Biotechnol Res 2017; Vol 3(3):65-70
eISSN 2395-6763

ORIGINAL RESEARCH

Isolation and identification of Probiotic Lactic Acid Bacteria from dairy products

Eman AMER[1], Hoda MAHROUS[1*], Laila A. SHEKIB[2]
[1]Industrial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt
[2]Food Science and Technology Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt
*Corresponding Author email: hmahrous7@yahoo.com

• *Received: 08 March 2017* • *Revised: 05 April 2017* • *Accepted: 10 April 2017* • *Published: 17 April 2017* •

ABSTRACT
Dairy products are a potential source of probiotic bacteria. Seven samples of different sources of dairy products were collected from different regions (Gharbiya & Minufiyah) of Egypt. The isolates were identified using morphological characteristics: gram staining, catalase test, growth at various temperatures (10, 37, and 45°C) and CO_2 production for all isolates. The identification was confirmed by determine the tolerance to Nacl (2, 4 and 6.5%) for cocci isolates and growth on *Streptococcus faecalis* medium (SF). Thirty isolates were tested for biochemical and morphological tests. Good growth was observed at 37°C and pH 6.2 for 100 isolates. After that the catalase positive isolates were removed and the results 3 isolates were *Lactobacillus;* (*L.parakefiri ; L.rhamnosus* and *L.paracasei*) and 2 cocci strains (*Lc.lactis subsp. cermoris* and *Lc.lactis subsp. lactis*) .All five isolates has properties of probiotics like tolerance to acidity and bile salts with ability for adhesion.

KEY WORDS: *probiotic bacteria, dairy products, biochemical tests, Lactic acid bacteria.*

INTRODUCTION

Lactic Acid Bacteria (LABs) as a major group of gram positive, catalase negative bacteria are the most important constituent of probiotics and have numerous applications in industry (Salminen *et al.,* 2004) According to FAO/WHO definition, probiotic “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001).

Most commonly used probiotics are from the genera *Bifidobacterium*, *Lactobacillus*, *Streptococcus thermophilus* and nonpathogenic strains of *Enterococcus*, *Bacillus, E. coli* and yeasts for example *Saccharomyces boulardii* (Dunne *et al.,* 2001). At the present, consumers are aware of the relation among lifestyle, good health, and diet, which explains the increasing request for products enhancing the health besides providing basic nutrition. Several therapeutic applications for probiotics can be cited protection against traveller's diarrhea, prevention of urogenital diseases, reduction of hypercholesterolemia, alleviation of constipation, prevention of food allergy and osteoporosis and protection against bladder and cancer (Lourens-Hattingh and Viljoen, 2001). In addition to, probiotics can be prevention or treatment of some disorders, such as lactose malabsorption, irritable bowel syndrome, acute diarrhea, mild forms of inflammatory bowel disease and necrotizing enterocolitis (Presti *et al*., 2015**)**. Furthermore, the effects of probiotics depend on the route of administration and dosage (Upadhyay and Moudgal, 2012). One of these mechanisms are production of bacteriocins, like nisin approved by the US Food and Drug Administration (FDA) since ago last decade for food preservation and shelf life extension (Collins *et al*., 2012; Yateem *et al*., 2008).

Yoghurt and other types of fermented milk product have been consumed to thousands of years. Yoghurt is

considered the best carrier of probiotics, one or more species of it, *L. acidophilus*, *Bifidobacterium* and *L. casei* (Shah, 2007). Curd is best source for probiotic bacteria especially *Lactobacillus* spp. (Kale, 2014). Lactobacilli are naturally present in raw milk, dairy products like (cheeses, yoghurts, and fermented milks), or added intentionally, owing to technological reasons or to provide a health benefit to the consumer (Oeuret *et al*., 2003). The aim of this work is isolation and identification of probiotic bacteria from different sources of dairy products which collected from different regions (Gharbiya & Minufiyah) of Egypt. The isolates were identified using morphological characteristics and API system. With study the properties of probiotics like tolerance to acidity and bile salts with ability for adhesion in the promising isolates.

RESULTS AND DISCUSSION

In this study, 112 strains were isolated from Seven samples from different sources of dairy products were collected from different regions (Gharbiya & Minufiyah), the dairy samples including regional Yoghurts, curd (cow milk), Crud (Buffalo milk) ,Ras Cheese, Quraysh Cheese, Whey (cow milk), Breast Milk. All strains were grown on MRS agar media at 37^{o}C and pH 6.2. Total count was showed in Table (1). Preliminary screening for the single isolated colony of bacteria was according to their morphological in plate and microscope, biochemical tests (gram and catalase reaction). When isolates Gram stained, found rod and cocci shaped and positive in Gram reaction. There were found 45 isolates negative in Gram reaction, 47 isolates positive catalase and 15 isolates were lost and five isolates Gram positive and catalase negative were stored for second screening.

Total count for samples of dairy products (Quraysh Cheese; Whey and Ras Cheese) was presented in table 1. There were significant differences among these samples. The least count in Ras Cheese which agree with reasons result were reported by Karimi *et al*., (2011) , Factors influencing the stability of probiotics count in cheese can be divided into three areas ,of which formulation factors (microbial interactions and strains of probiotic bacteria , pH and titrable acidity, molecular oxygen, growth promoters, hydrogen peroxide and ripening factors food additives, salt and microencapsulation), manufacturing factors (heat treatment ,incubation temperature, inoculation, and storage temperature), and materials of packaging and systems.

As regards kariesh cheese, results revealed that total bacterial count was present in all examined samples with a mean count of 1.1 x10^{9} ± 1.6 x 10^{9} cfu/g for open cheese and 2.4 x 10^{8} ± 7.4 x108 for packed cheese. Similar results were obtained (2.6 x10^{8}cfu/g) in Kafr ELSheikh city (Aman ,1994) While Moussa *et al*., (1984) obtained that total count bacteria in kariesh cheese (3.3x10^{7} cfu/g) in Monoufia.

Lower count (1.1 x10^{4} cfu/g) was recorded in Minia city (Kaldes *et al*., 1997) while Higher count (1.0 x10^{13} cfu/g) was recorded) in Zagazig city (Amer, 1982).

Table 1: Total count for samples of dairy products (Quraysh Cheese; Whey and Ras Cheese)

Sample	(CFU/ml) or (CFU/g)
Quraish Cheese	48.29 x10^{4} ±0.1
Whey (cow milk)	36.60 x10^{4}±1.01
Ras Cheese	14.34 x10^{4}±0.01

Data are presented as mean ±

The biochemical tests for the five isolates were showed in table 2&3. The cocci isolate from table 2 showed that the isolates Q13 may be *Lc. lactis subsp. cermoris* and the other isolate W1 may be *Lc. lactis subsp. lactis or L. lactis subsp. lactis biovar diacetylacti.* From table 3 the obtained results were M4 *L. parakefiri;* CR 11 *L. rhamnosus* and Y7 *L. paracasei* subsp. *tolerans.*

The Confirmatory identification for five isolates by API System showed in table 4 that the five isolates were: two cocci isolates: Q13 *L. lactis subsp. cermoris* 99.50% and W1 *L. lactis subsp. lactis* 99.95% *and* the three bacilli isolates*:* M4 *L. parakefiri* 99.85%; *CR 11 L. rhamnosus* 99.35% *and Y7 L. paracasei* 99.25%.

To determine the probiotic properties different tests were applied such as resistance to low pH; bile salt and adhesion tests. It is recorded that time at the first entrance to release from the stomach takes three hours. Strains need to be resistant to the stressful conditions of the stomach (pH 1.5-3.0) and upper intestine which contain bile (Çakır, 2003).

Acid tolerance

The effects of acid on the survival of the identified isolates were determined. The results indicated that *L. parakefiri* M4; *L. rhamnosus* CR11 and *L. paracasei* Y7 were more tolerant to the changing in pH in deferent levels than the flowed by *L. lactis subsp. cermoris* Q13 and *L. lactis subsp. lactis* W1.

Table 2: Biochemical identification of isolates (Cocci)

No.	Gram	Catalase	Growth at 10°c	Growth at 45°c	Production of CO_2	Growth on 2% salt	Growth on 4% salt	Growth on 6.5% salt	Growth on SF	Identification of strains according to Bergey's Manual
Q13	+	-	+	-	-	+	-	-	-	*Lc.lactis subsp. cermoris*
W1	+	-	+	-	-	++	+	-	-	*Lc.lactis* subsp. *lactis* or *Lc.lactis* subsp. *lactis* biovar diacetylactis

Table 3: Biochemical identification of isolates (Bacilli)

No.	Gram	Catalase	Growth at 10°c	Growth at 45°c	Production of CO_2	Identification of *Lactobacillus* spp. according to Bergey's Manual
M4	+	-	+	+	-	*L.parakefiri*
CR 11	+	-	+	+	+++	*L.rhamnosus*
Y7	+	-	-	++	-	*L.paracasei subsp. tolerans*

+ positive reaction, **-** negative reaction, **Y** = Regional Yoghurts (T11; B4), CR = curd (T14; B6), **M** = Breast Milk (T9; B2), =Whey (cow) (T15), **R** =Ras Cheese (T19; B1), **Q** =Quraysh Cheese (T18), **CB** = Crude (Buffalo) (T14; B2). T = Total isolates, B = Bacilli isolate

Table 4: Confirmatory identification for some isolates by API

No.	Identification using API System
Q13	*Lc.lactis subsp. cermoris 99.50%*
W1	*Lc.lactis subsp. lactis 99.95%*
M4	*L.parakefiri 99.85%*
CR 11	*L.rhamnosus99.35%*
Y7	*L.paracasei 99.25%*

These results were agreeing with Pereira and Gibson (2002) who recorded that human isolates of *L. fermentum* that could preserve viability at pH 2 .

Bile salts tolerance

The results showed the effect of different bile salt concentrations (0.2%, 0.3% and 0.4% w/v) on survival of isolates, generally, bile salt concentration 0.4%, exhibited the most suppression to all isolates compared to control values at 4 hrs. It is noted that all isolates could grow in the presence of 0.2% bile salts except *Lc.lactis subsp. lactis* W1.

Adhesion properties of some isolates on intestinal mucous

Five promising tested isolates were examined for their adhesion activity. Table 5 indicates that all isolates had the ability to adhere on intestinal mucous in different degree compared to the reference probiotic strain *Bifidobacterium bifidum* (B). These results were agreed with a lot of literatures (MacKenzie *et al*., 2009).

Table 5: Adhesion % for five isolates

No.	Adhesion (%)
Q13	1.32±0.20
W1	2.99±1.10
M4	1.19±0.21
CR 11	2.81±1.01
Y7	1.17±0.03
Bifidobacterium bifidum (B)	3.70±1.02

CONCLUSION

Probiotics are used in several food and dairy products, the main category being dairy products, but it present as food supplements in capsule or tablet form. Since the viability is an essential property of the probiotic organisms, the living probiotic must be occurring in an adequate amount in the final product. The present study aims to isolating and characterizing probiotics from some dairy products in Egypt. Five promising probiotics were identified and look good for screening for probiotic properties.

MATERIALS AND METHODS

Samples collection

Seven samples from different sources of dairy products were collected from different regions (Gharbiya & Minufiyah), the dairy samples including regional Yoghurts, curd (cow milk), Crud (Buffalo milk) ,Ras Cheese, Quraysh Cheese, Whey (cow milk), and Breast Milk. Samples were transferred in an icebox to laboratory, in sterile bottles, temperature (-4°C).

Culture media

MRS (deMan Rogosa and Sharpe) agar and broth were used for isolation of probiotic bacteria (De Man *et al*., 1960). Cysteine (0.05%) was added to this medium (Hartemink *et al*.,1997) .Sample (10 g) was dissolved into 0.1% sterile peptone water (90 ml) (Nice, India) and mixed up carefully. Then 1 ml of these serial dilutions was spread onto MRS agar. All plates were incubated at 37°C under anaerobic conditions for 48 h in anaerobic condition to remove unwanted bacteria. Colonies sub-cultured were isolated and transferred into new MRS agar plates by streaking to obtain single pure colonies. Isolates were kept in MRS broths 15% glycerol (v/v) to long time storage, at −20° to -80 °C as frozen cultures.

Total bacterial count

This test was carried out via pour plate technique (Kawo, *et al*., 2006). samples were plated on Nutrient agar, one mL of 3-6 dilution series of Ras Cheese, Quraysh Cheese, and Whey (cow milk) and incubated at 37°C for 48 h. Colonies that grown up were counted and expressed by (cfu/mL or g) colony forming units per milliliter (Dirisu, *et al*.,2015).

Biochemical characterization of Probiotic:

Gram staining

Isolates were identified as Gram positive by Gram's method and examined for purity and morphological characteristics under microscope (Bergey *et al*., 1994).

Catalase test

For further characterization, those isolates readily identified catalase negative, Fresh liquid cultures were grown overnight on MRS broth at 37°C (Bisen and Verma, 1998). It can be determined by adding hydrogen peroxide into culture broth (1 ml) on slides. Isolates did not release free oxygen bubbles were chosen (Sreenivasulu *et al*., 2015, Anandharaj and Sivasankari, 2013; Vasiee *et al*., 2014; Harrigan and MacCance, 1976; Allameh, 2012, Jeygowri *et al*., 2014, Bisen and Verma, 1998).

Growth at various temperatures

Optimal Growth at various temperatures (10, 37, and 45 °C) for 48 h was observed (Vasiee *et al*., 2014).

Production of CO_2 (Fermentation assay)

Phenol red dextrose broth

For gas detection, tubes containing Phenol red broth was inverted Durham's tubes. All tubes were sterilized for 15 min at 121 °C. These tubes were inoculated with the active bacteria under study. Positive reaction was indicated by production of gas and the changes in colour of the medium from red into yellow (Thoesen, 1994).

Determination of tolerance to NaCl

This test for cocci isolates only. Fresh liquid cultures were grown in MRS broth at 37°C for 24 h, Then 1 ml of these fresh cultures were added to 9ml of MRS broth in presence of (2, 4 and 6.5%) sodium chloride supplementation. All tubes were incubated at 37°C under anaerobic conditions for 48 h in anaerobic condition. Growth was indicative for NaCl tolerance (Mandal, 2015).

Growth on sf media

Sf **(***Streptococcus Faecalis* medium (**Difco** ™ SF Medium) is used for recognition of Enterococcus species, the Streptococcus group and other streptococci.

Microbiological characteristics by API-20E test kit STREB system, and API staph.

The API 50 CH strip (Biomerieux, Marcy l'Etoile France) was used to identify *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus thermophilus* cultures. It consists of 50 micro-tubes which contain an anaerobic zone (the tube portion), for the study of fermentation and an aerobic zone (the cupule portion), for the study of oxidation or assimilation. The first tube contains no substrate and is used as a negative control. The remaining tubes contain a defined amount of dehydrated substrate belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids).

Study probiotics properties

Acid tolerance

Overnight cultures were prepared by inoculated (0.1% v/v) into MRS broth and incubated at 37°C for 16 h to until logarithmic phase. Isolates were inoculated (10% v/v) into MRS broth previously adjusted to pH (2.0, 2.5 and 3.0 ±0.1) with HCl. Then were incubated at 37°C and growth development was recorded for 6 h by measuring the optical density at 650 nm (OD_{650} nm) using a spectrophotometer (780+UV/VIS Spectrometer PG Instruments Ltd). Comparison of isolates was based on development of growth in each broth and compared with MRS broth (pH 6.6) inoculated with the previous isolates. The experiments were repeated in duplicate.

Bile tolerance

All strains were tested for growth in MRS broth with and without bile salts at 37°C. Overnight cultures were inoculated 10% (v/v) into MRS broth and MRS broth containing 0.2, 0.3 and 0.4% (w/v) bile salts then incubated at 37°C. The bacterial growth was followed for 6 h by measuring the optical density at 650 nm (OD_{650} nm) using a spectrophotometer at one hour interiors. Comparison of isolates was based on their growth in each broth. The experiments were in duplicate.

Adhesion to human mucus

Five test strains were grown for 18-20 h without agitation at 37^{o}C to reach the early stationary phase. Isolates were harvested by centrifugation (15,000 x g, 10 min), and washed twice with phosphate buffer saline (PBS; pH 7.2). The optical density of the bacterial suspensions at 600 nm (OD_{600} nm) was adjusted with PBS to 0.5±0.02, giving approximately (10^{7}-10^{8}) $CFUml^{-1}$.

Statistical Analysis

Data are presented as the mean ± standard deviation, and n represents the number of samples from the replicates and the control.

REFERENCES

Allameh, S.K., Daud, H., Yusoff, F.M., Saad, C.R. and Ideris, A. 2012. Isolation, identification and characterization of Leuconostoc mesenteroides as a new probiotic from intestine of snakehead fish (Channa striatus). African Journal of Biotechnology 11(16): 3810-3816.

Aman IM. Microbiological quality of kariesh cheese in Kafr EL-Sheikh city. Assiut Vet Med J. 1994; 31:182-7.

Amer TA. Microbiological studies on local manufactured cheese in Zagazig markets. [Thesis.] University of Zagazig, Fac Vet Med. 1982.

Anandharaj,M., Sivasankari.B,2013. Isolation of potential probiotic Lactobacillus strains from human milk. International Journal of Research in Pharmacy and Life Sciences, 1: 26-29.

Bergey, David, H., John G. Holt, Noel R. Krieg, Peter H.A. Sneath, 1994. Bergey's manual of determinative bacteriology, 9th edn. Lippincott Williams & Wilkins.

Bisen, P. S. and Verma, K. (1998). Hand Book of Microbiology.1 st ed. CBS Publisher, New Delhi, India.

Çakır, İ. 2003. Determination of some probiotic properties on Lactobacilli and Bifidobacteria. Ankara University Thesis of Ph.D.

Collins B, Caitriona M, Guinane PD, Cotter CH (2012). Assessing the contributions of the LiaS histidine kinase to the innate resistance of Listeria monocytogenes to nisin, cephalosporins, and disinfectants. Appl. Environ. Microbiol. 78(8):2923-2929.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960). A medium for the cultivation of lactobacilli. Journal of Applied Bacteriology, 23:130 - 135.

Difco ™ SF Medium Difco™ & BBL™ Manual, 2nd Edition.

Dirisu ,C. G., Lily ,G., Igwe ,E.,2015. Microbiological load of yoghurt sold in Omoku schools, Rivers State, Nigeria. African Journal of Microbiology Research 9(34): 1960-1963.

Dunne, C. O'Mahony, L. Murphy, L. et al., 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr 73:386S–392S.

FAO/WHO, Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria (2001) Report of a Joint FAO WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, American Córdoba Park Hotel, Córdoba, Argentina.

Harrigan, W.F. and M.E.C. MacCance, 1976. Laboratory Methods in Microbiology. Academic Press, London and New York.

Hartemink, R., V.R. Domenech and F.M. Rombouts,1997 . LAMVAB - A new selective medium for the isolation of lactobacilli from faeces, J. Microbiol. Meth., 29: 77-84.

Jeygowri, N., Parahitiyawa, N. , Jeyatilake , S. , Ranadheera , S. and Madhujith ,T.2015 . Study on Isolation of Potentially Probiotic Lactobacillus species from Fermented Rice. Tropical Agricultural Research 26 (3): 428 – 440.

Kaldes YT. Microbiological examination of soft cheese manufactured in Minia city. Assiut Vet Med J. 1997; 75:39-47.

Kale, P.S., 2014. Isolation and identification of bacteria from curd and its application in probiotic chocolate. European Journal of Experimental Biology 4(6):95-97.

Karimi R, Mortazavian A.M., & Da Cruz A.G., (2011) Dairy Sci. & Technol. Viability of probiotic microorganisms in cheese during production and storage: a review. 91:283–308.

Kawo, A.H., Omole, E.M., Na'aliya, J., 2006. Quality assessment o f some processed yoghurt products sold in Kano Metropolis, Kano, Nigeria. BEST J. 3(1): 96-99.

Lourens-Hattingh, A., Viljoen, B.C., 2001. Yogurt as probiotic carrier food, Int. Dairy J. 11 1–17.

MacKenzie DA, Tailford LE, Hemmings AM and Juge N. (2009). Crystal structure of a mucusbinding protein repeat reveals an unexpected functional immunoglobulin binding activity. J. Biol. Chem., 284, 32444–32453

Mandal, S.,2015. Curd Lactobacilli with Probiotic Potentiality. iMedPub Journals 6 (2:8).

Ministry of Industry and Technological Development. The Egyptian standards No. 1008/2000 for Kariesh cheese. Egyptian Organization for Standardization and Quality Control.

Moussa AM, Zein GN, Nofel A, Gomaa EA. Studies on Kariesh cheese in the local markets of Monoufia. Egyptian J Dairy Sci. 1984; 12:117-2.

Oeuret,V.C., Ubernet, S.D., Ernardeau, M.B., Ueguen, M.G., Ernoux,J.P.V., 2003. Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products. INRA, EDP Sciences 83: 269–306.

Ottogalli G, Rondinini G, Pattano C. Microbial count in some soft cheese. LATTE. 1985; 10(6):556-62.

Pereira, D.I.A. and Gibson, G.R. 2002.Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. Appl. Environ. Microbiol. 68: 46-89.

Ram S, Khurana S, Khurana SB, Vadehra DV, Sharma S, Chhina RS. Microbiological quality and incidence of organisms of public health importance in food and water in Ludhiana. Indian J Med Res.1996; 103:253-8.

Salminen, S. and von Wright, A. Ouwenhand, A. Lactic Acid Bacteria. 2004, New York, Basel: Marcel Dekker Inc.

Scharlau Microbiology .2011 Technical Data Sheet, 064-PA0007. LABORATORIOS CONDA, S.A. CAT Nº: 1235. www.condalab.com.

Shah N.P. (2007). Functional cultures and health benefits. International Dairy Journal. 17: 1262-1277.

Sreenivasulu, P., Suman Joshi,D.S.D., Narendra, K., Venkata Rao ,G. and Krishna Satya, A. 2015. Isolation and Identification of Probiotic bacteria from River Banks of Krishna by Biochemical and Molecular Level Characterization . Int.J.Curr.Microbiol.App.Sci 4(11): 372 – 379.

Thoesen, J. C. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th edition. Version 1, fish Health Section, American Fisheries Society, 1994.

Upadhyay N, Moudgal V (2012). Probiotics: a review. J. Clin. Outcomes Manage. 19:76-84.

Vasiee, A. R., Tabatabaei Yazdi, F., Mortazavi, A. and Edalatian, M. R. (2014). International Food Research Journal ,21(6): 2487-2492.

Yateem A, Balba MT, Al-Surrayai T, Al-Mutairi B, Al-Daher R (2008). Isolation of lactic acid bacteria with probiotic potential from camel milk. Int. J. Dairy Sci. 3(4):194-199.