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Isolation and characterization of a lactic acid bacterium from infant Feces

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Abstract
The objective of this study was to isolate and evaluate the characteristics of a lactic acid bacterium from solely breast milk fed infant feces. The isolate obtained was Gram positive facultative anaerobic catalase negative, oxidase negative motile exopolysaccharide producing coccobacilli. The isolate had optimum growth at 37 ^{0}C and pH 8 and also shown moderate growth at pH 5 and 8% NaCl concentration. The isolate was a homolactic fermenter of lactose. Genotypic identification by 16SrRNA partial sequencing confirmed it as *Weissella confusa*. Inability to cause hemolysis and liquefy gelatin suggests the possible absence of virulent factors. Antibiogram study revealed the isolate ability to resistant Amoxycillin, Ampicillin, Bacitracin, Chloramphenicol, Ciprofloxacin, Co-Trimoxazole and Vancomycin with a multiple antibiotic resistance index of 0.7. The isolate exhibited an autoaggregation of 75% which is an indication of excellent aggregation potential and cell surface hydrophobicity of 45%.

Keywords: *Weissella confusa*, infant fecal resistance matter, multiple antibiotic Index, auto aggregation, exopolysaccharide

1. Introduction
Lactic Acid Bacteria (LAB) are extensively used in food industry. LAB are used as dairy starters and as well have diverse applications in biotechnology, drug delivery and production of food ingredients. They are catalase negative, oxidase negative facultative anaerobic Gram positive rods or cocci. They are reported to be present in milk, fruits, flowers, vegetables, soil, fecal matter, sewage water etc. (Lamont *et al.* 2017) [19]. Human breast milk recognized as the gold standard of infant feeding is also a contributor of lactic acid bacteria to infant gut (Kim *et al.* 2019) [11]. The integral role of breast milk in the development of infants are because of its nutrient composition and diverse microbiome that is unique for each mother (Martin *et al.* 2012) [22]. This varied microflora is an initial factor for the development of microorganisms in infant gut. There exist certain controversies on source of different species in human milk. Martin *et al.* 2004 [21] explains it as the endogenous transfer from maternal gut to mammary gland. There are also different factors that influence infant gut microflora such as feeding practices, exposure to drugs and mode of birth (Kim *et al.* 2019) [15].The microbiome of infant gut also plays an important role in immunity and infant metabolism (Bharadia *et al.* 2020) [5]. The present study was taken up to isolate, identify and characterize lactic acid bacteria from solely breast milk fed infant feces.

2. Materials and Methods
2.1 Isolation of the isolate
Infant fecal sample was collected in sterile sample containers under aseptic conditions. 10^{-1} dilutions of the sample was pre-enriched in nutrient media (HiMedia Laboratories Pvt. Ltd., Mumbai) followed by selective enrichment in DeMan Rogosa Sharpe -MRS (HiMedia Laboratories Pvt. Ltd., Mumbai) by incubating them at 37 ^{0}C for 24h in-between each steps. After the incubation, the turbid broth tubes were streaked on MRS agar. and incubated at 37 ^{0}C for 48h. Typical spindle shaped colonies were randomly selected and further streaked on MRS agar for purification. The colonies developed were further characterized. For long term preservation 70% glycerol stocks were made and stored in the deep freezer at -15 ^{0}C.

2.2 Phenotypic identification of the isolate
The colony morphology of the isolates developed on MRS agar plates was evaluated by

examining characteristics like shape, type of the colony, colony colour, margin, elevation, opacity and pigment production (Kumar and Kumar, 2014) [16]. Biochemical characterization was done using standard procedures (Guo *et al.* 2020) [13] Carbohydrates fermentation reactions were analyzed using API CHL 50 galleries (BioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Freshly activated culture with turbidity 2 MacFarland was added into wells containing 49 sugars and incubated at 37 °C for 48 h. The colour change was analyzed with help of manual provided with API CHL 50 galleries. Along with biochemical characters, the physiological characteristics like the effect of different pH (2, 5, 7, 8, 10), temperature (15 ^{0}C, 37 ^{0}C, 45 ^{0}C), NaCl percentage (2%, 4%, 6%, 8%, 10%) and presence of oxygen on the growth of isolate was also evaluated (Bohn *et al.* 2017) [6].

2.3 Genotypic identification of the isolate

Molecular identification of the isolate was done using 16S ribosomal RNA (16SrRNA) sequencing by outsourcing the samples to Rajiv Gandhi Centre for Biotechnology, Trivandrum. The 16SrRNA gene sequences obtained was searched with the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) for their closest relatives/reference strains with a homology of over or equal to 99%.

2.4 Ggregation Potential

2.4.1 Autoaggregation analysis

Autoaggregation assay was performed as per Collado *et al.* 2008 [8]. Bacteria were grown in MRS broth for 18 hours at 37ºC. After centrifugation at 5000x g for 15 minutes, cells were washed twice and suspended in phosphate buffered saline (pH 7.0) to give viable counts of 10^8 CFU/ml. Four ml of the cell suspension were mixed by vortexing for 10 s and autoaggregation was determined after 5 hour after incubation at room temperature. At hourly intervals, 100 µL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance was measured at 600nm. Autoaggregation was calculated according to the equation:

$\%\ \text{Aggregation} = (1 - (A_t / A_0) \times 100$

Where A_t represents the absorbance at time t = 5 hour and A_0 is the absorbance at t= 0.

2.4.2. Bacterial adhesion to hydrocarbons (BATH assay)

The bacterial adhesion to hydrocarbons test was performed according to the method of Rosenberg *et al* (1980) [25]. 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 0.25 ± 0.05 to standardize the number of bacteria (10^8 cells/mL) at 600 nm. Then, equal proportions of viable bacterial suspension and solvent (xylene) were mixed by vortexing for 5 minutes. The aqueous phase was removed after 1 hour of incubation at room temperature and its absorbance was measured. Results were reported according to the formula

BATH $\%=[(A_0–A)/A_0] \times 100$, where '$A_0$' and 'A' are absorbance before and after mixing with xylene, respectively.

2.5 Antibiogram of the isolate

Antibiogram of the isolate was evaluated by the Disc diffusion method following modified Kirby–Bauer procedure (Bauer *et al.* 1966) [4]. The antibiotics tested were Amoxycillin (30 µg), Ampicillin (10 µg), Vancomycin (30µg), Cephalothin (30µg), Ciprofloxacin (30 µg), Chloramphenicol (30 µg), Tetracycline (30µg), Erythromycin (10µg) and Bacitracin (10 U). The zone of inhibition was measured using an antibiotic zone scale and expressed in millimeter. The isolates were categorized based on zone of inhibition (Vlkova *et al.* 2006) [29].

2.6 Safety assessment of the isolate

Safety evaluation was done by looking at its ability to cause lysis of blood cells (Adetoye *et al.* 2018) [1] and liquefy gelatin (Sahu *et al.* 2019) [26].

2.6.1 Hemolytic prorty

The isolate was streaked on blood agar plate. Plate was examined for the presence of zones around the growth after incubation at 37ºC for 24 h. The isolate that produced green-hued zones around the colonies (alpha-hemolysis) or those that did not produce any zone on the blood agar (Gamma-hemolysis) were considered as non-hemolytic. Those producing zones of clearance (Beta- hemolysis) were classified as hemolytic (Adetoye *et al.* 2018) [1].

2.6.2 Gelatin liquefaction

The isolate was streaked on Gelatin agar (Hi-Media) slant and incubated at 37 ºC for 24 h. Uninoculated tube served as control. After incubation, both tubes were kept for 3h under refrigeration before reading the result to affirm that liquefaction is due to microbial action and not due to the incubation temperature employed. Partial or total liquefaction of the inoculated tube when compared to the control tube was taken as positive for gelatin liquefaction (Sahu *et al.* 2019) [26].

2.5 Exopolysaccharide (EPS) production by the isolate

EPS production potential was evaluated based on the 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 to Brain Heart Infusion agar containing five percent sucrose. The isolates were streaked on Congo red agar and incubated at 37 ^{0}C. Formation of slimy and shining black colonies within 24h of incubation was considered suggestive of EPS production.

3. Results and Discussions

3.1 Isolation of the isolate

Lactic acid bacteria are isolated from various sources such as milk, fruits, flowers, vegetables, soil, fecal matter, sewage water etc. (Lamont *et al.* 2017). There are reports on isolation of lactic acid bacteria from infant fecal sample (Xu *et al.* 2020) [31]. Infant fecal matter represents infant gut microflora and they derive from breast milk in condition that infant was fed solely breast milk (Martin *et al.* 2012) [22]. LAB found in gut microbiome of 5 days old infant consist 45% *Enterococcus faecalis*, 14% *E. faecium*, 11% *E. hirae*, 11% *Lactobacillus paracasei*, and 2% *L. gasseri* (Chotelersak *et al.* 2016) [7]. *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Enterococcus avium*, *Enterococcus faecium* and *Enterococcus lactis* species isolated from infant feces had shown wide range of probiotic potential and antimicrobial activity (Wang *et al.* 2020) [30]. *Weissella confusa* isolated by Nam *et al.* 2002 from infant feces found to inhibit *Helicobacter pyroli.* In this work, we intended to isolate a LAB from feces of breast milk solely

fed infant and genotypically identified it by 16SrRNA sequencing as *Weissella confusa.*

3.2 Phenotypic identification of the isolate

The isolate was Gram positive non-hemolytic, non- gelatin liquefying facultatively anaerobic catalase negative, oxidase negative motile EPS producing coccobacilli. The organism was positive for indole and methyl red test but negative to VP test and citrate utilization. The organism was able to grow at 45 ^{0}C but not in 15^{0}C with optimum growth at 37 ^{0}C. The isolate had shown optimum growth at pH 8 and a moderate growth at pH 5. But the growth was completely inhibited at pH 2 and pH 10. It has no ability to hydrolyse arginine when grown at specific media. The isolate had shown homolactic fermentation of lactose. The results of carbohydrate utilization is given in table 1 and we observed results similar to that of Mohammed *et al.* 2020 [23].

Table 1: (Sugar fermentation profile of the isolate)

Sl. No.	Sugar	Isolate 5
1	Arabinose	Negative
2	Cellobiose	Positive
3	Galactose	Positive
4	Glucose	Positive
5	Lactose	Positive
6	Mannitol	Positive
7	Mannose	Positive
8	Rhamnose	Positive
9	Salicin	Positive
10	Sorbitol	Positive
11	Sucrose	Positive
12	Trehalose	Positive
13	Xylose	Positive
14	Fructose	Positive

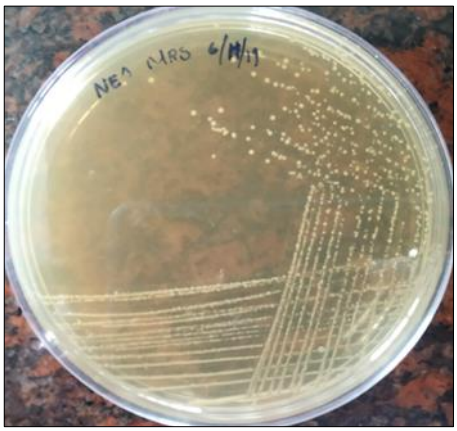

Fig 1: (MRS agar plate with the isolate)

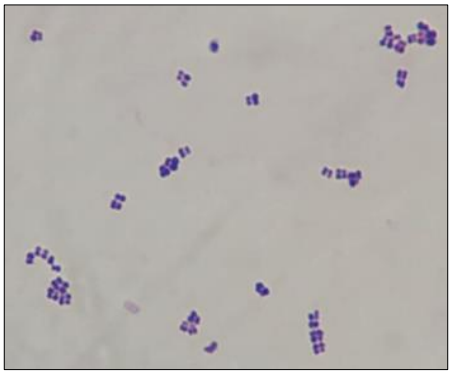

Fig 2: (Microcopic picture of Isolate)

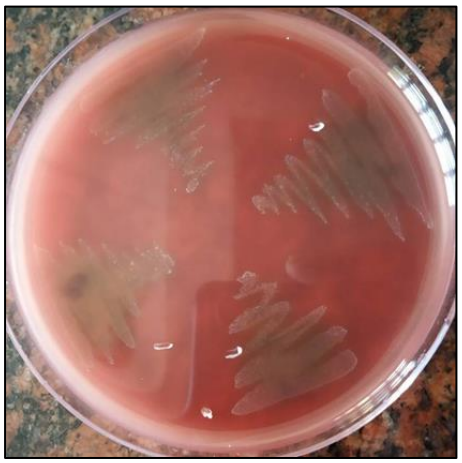

Fig 3a: Green hued zone in blood agar plate

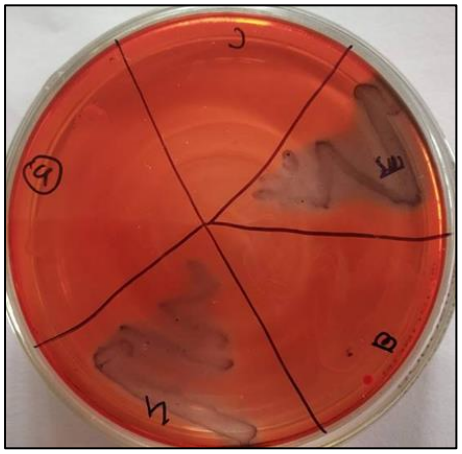

Fig 4: Black colored colony in Congo red agar

3.3 Genotypic identification of the isolate

Genotypic identification of the isolate by 16SrRNA partial sequencing confirmed it as *Weissella confusa.* The obtained nucleotide sequence was deposited in NCBI GenBank with accession numbers MT158671. Albesharat *et al.* 2011 [2] isolated *Weissella confusa* from fermented products, breast milk and infant fecal sample. The glycerol stock of the isolate was deposited in the culture collection Centre of Department of Dairy Microbiology, College of Dairy Science and Technology, Mannuthy, Thrissur, Kerala.

3.4 Aggregation Potential

Aggregation potential shows the potential of the oorganism to adhere to epithelial cells of gastrointestinal tract. The autoaggregation determines the ability of coaggregation and adhesion to hydrocarbon is evaluated by BATH assay. Higher value of autoaggregation and hydrophobicity indicates the ability to colonise inside human digestive tract (Sakandar *et al.* 2019[27]). *Weissella confusa* isolated from neonatal fecal matter exhibited an auto aggregation of 75% which is an indication of excellent adhesion potential. The cell surface hydrophobicity (CSH) was found out to be 45% for the isolate Lakra *et al.* 2020 [18] had reported a similar autoaggregation potential for *Weissella confusa* however CSH value with xylene was only 29.51%. This difference may be due to strain to strain variations in cell surface properties and experimental condition followed.

3.5 Antibiogram of the isolate

The isolate showed resistance to Amoxycillin (30µg),

Ampicillin (10µg), Bacitracin (10U), Chloramphenicol (30µg) Ciprofloxacin (30 µg), Co-Trimoxazole (25µg) and Vancomycin (30 µg) and sensitive to Cephalothin (30µg), Erythromycin (10µg) and Tetracycline (30µg). This is in agreement with the observation of Wang *et al.* 2020[30] for *Weissella confusa* from isolated human feces.*Weissella* is intrinsically resistant to vancomycin (Lee *et al.* 2011[20]; Quattrinni *et al.* 2019[24]). Sensitivity to tetracycline seen in our study is in line with Quattrinni *et al.* 2019[24]. The isolate exhibited a multiple antibiotic resistance (MAR) index of 0.7. MAR index greater than 0.2 is assessed by Davis *et al.* 2016[10] as an organism from a region of high antibiotic exposure and since multiple antibiotic resistant.

3.6 Safety assessment of the isolate

Weissella confusa from infant feces was a non-gelatin liquefier. It produced green-hued zones around the colonies when streaked on blood agar plates. The green-hued zones around the colonies indicate non-hemolytic nature of the organism. The organisms producing green-hued zones are considered as alpha-hemolytic while those do not produce any zone as non-hemolytic (Adetoye *et al.* 2018) [1]. LAB are generally non-hemolytic (Silva *et al.* 2019) [9] or alpha-haemolytic (Gunyakti and Ozusaglam, 2019) [12] indicating their the non-pathogenic nature (Somashekaraiah *et al.* 2019) [28].

3.7 Exopolysaccharide (EPS) production by the isolate

EPS production potential was evaluated based on Congo red assay. The Congo red is an EPS binding dye and those producing EPS is seen as distinctive black colored colonies (Arciola *et al.* 2002) [3]. EPS producing *Bifidobacterium* was isolated form infant stool by Kusharyati *et al.* 2020 [17]. EPS production by an isolate from infant is in agreement with the observations of Jin *et al.* 2019) [14], who had isolated polysaccharide producing organism *Weissella confusa* from young children's feces.

4. Conclusion

Isolate from solely breast milk fed infant feces had been identified using 16SrRNA sequencing as *Weissella confusa*. The isolate had shown moderate growth at pH 5 and 8% NaCl concentration. It had also exhibited homolactic fermentation of lactose and hence can be used in fermented milk production. The ability of this organism to produce EPS at pH 5 and temperature 37 ^{0}C can be explored for technological application. The absence of potent virulent factors shows amble scope for further studies in its probiotic potential. However well controlled biological studies and in depth molecular level work needs to be in place, before claiming the benefits.

5. Acknowledgement

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