Assiut Vet. Med. J. Vol. 61 No. 144 January 2015

EFFECT OF LACTIC ACID PRODUCING BACTERIA ON SOME POTENTIAL PATHOGENS IN SAUSAGE

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ABSTRACT

A total of 128 isolates of lactic acid bacteria (LAB) were isolated from hundred sausages samples collected from different supermarkets in Assiut City. The obtained isolates were identified bacteriologically as *Pediococcus cervisiae* (35), *Enterococcus faecium* (25), *Lactobacillus brevis* (12), *Lactobacillus* acidophilus (12), *Lactobacillus bulgaricus* (12), *Lactobacillus fermetni* (12), *Streptococcus thermophilus* (10) *Lactococcus garvieae* (10). Then they were screened for antagonistic activity against *listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella Typhi*murium using agar well diffusions method. All these isolates exhibited antibacterial activity against *L.monocytogenes* and *E.coli*, the most active strain against both pathogens was *Pediococcus cervisiae*, whereas *Enterococcus faecium*, *Streptococcus thermophilus* and *Lactococcus garvieae*. were not active against, *Staphylococcus. aureus*, also *Lactobacillus brevis*, *Lactobacillus acidophilus*, *Lactobacillus fermenti* and *Lactococcus garvieae* were not active against *Salmonella Typhi*murium.

Received at: 31/12/2014

Accepted: 15/2/2015

***Key words*:** lactic acid bacteria, *Lactobacillus acidophilus Staphylococcus aureus, Escherichia coli*.

INTRODUCTION

Lactic acid bacteria refers to a large group of beneficial bacteria that have similar properties and all produce lactic acid as an end product of the fermentation process. They are widespread in nature and are also found in our digestive systems. Although they are best known for their role in the preparation of fermented dairy products, they are also used for pickling of vegetables, curing fish, meats and sausages, in addition to flavoring foods and inhibiting pathogenic as well as spoilage bacteria in these products (Morsi *et al.*, 2003).

The general description of the bacteria included in this group is gram-positive, non-spore forming, cocci or rods, it is nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins. Recent taxonomic revisions of these genera suggest that the lactic acid bacteria comprise the following: *(Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lecunostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella)* (Rattanachaikunsopon and Phumkhachorn, 2010).

The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentration and acid or alkaline tolerance. LAB can be mainly divided into two groups based on the end-products formed during the fermentation of glucose. Homofermentative LAB such as *Pedicoccus*. *Streptococcus*, *Lactococcus* and some *Lactobacilli* produce lactic acid as the major or sole end-product of glucose fermentation. Heterofermetnive LAB such as *weissella*, *leuconostoc* and some *lactobacilli* produce equimolar amounts of lactate, CO_2 and ethanol from glucose. (De Vuyst and vandamme, 1993) and (Axelsson *et al.*, 1998).

The preservative action of starter culture in food system is attributed to the combined action of a range of antimicrobial metabolites produced during the fermentation process (Caplice and fizgerald, 1999). These include many organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms.

In addition to acids, starter strains can produce a range of other antimicrobial metabolites such as ethanol, H_2 O2 and diacetyl (Daeschel, 1989). Other examples of secondary metabolites produced by LAB

which have antagonistic activity include the compound reuterin (Axelsson *et al.,* 1989) and the recently discovered antibiotic reuterocyclin (Ganzle, 2000), both of which are produced by strains of *Lactobacillus* reuteri. Also L.A.B are known to produce compounds named "bacteriocins" (Geis *et al.,* 1983; Klaenhammer, 1988). Which are relatively small peptides, senstive to specific proteolytic enzymes, can be heat stable and have either bacteriocidal or baceriostatic activity against closely related, or in some casses a wide spectrum of microorganisms including food borne pathogens such as *Listeria monocytogenes* and some *lostridia*. This makes bacteriocin producers particulary for potential use in food preservation.

Nisin is the only bacterioicin with GRAS (Generally regarded as safe) status for use in specific foods and was further supported by the accumulated data indicating its nontoxic and nonallergenic nature, it is produced by strains of *Lactococcuslactis* and can prevent out growth of *Bacillus* and *Clostridium* spores (Daeshel, 1989).

Thus it is possible to use LAB for the treatment of different gastrointestinal disease (Ljungh and Wadstrom, 2009) and for foods preservation (Labioui *et al.,* 2005; Cocolim *et al.,* 2007; Dorlu and Thonart, 2009; Kouakou and Thonart, 2011) due to their antibacterial properties.

LAB have a very important role in the formation of the specific organoleptic characteristics of dry sausages, as well as the prevention of growth of pathogenic microorganism in this product (Zeljka *et al.,* 2012).

The objectives of this study were to isolate LAB as ferments present in sausages, evaluate the antibacterial activity of the isolates against *L.monocytogenes, S.aureus, E.coli,* and S.typhimurium and to select strains that could be used in sausages production.

MATERIALS and METHODS

Collection of samples:
Hundred sausage samples of various brands were collected from different supemarkets in Assiut City. The samples were freed aseptically from its casings and placed in clean bags and transferred to laboratory for microbiological study.

Isolation of LAB: (De Man *et al.,* 1960)

One gram of each sample was aseptically transferred to sterile MRS broth. Inculcated tubes were abaerobically incubated at 37ºC for 48-72 hours in Co_2 incubator, and next the broth was cultured on MRS-agar plates, then incubated under anaerobic conditions at 37ºC for 48-72 hours. Colonies of acid-producing bacteria identified by a clear zone around each colony, were randomly selected and purified by replating on MRS agar plates. After obtaining pure culture, Gram-stain, motility and biochemical tests were performed for identification purposes.

I-Identification of isolates:
Gram's stain (APHA, 1992):
LAB are Gram-positive, non-spore forming, cocci or rods.

Motility test (Baron *et al.,* 1994):
Tubes of semisolid medium were inoculated with a pure culture of suspected isolates by stabbing to a depth of approximately 2 cm with a bacteriological needle. After overnight incubation at suitable temperature, motility was evident as a haze of growth extending into the agar from the stabbing line. Growth of non-motile organism is restricted only to the stabbing line.

Π-Biochemical identification:
1- Catalase test (Land *et al.,* 1991):
A drop of 3% hydrogen peroxide was placed on a clean microscopic slide. A visible amount of bacterial growth was added with the inoculating loop. Both were mixed and observed for gas bubble production.

2-Oxidase test (Baron *et al.,* 1994):
By a sterile platinum loop, one colony was taken and rubbed in circles on a strip of filter paper impregnated with oxidase reagent (1% solution of tetramethy1-P-phenylene Diamine-Dihydrochloride). Appear-ance of dark purple color within 5-10 seconds indicated positive results while, no change in color means negative.

3-Indole production test (Koneman *et al.,* 1992):
Tryptone water tubes were inoculated with a pure culture of the tested organism. Inoculated tubes were incubated at 35ºC for 18-24 hours, and then drops of Kovac's reagent were added down the inner wall of the tubes. Development of a bright fuchsin red color at the interfere of the reagent and the broth within seconds indicated positive test, while no change in the broth indicated negative test.

4-Growth at 15ºC, 37ºC and 45ºC (Collins and Lynes, 1989):
Inoculate the isolated organism in MRS broth tubes and incubate at 15ºC, 37ºC and 45ºC and look for growth.

5-Carbohydrate fermentation (Collins and Lynes, 1989):
Inoculate the isolated organisms in MRS broth tubes containing the following sugars: lactose, sucrose,

mannitol, xylose, maltose and trehalose in concentration of 1% and with 0.05% phenol red as indicator. Tubes were incubated at 37ºC and results recorded daily up to 7 days. Appearance of yellow color indicated sugar assimilation.

6- Nitrate reduction test (Mackie and McCartney, 1989):
The isolated strains were inculcated into nitrate broth and incubated at 37ºC for 96 hours. After that 0.1 ml of the nitrate test reagent was added to the test culture. A red color developing within a few minutes indicated the presence of nitrite and hence the ability of the organism to reduce nitrate, while no change in color means negative results.

7- Arginine hydrolysis (Collins and lynes, 1989):
In MRS broth replaces the ammonium citrate by 0.3% arginine, the isolated strain was inoculated and incubated at 28-30ºC for 3-4 days. A positive reaction indicating hydrolysis of arginine with the formation of ammonia which causes alkalinity in the medium and denoted by changing the yellow medium into a distinct red or pink color.

8-Growth at 4% NaCI (Peter *et al.*, 1986):
MRS broth containing 4 g of NaC1 was inoculated with the isolated strains and incubated at 37ºC for 24 hours and examined for growth.

III-Detection of antibacterial activity:
The agar-well diffusion method was used to detect and determine the antibacterial activities of the isolated strains in which *Listeriamonocytogenes* (NCIB No 8588), *Escherichia coli* (NCPC No 12023), *Staphylococcus aureus* (NCPC No 7447), *Salmonella Typhi*murium (NCPC No 12241) were used as indicator bacteria for detection of the antibacterial activity, all strins mentioned were obtained from High Quality Meida Unit (HQM) in Animal Health Research Institute in Dokki, Egypt, the pathogens were maintained in Brain Heart infusion Agar (BHIA) butt– slants in screwcapped tubes kept at 4ºC.

Strains were propagated in a tryptone soya broth of 24 hrs culture at 37ºC.

IV- Bacteriocin activity assay (Geis *et al.*, 1983):
The isolated strains that were selected as potential bacteriocin producers were grown in MRS broth at 37ºC for 48 hours. Cells were separated by centrifugation at 5000 rpm for 10 minutes. The pH of the cell free supernatant was adjusted to 5.5 with sterile 0.2 N NaOH. Bacteriocin activity in the supernatant was tested by agar well diffusion assay.

Agar well diffusions method:
20 ml of molten nutrient agar medium were cooled at 47ºC and seeded with 1% overnight culture of each indicator organism separatly. Seeded agar was poured into sterile petridish and allowed to solidify at room temperature. Wells of 7mm diameter were cut in the solidified agar using a sterile metal cork borer and filled with 100µL of supernatant bacteriocin. The plates were left at 4-5ºC for 2 hours to allow diffusion of the substances and then incubated in Co_2 incubator for 24 hours at temperature optimum for the indicator organism 37ºC. Absence or presence of inhibition zones as well their diameters were recorded.

Another nutrient agar plates were seeded with 1% overnight culture of each indicator organism and used as control in which the wells were filled with sterile distil water and incubated at 37ºC for 24 hours. Absence or presences of inhibition zones were recorded.

RESULTS

Table 1: Incidence of isolated strains of LAB from sausages samples.

Number of samples tested (n)	*Lactobacilli SPP*								*Pediococcus cerevisiae*		*Enterococcus faecium*		*S.treplo coccustherm ophilus*		*Lactococcus garvieae*	
	L.brevis		*L.acidophilus*		*L.bulgaricus*		*L.fermenti*									
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
100	12	12	12	12	12	12	12	12	35	35	25	25	10	10	10	10

Table 2: Antibacterial activity of the isolated strains against some pathogenic microorganisms.

Isolated strains	Zone of inhibition(mm)							
	control	*Listeria monocytogenes*	control	*Staph.aureus*	control	*E.coli*	control	*S.typhim-urium*
L.brevis	**0**	**1.3**	**0**	**0.92**	**0**	**2.4**	**0**	**0.25**
L.acid	**0**	**0.79**	**0**	**0.53**	**0**	**1.84**	**0**	**0.1**
L.bulgaricus	**0**	**2.5**	**0**	**1.5**	**0**	**1.6**	**0**	**0.6**
L.fermenti	**0**	**1.5**	**0**	**1.86**	**0**	**1.9**	**0**	**0.36**
Ped.cerevisiae	**0**	**2.8**	**0**	**0.82**	**0**	**2.5**	**0**	**1.9**
Ecfaecium	**0**	**0.9**	**0**	**0.28**	**0**	**0.96**	**0**	**0.83**
S.thermophilus	**0**	**1.9**	**0**	**0.25**	**0**	**1.8**	**0**	**0.78**
Lc.garvieae	**0**	**0.7**	**0**	**0.34**	**0**	**0.81**	**0**	**0.2**

DISCUSSION

Lactic acid bacteria originally isolated from traditional sausages are probably the best candidates for improving the microbiological safety of these foods, because they are well adapted to the conditions in sausages and should there fore be more competitive than LAB from other sources (Salim *et al.,* 2006).

Naturally, different microorganisms derived from raw materials and the environment contaminate dry sausage mixtures. Among them, LAB are found to be the most active microorganisms in sausages (Sawitzki *et al.,* 2007).

In this study 8 strains were isolated from 100 collected samples of sausages. The strains were identified as showen in table (1) as *Pediococcus cerevisiae* (35), *Enterococcus faecium* (25), *Lactobacillusbrevis* (12) *Lactobacillus acidophils* (12), *Lactobacillus bulgaricus* (12), *Lactobacillus fermenti* (12), *Streptococcus thermophilus* (10) and *Lactococcus garvieae* (10). This indicated that the most isolated LAB was assigned to *Lactobacillas species* (48%), this observation is very close to the data of the (Erkkila *et al.,* 2001) who also pointed out that *Lactobacillus species* are the dominate microflora in fermented sausages. (Zeljka *et al.,* 2012) reported that the majority of the strains isolated from the Croatian dry fermented sausages were assigned to the species *L.plantarum* and *L.brevis.* Also other Lactobacilli such as *L.plantarumL. casei, L.brevis* and *L.alimentarius* could be isolated as well by (Rantsiou and cocolin, 2008).

Some authors indicated that the most frequent isolates of LAB in the fermentation process of dry sausages from European countries are *L.sakei, L.curvatus and L.plantarum* (Gurakan *et al.,* 1995; Santos *et al.,* 1998; Samelis *et al.,* 1998; Erkkila *et al.,* 2001; Aymerich *et al.,* 2003; Papamanoli *et al.,* 2003; Drosinos *et al.,* 2005; Gasparik- Reichardt *et al.,* 2005; Rantsiou *et al.,* 2006; Drosinos *et al.,* 2007; Kozacinski *et al.,* 2008; Mozzi *et al.,* 2010).

This variation in the recovered stains of *Lactobacilli* isolated by the previous authors may be due to the difference in the composition of dry sausages from region to region and there are also difference in the technological production process.

On the other hand both of *Streptococcus thermophilus* and *Lactococcus garvieae* were isolated from much fewer samples (10%) for each as showed in (table 1), also this table reported the percentages of *Pediococcs cerevisiae* and *Enterococcus faecium* they were 35% and 25% respectively.

In industries which process animal based products, overall *Listeria SPP*, are a major problem for this industry and although chemicals such as Na No_2 can inhibit *Listeria* and other pathogens in sausages, such substances may represent health risks for consumers and there is a constant demand for new preservative agents (Montville and Winkowski, 1997; Cleveland *et al.,* 2001).

Some authors (De martinis and Freilas, 2003; Tyopponen *et al.,* 2003 and Dicks *et al.,* 2004) have suggested the use of bacteriocinogenic LAB as starter cultures in food preservation.

For this reason the isolated strains of LAB in our study were screened for antagonistic activity against some pathogenic microorganisms (*Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*) to evaluate the

antibacterial activity of these isolates against the indicator organisms.

According to the results recorded in table (2) the most active strain against *L.monocytogens* was *Pediococcus cerevisioae* with an inhibition zone (2.8) mm followed by *Lactobacillus bulgaricus* with an inhibition zone (2.5) mm, while *Stryptoccus thermophilus*, *Lactobaccillus* and *Lactobacillusbrevis* showed an inhibition zones (1.9, 1.5 and 1.3) mm respectively.

Other strains, *Enterococcus faecium*, *Lactobacillus acidophilus* and *Lactococcus* garvieae showed a weak inhibition zones (0.9, 0.79 and 0.7) mm respectively.

As the results indicate, the zones of inhibition were varied, ranging between (0.7 to 2.8) mm. this revealed that all isolated strains inhibited *L.monocytogenes* according to (Schillinger and Lucke, 1989) who mentioned that inhibition was scored positive if the width of the *Pediococcus cervisiae* (35), *Enterococcus faecium* (25), *Lactobacillus brevis* (12), Also table (2) showed that most active strains against *Staphylococcus aureus* were *Lactobacillus fermetniand Lactobacillus bulgaricus* with an inhibition zones (1.86 and 1.5) mm respectively and the least active strains against this organism were *Lactobacillus brevis* (12), *Pediococcus cervisiae* and *Lactobacillus* acidophilus with an inhibition zones (0.92, 0.82 and 0.53) respectively, while the strains *Pediococcus cervisiae*, *Enterococcus faecium* and *Streptococcus thermophilus* showed no inhibitory effect against *Staphylococcus aureus*, according to (Schillinger and Lucke, 1989), that their inhibition zones less than 0.5mm. The fact that no inhibition was noticed by these strains against *Staphylococcus aureus*, may be an indication that their initial activities were due to the organic acid secretion such as lactic acid, while the activity of the other strains is an indication of the presence of other antibacterial substances such as peroxides, diacetyls and baceriocins.

These observations are in agreement with those reported by (Ogunbanwa *et al.,* 2003) who showed that *L.brevis* excreted other compounds such as bacteriocins that inhitited the growth of pathogens.

The ability of *Enterococcus faecium* species isolated from fermented sausages to display antibacterial activity against *Listeria* and *Staphylococcus aureus*, species was reported by several authors (Cintas *et al.,* 1997; Callewaert *et al.,* 2000 and Herranz *et al.,* 2001), this agreement with our study in which *Enterococcus faecium* showed antibacterial activity against *Listeria*. *monocytogenes*, and disagreement with our study in which the same strain showed no inhibitory effect against *Staphylococcus aureus*. Regarding the data presented in the same table it was found that all isolated strains were active against Gram-negative *E.coli*., the most active strain against this organism was *Pediococcus cervisiae* with an inhibition zone (2.5) mm. and the least active one was *Lactococcus garvieae* with an inhibition zone (0.81) mm. On the other hand 4 of the isolated strains were not active against Gram-negative *Salmonella typhimurium* they were *Lactobacillus. brevis Lactobacillus* acidophilus, *Lactobacillus. fermenti* and *Lactococcus garvieae*, in which their inhibition zones less than (0.5) mm. according to (Schillinger and Lucke, 1989).

The antimicrobial activity of LAB against Gram–negative organismses could not be detected by many authers (Steven *et al.,* 1991; Hechard *et al.,* 1992 and Mathieu *et al.,* 1993), also (Gao *et al.,* 1999) reported that the outer membrane of Gram-negative bacteria may protect the cytoplasmic membrane from the action of the antimicrobial compound, whereas (Jay 1982) reported that Diacetyl is an aroma component produced by strains within all genera of LAB and inhibits the growth of Gram-negative organisms by reacting with arginine utilization.

CONCLUSION

In this study the most active strain against *listeria monocytogene, E coli* and *Salmonella typhimurium* was *Pediococcuscerevisioae* while the most active strain against *Staphyloccocus asureus* was *Lactobaccillus. Fermenti*. This inhibition is possibly due to the fact that LAB produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin in the course of their metabolism.

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تاثير البكتريا المفرزة لحمض اللاكتيك علي بعض المسببات المرضية المحتملة في السجق

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لقد تم عزل ١٢٨عترة بكتيرية من بكتريا حمض اللاكتيك من ١٠٠ عينة سجق تم جمعها من محلات مختلفة بمدينة أسيوط وتم التعرف على هذه العترات بالتحليل البكتريولوجي والخصائص الظاهرية وإجراء الاختبارات الكيميائية لها وكانت كالآتي:
Lactobacillus bulgaricus (12), *Enterococcus faecium* (25).*Pediococcuscervisiae*(35), *Lactobacillus* acidophilus (12), *Lactobacillus fermetni* (12), *Streptococcus thermophilus* (10), *Lactococcusgarvieae* (10 *Lactobacillus brevis* (12))

وبدراسة التأثير المثبط لهذه العترات ضد ميكروب الليستيريا مونوسيتوجين والمكور العنقودي الذهبي والميكروب القولوني وسالمونيلا التيفود باستخدام طريقة الانتشار الجيد عبر الآجار. أوضحت النتائج أن كل العترات لها نشاط بكتيري مضاد لميكروب اللستريا مونوسيتوجين والميكروب القولوني وكانت سلالة *Pediococcuscervisiae* هي أقوى سلالة لها نشاط بكتيري ضد هاذين الميكروبين وكذلك ضد ميكروب سالمونيلا التيفود. بينما كانت السلالات
Lactococcusgarvieae,Streptococcusthermophilus, Enterococcus faecium ليسلها نشاط بكيتري ضد ميكروب المكور العنقودي الذهبي وكذلك السلالات *Lactobacillus fermetni, Lactobacillus acidophilus Lactobacillus brevis.,Lactococcus.garvieae* لم يكن لها نشاط بكتيري ضد ميكروب سالمونيلا التيفود. وكانت أغراض البحث هي عزل بكتريا حمض اللاكتيك كمعزز حيوي موجود بالسجق وتقييم النشاط البكتيري لها ضد ميكروب اللستيريا مونوسيتوجين والمكور العنقودي الذهبي والميكروب القولوني وسالمونيلا التيفود واختيار أفضل السلالات التي يمكن أن تستخدم في إنتاج السجق.