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Bacterial dynamics during yearlong spontaneous fermentation for production of *ngari*, a dry fermented fish product of Northeast India

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ABSTRACT

Ngari is the most popular traditionally processed non-salted fish product, prepared from sun-dried small cyprinid fish *Puntius sophore* (Ham.) in Manipur state of Northeast India. The microbial involvement in *ngari* production remained uncertain due to its low moisture content and yearlong incubation in anaerobically sealed earthen pots without any significant change in total microbial count. The culture-independent PCR-DGGE analysis used during this study confirmed a drastic bacterial community structural change in comparison to its raw material. To understand the bacterial dynamics during this dry fermentation, time series samples collected over a period of nine months through destructive sampling from two indigenous *ngari* production centres were analysed by using both culture-dependent and culture-independent molecular methods. A total of 210 bacteria isolated from the samples were identified by amplified ribosomal DNA restriction analysis (ARDRA) based grouping and 16S rRNA gene sequence similarity analysis. The dominant bacteria were *Staphylococcus cohnii* subsp. *cohnii* (38.0%), *Tetragenococcus halophilus* subsp. *flandriensis* (16.8%), a novel phylotype related to *Lactobacillus pobuzihii* (7.2%), *Enterococcus faecium* (7.2%), *Bacillus indicus* (6.3%) and *Staphylococcus carnosus* (3.8%). Distinct bacterial dynamics with the emergence of *T. halophilus* at third month (10^6 CFU/g), *L. pobuzihii* at sixth month (10^6 CFU/g), *S. carnosus* at three to six months (10^4 CFU/g) and *B. indicus* at six to nine months (10^5 CFU/g) in both the production centres was observed during *ngari* fermentation. However, the other two dominant bacteria *S. cohnii* and *E. faecium* were isolated throughout the fermentation with the population of 10^6 CFU/g and 10^4 CFU/g respectively. Culture-independent PCR-DGGE analysis further showed the presence of additional species, in which *Kocuria halotolerans* and *Macrococcus caseolyticus* disappeared during fermentation while *Clostridium irregulare* and *Azorhizobium caulinodans* were detected throughout the fermentation. Principal component analysis showed a drastic bacterial community structural change at the sixth month of fermentation. These identified dominant bacterial cultures of *T. halophilus*, *L. pobuzihii*, *S. carnosus* and *B. indicus* could be effectively utilised for designing starter culture and optimizing fermentation technology for industrialisation of *ngari* production.

1. Introduction

Northeast India is a home to wide varieties of unexplored indigenous fermented fish products, which are unique in organoleptic properties and their preparation processes. Thousands of metric tonnes of fermented fish products are prepared annually for the domestic consumption in this region. The major non-salted fermented fish products are *ngari* and *hentak* in Manipur, *shedal*/*berma* in Tripura and Assam, *ngawum* in Mizoram, *utong-ngari* in Nagaland and Assam, and *tungtap* in Meghalaya (Rapsang et al., 2013; Singh et al., 2014). The major salted fermented fish products are *elisa-ngari* in Assam and *lona-ilish* in Tripura and Assam (Majumdar and Basu, 2010). Similar types of fermented fish products are also consumed in Japan (Kuda et al., 2009; Matsui et al., 2010) and Southeast Asian countries (Paludan-Müller et al., 2002; Sangjindavong et al., 2008). *Ngari* is the most popular fermented fish product of Northeast India produced exclusively from sun-dried small cyprinid fish *Puntius sophore* Ham., which is locally known as *phabou*. Manipur valley is well known for the artisanal production of good quality *ngari*. It is produced by age-old practice of yearlong natural fermentation and used as an appetiser and flavour enhancer in different food preparations due to its excellent organoleptic properties (Jeyaram et al., 2009).

Association of a diverse group of microorganisms in various traditional fermented fish products has been reported. Among them, *Tetragenococcus halophilus*, *Tetragenococcus muriaticus* (Kobayashi et al., 2003), *Staphylococcus carnosus*, *Staphylococcus piscifermentans*, *Staphylococcus cohnii*, *Staphylococcus xylosus* (Zaman et al., 2011), *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus farciminis*, *Lactobacillus pentosus* (Matsui et al., 2010; Paludan-Muller et al., 2002), *Bacillus*

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amyloliquefaciens and *Bacillus licheniformis* (Toyokawa et al., 2010) have been frequently reported. Even, strictly anaerobic halophiles namely *Haloanaerobium praevalens*, *Haloanaerobium alcaliphilum and Haloanaerobium fermentans* were also isolated from fermented fish products (Kobayashi et al., 2000b).

According to previous studies, the dominant microbial species reported in *ngari* are *L. plantarum* (Thapa et al., 2004), *Micrococcus* sp. and *Bacillus* sp. (Sarojnalini and Suchitra, 2009). The present knowledge on microbial diversity of *ngari* is based on cultivation in limited media followed by phenotypic identification of dominant microorganisms present in the marketed finished product. To overcome the limitations of phenotypic identification, several DNA-based techniques particularly PCR-based ribotyping methods, such as amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA gene sequencing are used very effectively for authentic identification (Jeyaram et al., 2010). Though culture-dependent methods provide significant insight, it underestimates the microbial diversity of fermented foods (Ampe et al., 1999). To overcome this challenge, culture-independent molecular approaches using PCR-denaturing gradient gel electrophoresis/ temperature gradient gel electrophoresis (DGGE/TGGE) (Cocolin et al., 2006; El-Baradei et al., 2007) and next generation sequencing (NGS) (Ercolini, 2013) of rRNA gene directly from the metagenomic DNA of fermented foods have been widely used to study the complex microbial community structure of traditionally fermented foods (Cocolin and Ercolini, 2008). Moreover, both culture-dependent and culture-independent methods have been used together as an integrated polyphasic approach to describe the microbial community structure in various indigenous fermented foods (Martin-Platero et al., 2008; Rantsiou et al., 2005).

Although bacterial dominance has been reported in different fermented fish products, it has also been reported that some of the traditional fermented fish production were mediated by endogenous enzymes of fish rather than the associated microorganisms (Sanni et al., 2002). The microbial involvement during indigenous process of *ngari* production is uncertain due to different factors such as (i) moisture content of the processed raw material and *ngari* are lower (<30%, a_w < 0.84) than the other reported fermented fish products (72–78% moisture content) (Riebroy et al., 2004), (ii) pH is near neutral range (6.2–6.7), (iii) non-salted nature of the product, and (iv) incubation in anaerobically sealed pots for more than one year without any significant change in microbial count. Analysing the microbial community structure and its dynamics during spontaneous fermentation is a prerequisite for better comprehension of traditional food fermentation and the selection of efficient starter cultures. To the best of our knowledge, no extensive study had been taken up to demonstrate the microbial dynamics during yearlong spontaneous fermentation for production of the dry fermented fish product *ngari*.

The aim of this study was to confirm the microbial involvement in the traditional process of *ngari* production and to study the microbial dynamics during the yearlong spontaneous dry fermentation by using both culture-dependent and culture-independent molecular analyses.

2. Materials and methods

2.1. Sampling

The finished product of 9–12 months fermented *ngari* samples and the raw material *phabou* were used for comparative analyses of microbial structure and proximate composition. The *ngari* samples (100–200 g) were collected from 15 different artisanal production centres of Manipur situated at Singjamei, Keishampat, Thangmeiband, Khuyathong, Yaiskul, Keisamthong, Sekmai, Nagamapal, Mantripukri, Sagolband Tera, Samurou, Sagoltongba, Sangolband Salam Leikai, Tera Konjil Leikai and Kakwa. The *phabou* samples were sourced from five geographical regions namely Bangladesh, Gujarat, Siliguri (West Bengal), Assam, and Japan. Sampling was done during the period of October 2008 to December 2008. The samples were collected from different depth using sterile gloves and mixed thoroughly in a sterile 500 ml capacity sample container. Collected samples were brought immediately to the laboratory in cooler box, stored at 4 °C and subsequently used for analysis within 2–3 days of collection.

2.2. Chemical analysis

The samples were analysed for protein content by micro-Kjeldahl method, total lipid content by petroleum ether extraction method, carbohydrate content by Anthrone method, moisture content, water activity and ash content as per AOAC (William and George, 2000). The sample homogenate was used for measuring pH, titratable acidity by potentiometric titration and salt analysis by argentimetric method (William and George, 2000) using Autotitrator (LabIndia). Three independent measurements were performed for each sample during above analysis.

2.3. Traditional ngari preparation and sampling at different time intervals

Two artisanal *ngari* production centres were selected based on the pedigree record of good quality *ngari* production for microbial dynamics study. One production centre was located at Sekmai (24.87° N 93.85° E) and another at Singjamei (24.46° N 93.56° E) of Manipur in India. The raw materials used at Sekmai production centre originated from Lakhimpur of Assam valley, Gujarat and Bangladesh while that of Singjamei from Lakhimpur, Barpeta of Assam valley and Bangladesh. In both the production centres, the raw materials were processed and packed in traditional pots (*Ngari kharung*) as per the producer's instruction and incubated from January 2012 to October 2012 for spontaneous fermentation. For each production site, a total of 12 earthen pots (30–40 kg) were used for microbial dynamics study. Inside of earthen pots was repeatedly (3 times) pasted with mustard oil to make anaerobic environment conducive for fermentation (as per producer's instruction). Pre-treatment of the raw materials was done by brisk water washing, draining and heaping overnight. Unprocessed pond or stream water was used for this brisk washing and the same batch of water was repeatedly used for this washing. Next day, it was spread over and pressed hard using stone roller. The processed raw material was packed layer by layer and pressed hard manually using a wooden pestle. The fish scale residue formed during brief washing was used for topping the packed pot and sealed air tightly using clay paste. Samples were collected in triplicates before packing (processed raw material) and at different time interval (1st, 3rd, 6th and 9th months) during incubation at room temperature by destructive sampling. Before collecting the samples, the topmost layer of about 1 kg was removed. Sampling was performed in such a way that each sample was the representative of three different depths of the pot (top, middle and bottom layer). Samples (500 g each) at different time series were collected from both the production centres using sterile gloves and sample container, stored in cooler box (4 °C) and transferred immediately to the laboratory for further analysis. A total of 30 samples collected over a period of 9 months were analysed by using both culture-dependent and culture-independent molecular techniques.

2.4. Sample homogenate preparation

The samples (10 g) were chopped into small pieces using sterile scissors and transferred into a sterile stomacher bag and allowed to soak in 90 ml sterile physiological saline (0.1% w/v bacteriological peptone, 0.85% w/v NaCl, pH 6.1) for 20 min. The samples were homogenised using Stomacher® 400 circulator (Seward, UK) at 250 rpm for 3 min at room temperature. The homogenate was divided into two fractions, 50 ml was stored at −80 °C for metagenomic DNA extraction and the remaining was used for plating.