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Temporal variation in quality characteristics of nutritional constituents and bacterial community during dry salt-fermentation of hilsa (*Tenualosa ilisha*)

Md. Shirajul Islam Sarkar[1*], Md. Shahdat Hossain[2*], Muhammad Mehedi Hasan[3], Murshida Khan[1], Anas Al Islam[4] and Md. Kamal[5]

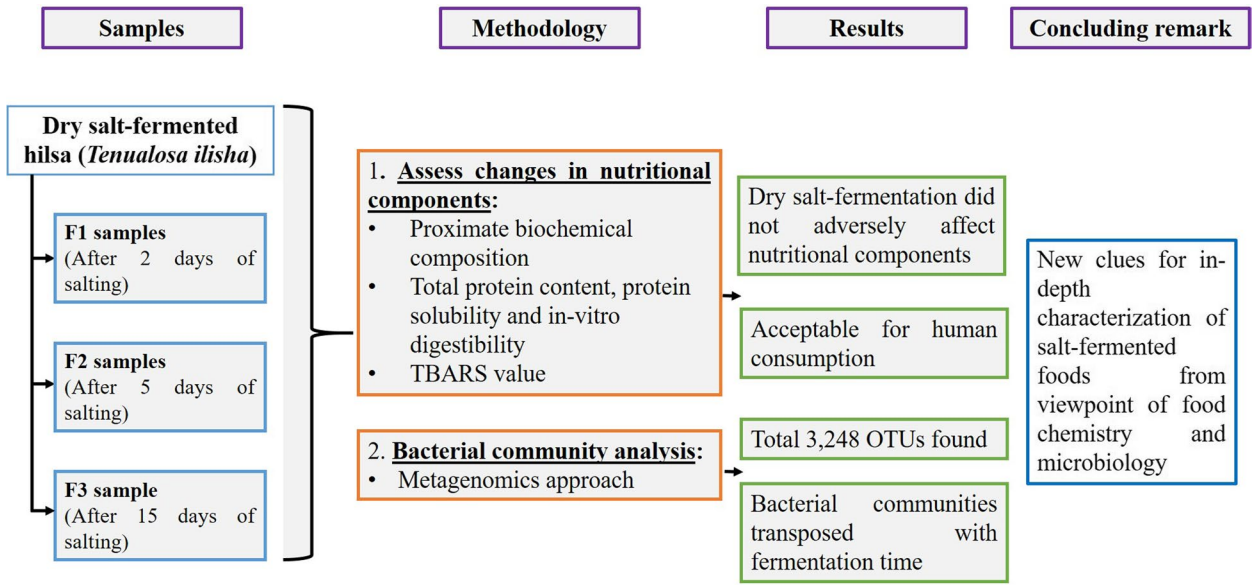
Abstract

Nutritional constituents and bacterial community of hilsa (*Tenualosa ilisha*) at different stages of dry salt-fermentation; namely, F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) was studied. Dry salt-fermentation did not negatively affect proximate biochemical composition. Total protein content in F1, F2 and F3 was 10.14, 22.30 and 16.21 mg/ml, respectively. With progression of ripening process, protein solubility gradually decreased. In all stages, about 98% protein digestibility was found. TBARS (Thiobarbituric acid reactive substances) values at F1, F2 and F3 stages was 0.30, 0.41 and 0.95 mg MDA/kg, respectively and within acceptable limit. A total of 3,248 OTUs were found. Of the identified 48 phyla, *Proteobacteria* (66%), *Firmicutes* (18%) and *Bacteroidota* (11%) were abundant at F1 stage but *Firmicutes* (82%) and *Proteobacteria* (9%) were dominant at F3 stage. Significant differences in the microbial β-diversity among initial, interim and ripe product were observed. There was no significant α-diversity difference at individual stages of dry salt-fermentation of hilsa. Functional gene profile revealed that, in the final product microbial genes related to organismal systems replaced human disease related genes found in initial and interim product. These findings provide new clues for in-depth characterization of salt-fermented foods from viewpoint of food chemistry and microbiology.

Keywords Hilsa, Salt-fermentation, Fish protein, Lipid oxidation, Bacterial community

*Correspondence:
Md. Shirajul Islam Sarkar
sislam@bsmrau.edu.bd
Md. Shahdat Hossain
shahdat.riad@yahoo.com
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

Various salt-fermented fish products are consumed worldwide, for example Surströmming in Sweden (Beddows 1997), Lona Ilish in Bangladesh and India (Majumdar et al. 2006) and Jeotgal in South Korea (Song et al. 2018). These products are source of important nutrients such as arginine, glycine, linoleic acid, eicosapentaenoic acid, docosahexaenoic acid, calcium and potassium (Park et al. 1996; Song et al. 2018). Considering antimicrobial activity, probiotic and organoleptic properties, antioxidant and bioactive peptides, fermented foods are often better than simple foods (Sharma et al. 2020). Salt-fermented hilsa (*Tenualosa ilisha*); locally known as Lona Ilish is very popular in Bangladesh and north-east India (Majumdar et al. 2006). This food is considered as delicacy and relished by people through different traditional recipes (Nowsad 2007). About 100 years ago the technology of salt-fermentation of hilsa was originated on the shore of Padma and Meghna river in Bangladesh due to an urge to preserve hilsa in glut catch period (Majumdar & Basu 2010). Salt-fermented hilsa is processed during June to September. Unlike drying, freezing and smoking, salt-fermentation process does not depend on sunny weather, electricity, expensive freezing equipment and provide relatively a long shelf life to product. There are two methods of salting- dry and wet salt-fermentation. In dry salt-fermentation, salt is mixed well with diagonally cut hilsa chunks (thickness 0.75–1.0 cm) and stored in bamboo baskets. In wet salt-fermentation process, either hilsa chunks are dry-salted and then placed in saturated brine solution or dressed whole hilsa is directly immersed in saturated brine solution and kept in a tin container (Nowsad 2007). In salt-fermentation processing, due to osmosis, salt and water exchange occurs within cells until endosomatic pressure exerted from water binding complex created by protein, sodium and chlorine ions and exosomatic pressure of surrounding salt concentration reach to an equilibrium. Ripening; also known as maturation, is the equilibrium state where due to completion of physico-biochemical processes, characteristic flavor and texture fully develop in a salt-fermented product and the product become ready for consumption (Horner 1992). For ripening of Hilsa, 7–15 days are required (Nowsad 2007).

Based on amount of salt used in fermentation, the proximate biochemical composition varies accordingly (Bakhiet & Khogalie 2012). After salting, although Hilsa may lost up to 20% of initial weight as muscle water leach out but within 8–12 days regain weight by salt uptake. Exudates released from salt-fermented fishes, drain out some water-soluble proteins (Nowsad 2007). Increase of salt concentration in fish muscle lead to protein denaturation, which in turn may alter protein properties of such as solubility (Feiner 2006). In ripe product, proteolysis and lipolysis generated compounds are predominantly found (Nowsad 2007). According to Jones (1962), the flavor of wet salted fish is substantially influenced by the Millard browning reaction.

Lipid oxidation and lipolysis greatly affects the quality of salt-fermented products, even in less fatty fishes (Mariutti & Bragagnolo 2017). Due to lipid oxidation, less lipid content is found in salt-fermented fish than fresh one (Alsaban et al. 2014). Lipid oxidation products further interact with proteins and degrade amino acids, generate carbonyl group which cause polymerization, thus reduce protein solubility and affect digestibility (Abraha et al. 2018). Studies on dry salt-feremnetd hilsa has been only limited to the following literatures-Majumdar et al. (2005), Majumdar et al. (2006), Nowsad (2007), Majumdar and Basu (2010), Mukit et al. (2016) and Sarkar et al. (2023). Among these studies, except Majumdar et al. (2006) there is no information on how much change occurred in nutritional constituents content in the course of dry salt-fermentation and nutrition finally remain available in the substrate at the end of processing is whether acceptable or not for human consumption. These aspects are significant form the viewpoint of food science and nutrition. Therefore, one of the objectives of this study was to observe changes of quality characteristics in nutritional constituents; especially changes occur in protein and lipid content with progression of time of dry salt-fermentation processing. To fulfill this objective, proximate biochemical composition, total protein content, protein solubility, in-vitro digestibility and TBARS (Thiobarbituric acid reactive substances) value were estimated at different stages during salt-fermentation of hilsa.

Appearance of appealing and trademark flavors of salt-fermented fish products are usually attributed to aeromatic compounds, small peptides and free amino acids produced by the catabolic activity of bacterial enzymes (Lee 1989). Bacterial communities regulate the maturation time and properties of fermented fish products (Alegria et al. 2009; Tu et al. 2010). It can be assumed that through competitive successions some bacterial communities establish themselves as final microbiota in the salt-fermented products. Although reports on bacterial communities found in final salted product is available, but no such information about what bacterial species become dominant at different stages of salt-fermentation is not studied yet. Conventional bacteria culture methods are unable to represent all bacteria remain present in a sample. In such circumstances, next generation sequencing (NGS) technology is appropriate, reliable and rapid. So, another aim of this reseach work was to 16S rRNA gene sequencing based metagenomics study of salt-fermented T. *ilisha* at different stages of ripening. To achieve this objective, high-throughput sequencing was performed to analyze bacterial community at different stages during salt-fermentation of hilsa.

Materials and methods

Sample collection

The processors from whom dry salt-fermented hilsa samples were collected, generally spend 15 days for ripening. At three different time interval during dry salt-fermentation which will cover the whole processing period, hilsa samples were collected randomly from Asadganj Shutki Palli (dried and salt-fermented fish products market), Chittagong, Bangladesh. The time interval/stages were; namely, initial salt-fermented fish (after 2 days of salting; F1), interim salt-fermented fish (after 5 days of salting; F2), ripe salt-fermented product (final product after 15 days of salting; F3). After mixing with salt, the processors kept the underprocessed hilsa in bamboo basket at ambient temperature and humidity. Mouth (top, open end) of bamboo basket is covered with gunny sack. Samples were transported to laboratory aseptically in iced condition and stored in -18 °C until further analysis.

Assessment of changes in nutrient constituents

Proximate biochemical composition analysis

Methods from AOAC (2002) was applied to determine crude protein, lipid, moisture and ash content. For crude fiber measurement, 1 g moisture and lipid free sample was digested with 150 ml of 0.1 M H_2SO_4 in a hot extraction unit (FT 122 Fibertec™, Foss, Denmark) for 30 min. Then sample was washed with 30 ml distilled water and again digested with 150 ml 2.0 M KOH for 30 min. The samples were dried at 130 °C for 2 h and then burnt at 500 °C for 3 h. Difference in weight of sample before and after ashing determined the fiber content. The following formula was used to calculate the carbohydrate content.

$$\%\ Carbohydrate = \{100 - (\%protein + \%lipid + \%moisture + \%ash + \%fibre)\} \times 100$$

Total protein content, protein solubility and in-vitro digestibility

Total protein content was determined by biuret method (Nowotny 1979). Fish muscle (5 g) was homogenized with phosphate buffered saline (Sigma-Aldrich, India) (1:1) and filtered with Whatman No. 1 filter paper. As working standard, 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of bovine serum albumin (Sigma-Aldrich, India) (5 g/ml) were used. Distilled water was added to the test tubes contained bovine serum albumin to make total volume 1 ml and then 3 ml biuret reagent

(Sigma-Aldrich, India) was added. After vortexing and incubating at 37 °C for 10 min, absorbance was recorded against 540 nm using Shimadzu UV-1601PC (Japan) spectrophotometer. Protein solubility was tested by the method described by Dewi 2002. For *in-vitro* digestibility test of salt-fermented fish protein, 1.5 mg pepsin and 15 ml 0.1 N HCl were added to 100 mg sample and incubated at 37 °C for 3 h. Then, 1 ml of toluene, 7.5 ml phosphate buffer containing 4 mg pancreatin (pH 8.0) and 0.2 N NaOH were added and incubated the solution for 24 h. After 24 h, 10 ml of 10% trichloroacetic acid was added to inactivate the enzymatic activity and undigested protein precipitated. After filtering out the precipitation, 100 ml of solution was collected, centrifuged at 5000 rpm for 30 min and nitrogen content of the supernatant was determined according to AOAC (2002).

TBARS (Thiobarbituric acid reactive substances) value estimation

Muscle sample (10 g) was taken from different parts of hilsa and then homogenized with 50 ml distilled water using a tissue homogenizer (Witeg HG-15D, Germany) for 2 min and then after addition of 50 ml 10% TCA (trichloroacetic acid) homogenized again. Homogenized muscle slurry was filtrated using Whatman No. 1 filter paper. To 8 ml of clear filtrate, 8 ml 2.9 mg/mL (w/v) thiobarbituric acid was added and incubated at 100 °C for 40 min. Absorbance was recorded at 530 nm in contrast to control sample using Shimadzu UV-1601PC (Japan) spectrophotometer. A solution of thiobarbituric acid (5 ml) and distilled water (5 ml) was used as control.

High-throughput sequencing and bacterial community analysis

The sliced tissue with skin was transferred to an extraction buffer (100 mM Tris–base, 100 mM EDTA, 1.5 M NaCl, pH 8.0) and then stored at -80 °C. The tissue with skin from hilsa of three salt-fermentation stages with three replications was chosen for metagenomic analysis. Samples were centrifuged at 1600 rpm for 4 min and then supernatant was collected and used at 11,000 rpm for 30 min to get the pellet. The pellet was mixed with double distilled water (ddH_2O) to dilute. This mixture was used for bacterial DNA extraction by combining Phenol–Chloroform-Isoamyl Alcohol (PCI), SDS and CTAB-DNA isolation protocol with minor changes (Larsen et al. 2014; Tina et al. 2014). The DNA quality and quantity were measured by 1% agarose gel and nano spectrophotometer. Isolated DNA was analyzed by Illumina NovaSeq 6000 platform. 16S rRNA gene (V4 region) was amplified by a specific primer (515F-806R) with the barcode. Raw sequences have been submitted in SRA, NCBI (Accession number: SRX15741855-SRX15741863). Samples were assigned as paired-ended reads based on their unique barcode and truncated by cutting off the barcode and primer sequence. The quality data were filtered according to the Cutadapt (Martin 2011). The UCHIME algorithm was used to eliminate the chimera sequence (Edgar et al. 2011). The Uparse software performed sequences analysis and ≥ 97% similarity was assigned to the same OTUs (Edgar 2013). The representative sequence for each OTU was screened for further species annotation by Silva Database (Quast et al. 2013). Multiple sequence alignments were performed using the MUSCLE software to study the phylogenetic relationship of the difference of the dominant species in hilsa at different satges of dry salt-fermentation (Edgar 2004). The α and β-diversity were calculated based on Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2010). LDA effect size (LEfSe) and biomarkers were identified to investigate differentially bacterial taxa abundances up to the genus level (Segata et al. 2011). PICRUSt aligned the sequences in the Greengene database to get the bacterial metabolic functions (Langille et al. 2013). Bacterial functional data were mapped and annotated at different KEGG levels (Kanehisa et al. 2016).

Statistical analysis

Data were presented as mean ± SD (standard deviation). One way analysis of variance followed by Duncan's multiple range test was conducted at 5% level of significance. Microsoft excel (2013) was used to calculate and visualize data of experimentations to assess amount of nutrient constituents changed.

Results

Change of nutrient constituents in dry salt-fermented hilsa

Proximate biochemical composition

Proximate biochemical composition of salt-fermented hilsa at different stages of dry salt-fermentation has been presented in Fig. 1. Protein content in F1 (after 2 days of salting) was 14.56% which varied significantly with F2 (after 5 days of salting) (14.88%) and in the final product protein content decreased to 13.28%. Lipid content varied significantly during all the salt-fermentation stages. Crude lipid content of F1 (after 2 days of salting) was 25.78% which decreased to 23.49% in F2 (after 5 days of salting), but a drastic change was observed in case of F3 (ripe product after 15 days of salting) where lipid content declined to 10.64%. A wide variation in moisture content was observed between F1 (after 2 days of salting) (50.96%) and F2 (after 5 days of salting) (43.93%), but in comparison to that, less variation was found between F2 (after

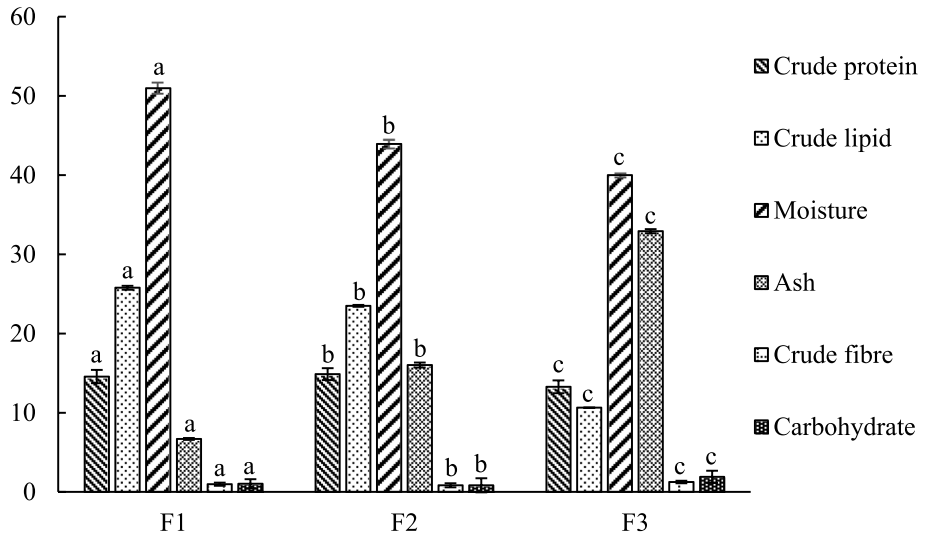


Fig. 1 Change in proximate biochemical composition of *T. ilisha* at different stages of salt-fermentation ($n=5$)

5 days of salting) (43.93%) and F3 (ripe product after 15 days of salting) (40%). Ash content varied about 2 to 5 times among F1 (after 2 days of salting) (6.69%), F2 (after 5 days of salting) (16.02%) and F3 (ripe product after 15 days of salting) (32.92%). Crude fiber content varied significantly among F1 (after 2 days of salting) (0.98), F2 (after 5 days of salting) (0.84) and F3 (ripe product after 15 days of salting) (1.25%). Carbohydrate content at F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) stages was found 1.03%, 0.84% and 1.91%, respectively.

Total protein content, protein solubility and in-vitro digestibility

Changes of protein content in hilsa subjected to dry salt-fermentation has been presented in Table 1. Total protein content in F1 (after 2 days of salting) was 10.14 mg/ml, which in next 3 days increased to 22.30 mg/ml in F2 (after 5 days of salting), then in the next 10 days total protein content reduced to 16.21 mg/ml in F3 (ripe product after 15 days of salting). In case of protein solubility, a significant decreasing trend was found with progression of salt-fermentation ripening process. Protein solubility of F1 (after 2 days of salting) was 0.9 mg/ml sample, which decreased to 0.54 mg/ml in F2 (after 5 days of salting) and further decreased to 0.40 mg/ml in F3 (ripe product after 15 days of salting). Throughout the study period *in-vitro* protein digestibility was high. About 98% of total protein was digestible throughout different dry salt-fermentation stages.

Lipid oxidation at different stages of dry salt-fermented Hilsa

TBARS (Thiobarbituric acid reactive substances) values observed during the study period has been shown in Table 2. TBARS value of F1 (after 2 days of salting) samples was 0.30 mg MDA/kg, which increased to 0.41 mg MDA/kg in F2 (after 5 days of salting) samples and then abruptly increased to 0.95 mg MDA/kg in F3 (ripe product after 15 days of salting) samples. So, lipid oxidation occurred throughout the study period but the rate was of lipid oxidation was higher toward the end of ripening process.

Change in microflora of dry salt-fermented hilsa

Microbial composition of the dry salt-fermented hilsa

Microbial communities of salted hilsa fish (*Tenualosa ilisha*) using a sequence-based assessment of the 16S rRNA gene (V4 region) at different stages; namely, initial

Table 1 Quantity and quality of hilsa protein at different stages of dry salt-fermentation ($n=5$)

Stages of dry salt-fermentation	Total protein content (mg/ml sample)	Protein solubility (mg/ml sample)	*In-vitro* digestibility (%)
F1 (after 2 days of salting)	10.14 ± 0.21^{c}	0.9 ± 0.05^{a}	98.03 ± 0.04^{a}
F2 (after 5 days of salting)	22.30 ± 0.03^{a}	0.54 ± 0.02^{b}	98.00 ± 0.12^{a}
F3 (ripe product after 15 days of salting)	16.21 ± 0.01^{b}	0.40 ± 0.09^{c}	97.98 ± 0.05^{b}

Different superscripts ($^{a, b, c}$) are significantly different ($P<0.05$)

Table 2 Lipid oxidation in hilsa at different ripening stages of dry salt-fermentation

Stages of dry salt-fermentation	TBARS value (mg monoaldehyde/kg)
F1 (after 2 days of salting)	0.30 ± 0.07^{c}
F2 (after 5 days of salting)	0.410 ± 0.02^{b}
F3 (ripe product after 15 days of salting)	0.95 ± 0.05^{a}

Different superscripts ($^{a, b, c}$) are significantly different ($P < 0.05$)

salt-fermented fish F1 (after 2 days of salting), interim salt-fermented fish F2 (after 5 days of salting) and ripe salt-fermented product F3 (ripe product after 15 days of salting) was investigated. The eDNA was collected from salt-fermented product and PCR-free libraries were constructed based on the Illumina Nova sequencing platform, followed by paired-end sequencing.

Using reads splicing, an average of 82,231 tags per sample was measured and an average of 79,945 valid data was obtained after cutting and filtering the sequence reads using a paired-end sequencing method (Fig. 2A). A total of 3,248 OTUs (Operational Taxonomic Units) were attained from 97% sequences clustered match with the Silva138 database for species annotation. The annotated proportion to the boundary level was 100.00%. In the annotation results, of which 2,273 (69.98%) were annotated to the kingdom level. The annotation ratio to the phylum, class and order level was 56.59%, 56.13% and 54.16%, respectively. Furthermore, the proportion of annotations at the level of family, genus and species was 48.92%, 37.81% and 10.59%, respectively. In the annotation results, a total of 1,228 OTUs were annotated at the genus level using the Silva138 database. The samples' rarefaction curves were drawn that reached a plateau at 44,000 reads (Fig. 2B). According to genus annotations, the additional calculations were performed on the α-diversity and the β-diversity of microbiota and their community structures were compared. The Venn diagram shows the unique and shared OTUs among initial, interim and ripe dry salt-fermented products microbiota. A total of 508 shared OTUs were identified among the groups. The highest 1,123 unique OTUs and lowest 379 unique OTUs were found in ripe and initial salt-fermented products, respectively (Fig. 2C and D).

Succession, diversity and richness of microbial communities

A total of 48 phyla, 121 classes, 46 order, 381 bacterial families and 636 genera were identified from the all samples. Highest 40 phyla were identified from ripe stage whereas 29 phyla were identified from initial stage. In the initial stage, Proteobacteria were abundant, but the Firmicutes replaced the both Proteobacteria and Bacteroidota in the final salt-fermented product. Proteobacteria (66%), Firmicutes (18%) and Bacteroidota (11%) were abundant at F1 (after 2 days of salting), similar abundance is also seen at F2 (after 5 days of salting). But, at F3 (ripe product after 15 days of salting) Firmicutes (82%) replaced other bacteria as the most abundant one with the Proteobacteria (9%) (Fig. 3A).

The highest 491 genera were identified from ripe stage, while 376 genera were identified from initial stage. The heat map exhibited a relative percentage of each abundant bacterial genus from the different stages of salt-fermentation (Fig. 3B). The abundance of *Enterobacter* (37%), *Shewanella* (11%), *Myroides* (8%) and *Kurthia* (6%) were higher at initial stage, but at the ripe stage, the abundance of *Cohnella* (11%), *Bacillus* (10%), *Enterobacter* (4%) and *Pseudarcobacter* (1%) were higher. Ripe salt-fermented product harbored more probiotic properties containing bacteria than the initial salted product, including *Bacillus*, *Bifidobacterium*, *Lactobacillus*, *Ligilactobacillus*, *Paenibacillus*, *Streptococcus*, *Enterococcus*, *Paracoccus* and *Limosilactobacillus*. Zoonotic *B. anthracis* was found in the final product.

The bar plot showed the relative percentages of fourteen major beneficial probiotic bacterial genera at the genus level at different stages of salt-fermentation of hilsa (Fig. 3C). *Lysinibacillus* and *Solibacillus* were dominant at F1 (after 2 days of salting) stage, where *Anoxybacillus* and *Brevibacillus* were the abundant at F2 (after 5 days of salting) stage, but *Bacillus* alone was differentially predominant at F3 (ripe product after 15 days of salting) stage. Bacterial circle graph showed the relative abundance of the 100 bacterial genera at different stages of salt-fermentation clustered at 97% sequence similarity (Fig. 4A). Moreover, the bacterial classification tree displayed the relative microbial abundance at different stages of salt-fermentation up to the species level (Fig. 4B).

(See figure on next page.)

Fig. 2 The number of total tags, taxon tags, unique tags, and OTUs were shown based on sample where F2R3 contained the top taxon tags and total tags. In addition, F3R2 harbored a higher 1,190 OTUs (**A**). Rarefaction analysis of the distinctive samples where OTUs rarefaction curves clustered at 97% sequence identity of the 16S rRNA gene. The OTUs (**B**) and observed spp. number (**C**) were higher in ripe than the initial salt-fermented product. The Venn diagram showed the unique and shared OTUs among the groups. A total of 508 shared OTUs were identified. The initial, interim, and ripe salt-fermented products harbored 1,123, 598, and 379 unique OTUs, respectively (**D**)

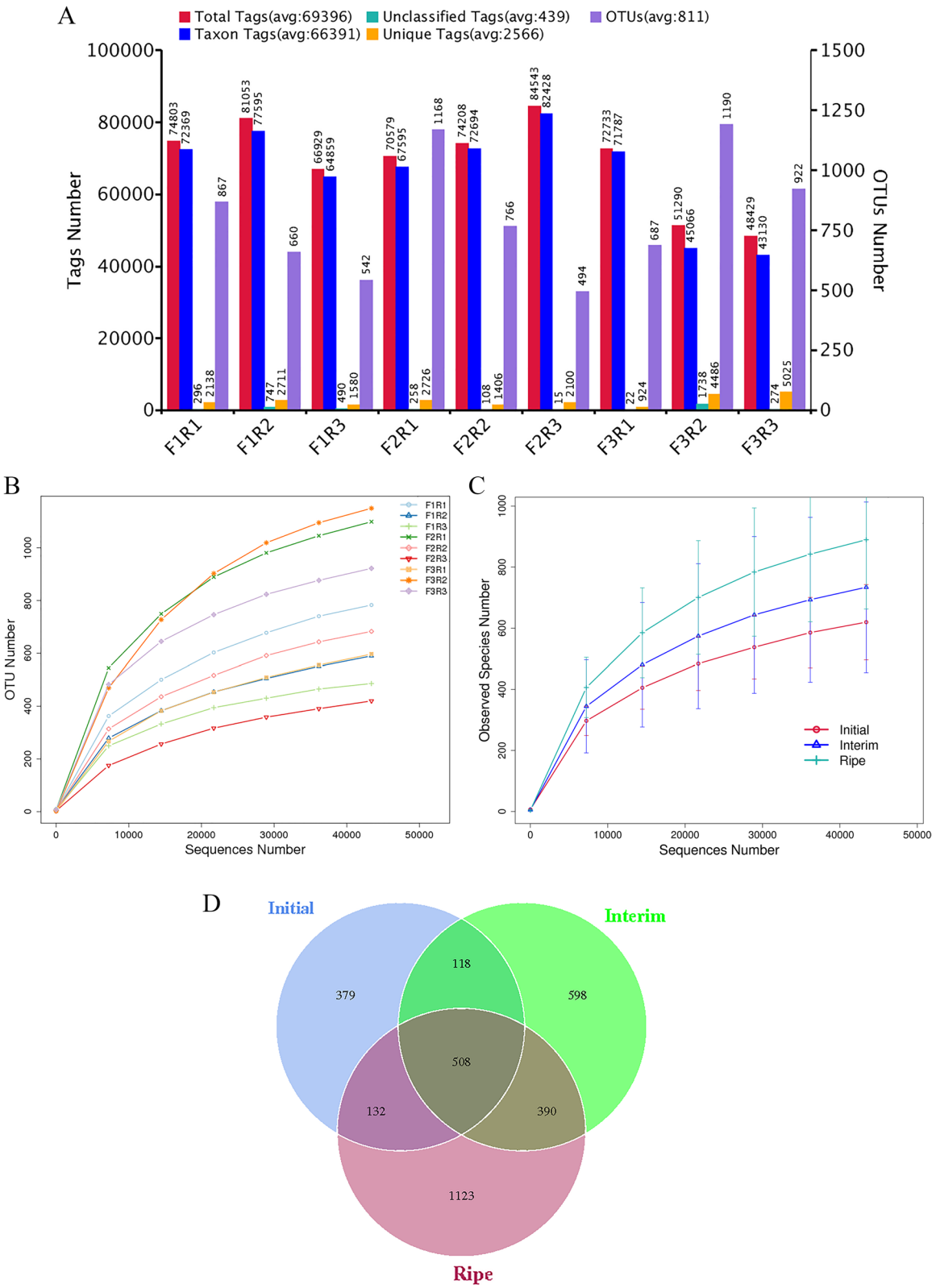


Fig. 2 (See legend on previous page.)

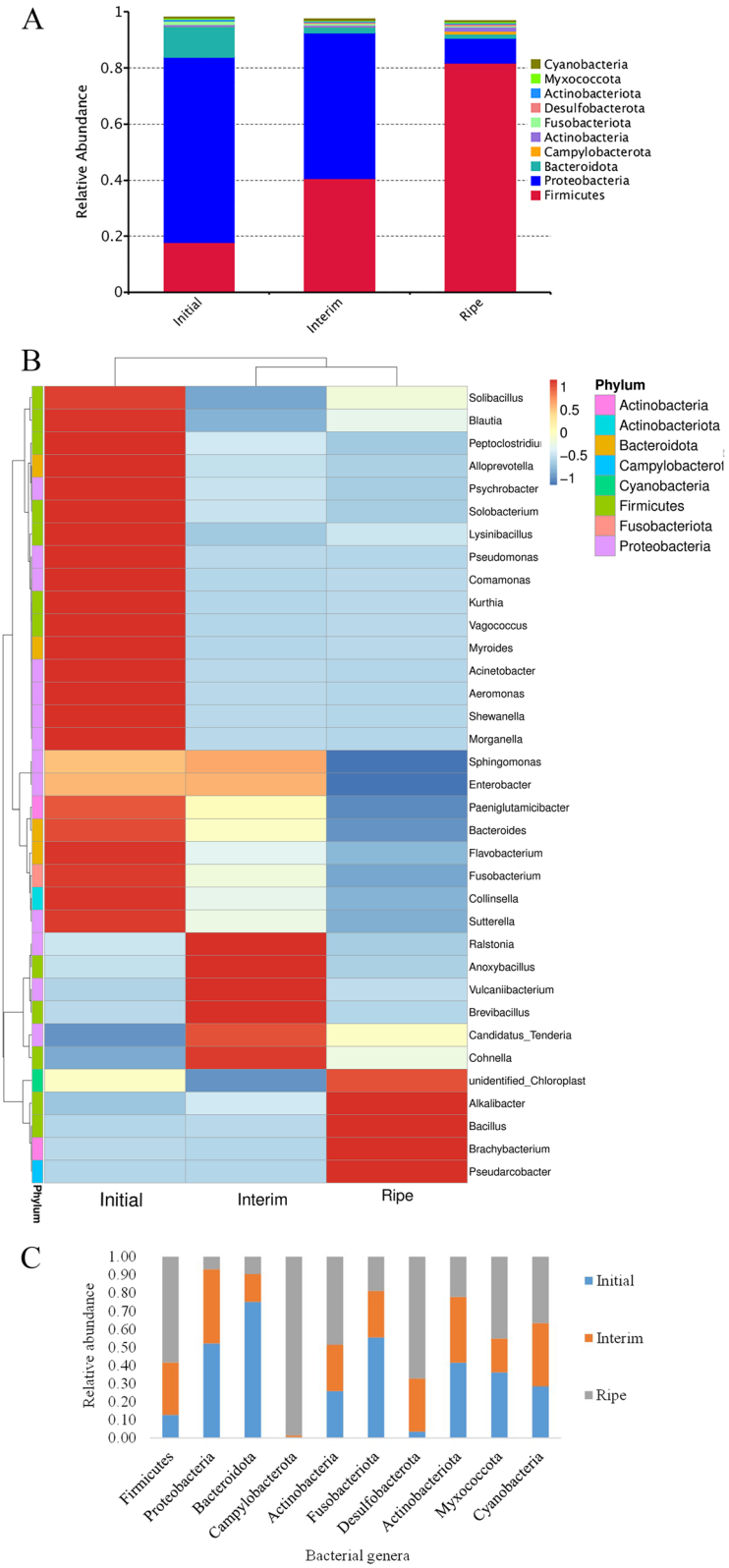


Fig. 3 The bar plot showed the relative abundance of bacterial phyla within the different groups of salt-fermented products (**A**). The heat map displayed the relative percentage of each bacterial genera, and the longitudinal and horizontal direction were the sample information and species annotation. The color intensity represents the corresponding values for the bacterial genus with the legend (**C**). The bar graph displayed the relative percentages of beneficial probiotic bacteria at the genus level in salt-fermented Hilsa (**B**)

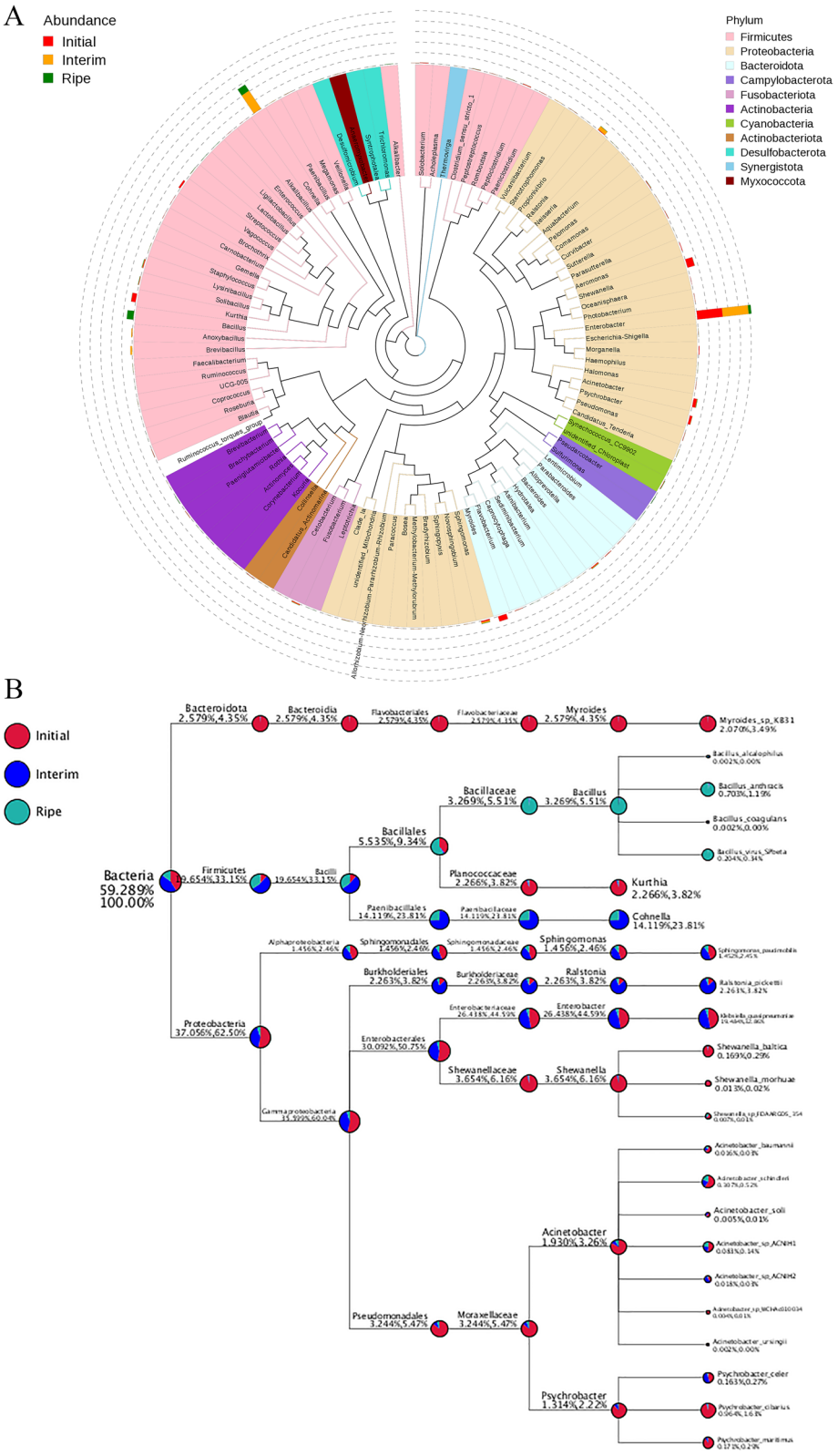


Fig. 4 A clustered circular diagram at 97% sequence similarity showed the relative abundance of the 100 most abundant genera of different groups of bacteria in salt-fermented product. In the circular phylogenetic tree, the color of the branch and the fan shape showed its corresponding gate, and the stacked column set outside the fan ring indicated the abundance of genus (**A**). The microbial taxa tree represented the most abundant bacterial taxa up to the species level. The circle size and color represented the relative abundance and groups, respectively (**B**)

According to the Shannon index, the bacterial diversity of the initial salt-fermented product was higher, but the bacterial richness and evenness of the ripe salted product were higher according to Chao1 index and Simpson index, respectively. The result also justified using ACE index (Table 3). Overall, the initial salt-fermented product harbored a higher abundance of microbes than ripe salted product. After salt-fermentation, similar types of beneficial bacteria were enriched in the ripe salted product. In general, there was no significant difference in the microbiota present in initial, interim and ripe salt-fermented products according to α-diversity index ($p < 0.05$). Based on AMOVA (Analysis of MOlecular VAriance) at $p < 0.05$, significant differences in the microbial β-diversity among initial, interim and ripe salted products was observed.

There were differences in β-diversity at different stages of salt-fermentation, in which bacterial β-diversity in mature salted product was gradually higher than that in interim and initial salted product (Fig. 5A). The β-diversity was higher within interim group, which was significantly different from microbiota of the initial salted product, but not the microbiota of the ripe salted product (Fig. 5B). The UPGMA clustering of groups was calculated using the weighted unifrac distance, where microbiota from initial and interim groups together formed cluster (Fig. 5C). A similarity analysis (ANOSIM) showed that differences between groups were higher than differences within groups. ANOSIM showed that diversity was higher in the interim and ripe compared to the initial product (R = 0.41 & 0.48). Microbiota was more diverse in inter group than intra-initial and intra-ripe salted products. Moreover, microbial diversity was higher in the ripe salted product compared to interim salted product (Fig. 5D). A statistically significant variation was found between initial-interim and initial-ripe products at the genus level ($p < 0.05$). *Myroides*, *Morganella*, *Aeromonas*, *Clostridium* and *Pseudomonas* were less in the interim salted product compared to the initial product (Fig. 5E). *Enterobacter*, *Myroides*, *Morganella*, *Aeromonas* and *Vibrio* were less in the ripe than the initial salted product (Fig. 5F).

Analysis of possible microbial function predicted by PICRUSt

PICRUSt analyzed the microbial functions at different stages of salt-fermentation. According to KEGG ortholog (KO) level-1 functional categories, microbial genes related to organismal systems, genetic information processing and cellular processes were higher in ripe product which replaced the human disease related genes at the initial salt-fermented product (Fig. 6A). The microbial genes related to infectious disease, immune system and metabolism were enriched in the initial product. Besides, lipid metabolism, membrane transportation, cancer and neurodegenerative disease related genes were higher in the interim product. However, in the ripe salted product, genes related to energy metabolism, cell motility, endocrine system and translation were enriched according to KO-2 functional categories (Fig. 6B). The microbial abundances were different between initial and ripe salt-fermenteded product and formed different cluster in the ordination table according to Principal Coordinate Analysis (PCoA) rank of the Bray–Curtis distances using weighted unifrac (Fig. 6C).

Discussion

Change in nutrient constituents of dry salt-fermented hilsa

Proximate biochemical composition

All the nutritional constituents significantly changed during salt-fermentation processing. In this study, 14.56%, 14.88% and 13.28% crude protein content was found in F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting), respectively. Majumdar et al. (2005) reported 17.56% protein in ripe salt-fermented *T. ilisha*. Proximate biochemical composition in fresh *T. ilisha* varies upon size, age, sex, geographical location of catch etc. (De et al. 2019). For this reason crude protein content detected in ripe product in this study varied from protein content determined by Majumdar et al. (2005). In this study, protein content at three different stages of dry salt-fermentation did not changed very much. So, in regard to protein content finally remain available in processed hilsa, salt-fermentation technology is acceptable.

Crude lipid content in F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) was 25.78%, 23.49% and 10.64%, respectively. So, after completion of salt-fermentation processing lipid content reduced about 2.5 times than initial *T. ilisha* samples F1 (after 2 days of salting). This phenomenon can be explained by leaching out of lipid content with water due to osmotic effect of salt. Majumdar and Basu (2010) found 9.41% lipid content in ripe salt- fermented *T. ilisha* which is consistent with the finding of this present study.

Moisture content of F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) was 50.96%, 43.93% and 40% while ash content was 6.69%, 16.02% and 32.92%, respectively. As salt-water osmotic interaction goes on in salt-fermentation process due to bipolarity characteristic of water and chemical potential difference of organic systems, so, salt enters into the muscle cells and simultaneously water drains out, which resulted in reduction in moisture and increase in ash content throughout the ripening period. Shenderyuk and Bykowski (1990) described that, in wet salt-fermentation processing,

Table 3 α-diversity of bacteria in *T. ilisha* at different time of dry salt-fermentation

Group	Shannon	Simpson	Chao1	ACE	Goods cov.	Observed Spp	PD whole tree					
Initial	4.67	0.89	814.46	851.71	1.00	619.00	166.64					
Interim	3.41	0.72	907.49	932.30	1.00	734.00	244.23					
Ripe	2.99	0.52	1083.99	1069.57	0.99	889.00	174.95					
Group differences	Shannon		Simpson		Chao1		ACE		Goods cov.		Obs. Spp	
	Difference	*P* value	Difference	*P* value	Difference	P value	Difference	*P* value	Difference	*P* value	Difference	*P* value
Initial—Interim	2.00	0.44	3.33	0.14	-1.00	0.70	-1.00	0.70	0.33	0.89	-1.00	0.67
Initial—Ripe	2.00	0.44	3.67	0.11	-2.00	0.45	-2.00	0.45	2.17	0.37	-3.00	0.23
Interim—Ripe	0.00	1.00	0.33	0.87	-1.00	0.70	-1.00	0.70	1.83	0.44	-2.00	0.41

fish starts to absorb water from brine and this water hydrolyzes proteins compounds. Along with leaching out of water, reason described by Shenderyuk and Bykowski (1990) may play a role in reduction of moisture content. Such reduction in moisture content directly affects water activity. Water activity governs food properties e.g. texture of final product (Offer & Trinick 1983) and dynamics in microbial composition (Martins et al. 2020). Mukit et al. (2016) reported similar finding that, after 6 days of salt-fermentation while the moisture content was reduced, simultaneously ash content was increased. Wheaton and Lawson (1985) and Hernandaz-Herrero et al. (2002) found similar pattern in dry salt-fermented anchovy. Majumdar et al. (2006) reported that moisture content in fresh hilsa and in dry salt-fermented hilsa at day 0, 15 and 30 days after salt application was 55.55%, 46.46%, 42.46% and 44.86%, respectively and inferred that, increase in moisture content in hilsa at day 30^{th} was due to absorption of moisture content in prolonged storage in brine.

Similar to this present study, Majumdar et al. (2006) found increase in ash content during dry salt-fermentation of hilsa. Increase in ash/mineral content and/or salt used in fermentation process, might somehow affect hypertension patients (Sivashanthini et al. 2012). Like other animals, fish generally contain very low content of carbohydrate and fiber content. This study has been presented crude fiber and carbohydrate content of dry salt-fermented hilsa for the first time ever.

In maintenance of proper diet and health, it is important to know carbohydrate and fiber content of a food. In available literature, there is no established reason why carbohydrate and fiber content in salt-fermented fish products vary at different stages during processing. Variation in carbohydrate content is may be due to microbial activity as because bacteria utilize carbohydrate as energy source during fermentation. Therefore endogenous or exogenous enzymatic activity or other complex chemical reactions occur during fermentation may be the reason for variation in carbohtdrate and fiber content in hilsa at different stages of salt-fermentation. Overall, dry salt-fermentation did not adversely affected proximate biochemical composition of hilsa.

Total protein content, protein solubility and in-vitro digestibility

In crude protein estimation, the nitrogen content from both protein and non-protein sources are calculated. Thus, crude protein provides a rough idea about protein content of a food. In this regard, total protein content estimted by biuret test determines actual protein content. Total protein content in F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) samples was 10.14, 22.30 and 16.21 mg/ml, respectively. Different chemical changes e.g., protein denaturation and various compounds e.g. free amino acids, alpha-amino acids, small peptides, non-protein nitrogen, volatile basic nitrogen formed in ripening process. Zhao et al. (2022) reported that in salt-fermented grass carp proteins larger than 100 kDa were most abundant. Dynamics of these chemical changes is not well-understood yet (Majumdar et al. 2006). Although no literature is available on why total protein content increased in F2 (after 5 days of salting) samples and decreased in F3 (ripe product after 15 days of salting) samples, but similar trend was also observed by Mathew and Raghunath (1996) and Majumdar et al. (2006). Liu et al. (2021) reported that use of selected strain of *Lactobacillus* in Chinese Suanyu fermentation improve nutritional profile by increasing non-protein nitrogen (NPN) and total free amino acid (FAA) contents.

Protein solubility of F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) samples was 0.9, 0.54 and 0.40 mg/ml, respectively. Decrease in protein solubility is reported by Meinke et al. (1972). At average salt concentration (10%) when electrolyte concentration reaches 2 M, bound water of myofibrillar protein decreases, which consequently lead to protein denaturation, protein precipitation and loss of solubility (Duerr & Dyer 1952). Towards the end of ripening process, as fish becomes saturated with salt, thus, in comparison with F1 (after 2 days of salting) and F2 (after 5 days of salting) samples, rate of decrease in protein solubility was slow in F3 (ripe product after 15 days of salting) samples. At more than 12% salt concentration such proteolysis stops in fish (Bilinski & Fougere 1959).

(See figure on next page.)

Fig. 5 A difference in beta diversity was observed among groups (**A**). Beta diversity was higher within interim group and differed significantly from microbiota of the initial salt-fermented product (**B**). The UPGMA clustering of groups was calculated using the weighted unifrac distance. Here, the relative abundance of different bacterial phyla for different groups. 'Others' indicated that OTUs could not be classified with the database (**C**). Analysis of similarity (Anosim) showed that differences between groups were higher than differences within the groups. The microbiota was more diverse in intergroup than intra-initial and intra-ripe salted products (R = 0.48) (**D**). Statistically significant variation was found between initial-interim and initial-ripe at the genus level ($p < 0.05$) (**E** &**F**)

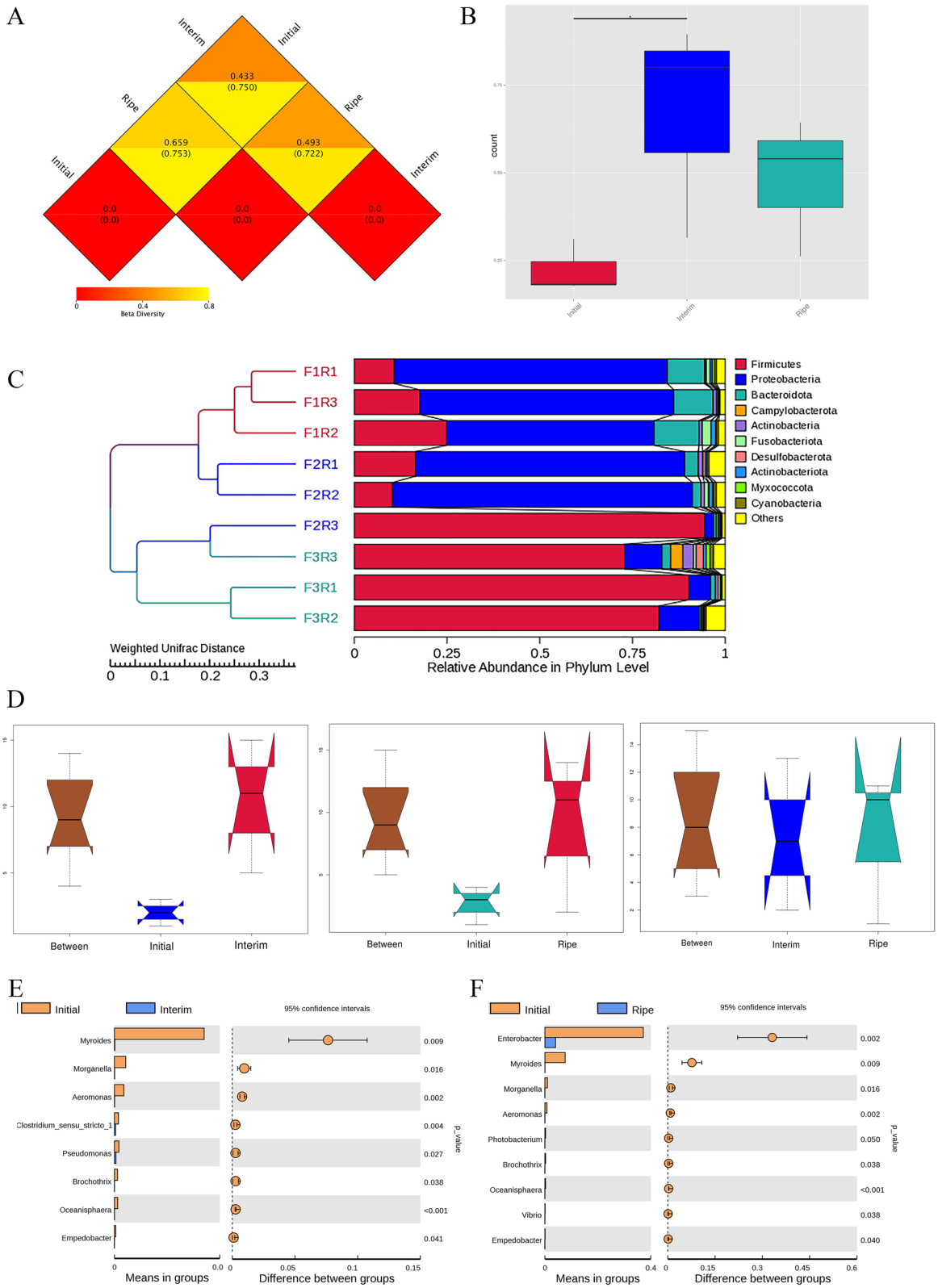


Fig. 5 (See legend on previous page.)

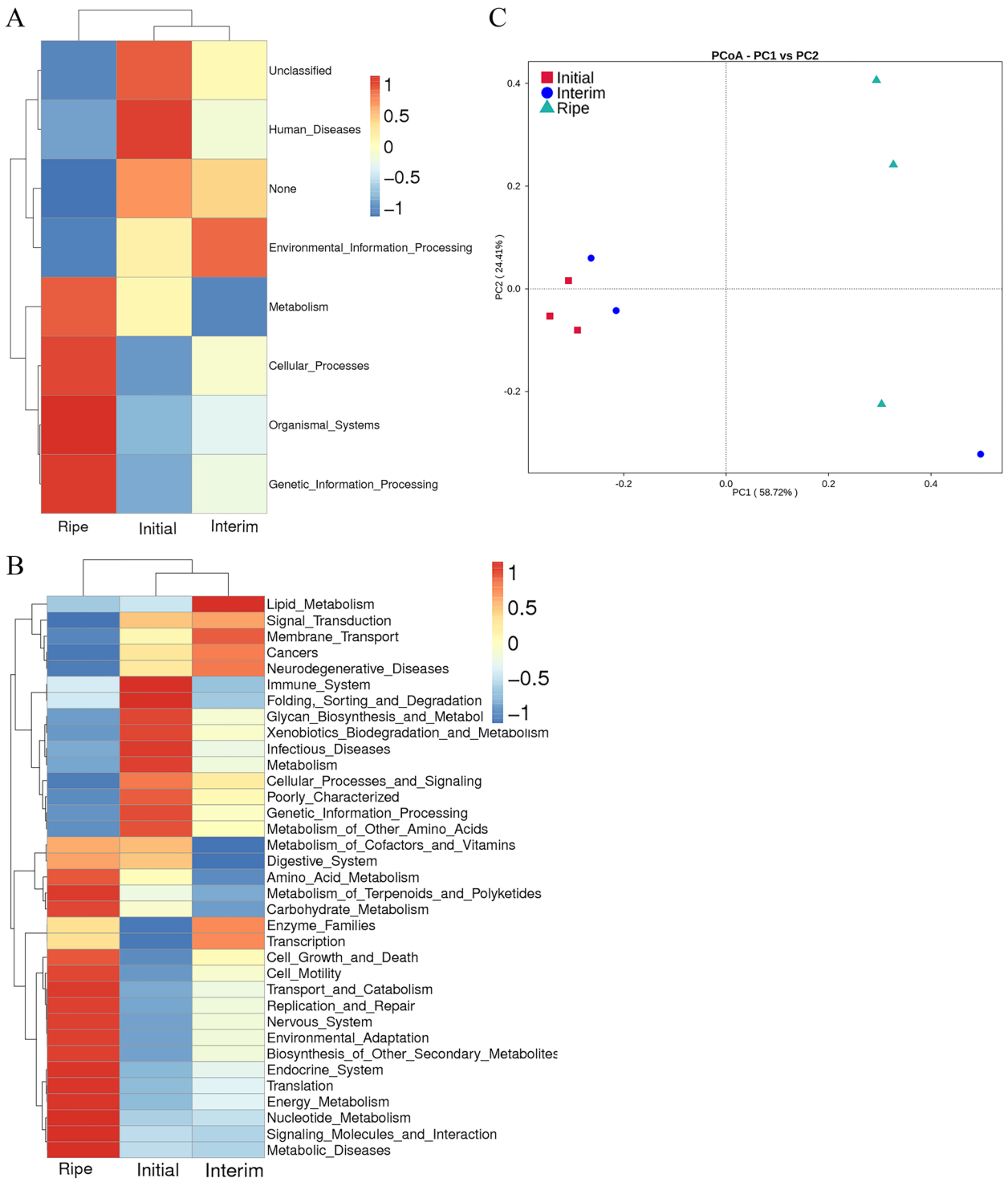


Fig. 6 The PICRUSt predicted the different metagenomics functions of microbiota among various salt-fermentation stages in the KEGG (KO-1) ortholog (**A**). The Predicted relative abundance of groups in KEGG level-2 by heat map plot, where the longitudinal and horizontal direction was the sample information and predicted bacterial functional annotation was based on the 16S rRNA gene. The color intensity represents the corresponding values for the gene function with legend (**B**). Group similarity and fingerprint variance were shown by Principal Coordinate Analysis (PCoA) rank of the Bray-Curtis distances using weighted unifrac. Each point on the graph represented a sample, and each group was marked with the same color in the figure. The distance between the points indicated the degree of difference (**C**).

In-vitro protein digestibility of F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) samples was 98.03%, 98% and 97.98%, respectively. As very little change was occurred among F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) samples, so, this variation is negligible, therefore, dry salt-fermentation did not adversely affect protein digestibility. Ismail (1996) reported that, after 2 days of wet-salting of morwong (*Nemadectylus macropterus*), shark (*Notogaleus rhinophanes*) and sardine (*Sardinops neopilchardus*), *in-vitro* protein digestibility was 99.7%, 98.2% and 98.l%, respectively.

Lipid oxidation at different stages of dry salt-fermented Hilsa

TBARS (Thiobarbituric acid reactive substances) value is the measure of secondary products produced from peroxide degradation e.g. monoaldehyde. TBARS value of F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) samples was 0.30 mg MDA/kg, 0.41 mg MDA/kg and 0.95 mg MDA/kg, respectively. Chaula et al. (2019) considered less than 6 mg MDA/kg as acceptable TBARS value for dried fishery products. Sarkar et al. (2023) reported 4.18 mg MDA/kg in dry-salt fermented hilsa collected from local market. Oxidation occurred for prolonged time during long term storage in retail marketing may be the reason for variation of TBARS value between this study and Sarkar et al. (2023) in dry salt-fermented hilsa. After 1 month of mixed salt-fermentation (both dry and wet salting) and dry salt-fermentaion, Nikiforova et al. (2020) found 1.2 mg MDA/kg and 1 mg MDA/kg TBARS value in Baikal omul (*Coregonus autumnalis migratorius*). Consumption of highly oxidized fish products might be responsible for brain dysfunction, heart disease, cancer and aging (Kinsella 1987). So, dry salt-fermented hilsa that is just ripened or has been stored for 4–5 weeks are safe for human consumption and expect to provide a pleasant and appealing aroma.

Change in microflora of dry salt-fermented hilsa

Microbial composition of the dry salt-fermented hilsa

This study reports the microbial composition in hilsa at different stages of dry salt-fermentation for the very first-time. In this study, average 623, 809 and 923 OTUs were found in F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting) samples, respectively. *Saeu-jeot* is salt-fermented small shrimp (*Acetes japonicus*) produced in Korea. Jung et al. (2013) reported 144, 134 and 200 OTUs in *Saeu-jeot* at 0, 5 and 15 days of salting, respectively. Every food has its own distinctive microflora. Leech et al. (2020) reported that microbiota of fermented food products varies with geographic location, processor, application of starter culture, consistency of food (solid/liquid/semi-solid) and substrate. Among the factors, substrate is the most determinant factor for microbial composition. For these reasons wide range of variation of OTUs in fermented fish products was found in existing literature. In this study, with progression of time OTUs were increased. *Kaburazushi* is a traditional Japanese sushi. Koyanagi et al. (2013) observed 4.2 times increase of bacterial cell number in *Kaburazushi* during 8 days fermentation period. So, increase of OTUs observed in this study is a common phenomenon.

Succession, diversity and richness of microbial communities

In this study, Proteobacteria and Firmicutes was most dominating phyla among all the stages. Salt-fermented *Jeotgal* is produced in Korea using anchovy (*Engraulis japonica*), sea squirt (*Halocynthia roretzi*) or shrimp (*Acetes japonicas*). In *Jeotgal* salt-fermentation processing, irrespective of raw material, Firmicutes and Proteobacteria was identified as most dominating species (Song et al. 2018). Lee et al. (2015) also observed dominance of Firmicutes and Proteobacteria in fish sauce known as *Myeolchi-aekjeot* in Korea. As we stated earlier that substrate is the main reason for why microbial composition varies, nevertheless in salt-fermentation processing some common bacteria is found. In different salt-fermented products, such common bacteria contribute similarly in ripening and flavor compounds formation through proteolysis and other biochemical reactions (Wang et al. 2022). Therefore, some common key characteristics are observed in different salt-fermented products and upon these common characteristics they are categorized as 'Salt-fermented' products. Jung et al. (2013) and Du et al. (2019) reported similar succession trend in *Saeu-jeot* as observed in this study. So, it can be inferred that, in salt-fermented fish products initial dominating bacteria become replaced by another dominating bacteria in final ripening stages. In case of genera wise distribution of bacteria, we have not found much similarity with existing literature except *Lactobacillus* was found in all fermented fish products (Koyanagi et al. 2011; Roh et al. 2009). Like this study Majumdar and Basu (2010) found *Bacillus* as a dominating genera in ripe product. In Thai fish sauce, halophilic *Bacillus* was found to be responsible for volatile fatty acids production. These volatile fatty acids are attributed to characteristic aroma and flavor of fermented fish products (Saisithi et al. 1966). Ji et al. (2017) also reported that *Streptococcus* and *Bacillus* are responsible for characteristic aroma and flavor of fermented fish

products through degradation of amino acids and production of aromatic compounds.

There was no significant α-diversity difference at individual stages of salt fermentation of hilsa. Jung et al. (2013) reported 3.07, 2.63 and 3.16 Shannon–Weaver index, while 216.73, 359.71 and 366.79 Chao1 index and 0.62, 0.54 and 0.60 Evenness for at 0, 5 and 15 days of salt-fermented *Saeu-jeot,* respectively. This result is more or less consistent with this study. Therefore it can be sated that, although substrate varies but some fermented fish products follow specific pattern i.e. all the products of a certain stage contain more or less similar bacteria. In this study, at the genus level significant ($p < 0.05$) β-diversity was observed among F1 (after 2 days of salting), F2 (after 5 days of salting) and F3 (ripe product after 15 days of salting). Du et al. (2019) observed variation in β-diversity among different sample groups taken at different times of salt-fermentation of fish sauce. From this study, although it is evident that what bacteria play role in fermentation at different stages and their community ecology, but to create cultured/tailored fermentation instead of wild fermentation, further research is required to understand their contribution in fermentation process e.g. characterization of catalytic activity of enzymes secreted by these bacteria to decompose substrate.

Analysis of possible microbial function predicted by PICRUSt

In this study, bacteria that might cause human disease was found in initial salt-fermentation stage. Jung et al. (2013) found *Vibrio*, *Photobacterium*, *Aliivibrio* and *Enterovibrio* at 0 days of salt-fermentation of *Saeu-jeot*. These bacteria are pathogenic but with progression ripening process, these bacteria gradually disappeared. With the progression of salt-fermentation, amino acid, nucleotide and carbohydrate metabolism increases (Wang et al. 2022). This statement supports the presence of genes associated with lipid metabolism, membrane transportation but there is no literature about genes associated with cancer and neurodegenerative diseases. Ji et al. (2017) reported that in fermented *Siniperca chuatsi* among 2,175 proteins, number of metabolic pathways related proteins were 1,217 and number of amino acid metabolism related proteins were 352. Zhang et al. (2016) reported that *Yucha* (fermented food made with cooked rice with fresh fish) fermentation, bacteria present in ripe *Yucha* was involved with following functions: energy generation and conversion, coenzyme transport and metabolism, inorganic ion transport and metabolism, lipid metabolism. In this study in the ripe salt-fermented hilsa, we also found bacterial genes associated with similar functions.

Conclusions

Throughout the study period, quality characteristics of nutritional constituents of hilsa changed significantly. Crude protein content decreased slightly whereas crude lipid content decreased drastically. There was an inverse relationship between moisture and ash content. Crude fiber and carbohydrate content of dry salt-fermented hilsa has been reported for the first time ever. High level protein digestibility observed and protein solubility was decreased gradually with progression of ripening. TBARS (Thiobarbituric acid reactive substances) value of final product was safe for human consumption.

Metagenomic study revealed that, dominant bacterial flora changed with progression of fermentation time. In the final salted product, zoonotic *B. anthracis* was found. Significant differences in the microbial β-diversity among initial, interim and ripe product were observed. There was no significant α-diversity difference at individual stages of dry salt-fermentation of hilsa. Functional gene profile category revealed that, in final product microbial genes related to organismal systems replaced human disease related genes found in initial and interim product. These findings provide new clues for in-depth characterization of salt-fermented fish products.

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Authors' contributions
Conceptualization: Md. Shirajul Islam Sarkar, Muhammad Mehedi Hasan, Md. Shahdat Hossain, Anas Al Islam Experimentation: Md. Shirajul Islam Sarkar, Md. Shahdat Hossain, Muhammad Mehedi Hasan, Murshida Khan, Md. Kamal Writing—visualization: Md. Shirajul Islam Sarkar, Md. Shahdat Hossain, Muhammad Mehedi Hasan, Murshida Khan, Anas Al Islam, Md. Kamal. Review-editing: Md. Shirajul Islam Sarkar, Md. Shahdat Hossain, Muhammad Mehedi Hasan, Murshida Khan, Anas Al Islam, Md. Kamal.

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There is no potential conflict of interest to disclose.

Author details
[1]Department of Fisheries Technology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh. [2]National Institute of Biotechnology, Ganakbari, Ashulia, Savar, Dhaka, Bangladesh. [3]Department of Fisheries Technology, Bangladesh Agricultural University, Mymensingh 2202,

Bangladesh. [4]Bangladesh Academy for Rural Development, Cumilla 3503, Bangladesh. [5]Chattogram Veterinary and Animal Sciences University, Khulshi, Chattogram 4225, Bangladesh.

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