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ANTIMICROBIAL AND LIPOLYTIC ACTIVITIES OF SOME STRAINS OF *Lactobacillus* SPP. FROM EGYPTIAN MISH CHEESE AND MILK

Samar A. Abd El-Hamid*, A. Osman, A.M. Gomaa and M.Z. Sitohy

Agric. Biochem. Dept., Fac. Agric., Zagazig Univ., Egypt

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ABSTRACT: The present study aims to elucidate the microbiota of Egyptian mish cheese and milk, to isolate new strains and to study their biochemical properties relevant to their potential application in food and dairy industry. Among the isolates from Egyptian mish cheese there were two strains identified to be *Lactobacillus fuchuensis* DSM 14340T DSM and *Lactobacillus paracasei* DSM 20006 DSM as well as *Lactobacillus acidophilius* isolated from milk. These strains were evaluated for potential probiotic properties. Absence of haemolytic activity and antibiotic resistance is considered as a safety prerequisite for the selection of a probiotic strains. All of the strains were γ-haemolytic (*i.e.* no haemolysis). All of the strains were recorded highly lipolytic activity on Tributyrin agar base plates. Anti-bacterial activity of the cell-free culture supernatants (CFCS) of the potential probiotic strains (*L. fuchuensis, L. paracasei* and *L. acidophilius*) was evaluated against *Salmonella enteritidis, Klebsiella pneumoniae, Aeromonas hydrophila, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Escherichia coli* O 157:H7, *Staphylococcus aureus* and *Enterococcus faecalis*. Supernatant culture obtained from *Lb. Fuchsensis* showed antimicrobial activity against all tested microorganisms except *Klebsiella pneumoniae*.Supernatant culture obtained from *Lb. paracasei* showed antimicrobial activity against all tested microorganisms except *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *E. coli* and *E. coli* O 157:H7. Supernatant culture obtained from *L. acidophilius* showed antimicrobial activity against all tested microorganisms except *Klebsiella pneumoniae, Aeromonas hydrophila, E. coli* O 157:H7 and *Staphylococcus aureus*. The commercial antibiotics sensitivity test against the potential probiotic strains (*L. fuchuensis, L. paracasei and L. acidophilius*) was evaluated.

Key words: *Lactobacillus*, lipase, mish cheese, milk.

INTRODUCTION

The demand for microbial industrial enzymes has attracted much interest owing to their novel and multifold applications in a wide variety of processes. Hydrolytic enzymes like lipases furnish the greatest share in the industrial enzyme market. Lipases are glycerol ester hydrolases that act on acylglycerols to liberate fatty acids and glycerol. Lipases can hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (**Gilham and Lehner, 2005; Angkawidjaja and Kanaya, 2006**). Lipases are ubiquitous enzymes which are widely. Lipase have been isolated and purified from fungi, yeast, bacteria, plant and animal sources but bacterial lipases are more economical and stable **(Snellman *et al.*, 2002**). Lipases find applications in many areas of biotechnology due to their ability to catalyze enantio selective reaction with a wide range of substrates and their stability over wide variations of temperature and pH. Lipids constitute a large part of the earth biomass and lipolytic enzymes play an important role. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat and cream (**Falch, 1991**). Lipases are also used in detergent industry as additive to washing

* **Corresponding author: Tel. :** +201065776313
E-mail address: samoraalaa18@gmail.com

powder (**Fuji *et al.*, 1986**), textile industry to increase fabric absorbency (**Sharma *et al.*, 2001**) and different transesterification reactions (**Hasan *et al.*, 2006**). In addition, the enzyme is used as a catalyst for production of different products used in cosmetic industry, in pulp and paper industry **(Bajpai, 1999),** in synthesis of biodiesel **(Noureddini *et al.*, 2005)** and in pharmaceutical industry **(Higaki and Morohashi, 2003).** Microorganisms with a potential for lipases production may be found in different habitats, including waste vegetable oils, dairy products, oil contaminated environment, seeds and spoiled food **(Sharma *et al.*, 2001**).Thus, the aim of the present study was to elucidate the microbiota of Egyptian mish cheese and milk, to isolate new strains and to study their biochemical properties relevant to their potential application in food and dairy industry.

MATERIALS AND METHODS

Sample Collection

Egyptian mish cheese and milk samples were collected from various locations in Sharkia Governorate, Egypt. The samples were collected in sterilized polythene bags and transported to the laboratory.

Isolation of Lactic Acid Bacteria

Ten grams of each sample (Mish cheese and milk) were homogenized in 90 ml of Man Rogosa and Sharpe (MRS) broth and incubated at 37°C for 48 hr. Then, 0.1 ml of the culture was spread on MRS agar and incubated for 48 hr., at 37°C. The obtained colony was selected for estimation lipolytic activity.

Screening of the Isolates for Lipase Activity

Lipolytic organisms were screened by qualitative plate assay. Isolates were grown on Tributyrin agar base plates and incubated at 36°C for 2 days. Zone of clearance was observed due to hydrolysis of tributyrin. The potential probiotic strains were conserved at -20°C in MRS broth with 30% glycerol.

Identification of the Selected Strains

Matrix –assisted laser desorption/ionization time of flight mass spectrometry (MALDI –TOF –MS) is a new technology for routine identification of bacteria in clinical or microbiological laboratories (**Biswas and Rolain, 2013**). Each mass spectrometer consists of three functional units : an ion source , to ionize and transfer analytic ions into the gas phase , a mass analyzer, to separate ions by their mass – to – charge ratio (M/Z); and a detection device, to monitor ions . MALDI –TOF –MS is known since 1996 but this is a new technology and it is a soft ionization method which allows desorption of peptides and proteins from both whole different cultured bacteria and crude bacterial extracts. Protein mass patterns can be used for identification of bacteria at the genus, the species and in some case, the subspecies level **(Graham *et al.*, 2007; Sauer and Kliem, 2010**). Identification of the selected ten isolates were conducted by this technique at Academic Park, Faculty of Medicine, Alexandria, University, Egypt. Shortly, one large colony or multiple small colonies (enough to fill about one half of a 10-µl inoculating loop) of a bacterial isolate to be tested was suspended in 70% ethanol in a 1.5- ml micro centrifuge tube. Extraction of bacteria, matrix preparation, spotting of the steel target plate, and calibration of the instrument were performed as previously described by **Stevenson *et al.* (2010)**. A Bruker MALDI-TOF MicroFlex LT mass spectrometer was used to generate spectra from the bacterial extracts, and the Biotyper software (Version 2.0.4) was used to analyze the results.

Hemolytic Activity

Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72 hr. Results were recorded based on the type of clear zone observed. *i.e.* α-hemolysis when the colony was surrounded by greenish zone β-hemolysis when the colony was surrounded by a clear white zone and γ- hemolysis when there was no change in the medium surrounding the colony (**Cheesbrough, 2006**).

Antibacterial Activity Against Pathogenic Bacteria

All strains were tested in triplicates for antimicrobial activity according to (**Argyri *et al.*, 2013**) against *Salmonella enteritidis, Klebsiella pneumoniae, Aeromonas hydrophila, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Escherichia coli* O 157:H7,

Staphylococcus aureus and *Enterococcus faecalis*. Fresh overnight bacterial MRS culture supernatants of the potential probiotic strains were collected by centrifugation (10,000 *xg*,15 min, 4°C), adjusted to pH 6.5 and filter-sterilised (0.22 mm). The cell-free culture supernatants (CFCS) of the potential probiotic strains were screened for inhibitory activity using the well diffusion assay. An initial inoculum of approximately $1x10^6$ cfu/ml of the target strain was incorporated into soft agar (1%, *W/V*) plates of the appropriate for the target strain medium. CFCS (50 µL) were transferred in holes (5 mm diameter) drilled into the agar. The plates were incubated at 37°C, depending on the target strain, and the antimicrobial activity was recorded as growth-free inhibition zones (diameter) around the well.

Antibiotic Resistance

For testing antibiotic resistance, LB agar plate was overlaid with 100 ml of LAB culture containing 10^8 CFU/ml and antibiotic discs containing Kanamycin (30 µg), Gentamycin (10 µg), Trimethoprim/ sulphamethoxazole (1.25/ 23.75 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Streptomycin (10 µg), Cefotaxime (30 µg) and Vancomycin (30 µg) were placed on inoculated plates under sterile conditions. After incubation for 24 hr., at 30°C, the diameter (mm) of inhibition zone was measured (**Angmo *et al.,* 2016**). The commercial antibiotics sensitivity test against bacterial isolates, measured by the inhibition zone diameter (mm) according to **(Charteris *et al.,* 1998; Han *et al.,* 2015**).

RESULTS AND DISCUSSION

Isolation and Identification of Lipolytic Lactic Acid Bacteria

Among the isolates from Egyptian mish cheese, two strains identified to be *L. fuchuensis* DSM 14340T DSM and *L. paracasei* DSM 20006 DSM and *L. acidophilius* isolated from milk (Table 1). These strains were evaluated for potential probiotic properties.

All of the strains were recorded highly lipolytic activity on Tributyrin agar base plates (Table 2).

Haemolytic activity

Absence of haemolytic activity and antibiotic resistance isconsidered as a safety prerequisite for the selection of a probioticstrain (**Joint, 2002**). None of the examined strains exhibited b-haemolytic activity when grown in Columbia human blood agar. All of the strains were γ-haemolytic (*i.e.* no haemolysis) (Table 2), Similar observations were made for all the strains of *L. paracasei* subsp. *paracasei* spp. and *L. casei* isolated from dairy products which showed γ-haemolysis except of few that showed α-haemolysis (**Maragkoudakis *et al.,* 2006**).

Antibacterial activity against pathogenic bacteria

Anti-bacterial activity of the cell-free culture supernatants (CFCS) of the potential probiotic strains (*Lb. fuchuensis*, *Lb. paracasei* and *Lb. acidophilius)* isolated from Egyptian mish cheese and milk against *Salmonella enteritidis, Klebsiella pneumoniae, Aeromonas hydrophila, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Escherichia coli O 157:H7, Staphylococcus aureus* and *Enterococcus faecalis* was observed in Table 3. Supernatant culture obtained from *L. Fuchsensis* showed antimicrobial activity against all tested microorganisms except *Klebsiella pneumoniae.* Supernatant culture obtained from *L. paracasei* showed antimicrobial activity against all tested microorganisms except *Klebsiella pneumoniae, Aeromonas hydrophila, E. coli* and *E. coli* O 157: H7. Supernatant culture obtained from *Lb. acidophilius* showed antimicrobial activity against all tested microorganisms except *Klebsiella pneumoniae, Aeromonas hydrophila, E. coli* O 157:H7 and *Staphylococcus aureus*. The production of antimicrobial compounds such as organic acids, short chain fatty acids and bacteriocins is one of the functional properties used to characterize probiotics (**Fuller, 1989**). The lowering of pH due to organic acids (especially lactic and acetic acids) produced by these bacteria in the gut has a bactericidal or bacteriostatic effect (**Shah, 2007**). The capacity to produce different antimicrobial compounds may be one of the critical characteristics for effective competitive exclusion of pathogen survival in the intestine and expression of a probiotic effect for the host (**Ouwehand and Salminen, 1998**). The acidic conditions in the

Table 1. Identification of lactic acid bacteria isolated from Egyptian mish cheese and milk

Strain No.	Source	Strain
1	Mish cheese	*L. fuchuensis* DSM 14340T DSM
2	Mish cheese	*L. paracasei* ssp *paracasei* DSM 20006 DSM
3	Milk	*L. acidophilius*

Table 2. Lipolytic and haemolytic activities of selected strains with probiotic potential activity

Strain	Lipolytic activity	Haemolytic activity
***L. fuchuensis* DSM 14340T DSM**	+	γ
***L. paracasei* ssp *paracasei* DSM 20006 DSM**	+	γ
L. acidophilius	+	γ

Table 3. Anti-bacterial activity of the cell-free culture supernatants (CFCS) of the potential probiotic strains (*L. fuchuensis*, *L. paracasei* and *L. acidophilius)* isolated from Egyptian mish cheese and milk against *Salmonella enteritidis, Klebsiella pneumoniae, Aeromonas hydrophila, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Escherichia coli* O 157:H7, *Staphylococcus aureus* and *Enterococcus faecalis* using the well diffusion assay

Microorganism	*L. fuchuensis*	*L. paracasei*	*L. acidophilius*
	Inhibition zone diameter (mm)		
Salmonella enteritidis	15 ±0.30	6 ±0.08	9 ±0.11
Klebsiella pneumoniae	0 ±0.00	0 ±0.00	0 ±0.00
Aeromonas hydrophila	7 ±0.10	0 ±0.00	0 ±0.00
Proteus vulgaris	13 ±0.20	7 ±0.07	8 ±0.10
Pseudomonas aeruginosa	20 ±0.22	13 ±0.11	15 ±0.23
E. coli	10 ±0.11	0 ±0.00	20 ±0.32
***E. coli* O 157:H7**	15 ±0.21	0 ±0.00	0 ±0.00
Staphylococcus aureus	12 ±0.13	9 ±0.11	0 ±0.00
Enterococcus faecalis	9 ±0.09	8 ±0.13	9 ±0.12

stomach may even enhance the activity of these antimicrobial compounds (**Gänzle *et al.,* 1999**). Furthermore, these probiotic characteristics may partly be based on the production of relevant concentrations of lactic acid in the microenvironment, which, in combination with a detergent such as bile salts, inhibits the growth of Gram-negative pathogenic bacteria (**Begley *et al.,* 2005**).

Antibiotic resistance

The sensitivity test was conducted for the three potential probiotic strains *(L. fuchuensis, L. paracasei and L. acidophilius*) against 8 commercial antibiotics and the results were recorded in Table 4. The strain *L. fuchuensis* showed antibiotic sensitivity, since it was affected by 7 antibiotics while it showed intermediate resistance to only one antibiotic (Erythromycin). The other two stains (*L. paracasei* and *L. acidophilius*) showed relatively higher antibiotic resistance and both were resistant to the same two antibiotics (Trimethoprim/sulphamethoxazole Cefotaxime). However, the first one showed intermediate resistance to two antibiotics (Erythromycin, Streptomycin) while the second one showed intermediate resistance to only one antibiotic (Chloramphenicol). In conclusion it can be stated that the strain *L. fuchuensis* is the most sensitive to the tested antibiotics while the other two strains (*L. paracasei* and *L. acidophilius*) were intermediary resistant. The sensitivity order can be arranged as follows: *L. fuchuensis* > *L. paracasei* > *L. acidophilius*. For safe food application the first strain is mostly recommended followed by the second and third one, respectively. World Health Organization characterizes antimicrobial resistance as a global public health crisis that must be managed with the utmost urgency **(WHO**, **2015)**. So, for food application it is advised to use the least antibiotic resistant bacteria to be always under control.

Table 4. The commercial antibiotics sensitivity test against the potential probiotic strains (*L. fuchuensis*, *L. paracasei* and *L. acidophilius)* isolated from Egyptian mish cheese and milk

Antibiotic	*L. fuchuensis*		*L. paracasei*		*L. acidophilius*	
	Inhibition zone diameter (mm)/sensitivity (S/I/R)*					
Kanamycin (30 µg)	21 ±0.30	S	20 ±0.11	S	18 ±0.11	S
Erythromycin (15 µg)	16 ±0.10	I	14 ±0.16	I	22 ±0.21	S
Chloramphenicol (30 µg)	34 ±0.50	S	22 ±0.21	S	16 ±0.15	I
Gentamycin (10 µg)	22 ±0.23	S	19 ±0.10	S	21 ±0.14	S
Vancomycin (30 µg)	22 ±0.14	S	18 ±0.20	S	18 ±0.20	S
Trimethoprim/sulphamethoxazole (1.25/23.75 µg)	25 ±0.27	S	0 ±0.00	R	0 ±0.00	R
Cefotaxime (30 µg)	30 ±0.26	S	0 ±0.00	R	0 ±0.00	R
Streptomycin (10 µg)	16 ±0.17	S	7 ±0.10	I	20 ±0.14	S

***** S/R/I: S: Sensitive, R: Resistant, I: Intermediate

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الأنشطة المضادة للبكتيريا والمحللة للدهون من بعض سلالات اللاكتوبسيليس من الجبن المصرى القديم والحليب

سمر علاء عبدالحميد - علي عثمان - أيمن محمد جمعه - محمود زكي سطوحي

قسم الكيمياء الحيوية - كلية الزراعه - جامعة الزقازيق - الزقازيق - مصر

الهدف من هذه الدراسة هو التعرف علي الميكروبات الموجودة في الجبن المصري القديم والحليب، من اجل عزل سلالات بكتيرية جديدة ودراسة خصائصها البيوكيميائية ذات الصلة بتطبيقاتها المحتملة في الصناعات الغذائية، من بين العزلات المتحصل عليها من الجبن المصري القديم عزلتان تم تعريفهم كالتالي : اللاكتوبسيليس فيوشينسيس١٤٣٤٠ت د س م واللاكتوبسيليس باراكاسي د س م ٢٠٠٠٦ د س م واللاكتوبسيليس أسيدوفيليس المعزوله من الحليب، هذه العزلات تم تقييمها من اجل الخصائص المحتمله للبروبيوتك، غياب نشاط الهيموليتك ومقاومة المضادات الحيوية تعتبر كمتطلب أمن من اجل اختيار عزلات البروبيوتك، كل العزلات كانت جاما هيموليتك (غير محلله)، كل العزلات سجلت نشاط تحللي عالي لليبز علي اطباق من بيئة ترايبيوترين، النشاط المضاد للميكروبات للخلايا الحره للبكتريا عزلات البروبيوتك المحتملة (اللاكتوبسيليس فيوشينسيس واللاكتوبسيليس باراكاسي بالاضافة الي اللاكتوبسيليس اسيدوفيليس) تم تقييمها ضد (سالمونيلا اينترايتديس)، كليبسيلا بنيومونيو، ايروموناس هيدروفيلا بروتيس فولجرس، ستافيلوكوكس ايروس، بسيدوموناس ايروجينوس، ايشريشيا كولايو ايشريشيا كولاي ع ١٥٧: ه ٧ ستافيلوكوكس ايروس واينتيروكوكس فايسلس، الرائق تم الحصول عليها من اللاكتوبسيليس فيوشينسيس أظهرت نشاط مضاد للميكروبات ضد كل البكتريا المختبرة ضد كليبسيلا بنيومونى، الرائق المتحصل عليها من اللاكتوبسيليس باراكاسي أظهرت نشاط مضاد للميكروبات ضد كل البكتريا المختبرة ماعدا كليبسيلا بنيومونى، ايروموناس هيدروفيلا، ايكولاي وايكولاي ع ١٥٧: ه٧، الرائق المتحصل عليها من اللاكتوبسيليس اسيدوفيليس اظهرت نشاط مضاد للميكروبات لكل سلالات البكتريا ماعدا كليبسيلا بنيومونى، ايروموناس هيدروفيلا، ايكولاي ع ١٥٧ :ه ٧ وستافيلو كوكس أيروس، المضادات الحيويه التجاريه الحساسه المختبره ضد عزلات البروبيوتك المحتمله (اللاكتوبسيليس فيوشينسيس اللاكتوبسيليس باراكاسي واللاكتوبسيليس اسيدوفيليس) تم تقييمها .

المحكمون :

١- أ.د. محسن محمد سليم عسكر أستاذ الكيمياء – المركز القومي للبحوث - الدقي - مصر

٢- أ.د. رجب عبدالفتاح المصري أستاذ الكيمياء – كلية الزراعة – جامعة الزقازيق.