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Antagonistic activity of probiotic (*Lactobacillus*) against food-borne pathogenic bacteria.

A Research Project

Submitted to the Council of the College of Education-Shaqlawa, Salahaddin University – Erbil in
Partial Fulfillment of the Requirements for the Degree of Bachelor in Biology

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CERTIFICATE

This research project has been written under my supervision and has been submitted for the award of the **BSc.** degree in **Biology** with my approval as a supervisor.

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Signature:

Date: 10 / 04/ 2023

DEDICATION

We declare that a research project entitled: **Antagonistic activity of probiotic (Lactobacillus) against food-borne pathogenic bacteria** is our own original work, and hereby certify unless stated, all work contained within this research project is our own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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ABSTRACT

Probiotics, live cells with different beneficiary characteristics, have been extensively studied and explored commercially in many different products in the world. Their benefits to human and animal health have proven in hundreds of scientific studies. Lactobacillus are one of the most commonly used probiotics in medical field. The aim of the present study was to examine the antagonistic activity of six Lactobacilli strains isolated from commercially available probiotic medicine including Advanced probiotic, Biolact, Lactoflor kids, Lactozinc, Probiodex and ProIBSand. All the selected Lactobacillus isolates were then characterized in vitro for their antimicrobial activities against five pathogenic bacteria divided to three enteric gram-negative bacterial species including *Klebsiella pneumoniae*, *Proteus mirabilis* and *Escherichia coli*, and two-gram positive cocci including *Streptococcus pneumoniae*, *Staphylococcus aureus*. Based on the inhibition zone measurement (mm), our results showed that the Biolact strain of Lactobacillus had the highest influence on the selected pathogens, (mean = 22, SD= 8.1) and Lactoflor Kids strain had the lowest influence, (mean = 16.6, SD= 5.46), One-Way ANOVA has shown no significant deference among the six probiotic bacterial strains ($P > 0.5$). In conclusion, Lactobacillus strains isolated in this study from the different probiotic medicine having in vitro properties that make them potential against pathogenic bacteria. The five tested clinical isolates were susceptible to all the six strains of Lactobacillus. These results collectively suggest that probiotics particularly the commercially available types are important for combating bacterial pathogens.

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LIST OF ABBREVIATIONS

- Lactic acid bacteria (LAB)
- [Generally Recognized as Safe (GRAS)]
- The American Type Culture Collection (ATCC)
- CAP Laboratory Accreditation helps laboratories: Maintain accuracy of test results and ensure accurate patient diagnosis.
- PCR. polymer chain reaction.
- MRS broth (De Man, Rogosa and Sharpe) is a liquid medium recommended for use in the cultivation of *Lactobacillus* species and in the differentiation of gram-positive cocci.
- BHI ... is a growth medium for growing microorganisms, can therefore be used to culture a variety of fastidious organisms.
- *K. pneumonia. Klebsiella pneumoniae*
- *P. mirabilis. Proteus mirabilis*
- *S. pneumonia. Streptococcus pneumoniae*
- *S. aureus...Staphylococcus aureus*
- *E. coli. Escherichia coli*

1. INTRODUCTION

Throughout the history of microbiology, most human studies have been focused on the disease-causing organisms found on or in people; whilst fewer studies have examined the benefits of the bacteria. However, we are surrounded by beneficial microorganisms that live in or on the human body Hotel and Cordoba (2001), (FAO/WHO 2002).

Probiotics are defined as live microorganisms when administered in adequate amounts confer a health benefit on the host. Victor, François et al. (2011) Lactic acid bacteria (LAB) are considered as [Generally Recognized as Safe (GRAS)] organisms and can be safely used as probiotics for medical applications.

Members of the genus *Lactobacillus* are the most common probiotics used in commercial fermented and non-fermented products, such as in some yogurts and fermented milk drinks, as well as dietary supplements, which are available in the market in the form of capsules, liquid/gel, and powdered, to combat the diseases due to their ability to inhibit the pathogenic microorganisms,(Sonomoto and Yokota 2011),(Ghodduzi 2012) *Lactobacilli*, primarily facultative or strict anaerobes generally have fastidious growth requirements. They prefer an acidic environment by producing lactic and other acids. In general, *Lactobacilli* have not been associated with disease and have been regarded as non-pathogenic and isolates were able to tolerate the acidic condition of the environment, NaCl concentration, and resistance to bile. The reported health benefits of probiotics include: boosting of the immune system, inhibition of the growth of pathogenic organisms, prevention of diarrhea from various causes, improvement of digestion of proteins and fats and synthesis of vitamins, (Prabhurajeshwar and Chandrakanth 2017).

Probiotics, a word derived from Latin, that means 'for life', have been with us for as long as people have eaten fermented milk, but their association with health benefits dates only from the turn of the last century. Growing awareness of the health benefits of

consuming microorganisms such as probiotics have encouraged consumers worldwide. These probiotic bacteria are essential for their beneficial effect on a particular organism's health and host nutrition for healthy gastrointestinal function. The original modern hypothesis of the Page 3/9 positive role played by certain bacteria was first introduced by Russian scientist Élie Metchnikoff, who in 1907 suggested that it would be possible to modify the gut microbiota and replace harmful microbes with useful microbes (Metchnikoff 2004).

On the other hand, the rise in antibiotic-resistant has awakened the scientific community to the prophylactic and therapeutic uses of probiotics and to reconsider them as alternatives to antibiotics (Fijan 2016) Antimicrobial activity of Lactobacillus strains against bacterial pathogens emerges to be multifactorial and includes the production of hydrogen peroxide, lactic acid, bacteriocin-like molecules, and unknown heat-stable, non-lactic acid molecules. (Servin 2004). Other mechanisms proposed for their activity are competition for nutrients (McFarland 2000, Reid and Burton 2002).

Adhesion inhibition of pathogens to the surface, and stimulation of the immune system (Gill, Rutherford et al. 2001). One of the important features of probiotic lactobacilli to achieve antagonistic activity against bacterial pathogens because of their capacity to produce lactic acid and other organic acids that lower the pH in the human intestine, and to produce H₂O₂ and bacteriocin, thereby establishing a hospital environment for the growth and survival of various human pathogenic bacteria. There are also other physiological benefits of probiotics that have been published as it helps in the removal of carcinogens, lowering of cholesterol, immune-stimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance. (Ouwehand, Salminen et al. 2002).

Therefore, since the important of studying the antagonistic activity of probiotic (Lactobacillus) against food-borne pathogenic bacteria, according to the aims in this study are:

- Obtaining and propagation of the selected pathogenic bacterial species. American ATCC strains of the bacterial isolates will be obtained from Media Diagnostic Center which is a CAP accredited Lab.
- Isolation of Lactobacillus species from commercially available probiotic medicine including (Lactoflor, Advanced Probiotics, ProIBS, etc...). We have already obtained MRS medium (broth and Agar) to isolate and enhance LAB.
- Molecular and Morphological identification of Lactobacillus using PCR and Vitek
- Evaluation of antagonistic efficacy of Lactobacillus against five species of pathogenic bacteria using agar diffusion assay.

2. METHODOLOGY AND RESEARCH DESIGN

Isolation and propagation of clinical isolates:

Probiotic Lactobacillus isolates from commercially available probiotic medicine were isolated using enrichment and selective media. Lactobacillus cultured in MRS broth and agar and incubated at 37°C under anaerobic conditions for 24-48 hours (anaerobic jar supplied with gas generating kits).



Figure 1: The probiotic sources used to isolate the six strains of Lactobacillus included: Advanced probiotic, Biolact, Lactoflor kids, Lactozinc, Probiodex and ProIBS.

The isolation of Lactobacillus species from commercially available probiotic medicine including (Advanced probiotic, Biolact, Lactoflor kids, Lactozinc, Probiodex and ProIBS). were carried out in Media center Laboratories in Erbil city, we have already obtained MRS (OXOID, UK) medium (broth and Agar) to isolate and enhance

LAB. Some of urgent works was performed in the both Laboratory of Microbiology/Biology department, college of Education/ shaqlawa, Salahaddin University-Erbil, Iraq and MLT Department, Shaqlawa Technical College, Erbil Polytechnic University, Erbil, Iraq.

Our research about activity of lactobacillus that type antibiotics, on each five types of pathogenic bacteria like gram positive 1. *Staphylococcus aureus* 2. *Streptococcus pneumonia* and gram negative 1. *Escherichia coli* 2. *Klebsiella pneumoniae* 3. *Proteus mirabilis*. The isolates were obtained by collaboration with Media Diagnostic Center and Nanakali Hospital for Hematology and Oncology and Medical Laboratory Technology Department of Shaqlawa Technical College.

Identification of the isolates was carried out to the species level. To identify the bacterial genera to species level, a 5-ml sample from pure BHI culture was centrifuged at $160 \times g$ for 5 min to pellet blood cells. The supernatant was then centrifuged at $650 \times g$ for 10 min to pellet bacteria. The turbidity of the bacterial suspension was adjusted with VITEK Densichek to match the McFarland 0.5 standard in 0.45% sodium chloride. Afterward, the VITEK 2 ID-GNB card, the AST-NO09 card, and the bacterial suspension were manually loaded into the VITEK 2 system.

The methods and elements that needed to procedure it's prepared of the media (Mueller-Hinton agar is a non-selective, non-differential microbiological growth medium. Mueller Hinton (Merk, Germany) Agar contains beef extract, acid casein hydrolysate, starch and agar. Beef extract and casein acid hydrolysate provide nitrogen, vitamins, carbon, amino acids, sulphur and other essential nutrients.

In the first step take 7.5 gram of agar 250 milliliter of water mixed well autoclaved at 121°C about 1 hour. we worked in lab of Microbiology and very well sterilize the equipment's and bench working near the Bunsen burner prepare 10 to 12 Petri dish. after cooling the media, prepare the probiotic powder that contain

lactobacillus. 1ml of lactobacillus to 5ml of broth ((type of broth) MRS Broth is a liquid medium recommended for use in the cultivation of Lactobacillus species and in the differentiation of gram-positive coccobacilli).

For all the Lactobacillus colonies following tests were carried out:

1. Gram stain

To determine the gram reaction of Lactobacillus isolates, pure colonies were isolated and smear was prepared.

At the room temperature, bacterial smear was flooded with Crystal violet for 1 minutes then rinsed with tap water.

After a quick drying using filter papers, the smear was flooded with iodine for 1 minute followed by a quick rinse. The smear was flooded with acid alcohol for 10 seconds then rinsed. Finally, the smear was flooded with the counter stain i. e. safranin for 1 minute and followed by last rinse and drying. The slides were later examined 100X oil immersion using an Olympus compound microscope and all the samples were photographed using iPhone 9 camera.

2. Catalase Test

Overnight cultures of Lactobacillus isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. The catalase test was conducted by dripping two drops of hydrogen peroxide (H₂O₂, 3%) on 24 h-old cultures on a glass slide. The catalase test showed positive reaction characterized by the formation of oxygen bubbles that indicate the production of catalase enzyme by the test bacterium. Therefore, the isolates, which did not give gas bubbles, were selected for subsequent activities.

3. Spore Staining

Gram-positive and catalase-negative isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. The spore-staining procedure was applied [20]. After the spore-staining technique, the endospore formulation was examined under light

microscopy using oil immersion objectives. The isolates which did not form endospores were selected for further analysis.

Ecological interaction (in -vitro):

Using *Lactobacillus* which grew in MRS broth against five pathogens (*K. pneumonia*, *P. mirabilis*, *S. pneumonia*, *S. aureus* and *E. coli*) in different concentrations of the probiotics with normal saline from (10^{-1} to 10^{-5}) then labeling the Muller Hinton agar plates with numbers from (0 to 5) circularly sequence and put sterile paper discs into each tube with different concentrations and put on numbers on blood agar, after that incubate for 24 hours at 37°C under aerobic condition.

3. RESULTS

By collaboration with Media Diagnostic Center and Nanakali Hospital for Hematology and Oncology and Medical Laboratory Technology Department of Shaqlawa Technical College, we could obtain five pathogenic isolates including three-gram negative bacilli *Klebsiella pneumoniae*, *Proteus mirabilis* and *Escherichia coli*, and two-gram positive cocci including *Streptococcus pneumoniae*, *Staphylococcus aureus*.

Following the Mahmud (2022)'s approach six probiotic *Lactobacillus* strains were isolated from commercially available probiotic medicine and named accordingly (Table 2).

Table 1: shows the biochemical and staining tests that were used to identify the bacterial isolates *Lactobacillus* spp from Suspected colonies.

Bacteria	Catalase test	Gram test	Spore stain
Lactobacillus spp	+ve	+ve	-ve

The clear zones of probiotics (*Lactobacillus*) seen on MHA agar in many concentrations which means that these probiotic bacteria could clearly inhibit the growth of the five isolates.

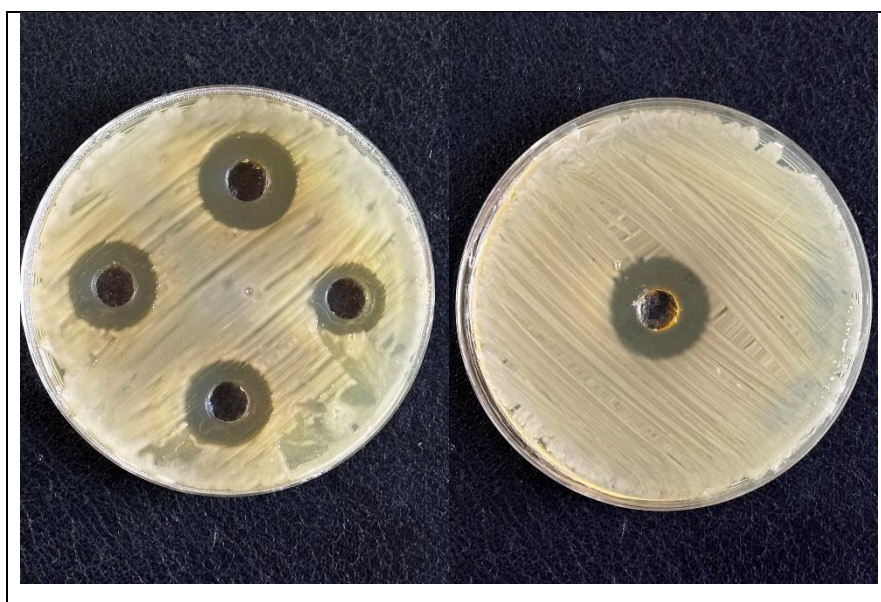


Figure 2: Inhibition growth zone of the two clinical isolates (*Klebsiella pneumonia*, *Proteus mirabilis*, *Streptococcus pneumonia*, *Staphylococcus aureus* and *Escherichia coli*) produced by *Lactobacillus* on MHA.

The antagonistic influence of the six probiotic *Lactobacillus* was expressed in form of zone of inhibition (table 1).

Table 2. Demonstrating the antagonistic influence of six *Lactobacillus* isolates against five pathogenic bacteria (*K. pneumonia*, *P. mirabilis*, *S. pneumonia*, *S. aureus* and *E. coli*). Growth of inhibition is given in mm.

Isolate	Antagonistic activity of <i>Lactobacillus</i> spp	<i>K. pneumonia</i>	<i>P. mirabilis</i>	<i>S. pneumonia</i>	<i>S. aureus</i>	<i>E. coli</i>
1	Advanced probiotic	22	14	19	13	25
2	Biolact	35	24	15	16	20
3	Lactoflor kids	23	18	10	20	12
4	Lactozinc	26	23	19	8	13
5	Probiodex	24	13	21	14	22
6	ProIBS	19	14	18	15	18

Table 3. Displaying the antagonistic influence of a mixture of six Lactobacillus isolates against the five pathogenic bacteria (*K. pneumonia*, *P. mirabilis*, *S. pneumonia*, *S. aureus* and *E. coli*). Growth of inhibition is given in mm.

Isolates/LB two-fold dilution	1:4	1:2	1:1
<i>Klebsiella pneumoniae</i>	16	18	18
<i>Proteus mirabilis</i>	16	20	18
<i>Streptococcus pneumoniae</i>	12	17	15
<i>Staphylococcus aureus</i>	24	25	26
<i>Escherichia coli</i>	16	17	19

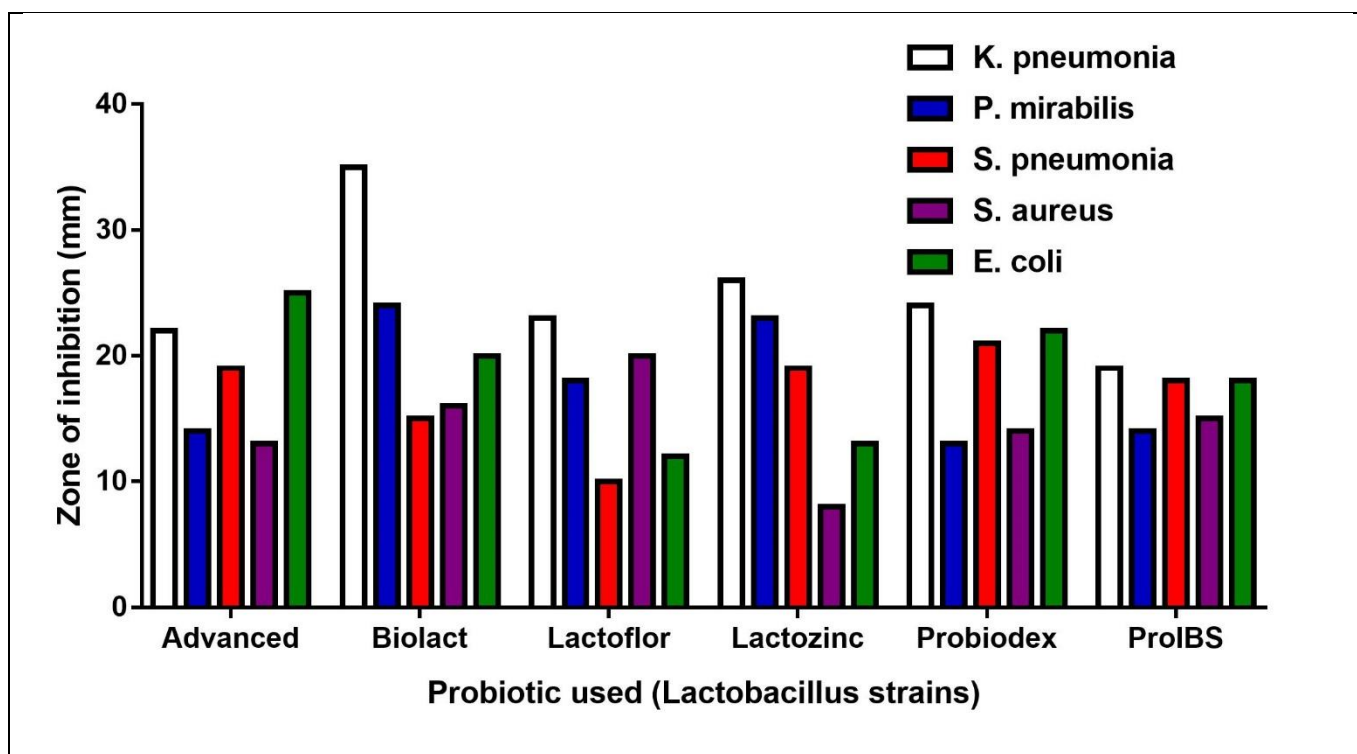


Figure 3: Displaying the antagonistic influence of each strain of Lactobacillus isolates against the five pathogenic bacteria (*K. pneumonia*, *P. mirabilis*, *S. pneumonia*, *S. aureus* and *E. coli*). Growth of inhibition is given in mm.

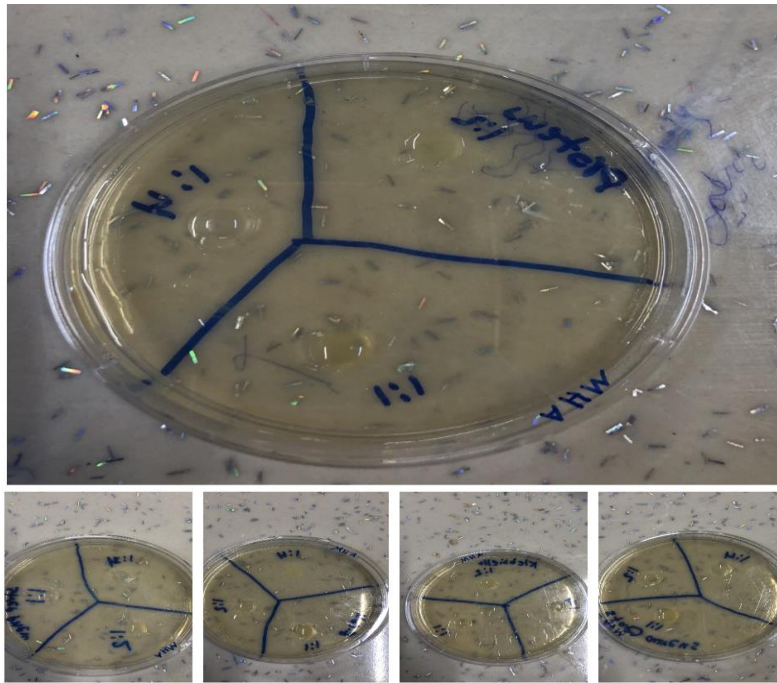


Figure 4: probiotic against Pathogenic bacteria (*Stapylococcus aureus*, *streptococcus pneumonia*, *Eshcherichia coli*, *proteus mirabilis*, *klebsilla mirabilis*)
(Befor Incubation)

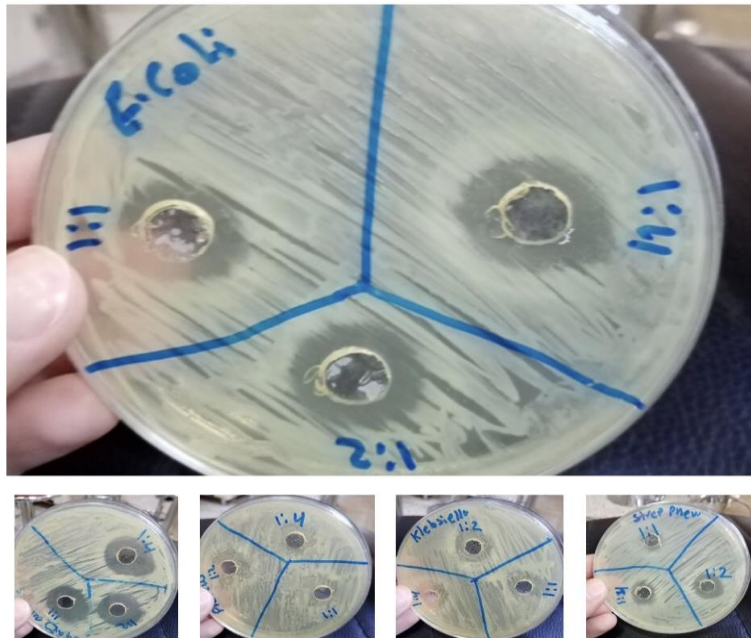


Figure 5: probiotic against Pathogenic bacteria (*Stapylococcus aureus*, *streptococcus pneumonia*, *Eshcherichia coli*, *proteus mirabilis*, *klebsilla mirabilis*)
(Aftar Incubation)

4. DISSCUSION

The goal of this project work was to isolate and characterize the potential antagonistic activity of probiotic *Lactobacillus* spp from commercially available probiotic medicine against two common gram positive and three-gram negative pathogenic bacteria. Our study describes that, *Lactobacillus* isolates from Advanced probiotic Biolact, Lactoflor kids, Lactozinc, Probiodex, ProIBS, considered as effective to assess their anti- bacterial activity against some common human pathogens.

The significant growth of the isolates at pH 6.5 on MRS agar plates in anaerobic conditions also confirmed their identification as *Lactobacillus* spp (Hotel, A. C. P. and A. Cordoba, 2001). Oxidase and catalase test of the selected isolates were exhibited same results as *Lactobacillus* spp. pH is an important factor which can consequentially affect bacterial growth. To be used as probiotic, organisms have to tolerate low pH of human gut. The isolated *Lactobacillus* spp. can tolerate a wide range of pH (2 to 8) and grow well at acidic pH (2 to 5).

Antimicrobial activity is one of the most crucial selection precedents for effective probiotics. Antimicrobial effects of all *Lactobacillus* isolates are sustained by producing some substances such as organic acids (Prabhurajeshwar et al., 2017). Probiotics including *Lactobacillus*, *Bifidobacterium* are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favourable effects against disease caused by an imbalance of the gut microflora, several experimental observations have revealed a potential protective effect of probiotic bacteria against the development of colon tumours (Hotel, A. C. P. and A. Cordoba, 2001).

In the study of Osuntoki et al., 2008, *Lactobacillus* spp. isolated from fermented dairy products showed antibacterial activity against some clinically important pathogens such as Enterotoxigenic *E. coli* (4.2mm), *Salmonella typhimurium* (4.3mm) and *Listeria monocytogenes* (5.0 mm). Isolates of the present study have better antimicrobial

capability than this *Lactobacillus* spp. isolates. Our isolates showed nearly similar antagonistic activity against *E. coli* and *S. typhimurium* as compared to *Lactobacillus plantarum* and *Lactobacillus salivarius* isolated by Murray et al., 2004, from a botanical probiotic. In the study by Gharaei-Fathabad and Eslamifar (2011) a strain of *Lactobacillus paraplantarum* isolated from tea leaves showed strong inhibitory activity against *S. typhii* (65 mm), *E. coli* (30 mm), *S. aureus* (56 mm), *E. faecalis* (55 mm) and *Citrobacter* spp. (60 mm). Isolates of the present study have almost similar antimicrobial capability. In our study, antagonistic activity of all selected *Lactobacillus* isolates against seven different test pathogens showed noticeable activity and achieved that the activity of these Lacto- bacillus isolates due to organic acid and low molecular weight antimicrobial substances produced from the isolates was the responsible.

Aggregation between microorganisms of the same strain (auto-aggregation) or between genetically different strains (co-aggregation) is of extensive importance in several ecological niches. Aggregating bacteria may achieve an adequate mass to form biofilms or adhere to the mucosal surfaces of the host and thus utilize their functions (De Man, and Rogosa Sharpe, 1960). The results of this study revealed that, auto-aggregation expanded as a concern of time and were highest at the 4 h of incubation time period.

5. CONCLUSIONS AND RECOMMENDATIONS

In conclusion, Lactobacillus strains isolated in this study from the different probiotic medicine having in vitro properties that make them potential against pathogenic bacteria. The five tested clinical isolates (*K. pneumonia*, *P. mirabilis*, *S. pneumonia*, *S. aureus* and *E. coli*) were susceptible to all the strains of Lactobacillus. These results collectively suggest that probiotics particularly commercially available types are important for combating bacterial pathogens.

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پوخته

پروبايوتیکهکان، خانه زیندوون که سوودمندی جیاوازیان همیه، به شیوهیهکی بهر فراوان لیکولینهوهیان لهسەر کراوه و به شیوهیهکی بازرگانی له زۆریک له بهرهمه جیاوازهکانی جیهاندا لیکولینهوهیان لهسەر کراوه. سوودهکانیان بۆ تهنروستی مرۆف و ئاژهلان له سهدان لیکولینهوهی زانستیدا سهلمینراوه. لاکتوباسیلۆس یهکیکه لهو پروبايوتیکانهی که زۆرتیرین بهکارهینانی همیه له بواری پزیشکیدا لهبهر ههبوونی سودی یهکجار زۆریان. ئامانجی ئهم توژیینهوهیه برتی بوو له جیاکردنهوهی شەش جۆری بهکتریای لاکتوباسیلۆس و پشکنینی توانای دژهمیکروبی له دژی بهکتریای نهخوشیدروستکەر. شەش جۆری دهرمانی پروبايوتیکی بهردهست له بازارهکانمان بهکارهینا بۆ جیاکراونهوهی لاکتوباسیلۆس که بریتی بوون له: **Advanced probiotic، Biolact، ProIBSand، Lactoflor kids، Lactozinc، Probiodef** . ههیهکیک لهه جۆرانهی لاکتوباسیلۆس تاقیکرايهوه لهدژی سی بهکتریای گرام نیگهتیفی نهخوشیدروستکەر لهنیوانیان: کلیسییلا نیومونیا، پروتیوس میرابیلیس و ئیشیرچییا کولای و دوو بهکتریا گرام پوزهتیف لهنیوانیاندا ستریتوکوکوس نیومونیا، ستافیلوکوکوسی ئاورپوس. ئهجامهکانمان دهريخست که به پشتهستن به پێوانهکردنی ناوچهی ریگریکردن لهگشه (ملم)، ئهجامهکانمان دهريانخست که لاکتوباسیلۆسی جۆری **Biolact** زۆرتیرین کاریگهری لهسەر بهکتریا زیانبهخشه ههلبژیردراوهکان ههبووه، (تیکرا = ۲۲، لادانی ستاندارد = ۸.۱) و جۆری **Lactoflor kids** کهمترین کاریگهری ههبووه، (تیکرا = ۱۶.۶، لادانی ستاندارد = ۵.۴۶)، بهلام پشکنینی ئهنوفا پيشانیدا که جیاوازیهکی ئاماری گرنگ نیه سهبارمت بهکاریگهريیهکان له دژی بهکتریا زیانبهخش له نیوان ئهو شەش جۆرهی بهکتریا پروبايوتیکهکاندا (**P>0.5**). له دهرئهجامدا، جۆرهکانی لاکتوباسیلۆس که لهه لیکولینهوهیهدا له دهرمانی جیاوازی پروبايوتیک جیاکراونهتهوه تایبهتمهندی همیه لهناو ئامیری پشکنیندا دژی بهکتریای زیانبهخش. پینج جیاکراوه کلینیکی بهکتریای زیانبهخش ههستیاری پيشاندا بۆ ههش جۆری لاکتوباسیلۆس. ئهم ئهجامانه بهکۆمهله پيشنیاری ئهوه دهکهن که پروبايوتیکهکان به تایبهتی جۆرهکانی بهردهست لهبازهراندا گرنگن بۆ بهرهنگاربوونهوهی بهکتریا زیانبهخشهکان.