Abstract

- This work was aimed to study the effect of some probiotic microorganisms on fungi causing diarrhea. Therefore a total of (45) stool sample were collected from children(2 – 10)years old with diarrhea. Results observed that 15(33.3%) isolates belong to genus *Candida*, and 4(8.8%) of isolates belong to *Candida albicans* according to the related biochemical tests.
- Another part of the study included isolation of *Lactobacillus* (LAB) isolates for using as probiotics to test there ability to inhibit growth and forming germ tube by *Candida albicans*. Seven isolates of LAB were obtained from (45) stool samples after culturing on (MRS) medium for 24 hr at $37C^0$.
- The minimum inhibition concentration of nystatin to the isolates of *Candida albicans* was 200 µg/ml liquid media and ($16*10^4$), While for the original sample ($133*10^4$) when cultured on SDA media.
- Upon such results, one isolate (*C*.2) of *Candida albicans* was chosen due to its ability to form high number of germ tube compared with other isolates, and secondly, three isolates of LAB: *Lactobacillus acidophilus* (*Lb*.1), *Lactobacillus plantarum* (*Lb*.5) and *Lactobacillus fermentum* (*Lb*.7) due to high number of colonies they formed when grown on MRS agar. No inhibitory activity was detected through the antagonism with *Candida albicans* when the novel methods "*Candida albicans* germ tube assay" was used.
- Minimum inhibition concentration was determined for the three – fold concentrated filtrate of LAB against germ tube formation by *Candida albicans* (*C*.2) isolate. Results showed that filtrates were able to decrease the extension of germ tube when compared with control. The average decreases were (1 – 5)%, (7 – 10)% and (20 – 30)% instead of 70% for the control growth.

الخلاصة

- هدفت الدراسة للتحري عن تأثير بعض النبيت المايكروبي المعوي على الفطريات المسببة للاسهال. لذا فقد جمعت (٤٥) عينة خروج من أطفال عمرهم بين (٢ – ١٠) سنة مصابين بالاسهال. اظهرت النتائج أن (٣٣,٣%) ١٥ عزلة كانت تابعة لجنس *Candida* و (٨,٨%) ٤ عزلة تكون من نوع *Candida albicans* و ذلك اعتمادا على فحوصات كيموحيوية ذات العلاقة.
- و تضمن الجزء التالي من الدراسة عزل عصيات اللاكتيك *Lactobacillus* و التي تستخدم كنبيت معوي طبيعي لاختبار قدرتها على تثبيط نمو و تكوين الانبوب الجرثومي من قبل المبيضات الفطرية *Candida albicans* . و كانت (٧) عزلات من عصيات حامض اللكتيك قد جمعت من(٤٥) عينة الخروج بعد زرعها وسط اكار(MRS) المحور و حضنها لمدة ٢٤ ساعة بدرجة حرارة ٣٧ ° م.
- كان اقل تركيز مثبط من النستاتين لعزلات خميرة *Candida albicans* هو ٢٠٠ مكغم/مل في الوسط السائل، و هي تمثل ($16*10^4$) بينما يمثل الزرع الاصلي ($133*10^4$) على الوسط الصلب.
- اعتمادا على ما ورد انفا من نتائج، فقد اختيرت احدى عزلات خميرة *Candida albicans* و هي (C.2) و ذلك لقابليتها على تكوين اعداد كبيرة من الانابيب الجرثومية مقارنة مع العزلات الاخرى ، كما و اختيرت ثلاث عزلات من بكتريا حامض اللكتيك العصوية و هي:

Lactobacillus acidophilus (*Lb*.1), *Lactobacillus plantarum* (*Lb*.5) and *Lactobacillus fermentum* (*Lb*.7) اعتمادا على اعطائها اعلى عدد مستعمرات لدى تنميتها على وسط اكار (MRS). لا تظهر اي فالية تثبيطية اثناء اجراء عملية العداء ضد خميرة *Candida albicans* و انما استخدمت طريقة حديثة تسمى:

" *Candida albicans* germ tube "

- اجري اختبار التركير المثبط الادنى لرواشح مزروع بكتريا حامض اللكتيك المركز لثلاث مرات على الانبوب الجرثومي المكون من خميرة *Candida albicans* . اظهرت النتائج قدرة الراشح على تقليل طول الانبوب الجرثومي عندما قورن مع طول الأنبوب قبل المعاملة. ان معدل النقصان في العدد يترواح بين %(5 – 1) %(10 – 7) و %(30 – 20) بدلا من (٧٠ %) للنمو الاصلي.

Conclusions

1. *Candida albicans* isolates were different in their ability to form germ tube depending on different signals and signaling pathways.
2. The effect of LAB directly on *Candida albicans*, no inhibition zone was formed neither in disk agar diffusion nor well diffusion methods. No effects of live LAB on *Candida albicans* morphogenesis.
3. *Lactobacillus* filtrates had considerable effects against the isolates of *Candida albicans* isolates.
4. Three – fold concentrated filtrates (12.5%) of *Lactobacillus acidophilus* isolates gave the highest inhibitory effect on the growth and production of germ tube of *Candida albicans* in comparison to *Lactobacillus plantarum* and *Lactobacillus fermentum* isolates.

Recommendations

Further studies are needs to: -

1. Extract, purify and identify of inhibitory substances produced by LAB isolates as probiotics.
2. Investigating the effect of LAB inhibitory substances against production of Secreted aspartyl proteinase (Sap) of *Candida albicans*.
3. Effect of LAB isolates against *Candida albicans* (In vivo).
4. The inhibitory effect on other fungi – causing diarrhea and there products.

1.1: Introduction

While most related references define diarrhea as an increase in the normal frequency of bowel movement with the passage of abdominally soft or watery faces. Emil *et. al.*, (2005) reported that diarrhea in cases is caused by direct infections of gastrointestinal tract, and also by systemic infections or infections that affect other organ systems. Several microorganisms are accused to cause diarrhea, including bacteria, fungi, virus and others. So many researchers declared the association of fungal overgrowth with diarrhoea. Adherence of fungi to host surfaces was an initial and important event in colonization and subsequent disease production for fungal as well as other enteropathogens. Many reports have indicated in vitro adherence of *Candida albicans* to epithelial cells of oral as well as intestinal origin. (Hasmukh *et. al.*, 1994). *Candida albicans* is an opportunist fungal pathogen that had the ability to adhere to host cell surface receptors via a number of adhesions. (Forbes *et. al.*, 2001). Stated that *Candida* species were frequently isolated from stools of children with diarrhoea but were not proven enteropathogens. It was hypothesized that faecal *Candida* causes diarrhoea.

Gastrointestinal microflora maintain a microbial barrier against the development of pathogenic bacteria in the digestive tract (Martine, 2000). Probiotics are nonpathogenic microorganisms that have positive influence on the health or physiology of the host. They can influence intestinal physiology either directly or indirectly through modulation of the endogenous ecosystem or immune system. They consist of either yeast or bacteria, especially lactic acid bacteria (Philippe *et. al*., 2001).

Lactic acid bacteria (LAB)such as *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus acidophilus* are the most common *Lactobacillus* species

isolated from the normal human intestine and used as probiotics. LAB may serve as probiotics by their ability to survive and grow in the gastrointestinal tract. Many theraputic and preventive effects of yogurt and lactic acid bacteria (which are commonly used in yoghurt production) were detected on various diseases such as cancer, infection, gastrointestinal disorders, and asthma (Simin and Woel–Kyu, 2000). (Tadao, 2004) referred such effects to the resistance of this bacteria to lysozyme in mouth, acidic conditions in stomach, and bile acids in intestines

Several metabolites (such as organic acids, hydrogen peroxide, and bacteriocins) are responsible for the inhibiting effects of lactic acid bacteria, as probiotic, on pathogens.(John *et. al.*, 1997). Probiotics appear to be useful in the prevention of several or treatment gastrointestinal disorders, including diarrhea.

Despite that too many investigations had been performed to evaluate the ability of LAB metabolites to inhibit microbial causes of diarrhea, fungi, including molds and yeasts, were not given such consideration diarrhea causes. Morever, *Candida albicans* is known to be able to colonize the epithelium of the gastrointestinal tract invasion and subsequent hyphal systemic infection. (Mairi and Gary, 2004).

Due to the previous reason, this study was performed aiming to :-

1. Isolation and identification of fungal causative diarrhea.
2. Isolation and identification of some probiotics microorganism.
3. Testing the antifungal sensitivity of the isolated fungi.
4. Testing the effect of probiotics on isolated fungi (in-vitro).
5. Possibility of testing the effect of probiotics on fungi in experimental animal.
6. Any other experiments needed.

1.2: Literature Review:

1.2.1: Gastrointestinal Tract Infection (GIT): -

Gastrointestinal complications (constipation, impaction, bowel obstruction, diarrhea, and radiation enteritis) are common problems for oncology patients (Culhane, 1983). The human gastrointestinal tract (GIT) is colonized by a vast and diverse community of microbes that are essential to its functions. These microbes have evolved in concern with their host to occupy specific regions and niches in the GIT (Simon and Gorbach, 1986). Approximately 85% of the intestinal microflora in a healthy person is beneficial bacteria and 15% is pathogenic bacteria. Microbial interactions contribute to the homeostasis of the gut bacterial flora and destabilization of this microorganism which contribute to these ecosystem results in various gastrointestinal disorders (Emily, 2005).

Diarrhea can be defined as the excessive loss of fluid and electrolyte in stool. The basis for all diarrhea is disturbed intestinal solute transport. Diarrheal illness, most of it attributable to enteric infection may continues to kill children. About 10 – 15% of cases of childhood diarrhea caused by bacterial pathogens *Escherichia coli, Salmonella, Shigella,* and others (Rishard, 1987). *Candida albicans* has been shown to be a cause of diarrhea (Timothy and Birdsall; 1997). Up to 40% of children with presumed infectious diarrhea, no recognized pathogen can be identified. This may be due to failure to appreciate the significance of certain intestinal microorganisms, such as yeasts (Forbes *et al*., 2001).

Candida is one of microorganisms found naturally in gastrointestinal tract. (Truss, 1981; Crook, 1984). Infections caused by *Candida* spp. are in general referred to as candidiasis (or candidosis or moniliasis) (Bodey, 1966;

Bielsa *et al.,* 1987; Bodey *et al.,* 1992). Some symptoms of these candidiasis are bloating, gas, diarrhea, and abdominal cramping. Others may occur such as headaches, sinusitis, palpitations, and environmental allergies and food allergies. Polysystemic candidiasis infection, occurs when *Candida* present as a fungi in the intestines, the rhizomes penetrate the walls of the intestines, and the endotoxins from the *Candida* overgrowth begin to invade the rest of the body. (Hilton, 1992). Candidiasis is the most frequently encountered fungus infection especially in condition with depression of immune system. Administration of antibiotics for a long duration and use of immunosuppresion agents, and patient with HIV infection, leads to high incidence of candidiasis (Heelan *et. al*., 1998; Herrero *et. al.,* 1999) .

1.2.2: Etiology of gastrointestinal tract infection: -

Candida is a conditionally pathogenic fungus. It is, normally found on skin, in mouth, gut, and other mucus membranes. It causes infection when antibiotics or other factors reduce our natural resistance to it's overgrowth. Most *Candida* infections are superficial, limited to mucus membranes. Some of microorganisms may interact synergistically with *Candida albicans* to enhance the pathological effect of infection. This phenomenon has been documented for *Cytomegalovirus* and *Candida albicans*, *Pseudomonas aerugenosa* and *Candida albicans, Salmonella typhimurium* and *Candida albicans*, and *Streptococcus faecalis* and *Candida albicans*(Odds, 1988).

Other names that have been given to *Candida* infection include:- (Candida-related complex, polysystemic candidiasis, chronic candidiasis, candidiasis hypersensitivity syndrome, and moniliasis)(Crook,1992; Cater,1995; Crook, 1997).

1.2.3: *Candida:* -

1.2.3.1: History of *Candida:* -

Early indication of yeast in gut was by Fleisher and Wachowiak 1923. In 1929 Wachowiak *et. al.* , isolated *Candida* that passed into the bloodstream from the gut. The possibility of dissemination of *Candida* to the blood from gut seems to be forgotten until 1969 when Nishikawa reported that cortisone-pretreated were related to *Candida* death of disseminated candidosis. Considerable progress has been made in discovery of the kinds of attribute that contribute collectively to the pathogenecity of *Candida* spp. and the response of the host component to the fungus(Odds,1988).

1.2.3.2: Genus *Candida:* -

The genus *Candida* belongs to the kingdom Fungi, division Eumycota (true fungi), which relates to class Deuteromycetes(fungi imperfectial) and the family Saccharomycetaceae(budding yeast) containing different genera of yeast , the genus of *Candida* which is one of the most common yeast.(Hannula, 2000).

Candida is a simple diploid eukaryote organism lacking the sexual cycle. It’s cells take different shapes (cocci, ovoid, cylindrical or elongate), which can be staining by Gram's and also by lacto phenol blue staining. Ovoid yeast cell ranging from 3 to 5 μm in size (Lodder, 1974). *Candida*, which are commonly part of the normal flora of mouth, skin, intestinal tract, and vagina, it is a necessary yeast as part of the normal flora for human health (Barefoot and Klaenhammer; 1983). *Candida* is dimorphic yeast (Fig. 1 – 1) exhibits a number of different morphological forms under different environmental conditions; such forms include budding yeast cells (blastospores, blastoconidia), pseudohyphae (elongated cells appear as

filamentous cell chains), true hyphae, and chlamydospores. Growth requirement of *Candida* are 20-38^{0}C within the range of pH 2.5 to 7.5 (Odd, 1988). The colonies of *Candida* spp. are cream colored to yellowish, which may be pasty, smooth, glistening or dry, wrinkled and dull, depending on the species.(Larone,1995). Ideal factors enhancing filamentation *Candida* (yeast – hypha) are: 35^{0}C temperature, pH 7.0, an inoculum of 1*10^6 blastospores/ml inoculum's size in addition to presence of serum, N-acetylglucosamine, and proline (Gloria *et. al.*, 1998).

Abi-Said *et al*., (1997) proved that the genus *Candida* including about 154 species. Among them, six species are most frequently isolates from human infections. The most abundant one of these species is *Candida albicans.* Other species involves *Candida tropicalls, Candida glabrata, Candida parapsilosis, Candida krusie,* and *Candida lusitaniae* are also isolated as causative agents of *Candida* infections.

There are two groups of methods to distinguish *Candida* strains. The first are phenotyping methods, which can be divided into two methods:

a. Biotyping: - based on differences in metabolic properties of yeast isolates. Recently, widely use of commercial test (API 20c) system for assimilation of 19 different carbohydrates.

b. Serotyping: - based on detecting reactions between the antigen and the antibody raised against it. A commercial slide agglutination test uses monospecific antisera raised with the polysaccharide antigens extracted from *Candida albicans.*

The second are genotyping methods, which include different molecular methods for detection of *Candida albicans* like karyotyping, restriction endonuclease analysis (REA), and restricted fragment length polymorphism (RFLP). While, Arbitrarily Primed Polymers Chain Reaction (AP – PCR) is the more recent methods for detection of *Candida albicans* strains (Hannula, 2000).

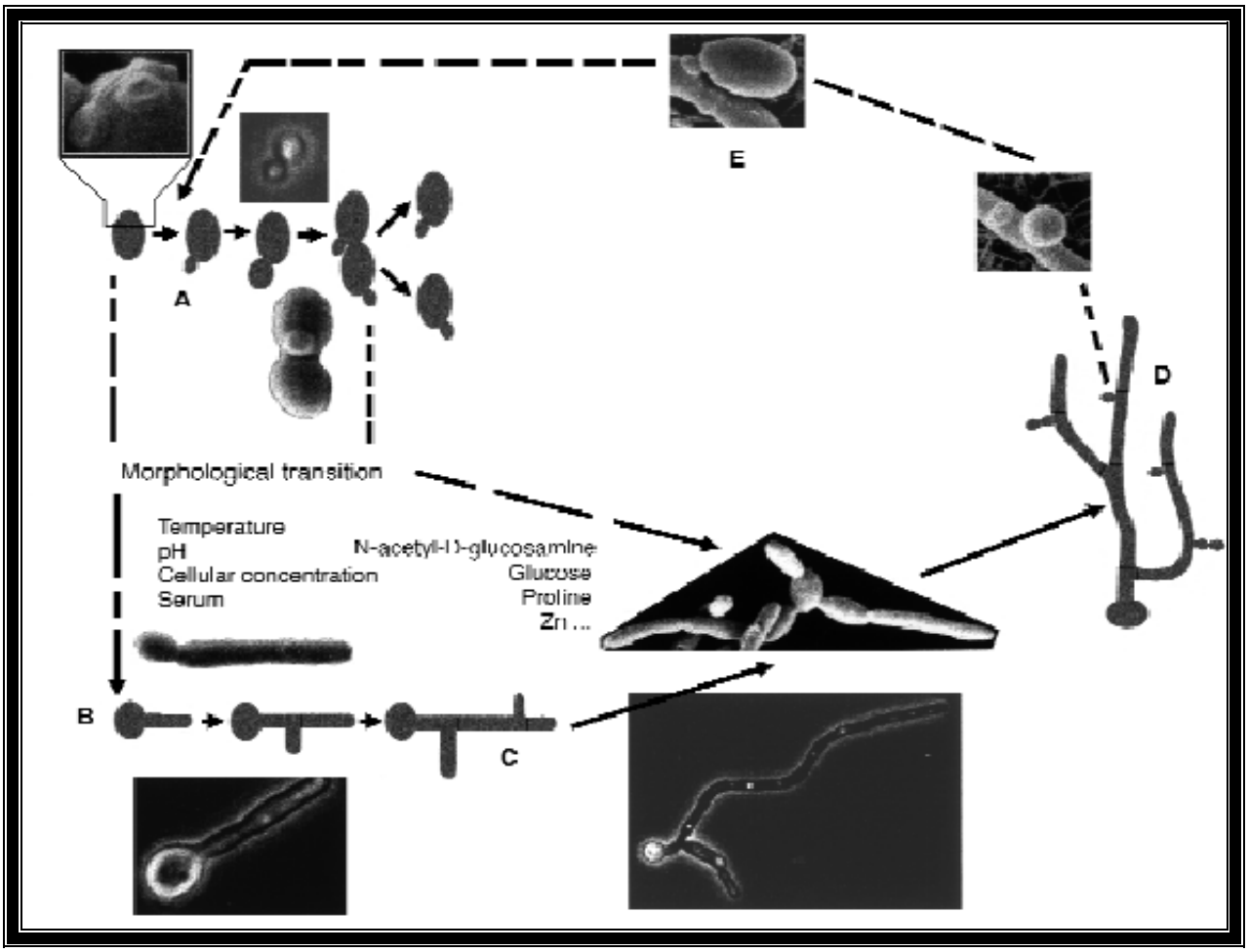

Figure (1 – 1): - *Candida albicans* dimorphism .

(A) Blastospores are unicellular forms of the fungus that divide by budding. (B) Cylindrical outgrowth is initiated on the surface of a blastospore forming a germ tube. (C) Germ tubes grow and septa are laid down behind the extending apical tip to form hyphae. (D) Hyphal branches and/or secondary branches are produced a mycelium. (E) Secondary blastospores become separated from the filament. (Gloria *et. al*., 1998).

1.2.3.3: Virulence factor of *Candida albicans:* -

Multiple factors have been implicated in the enhancement of *Candida albicans* pathogenicity; these include: -

1.2.3.3.1: Phospholipase production:-

Phospholipase D (PLD) activity is the first enzyme described in *Candida albicans* (CaPLD1). This enzyme was stimulated by inducers of dimorphic transition and an important regulatory to it's (Nealoo and Joseph, 1997). Activity of the enzyme is stimulated upon receptor ligation by agonists, resulting in of modification various lipid constituents of the membrane, degradation or

phosphorylation (membrane – damaging enzyme), and generation of one or more products that are able to recruit or modulate specific target proteins(Mordechai *et al*.,2000). Bernhard *et al*., (2001) found that these enzymes were responsible or essential for sporulation of *Candida albicans*.

1.2.3.3.2: Expression of drug resistance genes:-

Antifungal drug resistance is quickly becoming a major problem in the expanding population of immunocompromised persons. It has resulted in a drastic increase in the incidence of opportunistic and systemic fungal infections. Clinical resistance is defined as persistence or progression of an infection despite appropriate antimicrobial therapy. Resistance is considered primary when an organism is resistant to the drug before exposure, whereas secondary resistance is that which develops in response to exposure to the drug. Major genes that contribute to drug resistance are those coding for multidrug efflux pumps, the up – regulation of which can result in a multidrug-resistant phenotype. *Candida albicans* possesses different types of efflux pumps: adenosine triphos-phate–binding cassette (ABC) transporters

encoded by the CDR genes (CDR1 and CDR2) and major facilitators encoded by the MDR genes (Mary *et al*., 2004).

1.2.3.3.3: Production of an extracellularly Secreted aspartyl proteinase (Sap): -

Secreted aspartyl proteinases (Sap) appear to be a virulence-associated attribute of *Candida* species. These enzymes can cleave several proteins, which are important in host defenses, such as antibodies of both immunoglobulin G and A isotypes. Also, (Sap) may promotes colonization, penetration, and invasion by *Candida albicans* (Flavia *et al*., 1995). The function of (Sap) is through catalyzing the hydrolysis of peptide bonds (CO—NH) in proteins due to degradation of human proteins and structural analysis in determining (Sap) substrate specificity. Less pathogenic or nonpathogenic *Candida* species do not appear to produce significant amounts of these enzymes (Julian *et al*., 2003).The enzymatic activity of *Candida albicans* the secreted aspartic proteases(Saps) can be used as a tool for anti – *Candida* drugs (Backman, 2005).

1.2.3.3.4: Adhesion: -

Capacity of *Candida albicans* to adhere to many different host tissues is broadly considered a virulence trait to initiate invasive activity. Adhesion, either directly or by inference with the stated growth conditions, that hydrophobic *Candida albicans* cells adhere better and with greater site diversity than hydrophilic cells to endothelial cells, epithelial cells, and other host tissues (Pati *et al*., 2001).

1.2.3.3.5: Hyphal formation: -

Hyphae growth, initiation by germ tube formation, which increase adherence properties of yeast (Cutler, 1991). Switching between growth forms is influenced by many factors including temperature, pH, carbon source, nitrogen source, and cell concentration (Cheryl *et al*., 2001). The ability of the pathogenic fungus *Candida albicans* to switch from yeast to a hyphal morphology in response to external signals is implicated in its pathogenicity (Andre *et al*.2002). Hyphal cell walls are more adherent than yeast cell walls to human endothelial and epithelial cells and contain slightly more chitin. In addition, true hyphae express a specific constellation of genes (Eric *et. al*.2002). Hyphae are thought to play an important role in pathogenesis because their filamentous growth pattern can facilitate invasive growth. In addition to their unique morphology, hyphae are further distinguished from budding cells and pseudohyphae by the production of hypha-specific virulence factors. For example, hyphae produce adhesion proteins that facilitate attachment to host cells, and also secrete proteases that contribute to invasion into tissues (Stephen and James, 2004).

Formation of germ tubes can be triggered by a variety of inducers, including temperature, pH, and serum. Serum has been described as the 'magic potion' for induction of germ – tube formation by *Candida albicans* (Debbie *et al.*, 2004). On the other hand, interleukin-12 (IL-12) is a key promoter of protective immunity against human opportunistic pathogen (Paola *et. al*., 2000).

1.2.3.4: Antifungal agents for *Candida albicans:* -

Antifungal treatment strategies for *C. albicans* are limited to a small armamentarium of compounds, mainly Azoles, Polyenes, and Echinocandins.

1.2.3.4.1: Azoles: -

Azoles antifungal agents, have several advantages, such as an excellent oral bioavailability, a stable parenteral formulation, and minimal drug interactions. However, fluconazole, like other azole derivatives, is only act on ergosterol biosynthesis and it's fungistatic. Its efficacy relies on the function of the cellular host defenses (Oscar *et al*., 2000). Azole such as fluconazole. Emergence of resistance to azoles is an increasing problem (Gordon et al., 2002). Azoles included the imidazoles (clotrimazole, miconazole, and ketoconazole) and the triazoles (fluconazole and itraconazole) (Mary *et al*., 2004).

1.2.3.4.2: Polyenes: -

Polyene antifungal agents, which include nystatin and amphotericin B, are fungicidal. Nystatin inhibits the growth of many fungi including *Candida albicans* which may binds to sterols in the cell membrane and alters it's permeability so that potassium and small molecules are lost from the cell and metabolic processes, including glucose utilization are also inhibited(Doreen and Cawson, 1978). In 1950 Brown & Hazen discovered nystatin in a soil sample, obtained from a farm in Virginia, USA, containing a strain of *Streptomyces noursei*. Nystatin, was not absorbable after oral administration and could not be given parenterally, but was effective topically in the treatment of oropharyngeal candidosis. It was licensed for use in 1951 for superficial (mucosal) *Candida* infections of the oropharynx, oesophagus and intestinal tract (Vincent, 1999).

Amphotericin B, bind to membrane sterols, leading to membrane permeability, leakage, and cell death. These drugs have clinical drawbacks based on their toxicity (Gordon *et al*.,2002). Intravenous amphotericin B has

been the drug of choice for invasive fungal infections. The most serious side effect of amphotericin B therapy is nephrotoxicity(Mary *et al*.,2004).

1.2.3.4.3: Echinocandins:-

Echinocandins are new class of antifungal agents that inhibit the synthesis of 1,3-ß-D-glucan, which is a key component of the cell wall. Caspofungin, a member of this class, exhibits excellent pharmacokinetic characteristics, is fungicidal (against yeasts), and displays good safety profiles (Gordon *et al*., 2002).

1.2.3.5: Probiotics: -

1.2.3.5.1: Definition of probiotics: -

Early definition of probiotic suggested by Lilly and Stillwell (1965) were defined Probiotics as Substances produced by microorganisms, which inhibit the growth of other microorganisms. While Parker (1974) was defined it as Organisms and substances which contribute to intestinal microbial balance. Fuller (1989) described it as a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. While Havenaar and Huis (1992) definition was a viable mono- or mixed-culture of microorganisms, which applied to animal, or human beneficially affects the host by improving the properties of the indigenous microflora. However, Schaafsma (1996) stated that probiotics was a living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition. A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract(Naidu et al. 1999). Schrezenmeir and de Vrese (2001) defined probiotic as a preparation of or a product containing viable, defined

microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host. The newest definition of probiotics are live microbial food supplements or components of bacteria which have been shown to have beneficial effects on human health (Isolauri *et al*., 2002).

1.2.3.5.2: Effects of probiotics: -

A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth, activity, or both of one or a limited number of bacterial species already resident in the colon.

Another possibility in microflora management procedures is the use of synbiotic, in which probiotics and prebiotics are used in combination. The live microbial additions (probiotics) may be used in conjunction with specific substrates (prebiotics) for growth. This combination could improve survival of the probiotic organism, because its specific substrate is readily available for fermentation, and results in advantages to the host that the live microorganism and prebiotic offer (David *et al*., 1999).The most frequently used probiotic bacteria are lactic acid bacteria(LAB) and bifidobacteria. Data on probiotic bacteria imply that they have both up- and down regulatory effects on immune responses. Live probiotic bacteria have potentiate nonspecific immunity by stimulating cytokine production, which is beneficial in eradication of pathogens (Tanja *et al*., 1999).

Various nutritional and therapeutic effects of probiotics bacteria are detected (Semith *et al*., 2003): -

- Increasing nutritional value (better digestibility, increased bioavailability of minerals and vitamins).
- Promotion of intestinal lactose digestion (reducing symptoms of lactose intolerance and malabsorption).

- Promotion recovery from diarrhea (rotavirus, travelers).
- Inhibition of pathogen growth and translocation.
- Prevention of intestinal tract infection (bacteria or virus induced, *Candida enteritis*, *Helicobacter pylori*).
- Regulation of gut motility (constipation, irritable bowel syndrome).
- Enhancing specific and nonspecific immune system response.
- Stimulation of gastrointestinal immunity.
- Prevention of cancer and suppressing tumors and detoxification of carcinogens.
- Reducing of blood pressure in hypertensive.

1.2.3.5.3: Probiotic microorganisms: -

The probiotic species (bacteria and yeasts) are:

Lactobacillus species *L. acidophilus*, *L. casei*, *L. fermentum*, *L. bulgaricus*, *L. cremoris, L. lactis, L. plantarum*, *L. salivarius.*

Bifidobacterium species, *B. bifidum, B. breve, B. lactis, B. longum,*

Streptococcus species, *S. thermophilus.*

Enterococcus species, *E. faecium.*

Saccharomyces species, *S. cerevisiae* and *S. boulardii*.

1.2.3.6: Lactic Acid Bacteria (LAB): -

Lactic acid bacteria (LAB) were first described as milk-souring organisms, due to the sour milk that arose from their production of lactic acid. They are a relatively diverse group of bacteria, but related by a number of typical metabolic and physiological features. (Stiles and Holzapfel, 1997) LAB is a group of Gram-positive, nonspore-forming, strictly fermentative bacteria producing lactic acid as the major end product of sugar fermentation. They are devoid of cytochromes and are of a anaerobic or

microaerophilic nature but tolerate air. LAB are catalase-negative, although pseudo-catalase can be found in rare cases. Phylogenetically, they are a very diverse group of organisms and belong to the clostridial branch of Gram-positive bacteria. Two major pathways of hexose (e.g., glucose) fermentation can be distinguished among LAB. Glycolysis (Embden – Meyerhoff – Parnas Pathway) results in almost exclusively lactic acid as the end product (homofermentative) (Fig. 1 – 2.A). While the 6–phosphogluconate / phosphoketolase pathway results in significant amounts of other end products, such as ethanol, acetic acid and CO_2 in addition to lactic acid (heterofermentative) (Fig. 1 – 2.B). Based on the fermentative characteristics, lactobacilli can be divided into three groups: obligatory homofermentative, obligatory heterofermentative and facultatively heterofermentative. Obligatory homofermentative lactobacilli degrade hexoses exclusively to lactic acid and do not ferment pentoses or gluconate. Obligatory heterofermentative on the lactobacilli other hand, degrade hexoses to lactic acid and additional products such as acetic acid, ethanol and CO_2 and pentoses to lactic and acetic acid. While facultatively heterofermentative lactobacilli ferment hexoses to lactic acid and may produce CO_2 from gluconate but not from glucose. They also ferment pentoses to produce lactic and acetic acid (Ulrike, 2002).

Axelsson, (1989) illustrated the net fermentation reaction of (LAB)

Homofermentation

$$1\ \text{Hexose} + 2\text{ADP} + 2\text{Pi} \longrightarrow 2\ \text{Lactate} + 2\ \text{ATP}$$

Heterofermentation

$$1 \text{ Hexose} + 1 \text{ ADP} + \text{Pi} \longrightarrow \text{Lactate} + \text{Ethanol} + CO_2 + 1 \text{ ATP}$$

OR (if external e – acceptor)

$$1 \text{ Hexose} + 2 \text{ ADP} + \text{Pi} \longrightarrow \text{Lactate} + \text{Acetate} + CO_2 + 2 \text{ ATP}$$

While Tomas, (1997) illustrated the homofermentation and heterofermentation pathways of LAB as face:

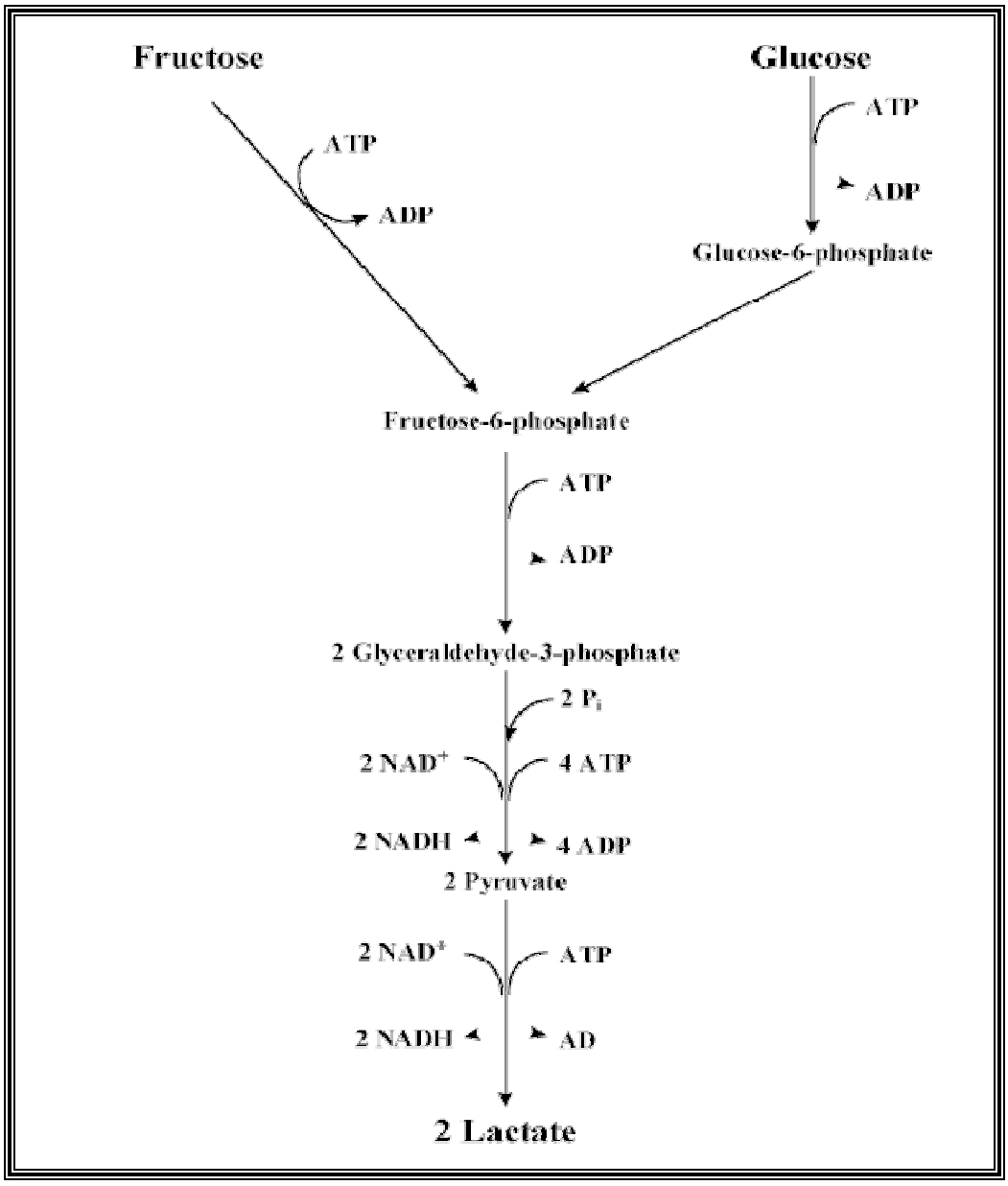

Figure (1 – 2)(A): The homofermentative pathway of lactic acid bacteria.

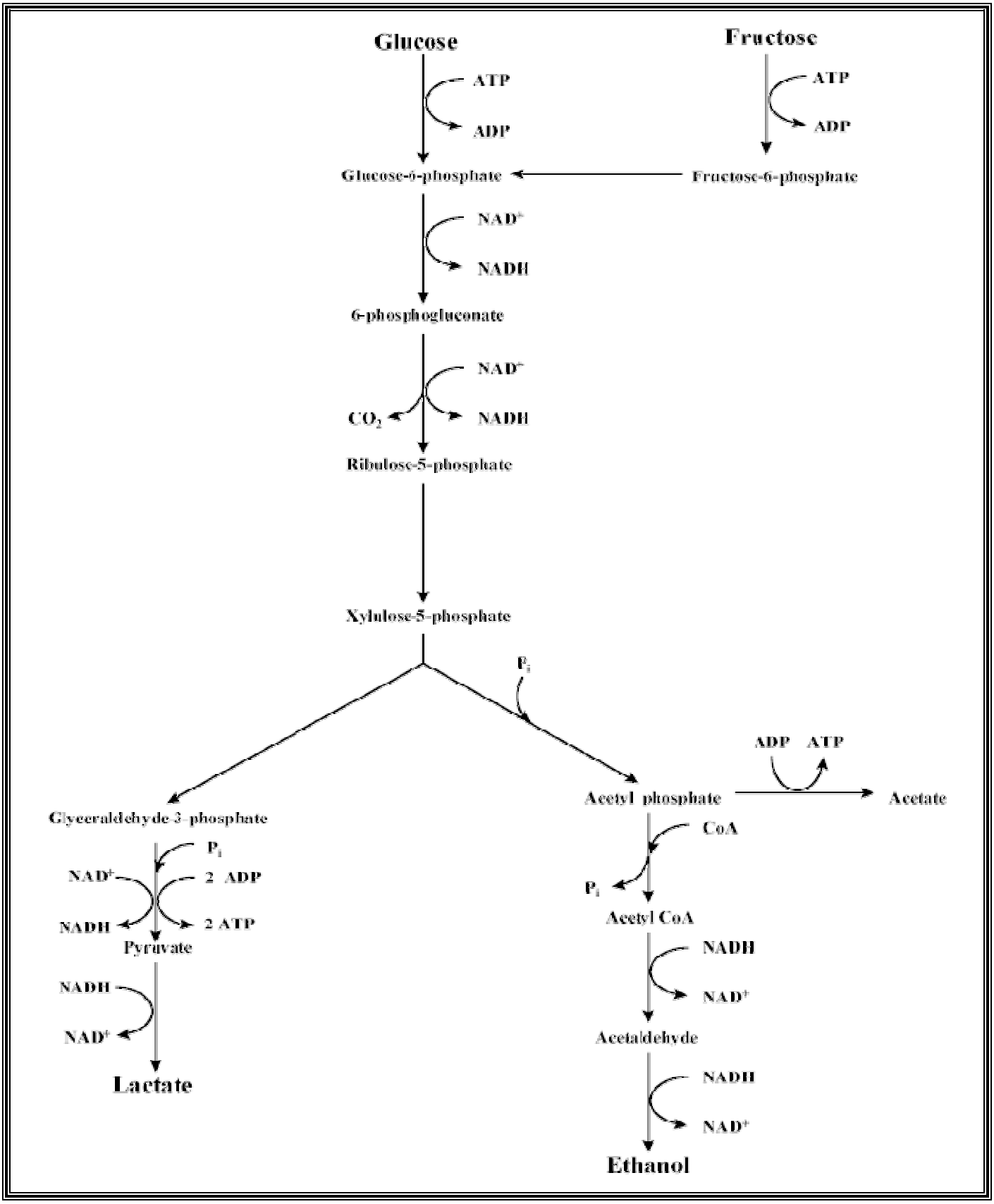

Figure (1 – 2)(B): The heterofermentative pathway of lactic acid bacteria.

Lactic acid bacteria are commonly found and known to proliferate in fermentation processes or as colonizers of mucosal surfaces of higher animals. They require a nutrient rich environment in order to establish. Besides carbohydrates, they also need to be supplied with amino acids, peptides, salts and vitamins among others (Hammes *et al.*, 1992; Carr *et al.*, 2002)

1.2.3.7: Antifungal activity of Lactic Acid Bacteria: -

The antimicrobial effect of the LAB (as described in Fig. 1 – 3) is mainly related to production of lactic and acetic acid, but for some strains, synthesis of bacteriocins is also of great importance. Due to the "Generally Regarded As Safe" status of LAB, the interest in using them for biopreservation has increased during recent years (Pitt *et al*., 1999). Katrin *et al*., (2002) found that production of antibacterial compounds by LAB, that effects on inhibition of yeasts and fungi are comparatively few. The precise mechanism of antimicrobial action is hard to be defined, because of a complex interaction between different compounds.

Important inhibitory compounds produced by LAB may be described as fallow: -

1.2.3.7.1: Fermentation products:

The major metabolite of lactic acid bacteria is lactic acid, which is responsible for the significant pH changes in their growth environment – sufficient to antagonize many microorganisms. (Darwin, 2003). Fermentation products contain a variety of fermented microorganisms belonging to various genera and species, all of which produced lactic acid (Simin and Woel-Kyu, 2000).

Yoghurt was defined as a coagulated milk product that results from fermentation of lactic acid bacteria in milk (Bourlioux *et. al.*, 1988). Potential therapeutic effects of LAB and yoghurt, including their immunostimulatory effect, which are primarily yogurt–induced changes in the gastrointestinal (GI) microecology. Increased amounts of LAB in the intestines could suppress the growth of pathogenic bacteria, which contributes in turn to reduce infection. (Simin and Woel-Kyu, 2000).

1.2.3.7.2: Proteinaceous compounds:-

Lactic acid bacteria were produced antibacterial, ribosomally synthesized peptides, generally termed bacteriocins. The bacteriocins are commonly divided into three groups: class I – the lantibiotics; class II – the heat stable unmodified bacteriocins; class III the larger heat stable bacteriocins. These compounds are generally only active against closely related bacterial species and there is no evidence that bacteriocins have any effect on growth of yeast or molds. (Darwin, 2003).

1.2.3.7.3: Low molecular weight inhibitory compounds:

Reuterin is one of the most low molecular weight inhibitory compounds. It is a broad-spectrum antimicrobial substance originally described from *Lactobacillus reuteri*. In addition, glycerol of this species resulted in a dramatic increase of the inhibitory effect against several filamentous fungi and yeast as well as toxicity towards a wide range of Gram-negative and Gram-positive bacteria because LAB lack the oxidative pathway of glycerol degradation, hence glycerol can not be metabolized as a sole carbon source. Reuterin is produced from glycerol by starving cells under anaerobic conditions and it is active against several different types of

icroorganisms including gram-positive and gram-negative bacteria, yeast and fungi. (Darwin, 2003).

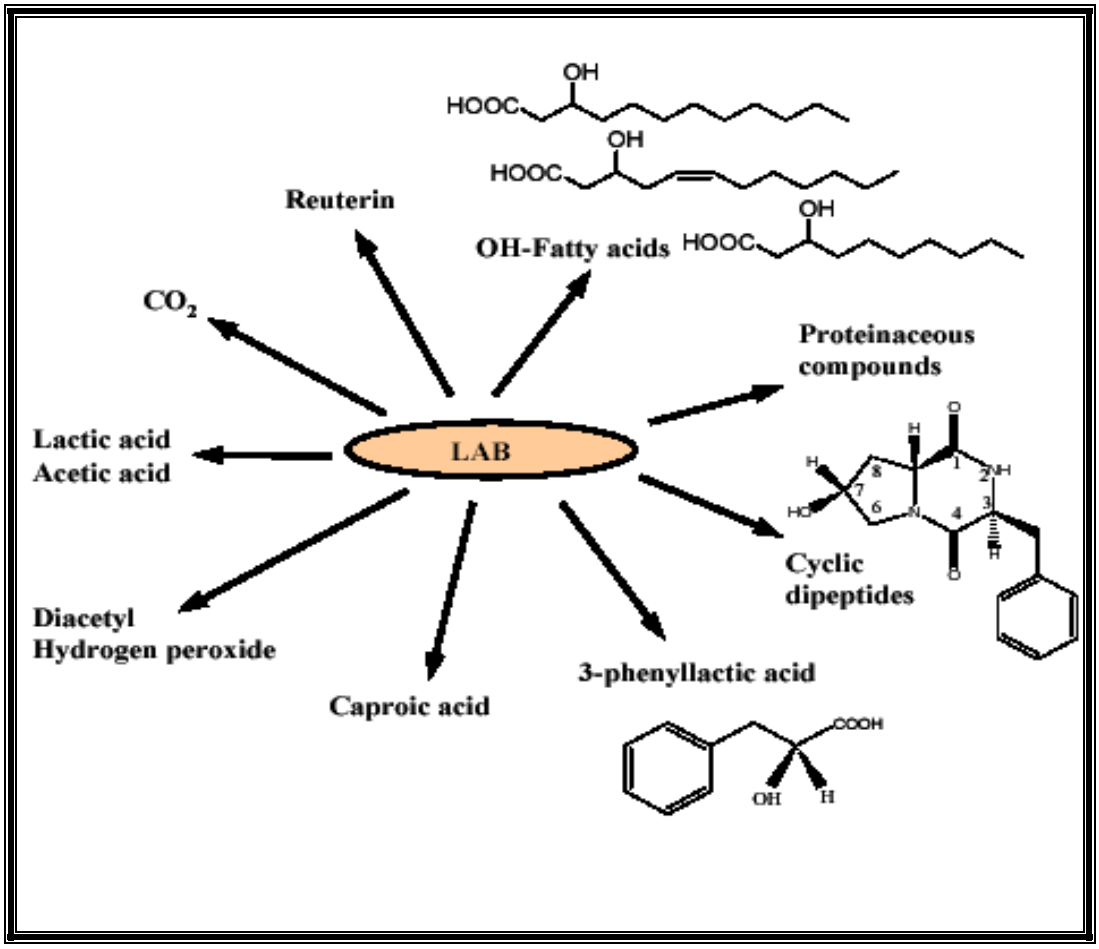

Figure (1 – 3): Production of antifungal products of lactic acid bacteria. (Darwin, 2003).

1.2.3.8: Using LAB as probiotics :-

Lactic acid bacteria (LAB) are present in intestine of most animals. The beneficial role played by these microorganisms in humans and other animals, including the effect on immune system. LAB are present in many foods and are frequently used as probiotics to improve some biological functions in the host. The activation of the systemic and secretory immune response by LAB requires many complex interactions among the different

constituents of the intestinal ecosystem (microflora, epithelial cells and immune cells). Through different mechanisms they send signals to activate immune cells (Gabriela *et. al.*, 2001). Lactic acid bacteria (LAB) have a long history in biotechnology, especially in the manufacture and storage of food ingredients by fermentation processes (Perdigon *et al*., 2001).

LAB are essential for yoghurt to exert immunostimulatory effects and that LAB cell walls contain the main immunomodulatory components. LAB cell wall is composed mainly of peptidoglycan, polysaccharide, and teichoic acid. The immunostimulatory activity in the host by cultured dairy products is mediated by glycopeptides in the bacterial cell wall. Yoghurt's microorganisms may prevent infections of GI tract by influencing microbial ecosystem. However, LAB that are colonized in human intestine, *L. acidophilus* and *Bifidobacterium* species, are more resistant to gastric acid than are LAB conventionally used for yoghurt fermentation. The inhibitory mechanisms of LAB against disease – causing bacteria are due primarily to two metabolites of lactic acid fermentation—organic acid and bacteriocin. In addition, oral microbial therapy with LAB can be effective in preventing antibiotic – induced GI disorders and in recovery from diarrhea (Simin and Woel-Kyu, 2000). *Saccharomyces boulardii*, a patented yeast preparation, is used in many countries as a preventive and therapeutic agent for diarrhea and other gastrointestinal disorders caused by the administration of antimicrobial agents. *S. boulardii* possesses many properties that make it a potential probiotic agent (Rial, 2000).

1.2.3.9: Effects of LAB on diarrhea: -

Probiotics can prevent or reduce diarrhea through their effects on the immune system. Probiotics can prevent infection because they compete with pathogenic viruses or bacteria or fungi for binding sites on epithelial cells.

Several probiotics are claimed to stimulate immune system. Their modes of action appear to be nonspecific, resulting in increased immune responsiveness to a wide variety of antigens.(Nicole and Martijn, 2000). Intestinal microflora maintain a microbial barrier against the development of pathogenic bacteria in the digestive tract. LAB have different properties, include restoration of a normal intestinal microflora, which contribution to elimination of pathogenic enteric bacteria (Martine**,** 2000). Probiotics microorganisms prevent or reducing detoxifying of harmful substances in gastrointestinal tract and other gastrointestinal side effects caused by cancer treatment. (Urbancsek *et. al.*, 2001).

1.2.3.10: Effects of LAB on fungi especially *Candida albicans* causing diarrhea: -

The microbiocidal action of LAB is based on both competition for nutrients and production of various compounds, such as organic acids, hydrogen peroxide, bacteriocins, and low molecular weight antimicrobial agents. Cell-free supernatants of *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* were grown in deMan Rogosa Sharpe (MRS) broth inhibited 95 – 100% growth of fungi. (Vina and Carol, 2005).

Lactobacilli (*casei*, *acidophilus, rhamnosus, plantarum* and others) produce lactic acid, which creates an acidic environment that is unfriendly to some harmful bacteria. *Lactobacillus acidophilus* has properties to prevent growth of pathogenic microorganisms, including *Candida albicans,* by producing both lactic acid and antibiotic compounds. Lactic acid produced by *L. acidophilus* act to block the receptors or adhesion sites of pathogens, creating a barrier against infectious organisms. They also prevent production of toxic amines by putrefactive bacteria, (Kilic *et. al.*, 1996). *Lactobacillus*

plantarum that produces broad-spectrum antifungal compounds, active against food- and feed-borne filamentous fungi and yeasts in a dual-culture agar plate assay.

Lactobacillus plantarum produced bacteriocins that had broad spectrum of inhibition against pathogenic bacteria, like *Escherichia coli* and *Enterococcus faecalis*, but did not inhibit *Candida albicans* and *Klebsiella* sp. (Ogunbanwo *et. al.*, 2003). Mairi and Gary (2004) stated that fatty acid metabolites and fatty acid pathways from LAB were up – regulate and down – regulate germination in *Candida albicans*.

Probiotics (*Lactobacillus* strains) are containing different mechanism(s) underlying antibacterial activity, includes lowering of pH and production of lactic acid and antibacterial compounds, including bacteriocins and non bacteriocin, and non-lactic acid molecules. (Domitille *et. al.*, 2005).

2. Materials And Methods

2.1: Materials: -

2.1.1: Equipment: -

Equipment	Company - Country
Anaerobic Jar	Rod Well – England
Autoclave	GallenKamp – England
Balance	Ohans – France
Distillator	GallenKamp – England
Freeze dryer	Vitris – USA
Incubator	GallenKamp – England
Light Microscope	Olympus – Japan
Micropipette	Witeg – Germany
Millipore filters	Millipore and Whatman – England
Oven	Memmert - Germany
Pasture pipette	Witeg - Germany
pH – meter	Metter – GmpH Tdedo - UK
Refrigerator Centrifuge	Harrier – UK
Sensitive Balance	Delta Range – Switzerland
Shaker Incubator	GallenKamp – England
Spectrophotometer	Aurora Instruments Ltd - England
Vortex	Buchi – Swiss rain
Water bath	GFL – England

2.1.2: Chemicals:-

Materials	Company – Country
Acetic acid	BDH – England
Ammonium Iron3 Citrate	
D – Glucose	
Esculine	
Maltose	
MgSO4.7H2O	
Peptone	
Potassium chloride	
Potassium dihydrogen phosphate	
Sodium acetate trihydrate	
Sodium dihydrogen phosphate	
Sucrose	
Xylose	
Agar	Difco – England
Lactose	
Raffinose	

Casein Peptone	Fluka – Switzerland
Chlorophenol Red	
Hydrogen Peroxide (3%)	
L – arginine monohydrochloride	
Tryptone	
Yeast extract	
Corn Meal Infusion	Oxoid – England
Disodium hydrogen phosphate	
Gelatin	
Meat extract	
Tween 80	
Cellobiose	Riedel-DeHaeny – Germany
Ethanol	
Methanol	
MnSO4.4H2O	
Sodium chloride	
Triammonium citrate	

2.1.3: Antibiotics and Antifungal: -

Nystatin and chloramphenicol were obtained from samara drug industries.

2.1.4: Solutions and Buffers:-

2.1.4.1: Normal saline :-

It was prepared by dissolving of 0.85g NaCl in 100 ml distilled water, pH was adjusted to 7.

2.1.4.2: SDS (Sodium Dodecyle Sulfate) solution: -

It was prepared by dissolving 0.25% SDS powder in distilled water.

2.1.4.3: Phosphate Buffer Saline (PBS): -

It was prepared by dissolving these materials in 1liter, which include:

NaCl	8 g
Na2HPO4	1.13 g
KH2PO4	0.2 g
KCl	0.2 g

pH was adjusted to 7.

2.1.4.4: Fixative solution: -

It was prepared by mixing of 50% Methanol and 10% acetic acid, then 40% of distilled water were added.

2.1.4.5: Nystatin stock solution: -

Solution **(**1**):-**It was prepared by dissolving 0.01g/10ml (3 ml DMSO + 7 ml D.W.). Solution **(**2**):-** It was prepared by dissolving 0.1g/ 10ml (3 ml DMSO + 7 ml D.W.). (Doreen and Cawson, 1978).

2.1.4.6: Chloramphenicol stock solution:-

It was prepared by dissolving of 100 mg of chloramphincol in 10 ml D.W. (Kevin, 2004).

2.1.5 Reagents: -

2.1.5.1: Kovac's reagent: -

It was prepared by dissolving of P – dimethyl – amino – banzaldehyde (5g) with (75 ml) butanol and then (25ml) of concentrated HCL was carefully and gradually added. It was used for indol test.

2.1.5.2: Phenol red reagent: -

It was prepared by dissolved 0.1g of phenol red in 10ml of distilled water.

2.1.5.3: Chlorophenol red reagent: -

It was prepared by dissolving 0.2g from reagent in 2ml distilled water. Volume was completed to 100ml with distilled water.

2.1.6 Culture Media: -

All following media was sterilized by autoclave at 121^0C for 15 min.

2.1.6.1 (SDA): (Ajello**,** 1957)

Sabouraud Dextrose Agar contain:

Peptone	10 g
Dextrose	20 g
Agar	15 g
D.W.	950 ml

pH was adjusted to 7. This media was used for growth and identification of *Candida* spp.

2.1.6.2. (SDB) medium: (Buffo *et. al.* 1984)

Sabouraud Dextrose Broth contain:

Peptone	10 g
Dextrose	20 g
D.W.	950ml

pH was adjusted to 7. This media was used for growth and identification of *Candida* spp.

2.1.6.3. Corn – Meal Agar: (Akisada *et. al.* 1983)

Corn – Meal infusion	50 g
Agar	15 g
D.W.	950 ml

pH was adjusted to 7. This media was used for identification of *Candida albicans*.

2.1.6.4. Fermentation basal medium: (Hassan *et. al*., 2004): -

Yeast extract	0.45 g
Peptone	0.75 g
Phenol red	5 ml
D.W.	95 ml

2% of Sugars (Glucose, Lactose, Maltose, and Sucrose) were added to fermentation basal media. pH was adjusted to 7 and autoclaved for 10 min. It was used this media for identification of *Candida* spp.

2.1.6.5. (MRS) medium: (Harrigan and McCacne, 1966)

Modified Regosa Sharp broth contain

Yeast extract	5 g
Peptone	10 g
Meat extract	10 g
D – Glucose	20 g
Sodium acetate rihydrate	5 g
Triammonium citrate	2 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.5 g
Tween 80	1 ml
D.W.	950 ml

pH was adjusted to 6.0. This media was used for growth *Lactobacillus* spp.

2.1.6.6. Aesculine Cellobiose Agar *:* (Hunger, 1986)

Casein Peptone	20 g
Yeast extract	5 g
Cellobiose	20 g
NaCl	4 g
Sodium acetate trihydrate	1.5 g
Tween 80	0.5 g
Esculine	1 g
Ammonium Iron III	0.5 g
Chlorophenol red	6.5 ml

Agar	15 g
D.W.	950 ml

pH was adjusted to 7. This media was used for growth and identification of *Lactobacillus acidophilus*.

2.1.6.7. MRS – Raffinose Agar: (Hunger, 1986)

It was prepared by adding raffinose to MRS broth instead of glucose. pH was adjusted to 7. It was used this media for identification of *Lactobacillus acidophilus*.

2.1.6.8.Carbohydrate fermentation medium: (De Man *et. al*., 1960)

It was prepared by using of MRS broth without glucose and meat extract. After that, 0.004% of phenol red reagent and 1% of each sugars (lactose, xylose, sucrose, mannitol, raffinose) was added to MRS broth. pH was adjusted to 6.5 and autoclaved for 10 min. It was used this media for identification of *Lactobacillus* spp.

2.1.6.9. Gelatin media : (Stolp and Gadkari, 1984)

It was prepared by dissolving of 12% (W/V) of gelatin in MRS broth. pH was adjusted to 6. This media was used for identification of *Lactobacillus* spp.

2.1.6.10. Litmus Milk Medium : (Baily *et. al.*, 1990)

Skim milk	100 g
Litmus media	5 g
D.W.	950 ml

pH was adjusted to 6 and autoclaved for 10 min. This media was used for identification of *Lactobacillus* spp.

2.1.6.11. MRS Arginine Broth : (Harrigan and McCance, 1966)

It was prepared by adding 0.3% (W/V) of L-arganine monohydrochloride to MRS broth. pH was adjusted to 6. This media was used for identification of *Lactobacillus* spp.

2.2 Methods:-

2.2.1: Collection of stool sample:-

Forty-five stool samples were collected from children with diarrhea. After samples were cultured on sabouraud glucose agar (SDA) (3.1.6.1) contained 0.5mg/ml chloramphenicol. Then, incubated at 37^0 C for 48 – 72 hours. Then, several steps of isolation were applied on colony for purity.

2.2.2: Isolation of *Candida* species: -

Candida species were isolated as follows: -

2.2.2.1: Morphology of colony: -

The morphology of colonies was examined and described after cultivation and incubation on SDA according to (Savage and Balish, 1971)

2.2.2.2: Microscopic examination:-

Part of suspected colony was fixed on slide, stained by gram's stain, then cells were described under oil – immersion lens of a compound light microscope. (Kreger-Van , 1984)

2.2.3: Identification of *Candida albicans*:-

2.2.3.1: Surface growth:-

This test was used for detection the ability of *Candida* spp to grow on surface of SDB (2.1.6.2) by transferring part of a colony to tubes contained this medium. After incubation at 37^0C for 24 – 72 hrs. Presence of gas bubbles was indicated a positive result. (Forbes *et. al.*, 1998).

2.2.3.2: Germ tube formation test:-

This test was used for detection the ability of *Candida albicans* to form germ tube. Formation of germ tube was examined by adding 1 ml of human serum in a sterile tube and re–suspended with part of colony then incubated at 37^oC for 2 – 4 hrs. After incubation, a drop from suspension was put by a pasture pipette on a slide. Result was recorded under light microscope by observed formation of germ tube.(Evans and Richardson, 1989).

2.2.3.3: Sugar fermentation test:-

This test was used for detection the ability of *Candida* spp to ferment 2% of certain sugar (glucose, sucrose, lactose and maltose) and production of CO_2. *Candida* isolates were incubation in fermentation basal media (2.1.6.4) contained different sugars in test tubes at 37^oC for 24hrs. The positive result was recorded by changing the red to yellow color and production of CO_2 gas bubbles, in comparison with control.(Fordes *et. al.*,1998)

2.2.3.4: Chlamydospore formation test: -

This test was used for detection and identification of *Candida albicans*. The isolates *Candida* were cultured on Corn – meal agar (2.1.6.3) with tween 80 and incubated at 37^0C for 5 – 7 days. Round blastoconidia bunched

together with pseudohyphae or true hyphea were observed under light microscope. (Barnett *et. al.*, 2000)

2.2.3.5: Growth in 45^0C: -

This test was used for differentiation *Candida albicans* from other species. *Candida* isolates were used to inoculate test tubes containing SDB, then incubated at 42 – 45^0C for 48 – 72 hrs. After incubation, *Candida albicans* should grow well on this temperature, while others give poor or no growth (Kim *et. al.*, 2002).

2.2.4: Preservation of *Candida albicans*: -

The isolates were inoculated on SDA slants contained 0.5mg/ml chloromphenicol. After incubation at 37^0C for 48 hrs, the slants placed in 4^0C as stock culture. These slants were recultured every three months (Oliver *et. al.*, 1982)

2.2.5: Isolation and identification of Lactic Acid Bacteria:-

2.2.5.1: Collection of stool samples: -

Forty-five stool samples were collected from children with diarrhea. After that, 1% of samples were cultured in 9ml MRS broth (2.١،٦،٥.) Then, incubated anaerobically at 37^0 C for 48 – 72 hrs. Serial dilution that was made on these samples in MRS broth. 0.1 ml of last dilution were spreaded on MRS – $CaCO_3$ (2.1.6.6) and incubated at 37^0C for 24 hrs. The arised colony, which surrounded by clear zone were transferred on MRS agar for purification. Then, several steps were applied on colony for purity.

2.2.5.2: Microscopic examination:-

A loopfull of lactic acid bacteria was fixed on a glass slide, then stained by gram's stain to examine cells shape, grouping, gram reaction and spore forming. Then cells were described under oil – immersion lens of a compound light microscope. (Garvie and Weiss, 1986).

2.2.5.3: Growth on aesculine cellobiose agar: -

This test was used for identification of *Lactobacillus acidophilus*. Part of a colony isolates (2.2.5.1) was streaked on aesculine cellobiose agar (2.1.6.6) and incubated at 40^0C for 48 hrs under anaerobic (using anaerobic jar) condition. The positive results were observed by changing color media to florescence green. (Hunger, 1986).

2.2.5.4: Growth on MRS – Raffinose agar: -

This test was used for identification of *Lactobacillus acidophilus*. Part of a colony isolates was cultured on MRS – Raffinose agar (2.1.6.7) and incubated at 37^0C for 48 hrs. Results were recorded by growing a colony on this medium. (Hunger, 1986).

2.2.5.5: Catalase test: -

It was performed by adding drops of hydrogen peroxide (H_2O_2) 3% over a part of a colony cultured on MRS agar for few min. Production of gaseous bubbles indicates a positive results.(Elaine *et. al.*, 2001).

2.2.5.6: Gelatin hydrolysis test: -

It was used this test for detection the ability of bacteria isolates to hydrolyzed gelatin by stabbing bacteria into tubes contained gelatin medium (2.1.6.9) and incubated at 30^0C for 5 – 7 days. The positive results were observed by gelatin liquification (Baron *et. al*., 1994).

2.2.5.7: Indole test: -

LAB isolates were inoculated with (2g trypton and 0.5g NaCl in 100 ml distilled water) and incubated at 37^0C for 3 days. After incubation period, drops of Kovac's reagent was added to detect the formation of red ring. (Harrigan and McCance, 1966).

2.2.5.8: Acid and curd production in litmus milk:-

Litmus milk was used for the maintenance of lactic acid bacteria and as a differential medium for determining the action of bacteria on milk. LAB were inoculated with litmus milk media (2.1.6.10) and incubated at 37^0C for 48 hrs. Positive results were recorded by changing a color to pink or purple and decrease in pH value (Fordes *et. al.*, 1998).

2.2.5.9: Production of ammonia from Arganine: -

It was used this test for detected production of ammonia from arganine. LAB were subcultured on MRS broth and incubated at 37^0C for 24hrs. 0.1 ml was transferred to MRS – Arganinie medium (2.1.6.11) and incubated at 37^0C for (5 – 7) days. The production of NH_3 from arginine was determined by adding Nessler's reagent and monitored the development of an orange to red color which indicator the positive results (Spano *et. al*., 2002).

2.2.5.10: Fermentation of carbohydrate test: -

This test was used for detected the ability of bacteria to ferment different sugars. LAB isolates was inoculated in tubes contained fermentation media (2.1.6.8**)** and incubated at 37^0C for 24 hrs. After that the results was compared with control tubes contained only fermentation media without bacteria. Changing of color from red to yellow indicated the positive results. (Elaine *et. al.,* 2001)

2.2.5.11: Growth at (15 – 45) ^{0}C: -

LAB isolates was inoculated in tubes contained MRS – broth and incubated at 15^0C or 45^0C for 24 hrs. After that, turbidity was observed and compared with control, which incubated at 37^0C. Positive results obtained by growth of LAB (Buck and Gilliland, 1995).

2.2.6: Maintaining of stool LAB isolates: -

2.2.6.1: Working culture: -

MRS – broth was inoculated with LAB isolates and incubated at 37^0C for 24 hrs. After incubation, 1% $CaCO_3$ was added and stored at 4^0C.

2.2.6.2: Primary Stock culture: -

Tubes containing MRS – broth with 20% glycerol were inoculated with LAB isolates. After incubation at 37^0C for 24hrs, they were stored at – 18 to – 20^0C.

2.2.7: Sensitivity of *Candida albicans* to antifungal agent and to determination of minimum inhibition concentration (MIC):-

Ten ml of SDB were inoculated by each *Candida* isolates and incubated at 30^0C for 24 – 48 hrs. After incubation period, their stationary phase was determined by (O.D. $_{490}$) in range from 1.0 to 1.2. Then serial dilutions was made to obtain 10^6 cell/ml. In other hand, an antifungal (Nystatin) was diluted to prepared different concentration, which were (10,20,30,40,50,60,70,80,90) µg/ml from stock solution (1)(2.١,٤,٦.)and (100,200,300,400, 500)µg/ml from stock solution (2) (2.1.4.6). All these dilution were made in tubes containing SDB at final volume 10ml in each tubes. 0.1ml of 10^6 cell/ml were added to each tubes, then incubated at 37^0C for 48 hrs. The result was recorded by determined the activity of antifungal drugs on *Candida* isolates. The suitable concentration was cultured on SDA. After incubation period (at 37^0C for 48 hrs) the result was recorded and compared with control (SDB with *Candida* isolates) to determine the minimum inhibition concentration of Nystatin. (Doreen and Cawson, 1978).

2.2.8: Determination of inhibitory effect of LAB:-

2.2.8.1: On solid media: -

LAB was cultured on MRS broth and incubated at 37^0C for 48 hrs. then, LAB was streaked on MRS agar, and incubated under anaerobic condition at 37^0C for 24 hrs. The agar disc diffusion method was employed for determination of antimicrobial activities of LAB on *Candida albicans*. After incubation, with the aid of cork porer (6 mm), disk of the grown culture were put on the surface of SDA that was inoculated previously with 0.1 ml of *Candida albicans* and incubated at 37^0C for 24 – 48 hrs. After that, the inhibition zone around the disk was measured in (mm). (Bektas *et. al.*, 2004).

2.2.8.2: In liquid media:-

LAB was inoculated in MRS – broth and incubated at 37^0C for 24 hrs. After incubation period, Cell – free supernatants were collected by centrifugation at 3000 rpm for 40 min. These supernatant was filtrated through Millipore filter unite (0.22 µm).

Then well diffusion method was used for detection the activity of LAB on *Candida albicans* by plate contain SDA cultured with 0.1 ml *Candida albicans* by using spreader. After that using cork pore (6 mm) wells were made in agar and filled by filtrate of LAB. Then, was incubated at 37^0C for 24 – 48 hrs. The inhibition zone around the well was measured by (mm) and compared with control, which contain MRS – broth without bacteria. The well diffusion method was repeated with different concentration of filtrate to detect the activity of LAB against *Candida albicans* and compared with control. (Bektas *et. al.*, 2004).

The filtrates of LAB were concentrated by freeze – dryer which has equal volume 100 ml of MRS broth inoculated with 1 ml LAB, concentrated to one fold (50 ml), two fold (25 ml), three fold (12.5 ml).

2.2.9: Germ tube assay (Mairi and Gary, 2004)*:* -

Candida albicans isolates was incubated in SDB in shaker incubator (150 rpm) at 37^0C for 48 – 72 hrs. Samples were washed in phosphate – buffered saline (PBS) and resuspended in 100% human serum. *Candida albicans* isolates was diluted in human serum to give a final concentration of 10^6 yeast cells/ml. Then (10 µl) of *Candida* isolates was plated into a 96-well flat-microtitter plate filled by human serum at a volume of 100 µl/well and plates were incubated at 37^0C for 2 hrs to induce germination. Adherent germ tubes formed were fixed by fixative reagents (2.1.4.4), and non – adherent yeast forms were removed by sequential washes with 70% ethanol

and 0.25% sodium dodecyl sulfate (SDS)(2.1.4.3). Plates were rewashed two or three times with distilled water. Plates were examined microscopically to ensure removal of non – adherent yeast cells.

2.2.10: Determination of inhibitory effects of LAB filtrates on *Candida albicans* morphogenesis :-

2.2.10.1: Effects of live LAB on *Candida albicans* morphogenesis: -

LAB was inoculated in MRS – broth and incubated under anaerobic conditions at 37^0C for 24 hrs. LAB were added {(10:1), (1:1) and (1:10)}µl of LAB / yeast to *Candida albicans* (was diluted in human serum to obtain 10^6 cell/ml) at final volume 100 µl of each well and incubated at 37^0C for 24 hrs.(Mairi and Gary, 2004). Results was recorded depending on above no effect of live LAB on *Candida albicans*.

2.2.10.2: Determination of minimum inhibition concentration (MIC) effect of LAB filtrates on *Candida albicans* morphogenesis: -

Different dilution of each concentrated filtrates were made in 96-well flat-microtitter plate containing 100 µl of human serum of each well. The various ratio (1,2,4,8,10,20,30,40,50,60,70,80,90)% were added to each well in the *Candida albicans* germ tube assay to give final volume 100 µl in each well. Then, incubation at 37^0C for 2 – 24hr. The germ tube formation was observed and minimum inhibition concentration was determined as the lower concentration of the filtrates that gave no germ tube formation. The results was recorded by observed that LAB filtrates was inhibited formation of germ tube of *Candida albicans* in human serum which represent of more efficient inducer for germ tube formation.

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المصادر العربية:-

- القصاب ، عبد الجبار عمر (١٩٨٨) التأثير المضاد لبكتريا حامض اللكتيك على بعض أنواع البكتريا المرضية ، رسالة ماجستير ، كلية الزراعة – جامعة بغداد

3.Results And Discussion

3.1: Isolation and identification of Yeast: -

Fourty five stool samples were collected from children suffering from (2 – 10) years old with diarrhea. It was found that 15(33.3 %) out of the total 45 samples gave positive for the microscopic examination. These results were near to those reported by Forbes *et. al*., (2001) who found that *Candida* species were frequently isolated from stool of children with diarrhea but were not proven to be enteropathogens. Identification of isolates were carried out according to culture, and morphology tests. Results showed that 15(33.3%) isolates belonged to genus *Candida*, and 4(8.8%) to *Candida albicans* according to the related biochemical tests. Forbes *et. al.*, (2001) reported that yeast species, predominantly *Candida*, were identified in 39% of the stools of children with diarrhea and 36% without diarrhea. Ormala *et. al*., (1992) found that 11% of faecal cultures of infants were positive for *Candida albicans*, while Pederson, (1969) reported that *Candida* was detected in up to a 25% stool samples of 5 to 12 years old infants. Moreover, Gorbach, (1967) proved that during childhood, 12 to 16% of children examined were carried *Candida* in the stool, and the proportion increased to 80% in adulthood. Concentration of *Candida* was positively related to the antibiotic use and the presence of another enteric pathogen, but not with patient age, nutritional status, or duration of diarrhea. (Forbes *et. al*., 2001). Odds *et. al*.,(1989) found that *Candida albicans* was increased in patients to reaches to 72% of faecal yeast isolates from cancer patients. Results of identification of *Candida albicans* could be more discussed according to the culture, microscopical and biochemical tests as follow: -

3.1.1: Morphological examination:-

Morphological examination of the suspected isolates showed that their colonies were appeared on SDA as white to cream, glossy, smooth, soft and circular colony. Such characteristics come in accordance with those belonged to genus *Candida*.

3.1.2:Microscopical examination: -

After parts of the suspected colony grown on SDA were, separately, gram-staining on slide and examined under oil immersion of the microscope, cells appeared to be violet oval have short extension shape, budding and sporeformer.

3.1. 3: Biochemical tests: -

Table (3 – 1) shown that all 4 isolates of *Candida* gave positive results to morphological tests performed (surface growth on SDB, formation of germ tube when cultured in human serum at 2 – 4 hrs., chlamydosopre formation and the growth in 42^0C). Such results are usually related to the yeast *Candida albicans*. Results in table (3 – 2) describe the ability of *Candida* isolates to ferment of glucose, lactose, maltose and sucrose. *Candida* isolates was able to ferment glucose and maltose during 24 hrs., but not lactose and sucrose. In this regard, Ping (2002) insisted on such above tests to differentiate of *Candida albicans* from other species by all these tests.

3.2: Isolation of lactic acid bacteria (LAB): -

From total forty five stool samples collected from children with diarrhea, 7 of them gave colonies surrounded by inhibition zone after cultured on MRS contained 1% $CaCO_3$. All isolates were negative to

catalase test except one isolate gave few bubbles when hydrogen peroxide was added.

Table (3 – 1) Morphological characteristics of *Candida* isolates.

Candida isolate	Surface growth	Germ tube formation	Chlamydospre formation	Growth in 42^0C
C. 1	+	+	+	+/–
C. 2	+	+	+	+
C. 3	+	+	+	+
C. 4	+/–	+	+	+/–

+ = Positive result

+/– = Positive / negative result

Table (3 – 2) Sugar fermentation by *Candida* isolates .

Candida isolate	Glucose	Lactose	Maltose	Sucrose
C. 1	+	–	+	–
C. 2	+	–	+	+/–
C. 3	+	–	+	+/–
C. 4	+	–	+	–

+ = Positive fermentation

– = Negative fermentation

+/– = Weak fermentation

3.3: Identification of LAB: -

3.3.1: Morphological characteristics : -

Colonies of LAB appeared pale, round shape, soft, mucoid, convex and surrounded by inhibition zone after cultured on MRS – $CaCO_3$. When the LAB isolates were appeared, colonies cultured on aesculine cellobiose agar and MRS – raffinose appeared specific media for identification of *L. acidophilus* small white round and fluorescent green, which are properties of *L. acidophilus* when grows on those media.

3.3.2: Microscopical examination: -

Lactobacillus grouped in chain {containing (3 – 8) cells, and appeared gram-positive bacilli, non-sporformers}

3.3.3: Biochemical tests: -

Table (3 – 3) contains biochemical tests of LAB. All suspected LAB isolates gave negative results for each of catalase test, gelatin test, and indole test. Adversely, they were able to produce clot on litmus milk medium and leading to decrease pH value from 6.5 to 4.5 or to 4. The LAB isolates were unable to produce ammonia from arginine when grown in MRS arginine broth in spite of addition of Nessler's reagent; color of broth still orange without any change. All isolates were unable to grow in 15^0C while, all grown in 45^0C except *Lb*. 5 and *Lb*. 10, in addition to *Lb*.4, which grew slowly at such temperature.

Table (3 – 3). Biochemical tests of Lactic acid bacteria isolated from stool samples.

isolates	Catalase	Gelatin hydrolysis	Indole	Nitrate reduction	Acid production	Growth in 45^0C	Growth in 15^0C
Lb. 1	–	–	–	–	+	+	–
Lb. 3	–	–	–	–	+	+	–
Lb. 4	+/–	–	–	–	+	+/–	–
Lb. 5	–	–	–	–	+	–	–
Lb. 7	–	–	–	–	+	+	–
Lb. 10	–	–	–	–	+	–	–
Lb. 11	–	–	–	–	+	+	–

– = Negative result

+ = Positive result

+/– = Slow growth

Carbohydrate fermentation test was performed to identify the seven species of *Lactobacillus*. The isolates were different in their ability to ferment carbohydrate sources used in this study. Table (3 – 4) shows that all isolates were able to ferment glucose and lactose. But unable to ferment xylose and mannitol. Isolates, which were ferment glucose and lactose in addition to raffinose, are identified as *Lactobacillus acidophilus*. While, those able to ferment all previous sugar (glucose, lactose, xylose, manitol, raffinose) were identified as *Lactobacillus plantarum*. On the other hand, isolates able to ferment all sugar used in this study except raffinose, were identified as *Lactobacillus fermentum*.

Table (3 – 4) Ability of Lactic acid bacteria to ferment carbohydrate source.

LAB isolate	Glucose	Lactose	Xylose	Manitole	Raffinose	*Lactobacillu*
Lb. 1	(1)+	(1)+	–	–	(1)+	*L. acidophilus*
Lb. 3	(1)+	(2)+	(1)+	(2)+	(1)+	*L. plantarum*
Lb. 4	(1)+	(1)+	(1)+	(2)+	(3)+	*L. plantarum*
Lb. 5	(1)+	(2)+	(1)+	(1)+	(3)+	*L. plantarum*
Lb. 7	(1)+	(1)+	(2)+	(1)+	–	*L. fermentum*
Lb. 10	(1)+	(3)+	(1)+	(1)+	–	*L. fermentum*
Lb. 11	(1)+	(1)+	(1)+	(2)+	–	*L. fermentum*

+ = Positive fermentation

– = Negative fermentation

( ) = No. of days to change the color.

3.4: Antifungal sensitivity of *Candida albicans:* -

3.4.1: Determination of minimum inhibition concentration (MIC):

Antifungal treatment for *Candida albicans* are limited to three compounds, mainly azoles, polyenes, and echinocandins. Azoles such as fluconazole act on ergosterol biosynthesis as fungistatic. Emergence of resistance to azoles is an increasing problem. Polyenes, such as amphotericin B and nystatin, bind to membrane sterols, leading to membrane permeability, leakage, and cell death. These drugs had clinical drawbacks based on their toxicity. Echinocandins were a new class of antifungal agents that inhibit the synthesis of 1,3-ß-D-glucan, a key component of the cell wall (Gordon *et. al.*,

2002). In this study was used nystatin to determine its inhibition activity against isolate (C.2) of *Candida albicans*, which produced highest number of germ tube. Table (3 – 5) shows the ability of different *Candida albicans* isolates to form germ tube from budding cells. MIC for nystatin were 200 µg/ml in liquid media. On solid media, number of colonies reached $16*10^4$ when compare with the original (control) culture $133*10^4$ after treatment by nystatin. Doreen and Cawson, (1978) reported that blastospores of *Candida albicans* contains high concentration of both acid phosphatase and succinic dehydrogenase which present in cell membrane, the activity of these enzymes are changed after 90 min. of exposure to nystatin and only trace activity remains.

Table (3 – 5) Ability of *Candida albicans* isolates to form germ tube in human serum.

Candida albicans isolate	No. of germ tube formed
C. 1	$50*10^4$
C. 2	$70*10^4$
C. 3	$63*10^4$
C. 4	$15*10^4$

3.4.2:Determination of inhibitory effect of LAB: -

3.4.2.1: On solid media: -

Studying the effect of LAB on fungi are complicated by the fact that fungi are sensitive to the normal by–products of LAB metabolism, like acetic and lactic acids. The observed effectiveness of an antifungal agent is dependent on the test used. Agar–diffusion methods was used for detecting the effect of LAB on *Candida albicans*. This method is usually used due to the fact that antimicrobial synthesis is sometimes dependent on direct contact between the indicator and test organism (Ress, 1997). In the following experiments, LAB isolates (*Lb.*1, *Lb.*3, *Lb.*4, *Lb.*5, *Lb.*7, *Lb.*10, *and Lb.*11) were used against isolate *C.*2 of *Candida albicans.* No inhibition zone was observed when LAB isolates were propagation on MRS agar against the yeast isolate cultured, previously on SDA. This result was agreed with that by Ress, (1997) who found that agar diffusion method was less effective method in investigating the inhibitory effect of LAB on *Candida albicans* because LAB may not be given sufficient time to express its antimicrobial activity and the action to replace the inverted agar tends to smear the streak of the test culture.

3.4.2.٢: in liquid media: -

Well–diffusion method was used to determinate the inhibitory effect of LAB isolates on *Candida albicans* by filling the wells with SDA palate which is cultured by the *C.*2 with the filtrate of three LAB isolates (*Lb.*1, *Lb.*5, *Lb.*7). Selection of these isolates depended upon high quality number of colonies produced. Plate (3 – 1) shows that no inhibition zone was formed. This result was disagreed with Ress, (1997) who found that the filtrate of a culture of *Lactobacillus acidophilus* was able to slightly retard growth of *Candida albicans*, while agreed with the result of Ogunbanwo *et.*

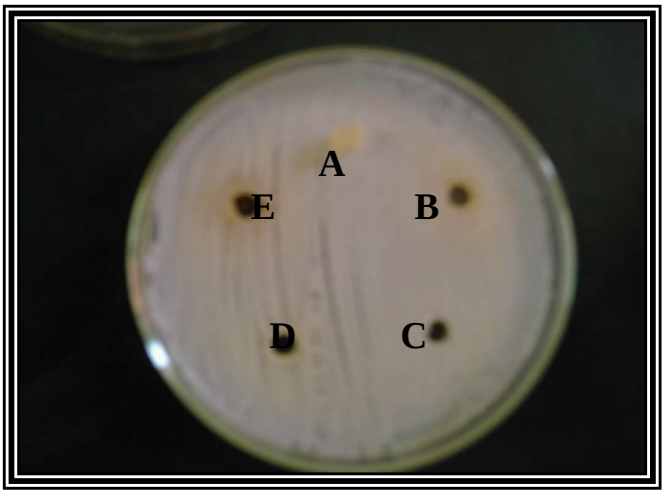
el., (2003) who reported that *Lactobacillus plantarum* and *L. brevis* were unable to inhibited *Candida albicans*.

3.5: Inhibitory effect of LAB filtrate on *Candida albicans:* -

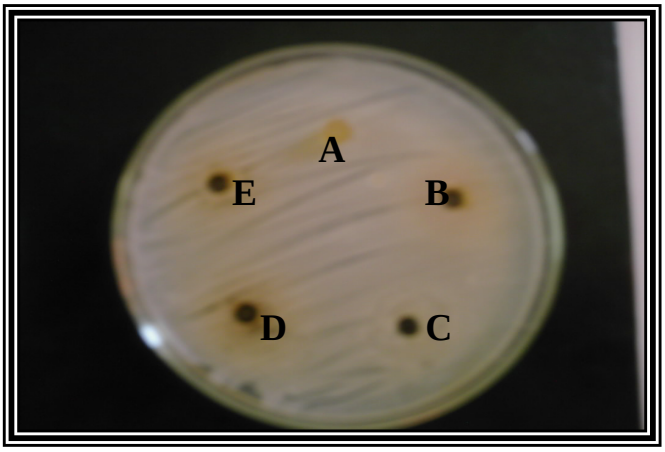
3.5.1: Effects of live LAB on *Candida albicans* morphogenesis: -

Candida albicans is a dimorphic yeast, with the ability to grow both as yeast and as a hyphae. Conversion to the hyphal form is required for virulence and invasiveness in vivo. *Candida albicans* morphogenesis is regulated by multiple signals and signaling pathways. However, signals that control morphogenesis in vivo are unknown. LAB culture supernatants as well as live LAB were inhibited *Candida albicans* morphogenesis (Mairi and Gary, 2004**).** When effect of LAB isolates on formation of germ tube by *Candida albicans* was investigated in induction media (human serum), results declared that no inhibitory activity was recorded by all lactic isolates tested on *C*.2 isolate of *Candida albicans* as shown in figure (3–1). This may be related to the inability of LAB cells to adhere to the receptor site of pathogen (germ tube) and block them. Such findings were agreed with those of Mairi and Gary, (2004**)** who reported that live LAB can inhibited *Candida albicans* morphogenesis. This inhibition was not due to competitive exclusion, because LAB do not adhere to *Candida albicans*, which cultured on tissue culture plastic.

L. acidophilus



L. plantarum



L. fermentum

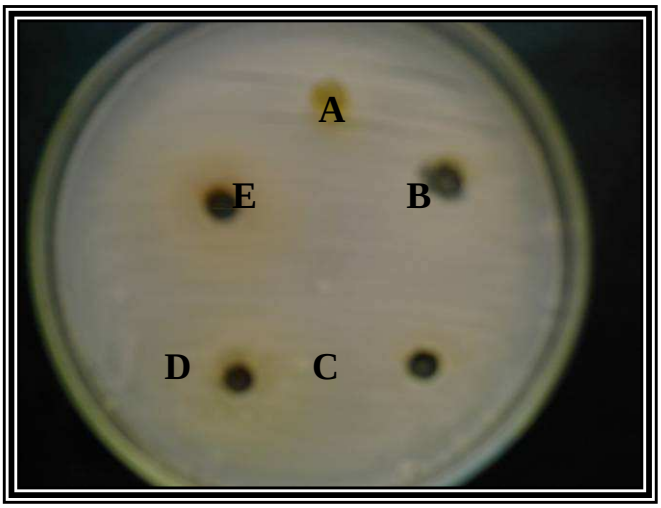


Plate (3 – 1) Inhibitory effect of *L. acidophilus, L. plantarum and L. fermentum* against *Candida albicans C.*2 isolates in liquid media (MRS broth) after 24 hrs.

A: Inhibitory effect of LAB isolates against *Candida albicans C.*2 isolates on solid media. B: MRS broth control. C: LAB isolates concentrated filtrate one fold. D: LAB isolates concentrated filtrate two fold. E: LAB isolates concentrated filtrate three fold.

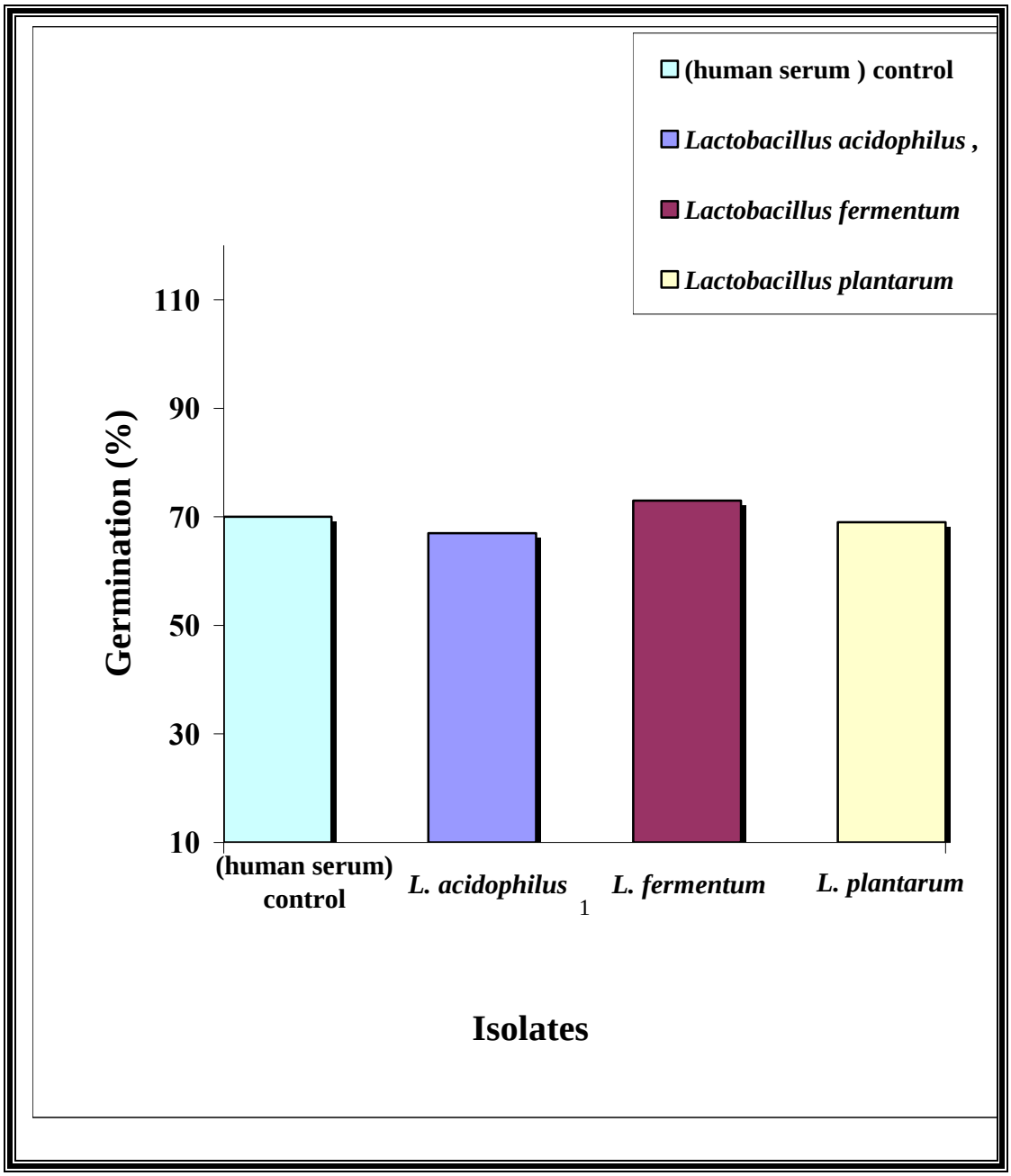


Figure (3 – 1) Effect of live LAB on *Candida albicans* morphogenesis.

Candida albicans isolate was grown in induction media (human serum) at 37^0C for 2 – 4 hr.

3.5.2:Effect of LAB filtrates on *Candida albicans* morphogenesis: -

LAB are known to inhibit *Candida albicans* colonization to the epithelium of the gastrointestinal tract and subsequent hyphal invasion and systemic infection. Depending on such property, *Candida albicans* germ tube assay was used to determine the MIC of LAB isolates on formation of germ tube (Mairi and Gary, 2004). For this purpose, various dilutions of the three – fold concentrate filtrates of LAB isolates (Lb. 1, Lb. 5, Lb. 7.) were prepared. Results of table (3 – 6) show that dilution of 1% to 10% filtrate had no effect on germ tube by the yeast. Filtrate of *Lb*. 1 in the dilutions 30% and up cause complete inhibition of germ tube formation by isolate C.2. Dilutions of 60% and above were needed of dilutions of *Lb*. 5 filtrate to gave similar effect on *C*.2 isolates. On the other hand, for *Lb*. 7 filtrate dilutions 40% were required for completely inhibited of growth of *C*.2 isolates. Depending on above results, filtrate dilutions; (20%) of Lb.1, (50%) of Lb.5, and (30%) of Lb.7 was recorded as the MIC of the LAB isolates against *Candida albicans* isolate *C*.2. Figure (3 – 2) illustrates occurrence of complete effect of LAB isolates on germ tube formation by *Candida albicans* isolate *C*.2 in comparison to the control (*Candida albicans* isolates previously grown in human serum). While Plate (3 – 2) shows formation of germ tube by *Candida albicans* isolate *C*.2 when grown in human serum as well as the effect of LAB on it after treatment with different dilutions of the LAB three – fold concentrate. This result was agreed with Timothy, (1997) reported that ingestion of probiotic organisms such as *Lactobacillus acidophilus* has been used for treating candidiasis, which caused by *Candida albicans*. Also Doug *et. al*., (1997) when their study the effect of probiotic bacteria to reduce candidiasis infection in mice, found that the prolonged survival of mice, decreased severity of mucosal and systemic candidiasis, modulation of immune responses, decreased number of *C. albicans* in the

alimentary tract, and reduced numbers of orogastric infections demonstrated not only that probiotic bacteria have biotherapeutic potential for prophylaxis against and therapy of this fungal disease but also that probiotic bacteria protect mice from candidiasis by a variety of immunologic (thymic and extrathymic) and nonimmunologic mechanisms in this model.

Table (3 – 6) Minimum inhibition concentration of LAB filtrates against *Candida albicans* isolate *C*.2.

MIC / LAB isolates	Percentage of three – fold concentrated filtrate											
	1	2	4	8	10	20	30	40	50	60	70	80
Lb. acidophilus	+	+	+	+	+	+	–	–	–	–	–	–
Lb. plantarum	+	+	+	+	+	+	+	+	+	–	–	–
Lb. fermentum	+	+	+	+	+	+	+	–	–	–	–	–

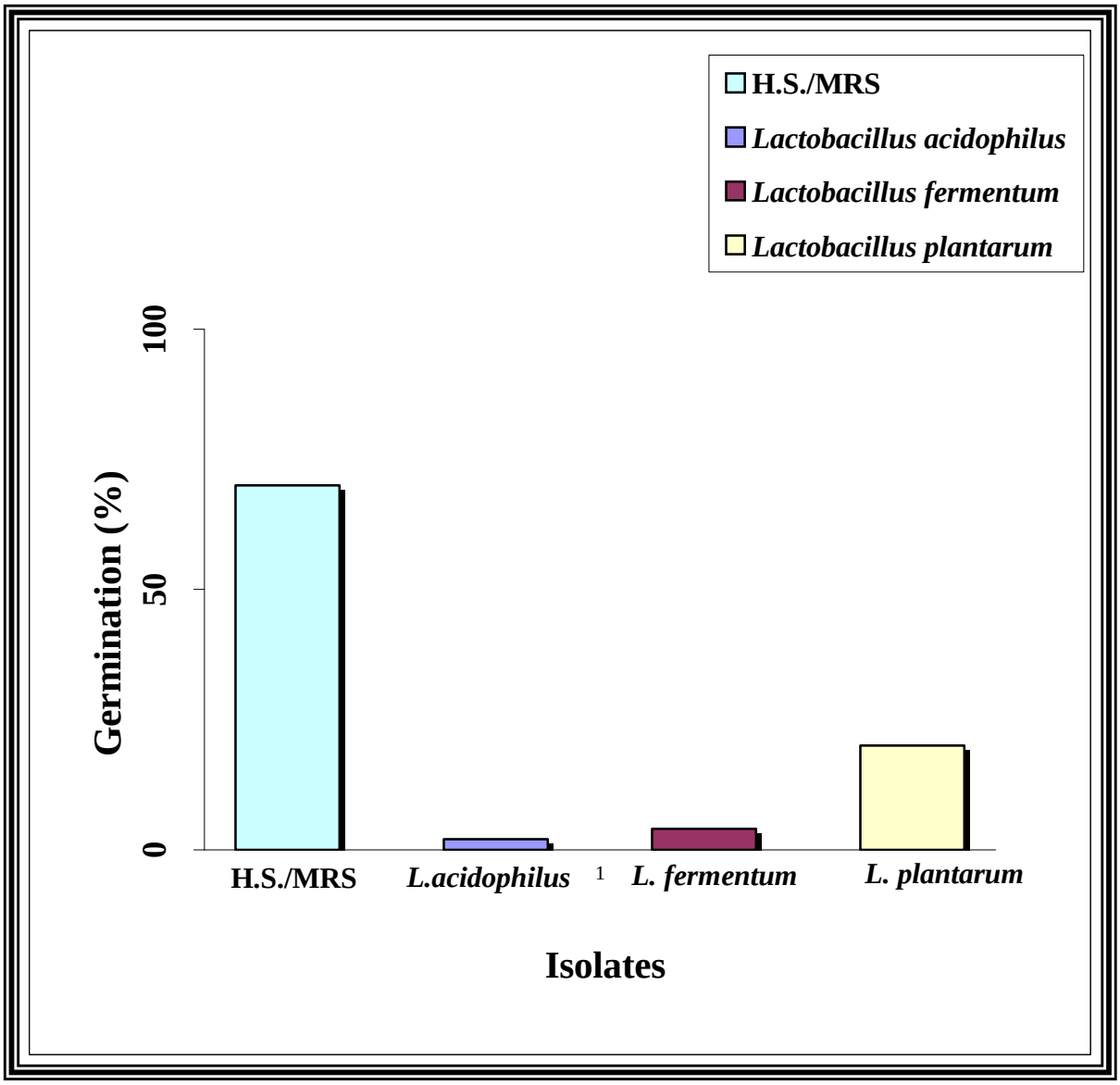
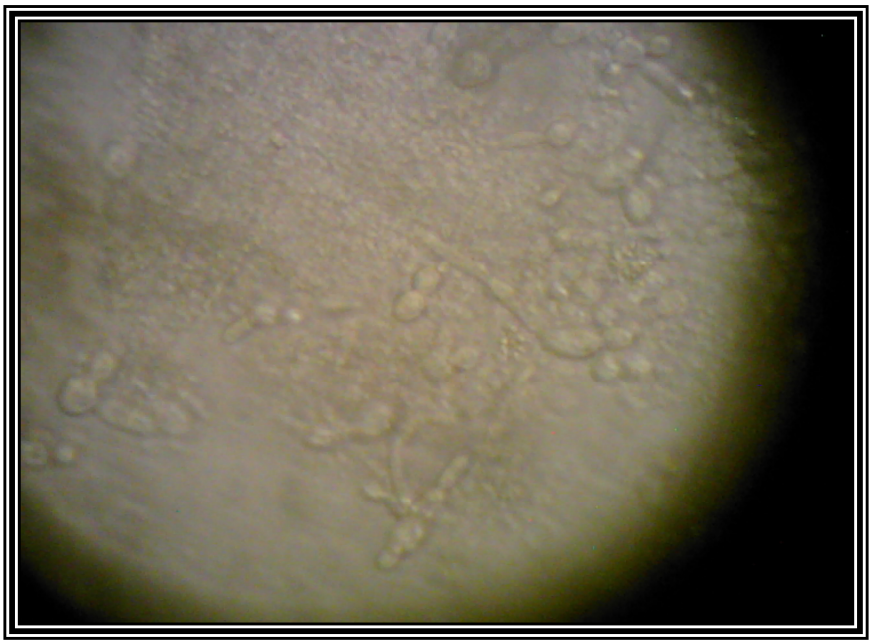


Figure (3 – 2) Effect of LAB filtrate on *Candida albicans* morphogenesis. *Candida albicans* isolate was grown in induction media (human serum) at 37^0C for 2 – 4 hr.

A/



B/

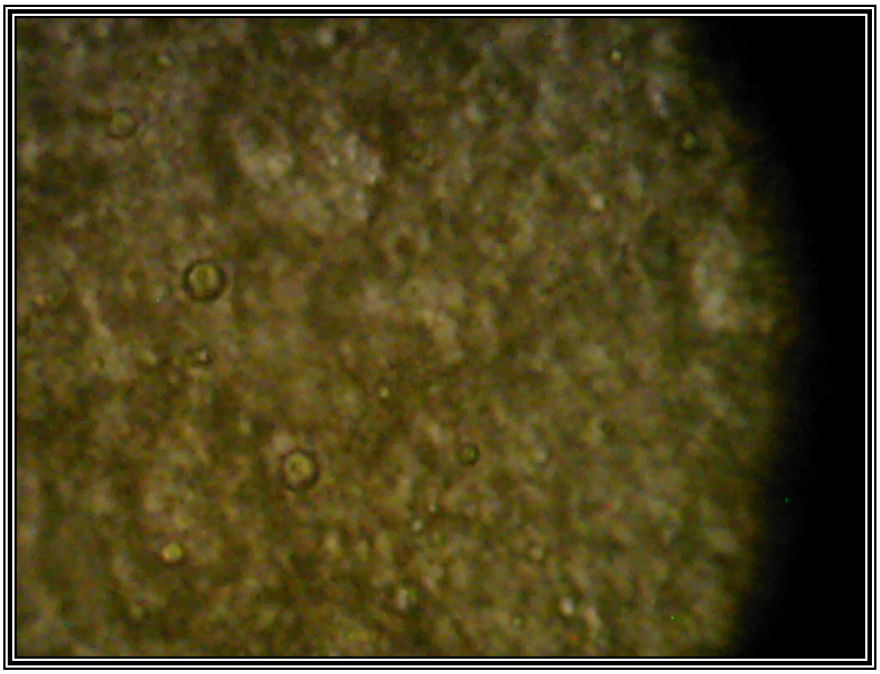


Plate (3 – 2) Inhibitory effect of LAB isolates on *Candida albicans* germ tube formation under light microscope at (400x).

A: Control (*Candida* isolate of grown in human serum). **B:** After treatment of C*andida albicans* isolate *C.* 2 with three – fold concentrated LAB filtrates

Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University / College of Science
Department of Biotechnology

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A thesis

submitted to the College of Science / Al – Nahrain University

In partial fulfillment of the requirements for the degree of

Master of Science in Biotechnolongy

By

Sura Ali Abdul lateef

B.Sc. Biotechnology/College of Science – 2003

Nov. /2006 Dhulqda/ 1427

جمهورية العراق
وزارة التعليم العالي و البحث العلمي
جامعة النهرين / كلية العلوم
قسم التقانة الإحيائية

تأثير بعض المعززات المجهرية على بعض المبيضات الفطرية المسببة للإسهال

رسالة

مقدمة إلى كلية العلوم جامعة النهرين

و هي جزء من متطلبات نيل درجة الماجستير علوم في التقانة الأحيائية

من قبل

سرى علي عبد اللطيف

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ذو القعدة / ١٤٢٧ تشرين الثاني /٢٠٠٦

بِسمِ اللهِ الرَحْمنِ الرَّحِيمِ

{ وَيَرَى الَّذِينَ أُوتُوا الْعِلْمَ الَّذِي أُنْزِلَ إِلَيْكَ مِنْ رَبِّكَ هُوَ الْحَقَ و ۘيَهْدِي إِلَى صِرَاطِ الْعَزِ يز الْحَمِيد}

صَدَقَ الله العَظيم

سورة سبأ– الآية "٦"

معلومات شخصية

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تاريخ المناقشة: ٢٠٠٦/١١/٢٧

عنوان الرسالة:

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