Analysis of 16S rRNA Gene of Lactic Acid Bacteria Isolated from Curd and Raw Milk

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Abstract: *Rawmilk and curdsamples from various dairy outlets in the city were collected and putative lactic acid bacteria were isolated on MRS agar. These were first screened using preliminary criteria and then identified by analysing the 16S rRNA gene. For the molecular identification, extraction and quantification of bacterial DNA was first performed and the isolated DNA was amplified by PCR. This PCR product was then purified and sequenced using specific primers. The DNA sequences were then analysed using BLASTn and the results were used to generate a phylogenetic tree. From a total of four samples of curd and six samples of raw milk, the organisms identified were Lactobacillus fermentum in all the four samples of curd and one sample of raw milk. The rest of the five samples of raw milk showed two of the isolates to be Weisella confusa (basonym Lactobacillus confusus) and three to be Weisella cibaria.*

Keywords: Lactic acid bacteria, *Lactobacillus*, *Weisella*, 16s rRNA gene sequencing

1. Introduction

Many species of lactic acid bacteria particularly, lactobacilli and bifidobacteria as well as certain species of yeast are now been widely used as probiotics by incorporating into food or using as health supplements [1]-[6]. A few species of *Weissella* are also now being suggested as being probiotic in nature [7], [8]. It is of advantage therefore to identify convenient and apparent sources of these organisms. This has prompted many researchers to investigate or screenseveral natural sources of these healthy bacteria.

Following isolation, proper identification of the isolated organisms is also warranted. Since the 16S rRNA gene has hypervariable regions which are species specific, the most dependable and widely used techniques for bacterial identification are based on the 16S rRNA gene [9]-[11].Since these hypervariable regions are also often flanked by strongly conserved regions, primers are designed to bind to the conserved regions and the variable regions can then be amplified. The 16S rRNA gene sequence has been determined for a large number of bacterial species and therefore, comparison of the unknown isolate with available sequences in databases such as the National Centre for Biotechnology Information (NCBI) can aid in proper identification of the organism.

In this study, molecular phylogeny of bacteria was determined by analysing genomic 16S rRNA region. The authors would like to also point out that not only can curd and raw milk be an easy to obtain source of putative lactic acid bacteria, but that with a little preliminary screening, the probability of obtaining lactobacilli from curd is very high as confirmed later by the 16S rRNA gene analysis.

2. Materials and Methods

Curd and raw milk samples were collected from various dairy outlets in Mumbai. Organisms present in these samples were isolated by streaking the samples directly onto deMann Rogosa Sharpe (MRS, HiMedia) agar plates followed by incubation at 37°C for 24-48 hours. Well isolated,typical colonies that represented putative lactobacilli according to Bergey's manual of systematic bacteriology (2009) were then chosen to undergo preliminary screening [12]. These tests were for catalase activity using H_2O_2, motility using stab method (0.4% agarin MRS broth) and gelatin liquefaction test using 12% gelatin in MRS broth [13], [14].

DNA extraction and quantification

DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505). DNA was precipitated by adding 200μl of ethanol to the lysate and mixed by vortexing. Lysate was then loaded on HiElute Miniprep Spin column and DNA purified by washing the column two times followed by elution using elution buffer. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation).

PCR amplification

The extracted DNA was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5μl of 10X buffer, 1μl of each primer (Table 1), 2.5μl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1μl Template DNA and 8.5μl nuclease free water. The PCR amplification cycle consisted of a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 50°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The reagents used were procured from GeNei.

Table 1: Primers used for 16S rRNA region amplification

Primers	Primer Sequence (5'-3')
519F (Forward)	CAGCAGCCGCGGTAATAC
1385R (Reverse)	CGGTGTGTACAAGGCCC

Gel electrophoresis

Gel electrophoresis was performed using 1.0% agarose to analyze the size of amplified PCR product.

DNA sequencing

The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). It was further sequenced using Applied Biosystems 3730xl DNA Analyzer, USA and chromatogram was obtained. For sequencing of PCR product,sequencing primer 519F-5'CAGCAGCCGCGGTAATAC3'was used.

Bioinformatics analysis

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of NCBI. The BLAST results were used to find out evolutionary relationship of bacteria. Altogether twenty sequences, including sample were used to generate phylogenetic tree. The tree was constructed by using MEGA 5 software [15]-[17].

3. Results and Discussion

Organisms that were Gram positive rods, non-motile, catalase negative and unable to liquefy gelatin were considered for molecular identification.The size of the amplified PCR products obtained was approximately 850bp for the 16S rRNA region.Comparisons of 16S region sequences obtained from the isolates with those present in GenBank enabled the identification of the isolates (Table2). The evolutionary history was inferred using the Neighbour-Joining method. Evolutionary analyses were conducted in MEGA5.

Table 2: Phylogenetic neighbours of isolates on the basis of similarity to the partial 16S rRNA sequence

Isolate	Source	Query Cover (%)	E value	Identity (%)	Species identification based on 16S sequence	Accession
MC1	Curd	100	0.0	100	*Lactobacillus fermentum*	KU761833.1
MC2	Curd	100	0.0	100	*Lactobacillusfermentum*	KU761833.1
MC3	Curd	100	1e-151	99	*Lactobacillus fermentum*	KU720301.1
MC4	Curd	100	0.0	99	*Lactobacillusfermentum*	KU761833.1
MM1	Raw milk	100	0.0	100	*Weissellacibaria*	KF023247.1
MM2	Raw milk	100	0.0	100	*Lactobacillusfermentum*	KU761833.1
MM3	Raw milk	100	0.0	100	*Weissella confusa*	LC127180.1
MM4	Raw milk	100	0.0	100	*Weissella cibaria*	KU533887.1
MM5	Raw milk	100	0.0	100	*Weissella confusa*	KT722822.1
MM6	Raw milk	100	0.0	99	*Weissella cibaria*	KT757266.1

The lactic acid bacteria mainly obtained from the present study were *Lactobacillus fermentum*, *Weisella confusa* and *Weisella cibaria*. A few strains of *L fermentum* have now been known to have probiotic potential [18]-[21]. Some of these are also being used for this purpose. *Weisella* on the other hand, has been only recently considered for its probiotic properties[7], [8], and [23].

4. Conclusions

Lactic acid bacteria are now been commonly used as probiotics. However, their sources vary from vegetables to meat, sourdough, dairy, animal or human faeces, as well as animal or human intestinal contents. Considering the health benefits that the probiotic lactic acid bacteria can offer, it is of value that the most appropriate sources be investigated. The curd samples used in this study showed the presence of *L fermentum* while raw milk seemed to contain *L fermentum*,*Weisella confusa*, *Weisella cibaria* as confirmed by the 16S rRNA gene sequence analysis.In studies not included in this work, these isolates have shown probiotic potential such as tolerance to acid and bile stress and antagonism to common enteric pathogens. Hence, the authors would like to suggest that dairy being easy to obtain and generally regarded as safe, is a good candidate for being an indigenous source of probiotic organisms. Also, that the probability of isolating lactobacilli from curd is greater than that from raw milk.

5. Acknowledgements

References

[1] **Paulo Sousa e Silva J., Ana Cristina Freitas (ed.)(2014).** Probiotic bacteria: Fundamentals, Therapy, and Technological aspects. Pan Stanford Publishing Pte Ltd.

[2] **Marcel B Roberfroid (2000).**Prebiotics and probiotics: are they functional foods? American Journal of Clinical Nutrition 71(suppl):1682S–7S.

[3] **Senok A. C, Ismaeel A. Y and Botta G. A. (2005).** Probiotics: facts and myths. Clinical Microbiology and Infection 11: 958–966.

[4] **Erika I (2001)**. Probiotics in human disease.American Journal of Clinical Nutrition 73(suppl):1142S–6S.

[5] **Sieladie D. V, Zambou N. F, Kaktcham P. M, Cresci A and Fonteh F (2011).** Probiotic properties of lactobacilli strains isolated from raw cow milk in the western highlands of Cameroon. Innovative Romanian Food Biotechnology 9:12-28.

[6] **Sirilun S, Chaiyasut C, Kantachote D and Lunxananil P(2010).** Characterisation of non-human origin probiotic *Lactobacillusplantarum* with cholesterol-lowering property. African Journal of Microbiology Research 4(10):994-1000.

[7] **Lee K. W, Park J. Y, Jeong H. R, Heo H. J, Han N. S, Kim J. H (2012).**Probiotic properties of *Weissella*

strains isolated from human faeces.Anaerobe18(1):96-102.

[8] **Funmilola A, Ayenia B, Borja Sáncheza, Bolanle A, Adeniyib, Clara G. de los Reyes-Gavilána, Abelardo M, Patricia Ruas-Madiedoa (2011)**. Evaluation of the functional potential of *Weissella* and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow's intestine. International Journal of Food Microbiology147(2):97–104.

[9] **Gerald W. T (1999).** Identification of Lactobacilli and Bifidobacteria. Current Issues in Molecular Biology 1(1): 53-64.

[10] **Chin M. L, Chin C. S., Norhani A. and Yin W. H (2008).** Estimation of 16S rRNA gene copy number in several probiotic *Lactobacillus* strains isolated from the gastrointestinal tract of chicken. FEMS Microbiology Letters 287(1):136–141.

[11] **Clarridge J. E. III (2004).**Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clinical Microbiology Reviews 17(4):840–862.

[12] Bergey's manual of systematic bacteriology; Volume 3: The Firmicutes: (464-513); Springer-Verlag, New York. 2009.

[13] **Collins C, Grange J, Lyne P, Falkinham J III(2004).**Collins and Lyne's Microbiological methods. 8th Edition; CRC Press.

[14] **Harrigan W. F(2013).** Laboratory methods in food Microbiology. 3rd edition; Academic Press; First Indian Print.

[15] **Saitou N, Nei M (1987).**The neighbor-joining method: a new method for reconstructing phylogenetic trees.Molecular Biology and Evolution 4(4):406-25.

[16] **Joseph F (1985).**Confidence limits on phylogenies: An approach using the Bootstrap. Evolution 39(4):783-791.

[17] **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011).**MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution (10):2731-9.

[18] **Gillian E. G, Christine H, Andrew W. B, BeuermanD, and Gregor R (2002).**Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. Clinical and Diagnostic Laboratory Immunology 9(1): 92–96.

[19] **Pan, Dao D, Xia Q. Z, and Tian Y (2011)**. Characterization of *Lactobacillusfermentum* SM-7 isolated from koumiss,apotentialprobioticbacteriumwithcholesterollo wering effects. Journal of the Science of Food and Agriculture91(3): 512-518.

[20] **John-Hwan, Park, Yeonhee L, Enpyo M, Seun H S, and Min-Won B (2005).** Safety assessment of *Lactobacillusfermentum* PL 9005, a potential probiotic lactic acid bacterium, in mice. Journal of MicrobiologyandBiotechnology15(3): 603-608.

[21] **Zeng, Xian Q, Dao D. P, and Pei D. Z (2010)**. Functional characteristicsof*Lactobacillusfermentum*F1.Current Microbiology62(1): 27-31.

[22] **Marika M and Mihkel Z (2009).** *Lactobacillusfermentum* ME-3 – an antimicrobial and antioxidative probiotic. Microbial Ecology in Health and Disease 21(1): 1-27.

[23] **Yeonhee L (2005).** Characterization of *Weissellakimchii* PL9023 as a potential probiotic for women FEMS Microbiology Letters 250(1):157–162.

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