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Dynamics of bacterial communities in alfalfa and mung bean sprouts during refrigerated conditions

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ABSTRACT

Sprouts are considered a healthy ready-to-eat food and has gained popularity in recent years. The objective of the present study was to determine the dynamics of sprouts' microbiome during cold storage to the end of their shelf-life at home. The microbiological quality of fresh alfalfa (*Medicago sativa*) and mung bean (*Vigna radiata*) sprouts from two commercial brands was tested and the number of APC ranges from 5.0 to 8.7 log CFU/g in alfalfa and 6.7 to 9.3 log CFU/g in mung bean sprouts. In the case of alfalfa, but not mung beans, there were differences in the mean numbers of APC between the two brands. The number of coliform bacteria ranges from 4.3 to 7.7 log CFU/g in alfalfa and 4.1 to 8.1 log CFU/g in mung bean sprouts. Four independent batches of sprouts were used for DNA preparation and were sampled immediately after purchase and once a week during subsequent storage in refrigerator until the end of their shelf-life. Microbial population of the sprouts was determined using next generation sequencing of 16S rRNA amplicons. Alfalfa sprouts were dominated by *Pseudomonas* throughout the storage time with relative abundance of > 60% at 3 weeks. Fresh mung bean sprouts were dominated by both *Pseudomonas* and *Pantoea,* but *Pantoea* became the dominant taxa after 2 weeks of storage, with > 46% of relative abundance. The bacterial communities associated with sprouts were largely dependent on the sprout type, and less dependent on the brand. The species richness and diversity declined during storage and the development of spoilage. Among the 160 genera identified on sprouts, 23 were reported to contain known spoilage-associated species and 30 genera comprise potential human pathogenic species. This study provides new insight into the microbiome dynamics of alfalfa and mung bean sprouts during cold storage.

1. Introduction

Sprouted seeds are increasing popularity due to their nutritional qualities and widespread availability (Yang et al., 2013). Sprouts are produced from a wide variety of plant species, mostly from *Leguminosae* family, such as alfalfa, mung bean, radish, and soy sprouts. Alfalfa (*Medicago sativa*) and Mung bean (*Vigna radiata*) are the most commonly consumed sprouts worldwide, due to their high nutritive contents as well as availability at low cost (Matos et al., 2002; Nagar et al., 2016). Sprouts are produced from seeds, often by small to medium-size firms under warm and moist environments, conditions that are ideal for growth of microorganisms (Barak et al., 2002; Fu et al., 2001; Jaquette et al., 1996; Taormina et al., 1999).

The consumption of edible sprouts is increased due to their healthy nutritional content (Yang et al., 2013), yet, sprouts are also considered a risky food as reflected by outbreaks of foodborne diseases associated with their consumption (Dechet et al., 2014; EFSA, 2012; WHO, 2011). Sprouts were involved in two of the largest and severe foodborne outbreaks that caused illness in thousands cases in Japan and in Germany, and resulted together in 62 death cases (Michino et al., 1999; WHO, 2011). More than 50 outbreaks implicated with sprouts consumption were reported in Europe from 1992 to 2012 (EFSA, 2012). In the USA, 33 outbreaks were associated with sprouts from 1998 to 2010 (Dechet et al., 2014). A majority of the outbreaks have been linked to ready-to-eat (RTE) alfalfa and mung bean sprouts (Bayer et al., 2014; Warriner et al., 2003; Ye et al., 2010). While many studies assess the presence of

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human pathogens, indicator- and culturable bacteria in sprouts (Abadias et al., 2008; Buchholz et al., 2011; Mahon et al., 1997; Sadler-Reeves et al., 2015; Saroj et al., 2006; Seow et al., 2012; Stewart et al., 2001; Viswanathan and Kaur, 2001), less data are available regarding the microbiome of fresh sprouts as well as regarding the effect of cold storage on the microbiome composition. Since bacteria readily flourish in the sprout's environment, knowledge regarding the nature of the autochthonous bacterial population may contribute to understanding of spoilage processes and possible association between microbiome composition and the presence of human pathogens.

Molecular approaches, e.g., Denaturing gradient gel electrophoresis (DGGE), clone library, rep-PCR, Terminal restriction fragment length polymorphism (T-RFLP), 16S rRNA gene sequencing, demonstrated diverse bacterial communities associated with sprouts (Landry et al., 2017; Loui et al., 2008; Molinos et al., 2009; Nagar et al., 2016; Paramithiotis et al., 2014; Weiss et al., 2007). High throughput next generation sequencing (NGS) based culture-independent techniques are increasingly employed to study microbial-population dynamics of food associated microbes with higher sensitivity in order to decipher dominant as well as rare taxa (Cocolin and Ercolini, 2015; Ercolini, 2013). Indeed, NGS approach was employed to analyse bacterial composition in various sprouts e.g., alfalfa, mung beans, radish, wheat, soybean, peanuts (Asakura et al., 2016; Huang et al., 2016, 2017a, 2017b; Leff and Fierer, 2013; Margot et al., 2016a, 2016b). However, to the best of our knowledge, none has examined the dynamics of microbial populations of commercial sprouts during cold storage until consumption. Sprouted seeds are transported from the producing farm to the retail points, where they are kept in cooled shelves up to several days and after purchasing, they are stored in home refrigerators. Since sprouts may be consumed following several days of storage in the production facility, transportation, retail store or in the consumer's house, it is of interest to understand the impact of storage time upon bacterial composition. This may provide important knowledge regarding the spoilage processes of sprouts, which determine sprout's shelf-life, as well as, information regarding the intake of major taxa by consumers. Consequently, the purpose of the present study was to determine the microbiome associated with fresh alfalfa and mung bean sprouts and elucidate the population dynamics during refrigerated conditions.

2. Materials and methods

2.1. Sample collection and processing

Commercial packages of 200 g Alfalfa and Mung beans sprouts were purchased from retailers in Rishon-LeZion city, Israel. Sprout were purchased from two national companies' brands designated c1 and c2 during the years 2014–2015. Sprouts were purchased from the supermarkets at the day of arrival or on the next day, and transported to the laboratory within half an hour under refrigerated conditions. Sprouts packages were opened aseptically by partially removing the thin plastic wrap, and samples of 25 g, were weighed and homogenized in 225 ml of buffered peptone water (BPW; Becton Dickinson, Sparks MD, USA) for 2 min using a stomacher (Interscience BagMixer 400, France). Samples from the homogenate were taken for microbial analyses. Four DNA preparation, from separate batches of each sprout and from each manufacturer were purchased at different times during 2 months and used as four biological replicates. Samples (25 g) were taken aseptically and homogenized as described before, and the homogenate was taken for DNA preparation, as described below. The packages were wrapped again with their original plastic wrap and stored in a refrigerator (4 °C) until initial signs of browning and/or decay appeared and the sprouts were spoiled (data not shown). The sprouts' shelf life under these conditions was 3 weeks for alfalfa and 2 weeks for mung bean sprouts. Each package was sampled, as described above, once a week (Supplementary materials, Table S1).

2.2. Bacteriological analysis

APC determination was performed using Plate Count Agar (Becton Dickinson) according to Bacteriological analytical manual (Maturin and Peeler, 2001). The plates were incubated for 48 h at 35 °C, and bacterial colonies were counted. Coliforms determination was performed using violet red bile agar (VRBA; Becton Dickinson) according to Bacteriological analytical manual (Feng et al., 2013). Typical large purple-red colonies were counted. *Escherichia coli* was determined by direct plating on Tryptone Bile X-Glucuronide (TBX) agar (Merck, Darmstadt, Germany). The plates were incubated at 44 °C for 20 h. Blue colonies were recorded as presumptive *E. coli*. Separate sprout packages were used for the detection of *Salmonella* and *Listeria*. For *Salmonella* determination, 25 g of each sample was diluted in 225 ml BPW and homogenized, as described above. Following 18–24 h of preenrichment at 35 °C, 0.1 ml of the suspension were further enriched in tetrathionate broth and Rappaport-Vassiliadis medium (both from Becton Dickinson) at 42 °C for 24 h. Detection of *Salmonella* in the enrichment broth was done according to ISO 6579. For *Listeria* detection, 25 g of sprouts were homogenized in 225 ml of *Listeria* enrichment broth, and incubated at 35 °C for 48 h. Identification of L. *monocytogenes* in the enrichment broth was done according to ISO 11290-1.

2.3. DNA preparation and sequencing

A sub-sample of stomached liquid (5 ml) was filtered through a sterile Whatman No. 1 filter (11 µm nominal pore size; Whatman, Maidstone, England) to remove residual sprouts' debris. The filtrate was centrifuged (12,800 × *g*, 10 min) to the pellet cells, and the moist pellet transferred to a 1.5 ml sterile microcentrifuge tube and frozen at −20 °C until DNA extraction. DNA was extracted using Mericon DNA bacteria plus kit (Qiagen, Germany) and genomic DNA was quantified, and checked for purity by NanoDrop spectrophotometer (Thermo Scientific, USA), before storing at −20 °C. PCR amplification was carried out in triplicates to amplify V3–V4 conserved regions of bacterial 16S rRNA gene sequences using adapted primers 515F and 806R (Liu et al., 2007). Library preparation, pooling and sequencing were performed at the DNA Services (DNAS) facility, Research Resources Center (RRC), University of Illinois at Chicago (UIC). Briefly, genomic DNA was PCR amplified using a two-stage targeted amplicon sequencing protocol (Green et al., 2015). Both primer sets were attached with Illumina adapter sequences. All PCR reactions were performed in 10 µl volumes, with a final concentration of each primer of 1 µM. Amplification of bacterial DNA was made using the MyTaq HS 2X master-mix, and 28 cycles of PCR were performed (i.e., 95 °C for 5′, followed by 28 cycles of 95 °C for 30"; 55 °C for 40"; 72 °C for 30″). All reactions were verified using agarose gel electrophoresis. Libraries were prepared from PCR amplicons and paired-end sequencing was conducted on an Illumina MiSeq platform, according to the manufacturer's instructions. Sequence data was obtained in FASTQ format files and was submitted to the National Center for Biotechnology Sequence Read Archive under Bio Project accession numbers PRJNA477703.

2.4. Sequence data analysis

Sequence processing was performed using Mothur software, version 1.36.1 (Schloss et al., 2009). Sequence analysis of each combined single FASTA file was processed using the Mothur MiSeq SOP accessed on the 20/08/17 (Kozich et al., 2013). The paired-end MiSeq Illumina reads from the 56 sprouts samples were aligned and converted to contigs yielding 2,673,317 reads. Sequences were quality checked and sequences having ambiguous characters, homopolymers longer than 8 bp were removed. High-quality sequences were aligned against the Mothur version of SILVA bacterial reference sequences (Pruesse et al., 2007). Unique sequences and their frequency in each sample were identified and then a pre-clustering algorithm was used to further denoise

Table 1

Microbial quality of retail alfalfa and mung bean sprouts from two Israeli brands.

	Bacterial count (log g^{-1})[a]			
	Alfalfa		Mung bean	
Brand	c1	c2	c1	c2
APC	7.53 ± 0.15A[a]	8.1 ± 0.07B[b]	7.63 ± 0.1C[a]	7.84 ± 0.08C[b]
Coliforms	6.86 ± 0.12A[a]	7.33 ± 0.05B[b]	6.84 ± 0.17C[a]	7.27 ± 0.09D[b]
E. coli [b]	1/30	0/30	0/30	0/30
S. enterica [c]	0/30	0/30	0/30	0/30
L. monocytogenes [c]	0/30	0/30	0/30	0/30

[a] Bacterial counts are presented as the logarithm of the mean bacterial counts per fresh weight and standard error of the means. Different capital and lower letters represent significant difference in mean bacterial counts of the same sprout between brands, and between the two sprouts of the same brand, respectively.

[b] Positive in 25 g of 1 sample out of 30.

[c] Absence in 25 g of each 30 samples.

sequences within each sample (Schloss et al., 2011). Singletons were removed from the dataset and chimera removal was performed using the UCHIME algorithm (Edgar et al., 2011). Finally, classification of the high quality filtered bacterial sequences (1,554,373) was done by the Mothur version of Bayesian classifier (Wang et al., 2007) with the full length sequences and taxonomy references from 128 release of NR SILVA database (Pruesse et al., 2007). Genera associated with spoilage of food products were determined based on published literature on spoilage microorganisms (Barth et al., 2009; Beuchat, 1995; Osman and Bozoglu, 2016; Tournas, 2005). Potential human pathogenic genera were determined on the basis of medically important bacterial genera listed in the most current edition of Manual of Clinical Microbiology (Jorgensen et al., 2015).

After normalizing the number of sequences in each sample (based on rarefied or subsampled data i.e., the minimum number of remaining sequences in any of the samples); alpha diversity was assessed by calculating the richness estimator Chao1 and the Shannon diversity index. Library coverage and species evenness were also calculated. The taxonomical classification and diversity indices of samples from respective replicates were averaged. Variation in community structure between samples (beta diversity) was determined using the same subsampling approach, an index that accounts for proportional abundances of both shared and non-shared Operational taxonomic units (OTUs). OTUs were defined at the cut-off of 0.03 dissimilarity.

2.5. Functional profiling of the bacterial community associated with sprouts

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt, release 1.0.0) was used to predict the functional profiling based on the 16S rRNA gene sequences of the bacterial communities associated with the sprout samples (Langille et al., 2013). For this analysis, OTUs were closed-reference picked against the Greengenes by Mothur (v.1.36). The functional taxonomies were normalized, predicted, and categorized according to online protocols of PICRUSt (http://huttenhower.sph.harvard.edu/galaxy) to generate the predicted KEGGs (Kyoto encyclopedia of genes and genomes) pathways. The biom table output as a final predicted metagenome is analysed with a software package, Statistical Analysis of Taxonomic and Functional Profiles (STAMP) v.2.0.9 (Parks et al., 2014) to test and visualize significant predicted functional differences in bacterial communities between the sprouts types, brand names and time of storage.

2.6. Statistical analysis

Bacterial counts were converted to log CFU/g and the means of triplicate of each APC and coliforms counts were calculated. The mean counts and standard error of the means were compared between the two manufacturers and between the two sprouts of the same manufacturer by one-way analysis of variance with Tukey-Kramer Multiple Comparisons Test using GraphPad Instat Software (version 3.0.6; San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

Differences in bacterial community composition among the groups of samples based on sprout types, brand name, weeks and repeats were assessed using Unifrac weighted distance metric (Lozupone and Knight, 2005). The randomization/permutation procedure analysis of similarities (ANOSIM) was used to evaluate the significant differences between groups (Clarke and Gorley, 2001). Two dimensional non-metric dimensional scaling (NMDS) plots were generated based on the weighted unifrac distance matrix calculated between different samples using the OTU table with OTU clustering at the species level. Unifrac and ANOSIM and were performed on subsampled OTU table using Mothur software, version 1.36.1 (Schloss et al., 2009). Diversity indices were compared with all pairs Tukey HSD test using software JMP pro 14.0.0 (SAS Institute Inc., NC, USA). Families and genera were tested for significance across the group of samples using software STAMP v.2.0.9 (Parks et al., 2014).

3. Results

3.1. Bacteriological evaluation of sprouts

The microbial quality of the sprouts is presented in Table 1. The number of APC ranged from 5.0 to 8.7 log CFU/g in alfalfa and 6.7 to 9.3 log CFU/g in mung bean sprouts. In the case of alfalfa, but not mung beans, the mean numbers of APC was higher in brand c1 (7.53 ± 0.15) compared to c2 (8.1 ± 0.07). The number of coliform bacteria ranged from 4.3 to 7.7 log CFU/g in alfalfa and 4.1 to 8.1 log CFU/g in mung bean sprouts. Both types of sprouts contained higher coliform counts in sprouts manufactured by c2 compared to c1. No significant differences in the mean coliform numbers were documented between the two sprouts produced by the same manufacturer. *E. coli* was detected in only 1 of the 60 sprout samples. None of the tested samples was positive for *S. enterica* or *L. monocytogenes*.

3.2. Species richness and diversity during cold storage

Paired-end sequencing of 16S rRNA gene yielded over 13,000 effective sequences per library at different time points in experimental storage of sprouts, except one sample of alfalfa (3 weeks, yielding only 248 sequences), which was removed from the downstream analysis. These effective sequences were normalized for the analysis of diversity indices. Overall, they were clustered into 1,375 OTUs at 97% similarity and the highest diversity indices were observed in alfalfa samples from brand c2 (Table 2). All of the rarefaction curves reached saturation (data not shown) and sequencing depth was considerable enough to cover the whole bacterial diversity. It is also supported by library

Table 2
Diversity indices of bacterial communities associated with Alfalfa (A) and Mung beans (M) sprouts originated from two companies (c1 and c2) under cold-storage conditions.

Samples[a]	OTUs[b]	Chao[b]	Shannon diversity index[b]	Shannon evenness[b]	Goods coverage
A_c1_0w	82.8 ± 25^{bcd}	91.5 ± 29^{cd}	1.92 ± 0.4^{abcd}	0.44 ± 0.1^{abc}	0.998 ± 0.0007
A_c1_1w	71.8 ± 11^{cde}	95.7 ± 17^{bcd}	1.83 ± 0.3^{bcd}	0.43 ± 0.1^{abc}	0.998 ± 0.0004
A_c1_2w	68.5 ± 13^{cde}	99.1 ± 32^{bcd}	1.73 ± 0.2^{bcd}	0.41 ± 0.0^{abc}	0.998 ± 0.0009
A_c1_3w	67 ± 24^{cde}	77.5 ± 24^{cd}	1.8 ± 0.3^{abcd}	0.43 ± 0.0^{abc}	0.999 ± 0.0005
A_c2_0w	137.8 ± 15^{a}	167 ± 27^{a}	2.64 ± 0.4^{a}	0.54 ± 0.1^{a}	0.997 ± 0.0013
A_c2_1w	108.3 ± 32^{abc}	122.8 ± 41^{abc}	2.31 ± 0.5^{abc}	$0.49 \pm 0.1^{a\ b}$	0.998 ± 0.0012
A_c2_2w	133.3 ± 24^{a}	170.9 ± 16^{a}	$2.43 \pm 0.3^{a\ b}$	$0.5 \pm 0.1^{a\ b}$	0.997 ± 0.0005
A_c2_3w	117.5 ± 26^{ab}	152.4 ± 23^{ab}	1.98 ± 0.1^{abcd}	0.42 ± 0.0^{abc}	0.997 ± 0.0005
M_c1_0w	41.3 ± 6^{de}	48.6 ± 13^{d}	1.46 ± 0.2^{d}	0.39 ± 0.1^{abc}	0.999 ± 0.0004
M_c1_1w	41.5 ± 7^{de}	66.9 ± 25^{cd}	1.43 ± 0.2^{d}	0.39 ± 0.1^{abc}	0.999 ± 0.0005
M_c1_2w	35.5 ± 11^{e}	52.5 ± 18^{d}	1.28 ± 0.3^{d}	0.36 ± 0.1^{bc}	0.999 ± 0.0004
M_c2_0w	72.8 ± 16^{bcde}	82.2 ± 19^{cd}	$1.52 \pm 0.5c^{d}$	0.35 ± 0.1^{bc}	0.999 ± 0.0005
M_c2_1w	57.8 ± 10^{cde}	73.3 ± 10^{cd}	1.3 ± 0.1^{d}	0.32 ± 0.0^{c}	0.999 ± 0.0003
M_c2_2w	64 ± 10^{de}	78.9 ± 17^{cd}	1.26 ± 0.3^{d}	0.3 ± 0.1^{c}	0.999 ± 0.0004

[a] 0w, denotes freshly purchased samples; 1w, 2w and 3w denotes samples stored in refrigerator for one, two and three weeks respectively.
[b] Respective indices with different superscript letter within each column are significantly different ($p < 0.05$; All pairs Tukey-HSD test).

coverage calculation, which was > 99% for all the samples.

The number of observed OTUs varied among the samples and highest number of OTUs were observed in samples of alfalfa from brand c2. The number of estimated species richness determined by Chao estimator was observed to be 613 in alfalfa from the c2 brand compared to 363 in alfalfa from the c1 brand, 234 in mung bean from the c2 brand and 167 in mung bean from the c1 brand, indicating that alfalfa sprouts of the c2 brand had the greatest species richness. The Shannon diversity index was also highest for alfalfa from c2 brand (mean = 2.34), lowest for mung bean from the c2 brand (mean = 1.35) and mean Shannon was 1.82 and 1.39 for alfalfa and mung bean from the c1 brand respectively. Shannon evenness index revealed that OTUs were more evenly distributed in alfalfa sprouts as compared to mung bean sprouts. The decrease in diversity and OTUs was observed in all the samples after cold-storage (Table 2).

NMDS was used to ordinate each sample in order to determine bacterial community similarity of bacterial populations associated with the sprouts. Two dimensional NMDS based on weighted unifrac scores was sufficient to account for community differences (stress = 0.05, $r^2 = 0.98$) (Fig. 1). The sprouts samples belonging to alfalfa and mung beans clearly clustered apart from each other. Alfalfa samples belonging to two different companies were more separated as compared to mung beans, which clustered close to each other. ANOSIM demonstrated statistically significant different in bacterial community composition between the sprout types ($R = 0.68$, $p < 0.001$), alfalfa companies ($R = 0.56$, $p < 0.001$), and mung bean companies ($R = 0.29$, $p < 0.001$). The bacterial community composition of replicates from respective companies as well as from subsequent weeks of storage were not significantly different ($p > 0.05$).

3.3. *Bacterial community composition associated with alfalfa and mung bean sprouts at the time of purchase*

The bacterial community composition in alfalfa and mung bean sprouts comprised of 12 different phyla along with unclassified bacterial members (Supplementary materials, Fig. S1). Overall, the

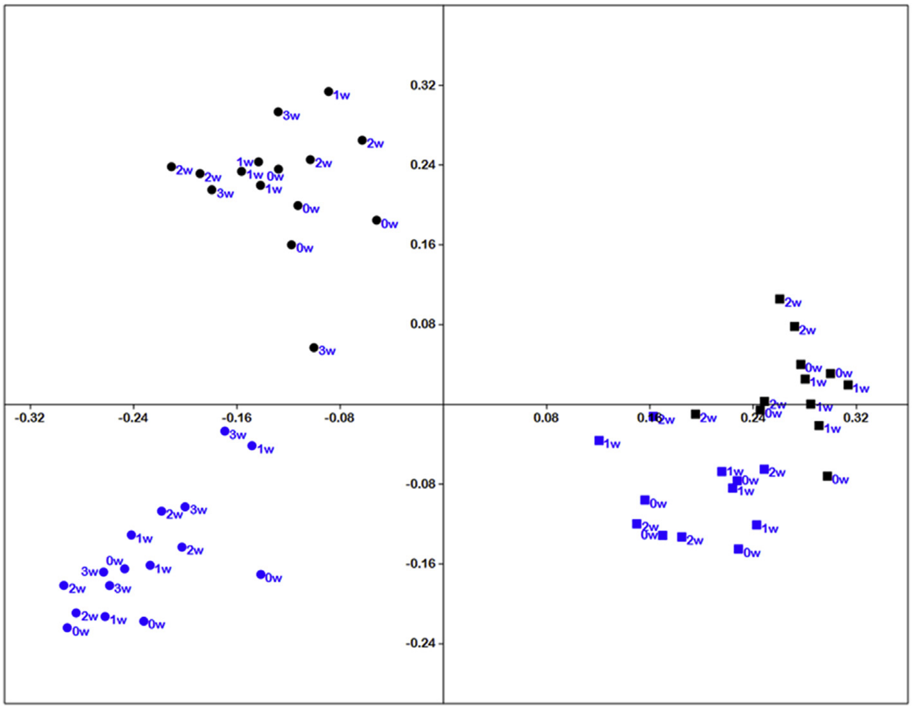

Fig. 1. Non-Metric Multidimensional Scaling (2D) plot of bacterial community composition associated with Alfalfa and Mung bean sprouts during cold-storage at 4 °C derived from 16S rRNA amplicon sequencing. Black and blue dots denotes bacterial community composition of alfalfa from brands c1 and c2, respectively; black and blue squares denotes bacterial community composition of mung beans from brands c1 and c2, respectively. Samples were stored up to 3 weeks for alfalfa (0w-3w) and up to 2 weeks (0w-2w) for Mung bean sprouts and experiments were replicated four times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sequences from both sprout's types could be classified into 18 classes, 43 orders, 68 families and 160 genera. Out of 160 observed genera, 56 accounted for more than 0.01% of the overall sequences, 25 were present at > 0.1% and only 10 genera were present at > 1% of the all sequences (Supplementary materials, Table S2). Alfalfa sprouts harbored more diverse genera and contained species from 154 genera, while mung bean sprouts harbor species from only 85 genera. At the time of purchase, microbial population of both alfalfa and mung bean sprouts from brand c2 were more diverse than in sprouts derived from brand c1 (Table 1). Alfalfa sprouts from brand c2 contained 107 genera compared to only 53 in from brand c1. Similarly, mung bean sprouts from brand c2 harbored 61 genera compared to 36 in spots from brand c1.

Proteobacteria was the most abundant phylum associated with fresh sprouts of both alfalfa and mung bean at the time of purchase. *Proteobacteria* relative abundance was 98.5% and 97.3% in alfalfa brands c1 and c2, respectively, and 99% and 91.7% in mung bean sprouts' brands c1 and c2, respectively. *Firmicutes* was the second most abundant phylum at the time of purchase representing relative abundance of 1.3% and 2.1% in alfalfa brands c1 and c2, respectively, and 0.74% and 8.1% of mung beans brands c1 and c2, respectively. Other 10 phyla namely, *Verrucomicrobia, Cyanobacteria, Bacteroidetes, Actinobacteria, Chlamydiae, Deferribacteres, Marinimicrobia (SAR406_clade), Acidobacteria, Planctomycetes, TM6 (Dependentiae),* were represented in relative abundance of less than 0.1%.

Variations at the family level were observed for both sprouts' type and brands (Fig. 2a). *Proteobacterial* species belonging to the genus *Pseudomonas* were the most abundant in alfalfa (48.8% and 29.5% in brand c1 and c2, respectively) and in brand c1 of mung bean (36.4%), while *Pantoea* species were most abundant in mung bean sprouts of c2 brand (33.5%). The next abundant genera in alfalfa were *Janthinobacterium* (9%) in brand c1, and *Duganella* (5%) in brand c2, while *Acinetobacter* was the third most abundant genus in both brands of mung bean (15.9% and 9.1% in brand c1 and c2 respectively).

3.4. Changes in bacterial community composition during cold storage

Variations in the abundance of the dominant genera were observed during cold storage for both type of sprouts and the producing companies (Fig. 2b). During the first week of storage of alfalfa*, Pseudomonas* dominance increased covering 56.8% and 46% relative abundance in brands c1 and c2, respectively. The abundance of *Pseudomonas* increased in successive weeks regardless of brand and at 3 weeks it represented more than 60% of the population (61% in brand c1; 64.4% in brand 2). The second most abundant genus, *Pantoea* decreased during cold storage and after 3 weeks it reached 6.2% and 3.2% in brand c1 and c2 respectively. The abundance of *Janthinobacterium* remained constant (9.7% at 0w and 10.4% after 3w), while it slightly decreased in c2 (from 2.3% at 0w to 1.4% after 3w). *Duganella* and *Acinetobacter* decreased in both brands after 3 weeks of storage (Fig. 2b).

In contrast, after 1 week of storage of mung bean, the abundance of *Pantoea* increased and it became the most dominant genus in both brands, representing 43.8% and 40.5% in brands c1 and c2, respectively. The abundance of *Pantoea* further increased after 2 weeks of storage (50.4% and 46% in brand c1 and c2 respectively) (Fig. 2b). The abundance of the second most dominant genus in mung bean, *Pseudomonas* decreased after 2 weeks in brand c1, but not in brand c2, where it increased to 21.2% after 2 weeks. The abundance of *Acinetobacter* decreased at 2 weeks of storage in both brands of mung bean sprouts.

3.5. Spoilage-associated genera

Among the 160 genera associated with sprouts, 23 were identified as spoilage-associated genera, based on published literature. Spoilage bacteria included the highly abundant genera, *Pseudomonas and Pantoea,* along with another 21 less abundant genera, which included *Leuconostoc, Erwinia, Pectobacterium, Xanthomonas, Flavobacterium* etc. The relative abundance of some of these genera increased during storage, while some have emerged only during the storage time without following any common pattern.

3.6. Pathogenic and potential pathogenic genera associated with sprouts

The abundance of some genera, which contain human pathogenic species increased during cold storage, while few genera harboring potential or opportunistic human pathogens decreased below the limit of detection during storage. Out of total 160 genera associated with sprout samples, 30 were determined which can include opportunistic, nosocomial or known human pathogenic species. Some of these genera were not detected in fresh samples but emerged only during cold-storage. For example, *Klebsiella*, *Streptococcus, Routella, Salmonella* etc. Notably, the foodborne pathogen *Salmonella,* was undetectable up to two weeks in alfalfa (c2), but was found at three weeks of storage at low abundance (0.004%).

3.7. Predicted function of sprouts' bacterial community

Knowledge regarding the sprouts' associated microbiome can shed light on the associated metabolic functions. The microbiome data was analysed by PICRUSt and a total of 294 metabolic pathways were predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG) homology, with some differences in abundance between sprout types. All Potential functions derived from the predicted metabolic pathways were analysed using PCoA plot (Fig. 3), which revealed distinct functions associated with alfalfa compared to mung bean sprouts. No separation in time- and replica-related clustering was observed (Supplementary materials, Fig. S3). Out of 5,708 descriptive predictive functions that were identified by PICRUSt, 64 were related to spoilage enzymes. These enzymes were grouped into 7 major types of spoilage associated enzymes, e.g., pectinolytic enzymes, amylases, alcohol dehydrogenases, lipases, decarboxylases, deaminases and lactate dehydrogenases (Supplementary materials, Table S3).

Overall, the most abundant predicted pathways in both sprouts were related to transporters (10.51%; including 4.03% of ABC transporters), poorly characterized pathways (3.58%), two-component systems (2.7%), secretion systems (2.3%) and DNA repair and recombination proteins (2.13%). Relatively less abundant pathways (2%–1%) were bacterial motility proteins, purine metabolism, transcription factors, peptidases, ribosome, ion-coupled transporters, arginine and proline metabolism, oxidative phosphorylation, pyrimidine metabolism, ribosome biogenesis, amino acid related enzymes, chaperones and folding catalysts, as well as unclassified (Supplementary materials, Table S4). The major metabolic pathways, which were significantly higher in alfalfa compared to mung bean sprouts were metabolisms of amino acids, lipids energy, terpenoids and polyketides, biosynthesis of secondary metabolites, metabolism and biodegradation of xenobiotics. Other functions like pores ion channel (cellular processes and signaling), cell motility (cellular processes) and transcription (genetic information processing) were also higher in alfalfa sprouts. In contrast, some major metabolic pathways were significantly higher in mung bean as compared to alfalfa e.g., metabolisms of enzyme families, co-factors, vitamins, glycans and nucleotides. Some other functions like membrane transport (environmental information processing), folding, sorting, degradation and translation (genetic information processing) were also high in mung bean sprouts. Among the 294 metabolic pathways, 15 were related to bacterial infection in both sprout types (Fig. 4). Relative abundance of these predicted pathways were significantly higher in mung bean sprouts as compared to alfalfa ($p < 0.01$) (Supplementary materials, Fig. S2), though they were represented by only 0.5%–0.55% of relative abundance of all predicted functions in alfalfa, and 0.59%–0.62% in mung bean.

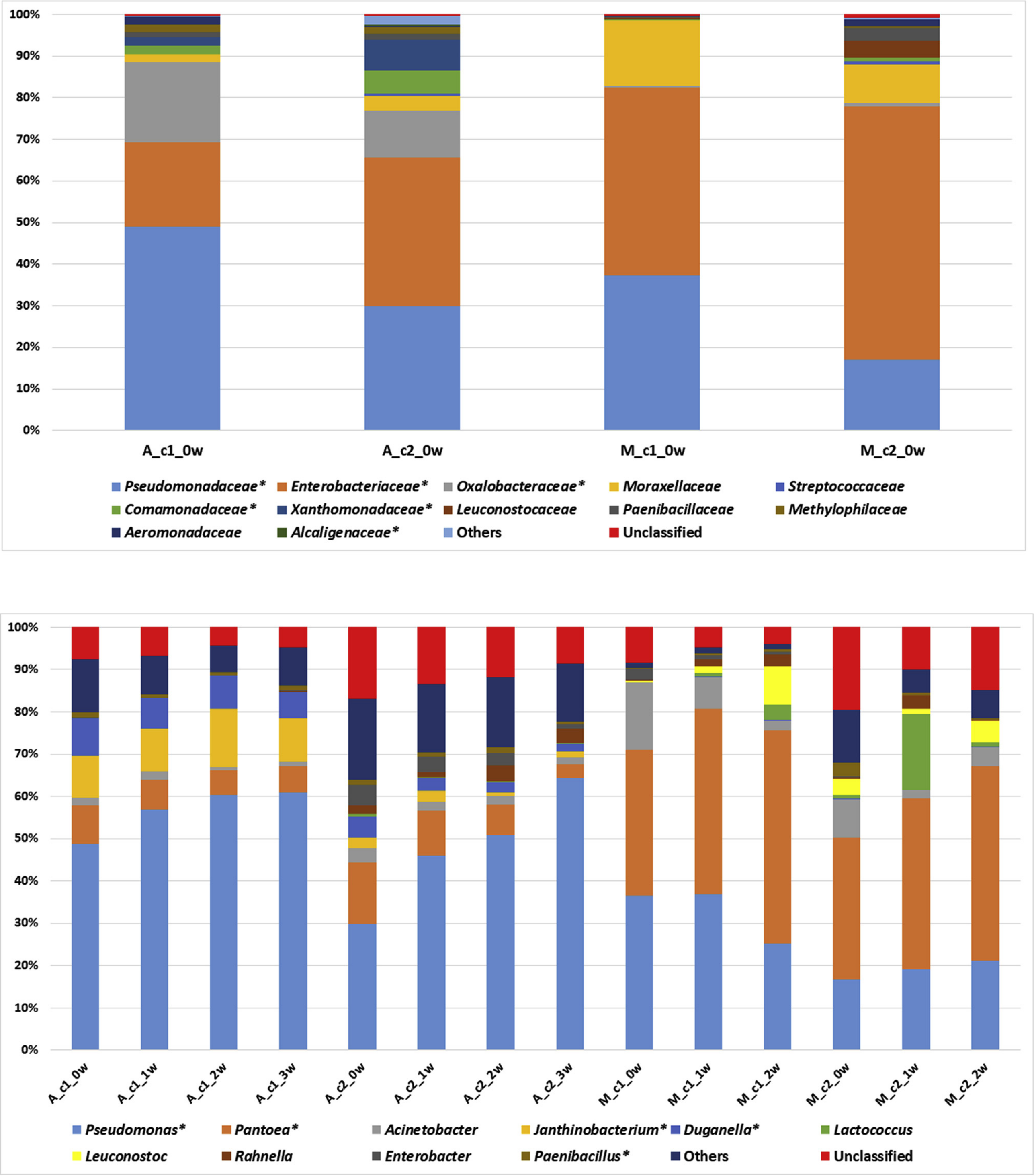

Fig. 2. Relative abundance of dominant families at the time of purchase (**a**) and dominant genera at the time of purchase and under refrigerated conditions (**b**), associated with Alfalfa (A) and Mung beans (M) sprouts originated from two companies' brands (c1 and c2). Families and genera having less than 0.1% and 1% of overall relative abundance respectively, were summed up and presented as others. *Denotes taxonomic group is significantly different across the group of samples ($p < 0.05$), Tukey-Kramer posthoc test and Boneferroni corrected *P* value.

4. Discussion

It has been proposed that consumption of fresh produce microbiome contributes to human health through stimulation of the immune system and contribution to the gut microbial diversity (Berg et al., 2014). Sprouts were reported to harbor the highest counts of aerobic mesophilic bacterial among other RTE foods, like leafy vegetables, non-pasteurized vegetable, fruit and fruit-vegetable juices (Berthold-Pluta et al., 2017). Surveys performed in different countries, revealed that APC, also known as total aerobic counts or total mesophilic bacteria, may range between 6 and 11 log CFU/g in various sprouts (Abadias et al., 2008; Althaus et al., 2012; D'Sa et al., 2015; Gabriel et al., 2007; Kim et al., 2013; Seo et al., 2010). In a recent study, APC was reported to be equal or less than log 5.5 CFU/g (Iacumin and Comi, 2019). In the present survey, we found that APC ranged between 5.0 and 8.7 log CFU/g in alfalfa and 6.7 to 9.3 log CFU/g in mung bean sprouts. The

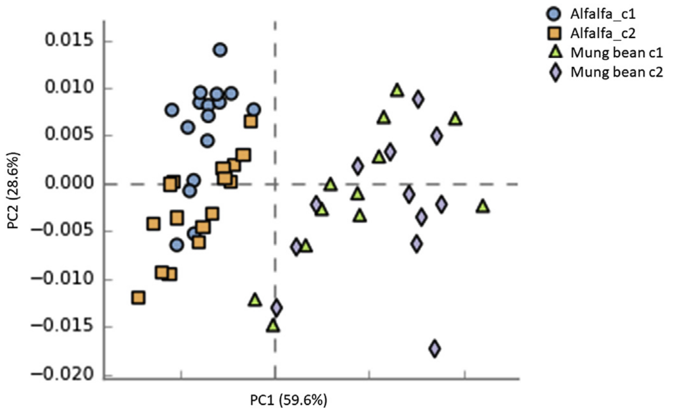

Fig. 3. PCoA (Principal Coordinate Analysis) plot of functional prediction of sprouts bacterial microbiome based on 16S rRNA sequences. Groupings were displayed between sprout types from two different companies (c1 and c2) for each sprout types.

average APC counts in alfalfa, but not in mung bean sprouts was higher in brand c1 compared to c2. Similarly, large variations in the population size of various sprouts, reported in other studies, may reflect, differences in the producing environment, seeds' source and water quality, as well as hygienic practice. The number of coliform bacteria ranged in our survey from 4.3 to 7.7 log CFU/g in alfalfa and 4.1 to 8.1 log CFU/g in mung bean sprouts. Similarly, coliforms count were reported in the range of 5.0–8.8 CFU/g (Seow et al., 2012); yet others have found lower than 3.5 CFU/g (Iacumin and Comi, 2019).

In the present survey, only one sample contained *E. coli* and none-contained *Salmonella* or *Listeria*. No pathogens were also reported by others (Althaus et al., 2012; Iacumin and Comi, 2019), though a number of studies have reported the presence of *E. coli* Abadias et al. (2008); Seow et al., 2012 and low incidence of *Salmonella*, and *Listeria monocytogenes* (Abadias et al., 2008; D'Sa et al., 2015; Gabriel et al., 2007; Seow et al., 2012).

Considering that the cultivable bacteria comprise only a small portion of the entire microbial population, consumption of sprouts is associated with high intake bacteria. Recent studies have utilized NGS to analyse sprout's bacterial populations (Asakura et al., 2016; Huang et al., 2016, 2017a; 2017b; Leff and Fierer, 2013; Margot et al., 2016a, 2016b).

Microbiome analysis of commercial sprouts, performed in the present study, revealed distinct bacterial communities in alfalfa and mung bean sprouts, suggesting that plant's species is the main factor determining sprout's microbial composition. Spouts' producing environment seems to be less important factor, since only minor differences in the microbiome were observed between the two producing companies tested in this study (Figs. 1 and 3). These results concur with those of a previous study reporting significant similarities in the microbiome of four different alfalfa sprouts samples produced by different growers, in distinct geographical regions, and purchased in different stores (Loui et al., 2008). Similarly, community level physiological profiling established that variability of microbial community among sprout types was more extensive than sprout-growing facilities and seed lots (Matos et al., 2002). Variation in the community structure of sprouts derived from different brands could be attributed to variation in the growing procedures, including water source used, as well as to different packaging and transportation conditions. In support of this notion, variation in the microbial composition was also observed between aseptically- and commercially-grown sprouts of mung beans, alfalfa and broccoli (Landry et al., 2017).

Alfalfa sprouts were inhabited by more diverse bacterial populations as compared to mung bean sprouts (Fig. 2) and as can be judged by the higher OTU richness in alfalfa sprouts as compared to mung bean sprouts (Table 1). Similar findings were reported previously, based on pyrosequencing data (Leff and Fierer, 2013). The sprouts'-associated microbiomes identified in the present study, as well as in other's (Huang et al., 2016, 2017a, 2017b; Margot et al., 2016a, 2016b) displayed a rather limited number of OTUs with lower species diversity compared to the phyllosphere of leafy vegetables (Dees et al., 2015; Leff and Fierer, 2013; Lopez-Velasco et al., 2011). This may be attributed to the potential sources of the sprouts' microbiota, e.g., dry seeds and irrigation water using a soil-free growth-environment, as compared to soil in the case of leafy green vegetables (Dees et al., 2015).

In our study, *Pseudomonadaceae* was the most dominant family in alfalfa, and the second most dominant family in mung bean sprouts (Fig. 2a). The genus *Pseudomonas* is a highly heterogeneous group of saprophytic species found in soil, containing opportunistic pathogens of plants, animals and humans, decomposers of organic matter, and vegetation (Cornelis, 2008; Lee et al., 2013). Members of the *Pseudomonadaceae* were previously reported to be highly abundant in RTE radish sprouts (Asakura et al., 2016), in alfalfa, mung beans and broccoli sprouts with significant differences in their abundance in aseptically- and commercially-grown sprouts (Landry et al., 2017). This finding infers that the sprouting environment, such as irrigation water, may affect the sprout-associated microbiome (Landry et al., 2017). Some species, such as, *P. fluorescens and P. viridiflava* can cause decay of plant tissues at temperatures at or below 4 °C (Barth et al., 2009), supporting a potential role for these and other psychrophilic species in the spoilage of alfalfa and mung bean sprouts during storage at 4 °C.

Enterobacteriaceae have been reported to be highly abundant in fresh produce including minimally processed vegetables (Abadias et al., 2008; Leff and Fierer, 2013; Oliveira et al., 2010), as well as during storage (Lopez-Velasco et al., 2011). *Enterobacteriaceae* contain known spoilage-associated genera (Barth et al., 2009; Osman and Bozoglu, 2016). In our study, *Enterobacteriaceae* appeared as the second most abundant family overall, covering 28.1% in alfalfa and 53.1% in mung beans at the time of purchase. After 3-weeks of cold storage, their relative abundance decreased to 15.2% in alfalfa, whereas it increased to 62.3% during the 2-weeks shelf-life of mung beans. It is possible that the shorter shelf-life of mung bean sprouts resulted from the proliferation of spoilage-causing *Enterobacteriaceae* during the simulation of cold storage. A potential candidate of spoilage-causing genus might be *Pantoea. Pantoea* were previously reported to be highly abundant in fresh mung bean sprouts (57.5%), spinach (32.5%), and pepper (11.5%) (Leff and Fierer, 2013) and species of this were reported to cause spoilage of plant based foods (Lopez-Velasco et al., 2011; Poubol and Izumi, 2005; Zhu et al., 2018). The abundance of *Pantoea* in mung bean sprouts increased during the 2-weeks storage up to 50.4% and 46% in brands c1 and c2, respectively, supporting a role for *Pantoea* species in mung bean spoilage. *Pantoea* sp. were also reported as a main fraction of cultured microbiota on hydroponically grown mung bean sprouts (Weiss et al., 2007). *Enterobacteriaceae* was earlier reported to be the most abundant family in alfalfa sprouts from two out of four brands based on 16S rDNA clone libraries (Loui et al., 2008), while in our study, *Pseudomonadaceae* was the most dominant family in all samples of alfalfa based on NGS approach. The different findings might be related to the different methodology used in both studies.

Understanding microbial succession during food storage is economically important since it is associated with spoilage. Overall bacterial diversity was reduced during storage in both sprouts and companies. A similar trend was previously reported in the case of the phyllosphere community of spinach, during storage at 4 °C and 10 °C up to 15 days (Lopez-Velasco et al., 2011). Indeed it was previously suggested that spoilage processes are associated with reduction of bacterial diversity in several food products (Berg et al., 2014; Kergourlay et al., 2015). Microbial succession during cold-storage has been studied in other food items (Del Arbol et al., 2016; Ercolini et al., 2006; Lopez-Velasco et al., 2011; Randazzo et al., 2009). Previous studies in sprouts, have analysed microbial succession from time of purchase up to 7 days,

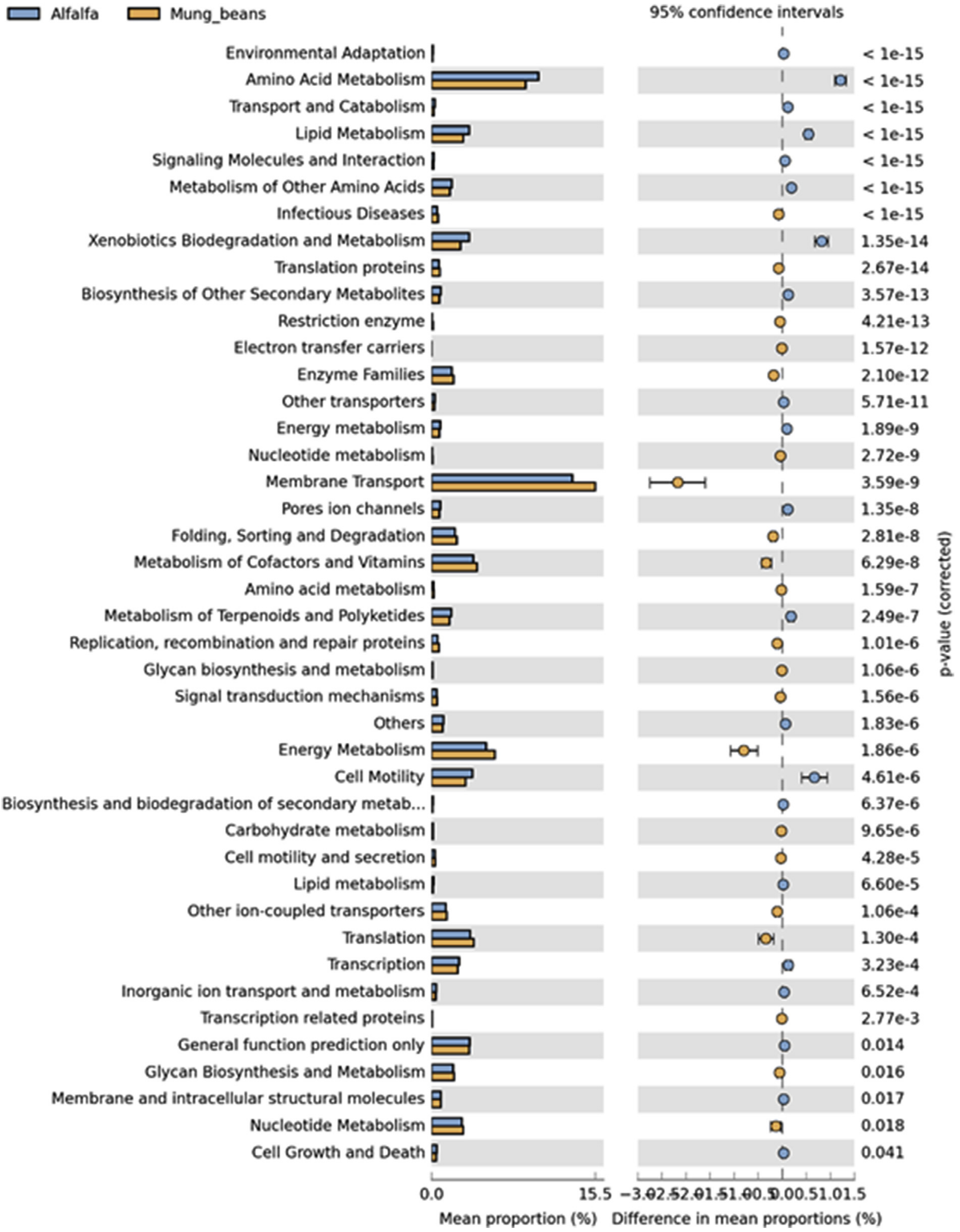

Fig. 4. PICRUSt prediction of functional profiling of the microbial communities based on the 16S rRNA gene sequences. Extended error bar plot indicating differences in functional profiles of the alfalfa and mung bean sprout associated microbiome (at taxonomic Level 2). All unclassified reads were removed and *P* value less than 0.05 is displayed. Categories are sorted by *P* value calculated using two-sided Welch's *t*-test.

using cultured- and molecular-based techniques, DGGE (Molinos et al., 2009) and up to 12 days using cultured and sequencing of 16S rRNA gene of the isolates (Nagar et al., 2016). The present study is the first to report microbial succession on sprouts, occurring during storage under refrigeration, using an NGS-based approach. Since, no “best before” date was marked on the sprouts' packages, we empirically followed the quality of the sprouts during cold storage, up to the end of their shelf-life, 3-weeks for alfalfa and 2-weeks for mung bean. The different times might be related to the dynamics of the microbial communities during cold-storage in the two sprouts.

Reduction of bacterial diversity and species richness has also been noticed in the case of spinach stored under refrigeration (Leff and Fierer, 2013). It is noteworthy that reduced species richness was previously linked to a better colonization of the human pathogen, *Salmonella enterica*, on lettuce (Klerks et al., 2007). It may be postulated that spoilage processes, associated with reduction of bacterial richness and diversity, pose a potential food safety risk by increasing the susceptibility of the food product to proliferation of human pathogens. Interestingly *Salmonella* also appeared in alfalfa during cold storage following reduction in bacterial diversity and species richness.

Storage under refrigeration resulted in temperature-dependent selection toward psychrotrophic taxa, such as *Pseudomonas, Pantoea, Leuconostoc, Erwinia, Rahnella*. Dominance of psychrotrophic bacteria, especially *Pseudomonas*, *Pantoea, Erwinia*, *Rahnella*, and lactic acid bacteria was also reported on vegetables following cold storage (Lopez-Velasco et al., 2011; Ragaert et al., 2007; Randazzo et al., 2009; Rudi et al., 2002). Many of these psychrotrophic bacteria were linked to spoilage of fresh vegetables (Kraft, 1992; Nguyen-the and Carlin, 1994). Potential role of psychrotrophic lactic acid bacterial (LAB), especially *Leuconostoc* and *Lactococcus,* in spoilage of refrigerated food products, were also established (Pothakos et al., 2014b). *Leuconostoc* spp. were the most dominant population in RTE salads at the end of their shelf-life, i.e., after one week of refrigeration at 4 °C (Pothakos et al., 2014a). Increased abundance of *Leuconostoc* after two weeks of cold storage was also observed in mung bean sprouts from both brands, supporting a potential role also in sprout's spoilage.

In order to learn about the potential functions associated with bacterial spoilage, we analysed the data using PICRUSt. Enzymes related to spoilage were predicted using KEGG orthology based on taxonomic profiling (Supplementary materials, Table S3). Most of the spoilage-associated enzymes were identified based on the spoilage mechanisms described by Osman and Bozoglu (2016), Barth et al. (2009), and Tournas (2005). Indeed, pectin methyl esterase, polygalacturonase and pectin lyase, three enzymes that involved in the depolymerization of the pectin chain, were predicted in both sprouts samples. The abundance of these enzymes, at the time of purchase, was higher in mung bean sprouts compared to alfalfa sprouts (Supplementary materials, Table S3). These findings may potentially explain the shorter shelf-life of mung bean compared to alfalfa sprouts.

It is notable that the most abundant predicted proteins in the two sprouts belong to 'Environmental information processing' function and consist of transporters (in general), ABC transporters and two-component signal transduction systems. These features enable bacteria to sense, respond and adapt to the dynamic changes in the sprout's environment. It is of interest to note that the 3rd most abundant function (Supplementary materials, Table S4) is 'poorly characterized'. It is likely proteins within this group have, a yet unknown, role in the colonization and deterioration of the sprouts.

The presence of alcohol dehydrogenases and lactate dehydrogenases in both sprout types suggests that spoilage was also carried out by anaerobic process. Species of the genus *Leuconostoc* can metabolize carbohydrates into either lactic acid, ethyl alcohol and CO_2 or acetic acid, acetoin, diacetyl and CO_2 (Aurand and Woods, 1973; Banwart, 1981). Members of the family *Enterobacteriaceae,* are associated with fermentation of fresh produce, forming biogenic amines from free amino acids with the activity of decarboxylase enzymes (Osman and Bozoglu, 2016). Several amino acid decarboxylases and deaminases were predicted in both sprout samples suggesting the breakdown of proteinaceous materials. Microbial lipases were also predicted in both sprouts, indicating potential lipid-degradation activity of the microbial communities of the sprouts. Overall, predicted enzymes related to spoilage were decreased in alfalfa sprouts after storage, while it increased in the case of mung bean sprouts, which also supports our finding regarding the shorter shelf-life of mung bean sprouts (Supplementary materials, Table S3).

Sprouts microbiome may also contain potential or opportunistic human pathogens, which may be detrimental for susceptible populations, such as immunocompromised, young children, older adults and pregnant women (USFDA, 2013). Sprouts harbor a dense bacterial population (Asakura et al., 2016; Huang et al., 2016, 2017a; 2017b; Margot et al., 2016a, 2016b), which usually doesn't pose health risk to consumers; nevertheless, they are one of the most common vehicles associated with foodborne pathogens (Abadias et al., 2008; Bayer et al., 2014; Buchholz et al., 2011; Mahon et al., 1997; Sadler-Reeves et al., 2015; Saroj et al., 2006; Seow et al., 2012; Stewart et al., 2001; Viswanathan and Kaur, 2001). The 16S rRNA amplicon sequencing has been recently employed to identify several genera associated with different food products (Chen et al., 2019; Kamimura et al., 2019; Ottesen et al., 2019; SantAnna et al., 2019; Ssepuuya et al., 2019). Our analysis has demonstrated the presence of 30 genera, which are known to contain potential and/or opportunistic human pathogens (Jorgensen et al., 2015). Nevertheless, the sequencing methodology used in this study does not allow the identification of bacteria at the species level, and therefore further studies using metagenomics and metatranscriptomics should be performed to characterize the sprouts bacterial community at the species level. The detection of *Salmonella* in alfalfa sprouts at a late storage time, underlines the potential risk of foodborne illness associated with sprouts' consumption. The failure of the standard microbiological test to detect *Salmonella* in alfalfa sprouts may be related to the low abundance of the pathogen (< 1 cell per 25 g) or to the presence unculturable or dead *Salmonella* cells.

While the sequencing methodology used in this study could not identify the taxa at sub-genera level, the metagenomics functional prediction analysis (PICRUSt) has identified metabolic pathways associated with infectious diseases, supporting the notion that some of the identified OTUs might be related to human pathogens (Fig. 4). These findings underpin the notion that decaying sprouts might pose a safety hazard to consumers. Still, it should be noted the PICRUSt data should be analysed cautiously, as the predictions are based on genomic data and 16SrRNA amplification, rather than on direct mRNA measurements (Gharechahi et al., 2017). Consequently, additional studies employing transcriptomics are needed in order to verify the role of the identified metabolic pathways in the spoilage processes.

Finally, further studies, analyzing the microbiome of alfalfa and mung bean sprouts from other locations and suppliers are needed in order to support our finding on a wider scale.

5. Conclusions

In summary, our study is the first to analyse temporal changes in bacterial population associated with commercial sprouts during storage under refrigerated conditions. Bacterial community associated with sprouts were dependent on the plant species but not on the manufacturing company. At the time of purchase, the dominant genera on both alfalfa and mung bean sprouts consist of *Pseudomonas* and *Pantoea*. In alfalfa, abundance of *Pseudomonas* increased after 3 weeks of refrigeration, while *Pantoea* abundance decreased; whereas in mung bean sprouts, *Pantoea* became the most dominant genus at 2 weeks of storage. Among 160 genera, 30 contain known pathogenic species and 23 contain spoilage associated species.

Conflicts of interest

The authors have no conflict of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103261.

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