Antimicrobial activity of *Lactobacillus spp.* metabolites on growth of two clinical isolates *in vitro*

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Abstract

Antimicrobial activity of secondary metabolites that produced by *Lactobacillus spp.* has been investigated against two clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa*.

Acidity effect of anaerobic fermentation of lactose in raw milk to lactic acid by *Lactobacillus spp.* was studied. The accumulation of acid in the medium lead to lowering pH of the yoghurt that prevent growth of *E. coli* and *P. aeruginosa*. The ability of two isolates was experimented for survival in acidic, alkaline, and neutral pH, the results showed that *E. coli* and *P. aeruginosa* have no growth in acidic pH while they have heavy growth in neutral and alkaline pH.

This study confirmed the presence of synergistic effect between the secondary metabolites of lactobacilli and the acidity in yoghurt, which lead to inhibitory effect on the growth of two clinical isolates.

Key ward: *Lactobacillus*, probiotics, secondary metabolites, therapeutic effect

Introduction

Lactic acid bacteria (Lab) are acid tolerant, Gram positive microorganisms, produced lactic acid as a main product during anaerobic fermentation of lactose in milk [1,2]. The most important genera are *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Leuconostoc,* and *Bifidobacterium*, among all Lab types, the genus *Lactobacillus* has beneficial characteristics make it more useful, the different *Lactobacillus* species are aerotolerant, non pathogenic and do not produce toxic substances or toxins [3]. Now, there is a growing interest in their use as probiotics which are living, health promoting microorganisms that are incorporated into various kinds of foods, when administered in adequate amounts confers a health benefit on the host [4]. It have beneficial therapeutic effect results from suppression pathogens and inhibit chemically carcinogens induce tumorization in the gastrointestinal tract. Modulation of the gut microflora and the enhancement of mucosal immunity of the gut are both mechanisms of probiotic function [5].

One important attribute of Lab is their ability to produce antimicrobial compounds called bacteriocins which constitute an important group of antimicrobials [4,6] , lactic bacteriocins are bioactive peptides with a bactericidal mode of action towards other Gram- positive and negative bacteria [7], the transient passage of Lab in the digestive tract may represent a microbial barrier against the development of pathogenic bacteria, due to release of these compounds contributing to the maintenance of colonization resistance to pathogens and by its inhibitory effects [8,9].

Lab also have another important feature that the ability to produce lactic acid that inhibit the growth of harmful microorganisms by lowering the acidity of the medium [10].

Continued overuse of antimicrobials is leading to problems with antimicrobials resistance, so there is a need for innovative measures to prevent and treat infectious diseases. The therapeutic use of microorganisms antagonistic to pathogens would have the potential to decrease antimicrobials use [11].

The main aim of this work was to investigate the antibacterial effect of metabolites produced by *Lactobacillus spp.* and therapeutic effect of probiotics in yoghurts against *E. coli* and *P. aeruginosa*.

Materials and Methods

Samples of yoghurts: Four types (10ml each) of commercial and home-made yoghurts were collected in sterilized screw capped bottles and stored under refrigeration conditions.

Bacterial isolates: two pathogenic bacterial isolates of *E. coli* (isolated from acute urinary tract infection) and *P. aeruginosa* (isolated from burns infection) used in this study, obtained from Laboratory Research in Pharmacology Department/College of Pharmacy.

First screening of yoghurts: 0.01ml from each type of used yoghurts was inoculated onto the surface of MRS (deMan, Rogosa and Sharpe) agar and nutrient agar plates by applying streak-plate technique, then incubated under anaerobic conditions at 37ºC for (24-48)hr., the obtained colonies from MRS agar plates were transferred on to new MRS agar plates by streaking [12]. Pure colonies thus obtained and checked for their morphological, cultural, and biochemical characteristics by the procedures described in the Bergey's Manual of Systematic Bacteriology [13].

Acidity effects (pH) on growth of *E. coli* and *P. aeruginosa*

1. The pH of all used yoghurts was measured before inoculation with clinical isolates, it will be found acidic (pH=5). From each type of used yoghurts, 10 ml was dispensed in screw capped bottles, then inoculated with 0.01ml of 10^6cfu/ml from bacterial suspension of *E. coli* according to Mcfarland standard scale [14]. The same step was repeated with *P. aeruginosa*, then screw capped bottles incubated at 37ºC for (24-48)hr.

2. From yoghurts that inoculated with clinical isolates of *E. coli* and *P. aeruginosa*, 0.01ml was inoculated onto the surface of MRS agar and nutrient agar plates, then incubated at 37ºC for 24hr. Control plates performed by streaking 0.01ml from bacterial suspension of *E. coli* and *P. aeruginosa* on nutrient agar plates.

3. In another step, 10ml from each type of used yoghurts was suspended in screw capped bottles, then the pH of all used yoghurts was adjusted to 7 by adding drops of 10% NaOH, after that yoghurts inoculated with 0.01ml of 10^6 cfu/ml from bacterial suspension of *E. coli* and *P. aeruginosa*, and incubated at 37ºC for 24hr.

4. 0.01ml was taken from each type of yoghurts (that pH=7 and inoculated with bacterial suspension) and streaked onto MRS agar and nutrient agar plates, incubated at 37ºC for 24hr. [15].

pH effects on the growth of *E. coli* and *P. aeruginosa* in water solution

1. The pH was adjusted to (acidic=5), (neutral=7), (alkaline=8) to three equal samples (10 ml) from sterilized distilled water that dispensed in screw capped bottles. Water samples then inoculated with 0.01 ml of 10^6 cfu/ml from bacterial suspension of *E. coli* and *P. aeruginosa*, all samples incubated at 37ºC for 24hr.

2. From inoculated water samples, 0.01ml was streaked on nutrient agar plates and incubated at 37ºC for 24hr.

Lactobacillus spp. production of secondary metabolites

This method employed according to [16] with modifications. *Lactobacillus* isolate selected for this test was isolated from the yoghurt sample No.1, three equal samples (10ml for each with two replications) from MRS broth medium in screw capped bottles, labeled with A, B, and C, then inoculated with pure *Lactobacillus* isolate and incubated an aerobically in water bath-shaker for 7 days at 37ºC. Extraction of the antimicrobial metabolites was carried out by centrifugation of *Lactobacillus* bacterial suspension at 10000 rpm at 4ºC for 20 min. The supernatant then filtered.

Antimicrobial metabolites activity

The pH of supernatant in samples with label A was adjusted to pH=5, while the pH of samples with label B and C was adjusted to 7 and 8 respectively by using 10% NaOH, then inoculated with 0.01ml of 10^6 cfu/ml

from *E. coli* bacterial suspension. The same step repeated with *P. aeruginosa,* all samples incubated at 37ºC for 24hr.

From the samples that inoculated with *E. coli,* 0.01ml was streaked onto nutrient agar plates. The same step repeated with *P. aeruginosa,* the results recorded after 24hr. at 37ºC. Control samples performed by inoculating three equaled groups of MRS broth (after adjusting the pH of each group to 5, 7, 8) with bacterial suspension of *E. coli* only, another group of MRS broth samples inoculating with bacterial suspension of *P. aeruginosa,* without addition the supernatant of metabolites product [16].

Secondary metabolites production of *Lactobacillus spp.* in water solution

1. 10ml from sterilized distilled water (pH=7) was inoculated with *E. coli* and *Lactobacillus spp.* at the same time. The same step repeated with *P. aeruginosa* and *Lactobacillus spp.*, samples were incubated at 37ºC for 24hr.

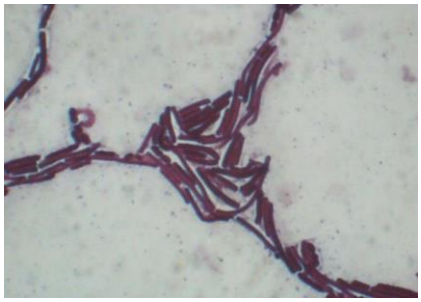
2. By streaking plate technique, 0.01ml from *E. coli* and *Lactobacillus,* *P. aeruginosa* and *Lactobacillus* suspensions were inoculated onto the surface of MRS agar and nutrient agar plates, the results recorded after (24- 48)hr, at 37ºC [16].

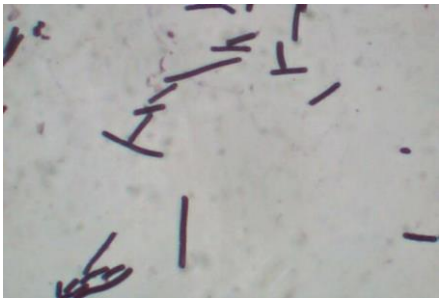
Results and Discussion

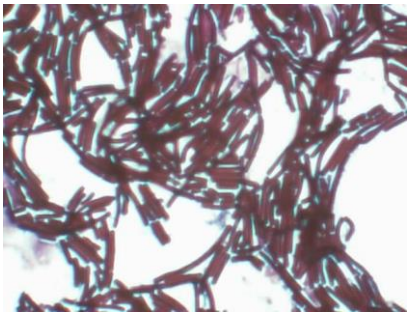
The results of primary cultivation of commercial and home-made yoghurts were showed the presence of *Lactobacillus spp.* as Gram-positive, catalase negative, glucose fermenter and elongated rods shape isolates Fig.(1), (2), (3) and (4), associated with *Saccharomyces* yeast Table(1).

Table(1). First screening of commercial and home-made yoghurts

Yoghurts samples	Microorganisms
1. Commercial	Pure *Lactobacillus spp.*
2. Commercial	*Lactobacillus spp.*
3. home-made	*Lactobacillus spp.*, *Saccharomyces* yeast
4. home-made	*Lactobacillus spp.*, *Saccharomyces* yeast







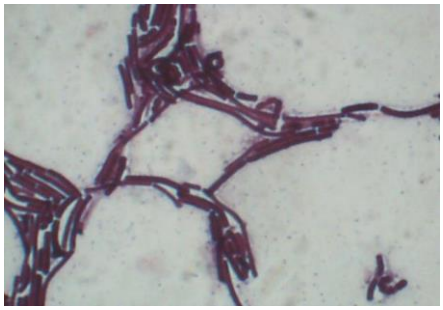


Figure (1), (2), (3) and (4) showed the *Lactobacillus spp.* isolated from yoghurt samples.

Acidic pH effect on the growth of clinical isolates

The pH 5 effect on the growth of clinical isolates of *E. coli* and *P. aeruginosa* were showed no growth of two clinical isolates on MRS agar and nutrient agar plates in all types of yoghurts, just *Lactobacillus spp*. exhibited a good growth Table(2).

Table (2). Acidic effect on growth of *E. coli* and *P. aeruginosa*

Yoghurts samples	pH before adjustment	Microbial growth on MRS agar	Microbial growth on Nutrient agar
1. Commercial	(pH=5)	Pure *Lactobacillus spp.*	Pure *Lactobacillus spp.*
2. Commercial	(pH=5)	*Lactobacillus spp.*	*Lactobacillus spp.*
3. home-made	(pH=5)	*Lactobacillus spp.*	*Lactobacillus spp.*
4. home-made	(pH=5)	*Lactobacillus spp.*	*Lactobacillus spp.*

This may be attributed to the lactic acid bacteria that utilized milk sugar (lactose) and release as much as 0.9 to 1.2% lactic acid as waste product, the accumulation of acid reduce the pH from 6.7 to below 4.6. The increased acidity causes prevent proliferation of other harmful bacteria [17].

The lowered pH caused by lactic acid preserves the milk by preventing the growth of putrefactive or pathogenic bacteria which do not grow well in acid conditions, the lower pH will not supply appropriate environment for growth and reproduction for most types of pathogens like *E. coli* and *P. aeruginosa* which prefer the alkaline medium that considered one of the important conditions for their growth [18]. The inhibitory action of organic acids produced by acidifying micro-organisms in dairy foods exhibited remarkable effect on pathogens and spoilage micro-organisms [19,20], on the other hand represented the typical environment for growth of *Lactobacillus spp*. which play a major role in lowering pH of yoghurts.

While the results of pH effect after adjustment to 7 showed the presence of *E. coli* and *P. aeruginosa* in the nutrient agar and MRS agar plates, beside *Lactobacillus spp.* Table(3).

Table(3). Neutral pH effect on growth of *E. coli* and *P. aeruginosa*

Yoghurts samples	pH after adjustment	Microbial growth on MRS agar	Microbial growth on Nutrient agar

1. Commercial	**(pH=7)**	**Pure *Lactobacillus spp.***	***E. coli* , *P. aeruginosa***
2. Commercial	**(pH=7)**	***Lactobacillus spp.***	***E. coli* , *P. aeruginosa***
3. home-made	**(pH=7)**	***Lactobacillus spp.***	***E. coli* , *P. aeruginosa***
4. home-made	**(pH=7)**	***Lactobacillus spp.***	***E. coli* , *P. aeruginosa***

This make sure the effect of acidity produced by *Lactobacillus spp*. to prevent the growth of most types of pathogenic bacteria, these fact supported by the results of pH effect on growth of *E. coli* and *P. aeruginosa* in water solution Table (4), in which the two clinical isolates will not grow in acidic medium (pH=5), while they exhibited heavy growth in neutral (pH=7) and alkaline (pH=8) media on nutrient agar plates.

Table(4). pH effect on growth of *E. coli* and *P. aeruginosa* in water solution

Clinical isolates	pH=5	pH=7	pH=8
E. coli	**No growth**	**Heavy growth**	**Heavy growth**
P. aeruginosa	**No growth**	**Heavy growth**	**Heavy growth**

Inhibitory activity of secondary metabolites

The results of secondary metabolites activity exhibited that both clinical isolates have no growth in MRS broth medium in pH= 5, 7 or 8 when inoculated on nutrient agar medium, in spite of the media with pH=7 and pH=8 will offer typical conditions for growth of two isolates, in comparison with control samples of MRS broth that lack presence of *Lactobacillus spp.* metabolites were *E. coli* and *P. aeruginosa* showed heavy growth in samples with pH=7 and pH=8 when inoculated on nutrient agar medium, but they have no growth in samples with pH=5 which influenced by the action of acidity, Table(5).

Table(5). Inhibitory effect of *Lactobacillus spp.* metabolites on clinical isolates

Clinical isolate	In presence of *Lactobacillus* metabolites			In absence of *Lactobacillus* metabolites		
	pH=5	pH=7	pH=8	pH=5	pH=7	pH=8
E. coli	No growth	No growth	No growth	No growth	Heavy growth	Heavy growth
P. aeruginosa	No growth	No growth	No growth	No growth	Heavy growth	Heavy growth

This may be rely on the presence of inhibitory action of secondary metabolites that produced by *Lactobacillus spp.* which play an inhibitory role against *E. coli* and *P. aeruginosa* besides the acidity.

These results will compatible with the work of [21], in which all *Lactobacillus spp.* in their experiment inhibited the growth of *E. coli* and *Staph. aureus*. Also, [22, 23] they get a good antibacterial activity of *Lactobacillus spp.* metabolites against *Staph. aureus, E. coli, H. pylori* and yeasts, they confirmed in their work that probiotic bacteria especially *Lactobacillus spp*. exert suppressive effect on pathogens.

The work of [24] showed the presence of antimicrobial activity of the probiotics isolated from the different milk products against *Staph. aureus, E. coli, P. aeruginosa, S. typhi, S. marcescens* and *Candida albicans*, they will be thinking that results may be due to the production of acetic and lactic acids

that lowered pH of the medium and the probiotic bacteria may also have competed for nutrients, simultaneously produced hydrogen peroxide and bacteriocins that acted as antibiotic agents. These bacteria produce peptides having inhibitory properties and the antimicrobial action is due to the potential of lactic acid bacteria to produce lactic acid and bacteriocins [25].

While the production of *Lactobacillus spp.* to secondary metabolites in water solution exhibited weak growth of *Lactobacillus* on MRS agar plates and a good growth for both *E. coli* and *P. aeruginosa* on nutrient agar plates, these results may related to the absence of suitable conditions for *Lactobacillus spp.* to produce metabolites that affect clinical isolates of *E. coli* and *P. aeruginosa* and affirmed the previous results.

Conclusions

From the results obtained, *Lactobacillus spp.* that isolated from commercial and home-made yoghurts have the ability to inhibit the growth of clinical isolates of *E. coli* and *P. aeruginosa* by two effective ways, acidity that resulted from lactic acid production which lowered pH of the medium, and production of antimicrobial metabolites.

Recommendations

Probiotics cultures using in yoghurts may be could decrease exposure to pathogens and improvement gut normal flora, therefore additional controlled studies are needed to clarify the safety and efficacy of these agents.

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References

1. Guessas, B. and Kihal, M. (2004). Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats milk. Afr. J. Biotechnol., 3:339-342.

2. Todar, K. (2004). Lactic acid bacteria. Scie. J., 304:1421.

3. Espirito Santo, M. L. P.; Beirao, L. H.; Sant Anna, E. S.; Dalcin, E. B. and Franco, B. G. M. (2003). Bacteriocinogenic effect of *Lactobacillus Sakei* 2A on microbiological quality of fermented *Sardinella braziliensis*. Braz. Arch. Biol. Technol., 46:553-561.

4. Gupta, V. and Garg, R. (2009). Probiotics. Indian J. Med. Microbiol., 27(3):202-209.

5. Ershidat, O. T. M. and Mazahreh, A. S. (2009). Probiotics bacteria in fermented dairy products. Pak. J. Nutr., 8(7):1107-1113.

6. De Vuyst, L.; Callewaert, R. and Crabbe, K. (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorous* and evidence for stimulation of bacteriocin production under un favourable growth conditions. Microbiol., 142:817-827.

7. Rodgers, S.; Kailasapathy, K.; Jacob and Peiris, P. (2002). Bacteriocin production by protective cultures. Food Service Technol., 2:59-68.

8. Heyman, M. (2000). Effect of lactic acid bacteria on diarrheal diseases. J. Ameri. Coll. Nutr., 19(2):137-146.

9. Altieri, C.; Speranza, B. M. A.; Del Nobile, M. A. and Sinigaglia, M. (2005). Suitably Bifidobacteria and thymol as biopreservatives in extending the shelf life of fresh packed plaice fillets. J. Appli. Microbiol., 99:1294-1302.

10. Aly, S.; Quattara Cheik, A. T.; Bassole Imael, H. N. and Alfred, T. S. (2006). Bacteriocins and lactic acid bacteria –A mini review. Afr. J. Biotechnol., 5:78-683.

11. Elmer, G. W. (2001). Probiotics: living drugs. Am. J. Health-Syst. Pharm., 58(12):1101-1109.

12. Harrigan, W. F. and McCane, M. E. (1976). Laboratory Methods in Food and Dairy Microbiology, Academic Press, London, UK. Pp. 19-20.

13. Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S.T. (1994): Bergey's manual of Systematic Bacteriology. 9th ed. Williams and Williams. Baltimore. pp. 566.

14. Collee, J. G.; Fraser, A. G.; Marmion, B. P. and Simon, A. (1996). Mackie and McCartney Practical Medical Microbiology. 14thed. Churchill Livingstone. New York. pp. 978

15. Awan, J. A. and Rahman, S. U. (2005). Microbiology Manual. Unitech Communications, Faisalabad, Pakistan, pp: 49-51.

16. Schillinger, V. and Luke, S. K. (1989). Antimicrobial activity of *Lactobacillus Sakei* isolated from meat. Applied Environment. Microbiol., 55:1901-1906.

17. Early, R. (1992). The Technology of Dairy Products. VCH Publishers , Inc. N. Y. pp. 1-298.

18. Van Der Berg, J. C. T. (1988). Dairy technology in the tropics and subtropics. Pudoc, Wageningen. The Netherlands. Pp. 146.

19. Garrote, G. I.; Abraham, A. G.; and De Antoni, G. L. (2000). Inhibitory power of Kefir: The role of organic acids. J. Food Protect., 63:364-369.

20. Pitt, M. W.; Harden, T.; and Hull. R. R. (2000). Behavior of *Listeria monocytogenes* in pasteurized milk during fermentation with lactic acid bacteria. J. Food Protect., 63:916-920.

21. Ashraf, M. ; Arshad, M. ; Siddique, M. and Muhammad,, G. (2009). *In vitro* screening of locally isolated Lactobacillus species for probiotic properties. Pakistan Vet. J., 29(4): 186-190.

22. Ibrahim, S. M. and Desouky, S. G. (2009). Effect of antimicrobial metabolites produced by Lactic Acid Bacteria (Lab) on Quality aspects of frozen Tilapia (*Oreochromis niloticus*) fillets. World J. of Fish and Marine Sci. , 1(1):40-45.

23. Behrad, S.; Yusof, M. Y.; Goh, K. L.; Baba, A. S. (2009). Manipulation of probiotics fermentation of yoghurt by Cinnamon and Licorice: effect on yoghurt formation and inhibition of Helicobacter pylori growth in vitro. World Academy of Science Engineering and Technology.

24. Eduardo, L.; Chuayana, Jr.; Carmina, V. P.; Ma. Rosanna, B. R. and Esperanza, C. C. (2003). Antimicrobial activity of probiotics from milk products. Phil J. Microbiol. Infect. Dis. , 32(2):71-74.

25. Strus, M.; Pakosz, K.; Gociniak, H.; PrzondoMordarska, A.; Roynek, E.; Pituch, H.; Meisel-Miko Ajczyk, F. and Heczko, P. B. (2001). Antagonistic activity of *Lactobacillus* strains against anaerobic gastrointestinal tract pathogens (*Helicobacter pylori, Campylobacter coli,Campylobacter jejuni, Clostridium difficile*). Med. Dosw. Mikrobiol., 53:133-142.

الفعالية ضد الجرثومية للنواتج الأيضية لجرثومة العصيات اللبنية على نمو عزلتين جرثوميتين خارج الجسم الحي

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درست الفعالية ضد الجرثومية للنواتج الأيضية الثانوية المنتجة من العصيات اللبنية ضد عزلتين مرضيتين لجرثومتي الأشريكية القولونية والزوائف الزنجارية وكان لها تأثيراً مثبطاً على كلا العزلتين. كما درس تأثير الحموضة الناتجة عن التخمر اللاهوائي لسكر اللاكتوز الى حامض اللاكتيك بواسطة العصيات اللبنية. أن تجمع حامض اللاكتيك في الوسط أدى الى خفض الأس الهيدروجيني للبن مما أدى الى منع نمو العزلتين المرضيتين.

أختبرت قابلية كلا العزلتين المرضيتين ضد الأس الهيدروجيني الحامضي، القاعدي والمتعادل، ولم تظهر النتائج أي نمو جرثومي لكلا العزلتين في الأس الهيدروجيني الحامضي بينما أظهرت كلا العزلتين نمواً كثيفاً في الأس الهيدروجيني القاعدي والمتعادل.

أن الدراسة الحالية تؤكد وجود تأثير تعاوني للنواتج الأيضية الثانوية للعصيات اللبنية والحموضة في اللبن أدت الى فعل تثبيطي على كلا العزلتين المرضيتين.