Intl. J. Food. Ferment. Technol. 8(1): 75-79, June 2018

DOI: 10.30954/2277-9396.01.2018.9

RESEARCH PAPER

Assessment of Probiotic Properties of *Lactobacillus fermentum* CM 36 Isolated from Camel Milk

Preeti and H. Joshi*

Department of Biotechnology, Vigyan Bhawan, Block B, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Corresponding author: hjbiotech@gmail.com

Paper No.: 208 **Received:** 19-11-2017 **Revised:** 14-02-2018 **Accepted:** 26-05-2018

ABSTRACT

Lactic acid bacteria (LAB) are known to possess probiotic potential and play a significant role to combat intestinal pathogens. The objective of the present investigation was to isolate, characterize and screen lactobacilli associated with camel milk for probiotic properties such as antibacterial activity, bile tolerance and antibiotic resistance. Among various isolates screened *Lactobacillus fermentum* CM36 showed remarkable antibacterial activity against *B.cereus*, *B.subtilis*, and *E.coli* in supernatant even after neutralization and protease treatment confirming bacteriocin activity. *L. fermentum* CM36 tolerated oxgall up to 0.4% (w/v) and showed resistance to various antibiotics used in the study. *L. fermentum* CM36 showed potential for antibacterial and thus, bile tolerance and may be further explored for its benefits towards animal and human health.

Keywords: LAB, camel milk, bacteriocin, probiotic, *Lactobacillus fermentum* CM36

LAB are widely used for the production of fermented foods and are considered as GRAS (generally recognized as safe) organisms. They are common inhabitants of gastrointestinal tract (GI) in animals and human beings. They produce organic acids such as lactic acid as a major by product and are prominently present in milk and fermented milk products (whey, yogurt, cheese etc). Lactic acid bacteria, particularly those belonging to beneficial and non-pathogenic genera (*Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc)* are widely used in food industry as biopreservative agents (Arokiyamary *et al*. 2011). Lactobacilli are characterized by the formation of lactic acid as main end product of carbohydrate metabolism. Lactobacilli are well known probiotic organisms and have been found to produce metabolic products that play an important role in controlling undesirable microflora in the gut. They prevent the proliferation of pathogenic bacteria in different ecosystems by production of various antimicrobial substances such as organic acids (lactic acid, acetic acid etc), hydrogen peroxide and bacteriocins (Jin *et al*. 1996). Bile tolerance and antibiotic resistance are crucial properties of lactobacilli which enable them to survive and perform their probiotic action in gastrointestinal tract. In present study, camel milk isolates were screened for their antibacterial activity. Bacteriocin producing probiotic properties such as antibacterial activity, bile tolerance and antibiotic resistance of lactobacilli were also studied and reported here.

MATERIALS AND METHODS

Collection of samples

Camel milk samples were collected from various regions (Teetardi, Jhadol, Thal and Dabok) of Udaipur, Rajasthan, India.

Isolation

Lactobacilli were isolated on MRS agar using standard pour plate method. The plates were incubated at 37°C +/- for 24 h.

Antibacterial Activity

The antibacterial activity was determined using well diffusion method as described by Ogunbanwo *et al*. (2003) against two gram-positive bacteria namely *Bacillus subtilis* and *Bacillus cereus* and a Gram-negative bacterium *E. coli*. The antibacterial activity was determined in cell free supernatants without neutralization and supernatant neutralized with 1N NaOH. Antibacterial activity due to bacteriocin production was determined by adding 20mg/ml protease to the cell free supernatant neutralized with 1N NaOH. The diameter of the zone of inhibition extending laterally around the well was measured with the scale.

Characterization of isolates

The isolates were characterized on the basis of cultural, morphological, biochemical and molecular analysis. Cultural characterization was based on the colony characteristics and the morphology was studied by Gram staining. Biochemical characterization was based on catalase reaction, growth on MRS supplemented with bromocresol purple (BCP), nitrate reduction, arginine hydrolysis, esculin hydrolysis, carbohydrate fermentation pattern using maltose, fructose, lactose, raffinose, mellibiose, galactose, mannose, sucrose and rhamnose.

The genomic DNA was extracted by Pospeich and Neumann's method (1995). Isolates were subjected to PCR using semi-universal *Lactobacillus* genus specific primer Lb1 (5'- AGAGTTTGATCATGGCTCAG-3') and Lb2 (5'-CGGTATTAGCATCTGTTTCC-3') based on variable loop of 16S rDNA sequence designed by Klijn *et al*. (1991). For sequencing, the amplified products were sent to SciGenome Labs Pvt Ltd. The partially sequenced data obtained were analyzed by BLAST and submitted to EMBL-EBI database.

Bile tolerance

The bile tolerance of isolates was studied according to the method suggested by Sirilun *et al*. (2010). MRS agar medium supplemented with oxgall at different concentrations such as 0.1, 0.2, 0.3, 0.4 and 0.5% were used. The plates were streaked and incubated at 37°C.

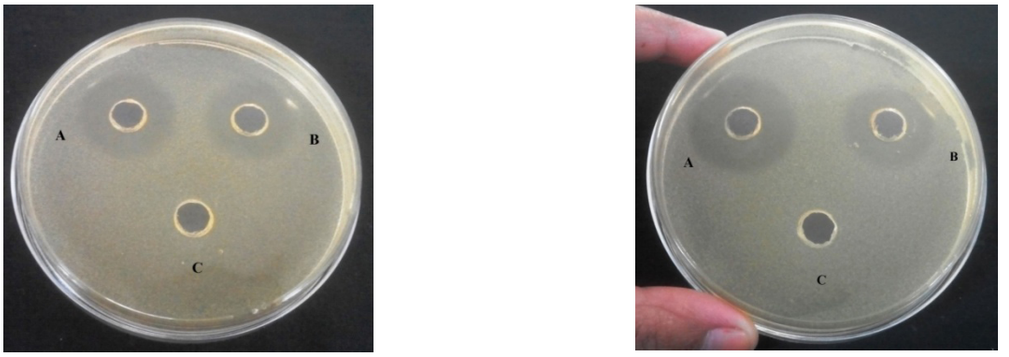
Antibiotic resistance

The antibiotic resistance of lactobacilli was evaluated using disc diffusion method (Bauer *et al*. 1966). The antibiotic discs (Himedia) used were of cefixime (5mcg/disc), amikacine (30mcg/disc), polymyxin (300unit/disc), kanamycin (30mcg/disc), trimethoprim (5mcg/disc), gentamycin (30mcg/disc), tetracycline (30mcg/disc), ampicillin (10mcg/disc), vancomycin (30mcg/disc) and ciprofloxacin (5mcg/disc). The plates were incubated at 37°C for 24 h and diameter of the inhibition zone was measured with a scale.

RESULTS AND DISCUSSION

Sampling: A total of 4 camel milk samples were collected from various regions (Teetardi, Jhadol, Thal, and Dabok) of Udaipur district. A total of 47 lactobacilli isolates were recovered on MRS agar using pour plate method.

Antibacterial Screening: A total of 22 out of 47 lactobacilli isolates showed antibacterial activity in cell free supernatants without neutralization against all the three test organisms namely *B.cereus*, *B.subtilis*, and *E.coli*. These 22 lactobacilli isolates were further tested for antibacterial activity using supernatant neutralized with 1N NaOH. A total of 5 lactobacilli isolates namely (isolates CM10, CM18, CM25, CM36 and CM44) out of 22 showed antibacterial activity against all the 3 test organisms. The neutralized cell free supernatants of these 5 lactobacilli isolates were subjected to enzyme treatment (protease) and were tested for bacteriocin activity against three bacteria. The zone of inhibition disappeared in only two isolates (namely CM36 and CM44), indicating bacteriocin activity of the isolates. Isolate CM44 was found to be cocci and Gram-negative. Isolate CM36



(a) **(b)**

Fig. 1: Antibacterial activity of *Lactobacillus fermentum* CM36 against **(a)** *Bacillus subtilis* **(b)** *Escherichia coli*

A = Supernatant without neutralization; B = Supernatant neutralization with 1N NaOH; C = Neutralized supernatant treated with protease.

was found to be gram- positive and rod shaped. The colonies of the isolate CM36 appeared as pin pointed, white, with entire margin and convex elevation. Antibacterial activity of isolate CM36 is shown in Table 1 and Fig. 1.

Table 1: Antibacterial activity of *Lactobacillus fermentum* CM36 without, with neutralization and after enzyme treatment (protease) against indicator organisms

Test organism	Diameter of inhibition zone (mm)		
	Supernatant without NaOH	Supernatant with NaOH	Neutralized supernatant with Protease
E. coli	18	13	NZ
B. cereus	13	12	NZ
B. subtilis	16	13	NZ

NZ= no zone of inhibition.

Characterization of isolates: Isolate CM36 was further characterized using biochemical tests and 16S rRNA partial gene sequencing. The isolate was found to be negative for catalase activity. Isolate CM36 showed yellow colored colonies on BCP-MRS medium. Isolate CM36 was unable to reduced nitrate and hydrolyze esculin. Isolate CM36 showed positive result for arginine hydrolysis. Isolate CM36 showed varied response for carbohydrate fermentation reaction, positive fermentation reaction was observed for maltose, fructose, lactose, raffinose, mellibiose and galactose. Negative reaction was observed for manose, sucrose and rhamnose.

The genomic DNA of the isolate was extracted by Pospeich and Neumann's method and was amplified by PCR using semi universal primers (Lb1 and Lb2). Isolate CM36 gave approximately 200bp product which was sequenced. The sequenced data obtained was analyzed by BLAST and was submitted to EMBL gene data base under the accession no. LTZ95042. Phylogenetic relationship was studied and tree was drawn using NCBI-BLAST neighbor- joining method, relationship between strain *L.fermentum* CM36 and other known sequences of *L.fermentum* is presented in Fig. 2.

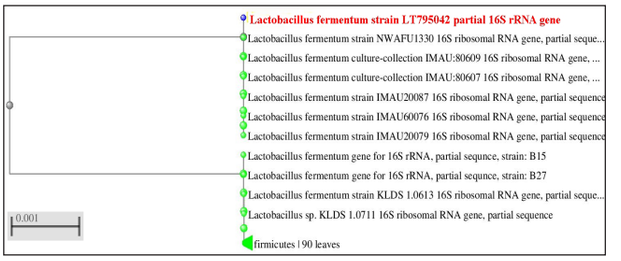


Fig. 2: Phylogenetic tree of *L.fermentum* CM36 based on 16S rRNA sequencing

Bile tolerance: *Lactobacillus fermentum* CM36 showed varied degree of growth in MRS agar supplemented with different concentrations of oxgall (0.1, 0.2, 0.3, 0.4 and 0.5%). The isolate tolerated three different

concentrations (0.1, 0.2 and 0.3%) of oxgall, as it showed maximum growth at all three concentration after 72h of incubation. The isolate also tolerated 0.4% of oxgall and showed comparatively less growth after 72h of incubation. Isolate was unable to tolerate 0.5% of oxgall and no growth appeared even after 72h of incubation. Data representing the tolerance of *L. fermentum* CM36 to different concentration of oxgall is shown in Table 2.

Table 2: Bile tolerance of *Lactobacillus fermentum* CM36

Sl. No.	Concentration of oxgall (%)	Growth of *Lactobacillus fermentum* CM36		
		Incubation period		
		24 h	48h	72h
1	0.1	+	++	++
2	0.2	+	++	++
3	0.3	+	++	++
4	0.4	-	-	+
5	0.5	-	-	-

+ = less growth; ++= maximum growth; - = No growth.

Table 3: Antibiotic resistance pattern of *Lactobacillus fermentum* CM36 against various antibiotics

Sl. No.	Antibiotics	*Lactobacillus fermentum* CM36
1	Cefixime (5mcg/disc)	Resistant
2	Amikacine (30mcg/disc)	Resistant
3	Polymyxin (300units/disc)	Resistant
4	Kanamycin (30mcg/disc)	Sensitive
5	Trimethoprim (5mcg/disc)	Resistant
6	Gentamycin (30mcg/disc)	Sensitive
7	Tetracycline (30mcg/disc)	Sensitive
8	Ampicillin (10mcg/disc)	Sensitive
9	Vancomycin (30mcg/disc)	Resistant
10	Ciprofloxacin (5mcg/disc)	Resistant

Antibiotic resistance pattern: Antibiotic resistance pattern of *L. fermentum* CM36 was evaluated against 10 different antibiotics. The isolate showed resistance to six antibiotics (namely ciprofloxacin, cefixime, polymyxin, trimethoprim, vancomycin and amikacine) and was found sensitive to four antibiotics (namely kanamycin, gentamycin, tetracycline and ampicillin).

The antibacterial activity of lactic acid bacteria has been widely studied and documented (Niel *et al*. 2002). In the present study, the *Lactobacillus fermentum* CM36 obtained from camel milk showed remarkable antibacterial activity (both in cell free supernatant with and without neutralization) against pathogenic bacteria. Abdelbasset and Djamila (2008) who studied antibacterial activity of lactic acid bacteria proposed that lactobacilli produces organic acids (such as lactic acids, acetic acids etc), hydrogen peroxides, diacetyls and bacteriocins during the process of fermentation and also concluded that the antimicrobial compounds produced by lactobacilli were fully or partially inactivated after the treatment of proteolytic enzymes, indicating proteinaceous nature. This may be the probable reason of antibacterial activity (without neutralization and with neutralization) observed due to bacteriocin production in the present study.

One of the important aspects of probiotic properties is the potential of the organism to resist the effect of bile salts to survive in small intestine. The normal level of bile salt in the intestine is around 0.3% (Mourad and Eddine, 2006). *L.fermentum* CM36 showed resistance to bile salt upto the concentration of 0.4% of oxgall. Halder and Mandal (2015) reported that *L.fermentum* and *L.casei* isolated from curd showed tolerance to bile salts (0.1 to 0.3% w/v) so, in this regards the results in present study is better as *L.fermentum* CM36 showed growth in MRS supplemented with 0.4% oxgall after 72h of incubation.

Another important probiotic property is antibiotic resistance to a broad spectrum of antibiotics so that lactobacilli can remain viable in gut and impart benefits to its host. *Lactobacillus fermentum* CM36 showed high resistance towards glycopeptides (such as vancomycin) and quinolones (such as ciprofloxacin, ofloxacin etc). Nawaz *et al*. (2011) reported high resistance towards glycopeptides and quinolones by lactobacilli and suggested the presence of intrinsic resistance mechanism against both the families. The results of the present study

are in agreement with the report of Nawaz *et al*. (2011). Various reports supported the resistance of lactobacilli from milk products regarding resistance to aminoglycosides (Ammor *et al*. 2007; Klare *et al*. 2007 and Nawaz *et al*. 2011). *Lactobacillus* species show sensitivity to cell wall synthesis inhibitors like ampicillin, these finding support the sensitivity of *L.fermentum* CM36 to ampicilin. These result are in agreement with the studies conducted by Coppla *et al*. (2005) and Khandelwal *et al*. (2014).

CONCLUSION

Lactobacillus fermentum CM36 showed remarkable antibacterial activity, bile tolerance and antibiotic resistance. The strain can play an important role as a probiotic supplement and starter culture for the production of fermented food products.

REFERENCES

Ammor, M.S., Florez, A.B. and Mayo, B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiol*., **24**: 559–570.

Arokiyamary, A. and Sivakumar, P.K. 2011. Antibacterial activity of bacteriocin producing *Lactobacillus* sp., isolated from traditional milk products. *Curr. Bot*., **2(3)**: 05-08.

Abdelbsset, M. and Djamila, K. 2008. Antimicrobial activity of autochthonous lactic acid bacteria isolated from Algerian traditional fermented milk "Raïb". *Afr. J. Biotechnol*., **7(16):** 2908-2914.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turch, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Patho*., **45**: 493-496.

Coppola, R., Succi, M., Tremonte, P., Reale, A., Salzona, G. and Sorrentino, E. 2005. Antibiotic resistance of lactobacilli isolated from two Italian hard cheeses. *Le lait*., **85**: 193-204.

Halder, D. and Mandal, S. 2015. Probiotic potentiality of curd lactobacilli. *Trans. Biomed*., **6**: 8.

Jin, L.Z., Ho, Y.W., Abdullah, N., Ali, M.A. and Jalaludin, S. 1996. Antagonistic effect of intestinal *Lactobacillus* isolates on pathogens of Chicken. *Lett. Appl. Microbiol*., **23**: 67-71.

Khandelwal, D., Joshi, H. and Chaudhary, B.L. 2014. Antibiotic resistance pattern of *Lactobacillus fermentum* CMU29 isolated from camel milk. *J. Herb. Med. Toxicol*., **8(1)**: 34-36.

Klare, I., Konstable, C., Werner, G., Huys, G. and Vankerckhoven, V. 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J. Antimicrob. Chemother*., **59**: 900-912.

Klijn, N., Weekamp, A.H. and de Vos, W.M. 1991. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction- amplified variable regions of 16S r RNA and specific DNA probes. *Appl. Environ. Microbiol*., **57**: 3390-93.

Mourad, K. and Eddine, K. 2006. *In vitro* pre-selection criteria for probiotic *Lactobacillus plantarum* strains of fermented olives origin. *Int. J. Probiotics Prebiotics*, **1**: 27-32.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X. and Moore, J.E. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Curr. Microbiol*., **62**: 1081–1089.

Ogunbanwo, S.T., Sannai, A.I. and Onilude, A.A. 2007. Characterization of bacteriocin produced by lactobacillus plantarum F1 and *Lactobacillus brevis* OGI, *Afr. J. Biotechnol*., **2(8):** 219-227.

Pospiech, A. and Neumann, B. 1995. Aversatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet*., **11**: 217-218.

Sirilun, S., Chaiyasut, C., Kantachote, D. and Luxananil, P. 2010. Characterisation of nonhuman origin probiotic *Lactobacillus plantarum* with cholesterol-lowering property. *African J. Microbiol. Res*., **4(10):** 994-1000.

Van Niel, C.W., Feudtner, C., Garrison, M.M. and Christakis, D.A. 2002. *Lactobacillus* therapy for acute infectious diarrhea in children: A meta-analysis. *Pediatrics*, **109**: 678–684.