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The Antimicrobial Activity of *Lactobacillus Acidophilus* Against Intestinal and Foodborne Pathogens

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Abstract

In this study, isolated of *Lactobacillus acidophilus* were evaluated for their anti-pathogenic bacterial activity. The antibacterial activity of the three isolated against intestinal and food borne pathogenic bacteria *in vitro* was determined by Well's Diffusion method, a total of three isolates of *Lactobacillus acidophilus* isolated from ten different brands of traditional yoghurts showed a various antibacterial activity against tested pathogenic bacterium, *Cronobacter sakazakii* isolated from stool samples was more sensitive to the inhibition(23mm)inhibtion zone than were *Helicobacter pylori* that isolated from stool samples (16mm) inhibtion zone and *Clostridium perfringens* that isolated from stool samples (15mm). These results may provide a basis for support therapies for the treatment of intestinal and foodborne pathogens.

Keywords: *L. Acidophilus*, Antibacterial, Intestinal and Food Borne Pathogens.

تأثير *Lactobacillus acidophilus* ضد مسببات الأمراض المعوية والتسمم الغذائي

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الخلاصة

في هذه الدراسة تم تقييم التأثير التثبيطي لعزلة*Lactobacillus acidophilus* في مكافحة البكتريا المرضية المسببة للأمراض المعوية والتسمم الغذائي خارج الجسم الحي وبطريقة Well's Diffusion method، حيث تم عزل ثلاثة عزلات ل*Lactobacillus acidophilus* من عشر ماركات مختلفة من الزبادي التقليدي وأظهرت قابلية متنوعة لتثبيط نمو البكتريا المرضية ،حيث اظهرت *Cronobacter sakazakii* حساسية عالية للتثبيط (23mm)مقارنة ب *Helicobacter pylori* (16mm)و *perfringens* Clostridium (15mm) و قد توفر هذه النتائج أساسا لعلاجات مساعدة لعلاج الامراض المعوية والتسمم الغذائي.

Introduction

Lactobacillus are member of lactic acid bacteria (LAB), defined by formation of lactic acid, in dairy products such as yoghurts, cheese and fermented milks, are naturally existent or added purposely due to their heath benefit for the consumer [1], Beneficent the microbial safety of food and also as a probiotic in animals and humans to improve the balance of microflora and to inhibit pathogenic bacteria in intestinal tract [2]. The health benefits presented by LAB can be nutritional or therapeutic including decrease the risk of diarrhea, immunomodulation, production of vitamins, and mutagenic

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activity. Lactic acid bacteria are able to create diverse antimicrobial including bacteriocins, hydrogen peroxide, organic acids and diacetyl [3]. Bacteriocins are proteinaceous antibacterial compounds that exhibit bactericidal activity, are the best alternatives to conventional antibiotics without raising the antibiotic resistance level [4], bacteriocin can be added to foods as bio preservative and can also be used to treat the acute childhood diarrhea, and regulating immune response,and antimicrobial activity against some human pathogenics microbes [5]. Increased use of antibiotics is a key factor in the emergence of antibiotic resistant pathogens. *Lactobacillus spp* are reported to have inhibitory activity against common intestinal and food borne human pathogens through their ability to produce antibacterial substances such as bacteriocins which have potential to be used in therapeutics and as food biopreservatives[6].

The aim of the present study was to isolate and characterize *Lactobacillus acidophilus* from traditional different yoghurt brands and to assess their antibacterial activity against *Cronobacter sakazakii*, *Helicobacter pylori* and *Clostridium perfringens*.

Material and Methods

Isolation and Identification of *Lactobacillus acidophilus*

Ten samples of different brands of traditional yoghurts were collected from a local market. Samples were serially diluted in the range of (10^1- 10^6). 100 µl from each dilution were added to MRS broth. Test tubes were incubated at 37 for 24 h condition under anaerobic condition. A loopful of each culture was streaked on to the MRS agar. Plates were incubated at 37C for 48 h under anaerobic condition [7].

Bacterial isolates were identified based on the colony and biochemical characterization(Gram staining, production of catalase, indole, starch hydrolysis, casein hydrolysis, gelatinase, growth at 15°C and 45°C in MRS broth, ability to ferment sugars[8], and confirmed identified by Api20a system .(BioMėrieux, France).

Preparation of cell free supernatant

The bacterial strains were grown in MRS broth that contained 1.5% glucose for 24h at 30°C. The cultures were harvested by centrifugation at (6000 rpm/ 15 min/ 4°C) to obtain a cell free supernatant. Supernatants were filter-sterilized by passing through a sterile 0.2 µl pore size filter. The pH of the supernatants was adjusted to 6.5 with 10 N of NaOH [9, 10].

Detection of antimicrobial activity of the supernatants

Screening for antimicrobial activity in the tested supernatants against indicator pathogenic bacterial was performed on *C. perfringens* and *H.pylori* and *C. sakazakii* isolated from stool samples.

Antimicrobial activity of *Lactobacillus acidophilus* against *pathogenic bacteria* (*in vitro)*

Screening for antimicrobial activity of *Lactobacilus acidophilus* in the tested supernatants against *C. perfringens*, *H.pylori* and *C. sakazakii*. Using agar well diffusion methods [11], 50 µl of the sterile supernatant were placed in 4 mm diameter wells on Muller- Hinton-agar plates previously cultivated with the indicator pathogenic bacteria isolates. After 18 h of anaerobic incubation at 37°C, the diameters of the zones of growth inhibition were measured.

Source of *Cronobacter sakazakii*

A clinical fecal swab from stool samples were especially collected from patients under 2 years of age with necrotizing enterocolitis NEC at Central Children Hospital and Children's Protections Educational Hospital, Isolation and Identification of *C.sakazakii* was done as described by [12], by selective media Hicrome *Enterobacter sakazakii* Agar (HESA) (Himedia,India), Gram stains , biochemical Tests and API 20E test strips(BioMerieux, France) [13,14]. Disk diffusion methods was performed with Oxoid disks (Oxoid, UK) for antimicrobial susceptibility testing to Cephalothin , Clindamycin, Streptogramins, rifampicin, fusidic acid , tetracyclines, Ampicillin , Gentamicin , chloramphenicol and Azetronam antibiotics.

Source of *Helicobacter pylori*

Collect Stool samples from dyspeptic patients (duodenal and gastric ulcers) in a clean and dry receptacle after they underwent endoscopy at Endoscopy unit of Baghdad Teaching Hospital in Baghdad. *Helicobacter pylori* was isolation and identification by:-1-Selective Brain Heart Infusion Agar [15]. 2-Gram Stain .3- Stool Antigen Test according to the manufacturer's instructions (Abon biofarm /UK).4- Biochemical Tests [16]: Rapid Urease Test (RUT) of Colonies, Catalase Test. Disk diffusion methods was performed with Oxoid disks (Oxoid, UK),for antimicrobial susceptibility testing to Amoxicylin , metronidazole, Rifampin , Gentamycin , Erythromycin and Tetracycline antibiotics.

Source of *Closrtridium perfringens* isolates

C. perfringens was isolated from stool samples collected from Iraqi patient suffering from food poisoning cases in Baghdad hospitals (Child protection teaching hospital, Baghdad teaching hospital ,Private Nursing Home Hospital ,Children teaching hospital). *C. perfringens* was isolation and identification from stool samples previously by: 1- blood agar and TSN agar (Tryptone Sulfite Neomycin) (SIGMA-ALDRICH, USA) with anaerobic jar (Oxoid Anaerobic Jar with Anaerogen (AN0025, OXOID, UK) gas back Kit. [17- 19]. 2-Gram stain ,Malachite green for spore stain.3-Api20A Kit(BioMerieux,USA). 4-detection of enterotoxines in stool samples by ELISA Kit (biopharm,Germeny, RIDASCREEN® *Clostridium perfringens* Enterotoxin . Disk diffusion methods was performed with Oxoid disks (Oxoid, UK),for antimicrobial susceptibility testing to Gentamicin , Erythromycin , Colistin , Clindamycin, Metronidazole , Ampicillin and Chloramphenicol antibiotics [20,21].The inhibition zone was measured for each antibiotic were determined according to BSAC methods for antimicrobial susceptibility testing [22].

Results and Discussion

Isolation and Identification of *Lactobacillus acidophilus*

Out of 10 yoghurt samples, three *Lactobacillus acidophilus* were isolated. The isolates were identification according to morphological and biochemical tests methods (MacFaddin, 2000) in Table-1. These isolates were confirmed identified by Api20A system in Figure-1. A total of three isolates of *Lactobacillus acidophilus* isolated from different comercial yoghurts in Table-2.

Table 1- Biochemical test for identification of *Lactobacillus acidophilus*.

Test	L1	L2	L3
Gram stain	+	+	+
Motility	–	–	–
Catalase	–	–	–
Indole	–	–	–
MR	–	–	–
VP	+	+	+
Nitrate reduction	–	–	–
Gelatinase	–	–	–
Casein hydrolysis	–	–	–
Growth at 15°C	–	–	–
Growth at 45°C	+	+	+
Glucose	+	+	+
Fructose	+	+	+
Ribose	–	–	+
xylose	±	±	±
Lactose	+	+	+
Sorbitol	–	–	–
Raffinose	+	+	+
Rhamnose	–	–	–
Mannitol	–	–	–
Galactose	+	+	+
Sucrose	+	–	+
– **negative, +positive, ± variable**			

Figure 1-Api20A system for Identification of *L. Acidophilus*.

Table 2- Different comercial yoghurts.

Name of products	Company
Rotas , Lezzet Süt , M life	Zaho,Iraq
Danone , Nart , Activia	Erbil,Iraq
Kanoon, Abu Ghraib	Baghdad,Iraq
Nebrase	Kirkuk,Iraq
Seven Kalleh	Iran

Isolation and Identification of *Cronobacter sakazakii* : colonies on HESA media appears blue-black to blue-gray, raised colonies 1-2 mm diameter , with and without halos after 18-24 hr. at 37°C and microscopic examination appears Gram -ve, rod shape, appeared as single or chains,Figure-2(A&B). The urease, oxidase, coagulase, indol and H2S production gives negative result, while catalase, citrate and motality test gives positive result in Table- 3 , and antimicrobial susceptibility testing showing resistance to Cephalothin , Clindamycin, Streptogramins, rifampicin, fusidic acid and appeared sensitive to tetracyclines, Ampicillin , Gentamicin , chloramphenicol and Azetronam .

Table 3- Identification tests of *C. sakazakii* isolates.

Test	Results
Gram stain	Negative
Shape of cell	rod
Urease , Oxidase , Coagulase , Indol , H2S production	Negative
Catalase , Citrate , Motility	Positive

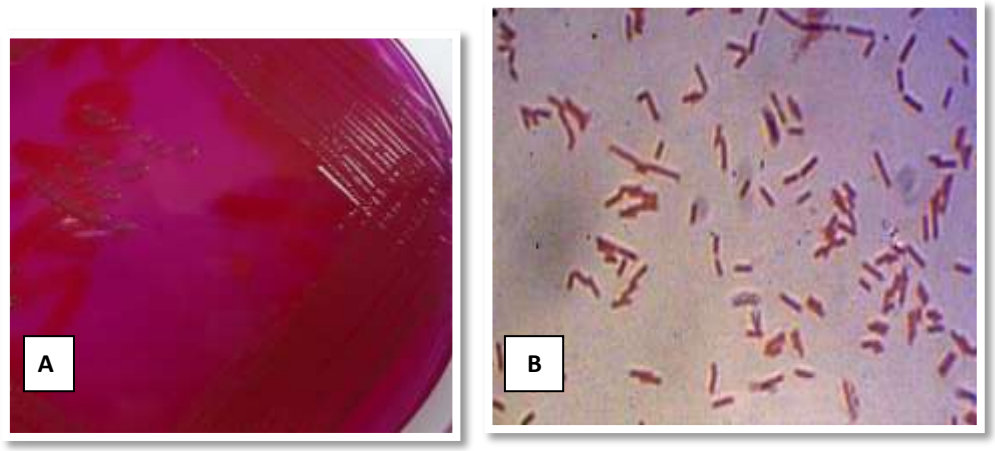

Figure 2-A: *C.sakazakii* on HESA media. **B**: Gram –ve, rod shape (100x).

Isolation and Identification of *Helicobacter pylori*:

colonies on Selective Brain Heart Infusion Agar media appeared tiny, glistering, translucent or gray and convex with entire edges, gram - negative ,cells appeared as slightly curved or straight rods to curved bacilli, Figure-3(A&B)., catalase positive, oxidase positive, urease positive, Stool Antigen Test positive in Table- 4 ,and antimicrobial susceptibility testing showing resistance to Amoxicylin, metronidazole, Rifampin,Gentamycin, Erythromycin and Tetracycline .

Table 4- Identification tests of *H. pylori* isolates

Test	Results
Gram stain	Negative
Shape of cell	straight to curved bacilli
catalase, oxidase, urease	positive
Stool Antigen Test	Positive

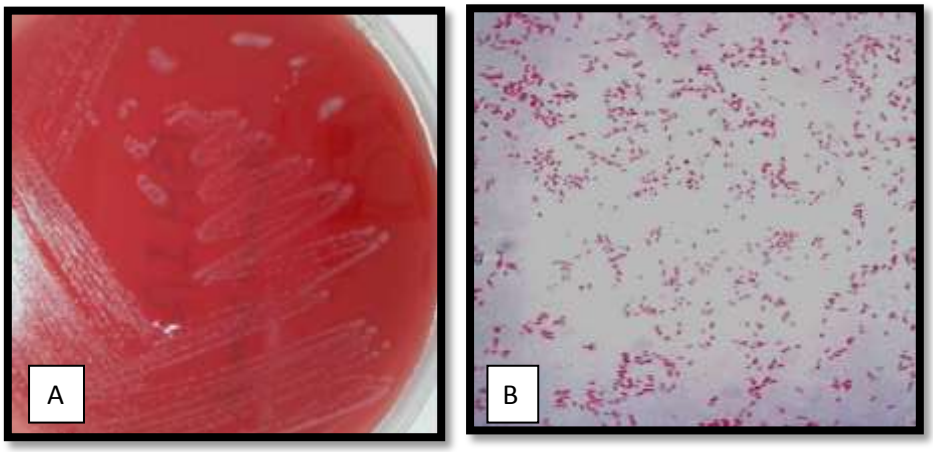

Figure 3-A: *H. pylori* on Selective Brain Heart Infusion Agar media. **B**: Gram –ve, straight to curved bacilli (100 x).

Isolation and Identification of *C. perfringens*:

On blood agar under anaerobic conditions colonies appeared with double zone of hemolysis were identified, after staining by Gram and Malachite Green stains, all isolates were gave gram positive, spore forming (sub terminal spores) rod shaped cells, occur in pairs or short chains in Figure- 4(A&B), identification by ELISA was presence of enterotoxin. Biochemical test to further confirmation with API 20A kits (bio Mérieux)was positive in Table- 5 , and antimicrobial susceptibility testing showing resistance to Gentamicin , Erythromycin , Colistin, Clindamycin, Metronidazole, Ampicillin and Chloramphenicol antibiotics.

Table 5-Biochemical tests of *C. perfringens* isolates.

Test	Results
Gram stain, spore forming	positive
Shape of cell	rod
Enterotoxin by ELISA	positive
API 20A	positive

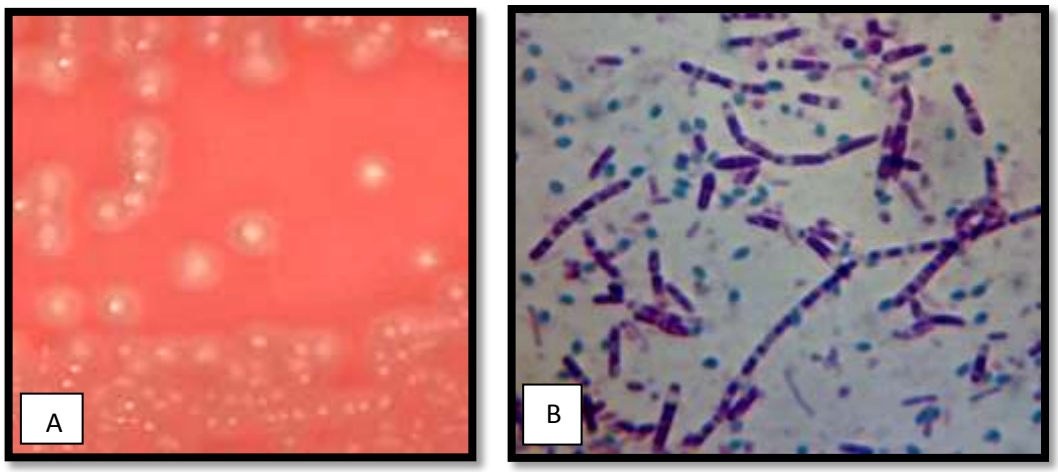

Figure 4-A: *C. perfringens* colonies appeared with double zone of hemolysis on blood Agar media. **B**: Gram +ve, spore forming, rod shape (100x).

Antimicrobial effect of *L.acidophilus* against three isolated pathogenic bacteria (*In vitro*).

The antibacterial activity of the *L. acidophilus* against pathogenic bacteria *C.perfringens in vitro* was determined by Well's Diffusion method. *L. acidophilus* showed antibacterial activity against selected local *C.perfringens* isolate that given resistance to many antibiotics used in sensitivity tested, the higher diameter of inhibition zone was (15 mm) at *L. acidophilus* L2 isolated,while lower inhibition zone at L1 isolated (13mm), and L3 (11mm), This indicates that the *Lactobacillus acidophilus* possessing of antibacterial activity, Figures- (5&6).

The antibacterial activity of the *L. acidophilus* against pathogenic bacteria *H.bylori in vitro* was determined by Well's Diffusion method. *L. acidophilus* showed antibacterial activity against selected local *H.pylori* isolate that given resistance to many antibiotics used in sensitivity tested, the higher diameter of inhibition zone was (16 mm) at *L. acidophilus* L2 isolated, while lower inhibition zone at L1 isolated (14mm), and L3 (13mm), This indicates that the *Lactobacillus acidophilus* possessing of antibacterial activity, Figures- (5&6).

The antibacterial activity of the *L. acidophilus* against pathogenic bacteria *Cronobacter sakazakii in vitro* was determined by Well's Diffusion method. *L. acidophilus* showed antibacterial activity against selected local *Cronobacter sakazakii* isolate that given resistance to many antibiotics used in sensitivity tested, the higher diameter of inhibition zone was (23 mm) at *L. acidophilus* L2 isolated, while lower inhibition zone at L1 isolated1(19mm), and L3 (18mm), This indicates that the *Lactobacillus acidophilus* possessing of antibacterial activity, Figures- (5&6).

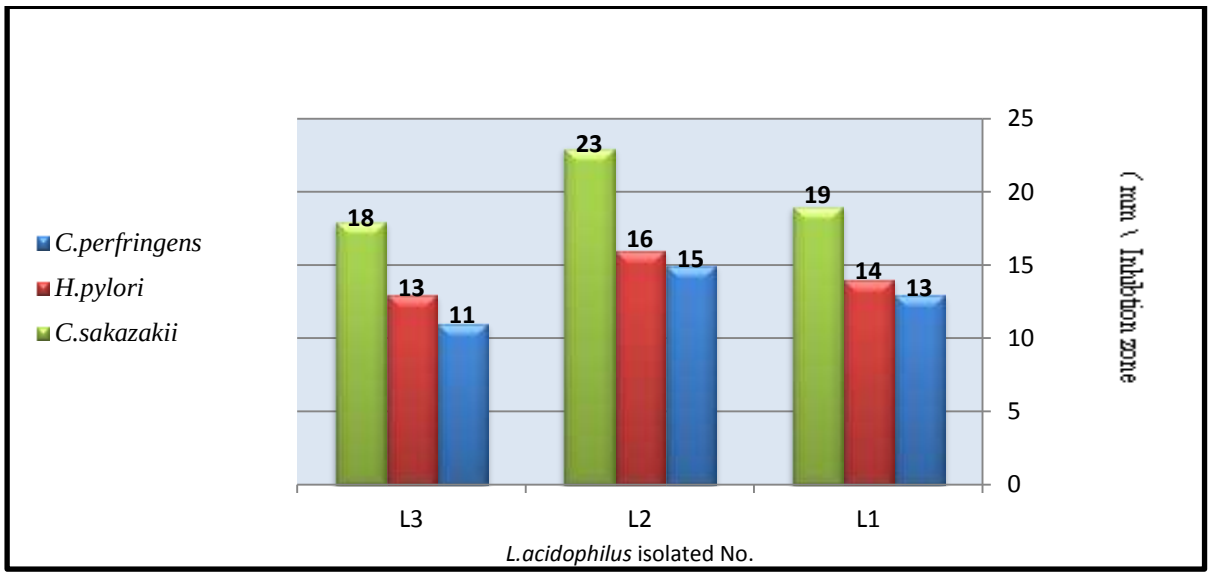

Figure 5- Antimicrobial activity of *Lactobacillus acidophilus* against 3 pathogenic bacterial *spp*.

Growth culture of the three *Lactobacillus acidophilus* isolates demonstrated wide antibacterial spectrum against indicator intestinal and food borne pathogenic bacteria

The results of present study, suggest that the cell-free-supernatants exerted varying inhibitory effect on the indicator pathogenic isolated. The highest inhibitory activity observed was against *Cronobacter sakazakii***.** This indicates that the *Lactobacillus acidophilus* possessing of antibacterial activity.

C.sakazakii it is an opportunistic human and food- borne pathogen. The organism can be found in broad range of food including powdered infant formula (PIF) , cheese, meat, vegetables, grain, herbs , spices , tomato , water and households[23, 24] These results coincides with pervious study which shown that *Lactobacillus acidophilus* has ability to inhibit and safe bio-preservative protect infants from *C.sakazakii* [25, 26].

Privious studies shown that higher doses of *L. acidophilus* pre-treatment reduce *H. pylori*- induced inflammation, Experimentally, *L. acidophilus* decreases the viability of *H. pylori in vitro* independent of pH and lactic acid levels.[27- 29],and *C. peifringens* were sensitive to the inhibition by *L. acidophilus*[30- 32].

For many decades, *lactobacilli* have been used as an effective therapy for treatment of several pathological conditions displaying an overall positive safety profile. *lactobacilli* can inhibit the growth of pathogenic bacteria *in vitro* and *in vivo* and have a favourable safety profile, shown that prolonged *lactobacilli* administration induces alteration in the human gastrointestinal microbial ecosystem with hopeful view in cancel pathology- related physiological and immunological changes.

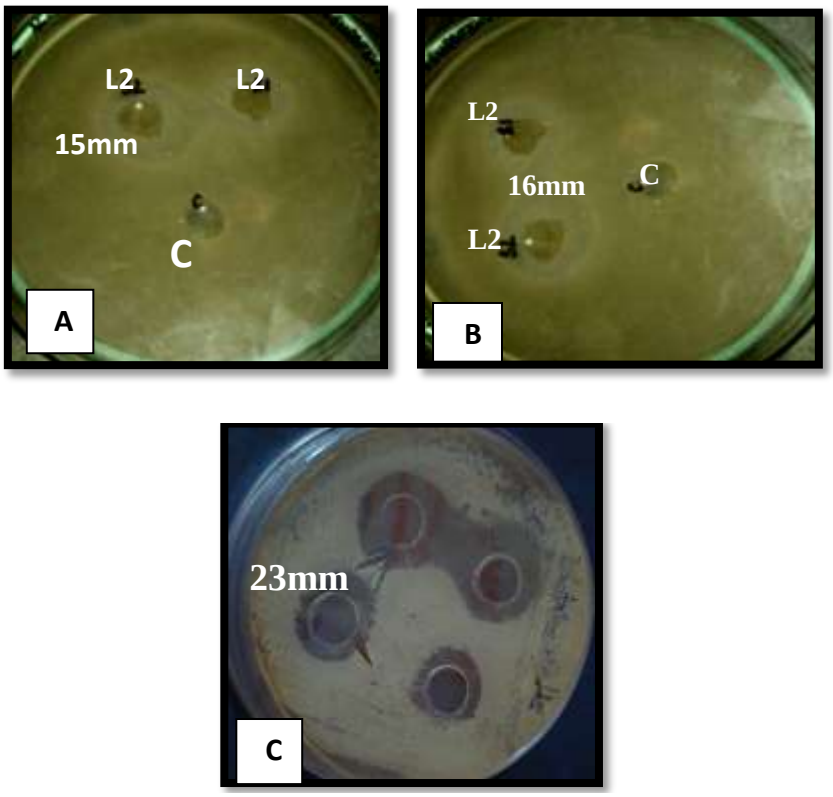

Figure 6- Antimicrobial activity of *L. Acidophilus* against 3 pathogenic bacterial *spp*.: **A**: *C.perfringense*, **B**: *H.Pylori* , **C**: *C.sakazakii* .

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