ISSN: 2278-5213

RESEARCH ARTICLE

Probiotic and antimicrobial activity of bacteria from fermented toddy of *Cocus nucifera*

M. Krishnamoorthy and P. Arjun
Microbiology, Plant tissue culture and Food safety lab, National Agro Foundation,
Anna University, Taramani Campus, Taramani, Chennai-600113
krish2285@gmail.com; +91 9962200868

Abstract

Two lactic acid bacteria isolated from 20 fermented samples of plant fermented beverages (PFB) were analyzed for probiotic properties. Acid tolrent (pH 3), thirty six *Lactobacillus* sp. and thirty *Streptococcus* sp. showed good resistance (2%) in bile salt even after exposure for 48 h. The test organisms showed high specific growth rate and inhibitory action against potent food borne pathogenic bacteria.

Keywords: Lactic acid bacteria, plant fermented beverages, probiotic, *Lactobacillus, Streptococcus*.

Introduction

Probiotics are growth promoting factors produced by microorganisms (Lilly and Stillwell, 1965). Parker (1974) defined probiotic as "Organisms and substances with beneficial effects on animals by influencing the intestinal microflora". Maintaining balance of bacteria residing in the intestine is necessary to healthy intestine. Many factors may change the balance away from potentially beneficial, health promoting bacteria like *Lactobacilli* and bifidobacteria to potentially harmful or pathogenic microorganisms like clostridia, sulphate reducers and bacteroides species. Use of probiotics help to protect the host from various intestinal diseases and disorders while increasing the number of beneficial bacteria and making the balance steady (Fooks *et al.*, 1999). It is believed that most probiotics do not permanently adhere in the intestine, but exert their effects as they metabolize and grow during their passage through the intestine (colonization). Thus, daily consumption of these bacteria is probably the best way to maintain their effectiveness. With the current focus on disease prevention and the quest for optimal health at all ages, the probiotics market potential is enormous. Health professionals are in an ideal position to help guide their clients toward appropriate prophylactic and therapeutic uses of probiotics that deliver the desired beneficial health effects.

Coconut palm (*Cocos nucifera*) produce fermented sap called toddy is popular among Asian countries. Toddy is a natural fermented food which contains microorganisms that are probiotic in nature. These probiotic microorganisms have the ability to act as antimicrobial agent so it can be used as natural food additive in order to increase immunity without side effects (Ashraf and Shah, 2011). The microorganisms on toddy fermentation produce lactic acid and CO_2 that make the toddy anaerobic and leaven the product. Both bacteria and yeasts are generally introduced by the two main ingredients and participate in the fermentation (Kadere *et al.,* 2008). In view of the above facts, this study was aimed to evaluate probiotic activity of isolated bacterial species from coconut toddy and antimicrobial activity.

Materials and methods

Chemicals: Ringer Martin salt, Nutrient agar, MacConkey agar, MRS agar, Nutrient broth, Phosphate Buffer Saline (PBS), ATCC culture of *Lactobacilli* was obtained from HiMedia lab Pvt. Ltd, India.

Sample collection: Fermented coconut toddy was collected in the early morning near Uthukottai, Tiruvallur. The container was pre-autoclaved with capacity of 300 mL. Samples were maintained between 0-5°C and brought to laboratory with in 2 h.

Saline preparation: Known volume of 8.9% saline was prepared in double distilled water and was autoclaved at 121^0C for 15 min at 15 Lbs.

Serial dilution of the sample: Toddy sample (25 mL) was transferred into 225 mL of saline (10^{-1}) from which, 10 mL was transferred to 90 mL of saline (10^{-2}) and from this 1 mL was transferred to 9 mL of saline (10^{-3}) and repeated up to 10^{-7} dilution.

Methodology of inoculation: Sample of 1 mL was taken from 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions and poured into separate petri plates. Nutrient agar (20 mL) was poured into 6 petri plates. It was then solidified and kept in incubator at 37ºC for 48 h in inverted position. Similarly 20 mL of MacConkey agar was solidified and kept for incubation at 37ºC for 48 h in inverted position.

Isolation and identification of strain from fermented coconut toddy

Coconut toddy (25 mL) was added to 225 mL saline and blended thoroughly. Appropriate serial dilutions of the blended mixture was plated onto PCA (Plate count agar) and MRS (de Man Rogosa and Sharpe) agar and incubated at 37°C for 48 h. The translucent/opaque colonies with 2-3 mm in diameter having entire margins were taken and suspended in nutrient broth and incubated at 37°C for 48 h. The process was repeated until pure cultures were obtained. These isolated organisms were maintained in nutrient agar slants, by sub-culturing them periodically and stored in at 37°C (Iyer and Ananthanarayan, 2008).

Microbial counts, laboratory isolation and identification

Ten milliliter of sample was homogenized in 90 mL sterile salt peptone solution containing 0.1% bacteriological peptone and 0.9% NaCl as the 1:10 dilution. After serial dilution, Aerobic mesophilic bacteria were enumerated by pour plate on Plate Count Agar (PCA) incubated aerobically at 37°C for 3 d. Then it was enumerated on MRS agar supplemented with cycloheximide (0.005%). Plates were incubated at 30°C for 2 d under aerobic conditions. The organisms were phenotypically characterized by Gram staining. Determination of morphology was done by phase-contrast microscopy. Only gram-positive, catalase negative, non motile rod and cocci isolates strains were selected. The presence of catalase activity was assessed by the formation of gas bubbles after the suspension of bacterial cells in a droplet of 3% hydrogen peroxide on MRS. Stock cultures of the isolates were stored in MRS broth containing 15% glycerol at 80°C. Carbohydrate fermentation pattern of lactic acid bacteria used for sap fermentation were determined according to the manufacturer's instructions (Amoa-Awwa *et al.,* 2007).

Determination of antimicrobial activity

Antimicrobial activity was assayed by an adaptation of the critical dilution assay method according to Mayr-Harting *et al*. (1972). The 48 h culture grown in medium was spread on 2% Nutrient agar (10 mL) was overlaid with nutrient agar 1% (5 mL) inoculated with overnight grown culture suspensions of the indicator organisms. The plates were allowed to solidify and wells of 6 mm diameter were punched into them with a sterile cork borer. Cell free extract (100 µL) was poured in each of the wells and the plates were placed in the refrigerator at 4°C for 20 min to enhance diffusion of sample. The plates were then incubated at 37°C. The plates were then incubated at 37°C for 24 h and examined for zone of inhibition.

Sugar fermentation test

Peptone water (5 mL) was taken in test tube with 1% sugar solution (Glucose and Lactose) and placed on Durham's tube in inverted position. The test tube was autoclaved at 121^0C for 15 min. The test culture was inoculated into Durham's tube and incubated at 37^0C. The results were confirmed based on the presence of turbidity and gas production.

Aerobic and anaerobic test

MRS agar (0.56 g) was dissolved in 10 mL of distilled water, and autoclaved at 121^0C for 15 min in 15 Lbs, then the agar was poured in two petri plates, one for aerobic growth and the other for the anaerobic growth of the organism. The test organisms were taken from the agar's land and was streaked on the MRS agars plates. The Aerobic plate was incubated at 48^0C and the anaerobic plate was incubated at 72^0C for 48 h in an anaerobic chamber (This slows down the growth). The colony morphology was then identified.

Sodium chloride tolerance test

One gram of 5% Sodium Chloride salt was mixed with 5 mL of MRS broth in a test tube, subsequently tube with 5 mL of MRS broth without salt was taken. With a circular loop, the test cultures were inoculated into the broth (with and without salt) and incubated at 37^0C for 48 h.

Bile tolerance isolates

The isolates were grown in MRS broth containing 2% (w/v) of bile salts mixture at 37°C for 24 and 48 h. The growth was checked using the pour plate technique (Seeley and VanDemark, 1981) wherein 1 mL of culture of appropriate dilutions was overlaid with MRS agar. The plates were incubated at 37°C for 48 h and the cell count was compared with that of the control MRS agar plates (containing cultures grown in MRS medium without bile salts mixture). Bacterial growth was expressed as colony forming units per mL (CFU/mL) and the survival percentage (% ± SD) of strains to bile salts was calculated as given below.

According to Mourad and Nour-Eddine (2006), the percentage survival ¼ log $CFUN_1$=log $CFUN_0$X100
Where, N_1 is viable count after exposure to bile salts
N_0 is viable count without exposure to bile salts

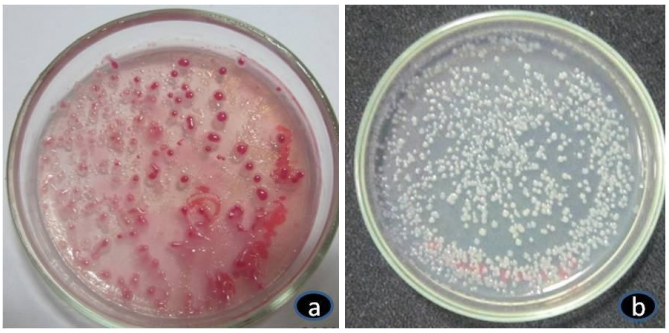
Tolerance to acidic pH values

Isolates were grown in MRS broth at 37°C for 48 h. The cultures were centrifuged at 8,000 rpm for 10 min at 4°C. The pellets were washed twice in sterile phosphate-buffered saline (PBS, pH 7) and re-suspended (1:100) in PBS to achieve a cell density of 1×10^{12} cells/mL. This was employed for setting up the experimental control and studying survival of isolates at low pH (pH 1, 2 and 3 prepared in PBS). The suspensions were incubated at 37°C and samples were removed after every 1 h to 4 h. Counts of surviving cells were determined by plating on MRS agar using the procedure followed in bile tolerance assay.

Results and discussion

The existence of two beneficial microorganisms were confirmed by different tests namely gram's staining, motility, catalase, oxidase tests, biochemical tests, indole methyl red, trible sugar iron, lactic acid confirmatory test, sugar fermentation, aerobic, anerobic, NaCl tolerant, probiotic confirmatory tests, bile salt tolerant and bile tolerant. Gram's staining results showed the isolated culture was purple coloured, non-sporulating and rod shaped. Gram's staining test showed positive for organisms 1 (*Lactobacillus*) and 2 (*Streptococcus*) (Fig. 1). A total of 10 organisms were isolated from different samples of fermented coconut toddy. Two bacterial strains which were clear, round, opaque, white to yellow colour colonies, 2-3 mm in diameter having entire margins from PCA and yellow colonies and pink colonies from MAC were taken for the study. The isolates were tested for the gram nature and catalase negative (Table 1; Fig. 1).

Fig. 1. Biochemical characterization of strains isolated from toddy.



a. *Lactobacillus* (Lactic fermenting); b. *Streptococcus* (Non-fermenting)

The isolates which are gram positive in nature and catalase negative were studied further. Some of the cultures were bacilli (short rods), the others were cocci and one was a coccobacilli. Based on the Gram nature, morphology and catalase test, the cultures were observed. Biochemical tests; citrate, indole showed negative, colour, ring was not observed and in methyl red test showed ring test was positive (Table 1). In trible sugar iron tests, H_2S showed negative for both 1 and 2, Gas test showed 1 postive and 2 negative (Table 2). Organisms 1 and 2 showed fermenting property with the production of gas, sugar fermentation test namely glucose and lactose showed positive (Table 3). No colour changes in citrate utilization test. In agar plug test, there is no gas formation and it was homo-fermentative. The isolates grown on MRS broth were treated with 5% of NaCl at 37°C for 48 h. Both the isolates showed good resistance to 5% NaCl even after exposure for 48 h (Table 1). The growth was checked using the pour plate technique (Seeley and VanDemark, 1971). One of the important criteria to be fulfilled and can be used as a probiotic is its ability to resist the effect of bile salts in the gastrointestinal tract (Lee and Salminen, 1995). However, there are no reports on the exact concentration to which a selected strain should be tolerant.

Table 1. Different types of confirmatory tests.

Types of tests	Observation	Sample 1 and 2
Motility test	Non motile	Non motile
Catalase test	Bubbles release was not observed	Negative
Oxidase test	Purple colour was not observed	Negative
Citrate test	Green colour was not observed	Negative
Indole test	Red ring was not observed	Negative
Methyl red test	Red ring was observed	Positive
Fermentation type	Glucose phosphate broth	MR positive (Mixed acid fermentation)
Agar plug test	Homo–hetero fermentative medium	Homo –hetero fermentative medium. No gas formation homo fermentative)
Triple sugar utilization	TSI agar	Yellow coloured slant and butt (Utilizes all sugar without H_2S and gas. No utilization of N source after exhaustion of C source
Citrate utilization	Simmon's citrate utilization. Agar test citrate as carbon source	No change in colour

Table 2. Trible sugar iron test.

Trible sugar iron tests	Results	
	Sample 1	Sample 2
H_2S	Negative	Negative
Gas	Negative	Positive
Butt	Acid	Acid
Slant	Alkaline	Alkaline

The physiological concentration of bile salts in the small intestine is anywhere between 0.2 and 2.0% (Gunn, 2000). Therefore, the isolates were treated with 2% bile as it is the highest concentration obtained in animal and human intestine during digestion process (Gotcheva *et al.*, 2002). Both the isolates showed good resistance to 2% bile salt even after exposure for 48 h. Resistance to bile is related to bile salt hydrolase (BSH), an enzyme which helps in hydrolyzing conjugated bile, thus reducing its toxic effect (Du Toit *et al.*, 1998). This differs significantly among the lab species and their strains. Similar results were also reported by Mourad and Nour-Eddine (2006) who found one of their isolated strains *L. plantarum* showed 65% survival rate on exposure to 2% bile salt.

Table 3. Sugar fermentation test.

Types of tests	Observation	Sample 1 and 2
Glucose	Gas production was observed	Positive
Lactose		Positive
Maltose		Positive
Sucrose		Positive
Mannose		Positive
Xylose		Negative
Sorbital		Negative
Rhaffinose		Negative
Aerobic	Growth was observed	Positive
Anaerobic	Growth was observed	Positive
NaCl tolerant	Growth was observed	Positive

Table 4. pH tolerant tests.

pH	1 H (No of organisms)		2 H (No of organisms)		3 H (No of organisms)	
	Org 1	Org 2	Org 1	Org 2	Org 1	Org 2
1	8	5	3	Nil	Nil	Nil
2	23	18	13	10	Nil	Nil
3	36	30	21	19	6	5

A probiotic strain should survive transit through the stomach where the pH is low around 1.5 to 3 (Table 4). Hence, tolerance to extremely acidic conditions is another important feature of probiotic strain (Dunne *et al*., 2001; Guo *et al*., 2009). It was observed that at pH 3.0, lactobacillus showed better survival, even after 4 h of incubation. However, it was noted that the percentage of survival decreased with decrease in pH.

Antimicrobial activity

An important feature of probiotic culture is its ability to kill pathogens which infect the gastrointestinal system. The isolates were checked for their antimicrobial activity against *B. cereus, L. monocytogenes* and *E. coli* which are common food borne pathogens that infect the gastro intestinal tract (Table 5). The results showed that two of the ten isolates could inhibit the indicator organisms, however, at different inhibition levels. Several researchers have observed that strains which can produce antimicrobial substances are active against pathogenic bacteria (Topisirovic *et al*., 2006). The differences in inhibition potential among the selected isolates could be due to different intrinsic factors induced by food origins (Klayraung *et al*., 2008).

Conclusion

Bacterial species isolated from fermented coconut toddy were confirmed by ATCC (American type colony control) gram staining, catalse, motility, sugar fermentation, aerobic/anaerobic test and salt tolerance. The isolated lactobacillus strain had the ability to tolerate high bile salt concentration and low pH. Based on these *in vitro* tests, there is a high possibility that the isolates would be able to reach the intestinal tract in good numbers.

Both the isolates were good lactic acid producers and also showed antibacterial activity against pathogenic microorganisms. The ability of the isolates to produce vitamin B12 and β-galctosidase should be investigated in future since this is essential in improving digestion and metabolism. This should be considered as a positive trait for microorganisms which are used as starter cultures and in manufacturing of probiotic and novel functional foods. However, the isolated strains need to be further investigated using *in vivo* experiments to establish their potential health benefits.

Table 5. Antimicrobial activity of the isolated strains.

Organism	MDA	MDB
Staphylococcus aureus	Positive	Positive
Micro coccus	Positive	Negative
salmonella typhi	Positive	Negative
Protease bulgaris	Negative	Positive
Listeria	Positive	Negative
Klebsiella	Negative	Positive
Bacillus cereus	Positive	Negative
E.coli	Positive	Positive
Shigella	Positive	Positive
Enterobacter	Positive	Positive

References

1. Amoa-Awua, W.K., Sampson, E. and Tano-Debrah, K. 2007. Growth of yeasts, lactic and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm (*Elaeis guineensis*) in Ghana. *J. Appl. Microbiol.* 102: 599-606.
2. Ashraf, R. and Shah, N.P. 2011. Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. *Int. Food Res. J.* 18(3): 837-853.
3. Du Toit, M., Franz, C., Schillinger, U., Warles, B. and Holzappfel, W. 1998. Characterization and selection of probiotic lactobacilli for a preliminary mini pig-feeding trail and their effect on serum cholesterol level, faeces moisture contents. *Int. J. Food Microbiol.* 40, 93-104.
4. Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G. C., Shanahan, F. and Collins, J.K. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Amer. J. Clinical Nut*. 73 (suppl): 386S–392S.
5. Fooks, L.J., Fuller, R. and Gibson, G.R. 1999. Prebiotics, probiotics and human gut microbiology. *Int. Dairy J.* 9: 53-61.
6. Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z. and Angelov, A. 2002. Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. *Food Biotechnol.* 16: 211-225.
7. Gunn, J.S. 2000. Mechanisms of bacterial resistance and response to bile. *Microb. Infect*. 2: 907-913.
8. Guo, J.J., Liu, K.F., Cheng, S.H., Chang, C.I., Lay, J.J., Hsu, Y.O., Yang, J.Y., Chen, T.I. 2009. Selection of probiotic bacteria for use in shrimp larviculture. *Aquacult. Res*. 40: 609-618.
9. Iyer, P.V. and Ananthanarayan, L. 2008. Enzyme stability and stabilization-aqueous and non-aqueous environment. *Proc. Biochem.* 43, 1019-103.

10. Kadere, T.T., Miyamoto, T., Oniang'o, R.K., Kutima, P.M. and Njoroge, H. 2008. Isolation and identification of the genera Acetobacter and Glucanobacter in coconut toddy (mnazi). *Afri. J. Biotech*. 7(16): 2963-2971.
11. Klayraung, S., Viernstein, H., Sirithunyalug, J. and Okonogi, S. 2008. Probiotic properties of Lactobacilli isolated from Thai traditional food. *Sci. Pharm*. 76: 485-503.
12. Lee, Y.K. and Salminen, S. 1995. The coming of age of probiotics. *Trend. Food Sci. Tech.* 6: 241-245.
13. Lilley, D.M. and Stillwell, R.H. 1985. Probiotics: Growth promoting factors produced by microorganisms. *Sci.* 147: 747-748.
14. Mayr-Harting, A. Hedges, A.J. and Berkeley, R.C.W. 1972. Methods for studying bacteriocins. In: Methods in Microbiology, 7A, Eds, J.R. Norris and D.W. Ribbons, pp. 315-422. Academic Press London, New York.
15. Mourad, K. and Nour-Eddine, K. 2006. *In vitro* preselection criteria for probiotic lactobacillus plantarum strains of fermented olives origin. *Int. J. Probiotics Prebiotics.* 1: 27-32.
16. Mourad, K. and Nour-Eddine, K. 2006. In vitro preselection criteria for probiotic lactobacillus plantarum strains of fermented olives origin. *Int. J. Probiotics Prebiotics.* 1: 27-32.
17. Mundt, J.O. 1986. Enterococci: Bergey's Manual of Systematic Bactriology. P.H.A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt (ed.). Williams and Wilkins, Baltimore. 2: 1063-1065.
18. Parker, R.B. 1974. Probiotics, the other half of the antibiotic story. *Animal Nutr. Health.* 29: 4-8.
19. Seeley, H.W. and VanDemark, P.J. 1981. Microbes in Action: A Laboratory Manual of Microbiology, 3rd Ed, W.H. Freeman and Company, USA.
20. Topisirović, L., Kojić, M., Fira, D., Golić, N., Strahinić, I. and Lozo, L. 2006. Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. *Int. J. Food Microbiol*. 112: 230-235.