IDENTIFICATION OF MICROBIAL DIVERSITY ASSOCIATED WITH POST-HARVEST STORAGE OF RICE IN INDIA

BY

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THESIS

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ABSTRACT

The microbial communities associated with eleven samples of milled and un-milled rice from various storage facilities and local trade markets of Haryana, India were analyzed using high-throughput pyrosequencing. Comparison of the microbial community compositions of freshly harvested paddy and stored rice led to identification of the dominant fungi and bacteria specifically present or enriched during storage. Greater microbial diversity of fresh paddy as compared to milled rice suggests that milling may be responsible for the removal of many microbes from paddy. *Lactococcus, Lactobacillus*, and *Leuconostoc* were the major bacterial genera specific to stored rice. *Clostridium,* although low in abundance, was significantly enriched during storage. The dominant fungus specific to stored rice was the well-known 'storage fungus' *Aspergillus.* It was present along with 'field fungi' *Fusarium, Alternaria* and *Cladosporium*. The wide range of temperature tolerance of lactic acid bacteria and *Aspergillus* may be leading to their high abundance at storage sites. The presence of lactic acid bacteria together with 'field fungi' is indicative of high moisture contents (>20%) and anaerobic conditions at storage sites. The lactic acid bacteria as well as *Clostridium* produce volatile organic compounds and biogenic amines which enhance spoilage of food grains. *Aspergillus* and *Fusarium,* on the other hand, are mycotoxigenic fungi known to produce toxins that are carcinogenic to humans. In conclusion, the microbes identified are suggestive of inappropriate post-harvest storage conditions leading to negative implications on grain quality and human health.

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CHAPTER 1

INTRODUCTION AND OBJECTIVE

Insufficient food supplies have always posed a significant challenge in developing nations worldwide. The major efforts aimed at coping up with the ever-increasing food demands have largely been directed towards means of increasing agricultural production (Bourne, 1977). Increasing production is certainly a desirable measure towards meeting demand, however, it not only increases the drain on environmental resources, which are limited, but, it, alone is also inadequate to keep up with the demand (Bourne, 1977; Hodges *et al.*, 2011). What is also needed is the presence of a robust system that would ensure the efficient processing and delivery of the fresh agricultural product to the point where it is consumable (FAO, 1978). Efficient delivery implies that any losses, whether qualitative or quantitative, must be minimized in the post-harvest chain. The post-harvest chain consists of all the operations carried out after the completion of harvest and before the point of consumption, like threshing, drying, milling, storage, transport, processing, packaging, etc. Any losses in quantity or quality of the food product that are incurred during the post-harvest chain are referred to as post-harvest losses (de Lucia and Assennato, 1994).

Post-harvest loss may be quantitative or qualitative, as mentioned above. Quantitative losses occur due to actual disappearance of the food product as a result of spillage or abrasion during a certain post-harvest operation, or consumption by organisms such as insects, pests or microbes. Qualitative loss, on the other hand, accounts for the loss in nutritional value of the food product caused due to biological degradation. Most of the developing countries have a tropical climate and biodegradation of food products is a major concern for them because the warm and humid tropical climate promotes the growth of microorganisms. Among all the

different types of food products, grain products are considered to be the most resistant and least perishable due to their low moisture contents (Bourne, 1977). However, due to poor post-harvest handling and storage practices in the developing countries, they still experience huge quantitative losses and losses in nutritional quality due to biodeterioration (Grolleaud, 1997; Boxall, 2002).

Nearly 20 to 30% of all food crops are estimated to have been lost in the post-harvest system, in developing nations (Hodges *et al.*, 2011). These losses are highly variable depending on the product being handled, the climatic conditions, the duration of storage and the post-harvest procedures employed. Rice post-harvest losses have been documented to be the highest among all the major crops grown in developing countries (FAO, 1977). Rice is also known to go through a greater number of post-harvest processing steps as compared to other grain products (Saunders *et al.*, 1980). It is alarming to note the high levels of post-harvest losses reported in case of rice since it is the major staple food crop of the developing countries and is also largely produced by them.

India, being the second largest producer of rice in the world after China, is also considerably lagging behind in terms of efficient post-harvest management of food grains. In India, the post-harvest losses in food grains are reported to be about 7-10% of the production from farm to market and about 4-5% at market level (World Bank, 1999). Among food grains, rice is a major staple crop of India. The rice post-harvest chain in India primarily consists of drying, threshing, milling, storage, packaging and transportation. Right after harvest, the rice or paddy (rice with the husk) is dried, harvested and transported to the local trade markets by the farmers. From this primary market, the paddy is transported to the milling and storage facilities by the millers, where it is milled and stored. The milled rice is then further processed, packaged and transported to the retailer for sale to the consumers. In a study that was conducted to evaluate

the relative losses along the different stages of the post-harvest chain in India, it was found that the losses were highest during the storage period (Basavaraja *et al.*, 2007). This finding is supported by another set of data representing post-harvest losses in rice in China, which also identifies storage as the major point of post-harvest loss (Grolleaud, 1997).

Rice in India has to be stored in large quantities in order to meet the demand throughout the year until the next harvesting season. The rice, after milling, is stored in many different ways. It may be packed in gunny bags which may be piled up out in the open or inside a storage facility. Less frequently, the rice is stored in metallic silos. It may also simply be stored in bulk in a storage facility. Nevertheless, whatever be the method of storage, temperature and humidity conditions are rarely controlled during storage and the stored rice is highly vulnerable to microbial contamination during the storage period, which may extend up to several months or even years. The original source of these microbial contaminants is the freshly harvested rice which is home to a wide range of microbes, including bacteria and fungi. Many times due to handling limitations, the freshly harvested rice, which has high moisture contents, is held for periods longer than 24 hours before it is dried. This temporary wet storage period promotes the growth of microbes found on the freshly harvested rice (Teunisson, 1954). Further, it is also known that drying is not completely effective for destroying these microorganisms (Wu, 2008). As a result, these microbial contaminants are carried all the way from the fields to the storage sites along with the rice.

Biodeterioration of rice due to microbial contamination during storage is a deep cause for concern because it is this stored rice that ultimately reaches the consumer. Consumption of such spoiled food leads to food-borne diseases and health issues in the developing world. Warm temperatures and high relative humidity in the storage sites combined with the high carbohydrate

content of rice make it prone to microbial attack. Microbial contamination not only results in losses in dry matter through carbohydrate utilization (Magan and Aldred, 2007) but also adversely affects the flavor and nutritional quality of rice due to release of a range of undesirable volatile organic compounds (Champagne *et al.*, 2004). Not only this, certain groups of storage fungi are also known to produce extremely harmful toxins which may even be carcinogenic (Reddy *et al.*, 2008; Wagacha and Muthomi, 2008) and such microbial groups and toxins have been detected in rice in India (Reddy *et al.*, 2009). Such studies together with the potential health risks due to microbial contamination of grains during storage highlight the need to develop suitable post-harvest measures to detect and monitor the onset of spoilage and select appropriate technologies to minimize it.

In order to be able to suggest post-harvest strategies to circumvent the spoilage issues during storage, it is essential to understand the various ecological factors which are at play in the stored grain ecosystem (Magan and Aldred, 2007). These factors have been categorized into implicit, intrinsic, extrinsic and processing factors. The implicit factors refer to the microbial community structure i.e. the types and relative abundances of microorganisms, which in turn depend on intrinsic factors like water activity, nature of substrate and nutrient composition of the grains, extrinsic factors like temperature and climatic conditions, and processing factors like drying conditions and addition of preservatives during storage. It is the implicit factors or the microbial consortia which are ultimately responsible for causing the biological degradation of stored grains and hence there is need to conduct inventory analysis to identify and characterize these microbial consortia at storage sites. Many studies have been conducted in this regard to identify the predominant fungal groups responsible for spoilage of rice grains during storage (Almeida *et al.*, 1991; Trung *et al.*, 2001; Taligoola *et al.*, 2004; Oh *et al.*, 2007; Oh *et al.*, 2008;

Reddy *et al.*, 2009; Gautam *et al.*, 2012; Uma and Wesely, 2013). However, current knowledge regarding the predominant bacterial groups associated with post-harvest grain storage is relatively limited (Oh *et al.*, 2007; Oh *et al.*, 2008; Cottyn *et al.*, 2001; Min-Cheol *et al.*, 2008; Ahn *et al.*, 2012). Besides, most of the aforementioned studies are based on culture-dependent methods which do not provide a complete picture of the microbial community composition (Ward *et al.*, 1990).

Bacteria belonging to the genera *Bacillus, Pectobacterium, Pantoea, Microbacterium, Sphingomonas,* and *Methylobacterium* have been isolated in studies of stored rice in Korea which used culture-based methods such as Biolog and fattty acid methyl ester (FAME) analyses (Oh *et al.*, 2007; Oh *et al.*, 2008) for identification. Further, a couple of studies which were conducted using raw rice-straw and freshly harvested rice grains, respectively, have found the following genera to be present: *Pantoea, Bacillus, Microbacterium, Enterococcus, Pseudomonas, Rhodococcus Enterobacter, Xanthomonas, Cellulomonas, Clavibacter, Burkholderia,* and *Paenibacillus* (Hong *et al.*, 2012; Cottyn *et al.*, 2000). These studies were conducted using culture-based and 16S rRNA fingerprinting methods. Additionally, studies that were carried out using soil from paddy fields have identified *Chloroflexi, Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes, Bacteroidetes, Planctomycetes, and Gemmatimonadetes* as the major bacterial phyla (Ahn *et al.*, 2012; Arjun and Harikrishnan, 2011) through 16S rRNA fingerprinting and sequencing methods.

Fungi belonging to the genera *Aspergillus, Penicillium, Cladosporium, Rhizopus,* and *Rhodotorula* were reported to be found in milled rice samples in Brazil (Almeida *et al.*, 1991). *Aspergillus*, *Fusarium,* and *Penicillium* were the major fungal genera identified in two independent studies of milled rice conducted in different parts of Africa (Taligoola *et al*., 2004;

Makun *et al.*, 2007). Other genera identified in these studies were *Eurotium*, *Cladosporium*, *Cochliobolus*, *Acremonium*, *Alternaria*, *Rhizopus*, *Trichoderma*, *Curvularia*, and *Helmenthosporium*. Another study conducted on rice samples from Vietnam also identified *Aspergillus*, *Fusarium,* and *Penicillium* as the dominant fungi. Studies of stored rice collected from rice processing complexes of Korea identified *Aspergillus* and *Penicillium* as the predominant fungi. *Aspergillus* is also the most predominant fungal genus isolated from rice samples across different states of India*, Penicillium, Fusarium, Alternaria,* and *Rhizopus* being a few others (Udagawa, 1976; Sundaram *et al.*, 1988; Reddy *et al.*, 2009; Uma and Wesely, 2013). From these studies, it is evident that *Aspergillus, Penicillium,* and *Fusarium* are currently known to be the most dominant fungal genera associated with stored rice. These are the major groups of mycotoxigenic fungi known to produce extremely toxic compounds known as mycotoxins (Wagacha and Muthomi, 2008). In the study by Reddy *et al.,* 1200 rice samples from 20 states across India were analyzed and majority of them were not only contaminated with different species of *Aspergillus*, but also contained aflatoxin B_1, a mycotoxin produced by *Aspergillus* that has been classified as a class I human carcinogen (IARC, 1993).

As mentioned earlier, a major limitation of the previous studies is that they rely on culture-based methods of identification. Besides, there has been no such study directed towards identifying potentially harmful bacterial groups associated with rice in India. Also, most of these studies did not provide any information regarding relative abundances of the various microbial groups identified. None of these studies performed a comparative analysis of microbial community structure of freshly harvested paddy and stored rice, belonging to a common post-harvest chain in one region. This comparison, being a distinct feature of the current study, is necessary in order to identify microbes which are specifically enriched during storage and hence

may be directly involved in grain spoilage. Although molecular methods of microbial community analysis are well developed, they have not yet been applied extensively to probe the stored grain ecosystem.

1.1 Objective

The objective of the present study was to perform a comparative analysis of the fungal and bacterial community structures of freshly harvested paddy from rice fields versus milled rice from storage sites, using high-throughput 'next-generation DNA sequencing' technology. The goal of this comparative analysis was to identify putative microbes involved in grain spoilage during storage of rice in the post-harvest chain in India.

CHAPTER 2

METHODOLOGY

2.1 Sample Collection

Rice samples collected from various storage facilities and local trade markets of the state of Haryana, India were provided by CCS Haryana Agricultural University, Hisar, India. The sample details are summarized in Table 1. The rice samples were stored at 4°C at the Environmental Engineering Laboratory of the Indian Institute of Technology, Kanpur, India, until DNA extraction was performed in January 2013. In total, eleven rice samples were used in this study, which included five samples of freshly harvested, un-milled rice, four samples of one year old, milled, stored rice, one sample each of freshly milled rice and packaged rice.

2.2 Microbial biomass collection

Two alternative methods were applied to collect biomass from every rice sample.

2.2.1 Heavy centrifugation method

Seventy-five grams of rice were thoroughly washed with sterile 1X Phosphate Buffered Saline (PBS) (Sigma) to facilitate the detachment of biomass associated with the surface of rice grains. The PBS was then collected and centrifuged at maximum speed for 10-15 min to facilitate the deposition of biomass in the form of a pellet. The supernatant was discarded and the pellet was processed further for DNA extraction

2.2.2 Light centrifugation method

Seventy-five grams of rice were thoroughly washed with sterile 1X Phosphate Buffered Saline (PBS) (Sigma). This PBS was then collected and centrifuged at low speed for 2 min to facilitate the deposition of the heavier material which was mostly expected to be the chaff

associated with rice grains. However, it was impossible to ensure the complete prevention of deposition of microbial biomass even at this low speed. Hence, the pellet from this initial low-speed centrifugation step was also processed for DNA extraction. The supernatant from this step was then centrifuged at maximum speed for 10-15 min to facilitate the deposition of biomass in the form of a pellet. The supernatant from this final centrifugation step was discarded and the pellet was processed further for DNA extraction. As a result of two alternative methods of biomass collection being employed, three different fractions of DNA were extracted corresponding to every rice sample.

2.3 DNA extraction, PCR and pyrosequencing

Genomic DNA was extracted from every biomass-containing pellet obtained in the previous step, following a protocol described previously (Zhou *et al.*, 1996) and stored at -20°C until further use. Bacterial-biased primers U515F (5'-GTGYCAGCMGCCGCGGTA-3') (Wang and Qian, 2009) and U1052R (5'-GARCTGRCGRCRRCCATGCA-3') (Wang and Qian, 2009) were used to amplify approximately 550 bp fragments of the V3 to V6 hypervariable regions of bacterial 16S rRNA gene. Fungal-biased primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) were used to amplify approximately 650-900 bp fragments spanning the fungal internal transcribed spacer (ITS) region. The primer pairs were modified for pyrosequencing by adding the 454 pyrosequencing adapter 'A' (CCATCTCATCCCTGCGTGTCTCCGACTCAG) followed by a 10-nucleotide barcode sequence at the 5' end of the forward primer (in case of 16S rRNA gene) or the reverse primer (in case of ITS region) and the 454 pyrosequencing adapter 'B'

(CCTATCCCCTGTGTGCCTTGGCAGTCTCAG) at the 5' end of the other primer. Bullseye Taq DNA Polymerase 2.0X reaction-mix (MIDSCI, St. Louis, MO, USA) was used to set up 25μL PCR mixture. The following thermal cycling conditions were used for PCR-amplification of the 16S rRNA gene: initial denaturation at 94˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 56˚C for 45 s, extension at 72˚C for 1 min, and a final extension at 72˚C for 10 min. The following thermal cycling conditions were used for PCR-amplification of the ITS region: initial denaturation at 95˚C for 10 min, followed by 30 cycles of denaturation at 95˚C for 30 s, annealing at 52˚C for 1 min, extension at 72˚C for 1 min, and a final extension at 72˚C for 10 min. The resulting PCR products were purified using Wizard SV gel and PCR clean-up system (Promega Corporation, Madison, WI, USA). Pyrosequencing of the purified PCR products was performed using the 454 GS FLX Titanium platform (Roche, Switzerland) at Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign.

2.4 Bioinformatic analyses

The bacterial sequence data obtained from GS FLX pyrosequencing was processed and analyzed through QIIME 1.6.0 (Caporaso *et al.*, 2010). The sequence reads were assigned to their respective samples through their unique nucleotide barcode identifiers. Along with this demultiplexing step, quality filtering was also performed through which sequences with a mean quality score below 25 and length outside of the bounds of 300 bp and 600 bp were removed. Forward and reverse primer sequences were trimmed. Chimera removal was performed. The non-chimeric sequences were clustered into operational taxonomic units (OTUs) at 97% similarity cut-off, via the UCLUST algorithm. The OTUs were then assigned taxonomic

affiliations using the Greengenes training set. Alpha rarefaction analysis and calculation of alpha diversity indices were also performed with QIIME. Further, weighted UniFrac distances between the different samples were computed and principal coordinates analysis (PCoA) was performed based on the resultant distance metric. The representative sequences of the most dominant bacterial OTUs were selected and their closest relatives were obtained through BLAST (Altschul *et al.*, 1990). These sequences were aligned through ClustalW (Larkin *et al.*, 2007) and evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000). The evolutionary distances were used to infer a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap test (Felsenstein, 1985) was performed for 1000 replicates. All phylogenetic analyses were conducted in MEGA6 (Tamura *et al.*, 2013). The phylogenetic relationships inferred from the tree for bacterial OTUs were consistent with the taxonomic assignments made through QIIME.

The fungal ITS sequences were also processed with QIIME but with different parameters and filtering criteria. Sequences with a mean quality score below 25 and length shorter than 200 bp were removed. Forward and reverse primer sequences were trimmed. The sequences were reverse-complemented since the pyrosequencing adapter 'A' was fused to the reverse primer. The sequences were then clustered into OTUs at 97% similarity, via the USEARCH algorithm which employs *de-novo* chimera removal for datasets without a reference set. Since the total sequencing reads were unevenly distributed among different samples, the number of reads per sample was normalized to the lowest common number of reads per sample by rarifying the OTU table. The rarified OTU table was used for downstream analyses which included principal components analysis (PCA). Alpha rarefaction analysis and calculation of alpha diversity indices were also performed. The representative sequences of the OTUs were identified and used for assigning

taxonomy to the corresponding OTUs through BLASTN searches against the UNITE (Abarenkov *et al.*, 2010) and GenBank (Benson *et al.*, 2005) databases. The phylogenetic tree construction was performed in a similar way as described earlier for bacterial sequences.

CHAPTER 3

RESULTS

3.1 Method of biomass collection did not affect the microbial community composition

Two alternative methods of microbial biomass collection, namely, the heavy- and the light centrifugation methods, were employed in this study (section 2.2). This was necessary to exclude any possible effects of extraneous DNA, contributed by the plant material washed off from the surface of rice grains during the process of biomass collection on the downstream analyses. The light centrifugation method was designed to be able to separate most of this extraneous rice material from the microbial cells during an initial low-speed centrifugation step. The heavy centrifugation method did not involve any prior separation step and the microbial biomass was collected along with the extraneous rice material during a single high-speed centrifugation step.

A comparison of the bacterial communities obtained from both methods for the five samples of freshly harvested, un-milled paddy is presented in Fig. 1. The differences between bacterial communities obtained from the two methods were tested e by using *t* tests on the relative abundances of taxa associated with the five samples for the two methods. No significant difference was found between the community compositions obtained from either method ($P > 0.05$). This finding was confirmed at three levels of taxonomic classification, namely, phylum, family and genus. In view of this finding and to maintain consistency, only the community composition data from the heavy centrifugation method was further analyzed.

3.2 Greater microbial diversity was associated with freshly harvested paddy as compared to milled rice

The number of bacterial taxa per sample of freshly harvested paddy varied from 183 to 276 while the number of bacterial taxa per sample of milled rice varied only from 51 to 119. Similarly, the number of fungal taxa per sample of freshly harvested paddy varied from 195 to 341 as opposed to the number of fungal taxa per sample of milled rice which varied only from 111 to 208 (Tables 2 & 3). As a result, the average alpha diversity index of the bacterial community associated with samples of fresh paddy was 238 which was significantly higher than 90.4, the corresponding value for milled rice samples ($P < 0.01$). The average alpha diversity index of the fungal community associated with samples of fresh paddy was 293.8 which was also significantly higher than 149.5, the corresponding value for milled rice samples ($P < 0.01$). The higher numbers of microbial taxa observed per sample of fresh paddy as compared to milled rice are also evident from the rarefaction curves for bacterial and fungal OTUs The rarefaction curves corresponding to fresh paddy samples begin to saturate at higher numbers of observed OTUs and greater sequencing depths as compared to those corresponding to milled rice samples (Fig. 2 & 3). Together, these findings clearly showed that freshly harvested, un-milled rice inhabited a greater microbial diversity as compared to milled rice.

3.3 Analysis of bacterial community structure of freshly harvested paddy and stored rice

The relative abundances of various bacterial taxa constituting the bacterial communities associated with the different types of rice samples are summarized in tables S1, S2, and S3. The community compositions were analyzed at phylum, family and genus levels. At phylum level, *Proteobacteria* was the most dominant bacterial phylum associated with freshly harvested paddy

comprising nearly 70% of the community followed by *Bacteroidetes* (18%), *Firmicutes* (10%), and *Actinobacteria* (2%) (Fig. 4). On the other hand, *Firmicutes* was the most abundant bacterial phylum identified in stored, milled rice comprising nearly 52% of the community followed by *Proteobacteria* which comprised the remaining 48%. Comparing the relative abundances of the dominant bacterial phyla between samples of fresh paddy and stored rice led to the emergence of *Firmicutes* as the only phylum that was significantly enriched in stored rice as compared to fresh, un-milled rice ($P < 0.05$).

At family level, nearly seventeen bacterial families were found to be associated with the rice samples in this study (Fig. 5). Within the phylum *Firmicutes,* three bacterial families of special concern are *Streptococcaceae, Lactobacillaceae,* and *Leuconostocaceae* because they were detected only in the stored rice samples and not in any of the freshly harvested rice samples. Amongst these, *Streptococcaceae* followed by *Lactobacillaceae* represented nearly 29% and 16%, respectively, of the bacterial community associated with stored rice. *Leuconostocaceae* was relatively less abundant constituting only about 2% of the community. In addition, *Clostridiaceae*, another member of *Firmicutes* was found to be significantly enriched in stored rice as compared to fresh paddy ($P < 0.05$), and it comprised only about 1% of the stored rice bacterial community.

At genus level, about twenty different bacterial genera were found to be present across the different types of rice samples (Fig. 6). Among these, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* are critical because they were specifically detected in the stored rice samples only and not in any of the fresh, un-milled rice samples. *Lactococcus* and *Lactobacillus* were the most dominant comprising nearly 29% and 16%, respectively, of the stored rice bacterial community at genus level, followed by *Leuconostoc* which formed only about 2% of the community. Besides

these *Clostridium* was found to be significantly enriched in the stored rice community as compared to the fresh paddy community ($P < 0.05$), although its relative abundance was only about 1%. *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Clostridium* were the major members of phylum *Firmicutes* specific to stored rice. The genera *Erwinia*, *Pantoea*, and *Pseudomonas* were detected almost throughout the post-harvest chain, being present in fresh paddy, stored rice as well as packaged rice samples. *Erwinia* ranged in abundance from about 1-11%, *Pantoea* from about 2-15% and *Pseudomonas* ranged between 2-30% of the communities at different stages along the post-harvest chain. *Serratia* was specifically most abundant in the packaged rice sample, comprising nearly 62% of the community.

3.4 Analysis of fungal community structure of freshly harvested paddy and stored rice

The relative abundances of various fungal taxa constituting the fungal communities associated with the different types of rice samples are summarized in Tables S1, S2, and S3. The community compositions were analyzed at phylum and genus levels. As fungal DNA could not be amplified from the packaged rice sample and one out of the four stored rice samples, the results presented here were obtained from analyzing the remaining nine samples of rice.

At phylum level, *Basidiomycota* was the most dominant fungal phylum associated with freshly harvested paddy comprising about 46% of the fungal community followed by *Ascomycota* which formed nearly 27% of the community (Fig. 7). In contrast, *Ascomycota* was the most dominant fungal phylum identified in the stored rice fungal community comprising about 56% of the community followed by *Basidiomycota* which constituted only about 11%. A little less than 2% of the stored rice community was also composed of the phylum *Zygomycota.* Additionally, comparing between fresh paddy and stored rice, phylum *Basidiomycota* was

significantly less abundant in stored rice than in freshly harvested rice ($P < 0.01$). *Ascomycota* was more abundant than *Basidiomycota* in stored rice ($P < 0.01$) and the freshly milled rice sample.

At genus level, about fifteen different fungal genera were found across all the rice samples analyzed (Fig. 8). The putative genera responsible for causing bio-deterioration of rice grains during storage are likely to be the ones which are detected specifically during storage. In contrast to bacterial community structure, comparison of fungal communities associated with freshly harvested and stored rice samples revealed only one genus, *Aspergillus,* which was relatively abundant, comprising about 11% of the stored rice fungal community, as well as specific to stored rice only. Most of the fungal diversity associated with stored rice was present in minute quantities. Besides *Aspergillus*, *Alternaria* and *Cladosporium* comprised about 15% and 7%, respectively, of the stored rice fungal community. However, they were also found to be present at comparable levels in freshly harvested rice (7% and 11%, respectively). Furthermore, *Cryptococcus* and *Pseudozyma* comprised about 16% and 22%, respectively, of the fresh, un-milled rice community. Genus *Fusarium* was quite abundant (22%) in the freshly milled rice sample and comprised about 3% of the stored rice community.

3.5 Variation in microbial community profiles among samples

The variations in bacterial and fungal community structures among the different rice types were investigated through ordination analyses (Fig. 9 & 10). The first ordination axis representing the primary axis of variation separated the bacterial as well as fungal communities of freshly harvested un-milled rice from those of stored and milled rice. This finding is also supported by the bacterial and fungal alpha diversity indices and rarefaction curves which

showed that microbial communities associated with fresh paddy were the most diverse, as described previously in section 3.2. The bacterial community of the only sample of packaged rice did not cluster together with any of the two major clusters, suggesting that its community composition was different not only from the fresh, un-milled rice samples but also from other milled rice samples.

3.6 Phylogenetic analysis of the dominant bacterial and fungal OTUs

Representative bacterial and fungal OTUs identified in the different rice samples and their closest relatives were used to construct phylogenetic trees (Fig. 11 & 12). Bacterial OTUs belonging to the most dominant and critical genera comprising greater than 15% of the bacterial community of any rice type, as described in section 3.3, were selected for bacterial tree construction. Fungal OTUs belonging to the most dominant and critical genera comprising about 10% or more of the fungal community of any rice type, as described in section 3.4, were selected for fungal tree construction. All of the selected bacterial OTUs either belonged to the phylum *Proteobacteria* or *Firmicutes*. The bacterial and fungal OTUs were closely related (≥ 94% sequence similarity of the partial 16S rRNA gene in case of bacteria and the ITS region in case of fungi) to the nearest species on the tree. The *Proteobacteria*-affiliated OTUs were closely related to species belonging to the genus *Serratia* or *Pseudomonas. Firmicutes*-related OTUs were clustered together with species from the genus *Lactococcus* or *Lactobacillus* and were absent in freshly harvested rice. The fungal OTUs belonging to phylum *Ascomycota* were closely related to the genera *Aspergillus, Fusarium* and *Alternaria* while those belonging to *Basidiomycota* were closest to the genera *Pseudozyma* and *Cryptococcus*.

CHAPTER 4

DISCUSSION

Next-generation sequencing techniques were used to survey the compositions of bacterial and fungal communities at different taxonomic levels leading to identification of the predominant microbial groups in every rice type. A comparison of the microbial communities associated with freshly harvested paddy and stored rice also identified dominant microbial groups specifically present or enriched during storage of rice.

4.1 Milling may facilitate reduction in microbial diversity of freshly harvested paddy

Significantly greater bacterial and fungal diversity of un-milled rice samples as compared to milled rice samples (section 3.2), suggests that the post-harvest milling process may be responsible for lowering the microbial diversity associated with freshly harvested paddy. The process of milling involves physical removal and separation of the outer husk and bran layers from paddy to produce white rice grains. This process is vigorous enough to even cause breakage of many rice grains. Hence it is expected that microbes associated with the outer layers of rice grains or those that are loosely bound to the surface of white rice may become detached and get removed, which explains the lower microbial diversity observed post milling.

4.2 Microbes associated with the different types of rice

Lactococcus, *Lactobacillus*, *Leuconostoc*, and *Clostridium,* the main bacterial genera specifically present or enriched in stored rice, have not been previously identified in any studies associated with rice or paddy field soils (Cottyn *et al.*, 2000; Oh *et al.*, 2007; Oh *et al.*, 2008; Hong *et al.*, 2012). Among other dominant genera identified, *Pantoea* and *Pseudomonas* have

been previously found in stored rice as well as paddy? (Cottyn *et al.*, 2000; Oh *et al.*, 2007; Hong *et al.*, 2012).

Among the dominant fungi identified, *Aspergillus* and *Fusarium* are two of the three most commonly found fungi in stored rice (Reddy *et al.,* 2008). *Penicillium* as the third most well-known fungal genus found during storage was not detected in any rice sample taken. This may be because *Penicillium* is usually known to dominate in cool, temperate climates (Magan *et al.,* 2003) while the rice samples for this study were obtained from a warm, tropical region. *Alternaria* and *Cladosporium,* which were quite abundant in both freshly harvested paddy and stored rice, have been frequently isolated from soils and rice samples (Reddy *et al.,* 2008; Bensch *et al.,* 2012; Almeida *et al.,* 1991). Species of *Cryptococcus* and *Pseudozyma* are also commonly isolated from soil and plant materials, but their presence may not be relevant to the stored grain ecosystem (Benham, 1956; Wei *et al.,* 2005). Majority of the remaining fungal diversity was present in very low abundance consistent with a previous study regarding eukaryotic microbes according to which there exist only a few functionally relevant species in any environment while others merely represent a 'seed bank' capable of surviving under variable conditions (Finlay, 2002).

4.3 Factors contributing to the dominance of specific microbes

Lactococcus, *Lactobacillus* and *Leuconostoc* from the families *Streptococcaceae, Lactobacillaceae,* and *Leuconostocaceae*, respectively, share some of the physiological features shared by these families. They are mostly gram-positive, facultative anaerobes which ferment sugars and polysaccharides, the major components of rice grains. Being facultative anaerobes, they can tolerate anoxic conditions which may develop due to poor aeration in the deeper layers

of stored grain. This may confer significant competitive advantage over other aerobic species of bacteria. They also have a wide range of growth temperatures ranging from 2°C to 53°C (Teuber, 2009; Holzapfel *et al.*, 2009; Hammes and Hertel, 2009). The region studied in India has a tropical climate with temperatures varying from about 5°C in the winter to 45°C in the summer (Harrington *et al.*, 1992). The temperature at storage facilities in India is not controlled and sometimes the grains are even stored in the open. As a result, the stored grains are expected to experience fluctuating temperatures of the local surroundings, and an ability to tolerate a wide range of growth temperatures is likely to be ecologically beneficial for microbes. Bacteria belonging to *Lactococcus, Lactobacillus,* and *Leuconostoc* are also known to produce organic acids as a result of their fermentative metabolism (Schleifer, 2009). Besides increasing acidity in the local environment, they can produce proteinaceous compounds known as bacteriocins which strongly inhibit a wide range of gram-positive bacteria from growing in the vicinity (Ogier *et al.*, 2008). These abilities may further promote their dominance in the community.

Serratia was particularly dominant in the packaged rice sample. It is a gram-negative, facultative anaerobe. A particular species of this genus, *Serratia marcescens,* can form spores which are known to persist into flours and also withstand baking processes (Tipples, 1995), suggesting it can survive very harsh treatments. This may be a reason for its persistence through the various processing steps of the post-harvest chain leading to high relative abundance in packaged rice. The bacterial community of the packaged rice sample was the least diverse. Likely, packaged rice, being most downstream of the post-harvest chain, harbors only the very few, highly persistent microbes which can survive through harsh processing conditions of the rice post-harvest system. However, only one packaged rice sample was analyzed in this study and more samples may need to be analyzed to confirm this finding.

Two distinct groups of fungi have been reported to invade the grains in the field and the storage sites (Christensen and Kaufmann, 1965). The 'field fungi' invade the seeds when they are developing on the plant before harvest. They require conditions of high relative humidity and their growth is usually inhibited post-harvest when the grain is dried and moisture contents are relatively lower. 'Storage fungi' represent the fungal groups which invade the grains during storage at low moisture contents. Spores of these fungi may be introduced into the grain from fields, processing and storage equipment and during post-harvest handling. These spores may then proliferate during storage. *Aspergillus* is classified as 'storage fungi' and specifically present in stored rice and not in fresh paddy in this study. It can grow at a wide range of temperatures from as low as 5°C to as high as 55°C (Christensen and Kaufmann, 1965). *Alternaria, Cladosporium,* and *Fusarium* are the major 'field fungi' identified in this study. Their presence in stored rice samples implies higher than usual moisture contents during storage. The growth of *Fusarium* in stored wheat has also been reported previously (Christensen and Kaufmann, 1965).

4.4 Implications of the presence of certain microbes on grain health

The lactic acid bacteria, identified during storage, ferment sugars and polysaccharides to lactate as the main fermentation product along with by-products like acetate, formate, ethanol, and carbon dioxide (Schleifer, 2009). *Clostridium* can produce organic acids and alcohols through carbohydrate metabolism. Such volatile organic compounds have been detected in rice stored at a high moisture content for long periods of time (Champagne *et al.*, 2004). They can add undesirable flavors and speed up the rate of spoilage, thus greatly lowering the nutritional quality of grain. Few species of *Leuconostoc* and *Lactobacillus* also induce spoilage by production of biogenic amines (Bernardeau *et al.*, 2008). Species of *Pseudomonas* are known to

cause a rice plant disease leading to grain discoloration (Cottyn *et al.*, 1996). *Erwinia* spp. and *Pantoea* spp. have also been reportedly involved in various plant diseases (Kado, 2006). *Serratia*, which was highly dominant in the packaged rice sample, is well known for the condition known as "bleeding bread" in which it produces a pigment that causes blood-like spots on food products (Grimont and Grimont, 2006). Among the fungi identified during storage, many produce toxins which directly affect human health, as discussed later.

On the positive side, such microbial secondary metabolites can serve as potential reliable indicators of onset of spoilage. This is important because bio-deterioration processes, being subtle during their early stages, are usually not apparent by visible inspection. This may lead to consumption of grain that appears to be healthy but has actually undergone significant degradation in quality.

4.5 Microbial community structure may be reflective of storage conditions

Grain moisture content and oxygen levels are key factors affecting microbial community structure during storage. Although bacterial endospores can survive low moisture contents due to desiccation-resistance, bacterial flora require moisture contents greater than 20% to grow (Tipples, 1995). Also, 'field fungi' growing in starchy grains such as rice typically require a moisture content of about 24 to 25%. Thus, the dominance of asporogenous and fermentative lactic acid bacteria along with 'field fungi' like *Alternaria* and *Cladosporium* in the stored grain microbial community is indicative of moisture content exceeding 20% and lack of proper aeration at the storage facilities. The range of moisture content usually recommended for safe storage of grain is only about 14 to 14.5 % (Christensen and Kaufmann, 1965). This shows how information regarding microbial community composition may be used to predict storage

conditions. However, definitive correlative measurements of microbial community structure with physico-chemical storage parameters are necessary to establish a reliable and accurate method of achieving this.

4.6 Potential risks to human health

Species belonging to *Aspergillus, Penicillium* and *Fusarium* are the only mycotoxigenic fungi known to be present and the associated mycotoxins have been reportedly found in rice (Abbas *et al.,* 1999; Liu *et al.,* 2006; Reddy *et al.,* 2009). The toxins produced by *Aspergillus* species are mainly aflatoxins with aflatoxin B_1being the most toxic and declared as a class I human carcinogen (IARC, 1993). Ochratoxin, a potent nephrotoxin is also produced by certain *Aspergillus* species, and a possible human carcinogen (IARC 1993). The major toxins produced by *Fusarium* species reported in rice are fumonisins, deoxynivalenol, zearalenone, and trichothecenes (Reddy *et al.,* 2008). All of these have been found to show a variety of toxic effects in animal studies while fumonisins have been classified as possible human carcinogens (IARC, 1993). The fungus *Alternaria,* a plant pathogen, produces certain phytotoxins, which can be toxic to humans (Moreno *et al.,* 2012). Co-occurrence of two or more of these toxins can take place and have synergistic effects on the carcinogenicity of these compounds (Ueno *et al.,* 1992). Amongst bacteria, *Clostridium* was the only genus associated with stored rice whose species are known to be pathogenic, some producing extremely harmful neurotoxins (Hatheway, 1990). Thus, grain spoilage induced by the growth of fungi is not only limited to grain discoloration, losses in dry matter and germination abilities, but also has more serious detrimental effects on human health.

CHAPTER 5

CONCLUSION

The bacterial and fungal community structures associated with different rice samples along the post-harvest chain were investigated. The major conclusions and implications of the study are summarized as follows:

- Two alternate methods of biomass collection were tested in this study and it was found that the choice of method did not affect the community composition of rice samples.
- Greater microbial diversity associated with freshly harvested paddy as compared to milled rice suggests that post-harvest milling may be responsible for the removal of many microbes from fresh paddy.
- *Lactococcus, Lactobacillus,* and *Leuconostoc* were the dominant bacterial genera specifically present in stored rice while *Aspergillus* being the dominant fungal genus specific to stored rice.
- The ability of these microbes to grow at a wide range of temperatures is likely to be a significant factor contributing to their dominance at storage sites. The lactic acid bacteria produce volatile organic compounds and biogenic amines which enhance bio-deterioration of grains. The fungi *Aspergillus* and *Fusarium,* on the other hand, produce carcinogenic mycotoxins which directly affect human health.
- These microbial secondary metabolites may serve as reliable early indicators of spoilage.
- The presence of fermentative, asporogenous bacteria along with major 'field fungi' like *Alternaria* and *Cladosporium* is indicative of high moisture content (>20%) and poor aeration of stored grain.

TABLES

Table 1. Details of rice samples collected from different sites in Haryana, India

Sample source/description	Rice variety
Milled rice from Haryana warehouse, Kaul, Haryana, stored since Nov 2011	PR family*
Milled rice from HAFED**-Vidhata Mill, Dhand, Haryana, stored since Aug 2011	PR family
Milled rice from Maheshwari Sheller, Dhand, Haryana, stored since Aug 2011	PR family
Milled rice from FCI***, Dhand, Haryana, stored since Jan 2011	PR family
Milled rice from FCI, Haryana - Fresh Procurement	PR family
Fresh, un-milled rice from local trade markets of Karnal, Haryana	PUSA Basmati 1121
Fresh, un-milled rice from local trade markets of Karnal, Haryana	PUSA Basmati 1121
Fresh, un-milled rice from local trade markets of Karnal, Haryana	PUSA Basmati 1121
Fresh, un-milled rice from local trade markets of Karnal, Haryana	PUSA Basmati 1121
Fresh, un-milled rice from local trade markets of Karnal, Haryana	PUSA Basmati 1121
Packaged rice from retail stores in Haryana – Pkg. date of Jan 2012	PUSA Basmati 1121

*PR family - family of coarse-grained rice developed by PUSA, a centre of ICAR in New Delhi. This is a mixed variety of rice and is procured for the public distribution system.
**HAFED - Haryana State Cooperative Supply and Marketing Federation Ltd
***FCI - Food Corporation of India

Table 2. Alpha diversity indices of bacterial communities associated with different rice types, based on the 'observed species' metric.

Rice type	Sample	Alpha diversity	Average alpha diversity per rice type
Fresh, un-milled	1	265	238
	2	276	
	3	234	
	4	232	
	5	183	
Stored, milled	1	51	85
	2	95	
	3	119	
	4	76	
Fresh, milled	1	111	-
Packaged	1	106	-

Table 3. Alpha diversity indices of fungal communities associated with different rice types, based on the 'observed species' metric.

Rice type	Sample	Alpha diversity	Average alpha diversity per rice type
Fresh, un-milled	1	289	294
	2	341	
	3	322	
	4	322	
	5	195	
Stored, milled	1	124	148
	2	111	
	3	208	
Fresh, milled	1	155	-

FIGURES

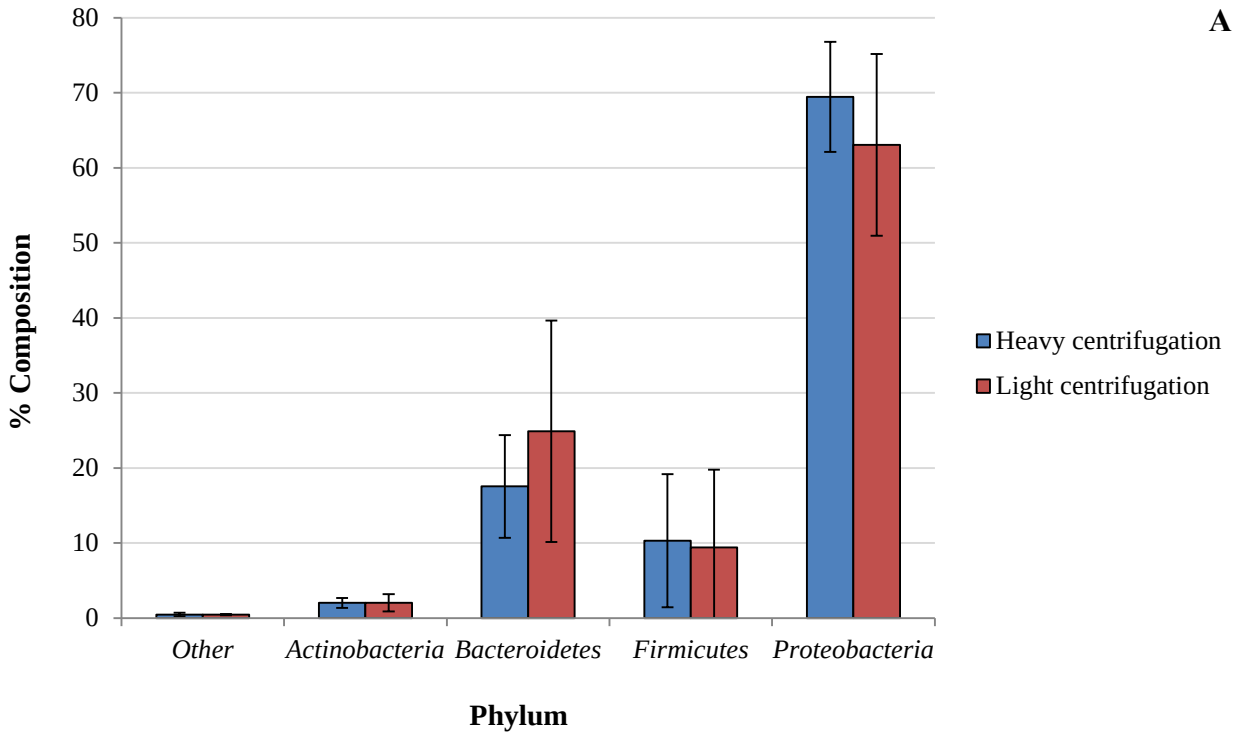
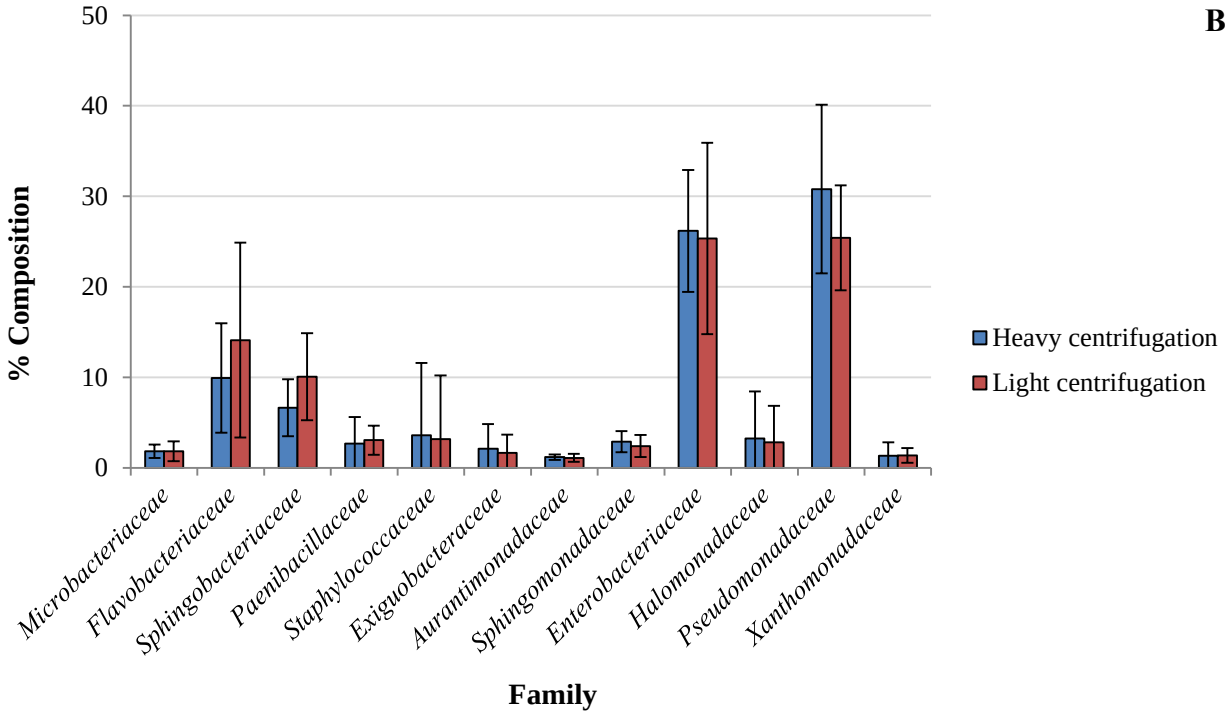
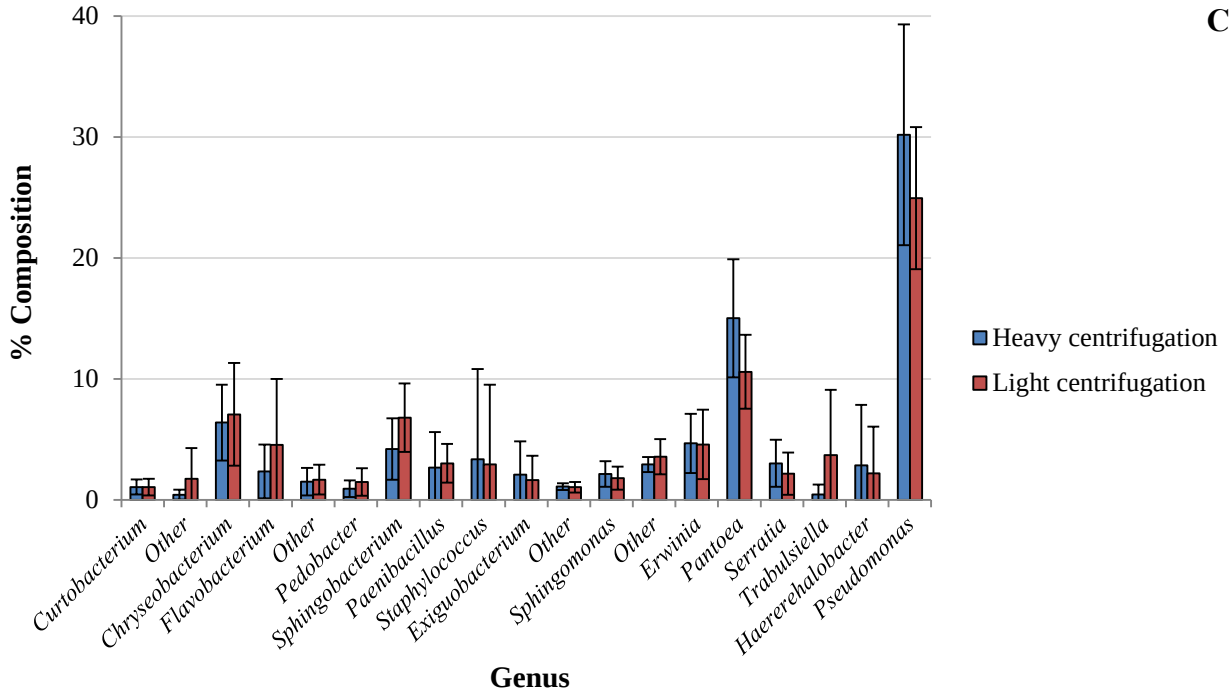


Figure 1. Comparison of bacterial community structure across two alternative methods of biomass collection from rice samples, namely, the heavy centrifugation method and the light centrifugation method. A) Comparison at phylum level. B) Comparison at family level. C) Comparison at genus level. The data presented represents the average over five samples of freshly harvested paddy.





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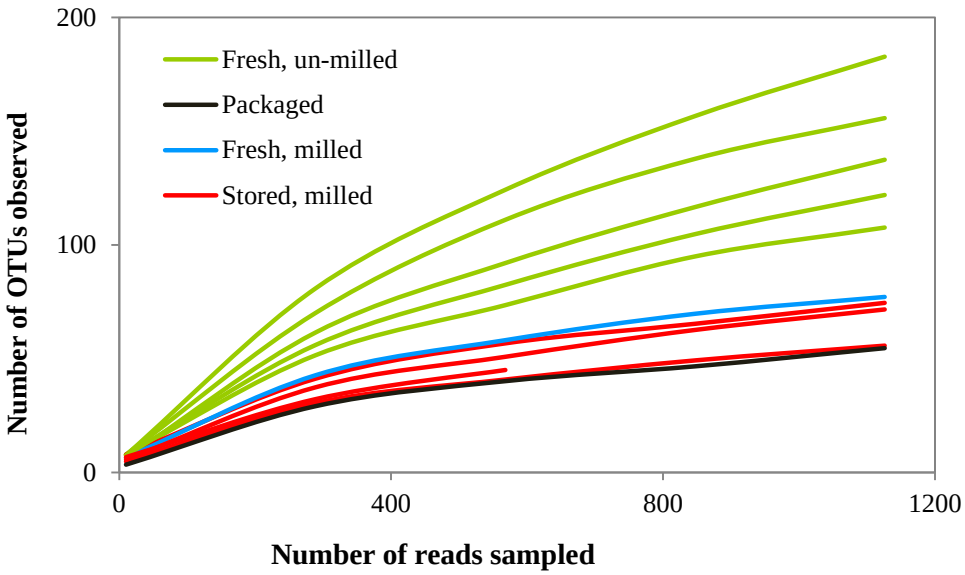

Figure 2. Rarefaction curves for bacterial OTUs (operational taxonomic units) clustered at 97% similarity cut-off. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage; 'packaged' rice simply corresponds to rice purchased from retail stores. Green represents un-milled rice samples while brown, orange and black represent milled rice samples.

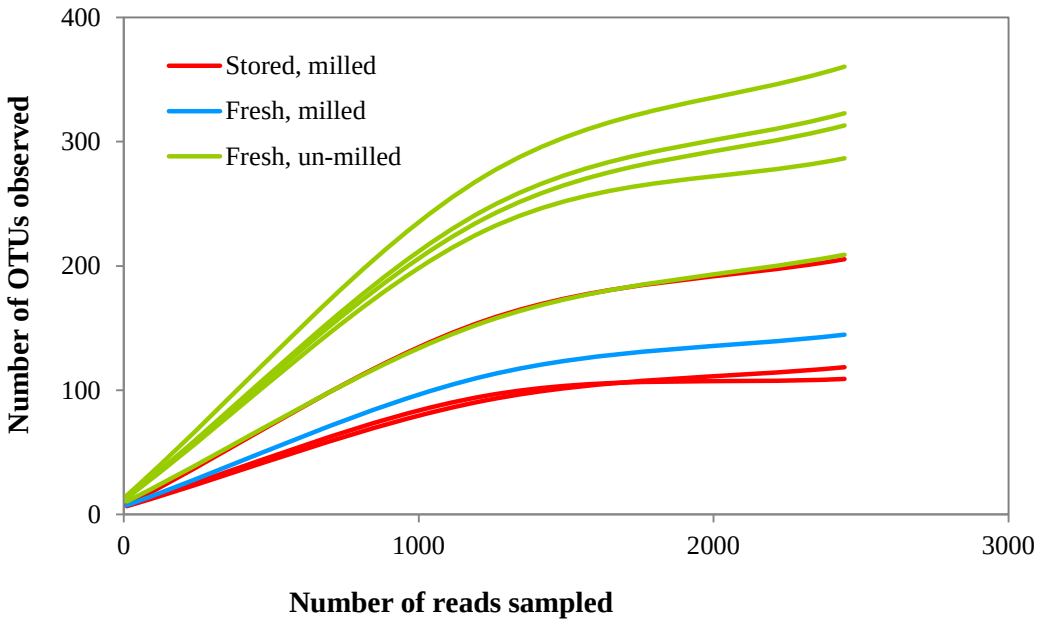

Figure 3. Rarefaction curves for fungal OTUs (operational taxonomic units) clustered at 97% similarity cut-off. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage. Green represents un-milled rice samples while brown and orange represent milled rice samples.

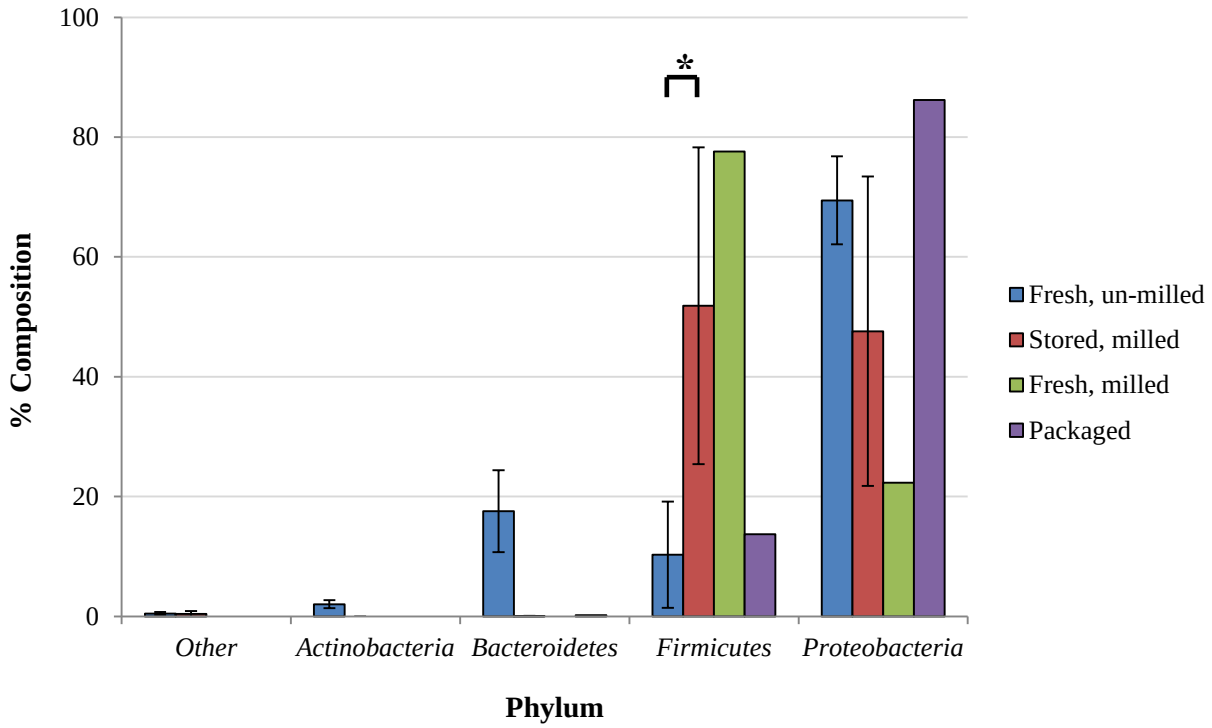

Figure 4. Comparative view of bacterial community structure, shown at phylum level, across four different rice types. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage; 'packaged' rice simply corresponds to rice purchased from retail stores. The data presented for fresh, un-milled rice represents the average over five samples while the data for stored, milled rice is averaged over four samples. The percentage compositions of bacterial groups were compared for significant differences using *t*-test. The * indicates $P < 0.05$.

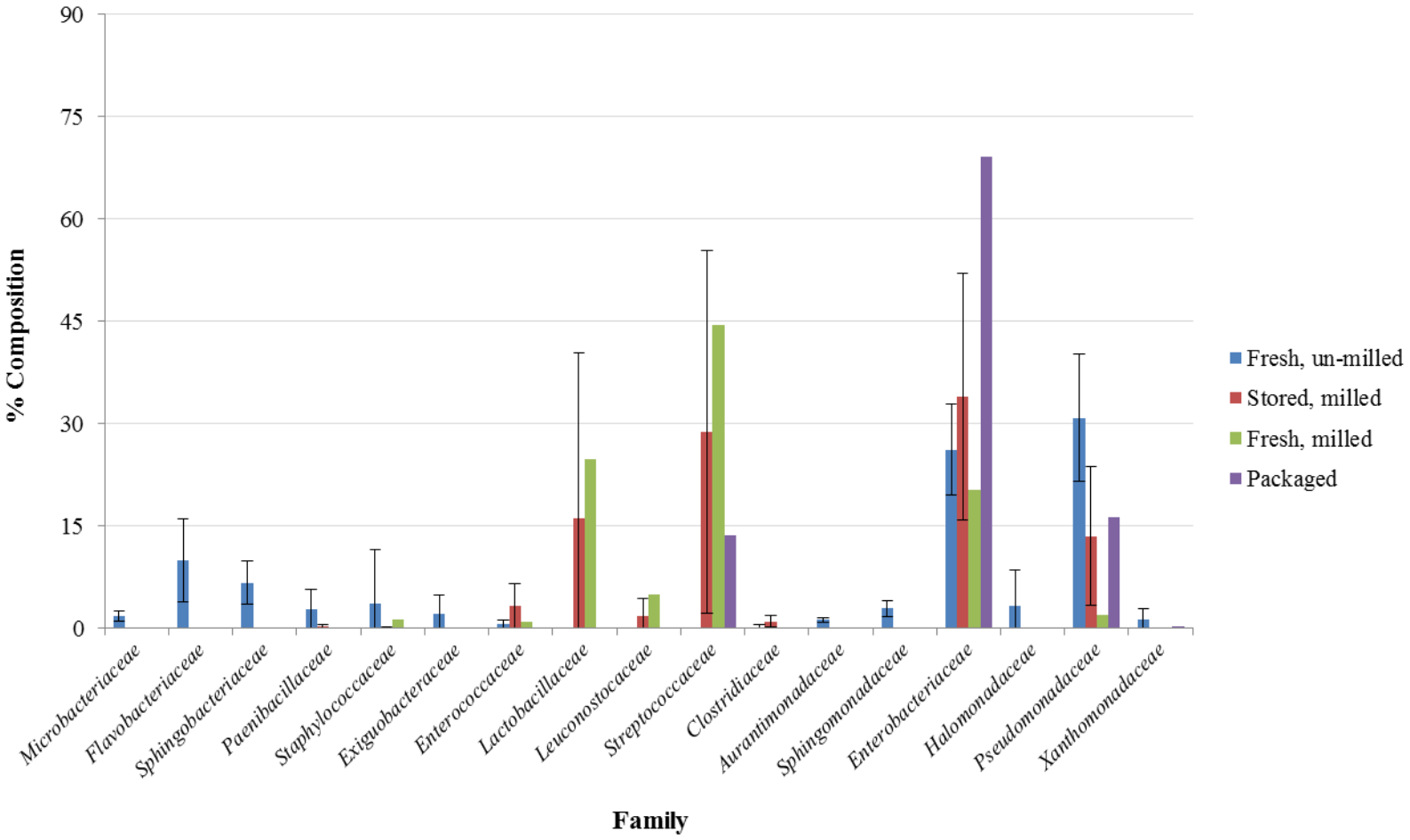

Figure 5. Comparative view of bacterial community structure, shown at family level, across four different rice types. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage; 'packaged' rice simply corresponds to rice purchased from retail stores. The data presented for fresh, un-milled rice represents an average over five samples while the data for stored, milled rice is averaged over four samples.

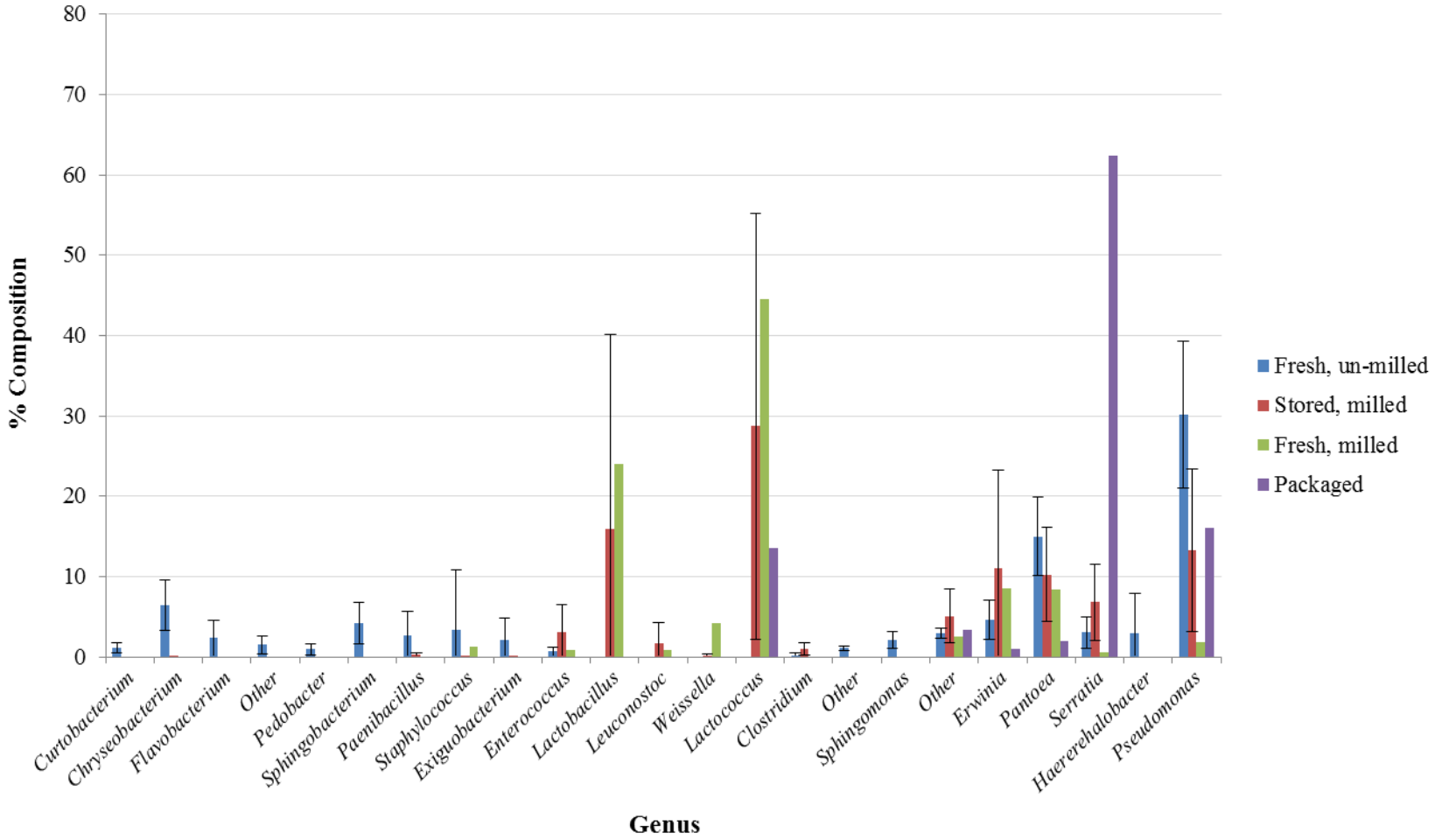

Figure 6. Comparative view of bacterial community structure, shown at genus level, across four different rice types. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage; 'packaged' rice simply corresponds to rice purchased from retail stores. The data presented for fresh, un-milled rice represents an average over five samples while the data for stored, milled rice is averaged over four samples.

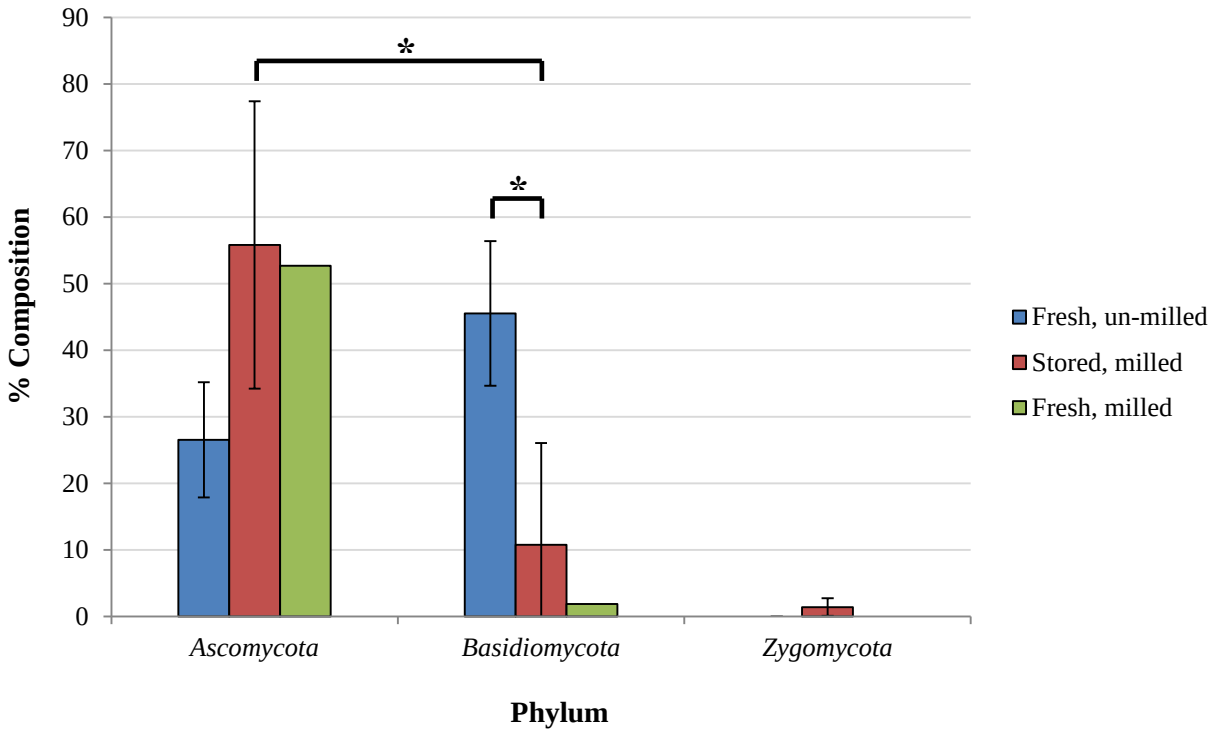

Figure 7. Comparative view of fungal community structure, shown at phylum level, across three different rice types. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage. The data presented for fresh, un-milled rice represents the average over five samples while the data for stored, milled rice is averaged over three samples. The percentage compositions of fungal groups were compared for significant differences using *t*-test. The * indicates $P < 0.05$.

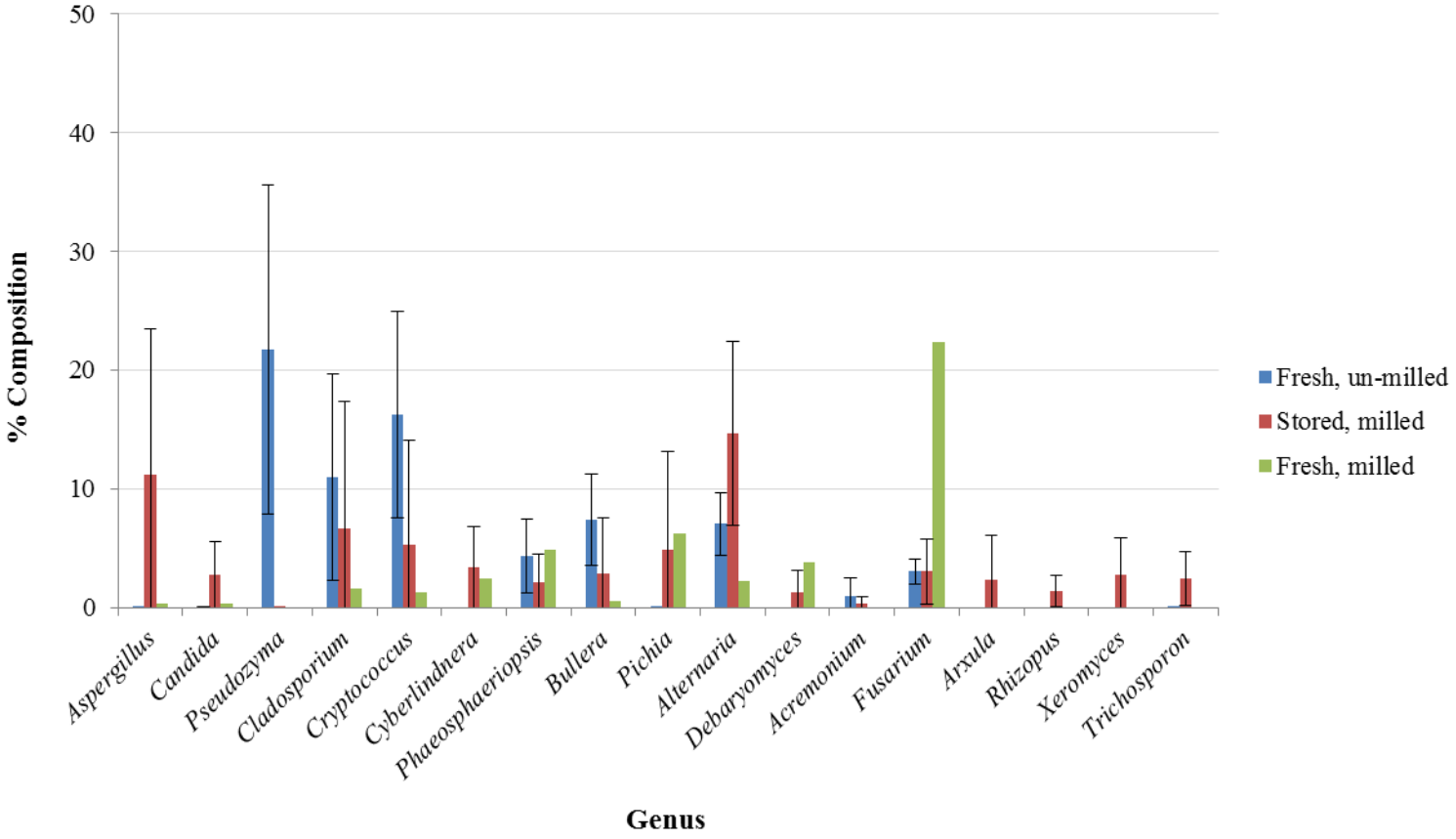

Figure 8. Comparative view of fungal community structure, shown at genus level, across three different rice types. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage. The data presented for fresh, un-milled rice represents an average over five samples while the data for stored, milled rice is averaged over three samples.

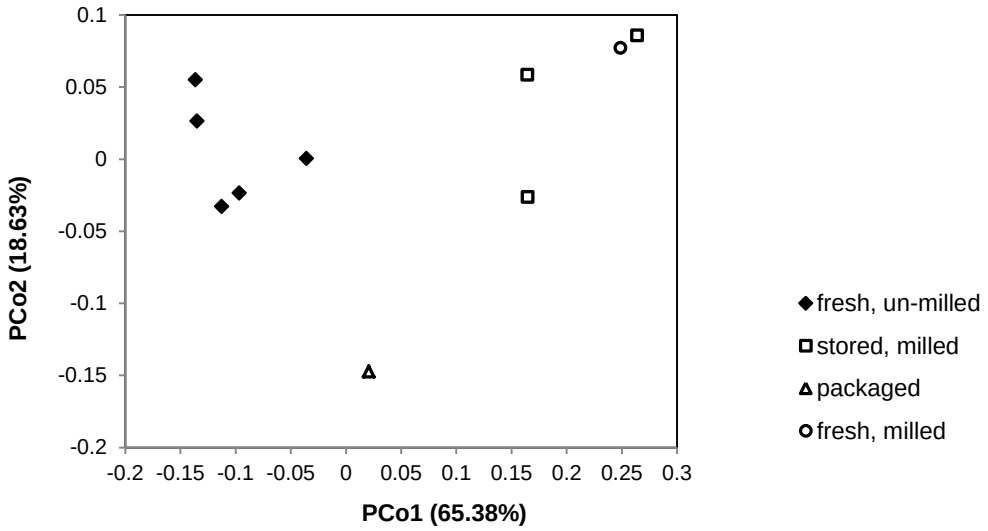

Figure 9. Principal coordinates analysis (PCoA) of the bacterial communities for the different rice samples. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage; 'packaged' rice simply corresponds to rice purchased from retail stores. The PCoA was based on the weighted UniFrac metric.

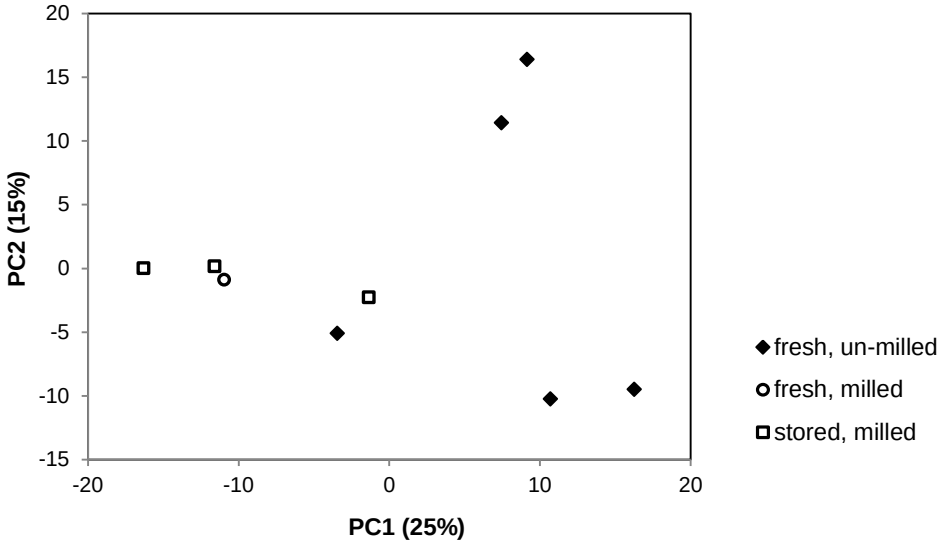

Figure 10. Principal components analysis (PCA) of the fungal communities for the different rice samples. 'Fresh, un-milled' corresponds to un-milled and freshly harvested paddy; 'Stored, milled' corresponds to milled rice that has been stored for one year at a storage facility; 'Fresh, milled' corresponds to rice that was collected right after the milling process before storage.

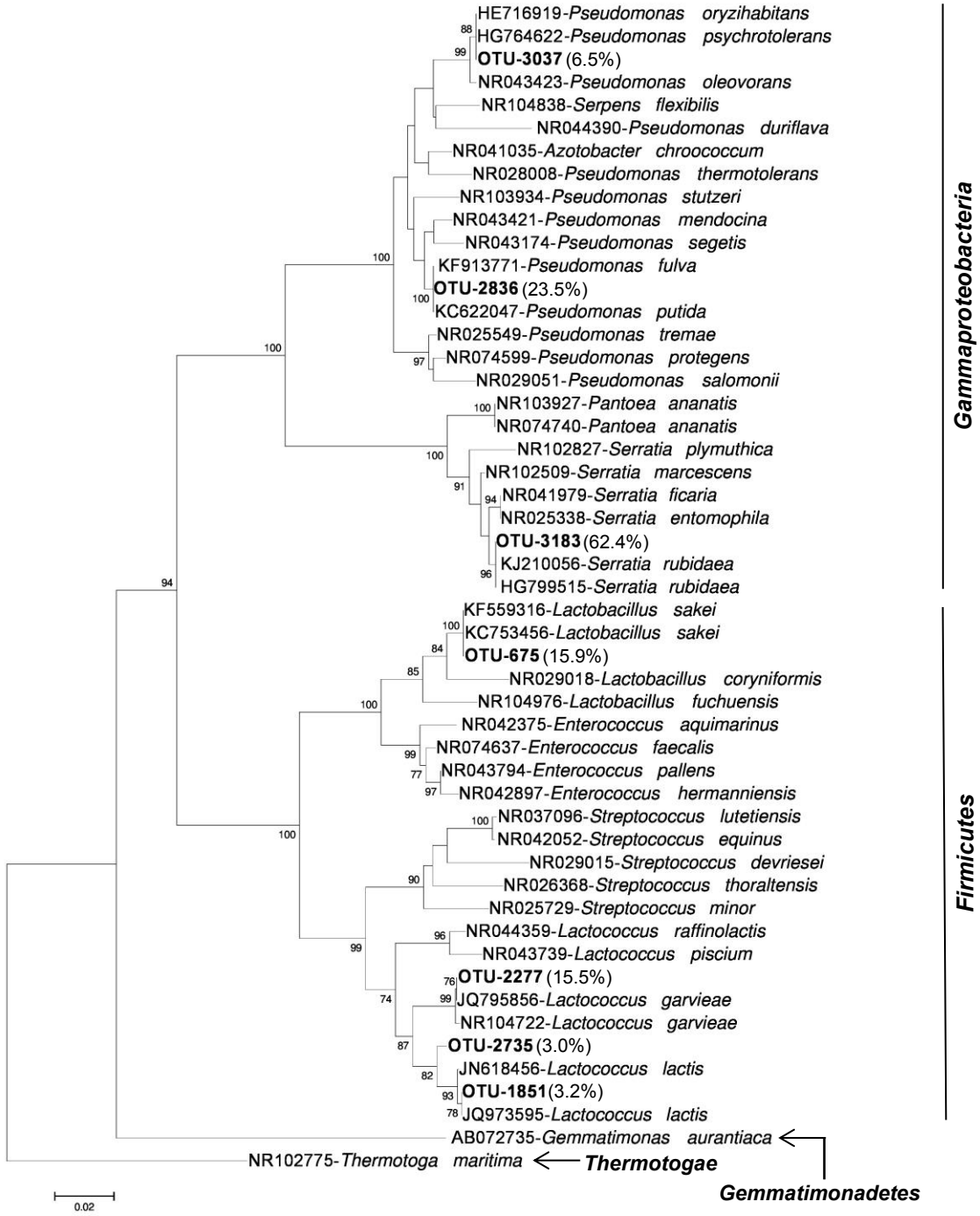

Figure 11. Phylogenetic tree of the dominant bacterial operational taxonomic units (OTUs) across different rice samples and their closest relatives (accession number indicated). The tree was inferred using the neighbor-joining method and based on the representative 16S rRNA gene sequence reads of the dominant OTUs and their closest relatives. *Thermotoga maritima* was selected as an outgroup. Bootstrap values greater than 70% are indicated next to the tree nodes and are based on 1000 iterations. The percentage abundance of the respective OTUs in the rice type in which they were dominant is indicated.

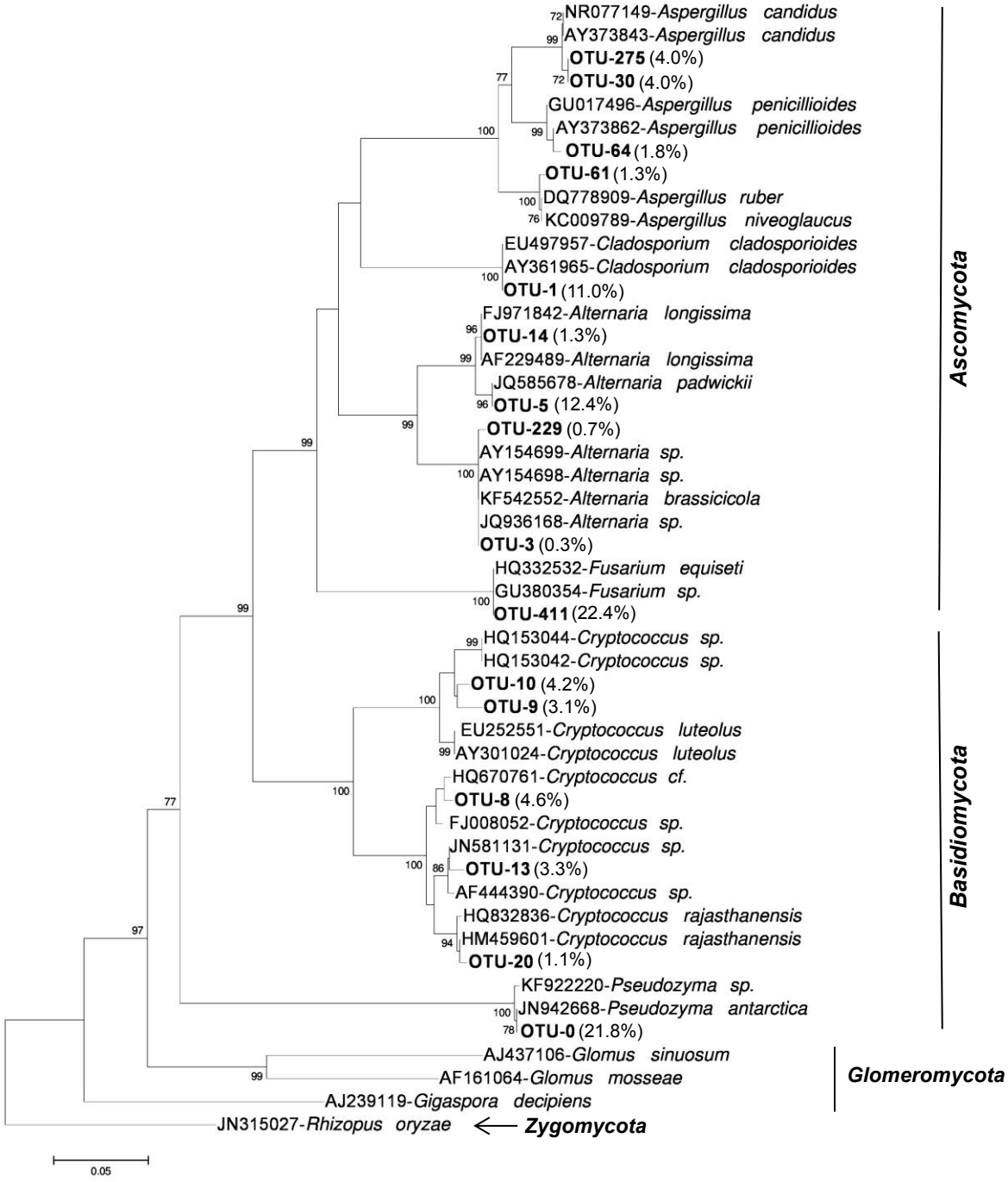

Figure 12. Phylogenetic tree of the dominant fungal operational taxonomic units (OTUs) across different rice samples and their closest relatives (accession number indicated). The tree was inferred using the neighbor-joining method and based on the representative ITS sequence reads of the dominant OTUs and their closest relatives. *Rhizopus oryzae* was selected as an outgroup. Bootstrap values greater than 70% are indicated next to the tree nodes and are based on 1000 iterations. The percentage abundance of the respective OTUs in the rice type in which they were dominant is indicated.

REFERENCES

Abarenkov K, Nilsson RH, Larsson K, Alexander IJ, Eberhardt U, Erland S, Hoiland K, Kjoller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Ursing BM, Vralstad T, Liimatainen K, Peintner U, Koljalg U. 2010. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytol. **186:**281-285.

Abbas HK, Cartwright RD, Xie W, Mirocha CJ, Richard JL, Dvorak TJ, Sciumbato GL, Shier WT. 1999. Mycotoxin production by *Fusarium proliferatum* isolates from rice with *Fusarium* sheath rot disease. Mycopathologia. **147:**97-104.

Ahn J, Song J, Kim B, Kim M, Joa J, Weon H. 2012. Characterization of the bacterial and archaeal communities in rice field soils subjected to long-term fertilization practices. J. Microbiol. **50:**754-765.

Almeida RMA, Gambale W, Correa B, Paula CR, Asavedo IG. 1991. Mycoflora and aflatoxigenic species of *Aspergillus* sp. isolated from rice. Microbiologia. **22:**161-163.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.

Arjun JK, Harikrishnan K. 2011. Metagenomic analysis of bacterial diversity in the rice rhizosphere soil microbiome Biotechnol. Bioinf. Bioeng. **1:**361-367.

Basavaraja H, Mahajanashetti SB, Udagatti NC. 2007. Economic analysis of post-harvest losses in food grains in India: a case study of Karnataka. Agri. Eco. Res. Rev. **20:**117-126.

Benham RW. 1956. The genus *Cryptococcus*. Bacteriol. Rev. **20:**189-201.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. 2005. GenBank. Nucleic Acids Res. **33:**D34-D38.

Bernardeau M, Vernoux JP, Henri-Dubernet S, Guéguen M. 2008. Safety assessment of dairy microorganisms: The *Lactobacillus* genus. Int. J. Food Microbiol. **126:**278-285.

Bourne MC. 1977. Post harvest food losses: the neglected dimension in increasing the world food supply. Cornell Int. Agri. Mimeo. **53:**1-42.

Boxall RA. 2002. Storage losses, p. 143-169. *In* Golob P, Farrell G, Orchard JE (eds.), Crop post-harvest: science and technology, volume 1: principles and practice. Wiley-Blackwell, Oxford.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneweld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. **7:**335-336.

Champagne ET, Thompson JF, Bett-Garber KL, Mutters R, Miller JA, Tan E. 2004. Impact of storage of freshly harvested paddy rice on milled white rice flavor. Cereal Chem. **81:**444-449.

Christensen CM, Kaufmann HH. 1965. Deterioration of stored grains by fungi. Annu. Rev. Phytopathol. **3:**69-84.

Cottyn B, Regalado E, Lanoot B, DeCleene M, Mew TW, Swings J. 2000. Bacterial populations associated with rice seed in the tropical environment. Phytopathol. **91:**282-292.

Cottyn B, Van Outryve MF, Cerez MT, De Cleene M, Swings J, Mew TW. 1996. Bacterial diseases of rice. II. Characterization of pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. **80:**438-445.

de Lucia M, Assennato D. 1994. Agricultural engineering in development: post-harvest operations and management of food grains. FAO Agricultural Services Bulletin No. 93. FAO, Rome.

FAO. 1978. Promotion of food security with special emphasis on reduction of postharvest food losses. FAO-DDD--FERC/78/6**.** FAO, Rome.

FAO. 1977. Analysis of an FAO survey of postharvest crop losses in developing countries. AGPP:MISC/27**.** FAO, Rome.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. **39:**783-791.

Finlay BJ. 2002. Global dispersal of free-living microbial eukaryote species. Science. **296:**1061-1063.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrihzae and rusts. Mol. Ecol. **2:**113-118.

Gautam AK, Gupta H, Soni Y. 2012. Screening of fungi and mycotoxins associated with stored rice grains in Himachal Pradesh Int. J. Theor. Appl. Sci. **4:**128-133.

Grimont F, Grimont PAD. 2006. The genus *Serratia*. Prokaryotes. **6:**219-244.

Grolleaud M. 1997. Post-harvest losses: discovering the full story. FAO, Rome.

Hammes WP, Hertel C. 2009. *Lactobacillus*, p. 465-511. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K, Whitman WB (eds.), Bergey's Manual of Systematic Bacteriology: The *Firmicutes*, 2nd ed. Springer, New York.

Harrington LW, Fujisaka S, Morris ML, Hobbs PR, Sharma HC, Singh RP, Chaudhary MK, Dhiman SD. 1993. Wheat and rice in Karnal and Kurukshetra districts, Haryana, India: farmers' practices, problems, and an agenda for action. International Maize and Wheat Improvement Center (CIMMYT), Mexico.

Hatheway CL. 1990. Toxigenic *Clostridia*. Clin. Microbiol. Rev. **3:**66-98.

Hodges RJ, Buzby JC, Bennett B. 2011. Postharvest losses and waste in developed and less developed countries: opportunities to improve resource use. J. Agri. Sci. **149:**37-45.

Holzapfel WH, Bjorkroth JA, Dicks LMT. 2009. *Leuconostoc*, p. 624-635. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K, Whitman WB (eds.), Bergey's Manual of Systematic Bacteriology: The *Firmicutes*, 2nd ed., . Springer, New York.

Hong SW, Choi JY, Chung KS. 2012. Culture-based and denaturing gradient gel electrophoresis analysis of the bacterial community from *Chungkookjang*, a traditional Korean fermented soybean food J. Food Sci. **77:**572-578.

IARC. 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. **56**.

Kado CI. 2006. *Erwinia* and related genera. Prokaryotes. **6:**443-450.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics. **23:**2947-2948.

Liu Z, Gao J, Yu J. 2006. Aflatoxins in stored maize and rice grains in Liaoning province, China. J. Stored. Prod. Res. **42:**468-479.

Magan N, Aldrid D. 2007. Post-harvest control strategies: minimizing mycotoxins in the food chain. Int. J. Food Microbiol. **119:**131-139.

Magan N, Hope R, Cairns V, Aldred D. 2003. Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. Eur. J. Plant Pathol. **109:**723-730.

Makun HA, Gbodi TA, Akanya HO, Sakalo AE, Ogbadu HG. 2007. Fungi and some mycotoxins contaminating rice (*Oryza sativa*) in Niger state, Nigeria. African J. Biotechnol. **6:**99-108.

Min-Cheol K, Ahn J, Shin H, Kim T, Ryu T, Kim D, Song H, Lee GH, Ka J. 2008. Molecular analysis of bacterial community structures in paddy soils for environmental risk assessment with two varieties of genetically modified rice, Iksan 483 and Milyang 204 J. Microbiol. Biotechnol. **18:**207-218.

Moreno MAP, Alonso IG, de Santos RM, Lacarra TG. 2012. The importance of genus *Alternaria* in mycotoxins production and human diseases. Nutr. Hosp. **27:**1772-1781.

Nei M, Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, Oxford.

Ogier JC, Casalta E, Farrokh C, Saïhi A. 2008. Safety assessment of dairy microorganisms: The *Leuconostoc* genus. Int. J. Food Microbiol. **126:**286-290.

Oh JY, Jee SN, Nam Y, Lee H, Ryoo MI, Kim KD. 2007. Populations of fungi and bacteria associated with samples of stored rice in Korea. Mycobiol. **35:**36-38.

Oh JY, Sang MK, Ryoo MI, Kim KD. 2008. Temporal changes of fungal and bacterial populations in rice under indoor storage conditions Plant Pathol. J. **24:**74-79.

Reddy KRN, Reddy CS, Abbas HK, Abel CA, Muralidharan K. 2008. Mycotoxigenic fungi, mycotoxins, and management of rice grains. Toxin Rev. **27:**287-317.

Reddy KRN, Reddy CS, Muralidharan K. 2009. Detection of *Aspergillus* spp. and aflatoxin B_1 in rice in India. Food Microbiol. **26:**27-31.

Saitou N, Nei M. 1987. The neighbor-joining method—a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406-425.

Saunders RM, Mossman AP, Wasserman T, Beagle EC. 1980. Rice postharvest losses in developing countries. ARM-W-12. U.S. Department of Agriculture, Oakland.

Schleifer K. 2009. *Lactobacillaceae*, p. 465-532. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K, Whitman WB (eds.), Bergey's Manual of Systematic Bacteriology: The *Firmicutes*, 2nd ed. Springer, New York.

Sundaram BM, Krishnamurthy R, Subramanian S. 1988. Aflatoxin-producing fungi in stored paddy Proc. Indian Acad. Sci. (Plant Sci.). **98:**291-297.

Taligoola HK, Ismail MA, Chebon SK. 2004. Mycobiota associated with rice grains marketed in Uganda. J. Biol. Sci. **4:**271-278.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. **30:**2725-2729.

Teuber M. 2009. *Lactococcus*, p. 711-722. *In* De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K, Whitman WB (eds.), Bergey's Manual of Systematic Bacteriology: The *Firmicutes*, 2nd ed. Springer, New York.

Teunisson DJ. 1954. Influence of storage without aeration on the microbial populations of rough rice. Cereal Chem. **31:**462-474.

Tipples KH. 1995. Quality and nutritional changes in stored grain, p. 325-351. *In* Jayas DS, White NDG, Muir WE (eds.), Stored-Grain Ecosystems. Marcel Dekker, Inc., New York.

Trung TSY, Bailley JD, Querin A, Bars PLE, Guerre P. 2001. Fungal contamination of rice from south Vietnam, mycotoxinogenesis of selected strains and residues in rice Revue. Med. Vet. **152:**555-560.

Udagawa S. 1976. Distribution of mycotoxin-producing fungi in foods and soil from New Guinea and southeast Asia Proc. Japanese Assoc. Mycotoxicol. **2:**10-15.

Ueno Y, Yabe T, Hashimoto H, Sekijima M, Masuda M, Kim DJ, Hasegawa R, Ito N. 1992. Enhancement of GST-P-positive liver cell foci development by nivalenol, a trichothecene mycotoxin. Carcinogenesis. **13:**433-437.

Uma V, Wesely EG. 2013. Seed borne fungi of rice from south Tamil Nadu. J. Acad. Indus. Res. **1:**612-614.

Umali-Deininger D, Ahluwalia D. 1999. India foodgrain marketing policies: reforming to meet food security needs. 18329-IN. Rural Development Sector Unit, South Asia Region, World Bank.

Wagacha JM, Muthomi JW. 2008. Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. Int. J. Food Microbiol. **124:**1-12.

Wang Y, Qian P. 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. Plos One. **4:**1-9.

Ward DM, Weller R, Bateson MM. 1990. 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. FEMS Microbiol. Rev. **6:**105-115.

Wei Y, Lee F, Hsu W, Chen S, Chen C, Wen C, Lin S, Chu W, Yuan G, Liou G. 2005. *Pseudozyma antarctica* in Taiwan: a description based on morphological, physiological and molecular characteristics. Bot. Bull. Acad. Sin. **46:**223-229.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., San Diego.

Wu VCH. 2008. A review of microbial injury and recovery methods in food. Food Microbiol. **25:**735-744.

Zhou J, Bruns MA, Tiedje JM. 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. **62:**316-322.

APPENDIX

SUPPLEMENTAL TABLES

Table S1. Composition of bacterial communities associated with different rice types, at phylum level

Phylum	% Composition			
	Fresh, un-milled rice	Stored, milled rice	Fresh, milled rice	Packaged rice
Proteobacteria	69.4	47.6	22.3	86.2
Firmicutes	10.3	51.9	77.6	13.7
Bacteroidetes	17.6	0.0	0.0	0.2
Actinobacteria	2.0	0.0	0.0	0.0

Table S2. Composition of bacterial communities associated with different rice types, at family level

Family	% Composition			
	Fresh, un-milled rice	Stored, milled rice	Fresh, milled rice	Packaged rice
Lactobacillaceae	0.0	16.1	24.7	0.0
Streptococcaceae	0.0	28.8	44.5	13.6
Enterobacteriaceae	26.2	34.0	20.3	69.2
Pseudomonadaceae	30.8	13.5	1.9	16.2
Leuconostocaceae	0.0	1.8	5.0	0.0
Clostridiaceae	0.2	1.0	0.0	0.0
Enterococcaceae	0.7	3.2	0.9	0.0
Xanthomonadaceae	1.3	0.0	0.0	0.2
Aurantimonadaceae	1.2	0.0	0.0	0.0
Sphingomonadaceae	2.9	0.0	0.0	0.0
Halomonadaceae	3.2	0.0	0.0	0.0
Microbacteriaceae	1.8	0.0	0.0	0.0
Flavobacteriaceae	9.9	0.0	0.0	0.1
Sphingobacteriaceae	6.6	0.0	0.0	0.0
Paenibacillaceae	2.7	0.2	0.0	0.0
Staphylococcaceae	3.6	0.0	1.3	0.0
Exiguobacteraceae	2.1	0.0	0.0	0.0

Table S3. Composition of bacterial communities associated with different rice types, at genus level

Genus	% Composition			
	Fresh, un-milled rice	**Stored, milled rice**	**Fresh, milled rice**	**Packaged rice**
Lactobacillus	0.0	15.9	24.0	0.0
Lactococcus	0.0	28.7	44.5	13.6
Leuconostoc	0.0	1.7	0.8	0.0
Enterococcus	0.7	3.1	0.9	0.0
Weissella	0.0	0.1	4.2	0.0
Serratia	3.0	6.8	0.6	62.4
Pseudomonas	30.2	13.3	1.9	16.0
Erwinia	4.7	11.0	8.6	1.0
Pantoea	15.0	10.3	8.4	2.0
Sphingomonas	2.2	0.0	0.0	0.0
Haererehalobacter	2.9	0.0	0.0	0.0
Clostridium	0.2	1.0	0.0	0.0
Curtobacterium	1.1	0.0	0.0	0.0
Chryseobacterium	6.4	0.0	0.0	0.0
Flavobacterium	2.4	0.0	0.0	0.0
Pedobacter	0.9	0.0	0.0	0.0
Sphingobacterium	4.2	0.0	0.0	0.0
Paenibacillus	2.7	0.2	0.0	0.0
Staphylococcus	3.4	0.0	1.3	0.0
Exiguobacterium	2.1	0.0	0.0	0.0

Table S4. Composition of fungal communities associated with different rice types, at phylum level

Phylum	% Composition		
	Fresh, un-milled rice	Stored, milled rice	Fresh, milled rice
Ascomycota	26.5	55.8	52.7
Basidiomycota	45.5	10.8	1.8
Zygomycota	0.0	1.4	0.0

Table S5. Composition of fungal communities associated with different rice types, at genus level

Genus	% Composition		
	Fresh, un-milled rice	Stored, milled rice	Fresh, milled rice
Aspergillus	0.0	11.2	0.4
Fusarium	3.0	3.0	22.4
Alternaria	7.1	14.7	2.3
Cladosporium	11.0	6.7	1.6
Pseudozyma	21.8	0.0	0.0
Cryptococcus	16.3	5.3	1.3
Candida	0.0	2.8	0.3
Cyberlindnera	0.0	3.4	2.5
Phaeosphaeriopsis	4.4	2.2	4.9
Bullera	7.4	2.9	0.6
Pichia	0.0	4.9	6.3
Debaryomyces	0.0	1.3	3.8
Acremonium	1.0	0.4	0.0
Arxula	0.0	2.4	0.0
Rhizopus	0.0	1.4	0.0
Xeromyces	0.0	2.8	0.0
Trichosporon	0.0	2.5	0.0