Current Journal of Applied Science and Technology

39(43): 28-37, 2020; Article no.CJAST.64166
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

In –Vitro Assessment of Probiotic Attributes of *Propionibacterium freudenreichii* Isolated from Dairy Cheese

K. Harish Kumar[1*], V. C. Suvarna[2], B. H. Sarvani[1], R. U. Abhishek[1] and Veena S. Anil[2]

[1]Department of Agricultural Microbiology, GKVK, University of Agricultural Sciences, Bengaluru, 560 065, India.
[2]Plant Biotechnology and Biochemistry, GKVK, University of Agricultural Sciences, Bengaluru, 560 065, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author KHK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors VCS and VSA managed the analyses of the study. Authors BHS and RUA managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2020/v39i4331137
Editor(s):
(1) Dr. Chen Chin Chang, Hunan Women's University, China.
Reviewers:
(1) Jerusa Souza Andrade, INPA - National Institute of Amazonian Research and Nilton Lins University, Brazil.
(2) Lucretia I .Barber, Rivers State University, Nigeria.
Complete Peer review History: http://www.sdiarticle4.com/review-history/64166

Original Research Article

Received 22 October 2020
Accepted 29 December 2020
Published 31 December 2020

ABSTRACT

Dairy propionibacteria possess innumerable advantages as probiotics, in food preservation and vitamin production. *Propionibacterium freudenreichii* is one among them and is known to produce cyanocobalamine (Vitamin B_{12}) in stable quantities. Deficiency of Vitamin B_{12} is a major health issue among vegetarians and vegans making it a global concern. Hence, an attempt has been made to isolate *Propionibacterium* from dairy products. *Propionibacterium* isolates were enumerated by employing standard dilution plate count technique using yeast extract lactone agar (YELA). Gram's reaction, pigmentation, catalase test, endospore production, nitrate reduction, gas production, fermentation of sucrose and maltose were performed to eliminate non-propionibacteria during isolation. Isolates that tested Gram positive, pigmented, catalase positive, non-endospore formation and could ferment sucrose as well as maltose were further used for identification by 16S rRNA

**Corresponding author: E-mail: harishkurmindla0@gmail.com;*

sequencing. One isolate was confirmed to be *Propionibacterium freudenreichii* (PF) as identified by 16s rRNA gene amplification and Sanger's sequencing. The confirmed isolate was subjected to various tests to check for its probiotic attributes. Haemolysis activity was tested to ensure non-pathogenicity using blood agar medium and isolate was found to be non-pathogenic. Antibiotic susceptibility pattern of the isolate was studied using currently prescribed seven antibiotics representing activity against both Gram-positive and Gram-negative bacteria. The isolate was found sensitive for all the seven antibiotics tested. The isolate was able to tolerate the pH till 2 and sustain by exhibiting minimal growth at 2.0 g L^{-1} of bile salts. Results reveal that PF, isolated native culture from cheese, proved to possess attributes for probiotic agent under *in -vitro* conditions.

Keywords: Propionibacterium freudenreichii; dairy propionibacteria; antibiotic sensitivity; probiotics; haemolysis.

1. INTRODUCTION

Dairy propionibacteria (DPB) are Gram positive, catalase positive, non-spore forming, non-motile, high G+C % containing, anaerobic to facultative anaerobic, pleomorphic actinobacteria with long generation time [1,2]. Ecology of dairy propionibacteria is not clearly elucidated; niches or sources were found to be soil, silage, grasses, vegetable fermentations, rumen of cattle and anaerobic digesters [3]. They are found in milk because of contamination [3]. DPB used as secondary fermenters in Swiss cheese result in characteristic eye formation. Dairy Propionibacteria can survive under severe nutrient stress conditions by utilizing the diversified carbon sources [4] and less intensive in nutritional requirements.

Dairy propionibacteria or classical propionibacteria are of such less conventional probiotics used for decades as food adjuncts, thickeners, cheese-ripening starters, food preservatives, as producers of propionic acid, bacteriocins, vitamins and exo-polysaccharides [4-6]. Recent studies on dairy propionibacteria were focused on their immense probiotic characteristics.

Probiotics are live microbial formulations which when administered in adequate quantities confer health benefit of host [7]. Last two decades witnessed the increased consciousness in probiotics, due to changed food habits of people that are leading to several gastro-intestinal related problems and immune disorders. In this context, market has seen a great boost in probiotic and pharmaceutical products. Along with the bulk of probiotics available as *Lactobacillus* and *Bifidobacterium*, less conventional probiotics as *Propionibacterium*, *Saccharomyces*, *Streptomyces*, *Enterococcus*, *Pediococcus* and *Leuconostoc* were also being analyzed for their immense potentials as probiotics [8].

A complete selective medium for the isolation of propionic acid bacteria is not available, added the propionibacteria are anaerobic to facultatively anaerobic [3], this may be a reason for lower number of isolations from known sources. Several specific media were developed for the isolation and identification of Propionibacteria based on the source type used for isolation; they possess varied degrees of selectivity. Drinan and Cogan (1992) used Sodium Lactate Agar (SLAC) (1.35% sodium lactate, 1% Yeast extract, 1% tryptone, 0.5% KH_2PO_4, 1% Oxoid No. 1 Agar and pH 7.0) added with Cloxacillin (4 µg / mL) for isolation of Propionibacteria in cheese [9]. Thierry and Madec (1995) utilized Pal Propiobac medium contained (pancreatic peptone, casein and Yeast extract) to enumerate the Propionibacteria present in milk [10]. Freitas *et al.* (2013) used Lithium Glycerol broth (LG) (10 g lythium lactate, 10 g peptone, 10 g yeast extract, 6 g glycerol, 1 g powdered milk, 50 mg bromocresol purple, 328 mg KH_2PO_4 and 56 mg $MnSO_4$) for selective enumeration of Propionibacteria in Emmental type of cheese [11]. Sodium lactate agar (SLA) is generally used medium for isolating propionic acid bacteria (PAB) containing yeast extract, tryptone, sodium lactate and phosphate [12-15].

The species of classical Propionibacterium, *Propionibacterium freudenreichii* is the only organism present in foods given the status of Generally Recognised as Safe (GRAS), Qualified Presumption for Safety (QPS) [16,17] and able to synthesize stable quantities of Vitamin B_{12}. The same species is also found to possess anti-virulence property against common food contaminant *Salmonella* spp. [18]. A few isolations of propionibacteria were cited in literature from sources of milk [10], olive mill

wastewater [19], Swiss cheese [20] *etc.* Isolation of dairy propionibacteria is difficult due to their anaerobic to facultative anaerobic nature and on the non-selective medium as their population is low in different sources especially, milk [3]. Further, the slow growing nature of propionibacteria, make them difficult to isolate on surface of medium as they are overgrown by other cultures. Hence, native isolates of propionibacterium may be of great significance for their usage either as a probiotic culture or as an industrial strain for the production of commercial Vitamin B_{12}. Hence, an attempt was made to isolate dairy propionibacteria from the locally available food and feed sources.

2. MATERIALS AND METHODS

2.1 Isolation of Test Culture

Various sources were used for isolation of dairy propionibacterium, like milk, curds, pickles, cheese and silage procured from the local markets of Bangalore, India. Standard plate count technique was adopted for isolation of propionibacteria. Different media were tried to get the propionibacterium isolate, *viz.*: Yeast Extract Lactone Agar (YELA) (Sodium lactate 20.0 g L^{-1} and yeast extract 5.0 g L^{-1}) [15], using pour plate method colony forming units in each source were analysed by counting the number of colonies in the YELA medium. As propionibacteria are anaerobic to facultatively anaerobic, to create favourable conditions for isolation, Petriplates were wrapped with Seran wrapper and incubated in the anaerobic gas jar containing gas-pack (HiMedia, Le002A), that helps in scavenging the free oxygen present in the anaerobic jar. Pigmented colonies of size larger than 1 mm diameter on YELA were tentatively considered as propionibacteria and were further used for isolation. Spread plate method was followed to grow and observe the colony morphology on YELA medium. Plates were incubated for five days at 30°C as optimum temperature for growth has been reported for growth of propionibacterium has been reported at 30°C.

2.2 Identification of Test Cultures

Tentative propionibacterial isolates were subjected to the following bio-chemical tests: Gram's staining, endospore staining, catalase activity, fermentation of sucrose, nitrate, maltose, lactose and gas production.

Bacterial genomic DNA isolation, 16s r RNA amplification and sequencing were performed to confirm the isolated culture as *Propionibacterium freudenreichii* sub sp. *shermanii.* Nucleotide sequence data was subjected to the Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnology Information (NCBI), the query cover match was found to be 99% and percent identity was found to be 96.47% confirming the isolate as *Propionibacterium freudenreichii*. Sequencing was performed by Barcode Bio-sequences, Bangalore, India.

2.3 Antibiotic Sensitivity of *Propionibacterium freudenreichii*

The antibiotic sensitivity of *Propionibacterium freudenreichii* was studied using seven antibiotics namely ampicillin (Class; penicillins; evaluated concentration: 10µg/ disc), azithromycin (Class: Macrolides; EC: 15 µg/ disc), chloramphenicol (EC: 30 µg/ disc), ciprofloaxin (Class: fluoroquinolones; EC: 5 µg/ disc), gentamycin (Class: aminoglycoside; EC: 10 µg/ disc), kanamycin (Class: Aminoglycoside; EC: 30 µg/ disc) and streptomycin (Class: aminoglycoside; EC: 10 µg/ disc). *Propionibacterium freudenreichii* culture was inoculated to YEL broth and incubated for five days at 30°C. Population of YEL broth was determined by standard plate count method and was found to be 5×10^5 cfu/ mL. YEL agar medium was seeded with YEL broth medium at the rate of 2.5 mL / 100 mL (Population of YEL). Plates were allowed to solidify and three antibiotic discs / plate were placed on the seeded agar medium using sterilized forceps. Plates were incubated for 7 days at 30°C and diameter of zone of inhibition was noted. The area of zone of inhibition was calculated using the formula $A= \Pi (R + r) (R - r)$.

2.4 Hemolytic Activity of *Propionibacterium freudenreichii*

Hemolytic activity of *Propionibacterium freudenreichii* was tested using agar well diffusion method [21] on Sheep blood agar medium. It was prepared with 5% sheep blood in Tripticase Soy Agar (TSA). Sheep blood was procured aseptically from a healthy sheep of Dairy unit, Hebbal Campus, Karnataka Veterinary Animal and Fisheries Sciences University, Bengaluru. In each blood agar plate, two wells were made such that it accommodates 100 µl of broth. Each organism was inoculated in triplicate number of plates, each plate with two wells. To

the control plate sterilized distilled water was added into the wells. Observation was taken at three days of incubation at 30°C.

2.5 Antibacterial Efficacy of Propionibacterium against Pathogenic *E. coli* and *S. aureus*

Antibacterial efficacy of *Propionibacterium freudenreichii* isolate was tested against pathogenic *Eshchericha coli* (NCIM 2065) and *Staphylococcus aureus* (NCIM 2079) using Nutrient agar medium. Pathogenic *E. coli* or *S. aureus* were streaked singly on nutrient agar medium perpendicular to *P. freudenreichii*. The *Propionibacterium freudenreichii* isolate was streaked (Approx. 6 cm) horizontally in the centre of agar plate and incubated to grow for 5 days, later pathogenic organisms were streaked vertically (Approx. 4 cm) exactly at the centre of the previous streak by leaving gap distance of 1 cm. Plates were incubated at 30°C for 2 days and observed for growth of pathogens.

2.6 Influence of Bile Salts and pH on Growth of *Propionibacterium freudenreichii*

The *P. freudenreichii* was tested for growth over a range of pH and bile salt concentrations in broth culture. The YEL broths of pH 2.0, 4.0, 6.0, 8.0 and bile salt concentrations *viz*., 0.25, 0.50, 1.0, 2.0 g L^{-1} were used. An initial population of 1 × 10^5 cells ml^{-1} was inoculated to each tube. Optical density observations and simultaneous plating of broth for populations count were taken during 12, 24, 48, 72, 96, 120 hours after initial inoculation.

3. RESULTS AND DISCUSSION

The varied characteristics for identification include, pleomorphic morphology of rods to cocci, range of pigmentation from brownish red, orange to yellow and creamish white colour. The other characteristics that helped in identification of dairy Propionibacteria were: their powdery texture of colony morphology, Gram positive organisms, pigmented. Bergey [22] used pigment production as a key to identification of species, even though that seemed impractical for Werkman et al. [23], specifically for species differentiation, characteristic of pigment production can be used for genus identification during their isolation from sources. Only Gram positive, pigmented and powdery colonies were used for screening as dairy Propionibacteria. Morphology was found to be very short rods, stretched cocci, 0.5-0.6 µ, non-motile and Gram positive that matched with the observations of Werkman and Brown [24].

High population of yeasts was also observed in cheese during the isolation of Propionibacteria. The typical characteristic of colonies obtained from milk is their pinpoint appearance such over numbering colonies were excluded from isolation and enumeration.

A total number of 45 cultures were isolated from all the five food and feed sources. Based on Gram staining (positive), pigmentation and microscopic observation of bacilli to coccobacilli, a total of 10 isolates were selected for further biochemical tests. The isolates performing positive for sucrose and maltose fermentation, nitrate reduction, catalase activity, gas production and negative for lactose fermentation, endospore formation were selected for further molecular identification. Similar tests (sucrose fermentation, maltose fermentation and nitrate reduction) were used by Cummins and Johnson [1], for the differentiation of propionic acid bacteria from lactic acid bacteria. Through the bio-chemical tests, isolates M15, M17, and C22 were selected for 16s rRNA isolation and sequencing, based on morphological characteristics that match dairy propionibacteria. C22 was found to be *Propionibacterium freudenreichii*, M15 was found to be *Bacillus* sp. and M17 was found to be *Lactobacillus* sp.

The characteristic G+C per cent of dairy propionibacteria is 53-68% and these Propionibacteria usually do not possess plasmids, rarely reported in *P. acidipropionici*, *P. freudenreichii* and *P. jensenii* [25]. The isolated strain's G+C content of 16s rRNA was found to 58%, confirming the high G+C per cent of the Propionibacteria. The 16s rRNA sequence of confirmed isolate was submitted to NCBI database and received the accession number (MT742831).

Surface colonies of *Propionibacterium freudenreichii* on YELA were 0.2 to 2.0 mm, convex, circular, entire, opaque, creamy white initially to bright yellow upon incubation for 5 days (Fig. 1). The isolated strain of *P. freudenreichii* was found to be non-haemolytic, as suggested by Vedamuthu et al. [26]. Two strains of *P. freudenreichii* differ by their ability to utilize nitrate and ferment lactose.

Propionibacterium freudenreichii subsp. *freudenreichii* can utilize nitrate and cannot ferment lactose whereas, *Propionibacterium freudenreichii* subsp. *shermanii* does the *vice versa*, it was confirmed by performing both tests. YEL broth cultures were slightly turbid, sedimented cells with yellow to creamish white colour was observed at the bottom at pH 7.0 ± 0.2. At times, a thread-like film of growth was observed in the broth that was maintained on rotary shaker conditions at 30°C for 5 days. The isolate *Propionibacterium freudenreichii* is a slow grower on solid medium but it grows at a faster rate in broth.

Haemolysis is a characteristic possessed by many Gram-positive cocci; these haemolytic bacteria produce exotoxins that lyse red blood cells (RBC's) and haemoglobin. The haemolysis is of three types α-haemolysis, β-haemolysis, and γ-haemolysis, α-haemolysis is partial haemoysis, here a greenish discolouration around colonies is observed, β-haemolysis is complete haemolysis here RBCs and haemoglobin are lysed creating hollow zone around colonies whereas γ-haemolysis is non-haemolysis only colony growth is observed no haemolysis takes place [27]. Among the two isolates (*Propionibacterium freudenreichii* and *Bacillus hyanessi*) that were tested for haemolysis, Bacillus being a fast grower, occupied full growth in plate by three days of incubation, no haemolysis was observed. *Propionibacterium freudenreichii* had growth and there was no haemolysis.

Propionibacterium freudenreichii being an actinomycete is a slow grower, in the present work to assess its antimicrobial activity against two pathogenic strains *Escherichia coli* and *Staphylococcus aureus*, that are fast growers, a modified cross streak method was used [28]. A space of 1 cm was left between horizontally streaked *Propionibacterium freudenreichii* isolate (6 cm length) and vertically streaked *E. coli* (3 cm length) and *S. aureus* (3 cm length) (on separate plates), in order to observe if these pathogens were able to grow beyond the inoculation point or the test organism was able to release substances into medium that inhibits pathogen growth beyond the point of inoculation. It was found that *Propionibacterium freudenreichii* was able to control growth of pathogenic strains beyond the points of inoculations and the pathogenic strains growth near to *P. freudenreichii* was also narrowed down (Plate. 3). The *P. freudenreichii* was found to possess better antagonistic activity against *S. aureus*, when compared to *E. coli*. Further, the pathogens were able to grow beyond the point of inoculation at the edges of Petri plate where the *Propionibacterium freudenreichii* influence would be less. The results are in accordance with data stating that *P. freudenreichii* possess higher biocontrol towards pathogenic *S. arureus* than *E. coli* [29]. Propionicin is a major bacteriocin known to be produced by majority of propionibacteria [30-32] that is responsible for inhibition of growth of various Gram-positive bacteria, Gram-negative bacteria and some molds.

A successful probiotic strain should sustain viability and adequate cell numbers through adverse conditions like, that are present in the gastro-intestinal tract, major factors are pH and tolerance to bile salts [33]. *Propionibacterium freudenreichii* was advocated to sustain through variable pH of 5.1 to 8.5 [1,2]. The current isolate *Propionibacterium freudenreichii* was able to sustain cell population till pH of 2, even though multiplication rate at this pH was very low (Table 1). The isolate exhibited the highest growth at pH of 8 the organism was able to equally multiply. The propionibacteria were known to tolerate high concentration of salts, this may be one of the reasons for high multiplication rate of *Propionibacterium freudenreichii* isolate at pH of 8 rather compared to the acidic pH range of 4 and below. With reference to bile salt tolerance the isolate was tested from a concentration range of 0.25 g L^{-1} to 2.0 g L^{-1}, the isolate was able to tolerate and sustain population till 2.0 g L^{-1} of bile salts, though cell number at 2.0 g L^{-1} bile salt concentration was not comparably equal with the cell number at 0.25 g L^{-1} bile salt concentration (Table 1). Leverrier *et al.,* 2003 [34], advocated that a concentration of 1.0 g L^{-1} is lethal for propionibacteria, but could sustain same concentration after an acclimatization exposure to 0.25 g L^{-1}. A decreasing trend in cell multiplication rate was observed over the bile salt concentration test range. A well-acclimatized and higher multiplication rate was observed with the bile salt concentration of 1.0 g L^{-1} compared to 2.0 concentration. The cellular mechanism in adapting to varied stresses and adverse conditions was shown to be associated with multi-copy of stress-induced genes [6]. Antibiiotic susceptibility testing was done Baur and Kirby method [35]. The *Propionibacterium freudenreichii* isolate was found to be sensitive for all the seven antibiotics tested (Table. 2 & Fig. 2).

Table 1. Influence of pH and bile salts on growth of *Propionibacterium freudenreichii* at different time intervals

Treatments		*Propionibacterium freudenreichii* population (log cfu /mL) at 24 h intervals					
		12h	24h	48h	72h	96h	120h
pH	2.0	4.52	4.84	5.1	5.25	5.33	5.34
	3.0	4.89	5.29	5.61	5.74	5.76	5.77
	4.0	5.16	5.51	5.83	5.91	5.93	5.93
	6.0	5.78	6.14	6.22	6.32	6.39	6.39
	7.0	5.83	6.14	6.39	6.44	6.47	6.49
	8.0	5.84	6.19	6.3	6.39	6.44	6.44
	9.0	5.75	6.05	6.23	6.15	6.16	6.16
Bile salt concentration (g /L)	0.25	4.87	5.28	5.51	5.66	5.69	5.70
	0.5	4.82	5.24	5.51	5.54	5.57	5.58
	1.00	4.15	4.51	5.32	5.4	5.21	5.01
	2.00	4.08	4.60	4.96	5.21	5.25	5.26

Table 2. Antibiotic sensitivity of *P. freudenreichii* against different broad-spectrum antibiotics

S. No	Antibiotics	Antibiotic concentration (µg/disc)	Diameter of zone of growth inhibition (mm)	Assessment of susceptibility
1	Ampicillin	10	34^{d}	Sensitive
2	Azithromycin	15	52^{a}	Sensitive
3	Chloramphenicol	30	45^{b}	Sensitive
4	Ciprofloxacin	05	32^{ef}	Sensitive
5	Gentamycin	10	32^{e}	Sensitive
6	Kanamycin	30	30^{f}	Sensitive
7	Streptomycin	10	40^{c}	Sensitive

Values are mean and values followed by the same letter in each column are significantly different from each other as determined by DMRT (p>0.05)

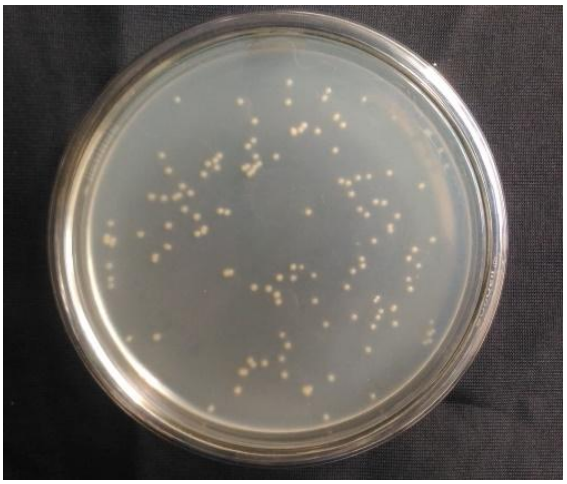

Fig. 1. *Propionibacterium freudenreichii* colonies isolated on yeast extract lactone agar

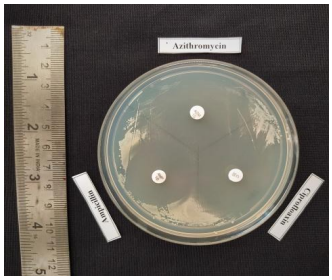

Plate (a). Top disc- Azithromycin
Right disc- Ampicillin
Left disc- Chloramphenicol

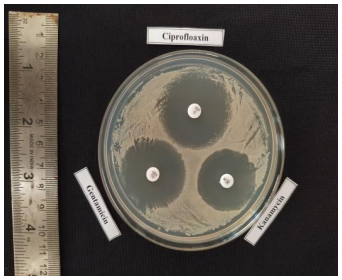

Plate (b). Top disc- Ciprofloxacin
Right disc- Kanamycin
Left disc- Gentamycin

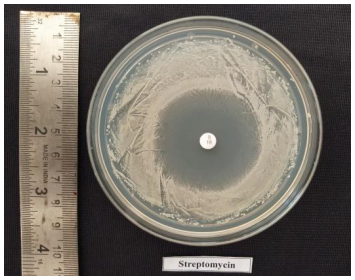

Plate (C). Disc- Streptomycin

Fig. 2. Antibiotic susceptibility of *P. freudenreichii*

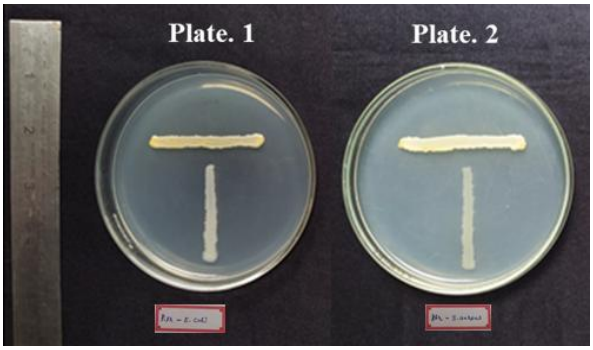

Fig. 3. Antibacterial activity of *Propionibacterium freudenreichii* against Pathogenic *E. coli* and *S. aureus*; Plate 1. Horizontal streak: *P. freudenreichii*, Vertical streak: *E. coli* ; Plate. 2: Vertical streak: *S. aureus*

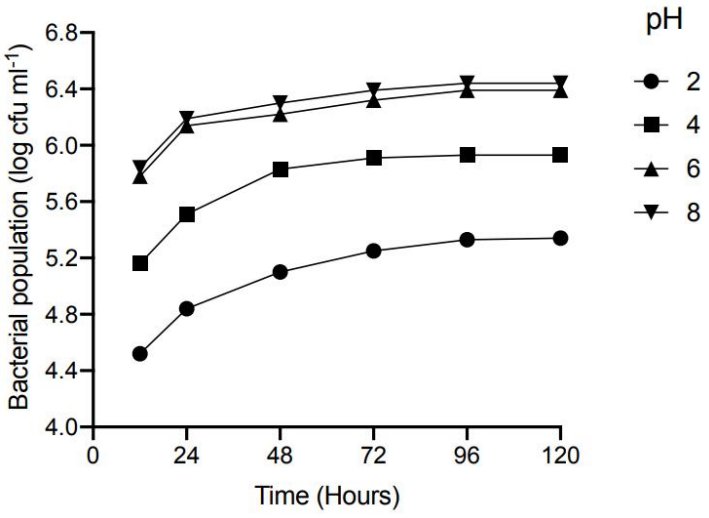

Fig. 4. Influence of pH levels on growth of *P. freudenreichii* at different time intervals

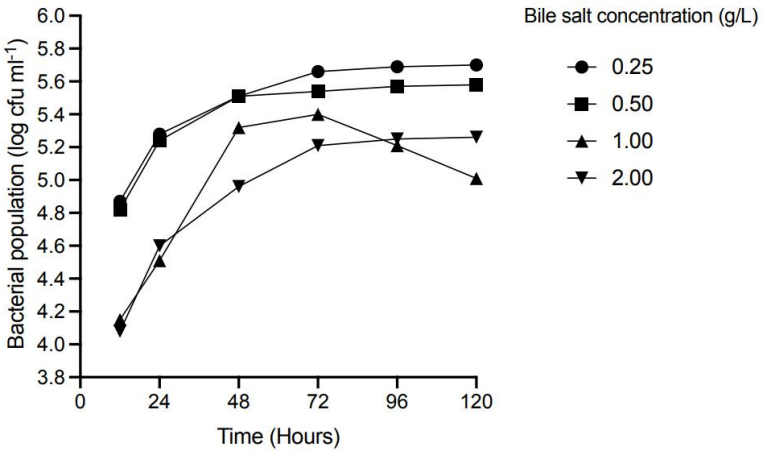

Fig. 5. Influence of bile salts (0.25-2.00 g/L) on growth of *P. freudenreichii* at different time intervals

4. CONCLUSION

The isolated *Propionibacterium freudenreichii* sub. sp. *freudenreichii* culture from an Indian origin cheese is a local strain. This isolation of dairy propionibacterium from Indian originated cheese sources, has been rarely reported from India. The isolate has been proven to possess good number of probiotic potentials in terms of pH and bile salt tolerance, antimicrobial and non-haemolytic activities but, found to be sensitive to frequently used antibiotics in the field of medicine. Further, this may lead to exploration of sources for dairy propionibacteria and would help in elucidation of their unclear ecology.

5. FUTURE STUDIES

The isolate, *Propionibacterium freudenreichii*, will be further used in future for development of Cobalamin rich nutraceutical product and the will be induced to mutations and such mutants will be screened for enhanced production of Cyanocobalamin.

6. FUNDING

Part of the work was financially supported by the Science and Engineering Research Board (New Delhi, India) in the form of National Post-Doctoral Fellowship (PDF/2017/001908) to RUA.

ACKNOWLEDGEMENTS

Authors are grateful to the Department of Plant Biotechnology and Biochemistry, GKVK, University of Agricultural Sciences, Bengaluru (India) for technical support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Cummins CS, Johnson JL. *Propionibacterium*, In: Holt JG. Ed., Bergey's manual of systematic bacteriology. Baltimore, MD, USA: The Williams & Wilkins Co. 1986;1346- 1353.
2. Stackebrandt ERKO, Cummins CS, Johnson JL. Family propionibacteriaceae: The genus Propionibacterium. The Prokaryotes. 2006;3:400-418.
3. Gautier M. Propionibacteria, Encyclopedia of food microbiology. Elsevier. 2014;3: 232-237.
4. Thierry A, Deutsch SM, Falentin H, Dalmasso M, Cousin FJ, Jan G. New insights into physiology and metabolism of *Propionibacterium freudenreichii*. Int. J. Food Microbiol. 2011;149:19-27.
5. Cousin FJ, Foligné B, Deutsch SM, Massart S, Parayre S, Le Loir Y, Boudry G, Jan G. Assessment of the probiotic potential of a dairy product fermented by Propionibacterium freudenreichii in Piglets. J. Agric. Food Chem. 2012;60(32): 7917-7927.
6. Falentin H, Deutsch SM, Jan G, Loux V, Thierry A, Parayre S, Maillard MB,

Dherbécourt J, Cousin FJ, Jardin J, Siguier P, Couloux A, Barbe V, Vacherie B, Wincker P, Gibrat JF, Gaillardin C, Lortal S. The complete genome of Propionibacterium freudenreichii CIRM-BIA1T, a hardy Actinobacterium with food and probiotic applications. PLoS One. 2010;5(7):11748.

7. FAO/ WHO Joint codex alimentarius commission and joint FAO /who food standards programme, *Codex alimentarius*. Bernan. Assoc. 2001;9.
8. Zarate G. Dairy propionibacteria: Less conventional probiotics to improve the human and animal health. Probiotic in Animals, Ed. Everlon Cid Rigabelo, Intech Open. 2012;153-202.
DOI: 10.5772/50320.
9. Drinan FD, Cogan TM. Detection of propionic acid bacteria in cheese. J. Dairy Res. 1992;59(1):65-69.
10. Thierry A, Madec MN. Enumeration of propionibacteria in raw milk using a new selective medium. Le Lait. 1995;75(4-5):315-323.
11. Freitas RD, Luiz LMP, Alves MP, Valence-Bertel F, Nero LA, Carvalho AFD. Selective enumeration of propionibacteria in Emmental-type cheese using PetrifilmTM Aerobic Count plates added to Lithium Glycerol broth; 2013.
12. Vedamuthu ER, Reinbold GW. Appl. Microbiol. 1975;29:807.
13. Hettinga DH, Vedamuthu ER, Reinbold GW. Pouch method for isolating and enumerating propionibacteria. J. Dairy Sci. 1968;51:1707-1709.
14. Peberdy MF, Fryer TF. Improved selective media for the enumeration of Propionibacteria from cheese. N. Z. J. Dairy Sci. Technol. 1976;11:10-15.
15. VAN NIEL, CB. The propionic acid bacteria JW. Bossevain and Co., Haarlem; 1928.
16. EFSA, EFSA panel on biological hazards (BIOHAZ) scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed: QPS update. EFSA J. 2013;11:3449.
17. FDA , Agency Response Letter GRAS Notice No. GRN000415; 2014.
Available:https://wayback.archive-it.org/7993/20171031010359/https://www.fda.gov/
Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm335742.html
18. Nair DVT, Kollanoor-Johny A. Effect of *Propionibacterium freudenreichii* on Salmonella multiplication, motility, and association with avian epithelial cells. Poult. Sci. 2017;96:1376-1386.
19. Koussémon M, Combet-Blanc Y, Patel BK, Cayol JL, Thomas P, Garcia JL, Ollivier B. A microaerophilic bacterium isolated from olive mill wastewater. Int. J. Syst. Evol. Microbiol. 2001;51(4):1373-1382.
20. Fessler D, Casey MG, Puhan Z. Identification of propionibacteria isolated from brown spots of Swiss hard and semi-hard cheeses. Le Lait. 1999;79(2):211-216.
21. Fernández-Garayzábal JF, Delgado C, Blanco M, Vázquez-Boland JA, Briones V, Suárez G, Dominguez L. Role of potassium tellurite and brain heart infusion in expression of the hemolytic phenotype of Listeria spp. on agar plates. Appl. Environ. Microbiol. 1992;58(1):434-438.
22. Bergey HD. Manual of determinative bacteriology, 3rd Ed. Williams and Wilkins, Baltimore, Md; 1930.
23. Werkman CH, Kendall, Sara E. Iowa State College. J. Sci. 1931;6:17-32.
24. Werkman CH, Brown RW. The propionic acid bacteria: II. Classification 1 J. Bacteriol. 1933;26(4):393.
25. Rehberger TG, Glatz BA. Characterization of Propionibacterium plasmids. Appl. Environ. Microbiol. 1990;56(4)864-871.
26. Vedamuthu ER, Washam CJ, Reinbold GW. Isolation of inhibitory factor in raw milk whey active against propionibacteria. Appl. Microbiol. 1971;22(4):552-556.
27. Leboffe MJ, Pierce BE. Microbiology: laboratory theory and application, Morton Publishing Company; 2015.
28. Velho-Pereira S, Kamat NM. Antimicrobial screening of actinobacteria using a modified cross-streak method. Indian J. Pharm. Sci. 2011;73(2):223.
29. Begunova AV, Rozhkova IV, Zvereva EA, Glazunova OA, Fedorova TV. Lactic acid and propionic acid: The formation of a community for the production of functional products with bifidogenic and hypotensitive properties. Appl. Biochem. Microbiol. 2019;55(6):660-669.
30. Brede DA, Faye T, Johnsborg O, Ødegård I, Nes IF, Holo H. Molecular and genetic characterization of propionicin F, a bacteriocin from Propionibacterium freudenreichii. Appl. Environ. Microbiol. 2004;70(12):7303-7310.
31. Gwiazdowska D, Trojanowska K. Antimicrobial activity and stability of partially purified bacteriocins produced by

Propionibacterium freudenreichii ssp. freudenreichii and ssp. shermanii. Le Lait. 2006;86:141-154.
32. Ratnam P, Barefoot SF, Prince IU, Bodine AB, McCaskill LH. Partial purification of the bacteriocin produced by Propionibacterium jensenii B1264. Lait. 1999;79:125-136.
33. Harimurti S, Hadisaputro W. Probiotics in poultry. in Beneficial Microorganisms in Agriculture, Aquaculture and Other Areas, ed. M.T. Liong, Basel: Springer International Publishing. 2015;1–19.
34. Leverrier P, Dimova D, Pichereau V, Auffray Y, Boyaval P, Jan GL. Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii:* Physiological and proteomic analysis. Appl. Environ. Microbiol. 2003(69):3809-3818.
35. Bauer AW, Kirby WMM, Sherris JC, Turck M., Antibiotic susceptibility testing by standardized single disc test methods. Am. J. Clin. Pathol. 1966;36:493-496.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/64166