Comparison of bacterial communities in gliadin-degraded sourdough (*Khamir*) sample and non-degraded sample

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Abstract The study was undertaken to investigate the comparison lying between bacterial communities in autochthonous gliadin-degraded sourdough sample (D13) and non-degraded sample (D50). Degree of gliadin degradation in various samples was determined by Fourier transform infrared spectroscopy and represented samples were selected for 16 S rDNA sequence analysis by Illumina Miseq platform. It was observed, that Proteobacteria (50.65%) and Actinobacteria (6.70%) phyla were more abundant as compared with Firmicutes (42.53%) in D13, however, Firmicutes (83.44) were more abundant, comparatively, in D50 than Proteobacteria (14.97%). Lower taxonomic levels surfaced its more prominent effects. It had been also observed that *Lactobacillus* genera was the core genera (50.37%) followed by *Weissella* (27.15%) and *Psychrobacter* (21.53%) in D50 and D13, respectively. Shannon and Simpson indices indicated that degraded

sample had more bacterial diversity and richness compared with non-degraded sample.

Keywords Bacterial community · Sourdough · Gliadin · Metagenomics

Introduction

Sourdough is an important technique for the fermentation of cereals, applied in the production of different cereal-based food products such as breads, biscuits, cakes, and crackers (Hayta and Hendek 2018). Gliadin/gluten free bread and other fermented baked products have been consumed by patients suffering from celiac disease (1–2% of world population), non-celiac-gluten sensitivity (6–7%) and irritable bowel syndrome patients (20–25%) (Scherf et al. 2016). Sourdoughs are considered as an effective tool for improvement of gliadin/gluten free breads (Brandt 2019). The microbial diversity of artisanal-fermented sourdoughs is determined by plethora of ecological factors, *inter alia*, the most important is the environmental factor, processing technology, the type and physico-chemical composition of raw material, process parameters (temperature, pH, ratio of mixing flour and water), and fermentation duration (De Vuyst et al. 2009).

It has been observed that many ecological factors i.e. ionic strength, temperature and microbial products affect the yield and growth rate of micro-organisms (Rinaldi et al. 2015). Micro-organisms from traditionally fermented sourdoughs cannot be thoroughly identified by culture-dependent method (Nionelli et al. 2018). However, to analyze microbial diversity of sourdough, various molecular methods are already in use (Corsetti et al. 2003; Zhang et al. 2018). Many studies have been done on the microbial

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diversity of various sourdoughs especially in China and Europe (Liu et al. 2018a, b; Palla et al. 2019; Raimondi et al. 2017; Yan et al. 2019; Zhao et al. 2019). These analyses revealed a huge diversity in bacterial community among various sourdough samples i.e. Chinese sourdough, Chinese steamed dough and Belgian sourdough.

Sourdough bread produced by traditional and modern methods varies in taste and nutritional value. Sourdough bread production by traditional methods have substantial variations, such as Chinese sourdough bread is produced by different processing conditions in various geographical regions of China like southern, northern and western regions. Therefore, there is no standard traditional method for sourdough bread production (Liu et al. 2018b). However, three kinds of sourdough fermentation processes could be distinguished, on the bases of inoculum used for their production (Dastmalchi et al. 2016; De Vuyst et al. 2009). Type I sourdoughs are manufactured by a traditional method and baker's yeast is not added separately, contrary to Type II and Type III sourdoughs. Type II sourdoughs are prepared through long fermentation times in silos in the form of semi-fluid and used as starters. Type III sourdoughs are consist of instant starters consist of mainly lactic acid bacteria (LAB) and yeast (De Vuyst and Marc 2007). Pakistani traditional wheat sourdoughs (*Khamir*) belong to type I, produced by traditional method and characterized by daily propagations at room temperature. Wheat is staple food in Pakistan which is commonly used for making *Chapati*, *Khamiri roti* (sourdough bread) and other bakery products. Population of Pakistan mainly hinges on *Chapati* and *Khamiri roti* which comprises 50–60% of their diet (Sakandar et al. 2019). It is produced by the traditional fermentation of wheat flour, consist of diverse Lactic acid bacteria and yeast species (Sakandar et al. 2018). Studies on the composition of the microflora of the spontaneously fermented sourdough showed that LAB (*Lactobacillus plantarum*, *L. sanfranciscensis*, *L. brevis*, *L. fermentum* etc.) the dominant group of bacteria while *Saccharomyces cerevisiae* was most abundant yeast present in sourdough (Corsetti and Settanni 2007; De Vuyst and Neysens 2005). In contrast, Pakistani sourdough (*Khamir*) revealed the abundance of *Enterococcus* species and *Wickerhamomyces anomalus* yeast species (Sakandar et al. 2018). The *W. anomalus* has also been reported to be the most abundant yeast specie in Belgian sourdoughs (Daniel et al. 2011).

However, to the best of our knowledge, no such study has been conducted to evaluate the bacterial diversity of artisanal-fermented Pakistani sourdough (*Khamir*). The objective of present study was to compare the bacterial communities in gliadin-degraded and non-degraded sourdough (*Khamir*) based on 16S rDNA gene sequence by Illumina Miseq platform.

Materials and methods

Sourdough sampling

In total, sixty sourdough (*Khamir*) samples (D1–D60) were collected aseptically by sterilized disposable gloves from different local shops (bakeries) of Rawalpindi and Islamabad (Pakistan), involving wheat dough fermentation, produced by traditional fermentation process used for making *Naan* or bread. The samples were immediately stored in refrigeration at 4 °C until further analysis.

Biochemical analysis of sourdough samples

Total titratable acidity (TTA), pH and moisture content of sourdough samples were analyzed (AOAC 2006).

Determination of degree of gliadin degradation by Fourier transform infrared (FTIR) spectroscopy

The lyophilized sourdough samples were blended with potassium bromide (1% w/w) for the preparation of thin discs and the spectra were obtained by using Spectrum One FTIR spectrometer (PerkinElmer, Waltham, MA, USA). Second derivative function was used to analyze the amide III regions (1350–1200 cm⁻¹) and Omnic software 8.2 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and was also used for the interpretation of FTIR spectra. The derivative spectra were obtained from the eleven points, two-degree polynomial function and then inverted by multiplying with 1. Finally, the agglomerative hierarchical clustering (Pearson correlation coefficient for dissimilarities) was done with XLSTAT (Addinsoft, NY, USA, version 2016.01) (data not shown).

DNA extraction from sourdough samples

Extraction of DNA from sourdough samples was done according to the method of Randazzo and coworkers (2005).

Metagenomics and data analysis

The 16S rRNA gene fragments of V4 for bacteria were generated by PCR, using 515F (GTGCCAGCMGCCGCGGTAA)/806R (GGACTACHVGGGTWTCTAAT) which were adjusted by adding ligation barcode. PCR was operated in three replicates with 20 µl reaction mixture using the Hot Star Taq Plus Master Mix Kit (Qiagen, USA) under the following conditions: initial denaturation, for 4 min at 96 °C, followed by 34 cycles, denaturation for 40 s at 96 °C, annealing at 56 °C for 50 s and extension was done

for 1 min at 75 °C. Success of amplification and corresponding bands intensity was analyzed by using agarose gel (2%), afterwards same proportion of all samples were pooled together and purified by using calibrated Ampure XP beads. Then the PCR product which was purified and pooled was used to prepare Illumina DNA library. Sequencing was performed at MR DNA (Shallowater, TX, USA) by using Illumina Miseq platform according to manufacturer's protocol. Sequenced data was analyzed statistically and bio-informatically according to the method of DeSantis et al. (2006).

Statistical analysis

Principal component analysis of bacterial genera of D50 and D13 was checked by Pearson's correlation using XLSTAT (Addinsoft, NY, USA, version 2016.01) for windows 10. Alpha diversity was determined to analyze the complexity of genera diversity between samples by using operational taxonomic units (OTUs). Simpson and Shannon indices were used to evaluate the species diversity in both sourdough samples. The hierarchically clustered heatmap figures, for pictorial explanation of bacterial diversity in samples, were generated by using Heatmap Illustrator (Cuckoo work group, China, Hemi, version 1.0) for windows according to the method of Deng et al. (2014).

Results and discussion

FTIR analysis of sourdough samples

Sixty sourdough samples were analyzed for the determination of degree of gliadin degradation by FTIR spectroscopy. Among all sourdough samples, D13, D18, D41, and D58 revealed significant gliadin degradation. While, among these four samples, D13 had maximum degradation of gliadin, and remaining 56 samples had no significant gliadin degradation (data not shown). These were compared to the standard gliadin as control. Therefore, D13 and D50 were selected, on the basis of degree of gliadin degradation, so that metagenomical comparison between these two sourdough samples could be evaluated.

Biochemical analysis of sourdough samples

The median values of pH, total titratable acidity and moisture were 5.14, 1.38 and 51.60, respectively. The results were comparable with findings of previous researches (Zhang et al. 2011). They worked on Chinese sourdough but pH value of our samples was high, and acidity was low compared to the findings of Liu and coworkers

(Luangsakul et al. 2009). The rationale for this likely due to the different acid producing microbiota capacity and level present in the samples as well as the variable buffering capacity of the flour used in the production of sourdoughs (De Vuyst et al. 2002).

Distinct bacterial diversity between gliadin-degraded (D13) and non-degraded (D50) samples

After the trimming of PCR primers and removal of the low quality, chimera reads, 70,833 reads were corresponded to high quality partial 16S rRNA gene sequences longer than 150 bp. The alpha diversity in both samples was analyzed by Shannon and Simpson indices. It was observed that bacterial diversity in D13 (Shannon = 2.48 and Simpson = 0.86) was higher than in D50 (Shannon = 2.31 and Simpson = 0.82). This is presumably due to the production of diverse compounds in gliadin-degraded sample which caused succession of more diverse bacterial community (De Vuyst and Neysens 2005).

Bacterial community structures between gliadin-degraded (D13) and non-degraded (D50) samples

Altogether 6 bacterial phyla were observed in D50 and D13 samples. Five of these phyla were shared by both samples while 6 were present in D50 and 5 in D13, accounting for 99.91–100% of the total abundance of bacterial communities in each sample. It was observed that Proteobacteria (50.65%) was the most dominating bacterial phylum in D13 while, Firmicutes (83.44%) was most abundant in D50. The second most abundant phylum in D50 and D13 was Proteobacteria (14.97%) and Firmicutes (42.53%), respectively. It was revealed that the diverse effects were more prominent with lower taxonomic levels, indicating probable functional redundancy of the sourdough samples.

At the genus level, only the genus that appeared more than twice in one sample type was considered as a valid genus. Thus, totally, 65 genera were detected in both samples, and 35 of these genera were clearly classified. Among them, twenty-four genera were with an average relative abundance over 0.01% in at least one type of sample. These genera held 99.11–99.47% of the total bacterial community. Only ten genera were with an average relative abundance over 1% in D50, including *Lactobacillus* (52.13%), *Weissella* (27.15%), *Proteus* (4.08%), *Serratia* (2.70%), *Leuconostoc* (2.26%), *Psychrobacter* (1.97%), *Providencia* (1.73%), *Alcaligenes* (1.58%), *Brevibacterium* (1.36%) and *Acinetobacter* (1.10%). While, eleven genera were observed in D13 with an average relative abundance more than 1%, including *Lactobacillus* (33.73%), *Psychrobacter* (21.53%), *Providencia* (13.62%), *Proteus* (8.30%), *Brevibacterium* (6.09%), *Alcaligenes*

Fig. 1 Relative abundance of genera in gliadin degrading and non-degrading sourdough samples

(3.80%), *Bavariicoccus* (2.96%), *Carnobacterium* (1.76%), *Oceanobacillus* (1.69%), *Brevundimonas* (1.57%) and *Weissella* (1.10%). These genera accounted for 96.12% and 96.21% in D50 and D13, respectively. Moreover, *Bavariicoccus*, *Carnobacterium*, *Oceanobacillus* and *Brevundimonas* were only present in D50, and *Providencia*, *Serratia* and *Acinetobacter* were only observed in D13 (Fig. 1). Bacterial community in D50 was in synch with the findings of Luangsakul et al. (2009) and Zhang et al. (2011), but the bacterial community in D13 was inconsistent with their findings. This inconsistency in our findings is presumably due to different ecological factors (De Vuyst and Neysens 2005).

Correlation of bacterial communities between gliadin-degraded (D13) and non-degraded (D50) samples

Fifty-two genera were shared by D50 and D13, which accounted for 99.76–99.88% of the total abundance. Fifteen different genera were found in D13 and seven were found in D50.

Key bacterial genera present in gliadin-degraded sample were *Proteus* and *Psychrobacter*, and it has been reported that these genera have substantial proteolytic activity (Denner et al. 2001; Kushwaha et al. 2014). These were found in high concentration in D13 while, *Lactobacillus* abundance was significantly decreased. It could be hypothesized that *Proteus* could be the responsible genera for gliadin degradation in D13. *Proteus* species has been reported in fermented foods (Imran et al. 2019). It was also observed that more genera were observed in D13

Fig. 2 Principal component analysis of bacterial genera in gliadin degraded samples and non-degraded samples

compared to D50 was due to the production various amino acids gliadin degradation. Principal component analysis (PCA) was performed to analyze comparatively the relationship of the bacterial communities in D50 and D13 sourdough samples. It was revealed that the bacterial community in D13 was distinct from that in D50 (Fig. 2). The bacterial community in the D13 was bracketed to the left of the graph along F1. However, the bacterial community in the D50 varied considerably. The variation in the relative abundance of the bacterial genera between D50 and D13 was found highly variable (Fig. 3). Similar pattern of grouping, as in the case of PCA, was also observed in the heatmaps. These results were in corroboration with the findings of Zhang et al. (2011) and Liu et al. (2016).

Fig. 3 Comparison of bacterial community in gliadin degraded and non-degraded sourdough samples profile at genera level. Heatmaps were constructed with the abundant bacteria at genus level

Conclusion

In conclusion, this research had some insights of the bacterial community in Pakistani sourdough, and the responsible bacterial community in gliadin-degraded sample. Additionally, an unprecedented study on Pakistani sourdough samples was conducted to characterize the bacterial community. Firmicutes were dominating phyla in sourdough samples and Proteobacteria was the most abundant phyla in gliadin-degraded sample. At genera level, *Lactobacillus* was most abundant genera while second most abundant genera in gliadin-degraded and non-degraded sourdough sample was *Psychrobacter* and *Weissella*, respectively. This study could help to track the complexity of natural bacterial communities responsible for gliadin degradation. This preliminary study could lead to develop specific starters for sourdough fermentation at industrial level.

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