrates of *Lb. acidophilus*, and *S. boulardii*), enzyme activity was completely inhibited by the bacteriocin while partial inhibited by *S. boulardii* as shown in Fig (3-8).

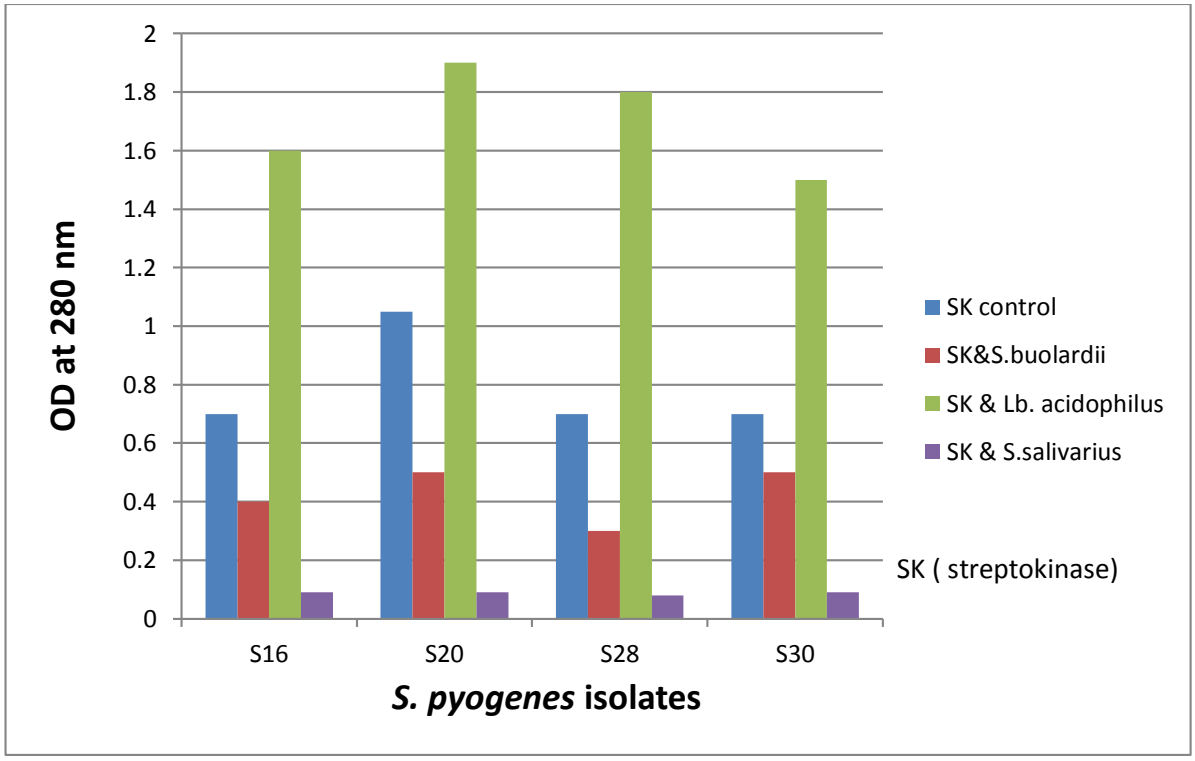


Figure (3-8) Effect of probiotic microorganisms on streptokinase activity produced by *S. pyogenes*.

Adversely, streptokinase activity was not affected by the filtrate of *Lb. acidophilus*. This may be related to the fact that this probiotic bacteria and Lb. acidophilus produced protease has also activity on casein the substrate of streptokinase, as shown by Banina *et al.*, (1998) who found that *Lb.acidophilus* was able to completely hydrolysed casein fractions regardless to the source of cells whether they were originally grown in MRS broth or on MRS agar plates.

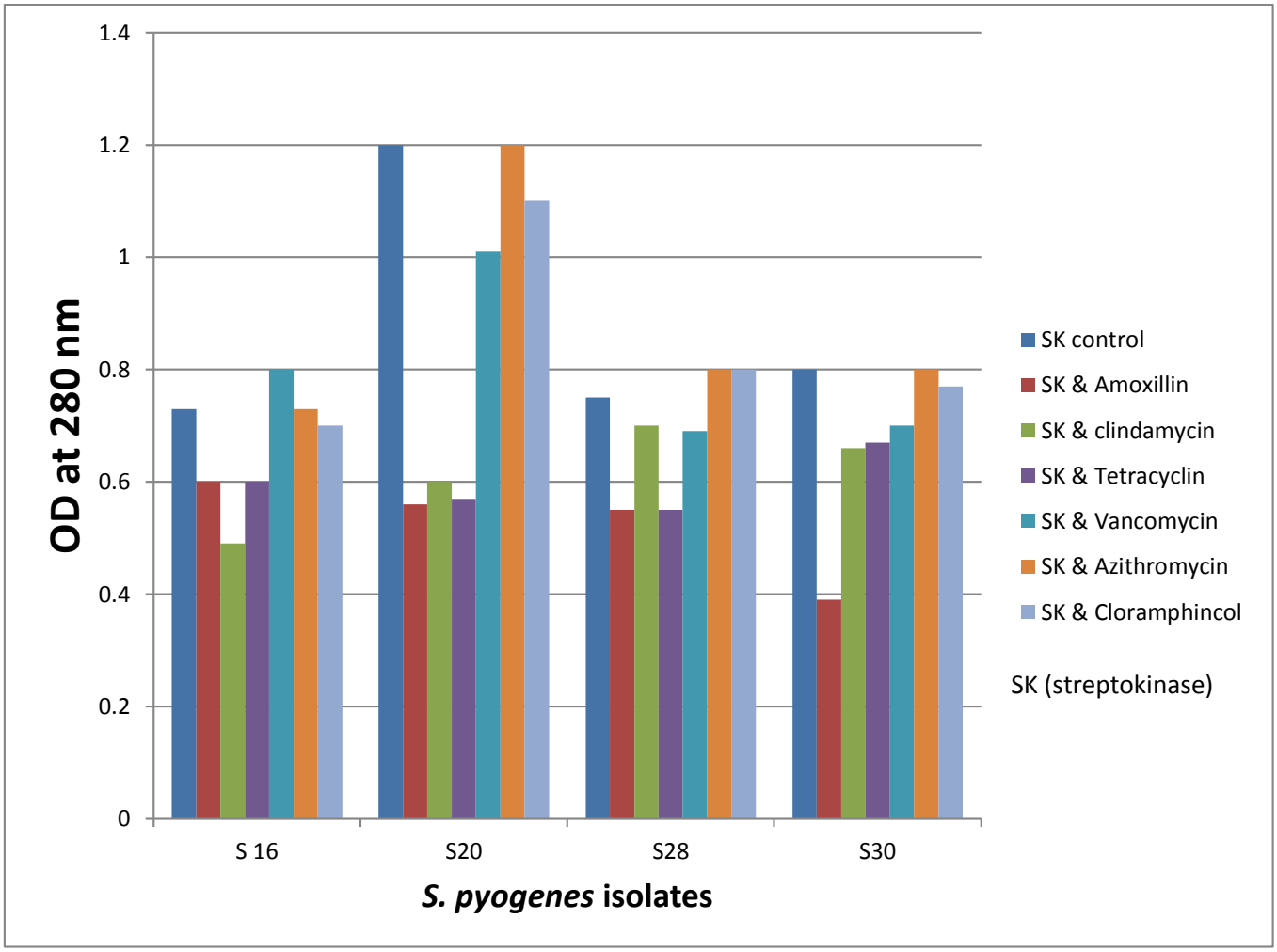


Figure (3-9) Effect of antibiotics on streptokinase activity produced by *S. pyogenes*.

Three of the antibiotics (Amoxillin, Tetracyclin and Cephalexin) from those used in this study caused partial inhibition in the activity of streptokinase, while no any other antibiotics did so as shown in fig (3-9) .

Carapetis *et al.* (2005) reported that severe infections including (bacteraemia, necrotizing fasciitis and streptococcal toxic shock syndrome) is caused by *S. pyogenes.* Estimates suggested that there are over 660,000 cases of invasive *S. pyogenes* infections per year which results in over 160,000 deaths world wide.

Stevens *et al.* (2005) found that the recommended treatment to reduce *S. pyogenes* production of pyrogenic exotoxins and superantigens consists of surgical debridement, fluid replacement/blood pressure support and high-dose intravenous antibiotic therapy (commonly penicillin combined with clindamycin).

Despite that Sriskandan *et al.* (2006) pointed out that using polyclonal human intravenous immunoglobulin (IVIG) for the treatment of invasive infections has been demonstrated in numerous *in vitro* studies, Shah *et al.* (2009) found the use of IVIG as an adjunctive treatment in a clinical setting has been less promising. Therefore, there is need to develop additional therapeutics that may complement current treatments and can restrict the systemic spread of *S. pyogenes* during the early stages of infection.

Streptokinase in the cell surface of *S. pyogenes,* which is critical for invasive disease initiation, was considered to be as the spreading factor for pathogen, therefore, the possibility exists to target the bacterial factors involved in this process as a mean to prevent systemic infection (McArthur *et al.*,2012).

One possible strategy would be to inhibit streptokinase mediated plasminogen activation without affecting physiological plasminogen activation mechanisms. This could be achieved by designing specific inhibitors that prevent the initial interaction between streptokinase and plasminogen also prevent the streptokinase mediated conformational rearrangement of plasminogen that exposes the active site or prevent the plasminogen-streptokinase activator complex from binding substrate plasminogen (McArthur *et al.*,2012).

In the recent human research, studies have looked at the role of probiotics in augmenting the use of antibiotics in the management of various infections (Reid, 2006).

Resta-Lenert *et al.* (2003) reported that the mechanisms of action have been studied, and one of the mode of action explanation referred that to the ability of probiotic bacteria to interfering with the invasion and adhesion of the pathogens. While other referred that to the ability of probiotic bacteria to protect the gut epithelium from further invasion to stop the bacteria infecting cells already exposed. Benno *et al.* (1996) declared that probiotics have beneficial effects on intestinal immunity by increasing the numbers of cells that secrete immunoglobulins in the intestinal mucosa and stimulating the local release of interferon.

3.10 Effect of probiotics and antibiotics on hyaluronidase activity:

When the crude hyaluronidase enzyme was incubated at room temperature for 10 min with the crude extraction of bacteriocin from *S. salivarius* and with the probiotics (concentrated filtrates of *Lb. acidophilus,* and *S. boulardii*), enzyme activity was completely inhibited by the bacteriocin while partial inhibited by *S. boulardii* and *Lb. acidophilus* as shown in Fig (3-10).

Generally, all antibiotics used in this study had not effect on the hyaluronidase activity as shown in Fig (3-11). However, the enzyme still kept working on the substrate and the reaction mixture became less turbid because of the action of enzyme in hyaluronic acid degradation.

The key role of the hyaluronidase had been recognized in a number of physiological and pathological processes such as embryogenesis, angiogenesis, inflammation, wound healing, bacterial pathogensis. Therefore identification

and characterization of hyaluronidase inhibitors would be valuable for developing contraceptives, antitumor agents, and antimicrobial agents (Isoyama *et al.*, 2006).

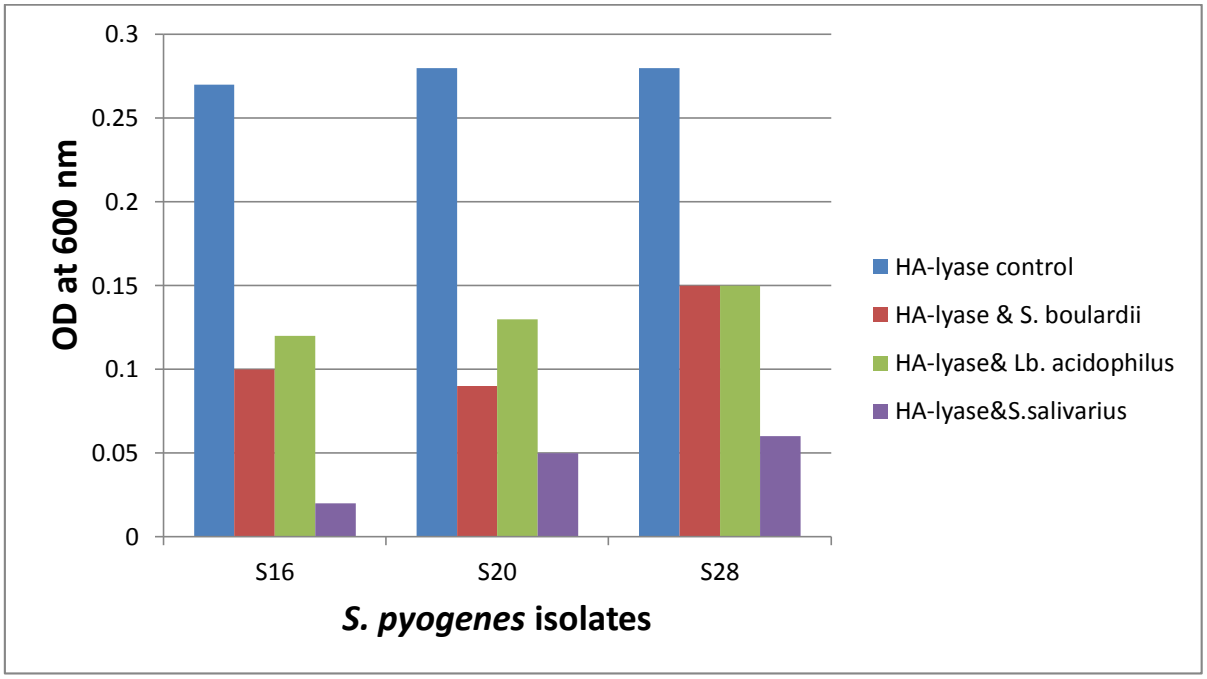


Figure (3-10): Effect of probiotic microorganisms on hyaluronidase activity produced by *S. pyogenes*.

Mio and Stern in (2002) reported that certain anti-inflammatory drugs, including salycylates, indomethacin and dexamethasone, also exert anti-hyaluronidase activity. These drugs may prevent HA degradation. In these experiments tested, the activity of probiotics to inhibition of hyaluronidase activity by hyaluronidases, though the degradation products of HA are potent inducers of inflammatory cytokines.

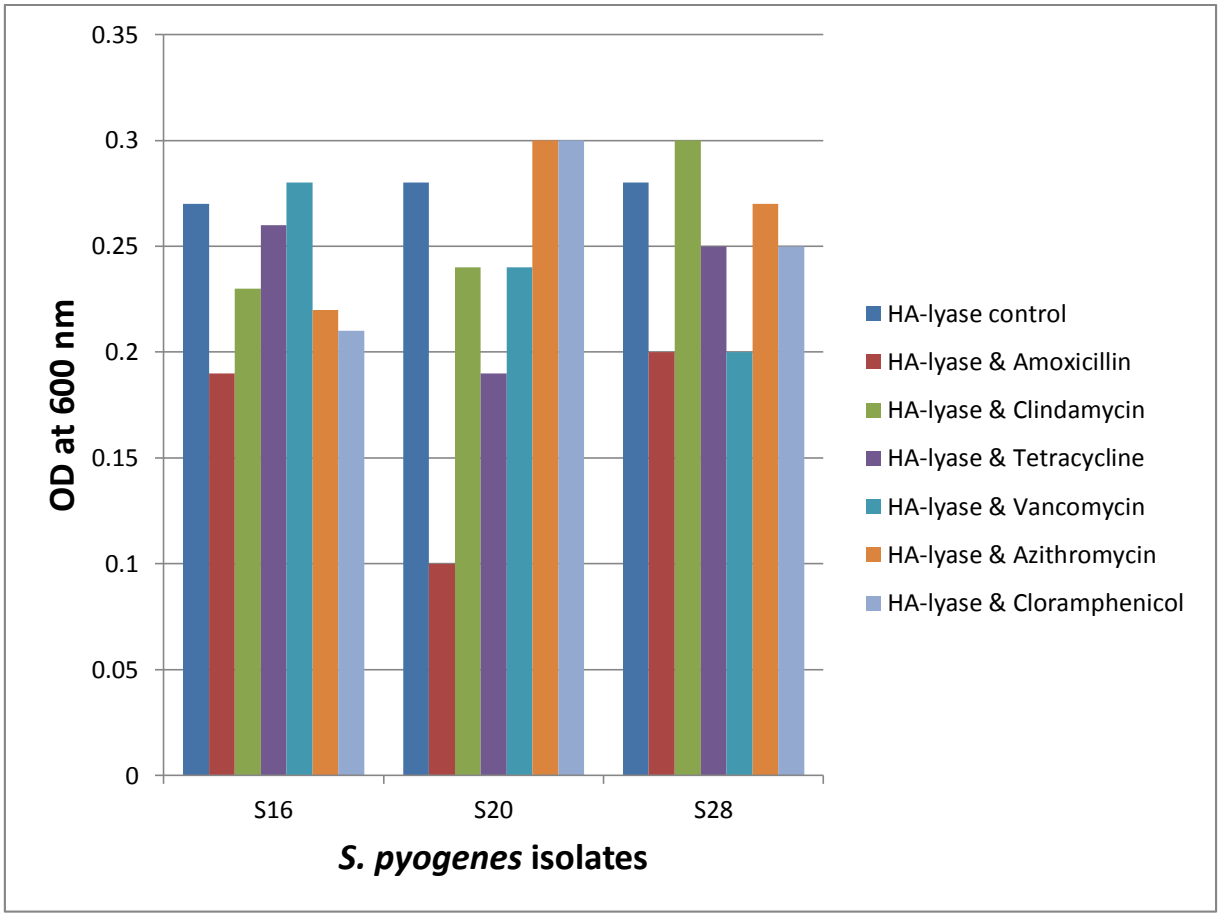


Figure (3-11): Effect of antibiotics on hyaluronidase activity produced by *S. pyogenes*.

4. Conclusions and Recommendations

4.1 Conclusions:

- Isolates of *Streptococcus pyogenes* obtained from children infected with pharyngitis had the ability to produce streptokinase and hyaluronidase.
- As probiotics, *Lactobacillus acidophilus* and *Streptococcus salivarius* exhibited inhibitory effect against *Streptococcus pyogenes*, while *Saccharomyces boulardii* did not do so.
- Despite that all the three probiotic microorganisms were able to inhibit hyaluronidase activity, only two of them (*S. salivarius* and *S. boulardii*) inhibited activity of the streptokinase.
- When isolates of *S. pyogenes* were subjected to treatment with six of the commonly used antibiotics, none of the antibiotics was able to show efficient inhibitory effect on activity of both enzymes as the probiotics did.

4.2 Recommendations:

- Investigating the effects of probiotics against *Streptococcus pyogenes* and other pathogenic bacteria *in vivo*.
- Immunologically and histopathological study on the effect of probiotics on experimental animals after ingestion *S. pyogenes* is needed;
- An attempt to develop more therapeutic and prophylactic commercial medicines from efficient species of *Lactobacillus*, *Streptococcus* and *Saccharomyces* against *S. pyogenes* is recommended.

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Appendix : VITEK 2

The VITEK 2 is an automated microbiology system utilizing growth-based technology. The system is available in three formats (VITEK 2 compact, VITEK 2, and VITEK 2 XL) that differ in increasing levels of capacity and automation. All three systems accommodate the same colorimetric reagent cards that are incubated and interpreted automatically

- **Reagent Cards**

The reagent cards have 64 wells that each can contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation (described below). Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system.

There are currently four reagent cards available for the identification of different organism classes as follows:

GN: Gram-negative fermenting and non-fermenting bacilli.

GP: Gram-positive cocci and non-spore-forming bacilli.

YST: yeasts and yeast-like organisms.

BCL: Gram-positive spore-forming bacilli.

- **Suspension preparation:**

Table (2-1): Suspension turbidities used in the VITEK 2reagent cards inoculation.

Product	McFarland turbidity reagent
GP	0.50-0.63
GN	0.50-0.63
YST	1.80-2.20
BCL	1.80-2.20

A sterile swab was used to transfer a sufficient number of colonies of a pure culture and to suspend them into 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to pH 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted accordingly (as in table 1) and measured using a turbidity meter called the DensiChekTM.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

دراسة تأثير المعززات الحيوية على فعالية النسبيه لانزيمي الهيلوروندیز والستربيتوكاينيز المنتجة من عزلات محلية لبكتريا المكورات المسبحيه القيحية

رسالة

مقدمه الى مجلس كلية العلوم / جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير في علوم التقانة الاحيائية

من قبل

ياسمين ثائر احمد

بكالوريوس 2012

اشراف

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الملخص

هدفت هذة الدراسة للتحري عن التاثير التثبيطي لبعض الاحياء المجهرية العلاجية ضد بكتريا *Streptococcus pyogenes*. تم لهذه الغرض جمع مسحات بلعوم من 85 من الأطفال المصابين بالتهاب البلعوم. وامكن الحصول على اربع عزلات من هذه البكتريا, حيث تقويم نشاط انزيمي الستربتوكاينيز و الالهيلويورنيز اللذين تنتجهما كعوامل انتشار لأحداث الأصابة .

اوضحت النتائج أن الراشح المركز لبكتريا *Lactobacillius acidophilius* (كائن مجهري علاجي) والذي ,تم الحصول عليه من خلال تركيز الراشح الناتج من تنميتها في وسط مرق ال MRS , أظهر فعالية نثبيطية ضد ثلاث (S16 , S20, S28) من عزلات *S. pyogenes* ألأربع, فيما لم تتأثر العزلة الرابعة S30 به.

لدى استخدام *Streptococcus salivarius* كائن مجهري علاجي ايضا، اظهرت النتائج ان راشحها قد امتاز بفعاليته التثبيطية ضد كل العزلات الأربعة من بكتريا *S. pyogenes* وذلك عند استخدام الطريقة المعروفة بأسم Deferred. antagonism test

وعلى العكس من ذلك, فلم يكن لراشح خميرة *Sacchromycse boulardii* المركز أي تأثير كابح للنمو ضد أي من العزلات الأربع لبكتريا *S. pyogenes* المرضية التي استخدمت في الدراسة.

عند اختبار نشاط انزيمي streptokinase و hyaluronidase اللذان تنتجهما بكتريا *S. pyogenes*, أظهرت النتائج امتلاك جميع عزلاتها الأربع القدرة على إنتاج هذين الأنزيمين بالطريقتين المعروفتين بأسمي streptokinase the casinolytic assay و radial caseinolysis assay.

وفيما يتعلق بأنزيم hyaluronidase , فقد أظهرت النتائج أن ثلاثة فقط (S16، S20 S28) من بين عزلات *S. pyogenes* الأربع كانت قادرة على انتاج الانزيم عند الكشف عنه بطريقتي plate assay و turbidity reduction assay ، في حين لم تكن العزلة S30 قادر على انتاج هذا الأنزيم .

بعد إخضاع مستخلصي انزيمي (streptokinase و hyaluronidase) الخام المنتجة من *S. pyogenes* للمعاملة مع كل من المرشح المركز لبكتريا *Lb. acidophilus* ، ومستخلص ال bacteriocin الخام من *S. salivarius* و راشح *S. boulardii* المركز وستة من المضادات الحيوية (erythromycin, azithromycin, chloramphenicol, clindamycin, amoxicillin, tetracycline, and vancomycin) ، ويمكن تلخيص النتائج التي تم الحصول بالأتي:

• تم تثبيط نشاط streptokinase تماما باستخدام مستخلص bacteriocin بينما كان التثبيط جزئيا من قبل *S. boulardii*.

• لم يتأثر نشاط انزيم streptokinase بالراشح المركز لبكتريا *Lb. acidophilus* .

•أظهرت مضادات amoxillin و tetracycline و cephalexin من بين تلك المضادات الحيوية الست المستخدمة في هذه الدراسة تأثير تثبيط يجزئي في نشاط انزيم streptokinaseا .

• تم تثبيط نشاط hyaluronidase تماما من قبل ال bacteriocins المنتجه من بكتريا

S. salivarius.

• تم تثبيط hyaluronidase جزئيا عن طريق معاملته مع كل من الراشحبن المركزين ل *S. boulardii* و *Lb. acidophilus*.

• لم يكن لأي من المضادات الحيوية الستة المستخدمة في هذه الدراسة تأثير على نشاط انزيم hyaluronidase.