Molecular Identification of Lactic Acid Bacteria as a predominant probiotic microorganism found in indigenous fermented pig fat of Assam, India

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Abstract. The study was focused on identifying probiotic bacteria from traditionally fermented pig fat (Sathu) procured from Karbi Anglong Assam. Two types of bacteria were isolated (KJc8_C8 and KJR2_C9) from the food sample. The isolates were morphologically found to be cocci and rod-shaped. The isolated strains showed resistance to inhibitory compound such as NaCl (1-10%), Bile salt (0.1-1%) that exhibit better growth in acidic condition which were the important characteristics for bacteria to be probiotic. In addition the isolates showed positive results in metabolizing different carbohydrates sources. Molecular identification using 16SrDNA gene sequencing confirmed the presence of probiotic bacteria namely Bacillus and Lactobacillus species. The experimental results further indicated the absence of any other spoilage bacteria in the food sample which predominated only Lactobacillus and Bacillus species. This strongly supports the food product as a potential probiotic. Further studies of the food product will be a milestone for economic growth of the society beyond its nutritional effects.

1. Introduction

Northeast represents one of the biodiversity hotspots region in India with a huge reserve of traditional fermented foods. Among various fermented food products, the fermented pork fat is one of the ethnic fermented food which is traditionally prepared from animal source. It was consumed by the diverse groups of people and the tribes such as Dimasa, Hmar, Zeme, Naga, Rangmei, Baite, Vaiphei, Hrangkhol, Karbi, Kuki, Khasi, Jayantia and Khelma living in North Cachar district of Assam [1,2].

Since eating of meat is a habitual culture among indigenous people in Northeast. Domestic animals are slaughtered on festivals and special occasion, the meat is cooked and the fat portion are preserved by fermentation for future consumption.

Some of the common traditionally processed fermented meat products which were lesser known and scientifically documented from Assam were Mogonggrain (meat of buffalo/pig, boar/ deer), Honoheingrain (pig/boar meat), Sathu (pig fat), Gwai-Rou (cow/ buffalo fat and intestine), Saphak (pig meat with fats), Gwag –Ruum (pork fat and meat) Cheu(Semi-boiled pork pieces), Dohklong(boiled pork), Doh Khlei(pig brain), Doh Kpu (Finely minced(pork), smoked dried pork/beef Egd-adin banum(pork pieces mild cooked), Saphak Assam(Pork) [1,2]. These fermented meats are at low cost and use for socio-cultural reasons by different ethnic people of Assam. In making these fermented...
food and marketing of products, ethnic women with traditional knowledge play a major role. However preparation methods may vary due to diversities in ethnicity and specific region. The climatic condition, geographical location, food preference and seasonal availability of plants and animals were the important factors responsible in producing varieties of indigenous foods and availability within the North East States [3]. Fermented foods are an essential part in the daily meals for the people of northeast eastern region. Not only Northeast, traditional fermented products have played an integral part in the diet of developing nation and far East because of health benefits driven by the probiotic organisms in fermented foods. In the world, it has assumed around 50-400g of fermented foods and alcoholic beverages have consumed per day which represented 5-40%of the daily meals [4]. This is because fermented foods harbor major sources of probiotic bacteria that have been associated with human diet from traditional times. By probiotic means live microorganism that confer health benefits to hosts by modulating mucosal and innate immunity when taken in adequate amount thereby enhancing dietary and microbial stability in the intestinal tract [5,6]. Among them Lactic acid bacteria (LAB) including Lactobacillus species have accepted as probiotics and Generally Recognized as Safe (GRAS) organisms above its functional property of fermenting food materials [7,8]. In fact most strains of Bifido bacterium and LAB isolated from fermented food materials increased the survival of compatibility with the human gut micro flora and improved their chances of survival and showed effective in many ways. In general the lactobacillus and Bifido bacterium are predominant bacterial strains used as probiotics microbial flora. Among them Lactic acid bacteria including Lactobacillus Lacto cocci, Enterococcus, Bacillus, Pedi cocci, Saccharomyces were commonly occured microorganisms in raw meat as well as also representative probiotics [9,5,10]. This means of probiotic has used in food and feed industry as starter culture and research has proven their exert beneficial effects to the host that include improving intestinal microbial balance, production of bacteriocins or bactericidal proteins compounds, moreover produces bioactive molecules such as ethanol fatty acids, diacetyl, hydrogen peroxide as lactic acid thus decreasing toxicity level , lowered the luminal pH that has strong antagonistic effects in the inhibition of spoilage and other pathogenic bacteria[10]. Thus occurrence of probiotic bacteria to the fermented meat products can benefit in improving health through balancing activities of intestinal microbial flora in association with LAB. Moreover the connection between probiotic characteristics and the dairy products has scientific evidence. So, probiotic properties from animal sources provided by microorganisms has also becoming a burning research during recent years. With the present growing concern regarding healthy lifestyles, having of good food becomes the first priority. For this, isolation of probiotic bacteria from potent food sample is the first step for production of probiotic food products. Thus the study aims to identify probiotic microorganisms by 16s rDNA sequencing and analysis of probiotic activities responsible in changing the meat products with the help of advance biotechnological tools. Processing such fermented pig fat can largely contribute to the regional economy as well as can provide good benefits towards the health sector of the society.

2. Materials and Methods

2.1 Collection of sample and isolation of bacteria.
The fermented product of pig fat was collected from local kuki lady Karbi Anglong, Assam. The sample was aseptically transferred to a sterile container and stored at 4°C refrigerator. In 10ml of Mann Rogosa Sharpe (MRS Himedia) broth 0.1 gm of sample was suspended and homogenized well. The broth was incubated anaerobically at 37°C for 24 hrs. at 200 rpm and stored in refrigerator as initial mother stock. From the stock solution, 1ml was taken and inoculated in 9ml of sterilized MRS broth which was defined as 10⁻¹ dilution. Appropriate serial dilution up to 10⁻⁵ were prepared. 100µL each of the broth cultures from different dilution were spread plated on MRS agar plate for 48 hrs. at 37°C. The heterogeneous bacterial colonies were isolated by streaking in different MRS agar plates and incubated 24hrs at 37°C.
2.2 Morphological and biochemical characteristics
From the isolated colonies, colony morphology and Gram nature were studied. The purified isolates were preserved at 4°C for immediate use and in 30% glycerol at -20°C for future studies. The biochemical tests such as catalase, motility, Methyl Red (MR), Voges Proskauer (VP), Indole and Simmons’s Citrate Slant test (IMVIC test) and carbohydrates utilization tests were performed.

2.3 Physicochemical characteristics of the isolates

2.3.1 Determination of optimum pH
To determine optimum pH, 100µL of overnight grown cultures were inoculated into each sterilized 10 ml of MRS broth of different pH ranging from 2, 3, 4, 5, 6, 7, 8 and 9 by adjusting the pH with 1N HCl and 0.5 M NaOH for acid and base. The inoculated broth were incubated anaerobically for 24 hrs. at 37°C. After 24 hrs. incubation, the optical density of the bacterial isolates were recorded using spectrophotometer at 600 nm along with uninoculated controlled broth [11].

2.3.2 Determination of optimum temperature
From overnight grown culture of isolated bacteria, 100µL was inoculated into different 10ml of sterilized MRS broth and incubated anaerobically at different temperature such as 25°C, 30°C, 35°C and 45°C for 24 hrs. at 200rpm. After incubation, the bacterial growths were recorded by spectrophotometer at 600 nm along with uninoculated controlled broth [12].

2.3.3 NaCl tolerance test
The isolates were investigated for NaCl tolerance by inoculating 100µL of freshly prepared overnight grown cultures into each sterilized MRS broth with different concentration of NaCl (0, 2%, 4%, 6%, 8%, 10%) and incubated for 24 hrs. at 37°C. After incubated optical density of the bacteria were measured at 600 nm [13].

2.3.4 Bile salt tolerance test
Tolerance to bile salt was performed by measuring the optical density of isolates at 600 nm of sterilized MRS medium containing different concentration (0, 0.1%, 0.3%, 0.6%, 0.8%, and 1%) of bile salts in which 100µL of overnight isolated cultures were inoculated and incubated for 24 hrs. at 37°C [11].

2.4 Molecular Identification

2.4.1 Extraction of genomic DNA form isolates
Bacterial genomic DNA was isolated using HiMedia HiPurATM Bacterial Genomic DNA purification Kit with spin-column technology. 1.5ml of each bacterium Lactobacillus MRS Broth culture was spun down at 10000rpm for 2min and the supernatant was discarded followed by lysis of cells. The lysis was done according to the protocol based on Gram characteristics. For Gram positive bacteria, 45 mg/ml lysozyme solution was used instead of AL solution (provided with the Kit). RNase A and Proteinase K solution was added every time after cell lysis to avoid RNA ad protein contamination. After addition of Elution Buffer, the spin-columns were incubated for 30 min to obtain greater yields of bacterial genomic DNA. 10 µL of each genomic DNA sample was electrophoresed on 0.6 % Agarose gel and visualized with pro-stained Ethidium bromide under Ultraviolet light at 254nm along with 1kbDNA ladder in Gel Documentation machine. The purified DNA was stored at -20°C refrigerator.
2.4.2 PCR Amplification of 16S-rDNA gene
Purified DNA was used as a template to amplify a segment of the 16S-rDNA gene by the PCR techniques using the two universal prokaryotic primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-CGGTTACCTTGTTACGACTT-3') 100ng of genomic DNA isolated from each bacterial strain was amplified in the PCR reaction. A total volume of 25 ml of each PCR reaction mixture was prepared where 2XPCR Tag mixture (HiMedia) was added and final concentration was made to 1X by half dilution. The program of PCR reaction was set as initial denaturation for 2 minutes at 95°C followed by denaturation step of 35 cycles at 95°C for 30 seconds, annealing step of 30 seconds at 52°C and 2 minutes extension step at 72°C with a final extension step at 72°C for 15 minutes. The amplified PCR products were checked on 1% Agarose gel with Ethidium bromide dye under ultraviolet light in Gel DOC machine and image was captured [7].

Sequencing of 16S rDNA gene of the isolates were carried out to verify the strains within each cluster. The amplified DNA was sequence with primers 785F(5'-GGATTAGATACCCTGGTA-3') and 907R(5'-CCGTCAATTCTTTAGTTT-3') by Aakaar Biotechnologies Private Limited R&D Unit 9/322 Jankipuram Extension, Lucknow, 226031, India. The sequences were aligned by using the sequence alignment and cluster W. Its homology was analyzed through BLAST algorithm to find the most related sequence in the NCBI nucleotide sequence database. After sequencing Phylogenic tree construction was performed with mega X by neighbor-joining analysis

3. Result
3.1 Isolation of Bacteria
Two types of bacteria were isolated from the fermented pork fats named as KJc8_C8 and KJR2_C9. Both the isolates produced round white and small brownish color on MRS agar plate. The isolate KJR2_C9 was found dominated on the MRS agar plate.

3.2 Morphological and Biochemical Characterization
When gram stained and examined under microscope a gram positive cocci, chain and short rod shape were found in the medium (Fig 2 and 4) typically similar to be reference lactobacillus strain MTCC 9748. The isolates were further taken for biochemical characterization, and were found negative for catalase, oxidase, indole MR, VP, Citrate test but positive in case of KJc8_C8 in MR and citrate test. Carbohydrate utilization tests were also performed to investigate the ability to ferment Glucose, sorbitol fructose, maltose, Lactose, Inositol sucrose mannitol, arbutol. The isolate KJR2_C9 was able to ferment the given sugars but in KJc8_C8 except sorbitol all the sugars were fermented (Table 1).
Fig. 3 Typical colony characteristic of isolate KJR2_C9 on MRS agar

Fig. 4 Microscopic view of KJR2_C9 after Gram staining

Table 1 - Morphological and Biochemical characteristic of the isolates

| Test parameter               | KJc8_C8 | KJR2_C9            | Reference            |
|------------------------------|---------|--------------------|----------------------|
| Gram stain                   | +       | +                  | Bansal et al 2009    |
| Colony morphology            | Round white color | Small and brownish | Bansal et al 2009    |
| Microscopic view             | Cocci chain | Short Single rod   | Bansal et al 2009    |
| Catalase test                | +       | -                  | Menconi et al 2004   |
| Methyl Red(MR)Test           | +       | -                  | Menconi et al 2004   |
| Indole test                  | -       | -                  | Menconi et al 2004   |
| Citrate test                 | +       | -                  | Menconi et al 2004   |
| Voges Proskauer(V) test      | -       | -                  | Menconi et al 2004   |
| Glucose fermentation         | +/-     | +                  |                      |
| Sorbitol Fermentation        | -       | +                  |                      |
| Fructose Fermentation        | +       | +                  |                      |
| Maltose Fermentation         | +       | +                  |                      |
| Lactose Fermentation         | +       | +                  |                      |
| Inositol Fermentation        | +       | +                  |                      |

+: Positive reaction, -: Negative reaction, +/- : weak reaction

3.3 Physio-chemical Characteristics of the isolates

The isolated bacteria were determined for the following physiochemical characteristics.
3.3.1 Determination of optimum pH:
The isolates were able to grow between pH 4 to 8. Maximum growth was found at pH 6 for both the isolates. However growth of KJc8_C8 decreases drastically after pH 6 in MRS broths at 35°C in Fig. 5. The result showed KJR2_C9 was tolerant to high pH condition. Thus the optimum pH of KJc8_C8 and KJR2_C9 were pH 6 and pH 8 respectively which means both the isolates could tolerate in high pH condition.

![Fig. 5 Effect of pH](image)

3.3.2 Determination of Optimum temperature:
Among the different temperature such as 25°C, 35°C, 40°C and 45°C, the best growth temperature of KJc8_C8 and KJR2_C9 were found at 35°C. After 35°C growth of both the isolates decreases as in Fig. 6.

![Fig. 6 Effect of temperature](image)

3.3.3 NaCl tolerance test
The isolated species from fermented pig fat grew well in 2% NaCl concentration and started decreases their growth rate with increasing concentration. The result showed KJc8_C8 was more tolerant to NaCl than KJR2_C9.
3.3.4 Bile salt tolerance result
Both the isolates were able to endure in 0.1 to 1% bile salt but the growth rate decreases with increase concentration of bile salt. The result showed the isolate KJR2_C9 was more inhibited by bile salt than KJc8_C8. This indicates that bile salt act as inhibitory effect as illustrate in Fig.8.

3.4 Molecular identification of the isolates
The amplification products of 16s rDNA sequences were run through 1% agarose gel. A sharp band at around 1.5kb of ladder sequence was observed which the size of the sequence of interest (Fig 9).
After DNA sequencing of the 16s rDNA sequences, the sequences were blasted with known sequences using NCBI nBLAST. The result showed that KJc8_C8 has highest similarity (but not 100% identical) with *Bacillus australimaris* (Table 2) whereas; KJR2_C9 has highest similarity (but not 100% identical) with *Lactobacillus brevis* (Table 2). This concludes that the isolates belong to new strains which are to be named as *Bacillus australimaris* KJc8_C8 and *Lactobacillus brevis* KJR2_C9. The new sequences were submitted to NCBI Gene Bank and accession number of MK720731.1 and MK729004.1 were given for *Bacillus australimaris* KJRe8_C8 and *Lactobacillus brevis* KJR2_C9 respectively as shown in table 2.
Fig. 9 Agarose gel visualization on UV trans illuminator showing PCR band of C8 and C9. C9 =KJR2_C9, M= DNA ladder ,C8 =KJe8_C8 respectively.

Table 2 Details submission of sequences in NCBI GeneBank of identified bacteria from fermented pig fat based on I6s rDNA sequencing

| Name of isolate | Sequence identified       | Sequence length | % identity with closest species | Gene Bank Strain accession no. |
|-----------------|---------------------------|-----------------|---------------------------------|--------------------------------|
| KJRc8_C8        | Bacillus australimaris    | 979             | 97%                             | MK720731.1                     |
| KJR2_C9         | Lactobacillus brevis      | 1361            | 99%                             | MK729004.1                     |
Table 3 Nucleotide BLAST result of KJc8_C8 showing the top sequences with the name of organism

| Description                                      | Max score | Total score | Query cover | E value | Ident | Accession     |
|-------------------------------------------------|-----------|-------------|-------------|---------|-------|---------------|
| Bacillus australimaris strain MCCC 1A05787      | 1751      | 1751        | 99%         | 0       | 99%   | NR_148787.1   |
| Bacillus xiamenensis strain MCCC 1A00008        | 1746      | 1746        | 99%         | 0       | 99%   | NR_148244.1   |
| Bacillus stratosphericus strain 41KF2a          | 1742      | 1742        | 99%         | 0       | 99%   | NR_042336.1   |
| Bacillus zhangzhouensis strain MCCC 1A08372     | 1740      | 1740        | 99%         | 0       | 99%   | NR_148786.1   |
| Bacillus aerius strain 24K                       | 1736      | 1736        | 98%         | 0       | 99%   | NR_118439.1   |
| Bacillus safensis strain NBRC 100820             | 1735      | 1735        | 97%         | 0       | 99%   | NR_113945.1   |
| Bacillus altitudinis strain 41KF2b               | 1724      | 1724        | 97%         | 0       | 99%   | NR_042337.1   |
| Bacillus atrophaeus strain JCM 9070              | 1696      | 1696        | 99%         | 0       | 98%   | NR_024689.1   |
| Bacillus subtilis subsp. subtilis strain 168     | 1668      | 1668        | 99%         | 0       | 98%   | NR_102783.2   |
| Bacillus mojavensis strain NBRC 15718            | 1657      | 1657        | 97%         | 0       | 98%   | NR_112725.1   |
| Bacillus drentensis strain LMG 21831             | 1526      | 1526        | 98%         | 0       | 95%   | NR_118438.1   |

Fig.10 Phylogenetic tree (Neighbor joining method) of KJc8_C8 based on 16s rDNA sequence
Table 4 Nucleotide BLAST results of KJR2_C9 showing the top hits sequences with the name of organism

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|-------------|-----------|-------------|-------------|---------|-------|-----------|
| *Lactobacillus brevis* strain ATCC 14869 | 2503 | 2503 | 100% | 0 | 99% | NR_116238.1 |
| *Lactobacillus senmaizukei* strain NBRC 103853 | 2394 | 2394 | 100% | 0 | 98% | NR_114251.1 |
| *Lactobacillus yonginensis* strain THK-V8 | 2390 | 2390 | 100% | 0 | 98% | NR_109452.1 |
| *Lactobacillus hammesii* strain TMW 1.1236 | 2375 | 2375 | 100% | 0 | 98% | NR_042243.1 |
| *Lactobacillus koreensis* strain DCY50 | 2364 | 2364 | 100% | 0 | 98% | NR_116854.1 |
| *Lactobacillus cerevisiae* strain TUM BP 140423000-2250 | 2357 | 2357 | 100% | 0 | 98% | NR_158030.1 |
| *Lactobacillus zymae* strain R-18615 | 2329 | 2329 | 99% | 0 | 98% | NR_042241.1 |
| *Lactobacillus paucivorans* strain TMW 1.1424 | 2266 | 2266 | 99% | 0 | 97% | NR_116943.1 |
| *Lactobacillus oryzae* strain SG293 | 2174 | 2174 | 99% | 0 | 96% | NR_114339.1 |
| *Lactobacillus parabuchneri* strain LMG 11457 | 2156 | 2156 | 99% | 0 | 95% | NR_114962.1 |
| *Pediococcus ethanolidurans* strain Z-9 | 2045 | 2045 | 99% | 0 | 94% | NR_043291.2 |

Fig. 11 Phylogenetic tree (Neighbor joining method) of KJR2_C9 based on 16s rDNA sequence
4. Discussion
According to our finding, fermented pig fat contained a rich microbiota mainly composed of two types of bacteria, *lactobacillus Brevis* KJR2_C9 and *Bacillus australimaris* KJc8_C8 without the occurrence of food borne pathogens and spoilage bacteria [1]. The identification of KJc8_C8 and KJR2_C9 were carried out through phenotypic, biochemical and genotypic methods to give polyphasic approach. The combination of this method has provided greater accuracy for identification of new bacterial strain. It is also a major criterion during the selection of potential probiotic bacteria. The physiochemical characteristics tests parameters of KJc8_c8 and KJR2_C9 were listed in Table 1. The result of gram positive reaction with morphological shape of rod and cocci with brownish and whitish colour indicated that the isolates have belonged to Lactobacillus species. The momentous growth of isolates at pH ranging from 6 to 8 at 35°C more confirmed their identification as Lactobacillus along with the results of IMVIC tests and carbohydrates fermentation tests [14]. The tested isolates showed maximum growth at 35°C while KJR2_C9 could grow up to 40°C. The ability to grow at high condition is a good characteristic for lactic acid production which decreases chances of contamination by other microorganism. The ability to grow in acidic and bile salts condition are also an in vitro tests guidelines to evaluate during the selection of potential probiotic bacteria. The strains were also determined for optimum pH. Both isolates exhibited the ability to grow in between pH 4 to 8 but favourable growth was found at pH 6. The results indicated growth rate decreases with increase in pH which has in correlation[15]. However the isolates KJR2_C9 could grow up to pH 8. This indicates Lactobacillus spp. were strain dependant. The isolated strains were able to resist (1-9 %) w/v concentration of NaCl MRS broth. Both isolates maintain good growth at 2% concentration of NaCl and declined sharply with increase of NaCl concentration in the medium. In bile salt tolerance test, the isolates showed good growth upto 0.3% w/v of bile in the MRS broth and continued resistance up to 0.8% concentration of bile salt. Tolerance to lower concentration depicted the isolates has the potential to colonize and balancing the intestinal microbial inside the small intestine [11,16]. Both the strain deceases their growth rate with increased concentration of bile salt and NaCl. In some research articles, tolerant to 0.3% bile concentration was the first property of a probiotic bacterium required for human consumption. Based on the results the isolated strains were able to live through the harsh condition of toxicity [13] therefore can be used as a potential probiotic bacteria. As reported by Lilis Nuraida not only LAB in fermented food some species of bacillus have also potential probiotics [17]. Further, 16s rDNA sequencing revealed both the isolates as a new strains of *lactobacillus Brevis* and *Bacillus australimaris* which were named as *Bacillus australimaris* KJc8_C8 and *Lactobacillus brevis KJR2_C9*.

5. Conclusion
Two predominant probiotic bacteria with new strains have found in the ethnic fermented pig fat. The two bacterial strains belong to *Lactobacillus brevis* and *Bacillus australimaris* and are named as *Bacillus australimaris* KJc8_C8 and *Lactobacillus brevis KJR2_C9*. Both the isolates assure the capability and functional properties of probiotic, resistance to harsh conditions such as low pH, high salt and bile concentration. In this regards, fermented pig fat may be a potent sample for the development of probiotic food. Our study has established documented proof of the beneficial effects that may exert health promotion in human by consumption of fermented pig fat. This study confirms the occurrence of potential probiotic bacteria with new strains present in fermented meat pig fat. Further studies will provide a base for the recognition of such products as healthy foods and a supplementary for beneficial bacteria.

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