Food Microbiology 38 (2014) 16–25

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Effects of temperature on microbial succession and metabolite change during saeu-jeot fermentation

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ARTICLE INFO

Article history:
Received 1 March 2013
Received in revised form
3 June 2013
Accepted 10 August 2013
Available online 22 August 2013

Keywords:
Seafood fermentation
Saeu-jeot
Shrimp
Temperature effects
Microbial succession
Metabolites

ABSTRACT

To investigate the effects of temperature on saeu-jeot (shrimp) fermentation, four sets of saeu-jeot samples with approximately 25% (w/v) NaCl were fermented at 10 °C, 15 °C, 20 °C, and 25 °C. The pH values of the 10 °C and 15 °C samples were relatively constant during the entire fermentation period, whereas those of the 20 °C and 25 °C samples gradually decreased after 25 days of fermentation. Quantitative PCR showed that the maximum bacterial abundance was greater in higher temperature samples, and the bacterial abundance in the 10 °C samples steadily decreased during the entire fermentation period. Community analysis using pyrosequencing revealed that the initially dominant *Proteobacteria* including *Pseudoalteromonas*, *Photobacterium*, *Vibrio*, *Aliivibrio*, and *Enterovibrio* were replaced rapidly with *Firmicutes* such as *Psychrobacter*, *Staphylococcus*, *Salimicrobium*, *Alkalibacillus*, and *Halanaerobium* as the fermentation progressed. However, *Vibrio*, *Photobacterium*, *Aliivibrio*, and *Enterovibrio*, which may include potentially pathogenic strains, remained even after 215 days in the 10 °C samples. Metabolite analysis using ^{1}H NMR showed that amino acid profiles and initial quick increases of glucose and glycerol were similar and independent of bacterial growth in all temperature samples. After 25 days of fermentation, the levels of glucose, glycerol, and trimethylamine N-oxide decreased with the growth of *Halanaerobium* and the increase of acetate, butyrate, and methylamines in the 20 °C and 25 °C samples although the amino acid concentrations steadily increased until approximately 105 days of fermentation. Statistical triplot analysis showed that the bacterial successions occurred similarly regardless of the fermentation temperature, and *Halanaerobium* was likely responsible for the production of acetate, butyrate, and methylamines. This study suggests that around 15 °C might be the optimum temperature for the production of safe and tasty saeu-jeot.

1. Introduction

The Korean fermented salted seafood, called jeotgal in Korean, is made by the fermentation of highly salted sea animals such as shrimp, anchovy, squid, oyster, and fish roe. Jeotgal is classified into more than 150 different varieties based mainly on the sea animal used (Hur, 1996). Natural fermentation without the use of starter cultures or the sterilization of raw materials gives rise to the growth of diverse microorganisms during jeotgal fermentation and which are primarily derived from the sea animals themselves and/or the solar-salts used in the preparation of the fermented seafood. It has been suggested that diverse microorganisms may be primarily involved in the development of different jeotgal tastes and flavors (Lee, 1993; Roh et al., 2010). Therefore, many studies have been performed to isolate microorganisms for an understanding of this complex seafood fermentation (Paludan-Müller et al., 2002; Kim et al., 2009; Guan et al., 2011). Currently, more than 20 novel bacterial and archaeal species have been isolated from jeotgal (http://bacterio.net) (Euzéby, 1997).

Saeu-jeot, made by the fermentation of tiny salted shrimp (*Acetes japonicus*), is the most representative and best-selling fermented jeotgal in Korea. Because saeu-jeot fermentation occurs under uncontrolled conditions, it may bring about the growth (or survival) of pathogens derived from raw materials or saeu-jeot putrefactions during fermentation. Saeu-jeot is usually processed at high salt concentrations [approximately 25% (w/v)] and low temperatures (0 °C–10 °C) to ensure proper fermentation without pathogenic growth or putrefaction. However, saeu-jeot fermentation under high salt and low temperature conditions leads to a long fermentation time, which is an important obstacle to produce saeu-

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http://dx.doi.org/10.1016/j.fm.2013.08.004

jeot economically. Therefore, many companies in Korea have taken an interest in the acceleration of the saeu-jeot fermentation process. Because temperature is one of the most important factors in microbial growth and enzyme activity (Mok et al., 2000; Gildberg and Thongthai, 2001; Yongsawatdigul et al., 2007), temperature elevation has been considered as an important way for the acceleration of the saeu-jeot fermentation process. However, to the best of our knowledge, the effects of temperature on saeu-jeot fermentation have not yet been fully explored.

Culture-dependent approaches have been limited to apply to the community analyses of fermented seafood due to the unculturability of many bacteria (Kim et al., 2009; Guan et al., 2011). Culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries also have many limitations in investigating microbial successions during seafood fermentation because they involve time-consuming and laborious steps and produce a limited amount of information (Ercolini, 2004; An et al., 2010; Masoud et al., 2011). Recently, pyrosequencing has emerged as a powerful tool to unveil complex microbial communities (Humblot and Guyot, 2009; Roh et al., 2010; Sakamoto et al., 2011). Because multiplex barcoded pyrosequencing strategies allow for the analysis of multiple samples in a single run, they have been more inexpensively applied to investigate microbial successions during food fermentation processes (Jung et al., 2012, 2013; Kiyohara et al., 2012; Jeong et al., 2012).

It has been suggested that microorganisms as well as diverse endogenous enzymes such as proteinases and lipases originating from fish muscle and digestive tracts may be responsible for saeu-jeot and other salted seafood fermentation (Saisithi, 1994; Choi et al., 1999; Yongsawatdigul et al., 2007). Because the metabolite composition of fermented seafood reflects a more collective phenotypic view of related microbial populations and endogenous enzymes during the seafood fermentation process, parallel studies of microbial successions and metabolites are indispensable to better understand seafood fermentation (Ercolini et al., 2011; Jung et al., 2013). Proton nuclear magnetic resonance (^{1}H NMR) is one of the most comprehensive, easy, and powerful tools used to simultaneously measure diverse metabolites present in a sample, especially in fermented foods (Figueiredo et al., 2006; Jung et al., 2011, 2012; Jeong et al., 2012). The main objective of this study was to investigate the effects of fermentation temperature on saeu-jeot fermentation through the comparison of bacterial communities and metabolites of saeu-jeot samples fermented at different temperatures (10 °C, 15 °C, 20 °C, and 25 °C), which may suggest the optimum temperature for the production of safe and tasty saeu-jeot. Here, we applied the combination of a barcoded 454-pyrosequencing strategy and a ^{1}H NMR technique to investigate bacterial successions and metabolite changes and the relationships between them during an entire saeu-jeot fermentation period.

2. Materials and methods

2.1. Preparation of saeu-jeot and sampling

To investigate the effects of fermentation temperature on saeu-jeot fermentation, four sets of saeu-jeot samples with approximately 25% (w/v) salt were prepared in triplicate using shrimp (*A. japonicus*) according to a traditional manufacturing method as described previously (Jung et al., 2013). Briefly, fresh tiny shrimp, about 4–6 cm in length, caught from the Yellow Sea of South Korea were equally dispensed into 12 plastic containers to include 1.5 kg shrimp and 450 g solar salt (Shinan, Korea). An additional 600 ml of 25% (w/v) solar salt solution was poured into each container to soak the shrimp completely, and three containers were stored at each 10 °C, 15 °C, 20 °C, and 25 °C, respectively. Four milliliter aliquots from each of the saeu-jeot soups (liquid parts of the saeu-jeot) were intermittently sampled from each container, and their pH values were immediately measured. The saeu-jeot soups were filtered using four layers of sterile coarse gauze (Daehan, Korea) to remove large particles, and the filtrates were centrifuged (8000 rpm for 20 min at 4 °C) to harvest microorganisms. Three centrifuged pellets from the same storage temperature were combined and stored at −80 °C for microbial community analyses. The supernatants were stored separately at −80 °C for respective metabolite analyses.

2.2. Quantitative real-time PCR (qRT-PCR) to estimate bacterial abundance

To estimate bacterial abundance in saeu-jeot samples, the total genomic DNA from the pellets derived from 2.0 ml of saeu-jeot soups was extracted using a FastDNA Spin kit (MPbio, Solon, OH) according to the manufacturer's instructions. A qRT-PCR primer set, bac340F (5′-CCTACGGGAGGCAGCAG-3′)/bac758R (5′-CTACCAGGGTATCTAATCC-3′) was used for the measurement of the 16S rRNA gene copies of *Bacteria* (Juck et al., 2000). The qRT-PCR was performed as described previously (Jung et al., 2013). A standard curve for the calculation of bacterial 16S rRNA gene copies was generated on the basis of the number of pCR2.1 vectors (Invitrogen, USA) carrying a bacterial (*Staphylococcus*) 16S rRNA gene derived from a saeu-jeot sample. The number of bacterial 16S rRNA gene copies from each sample was calculated as described previously (Ritalahti et al., 2006; Jung et al., 2013).

2.3. Barcoded pyrosequencing for bacterial community analysis

The genomic DNA from the combined pellets of the triplicate samples was extracted using the FastDNA Spin kit according to the manufacturer's instructions. Bacterial 16S rRNA genes were amplified using the universal primer sets, Bac27F (5′-adaptor B-AC-GAG TTT GAT CMT GGC TCA G-3′)/Bac541R (5′-adaptor A-*X*-AC-WTT ACC GCG GCT GCT GG-3′) (Lee et al., 2012), where *X* denotes unique 7–11 barcode sequences inserted between the 454 Life Sciences adaptor A sequence and the common linker, AC (Supplementary Table 1). All PCR amplifications were carried out as described previously (Lee et al., 2012) and the PCR products were purified using a PCR purification kit (Bioneer, Korea). Purified PCR product concentrations were carefully assessed using a Synergy MX ELISA reader equipped with a Take3 multi-volume plate (BioTek, USA), and a composite sample was prepared by pooling equal amounts of PCR amplicons. The pyrosequencing of the pooled sample was performed using a 454 GS-FLX Titanium system (Roche, Germany) at Macrogen (Korea).

2.4. Sequence processing and data analysis

The pyrosequencing reads were processed and analyzed primarily using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/) (Cole et al., 2009). Briefly, the sequencing reads were assigned to each saeu-jeot sample based on their unique barcodes, and then the barcodes were removed. Sequencing reads with 'N' (undetermined nucleotide), shorter than 300 bp, and average quality values below 20 (error rate 0.01) were removed from the data set. Putative chimeric sequences from the remaining sequencing reads were removed using the chimera.slayer command as implemented in MOTHUR (Schloss et al., 2009). The processed sequencing reads with high quality were aligned using the fast, secondary-structure aware INFERNAL aligner (Nawrocki and Eddy, 2007), and their operational taxonomic units (OTUs) and rarefaction curves (Colwell and Coddington, 1994) were generated using the complete-linkage clustering tool at a 3% dissimilarity