

**EFFECT OF PROBIOTICS BACTERIA ISOLATED FROM  
YOGHURTS PRODUCED IN DAMIETTA CITY ON SOME  
PATHOGENIC BACTERIA**

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**ABSTRACT**

**A** total of 200 samples of different brand of traditional (balady) yoghurts were randomly collected from local markets and supermarkets in various districts of Damietta city.

Probiotics were isolated from yoghurts samples anaerobically in the presence of 10% CO<sub>2</sub> and identified. In this study a total of 17 lactic acid bacteria were isolated. For the antimicrobial activity of the isolated probiotics organisms were used as the inhibitory substances against some common foodborne pathogens (E.coli, Salmonella spp. and Staphylococcus aureus) using the agar-well diffusion assay.

In conclusion, yoghurts probiotics has an antimicrobial activity in vitro against the tested indicator pathogens and have better antimicrobial activity at concentration of 10<sup>9</sup> CFU/g .

**Keywords:** probiotics, traditional (balady) yoghurts, antibacterial, E. coli, Staphylococcus aureus and Salmonella spp.

## INTRODUCTION

Probiotics are widely used in fermented food production and are considered as generally recognized as safe organisms which are safely applied in medical and veterinary functions. In the food industry, probiotics are widely employed as starter cultures and have been indexed as part of human microbiota. Yoghurts, cheese and fermented milk products are mentioned as the main food sources of probiotics. The lactic acid that is produced from the fermentation of lactose contributes to the sour taste of yoghurts by decreasing pH and allows for the characteristic texture by acting on the milk proteins **Heller, (2001)**. Probiotics are alive and nonpathogenic microorganisms that have beneficial effects on their host's health. Traditional dairy products have been used for many centuries among the natives and are the main source of potentially probiotics bacteria.

Probiotics are living, health-promoting microorganisms that are incorporated into various kinds of foods. The ability of probiotics to withstand the normal acidic conditions of the gastric juices and the bactericidal properties of the bile salts, as well as the production of lactic acid that inhibits the growth of other microorganisms, allow them to be established in the intestinal tract (**Oskar et al., 2004**). Probiotics can produce a wide range of antimicrobial metabolites, i.e. organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins. These antimicrobial activities can contribute in the microbiological safety by controlling the growth of other microorganisms, and inhibition of pathogenic bacteria **Hobbs, (2000)** and **Ouweh and Vest (2004)**. The reported health benefits of probiotics include: boosting of the immune system, inhibition of the growth of pathogenic microorganisms, prevention of diarrhea from various causes, prevention of cancer, reduction of the risk of inflammatory bowel movements, improvement of digestion of proteins and fats, synthesis of vitamins, and detoxification and protection from toxins **Sonomoto and Yokota (2011)**. Members of the genera *Lactobacillus* and *Streptococcus* are the most common probiotics used in commercial fermented and non-fermented dairy products today (**Reid and Bruce 2006**).

The aim of this study was to determine the antibacterial effects among the probiotics bacteria isolated from different yoghurts marketed in Damietta Governorate against some common bacterial food pathogens.

## MATERIALS AND METHODS

### Materials:

#### Samples collection:

Two hundred traditional (balady) yoghurts samples of (50 ml) were randomly collected from different shops and super markets at Damietta city and kept under refrigeration temperature using an ice box and brought to the laboratory.

**Methods:**

**Plating and isolation of lactic acid bacteria (LAB) according to the standards IDF, International Standards (1996):**

The samples of the yoghurts were shaken vigorously to suspend the bacterial contents. Then, 10 g of each yoghurts sample was separately mixed in 50 ml of MRS broth at pH 6.5. After mixing into MRS broth, they were shaken to obtain a homogeneous suspension. Anaerobic condition in the presence of 10% CO<sub>2</sub> was created for removal of unwanted bacteria. The plates were incubated anaerobically on jars using BBL, GasPak Plus at 37°C for 72 h. Finally, the single colony of bacteria was isolated by observing their colonial morphology and some physiological tests (Gram staining and catalase reaction).

**Identification of isolates (Holt, 1984 and Ali 2011):**

The suspected probiotics were further pure cultured for morphological and biochemical identification. Gram stain and microscopic study were performed for the isolates of 18 hrs. culture from MRS agar plates.

**Acid tolerance test ( Laroia and Marin, 1991):**

The isolated Lactobacilli were subjected to primary screening for acid tolerance in MRS broth adjusted to pH 2.5 with 1N HCl for 90 min at 37°C. The determination of survival was performed by single streaking on MRS agar plates, and the growth was observed after 24-48 h after anaerobic incubation at 37°C. Isolates which were growing on the agar were considered to be acid tolerant strains.

**Pathogenic bacterial strains:**

The pathogenic strains used in this work were obtained from microbiology laboratory, Animal Health Research Institute Giza, Cairo, Egypt. The pathogens were maintained in brain heart infusion agar (BHIA) butt-slants in screw-capped tubes kept at 4°C. They included both Gram-negative (*Escherichia coli*, *Salmonella* spp.) and Gram-positive strains (*Staphylococcus aureus*) (Laboratory strains).

**Agar-well diffusion assay according to (Cardirici and Citak (2005); Raczyńska-Cabaj et al., (2005) and Lalitha, (2007):**

- The pathogenic bacterial strains from the stock cultures were subculture aerobically in brain heart infusion broth (BHIB) medium at 37°C for 18hrs., mixed at 1% with about 25ml of BHIA medium held at 45°C (to give approximately 10<sup>6</sup> CFU/ml), and then poured into sterile Petri dishes and stored at 4°C for 1h. to solidify the media.
- A 6mm wide well was cut in the center of each solidified agar plate, using a sterile metal borer.
- Culture supernatants of probiotics isolates were prepared by cultivation anaerobically in MRS broth overnight at 37°C and removal of the cells by centrifugation at 2000×g for 10 minutes. Culture supernatants pH was adjusted

to 7.0 using (1M NaOH). Culture supernatant was passed through a sterile microbiological filter (Millipore 0.22  $\mu\text{m}$ ) for sterilization, and then aliquots of 100  $\mu\text{l}$  were poured in each well of BHIA medium.

- The plates were first incubated at 4°C for 1-2h. to allow the test material to diffuse into the agar and, then incubated under aerobic conditions at 30°C for 18-24hrs.
- The previously prepared neutralized filtrate of cell-free supernatants were 2-fold serially diluted in MRS broth and examined for their antimicrobial activity as described before in order to determine the minimum inhibition concentration (MIC) induced by each probiotics strain. MIC was expressed as arbitrary unit (AU/ml), (one arbitrary unit was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition on the indicator lawn).
- After 24h. of anaerobic incubation, each plate was examined for zones of inhibition around the wells. Inhibition was considered positive when the width of the clear zone around the was 0.5mm or more where, resistance was defined as the absence of a growth inhibition zone around the well.
- Finally, the diameter of the zones of complete inhibition was measured, including the diameter of the disc. Zones are measured to the nearest whole number in millimeter, using transparent ruler and recorded.
- To further determine whether the selected pathogens were inhibited or killed by probiotics, the growth inhibition zone was swabbed. The swab was then inoculated into BHIB medium and incubated aerobically under 37°C for 24 h. The BHIB medium were then checked for growth. The presence of growth in the broth was interpreted as an inhibitory property in the agar plate, while no growth was interpreted to be as a result of the bactericidal effect.
- Each of the tests in the determination of antibacterial effects of the probiotics was conducted in two trials, and in duplicate (**Lim and Dond-Soon, 2009**).
- Parallel work was done as control without using the pathogenic bacteria.
- Each of the tests in the determination of antimicrobial activity of the probiotics was conducted in two trials, each in triplicate.

**Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) determination by broth dilution according to Abdelbasset and Djamil (2008):**

For MIC and MBC determinations, serial dilutions of culture supernatants of probiotics in 5 ml of broth inoculated with 50  $\mu\text{l}$  of fresh indicator pathogens (inoculum,  $\sim 10^8$  CFU/ml). The tubes were incubated at 37°C overnight with shaking and the highest dilution in which there were no growths was recorded as the MIC. For MBC testing, aliquots (20  $\mu\text{l}$ ) of broth from tubes containing no growth were plated onto solid medium and again incubated overnight at 37°C. The highest dilution in which there were no survivors was recorded as the MBC. In the above method, controls for each organism were performed using the sterile liquid medium without aqueous crude extract. All MICs and MBCs were confirmed by triplicate assays.

**Determination of optimal growth and pH according to Kim et al., (2003):**

For the determination of optimal growth and pH of *Lactobacillus* 1% (v/v) fresh overnight culture of *Lactobacillus* were inoculated into MRS broth with varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5N NaOH. The inoculated broths were incubated in anaerobic condition 24 h. at 37°C in the presence of 10% CO<sub>2</sub>. After 24 h of incubation growth of the bacteria were measured using a spectrophotometer, reading the optical density at 560 nm (OD) against the uninoculated broth.

**Statistical analysis:**

Statistical analysis was performed using SPSS statistical program for windows (Version 16) (SPSS Inc., Chicago, IL, and USA).

## RESULTS AND DISCUSSION

After morphological examination in plates and Gram staining it could be concluded that the isolated bacteria were rod shaped, convex, smooth, shiny, irregular, circular, Gram positive, facultative anaerobic, non-spore forming which indicate them to be the member of *Lactobacillus* spp (Holt, 1994). These isolates were subjected to grow on selective MRS agar media and produced round shape, off-white to cream color, shiny colonies those were quite similar to the reference *Lactobacillus* spp. grown on MRS agar media. Isolates when Gram stained, found rod shaped, short-medium chain and positive in Gram reaction those all are typical characteristics of *Lactobacillus* spp. according to Bergey's Manual of Determinative Bacteriology (Holt, 1984).

**Table (1): The number of rod-shaped (*Lactobacillus* isolates) after biochemical identification and screening for acid tolerance.**

Dairy Products	Samples number	Number of isolates
Group 1	50	2
Group 2	50	5
Group 3	50	3
Group 4	50	7
	200	17

**Table (2): Diameter of zone of inhibition (mm) produced by sterilized crude extract of probiotics isolates on pathogenic bacteria using well diffusion assay technique.**

<b>Group 1 isolates</b>	<b>(1)</b>	<b>(2)</b>	-	-	-	-	-
<b>E. COLI</b>	12	9	-	-	-	-	-
<b>SALMONELLA</b>	20	12	-	-	-	-	-
<b>S. AUREUS</b>	9	10	-	-	-	-	-
<b>Group 2 isolates</b>	<b>(1)</b>	<b>(2)</b>	<b>(3)</b>	<b>(4)</b>	<b>(5)</b>	-	-
<b>E. COLI</b>	10	13	11	10	13	-	-
<b>SALMONELLA</b>	27	16	14	20	16	-	-
<b>S. AUREUS</b>	9	12	11	10	11	-	-
<b>Group 3 isolates</b>	<b>(1)</b>	<b>(2)</b>	<b>(3)</b>	-	-	-	-
<b>E. COLI</b>	13	12	13	-	-	-	-
<b>SALMONELLA</b>	17	14	14	-	-	-	-
<b>S. AUREUS</b>	9	10	9	-	-	-	-
<b>Group 4 isolates</b>	<b>(1)</b>	<b>(2)</b>	<b>(3)</b>	<b>(4)</b>	<b>(5)</b>	<b>(6)</b>	<b>(7)</b>
<b>E. COLI</b>	13	13	12	11	12	9	11
<b>SALMONELLA</b>	18	14	14	17	13	17	14
<b>S. AUREUS</b>	10	9	10	8	9	11	9

\*\*Values between brackets denoted the probiotics isolates /examined groups

**Table (3): Antibacterial activity of probiotics isolates against three pathogenic bacteria.**

<b>Antibacterial</b>			
<b>Group</b>	<b>Effect</b>	<b>MIC</b>	<b>MBC</b>
<b>Group 1</b>			
<b>E. COLI</b>		<b>10<sup>7</sup></b>	<b>+ Ve</b>
<b>SALMONELLA</b>		<b>10<sup>6</sup></b>	<b>+ Ve</b>
<b>S. AUREUS</b>		<b>10<sup>9</sup></b>	<b>- Ve</b>
<b>Group 2</b>			
<b>E. COLI</b>		<b>10<sup>7</sup></b>	<b>+ Ve</b>
<b>SALMONELLA</b>		<b>10<sup>6</sup></b>	<b>+ Ve</b>
<b>S. AUREUS</b>		<b>10<sup>9</sup></b>	<b>- Ve</b>
<b>Group 3</b>			
<b>E. COLI</b>		<b>10<sup>7</sup></b>	<b>+ Ve</b>
<b>SALMONELLA</b>		<b>10<sup>6</sup></b>	<b>+ Ve</b>
<b>S. AUREUS</b>		<b>10<sup>9</sup></b>	<b>- Ve</b>
<b>Group 4</b>			
<b>E. COLI</b>		<b>10<sup>7</sup></b>	<b>+ Ve</b>
<b>SALMONELLA</b>		<b>10<sup>6</sup></b>	<b>+ Ve</b>
<b>S. AUREUS</b>		<b>10<sup>9</sup></b>	<b>- Ve</b>

The lactic acid bacteria (LAB) comprise a clade of Gram-positive, acid-tolerant, generally non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria, usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Furthermore, lactic acid and other metabolic products contribute to the organoleptic and textural profile of a food item. The industrial importance of the LAB is further evinced by their generally recognized as safe status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces. Antimicrobial activity is one of the most important selection criteria for probiotics. Antimicrobial effects of lactic acid bacteria are incurred by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (**Dunne et al., 2001** and **Ammor et al., 2006**).

Data in table (1), showed the number of probiotics isolates which were 2, 5, 3 and 7 in group 1, 2, 3 and 4 respectively where all the isolates chosen were of the *Lactobacillus* species without further confirmation by advanced methods such as polymerase chain reaction.

The results showed in table (2), revealed that *Lactobacilli* probiotics inhibited all of the test indicator microorganisms either Gram positive (*S. aureus*) or Gram negative (*E. coli* and *Salmonella*) indicator pathogens and this data were in coincide with that reported by **Lei and Jacobsen, (2004)** and **Olivares et al., (2006)**.

From table (3), the results showed that isolated probiotics inhibited all of the test indicator microorganisms; *Lactobacilli* spp were bactericidal for *E. coli* and *Salmonella* spp., but inhibitory for *S. aureus*, the diameters of inhibition zones and it could be concluded that the probiotics could inhibit the growth of indicator microorganisms in different degrees as it had the strongest effect on *Salmonella* followed by *E. coli* then at last was the effect on *S. aureus* where the widest inhibition zone (27mm) was observed in case of *Salmonella* organisms subject to the inhibitory effect of the first probiotics isolated from yoghurts sample in group 2 and in the same time the narrowest inhibition zone was observed in case *S. aureus* subjected to the inhibitory effect of probiotics isolate from yoghurts samples in group 4. These data were nearly as previously obtained by **Ayad et al., ( 2004)** and **Lei and Jacobsen, (2004)** in the study on the antimicrobial activity of the probiotics isolated from yoghurts. Many authors supposed the antimicrobial effect of probiotics may be due to the production of acetic and lactic acids that lowered the pH of the medium **Bezkorovainy (2002)** and **Mobarez et al., (2008)**. The probiotics bacteria may also have competed for nutrients, **Marteau (1990)**, simultaneously produced hydrogen peroxide and bacteriocins that acted as antibiotic agents. Other than bacteriocins, some are also capable of reuterine production that is known to act as an antibacterial compound **Ray (2000)** and **Meyer et al., (2007)**.

The spectrum of antimicrobial activity for the species suggested that the inhibitory components were different (**Raja et al., 2009**). Similarly (**Cadirici and Citak, 2005**; **Girum et al., 2005** and **Hami, (2011)** observed varying degree of inhibition of various foodborne pathogens by the culture filtrate of lactic acid bacteria,

although these inhibitory substances produced by the lactic acid bacteria strains acts differently on the pathogenic reference indicator strains, inhibitive substances produced by the lactic acid bacteria can be generally protein (**Vandenberg 1993** and **Moghaddam et al., 2006**). Inhibition caused by hydrogen peroxide and organic acids was ruled out as the producer strains were cultured anaerobically and the culture supernatant was neutralized before assaying the antimicrobial activity. However, the importance of the inhibition effect varies according to serotypes (**Savado go et al 2004** and **Dhanasekaran et al., 2010**).

**Kurmann** and **Rasic (1991)** suggested to achieve optimal potential therapeutic effects, the number of probiotics organisms in a probiotics product should meet a suggested minimum of  $>10^6$  CFU/ml<sup>-1</sup> while other authors agreed that  $>10^7$  (**Davis et al., 1999** and **Raczynska-Cabaj et al., 2005**) could do the same effect mean while  $10^8$  CFU/ml<sup>-1</sup> was satisfactory levels by (**Kailasapathya** and **Rybka 1997**).

Probiotics concentration of  $10^9$  CFU/g in our study was the more effective probiotics concentration. Also, most of the probiotics lactobacilli in human foods are supplied in highly concentrated forms containing more than  $10^9$  CFU/g. These finding was in line with the observations of others who worked on fermented milk, **Almaz et al (1999)** in Ethiopia, **Savado go et al., (2004)** in fermented milk of Burkina Faso, **Ayad et al., (2004)** in Egypt.

In this work the sterilized crude extracts of the isolated probiotics organisms were used, therefore its action on pathogenic bacteria could be attributed to all of the previously mentioned substances.

Finally, the capability of the probiotics incorporated in bioyoghurts to inhibit the growth, or even kill certain selected pathogens confirms the health benefits derived from the consumption of these yoghurts as indicated from table (3), that showed that all probiotics isolates had a bactericidal effect on E. coli and Salmonella species but had a bacteriostatic effect on S. aureus with a minimum inhibition concentration of  $10^6$ ,  $10^7$ , and  $10^9$  for E. coli, Salmonella and S. aureus successively.

#### **Public health significance:**

Probiotics are intended to assist the body's naturally occurring gut microbiota. Some probiotics preparations have been used to prevent diarrhea caused by antibiotics, or as part of the treatment for antibiotic-related symbiosis **Soomro et al., (2002)**. Studies have documented probiotics effects on a variety of gastrointestinal and extraintestinal disorders, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), vaginal infections, and immune enhancement (**Shah and Dave, 2002**). Some probiotics have also been investigated in relation to atopic eczema, rheumatoid arthritis, and liver cirrhosis (**Ong et al., 2006**). Although there is some clinical evidence for the role of probiotics in lowering cholesterol, the results are conflicting. In general, the strongest clinical evidence for probiotics is related to their use in improving gut health and stimulating immune function **Tharmarja and Shah (2004)**. A number of studies have found probiotics consumption to be useful in the treatment of many types of diarrhea, including antibiotic-associated diarrhea in adults, travelers' diarrhea, and diarrheal diseases in young children caused by rotaviruses. Lactobacillus and Streptococcus are the most common probiotics used in

commercial fermented and non-fermented dairy products today (**Heller, 2001**). Antimicrobial effects of lactic acid bacteria are formed by producing some substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (**Quwehand and Vesterlund, 2004**).

Lactic acid bacteria (LAB) have been used successfully, with few adverse effects, to prevent antibiotic associated diarrhea, to treat acute infantile diarrhea and recurrent Clostridium disease and to treat various diarrheal illnesses, the antagonistic property is attributed to the lowered pH, the undissociated acids and production of other primary and secondary antimicrobial metabolites produced by LAB. The metabolites produced by the fermentation process, except the volatile ones, are kept in the foods and result in growth inhibition of food spoilage or poisoning bacteria and detoxification of noxious compounds of plant origin (**Reidand and Bruce 2006**). The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH. In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), carbon dioxide (CO<sub>2</sub>), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocin (9-12). All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods (**Catanzaro and Green, (1997)**; **Moghaddam et al., (2006)** and **Aweenet et al., (2012)**).

In general the reported health benefits include: boosting of the immune system, inhibition of the growth of pathogenic microorganisms, prevention of diarrhea from various causes, prevention of cancer, reduction of the risk of inflammatory bowel movements, improvement of digestion of proteins and fats, synthesis of vitamins, and detoxification and protection from toxins (**Hobbs, 2000**).

## CONCLUSION

The probiotic bacteria isolated in our study possess varying degrees of inhibition towards tested enteric pathogenic bacteria to indicate that there is potential for the derivation of health benefits from consuming traditional yoghurts containing specific strains of probiotics are safe for human use and able to confer some health benefits on the host. The health benefits for which probiotics can be applied include conditions such as gastrointestinal infections, certain bowel disorders, allergy, and urogenital infections, which afflict a large portion of the world's population.

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## "تأثير بكتيريا البروبيوتك المعزولة من الزبادي المنتج بمحافظة دمياط على بعض الميكروبات الممرضة"

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### المُلخَص

جمعت ما مجموعه ٢٠٠ عينة من مختلف العلامات التجارية من الزبادي التقليدية (بلدي) بشكل عشوائي من الأسواق المحلية ومحلات السوبر ماركت في أحياء مختلفة من مدينة دمياط.

تم عزل ما مجموعه ١٧ من معزولات بكتيريا البروبيوتيك من عينات الزبادي التقليدية لاهوائياً في وجود ١٠٪ ثاني أكسيد الكربون، تم الكشف عن النشاط البكتيري لبكتيريا البروبيوتيك المعزولة عن المواد المثبطة ضد بعض مسببات الأمراض التي تنقلها الأغذية الشائعة مثل السالمونيلا والإي كولاي والميكروب العنقودي الذهبي باستخدام اختبار نشر الآجار، وقد أظهرت هذه الاختبارات عن التأثير التثبيطي الجيد لمعزولات البروبيوتيك ضد المكروبات المرضية محل البحث بشكل جيد، أظهرت النتائج أن بكتيريا البروبيوتيك الموجودة في عينات الزبادي المختبرة لها تأثير مثبط جيد في المختبر ضد بعض مسببات الأمراض الممرضة المختبرة ويكون أفضل تأثير لها عند تركيز ١٠<sup>٨</sup> /جم.

**كلمات البحث الدالة:** بكتيريا البروبيوتيك، التقليدية، الزبادي البلدي، مضاد للجراثيم، الإي كولاي القولونية، المكورات العنقودية الذهبية، السالمونيلا، محافظه دمياط.