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Amrutha TA
M. Tech scholar, Dept. of Dairy Microbiology College Of Dairy Science and Technology Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India

AK Beena
Professor and Head, Dept. of Dairy Microbiology College of Dairy Science and Technology Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India

Aparna Sudhakaran V
Asst. Professor, Dept. of Dairy Microbiology College of Dairy Science and Technology Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India

Rejeesh R
Asst. Professor, Dept. of Dairy Microbiology College of Dairy Science and Technology, Thiruvananthapuram Kerala Veterinary and Animal Sciences University, Kerala, India

Archana C
Asst. Professor, Dept. of Dairy Microbiology College of Dairy Science and Technology, Pookkode Kerala Veterinary and Animal Sciences University, Kerala, India

Vinod V
College of Dairy Science and Technology, Mannuthy Kerala Veterinary and Animal Science University, Kerala, India

Correspondence
Amrutha TA
M. Tech scholar, Dept. of Dairy Microbiology College Of Dairy Science and Technology Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India

Antioxidant property of *Weissella cibaria* DMA 18 isolated from tender coconut water

Amrutha TA, AK Beena, Aparna Sudhakaran V, Rejeesh R, Archana C and Vinod V

Abstract
The objective of this study was to evaluate the functional properties of a lactic acid bacteria isolated from tender coconut water. On the basis of biochemical characteristics and 16S rRNA sequencing, the isolate was identified as *Weissella cibaria*, the sequence of which is deposited in NCBI with accession number MH782084. Acid tolerance, bile tolerance and adhesion potential were assessed *in vitro*. *Weissella cibaria* DMA 18 strain was found to withstand pH 3.0 and 0.6% (w/v) concentration of bile salt environment for a period of 3h. Auto-aggregation and cell surface hydrophobicity of 83.78% and 89.1% is suggestive of the good adhesion potential of the isolate. The non-haemolytic nature of the strain indicates the possible absence of virulence factors. Both the culture and crude EPS displayed remarkable antioxidant DPPH assay revealed antioxidant activity of 27% and 33.55% respectively with sample volumes as low as 10 µl and 6µl respectively. The results confirm the potential of *Weissella cibaria* DMA 18 to be used as a functional culture for designing foods to ameliorate disorders induced by oxidative stress.

Keywords: *W. cibaria*, exopolysaccharides, antioxidant activity, coconut water

Introduction
Oxidation is an integral metabolic reaction in all living cells. Presence of free radicals leads to oxidative stress that is evidently harmful to human beings. The reactive oxygen species (ROS) are highly destructive to cells and for the same reason they play a crucial role in the initiation and progression of different disease conditions such as cancer, heart diseases, and inflammatory bowel disease (Valko *et al*., 2001) [27]. With free radical species being identified as responsible for augmented number of diseases, antioxidants are gaining more attention these days. Antioxidants are those substances, which can reduce the oxidative stress either by preventing the formation of radicals, or by scavenging these radicals or by destroying them. Living organisms are well protected against free radical damage by enzymes such as, superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherol and glutathione (Niki *et al*., 1994) [20]. Synthetic antioxidants such as butylated hydroxyanisole (BHA), n-propyl gallate (PG) and butylated hydroxytoluene (BHT) also exhibit strong antioxidant activity but their toxic nature pose potential risks. Antioxidants from natural sources are likely to be of more demand as the use of synthetic antioxidants food is restricted or prohibited in many (Tayo *et al*., 2018) [26].

Lactic acid bacteria and their metabolites are reported to impart numerous health benefits when consumed. Major share of probiotic LAB mainly include members of the genera *Lactobacillus* and *Bifidobacterium*. Members of *Pediococcus, Weissella, Lactococcus and Enterococcus* are also being recognized for their health-promoting effects (Zommiti *et al*., 2018) [32] Lactic acid bacteria like *W. confusa, L. plantarum, L. rhamnosus* and *L. mesenteroides* have been proved to be capable of reducing free radicals in our body (Sharma *et al*., 2018, Le and Yang, 2018, Xing *et al*., 2015, Abubakr *et al*., 2012) [23, 16, 30, 1].

Exopolysaccharides, referred to as natural antioxidants can substitute synthetic antioxidants in the proactive management of lifestyle diseases like heart disease, obesity, stroke and diabetes (Nampoothiri *et al*., 2016) [19]. EPS produced by LAB are reported to have emulsifying, gelling and stabilizing properties. Health conscious population prefer low fat products but consumer appeal of these products are very much affected because of poor 'mouth feel'. EPS being a natural bio thickener, exopolysaccharides producing lactic acid bacteria has ample scope to be used in food industry for improving the rheological property of fermented milk products.

This work attempted to evaluate the probiotic temperament of a LAB isolated from tender coconut water, a nutritious thirst quenching fruit drink popular in the state of Kerala.

Materials and methods

Isolation and identification of lactic acid bacteria

Coconut water was collected aseptically from freshly harvested tender coconut. A pre-enrichment was done initially by transferring one millilitre of sample to five millilitre of MRS broth and incubating at 37 °C for 24h. Appropriate dilutions of these samples were pour plated in De Man, Rugose and Sharpe (MRS) agar and incubated at 37 °C for 48h so as to get well isolated discreet colonies. Morphological and biochemical identification at preliminary level was performed according to the methods described in Berge's Manual of systematic Bacteriology for identification of LAB (Holt *et al*., 1994) [15]. All the tests for identification was carried out using freshly activated cultures in MRS broth. Molecular level confirmation of the isolate was done by 16S rRNA sequencing. The primers used were 16S-RS-F Forward 5'CAGGCCTAACACATGCAAGTC3' and 16S-RS-R Reverse 5'GGGCGGWGTGTACAAGGC3'.

Hemolytic property

The LAB strains were streaked on blood agar and incubated at 37 °C for 24h.. The LAB strains that produced green-hued zones around the colonies (alpha-haemolysis) or those that did not produce any effect on the blood agar (Gamma- hemolysis) were considered non hemolytic. Those producing clear zones of RBC lysis around the colonies (Beta- hemolysis) were considered as haemolytic in nature (Adetoye *et al*., 2018) [2].

Probiotic characterization of isolates

Probiotics bring about its beneficial effects by altering the intestinal microflora in a way advantageous to the consumer. So for an organism to be a potential probiotic candidate, it should have the ability to withstand and surpass the harsh acid and bile environment. Apart from these, ability to get attached to the intestinal mucosa is also equally important. These properties of the indigenous isolates obtained in this work were evaluated in terms of acid tolerance, bile tolerance and adhesion potential.

Acid tolerance

To assess the acid tolerance, the isolate was exposed to pH 2.0 and 3.0 by inoculating into MRS broth tubes whose pH was adjusted using 1N HCl. Incubation was done at 37 ^{0}C (Pundir *et al*., 2013) [22]. The number of survivors were qualitatively assessed by streaking on MRS agar plates at hourly intervals for three hours.

Bile tolerance

To assess the bile tolerance, the isolate was exposed to 0.3 and 0.6 percent bile by inoculating into MRS broth tubes containing bile salts. Incubation was done at 37 ^{0}C (Pundir *et al*., 2013) [22]. The number of survivors were qualitatively assessed by streaking on MRS agar plates at hourly intervals for three hours.

Adhesion Potential

The isolate was further evaluated for their adhesion potential in terms of Cell surface hydrophobicity (CSH value) and auto aggregation (%).

Bacterial cell surface hydrophobicity

The adhesion potential was measured in terms of Cell surface hydrophobicity by BATH (Bacterial Adhesion to Hydrocarbons) assay (Collado *et al*., 2008) [6]. The isolates were grown in MRS broth for 16-18 h at 37 °C. Bacterial cells in the stationary phase were harvested as pellets by refrigerated centrifugation at 4 ^{0}C for 12000rpm for 10 minutes. After washing the cell pellet with phosphate buffered saline (Himedia) thrice, pellet was suspended in the same buffer to have an optical density of 0.25±.05 at 600 nm. To 5ml of this suspension, equal volume of xylene was added and the two phase system was mixed thoroughly by vortexing for five minutes. Immediately after vortexing, OD at 600nm was recorded. The vortexed samples were then kept at 37 ^{0}C for 1h to allow phase separation. The aqueous phase on the top was pipetted out and OD at 600nm was determined. The percentage decrease in absorbance of the original suspension due to partitioning of cells was taken as the CSH value. The average of triplicate observations were recorded as result. The CSH value was calculated using the formula.

$$\frac{\text{Initial OD- Final OD}\times 100}{\text{Initial OD}}$$

Auto-aggregation

MRS broth was inoculated with freshly activated culture at a level of one percent. After incubation at 37 °C for 18h, the cells were harvested by refrigerated centrifugation at 5000 g for 15 min, The cell pellet was washed twice with phosphate buffered saline (PBS) and then resuspended in same buffer so as to get an optical density of 0.60±0.02 at 600 nm. Four milliliters of this cell suspension was vortexed and 0.1 ml of this suspension was transferred to another tube with 3.9 ml of PBS and the absorbance was measured at 600 nm (A1). The sample was kept undisturbed at 37 °C and OD of the sample (A2) was determined again at 6h.

The auto-aggregation percentage was expressed as:

(Auto-aggregation) = [(A1 - A2)/ (A1) x 100]

Where A1: initial optical density, A2: optical density after incubation.

Auto aggregation was calculated from three replicates as the percentage decrease in absorbance of the original suspension due to aggregation and sedimentation

Screening of cultures for EPS production

The mucoid /slimy colonies were microscopically examined for presence of capsule. Capsular staining was done as per Anthony (1931) [3]. A thin smear of active culture was prepared and air dried without heat fixing, One percent Crystal violet was allowed to act for two minutes and then rinsed with 20% (w/v) copper sulphate solution. After air drying, smears were examined under oil immersion. Crystal violet get dislodged easily from the non-ionic capsule when washed with copper sulphate solution. Light blue to colourless halo around deeply stained bacterial cells indicated the presence of capsules.

EPS production potential was evaluated based on colony characteristics when streaked on Congo red agar (Freeman *et al.*, 1989) [11]. Congo red agar was prepared by adding 0.1% Congo red solution at a level of nine percent and sucrose at a level of five percent to Brain Heart Infusion agar. The presumptive colonies were streaked on Congo red agar and

incubated at 37 ^{0}C. Formation of slimy and shining black colonies within 24 h of incubation was suggestive of EPS production.

Extraction of EPS

Nutrient broth (200ml) supplemented with two percent sucrose was inoculated with two percent freshly activated culture. After incubation at 37 ^{0}C for 24h, the cells were removed by refrigerated centrifugation at 6000 rpm / 20 min. The cell free supernatant (CFS) was mixed with equal volume of chilled ethanol and thoroughly vortexed. The mixture was kept at 4 °C overnight to precipitate out the EPS. This mixture was further subjected to centrifugation at 6000 rpm/ 20 min/ 4 °C. The supernatant was decanted and the precipitate obtained was dissolved in five millilitres of deionized water. Crude EPS was quantified as described by Dubois *et al*., (1956) [9].

Isolation and identification of LAB

Anti-oxidant assay

Antioxidant activity of LAB

The radical scavenging activity of the isolate was determined by DPPH assay (Son and Lewis, 2002) [25]. To assess the antioxidant potential of the isolate, different volumes of CFS (2, 4, 6, 8 and 10µl) was added to 0.375 ml of freshly prepared 0.1mM DPPH and volume was made up to two millilitre with ethanol. Control was prepared by using uninoculated MRS broth instead of CFS. The mixture was kept in a dark room at room temperature for 20 min for reaction to take place. After 20 min, the absorbance of the mixture was read at 517 nm. 0.1Mm DPPH as such as was used as blank. Antioxidant activity of crude EPS (extracted from 200 ml growth media) was determined in the same way as that of lactic culture using sample volumes ranging from 1 to 6 µl. The radical scavenging activity of the samples was calculated using the following formula and expressed as percentage

% DPPH scavenging activity: $(OD_{blank} - OD_{sample} / OD_{control}) X100$

Result and discussion

Tender coconut water is a very popular drink available throughout Kerala. The present study attempted to to explore coconut water as a source of LAB. Interestingly, pour plating in MRS agar revealed only one type of colony. The mucoid and slimy nature of colony was suggestive of EPS production. The ropy nature for the colonies of EPS producers has been reported previously (Ortega-Morales *et al*., 2007) [21].The phenotypic and biochemical characteristics of the isolate were similar to LAB. The supportive nature of tender coconut water for the growth of LAB have been confirmed by (Lee *et al*., 2013, Giri *et al*., 2018) [18, 13] who had used tender coconut water for development of probiotic beverages. Being rich in vitamins and minerals coconut water, favours the growth of LAB, that are fastidious in their nutrient requirements. The ability of the isolate to utilize a wide variety of sugars including glucose and fructose (Table 1) justifies the presence of this isolate in coconut water. This is the first report of isolation of LAB from tender coconut water. The absence of hemolysis is suggestive of the non-pathogenicity of the isolate.

16S rRNA sequencing confirmed the isolate to be a member of Weissella, a genus that is reported to be prevalent in plants and vegetables, and also in different traditional fermented products like kimchii, idli batter and dairy products. (Dahunsi *et al*., 2017, Fusco *et al*., 2015)[8, 12].*Weissella cibaria* DMA 18 obtained in this study is deposited in NCBI with accession number MH782084. The phylogenetic tree was constructed based on the gene sequence of *W. cibaria* DMA 18 depicted in the Fig 1.

Weissella are Gram-positive, catalase-negative, non-endospore forming coccoid /rods belonging to the Phylum Firmicutes, Class Bacilli, Order Lactobacillus's and Family Leuconostoc ceae (Collins *et al*., 1993) [7].

W. cibaria DMA 18 was found to withstand 6.5% NaCl. It was also found to be capable of multiplication at 15, 37 and 45 ˚C. This is in agreement with taxonomical description of *W. cibaria.* Björkroth *et al*. (2002) [5] Characteristics of *W. cibaria* DMA 18 is illustrated in Table 1.

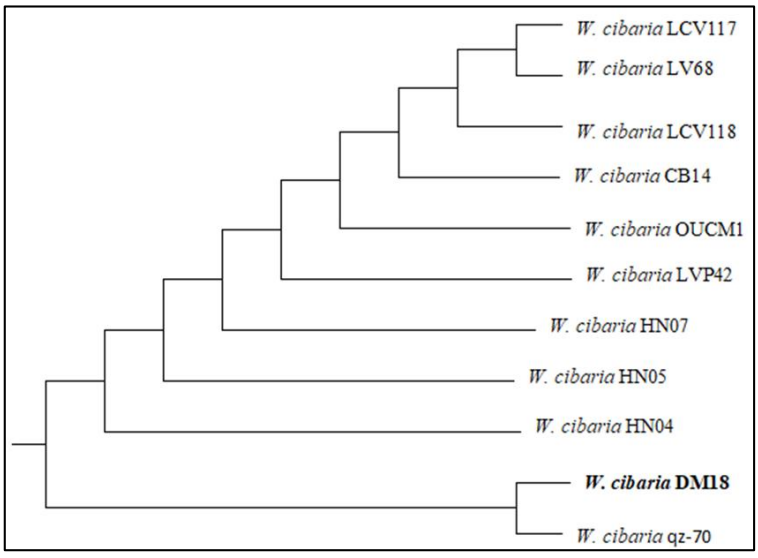

Fig 1: Phylogenetic tree of *W. cibaria* DMA18.

Phylogenetic tree was constructed using t Neighbour joining method implemented in multiple sequence alignment –using UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X.

Table 1: Characteristics of *W. cibaria* DMA18

Characterization	**DMA18**
Gram' s reaction	+
Catalase reaction	-
Oxidase reaction	-
Exopolysaccharide production on congo red agar	
Presence of capsule	+
Hemolysis	+
Growth at	-
15 °C	+
37 °C	+
45 °C	+
6.5% NaCl	+
Acid production from	
Arabinose	-
Cellobiose	+
Galactose	+
Fructose	+
Lactose	+
Glucose	+
Maltose	+
Mannitol	+
Melibiose	+
Raffinose	-
Salicin	+
Sucrose	+
Trehalose	+
Xylose	+

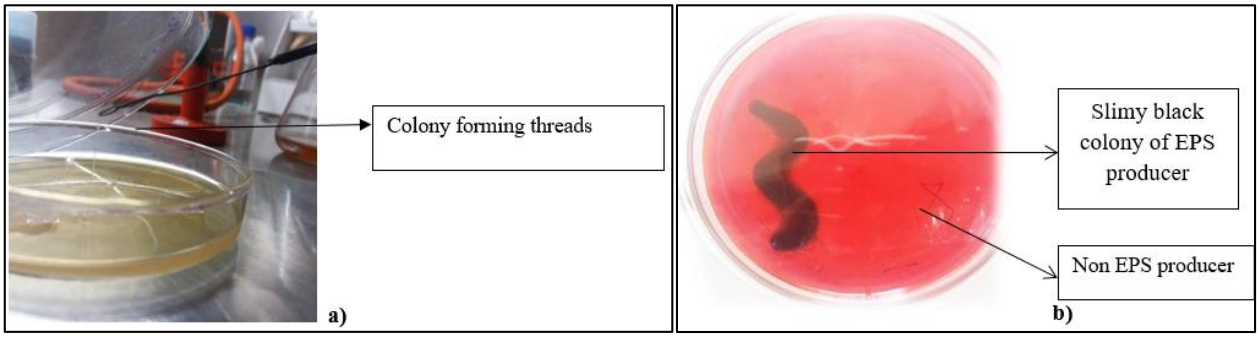

Fig 2: Colony charecteristics of *W. cibaria* DMA18 on a) MRS agar b) Congored agar

Probiotic properties

Acid and bile tolerance

Probiotic organisms should survive the stress environments of stomach (acidic) and intestine (bile and hydrolytic enzymes) through which they transit. The pH of gastric juice varies from 2.0 to 3.0 and food has to travel 2–3 h before they reach intestine (Vidhyasagar and Jeevaratnam, 2013) [28]. Goldin *et al.*, (1992) [14] opined that intestinal bile concentration is 0.3%. The transit tolerance of the isolate was evaluated by exposing the standardized inoculum of viable cells (10^8 cfu/ml) to pH 2.0, pH 3.0, 0.3 percent bile and 0.6 percent bile salt. The number of survivors decreased with increase in exposure time. At pH 3.0 remarkable growth was evident even after 3h.At pH 2.0, the cells got completely inhibited at 2h.In general the growth was significantly less at pH2.0 when compared to pH 3.0.Even in 0.6% bile concentration, the count obtained was comparable to that of control. Similiar observations have been reported by Elavarasi *et al*. (2014) [10] for *Weissella cibaria* KTSMBNL 28. The capsulated nature of the isolate would have provided additional protection against harsh environment.

Adhesion potential

The determination of microbial adhesion is a way to estimate the ability of a strain to adhere to epithelial cells. Auto aggregation assay and BATH assay are valid qualitative phenomenological approaches for estimating adhesion potential. Adherence of bacterial cells is usually related to cell surface characteristics. Cell surface hydrophobicity is a non-specific interaction between host and microbial cells.

Auto aggregation

According to Wang *et al.*, (2010) [29], an auto an auto aggregation value higher than 40% is exceedingly good. A high auto aggregation value of 83.78% was showed by DMA18. In contrast to this observation, Le and Yang. (2018) [16] Reported only 25% auto aggregation for strains of *Weissella*. Hydrophobicity is an important cell surface property that influence adhesion of bacteria to different surfaces (Balakrishna, 2013) [4]. The CSH value of the isolate in this study was 89.1% which is high when compared to the CSH value of Weissella strains as reported by Elavarasi *et al.*, (2014) [10] when a non-polar solvent like normal hexadecane was used. CSH value as high as 95.16% has been reported for *W. confusa* with xylene (Sharma *et al*., 2018) [23].

The probiotic potential *Weissella cibaria* has been reported in several studies (Silva *et al.* 2017, Lee *et al.*, 2012) [24, 17].

Antioxidant activity

The DPPH scavenging ability of *W. cibaria* DMA18 was found to be 27 percent (Fig 3) when the sample volume used was 200 µL *Weissella cibaria* JW15 showed an antioxidant activity 23.53% (Yu *et al.*, 2018) [31]. It is noteworthy that a comparable value was obtained even with a sample volume as low as 10 µl for the coconut water isolate.

As exopolysaccharides are linked to various beneficial biological activities, the antioxidant activity of EPS extract was also determined. (Fig 4) clearly demonstrates that antioxidant activity increases with increase in EPS concentration. Such dose dependent relation has been recorded by by Dahunsi *et al.*, (2017) [8]. In this study the antioxidant activity of 33.5% was observed with an EPS concentration as low as 0.016mg /ml. A direct comparison with earlier reports is not logical because of the different sample sizes, different radical concentrations and different assay methods. The observations of this study endorse the superior antioxidant activity of the EPS.

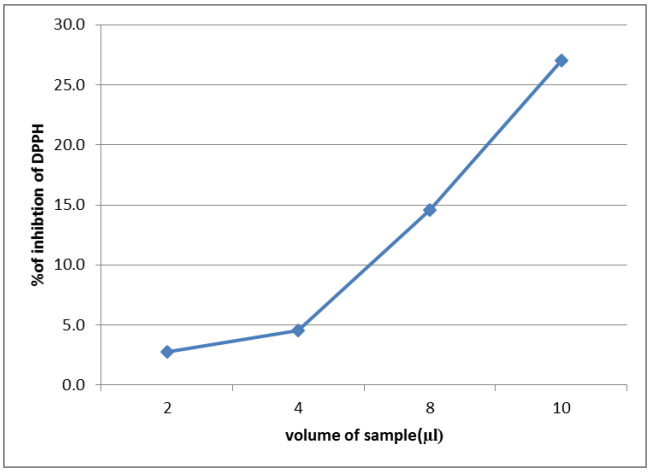

Fig 3: DPPH scavenging activity of *W. cibaria* DMA18

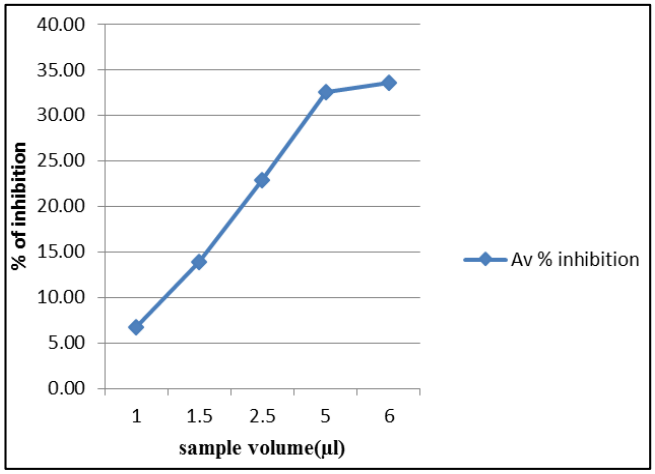

Fig 3: DPPH scavenging activity of EPS produced by *W. cibaria* DMA 18

Conclusion

The observations in this study endorses *W. cibaria* DMA18 as a prospective functional probiotic strain with remarkable antioxidant potential. This isolate has ample scope to be industrially exploited.

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