Determination Of some organic acids produced from probiotic bacteria during fermentation processes

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Abstract

Lactic acid production during growth of probiotic bacteria (*L.acidophilus &Bifidobacterium sp.)* in model MRSB media has been monitored using HPLC. HPLC evaluation showed that *L.acidophilus* achieved lactic acid concentration (44.6)%and *Bifidobacterium* achieved concentration (2.6)% (after 24 h of fermentation) .The acetic acid content was higher in MRS broth with *Bifidobacterium sp.* (4.1)% while the MRS broth with *L.acidophilus* nil from acetic acid .The inhibition activity of this probiotic bacteria filtrates was studied against some pathogenic bacteria included *Proteus sp., Staphylococcus epidermidis, Pseudomonas aeruginosa ,Klebsiella sp.* The results showed inhibition activity of both *L.acidophilus &Bifidobacterium sp.* filtrates against pathogen bacteria.

Introduction

Nonpathogenic *Lactobacillus acidophilus* which is a member of the normal intestinal microflora is widely used in fermented dairy products and is of considerable industrial and medical interest because it has been reported to aid in the reduction of the levels of harmful bacteria and yeast in the small intestine and to produce lactase ,an enzyme which is important for the digestion of milk (1).Therefore *L. acidophilus* group *o*f lactic acid bacteria (LAB) is added as dietary adjuncts to commercial fermented milk products and the intake of these bacteria may have beneficial effects on human health (2). *L. acidophilus* strains have been widely utilized as a dairy starter culture for their therapeutic activities associated with an intestinal microbial balance, and has been used in fermented foods, and as a probiotic in dietary supplement (3).

Lactic acid bacteria produce various compounds such as an organic acid, diacetyl, hydrogen peroxide and bacteriocins or bactericidal proteins during lactic acid fermentation (4). Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (5).*Bifidobacterium is* a genus of Gram-positive, non motile, strictly anaerobic bacteria, inhabiting the

gastrointestinal tract and vagina. *Bifidobacteria* aid in digestion associated with a lower incidence of allergies and also prevents some forms of tumors growth. Some Bifidobacteria are used as probiotics .This type of bacteria is considered beneficial due to the acid it produces such as lactic acid and acetic acid (6).The pH may drops to as low as 4.o ,low enough to inhibit the growth of most other microorganism including the most common human pathogens ,thus allowing some foods to prolong shelf life (7).

Organic acid occurs in fermented products as a result of hydrolysis ,biochemical metabolism, and microbial activity .Quantitative determination of these acids is important in fermented food for technical, nutritional, sensorial and microbial reasons .Titrimetric methods, gas chromatography colorimetric analysis and enzymatic methods are examples of techniques that are used for analyses of organic acid in foods .The HPLC technique is an attractive method because of simplicity and speed of analysis which requires a minimum of sample preparation prior to separation and permits quantitative determination of organic acids in short time (8).

The objective of this study was to extracted and purified an organic acid (lactic &acetic acids) from probiotic bacteria such as *Bifidobacteria* and *Lactobacillus acidophilus* and show their antimicrobial activity toward harmful bacteria such as *Proteus sp., Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella sp.*

Material and Methods

Bacterial strains and culture media

pathogenic bacteria (*Staphylococcus epidermidis ,Pseudomonas aeruginosa , Proteus sp. ,klebsiella sp.*) were obtained from the AL-Kindi Teaching Hospital and were identified by cultural characters, morphology and standard biochemical tests according to the method of (9). *L. acidophilus* obtained from laboratory of Biology department-Science college-AL-Mustansiriya University , *Bifidobacterium sp* .was isolated from probiotic yogurt (Activia) and identification by cultural characters, morphology and standard biochemical tests according to the method of (10).

Preparation of the cell-free supernatants (CFS) from *Bifidobacterium sp.and L.acidophilus*

Cell -free supernatant (CFS) of probiotic bacteria was obtained from 18h old cultures in MRS broth (de Man ,Rogosa ,Sharpe).The culture broth was centrifuged at 6000 rpm for 15 min .The resultant supernatants were then filter-sterilized using a 0.22µm membrane filter (Sartorius Stedim).(11) .Supernatants were freezed until used .

Measurement of Antimicrobial Activity Against pathogenic bacteria

An agar Well-Diffusion Assay was used for detection of antimicrobial activity according to(12).Nutrient agar plates spread with 0.1 ml from cultures of pathogenic strains (10^5)cell/m. Wells cut into the (N.A)plates with 5mm sterile cork borer were filled with 50 µl of the culture supernatant . The plates were incubated at $37C^0$ for 24 hours. Inhibition was detected by a zone of clearing around the supernatant well (13). .

Lactic acid and acetic acid content

Supernatant was filtered and sterilized using a 0.22µm membrane filter after then was analyzed by HPLC(Shimadzu LC-2010AHT) ;250×4.6mm column lona 5u C18 ,UV detector 220nm and mobile phase was methanol +water 70:30;pH :2.2 flow 0.5ml/min ;comp :120 bar (14).

Statistical Analyses

Analysis of variance (ANOVA) was applied to all data for the production of lactic acid in these strains, followed by discrete variables as percentages.

Result and discussion

HPLC quantification

The lactic acid and acetic acid of MRS broth indicated these acidity table 1, presented the results of these acids content (%) for each growth *Lctobacillus acidophilus &Bifidobacterium sp.)*.The concentration of lactic acid in MRSB using *L. acidophilus* was (44.6)% higher than the MRSB with *Bifidobacterium sp.* (2.6)%,contrast the acetic acid concentration it was shown higher in MRSB with *Bifidobacterium* sp.(4.1)% when the MRSB with *L. acidophilus* it was nil from acetic acid .To detection the organic acids in MRSB with *L.acidophilus* determinate the retention times for lactic acid ,it was 5.738 min and 5.674 for the standard (figure 3,4) .In MRSB with *Bifidobacterium. sp.* The retention times for lactic acid and acetic acid were 5.277 ,5.277 min respectively and 5.143 ,5.296 min for the standard (figure 5,6).This results depended on pathway for each bacteria ,*L.acidophilus* it was homolactic fermentation ,one molecule of glucose is converted to two molecules of lactic acid (15), while *Bifidobacterium sp.*it was heterofermentative which producing 40% lactic acid and 60% acetic acid (13). Degnan and Macfarlane (16) studying the impact of source of carbon on *B.breve* NCFB2257 fermentation showed that at limited glucose availability the cultures produced mainly acetic and formic acid ,were as in the excess of glucose –lactic and acetic acid .In the opinion of Macfarlane and Gibson (17) lactate frequently appears following rapid fermentation of carbohydrate ,and acts as an electron sink to dispose of excess reducing power ,when substrate is plentiful .Scardovi (18) noticed, that although the theoretical ratio of acetate to lactate is 3:2 ,it is scarcely ever found in growing cultures of bifidobacteria .

Table 1 . The effect treatment on lactic acid and acetic acid

No.	Growth in MRS broth	lactic acid (%)	acetic acid(%)
1	*Bifidobacterium spp.*	2.6	4.1
2	*L.acidophilus*	44.6	----

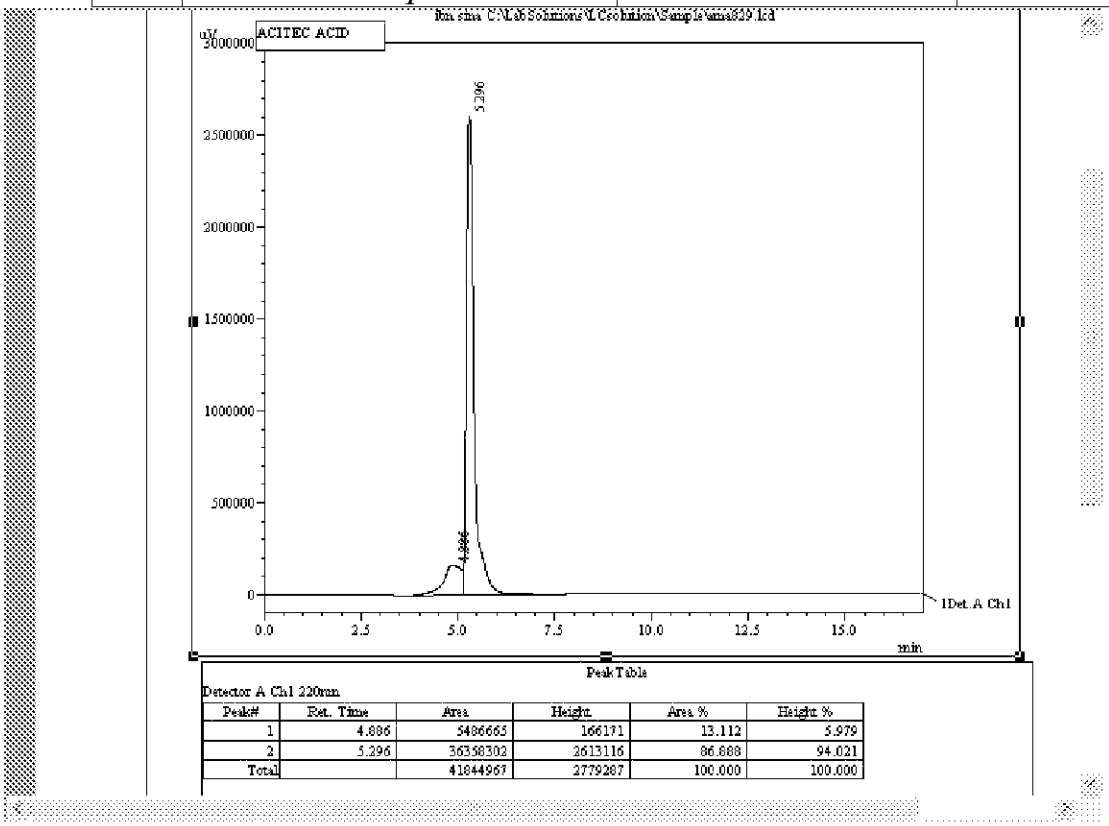


Figure -1-Standard chromatograph of acetic acid

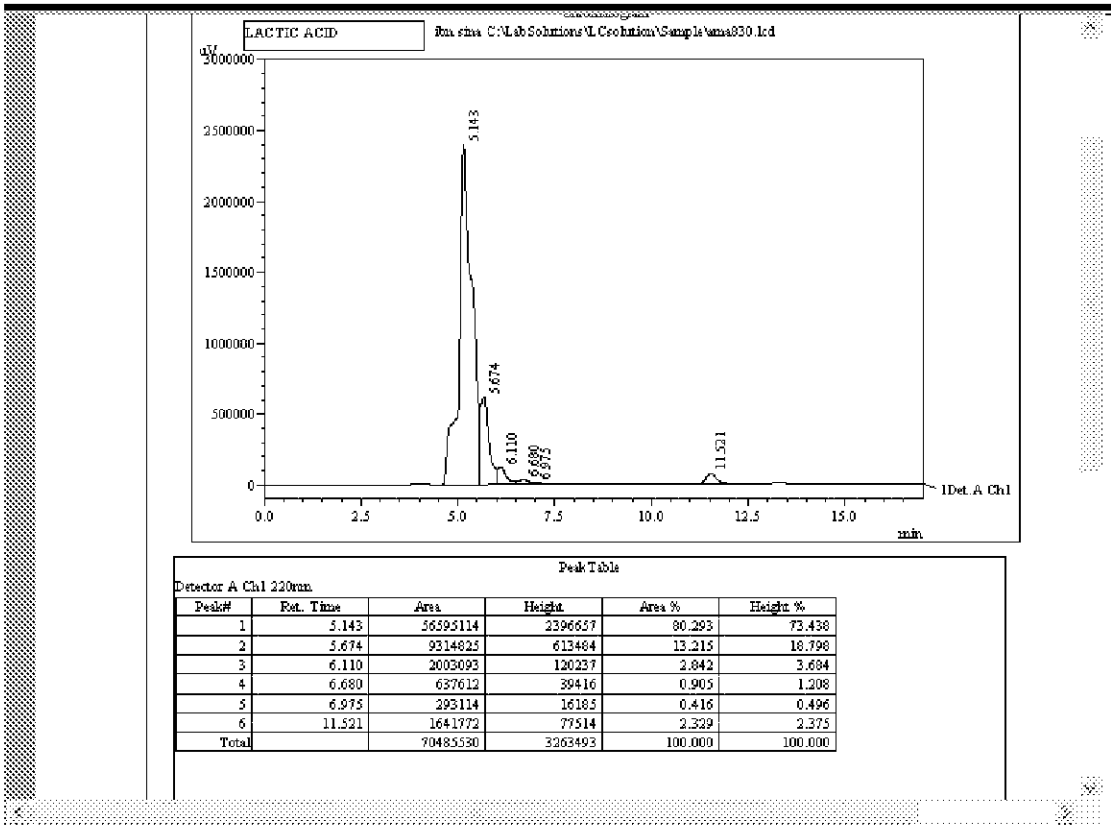


Figure -2- Standard chromatograph of lactic acid

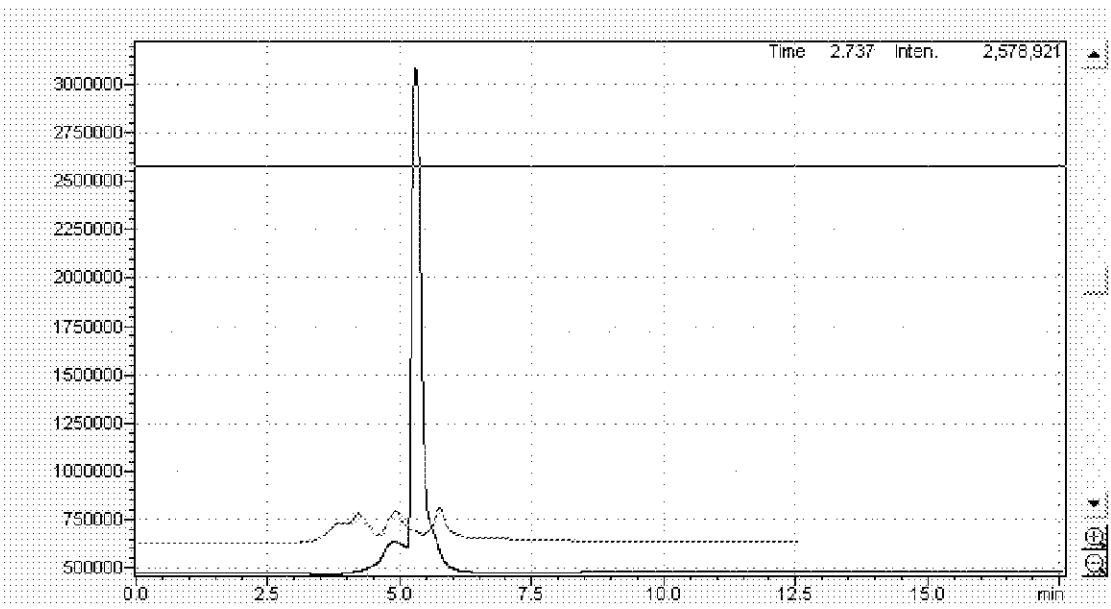
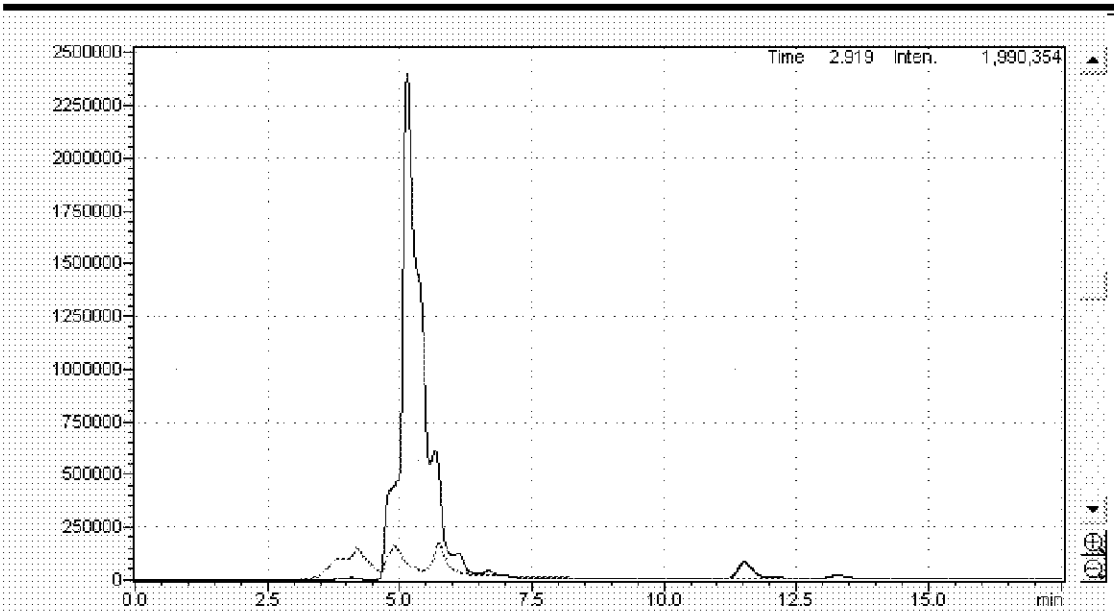


Figure -3- ST1 +A

ST1 : Standard curve of acetic acid
A : *L.acidophilus*



A

Figure -4- ST2 +

ST2 : Standard curve of lactic acid

A : *L.acidophilus*

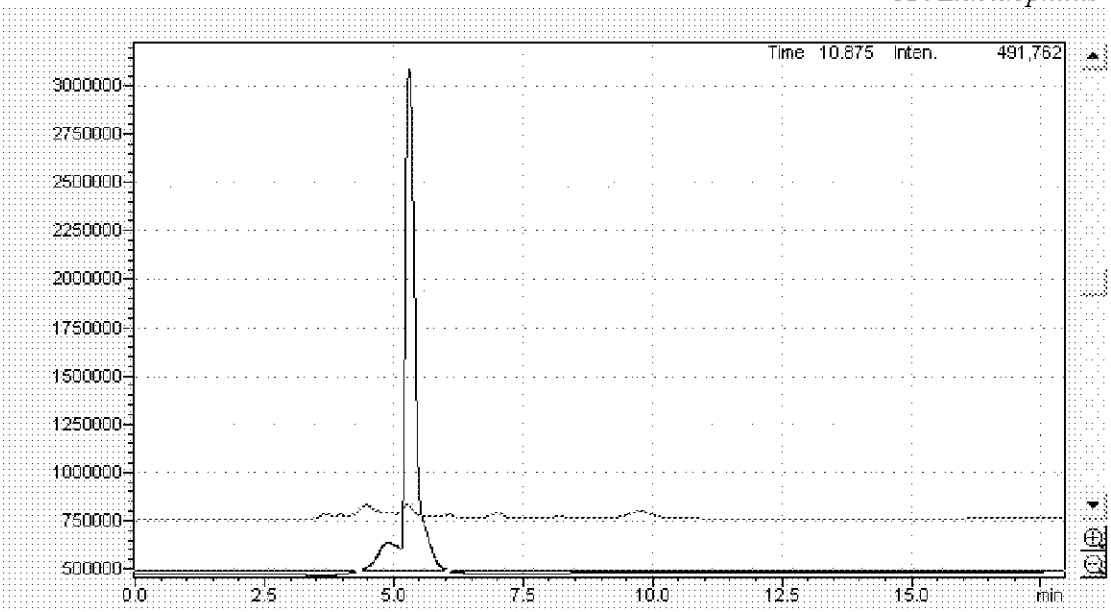


Figure -5- ST1+B

ST1 : Standard curve of acetic acid

B : *Bifidobacterium sp.*

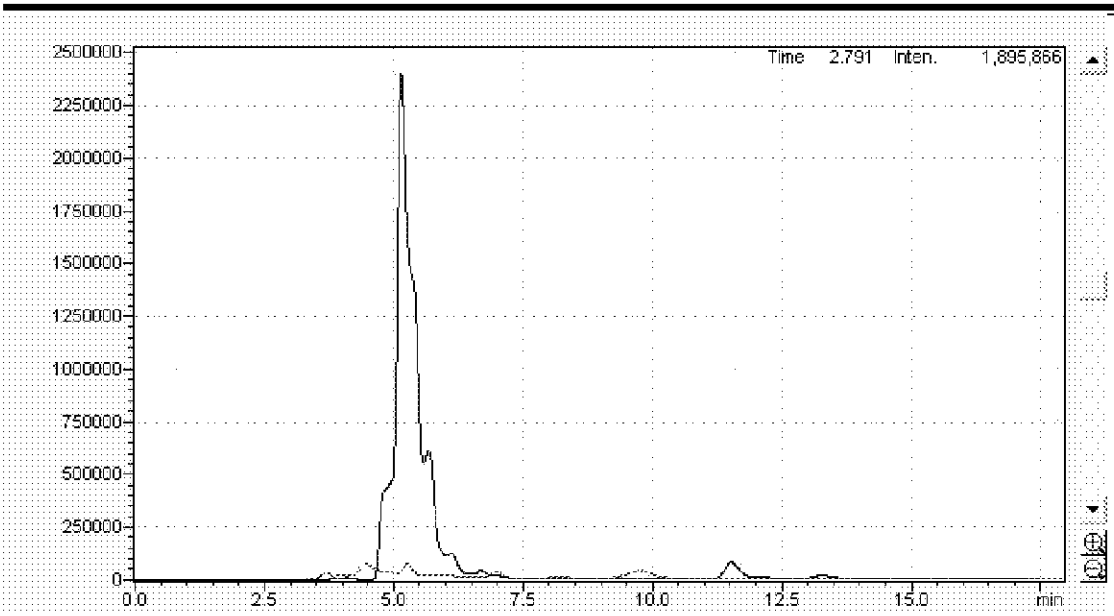


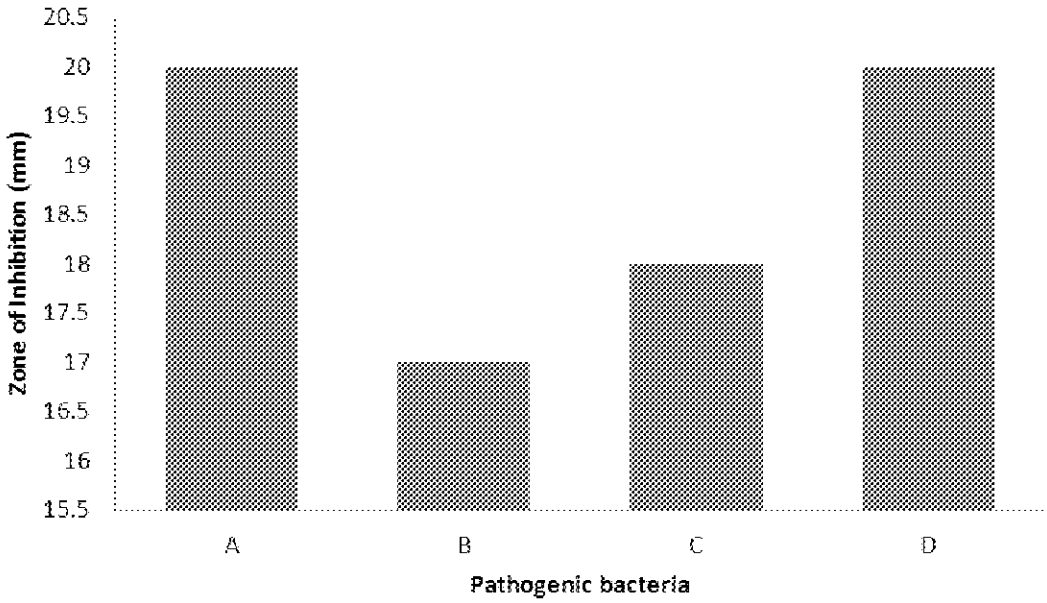
Figure -6- ST2+B

ST2 : Standard curve of lactic acid

B : *Bifidobacterium sp.*

Antibacterial activity

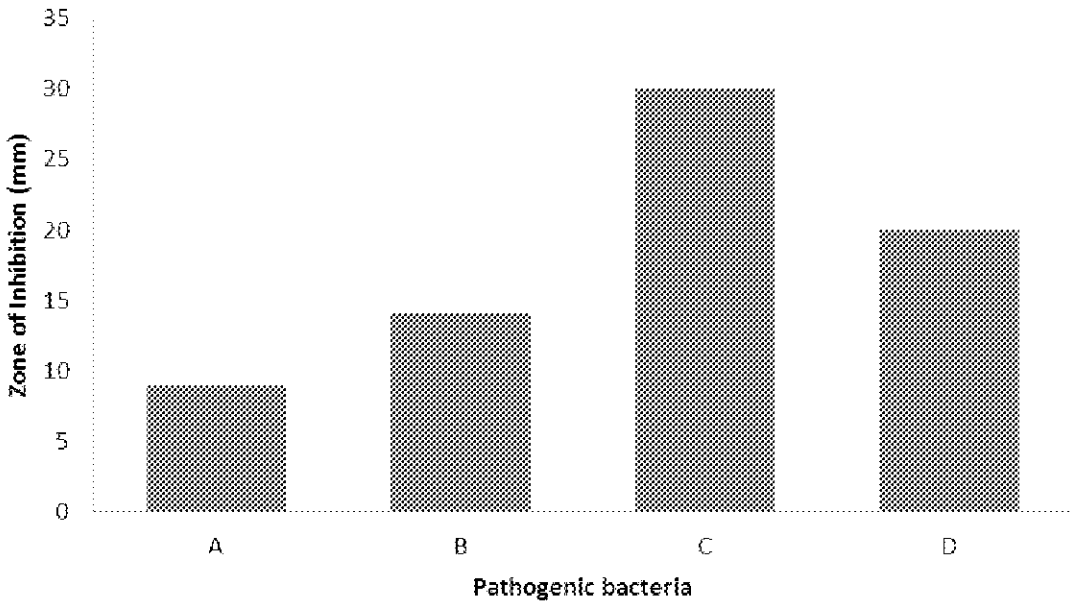
Supernatant of *l.acidophilus* was shown to produce inhibition zones against some pathogenic bacteria were tested (*S.epidermidis* , *Proteus sp, P.aeruginosa ,klepsiella sp.)* .Inhibitory spectra of this supernatant was presented in graphic 1.All of these pathogenic bacteria were inhibited ,displayed zone of inhibition (20,17,18,20)mm respectively. Antibacterial activity of supernatant for *l.acidophilus* observed against awide range of Gram-negative and Gram – positive pathogenes ,such as *Staphylococcus aureus ,Listeria monocytogenes ,Salmonella typhimurium ,Shigella flexneri ,Klebsiella pneumonia ,pseudomonas aeruginosa* and *Enterobacter cloacae* (19). Antibacterial activity for supernatant of *Bifidobacterium sp.* againts the same pathogenic bacteria displayed zone inhibition (9,14,30,20)mm respectively shown in graphic 2 . This supernatant was active against gram-positive and gram-negative bacteria and yeast relevant to food safety and human health (20).Supernatant of *Bifidobacterium sp.* showed the highest antimicrobial activity against *E.coli* and *Bacillus cereus* it was mainly due to the presence of the organic acids (21).



antibact

erial activity for supernatant of *L.acidophilus* on pathogenic bacteria -Graphic 1

A :*S.epidermidis*
B :*P.aeruginosa*
C :*Proteus sp.*
D :*Klepsiella sp.*



Graphic 2-antibacterial activity for supernatant of *Bifidobacterium* on pathogenic bacteria.

A :*S.epidermidis*
B :*P.aeruginosa*
C :*Proteus sp.*
D :*Klepsiella sp.*

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تحديد بعض الأحماض العضوية المنتجة من المعززات الحيوية أثناء عمليات التخمر

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الخلاصة

رصد حامض اللاكتيك المنتج من بكتريا المعززات الحيوية (*L.acidophilus &Bifidobacterium sp* في وسط MRSB بأستخدام جهاز HPLC أعطت نتائج الHPLC ان بكتريا *L.acidophilus* حققت تركيز (6و44)% لكن بكتريا. *Bifidobacterium sp* أعطت تركيز (6و22)%(بعد مرور 24 ساعة للتخمر).وجد حامض الخليك بنسبة عالية في الوسط MRSB الملقح ببكتريا *Bifidobacterium sp*(1و4)%بينما وجد وسط MRSB الملقح ببكتريا *L.acidophilus* خالي من الحامض .درست الفعالية التثبيطية لرواشح هذه المعززات الحيوية تجاه بعض البكتريا المرضية والتي تضمنت : *Klebsiella sp., Pseudomonas aeruginosa, Staphylococcus epidermidis, Proteus sp.* اظهرت النتائج ان هناك فعالية تثبيطية لرواشح بكتريا (*L.acidophilus &Bifidobacterium sp.*) تجاه البكتريا المرضية .