RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2023.54.3.:808-816

Fructobacillus trapaeoli - A fructophilic lactic acid bacteria isolated from *Manilkara zapota*[#]

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Citation: Divanshi, A.S.M., Aparna, S.V., Beena, A.K., Ligimol, J. and Aysha, C.H. 2023. *Fructobacillus trapaeoli* - A fructophilic lactic acid bacteria isolated from *Manilkara zapota. J. Vet. Anim. Sci.* **54**(3):808-816
DOI: https://doi.org/10.51966/jvas.2023.54.3.:808-816

Received: 08.06.2023 *Accepted: 29.07.2023* *Published: 30.09.2023*

Abstract

The study was undertaken to isolate and characterize fructophilic lactic acid bacteria from the fruit Manilkara zapota (chikoo). Microscopic, phenotypic, biochemical and molecular characterisation of the isolates was done. The Gram-positive thin rods (5 µm length and 0.25 µm thickness) were catalase and oxidase negative and did not hydrolyze arginine. The carbohydrate fermentation studies showed that the isolate preferred fructose similar to glucose as an energy source. The isolate had an optimum growth at 37°C and also had grown at 45°C. 16S rRNA sequencing confirmed the isolate as Fructobacillus tropaeoli DMM03 (NCBI accession number MT158673). There are very few reports of isolation and characterization of Fructobacillus from India. The osmotolerant nature of the isolate was established by its ability to grow in pH 5, 8 per cent NaCl, 35 per cent D-Fructose and 50 per cent D-Glucose. The isolate had also shown growth in the presence of heavy metals with a maximum concentration of 60 ppm cadmium and 2425 ppm lead. An autoaggregation of 46.3 per cent and cell surface hydrophobicity value of 22 per cent with apolar solvent xylene suggested the high adhesion potential. The isolate was found to be non-haemolytic and not liquefying gelatin which implies its safety in use for product preparation. The results point out a prospective area for future research in Fructobacillus tropaeoli DMM03 for tapping its potential in the field of probiotics and bioremediation.

Keywords: *Fructobacillus, Lactic Acid Bacteria, Manilkara zapota, osmotolerant*

[#]*Part of M.Tech. thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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Lactic acid bacteria (LAB) have been universally isolated from different sources like milk, milk products, fermented foods, breast milk, fruits, flowers, and gastrointestinal tracts of humans and animals. Due to its diverse potential, LAB is a prospective candidate for use in the development of functional foods. The role of LAB as a functional starter in fermented food especially dairy-based and as a biotechnological tool for producing bioactive compounds has been widely recognized (Leroy and De*,* 2004). Now their technological application has expanded to agro-industry, nutraceutical production, food ingredients manufacture, texturing agent production, chemical synthesis and even in the medical arena (Gaspar *et al.,* 2013). The wild strains of LAB isolated from different niches possess unique potential.

The fruits and flowers harbour unique lactic acid bacterial communities, including fructophilic lactic acid bacteria (FLAB), which prefer D-fructose over D-glucose (Sakandar *et al.,* 2019). The newly explored FLAB is reported to possess specific biofunctional properties like probiotic potential which could be exploited in the development of fruit-based functional foods (Sakandar *et al.,* 2019). They have been reported to have the unique property of producing low glycemic index products from high glycemic index carbohydrates (Rodriguez *et al.,* 2017). The activity of LAB in wine has an impact on its flavour. Their ability to grow in high concentrations of fructose and glucose makes FLAB pertinent in the wine industry too (Endo *et al.,* 2009).

Fructobacillus, an evolving member of FLAB comes under the family *Leuconostocaceae* based on its phylogenetic position, and biochemical and morphological characteristics (Sakandar *et al.,* 2019). The genus *Fructobacillus* has 90.4 to 94.4 per cent sequence similarity with the genus *Leuconostoc*, 82.8 to 83.3 per cent with *Oenococcus*, and 85.4 to 89.1 per cent with *Weissella* (Endo and Okada, 2008). The genus *Fructobacillus* mainly has five species *Fructobacillus fructosus, F. durionis, F. ficulneus, F. pseudoficulneus* and *F. tropaeoli.* (Endo *et al.,* 2014). The first four species previously belonged to the *Leuconostoc* genus. *F. tropaeoli* isolated from the flower *Tropaeolum majus* by Endo *et al.* in 2011 is now included under the genus *Fructobacillus*. The five species from the genus *Fructobacillus* and two species from the genus *Lactobacillus* (*Lb. kunkeei, Lb. florum*) constitute the FLAB (Gustaw *et al.,* 2018). To the best of the authors' knowledge, there are no reports of isolation and characterization of *Fructobacillus* from Kerala. Hence in this study, the fructose-rich fruit *Manilkara zapota* (*chikoo*), was chosen for the isolation of LAB.

Materials and methods

Isolation and identification

The fruit sample *Manilkara zapota* (*chikoo*) was purchased from the local market. The dilutions by adding 11gm sample to 99 ml 0.85 per cent saline for 10^{-1} dilution and 1 ml previous dilutions to fresh 9 ml saline tubes for subsequent dilution till 10^{-3} followed by pre-enrichment in nutrient broth (37ºC / 24h) (HiMedia Laboratories Pvt. Ltd., Mumbai) and successive selective enrichment in DeManRogosa Sharpe - MRS (HiMedia Laboratories Pvt. Ltd., Mumbai) media at 37ºC for 24h. After the selective enrichment, the turbid broth tubes were streaked on MRS agar and incubated at 37ºC for 48h (DeMan *et al.,* 1960). The particular spindle-shaped subsurface colony developed were further characterised.

Characterisation of the isolate

The isolate was further studied for its morphological, biochemical and physiological characteristics. Morphological and biochemical identification at the preliminary level was performed according to the methods described in Bergey's Manual of Systematic Bacteriology (2009). All the tests for identification were carried out using freshly activated cultures in MRS broth.

The colony morphology of the isolates developed on MRS agar plates was assessed by examining their different characteristics like shape, type of the colony, colony colour, margin, elevation, opacity and pigment production (Kumar *et al.,* 2015). The purity

and tentative identity of the isolate as rods or cocci were ascertained by Gram's staining The microscopic structure was characterized by trinocular and Scanning Electron Microscope (SEM). For SEM, the cell pellets after washing in molecular biological grade water were subjected to a series of dehydration in 15-70 per cent ethanol for 10 min each; 80 per cent and 90 per cent for 15 min; and 100 per cent for 1 h. Eventually, after vacuum drying, the samples were fixed in aluminium stubs on a double-faced metallic tape, sputter-coated with gold and observed using Tescan Vega 3 (Czech Republic) SEM operated at 10.0KV at different magnifications (Ameen *et al.,* 2020). The motility of the isolate was determined by the Hanging drop method, Motility agar method and Phase contrast Microscopy.

The biochemical characteristics of the organism were evaluated by a series of tests *viz*. catalase test, oxidase test, indole test, methyl red test, VogesProskauer (VP) test, citrate utilization, arginine hydrolysis and carbohydrate fermentation test. The carbohydrate utilisation test was checked by both conventional method and API CHL™ 50 galleries (BioMerieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Briefly, inoculum from all isolates with turbidity 2 MacFarland was added into wells containing 49 sugars and incubated at 37°C for 24 and 48 h. The obtained results were analyzed with the help of the manual provided with API CHL 50 galleries. The physiological characterization of the isolates were further determined in terms of their growth characteristics (temperature, pH, salt tolerance, aerobic/anaerobic). The potential of the isolates to form biofilms was evaluated by Congo red assay (Freeman *et al.,* 1989). The molecular level confirmation of the isolate was done by 16S rRNA sequencing by using the species-specific universal 16S rRNA primer, forward 5'CAGGCCTAACACATGCAAGTC3' and reverse 5' GGGCGGWGTGTACAAGGC3' primers (Neveling *et al*., 2012). The PCR was carried out in a 10 µL total reaction volume with 5µL of 2X Phire Master Mix and 0.5 µmol each of forward and reverse primers. The cycling conditions included an initial denaturation of 95°C for 5min, followed by 35 cycles of denaturation (95°C, 30s), annealing (60°C, 40s), extension (72°C, 40s) and final extension at 72°C for 7min. The amplicons were sequenced. The 16S rRNA gene sequences obtained were searched in the GenBank database NCBI (www.ncbi.nlm.nih.gov) using the BLAST (Basic Local Alignment Search Tool) software (http://www.ncbi.nlm.nih.gov/BLAST) for their closest relatives/reference strains with a homology of over or equal to 99 per cent.

Determination of the adhesion potential

The adhesion potential was judged by determining the autoaggregation percentage and cell surface hydrophobicity (CSH) value by bacterial adhesion to hydrocarbons (BATH assay). Autoaggregation assays were performed according to Collado *et al*.(2008). Briefly, the bacteria were grown in MRS broth for 18 h at 37 °C. After centrifugation at 5000*g for 15 minutes, cells were washed twice and suspended in phosphate-buffered saline (pH 7.0) to give viable counts of approximately 10^8 CFU/ml. Four ml of the cell suspension were mixed by vortexing for 10s and autoaggregation was determined during 5h after incubation at room temperature. At hourly intervals, 100µl of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance was measured as OD_{600} (Optical Density at 600 nm in a UV Visible Spectrophotometer). Autoaggregation was calculated according to the equation:

$$\%Autoaggregation = \left(1 - \left[\frac{A_t - A_0}{A_t}\right]\right) * 100$$

Where A_t represents the absorbance at time t = 5 hours and A_0 is the absorbance at t= 0. All experiments were performed in triplicate.

The bacterial adhesion to hydrocarbons test was performed according to the method of Rosenberg *et al.* (1980). A 24h culture of the isolate was centrifuged and the pellet was washed with PBS (Phosphate Buffered Saline) buffer twice and resuspended in the same buffer. Absorbance was adjusted to OD_{600} 1.0. Then, equal proportions of viable bacterial suspension and solvent (xylene) were mixed by vortexing for five minutes. A two-phase system appeared and the aqueous phase was removed after 1h of incubation at room temperature and its OD_{600} was measured.

Results were reported according to the formula

$$\%BATH = \left(1 - \left[\frac{A_0 - A}{A_0}\right]\right) * 100,$$

where 'A_0' and 'A' are absorbances before and after mixing with xylene, respectively.

Osmotolerance of the organism

The osmotolerance of the isolate was evaluated by monitoring its growth in media containing high concentrations of fructose and glucose as per the modified procedure of Gustaw *et al*. (2018). Filter sterilized D-fructose (Nice Chemicals, Kochi, Kerala) was added aseptically to Fructose Yeast Peptone (FYP) broth so as to have a fructose concentration of 1-50 per cent. D- Fructose in the media was replaced with D-Glucose (Nice chemicals, Kochi, Kerala) to find the glucose tolerance of the isolate. The freshly activated culture (10^7 CFU/ml) was added at a level of one per cent to each tube. Incubation was done at 37°C for 24h. The growth of the organism at different concentrations was measured as OD_{600}. The FYP broth without inoculum served as blank. Also, the growth of bacteria in the presence of heavy metals, cadmium and lead was also monitored.

Safety evaluation of the isolate

The safety of the isolate was evaluated by assessing the haemolytic activity and gelatin liquefaction potential. The isolate was streaked on blood agar and incubated at 37°C for 24 h. Plates were examined for the presence of particular zones around the growth that differentiate α, β, γ - hemolysis. (Adetoye *et al.,* 2018). The isolate was also streaked on Gelatin agar (HiMedia Laboratories Pvt. Ltd., Mumbai) slant and incubated at 37°C for 24 h. Tubes were examined after 3h of refrigeration to see if gelatin is in liquid or solid form (Sahu *et al.,* 2019).

Results and discussion

Characterisation of the isolate

Fruits are potent sources of FLAB. *Manilkara zapota* (Chikoo) is rich in fructose (National Nutrient Database for Standard Reference Release, 2016) and was selected for the isolation of FLAB. The isolate *Fructobacillus trapeoli* DMM03 was obtained from *Manilkara zapota* (Chikoo). The organism was Gram-positive rods (Fig.1) and seen as small pinpoint colonies over MRS agar as illustrated in Fig 2. The microscopic characterisation observed under trinocular and SEM is illustrated in Figs 3, 4 & 5 showing isolate as thin rods of 5 µm length and 0.25 µm thickness. FLAB species are rod-shaped resembling lactobacilli cells (Endo *et al.,* 2009).

The characteristics of the isolate analysed revealed a similarity towards LAB. The isolate was catalase and oxidase negative and did not hydrolyse arginine when grown in

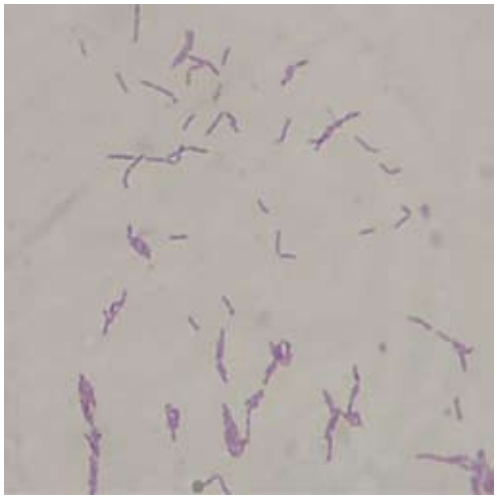

Fig.1. Gram's staining of the isolate

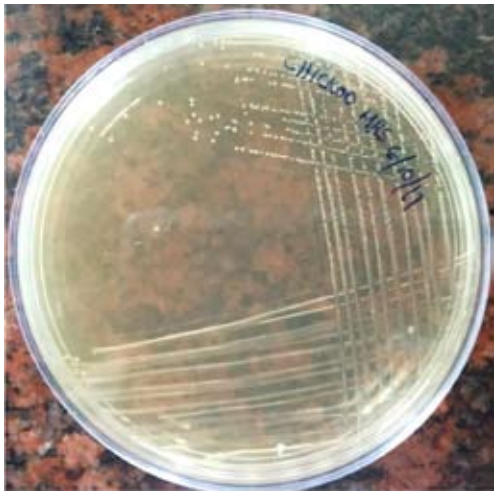

Fig. 2. Colony obtained on MRS streak plate

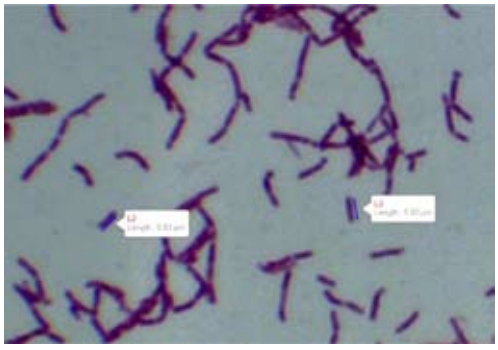

Fig. 3. *Fructobacillus tropeoli* observed in trinocular microscope (100x)

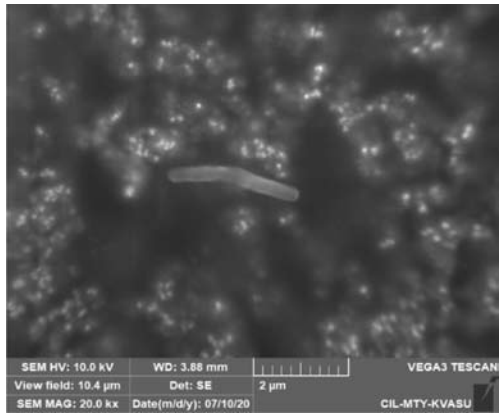

Fig. 5. *Fructobacillus tropeoli* rod observed in Scanning Electron Microscope

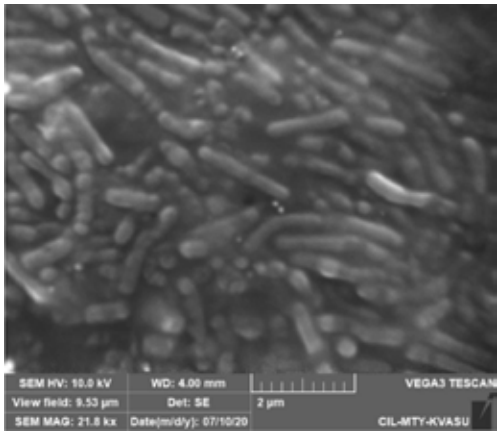

Fig. 4. *Fructobacillus* observed in Scanning Electron Microscope

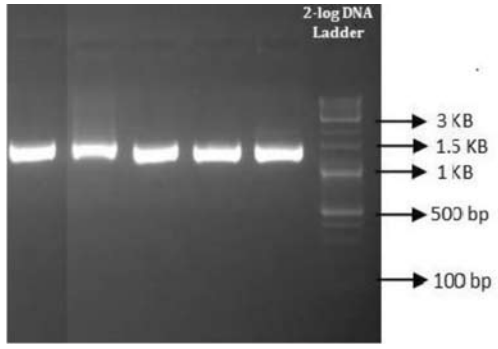

Fig. 6. Growth of *Fructobacillus tropaeoli* at different concentration of fructose

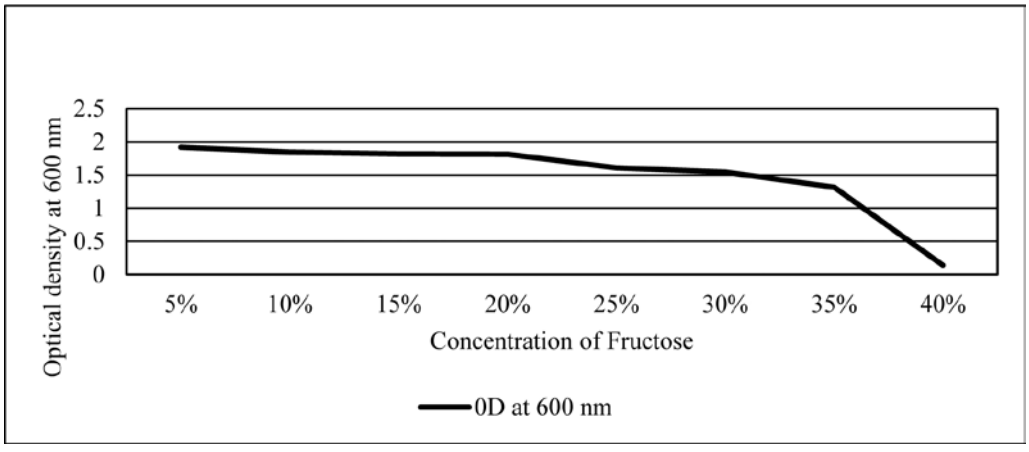

Fig. 7. The PCR amplified 16S rRNA sequence for the isolate

specific media as detailed in Table 1. The FLAB isolate obtained in this study only fermented glucose, fructose, tagatose, fucose, gentibiose, trehalose and mannitol but failed to ferment several sugars out of 49 test sugars in API 50 CHL medium. It is reported that *Fructobacilli* only utilises limited carbon sources like fructose, glucose (Chambel *et al.,* 2006; Endo *et al.,* 2009) and mannitol (Rodriguez *et al.,* 2017). It may be due to the presence of fewer carbohydrate metabolism genes in FLAB than usually seen in other LABs; especially it lacks

Table 1. Biochemical characteristics of the isolate obtained from *Chickoo*

Characteristics		Isolate
Colony Characteristics	Colony Shape	Spindle
	Colony type	Smooth
	Colony colour	Yellowish
	Margin	Entire
	Elevation	Convex
	Opacity	Opaque
	Pigment	Negative
Morphological characteristics	Gram stain	Positive
	Shape	Thin rodes
	Motility	Non motile
Biochemical Tests	Catalase test	Negative
	Oxidase test	Negative
	Indole test	Positive
	Methyl red test	Positive
	VP test	Negative
	Citrate utilization	Positive
	Arginine test	Negative

Table 2. Optical density of the isolate at various pH

pH (Optical Density at 600nm)	**2**	0.035±0.01
	5	1.904±0.12
	7	0.612±0.11
	8	1.915±0.01
	10	0.023±0.24

Table 3. Effect of Oxygen on growth characteristics of *Fructobacillus trapeoli* DMM03

Incubation condition	Optical Density at 600 nm under UV-Visible Spectrophotometer
Growth at the aerobic condition	2.285
Growth at the anaerobic condition	2.134

transporters for the phosphotransferase system (PTS) (Endo *et al.,* 2018). It was observed that just as glucose, fructose was also equally utilized by the fructophilic isolate obtained from *chikoo*. This affinity to fructose is one of the distinguishing characteristics of *Fructobacilli* (Endo *et al.,* 2011). FLAB is classified mainly into two groups – the obligate FLAB which grows well on fructose but poorly on glucose and the facultative FLAB which grows both in fructose and glucose-containing media but less growth observed in glucose (Endo *et al.,* 2009).

Fructobacillus trapeoli DMM03 when assessed for their growth at different temperatures, showed good growth at 37ºC (OD_{600}- 2.285±0.03) as well as 45ºC (OD_{600}- 1.083±0.02) under aerobic conditions. Similar to the observation, Endo, 2011 reported the optimum growth temperature as 35-37ºC. The isolate showed good growth at pH 5 (OD_{600}- 1.904) and pH 8 (OD_{600}-1.915) (Tables 2 and 3) which is similar to the observations of Endo *et al.* (2011). The isolate showed maximum tolerance to 8 per cent NaCl (OD_{600} -1.132±0.24) in media which was higher than the reports of Endo *et al.* (2011) who illustrated that the *F. trapeoli* tolerated up to 2.5 per cent NaCl. The LAB usually gets inhibited in aerobic conditions whereas FLAB seemed to grow more in aerobic conditions than anaerobic (Endo *et al.,* 2018). This study observed similar growth in both aerobic conditions (OD_{600}-2.285) and anaerobic conditions (OD_{600}- 2.134) after 24h of incubation. Molecular characterisation of the isolate by 16S rRNA by PCR following the sequencing of 1.1 Kbp fragment (Fig. 7)

Table 4. Optical Density obtained for growth of *Fructobacillus trapeoli* DMM03 different fructose concentration

Fructose Concentration (%)	OD at 600nm under UV-Visible Spectrophotometer
1	1.946±0.03
5	1.921±0.02
10	1.845±0.01
15	1.819±0.01
20	1.815±0.09
25	1.609±0.07
30	1.545±0.03
35	1.317±0.09
40	0.141±0.01
45	0.040±0.03
50	0.059±0.04

confirmed the isolate as *Fructobacillus trapeoli* DMM03 with 99.99 per cent similarity with reported sequences. The sequence obtained was deposited in NCBI with accession number MT158673. The isolate *Fructobacillus tropaeoli* DMM03 exhibited growth tolerance to cadmium and lead concentration. The cadmium and lead tolerance of the isolate observed in our study was comparatively less than the values reported for other genera of LABs (Divanshi, 2020).

Adhesion potential

Adhesion potential is indicative of the organisms' capability to adhere to the surface mucosa of the gastrointestinal tracts. The adhesion potential is determined by the autoaggregation and cell surface hydrophobicity of the cells. Adhesion potential is proportional to the autoaggregation of the isolate which is indicative of probiotic characteristics. The greater the autoaggregation potential and hydrophobicity, the greater will be the stability and chances of colonisation in the gastrointestinal tract (Sakandar *et al.,* 2019). *Fructobacillus tropaeoli* DMM03 had shown 46.3 per cent autoaggregation value which indicates a good adhesion potential. Sakandar and coworkers (2019) reported 31 per cent autoaggregation for *Fructobacillus.* Zommiti *et al.* (2018), stated that bacteria with more than 40 per cent autoaggregation can be considered as having good adhesion potential. The autoaggregation and CSH results indicated adhesion potential, which is a favourable attribute of probiotic cultures.

Osmotolerance of the organism

Growth of the fructophilic isolate in the high concentration of D glucose and D fructose was determined in FYP broth containing increasing concentrations from 1 to 50 per cent (w/v) of both sugars. The result obtained is illustrated in Fig 6 and Tables 4 & 5. The isolate exhibited growth up to 35 per cent (w/v) D- Fructose beyond which the growth was inhibited. The isolate also had growth in the presence of 50 per cent D-Glucose (w/v) in the media. Gustaw *et al.* (2018) reported about *Fructobacillus trapaeoli* that can tolerate up to 40 per cent glucose (w/v). Endo *et al.* (2009) reported that their isolate could not tolerate

Table 5. Optical Density obtained for growth of *Fructobacillustrapeoli*DMM03 different glucose concentration

Glucose Concentration (%)	Optical Density at 600 nm under UV-Visible Spectrophotometer
1	1.818±0.04
5	1.909±0.03
10	2.037±0.12
15	2.011±0.10
20	1.740±0.09
25	1.840±0.02
30	1.870±0.00
35	1.808±0.04
40	1.707±0.05
45	1.685±0.23
50	1.520±0.25

even 30 per cent (w/v) D-fructose. The isolation of FLAB from the intestinal tract of honeybees was possible due to its high osmotolerance to the high amount of fructose in nectar (He *et al.,* 2011). This property of the isolate can be further explored for the development of functional foods, and fermented products from fruits. The malo-lactic fermentation by FLAB including *Fructobacillus*, immediately after alcohol production will decrease wine pH and this has a role in maintaining keeping quality of the wine (Ines and Falco, 2018).

Safety evaluation of the isolate

The evaluation of isolate for its safety to use is obligatory. No clear zone was observed on blood agar plates and gelatin liquefaction was also not there for this isolate which suggested the possible absence of any virulent factors present and safety for application in the food industry.

Conclusion

In the present study, we isolated a less explored group of LAB, the fructophilic lactic acid bacteria. *Fructobacillus tropaeoli* DMM03 from *Manilkara Zapota* (*Chikoo*) utilized fructose at a higher level unlike other LABs indicating their role in designing new plant-based products. It can be also explored for the development of cereal-fermented food to ameliorate Irritable Bowel Syndrome (IBS). The increased aggregation potential of the isolate *Fructobacilli tropaeoli* is an indication of its probiotic potential. FLAB can be considered a promising candidate for probiotic culture. There are reports of FLAB producing large amounts of a low-calorie sweetener such as mannitol which can very well be exploited industrially. The high osmotolerance makes it a good candidate in the food processing sector. Further in-depth studies need to be in place before recommending this FLAB for bioremediation and use in the food industry.

Acknowledgements

We acknowledge the support provided by the Kerala Veterinary and Animal Sciences University, Kerala, India for providing the funds and facilities and the Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, India for providing the facility for genome sequencing.

Conflict of interest

The authors declare that they have no conflict of interest in the publication

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