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Characterization of the bacterial biodiversity in Pico cheese (an artisanal Azorean food)

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Abstract

This work presents the first study on the bacterial communities in Pico cheese, a traditional cheese of the Azores (Portugal), made from raw cow's milk. Pyrosequencing of tagged amplicons of the V3–V4 regions of the 16S rDNA and Operational Taxonomic Unit-based (OTU-based) analysis were applied to obtain an overall idea of the microbiota in Pico cheese and to elucidate possible differences between cheese-makers (A, B and C) and maturation times.

Pyrosequencing revealed a high bacterial diversity in Pico cheese. Four phyla (Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes) and 54 genera were identified. The predominant genus was *Lactococcus* (77% of the sequences). Sequences belonging to major cheese-borne pathogens were not found. *Staphylococcus* accounted for 0.5% of the sequences. Significant differences in bacterial community composition were observed between cheese-maker B and the other two units that participated in the study. However, OTU analysis identified a set of taxa (*Lactococcus*, *Streptococcus*, *Acinetobacter*, *Enterococcus*, *Lactobacillus*, *Staphylococcus*, *Rothia*, *Pantoea* and unclassified genera belonging to the Enterobacteriaceae family) that would represent the core components of artisanal Pico cheese microbiota. A diverse bacterial community was present at early maturation, with an increase in the number of phylotypes up to 2 weeks, followed by a decrease at the end of ripening. The most remarkable trend in abundance patterns throughout ripening was an increase in the number of sequences belonging to the *Lactobacillus* genus, with a concomitant decrease in *Acinetobacter*, and *Stenotrophomonas*. Microbial rank abundance curves showed that Pico cheese's bacterial communities are characterized by a few dominant taxa and many low-abundance, highly diverse taxa that integrate the so-called "rare biosphere".

1. Introduction

Pico cheese is an artisanal cheese manufactured in small dairy units in Pico Island (Azores, Portugal). It is manufactured from raw cow's milk, animal rennet and salt, without addition of starter cultures. Pico cheese is a soft, yellowish, mildly compact, unctuous paste, that is obtained by slow drainage of the curds, which results from the addition of animal rennet to raw cow's milk. Pico cheese is one of the two Portuguese cow milk cheeses granted a Protected Designation of Origin status and it has been in high demand in the local market, being greatly appreciated by the consumers. It is round and relatively thin, with a typical diameter of 16–17 cm and a height of 2–3 cm. By the end of ripening, cheeses typically weigh 650–800 g. Ripening does not usually exceed 20 days and is carried out in rooms maintained at a temperature of 10–14 °C and a relative humidity of 80–85%.

In spite of its popularity among consumers, this cheese has failed in keeping its share in the national market, and the number of artisanal Pico cheese producers has quickly dwindled from 10 units in 2001 to a mere three in 2011 (IDRHa, 2001). Studies on Pico cheese microbiota are thus urgent and rather important too in attempts to preserve its bio- diversity, promote its safety, enhance its marketability and, ultimately, ensure its survival as a gourmet product. To our knowledge, this is the first study ever addressing the profile of the microbial population in Pico cheese. Studies focused on the bacterial biodiversity of raw milk cheeses are a useful tool in evaluating their hygienic status (Alegría et al., 2009), and may be the starting point for the development of specific starter and adjunct cultures containing technologically relevant microorganisms from the cheese under study. The bottom line is increasing the reliability of the production protocols, while pre- serving as much as possible their typical flavor (Albenzio et al., 2001; Parente and Cogan, 2004). Traditional foods are indeed a promising reservoir of new strains of lactic acid bacteria (LAB) that might be an asset for the dairy industry, while bearing traits such as bacteriophage resistance, antimicrobial activity against food spoiling microorganisms and pathogens, generation of unique flavors and pro- biotic features (Leroy and de Vuyst, 2004).

The main objective of this study was to characterize the bacterial community diversity throughout the ripening of a raw milk cheese (Pico cheese) to serve as a basis to improve the production, safety and quality of this unique cheese variety. Compared to other strategies such as DGGE, next generation sequencing methodologies provide an excellent tool to study microbial community structure and composition shifts at different stages of ripening, allowing the detection of minor bacterial populations. These methods have been recently used in other dairy products, demonstrating the feasibility of generating deeply sequenced metagenomes for microbial identification and to study the dynamics of microbial communities (review by Bokulich and Mills, 2012; Ercolini, 2013). Recent studies in microbial diversity in cheeses (De Filippis et al., 2014; Ercolini et al., 2012; Masoud et al., 2011) and more specifically artisanal cheeses (Aldrete-Tapia et al., 2014; Alegría et al., 2012; Fuka et

al., 2013; Quigley et al., 2012) have validated the application of this methodology in community screening to unravel the microbial structure and to detect determinants of safety and quality in cheese (Lusk et al., 2012; Montel et al., 2014; O'Sullivan et al., 2013). Pyrosequencing was chose for its sensitivity, which provided great sequence coverage and allowed to obtain a complete picture of the diverse microbial communities involved at the different fermentation times. High-throughput sequencing has allowed to detect both the core microbiome (De Filippis et al., 2014) and low-abundance microbiota (Quigley et al., 2012) in cheese samples. Low-abundance OTUs in environmental complex microbial communities make up what has been referred in the literature as the microbial rare biosphere (Pedrós-Alió, 2007; Sogin et al., 2006).

2. Materials and methods

2.1. Cheese-making and sampling

Pico cheese was manufactured by experienced cheese-makers in three artisanal units (A, B and C), following the traditional protocol. Raw milk was curdled by adding animal rennet. Curds were cut manually, drained and placed in moulds. Salt was then rubbed onto the surface; the topmost layer was worked into fine crumbs to ensure a smooth surface, and pressed by hand. Cheeses were placed without further pressing into maturation rooms, where they were left for 21 days, at ca. 11 °C.

Two cheeses from three different batches (1, 2 and 3) were obtained from each dairy at days 0, 14 and 21 of maturation. A radial slice of each of the two cheeses from each batch of each dairy at each sampling time was obtained, and both slices were combined to provide a composite sample. Samples were placed in sterile plastic containers that were transported to the laboratory under refrigeration.

One whole industrially-produced cheese, produced in Pico Island, in a small industrial unit, according to the same protocol as the artisanal cheeses, but using pasteurized cow's milk with the addition of a commercial starter culture (DELVO®TEC DX-33, DSM, The Netherlands, consisting of *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*, and *Leuconostoc* sp.), and matured for 21 days under similar conditions was also purchased at the local market.

2.2. Total DNA extraction

A total of 28 samples, 9 corresponding to each cheese-maker, as well as an industrial Pico cheese sample were subjected to analysis. They were homogenized with a Stomacher Lab-Blender 400 (Seward, London, UK) for 4 min. A portion of 60 mg was sectioned from the homogenate sample, and total bacterial DNA was extracted with an Invisorb Spin Tissue mini-kit according to manufacturer's protocol.

2.3. 16S rRNA gene amplicon library preparation and sequencing

The small subunit rRNA gene was amplified from community DNA targeting the V3 and V4 hypervariable region, with barcoded fusion primers containing the Roche-454 A and B Titanium sequencing adapters, a six-base barcode sequence, the forward primer 5′-ACTCCT ACGGGAGGCAG-3′ and the reverse primer 5′-TACNVRRGTHTCTAATYC- 3′ (Wang and Qian, 2009). Two replicate PCR amplifications were per- formed from the same sample in 25 μL reaction volumes with Fast Start polymerase (Roche, NJ, USA) using 1.8 mM $MgCl_2$, 0.2 μM each primer, 200 mM dNTPs, 5 U of polymerase and 2 μL of template DNA. The PCR conditions were 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 44 °C for 45 s and 72 °C for 60 s and a final elongation step at 72 °C for 2 min. The amplicons were quantified by fluorimetry with PicoGreen (Invitrogen, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA) at Biocant (Cantanhede, Portugal).

2.4. Bioinformatics and OTU-based analysis

The raw pyrosequencing reads were processed using version 1.25 of the mothur software package (Schloss et al., 2009). Sequencing reads were assigned to the appropriate samples based on the corresponding barcode and were quality filtered to minimize the effects of random sequencing errors, by eliminating sequence reads b 200 bp, sequences that contained more than one undetermined nucleotide (N) and sequences with a maximum homopolymer length of 8 nucleotides. Identification and removal of chimeras was performed with Chimera.uchime (Schloss et al., 2011). Sequences not passing these quality controls were discarded.

Operational Taxonomic Units (OTUs) were assigned from the uncorrected pairwise distances between aligned 16S rRNA gene sequences, using the average neighbor clustering (Schloss and Westcott, 2011), considering a cut-off value of 97% similarity. Rarefaction curves, diversity indices Shannon (Shannon, 1948), Simpson (Simpson, 1949) and Good's Coverage (Good, 1953), as well as, richness estimators Chao1 (Chao, 1984) and Abundance Coverage Estimator (ACE) (Chao and Lee, 1992) were calculated to infer on richness, evenness and to evaluate sampling efforts in the samples. All OTU-based approaches were performed with software package Mothur 1.25.1 (Schloss et al., 2009) as well as the taxonomic assignment of the sequences, performed by the Silva-based alignment using default parameters. Statistical non-metric multidimensional scaling (nMDS) was performed (Schiffman and Reynolds, 1981) on distance sequence matrix from each sample, after normalization of the data. ANOVA was used to examine statistical significance between samples. Statistical methods for detection of differential abundance between samples were applied using the Metastats program (White et al., 2009), implemented in Mothur software. Statistical significance was considered at P b 0.008. Venn diagrams were generated using Mothur 1.25.1 (Schloss et al., 2009).

2.5. Statistical comparisons between libraries

In order to check the reproducibility of our methods, the similarity between libraries was estimated for the microbial communities. All approaches were performed with the software package Mothur 1.25.1. Tree files describing the dissimilarity among multiple groups were constructed. Groups were clustered using the distance between communities by the BrayCurtis, Yue & Clayton and Jaccard calculators, describing the similarity in community membership (Jaccard) or structure (BrayCurtis and Yue & Clayton). To determine whether the clustering within the tree was statistically significant, the weighted UniFrac method was calculated. The significance of the test statistic indicates the probability that the communities have the same structure by chance. The libraries were considered significantly different if the P value was b 0.05. Analysis of molecular variance (AMOVA) (Anderson, 2001; Excoffier et al., 1992; Martin, 2002) and Homogeneity of molecular variance (HOMOVA) (Stewart and Excoffier, 1996) was used to test whether population genetic heterogeneicity is significantly different be- tween populations (Schloss, 2008). With an error rate of 0.05 and taking into account a Bonferroni's correction for multiple comparisons, the libraries were considered significantly different if the P value was b 0.0167.

3. Results and discussion

3.1. Characteristics of pyrosequencing data

After quality control and filtering of the crude pyrotags, 207,762 sequences with good quality were retained, consisting of 4593 unique sequences. The average sequence length was 265.82 bp (range of 239-277; median 266; sd 1.78). Reads corresponded to 65,746, 72,592 and 63,371 from cheese-makers A, B and C respectively. Sampling completeness assessed by Good's coverage estimator for each data set returned values above 99% in all cases (Table 1). Rarefaction curve analysis, which assesses species richness from sampling, showed a trend to level-off at genus level, thus suggesting sufficient sampling of the microbial cheese communities both for producers and ripening times (Fig. S1 in the supplemental material). Simpson's and Shannon's diversity indexes revealed a higher diversity at initial stages of cheese manufacture for cheese-makers A and C; the B producer's initial and final stage showed a similar value of estimation with higher diversity by 14 days (Table 1). Richness estimators exhibited a trend of increasing richness at intermediate stages of maturation and a decrease tendency at the end of ripening time.

To determine the reproducibility of the methods, the similarity be- tween batches from the different cheese-makers was estimated in terms of microbial community membership and community structure. AMOVA and HOMOVA results indicated that both structure and membership were not the same for the batches from each cheese-maker at early times of the ripening process while homogeneity was observed at later

stages, supporting their analysis as replicate units (Table 2). Dis- similarity tree files confirmed differences at the level of community membership between early communities in cheese-makers A and B and at the level of structure in cheese-makers A and C (see Fig. S2 in the supplemental material). The apparent similarity between individual batches at 14 and 21 days may indicate that the replicates accurately reflect the overall community structure and membership. These results suggest that communities evolve towards more stable and predictable ones. Many or most organisms derived from the environment or the cow's mammary glands do not survive during cheese ripening and are not present in the final population. This could account for the observed differences in the early maturation of Pico cheese. However, we are aware that the community, as described by the analysis of samples taken in early batches might be biased.

3.2. Identification of core bacterial community members and abundance patterns

Four phyla were disclosed in Pico cheese from the taxonomic classification of our sequences, i.e. Firmicutes (86.7%), Proteobacteria (12.9%), Actinobacteria (0.3%) and Bacteroidetes (0.1%), which is in agreement with the findings of other works on artisanal cheese (Aldrete-Tapia et al., 2014; Alegría et al., 2012; Fuka et al., 2013; Quigley et al., 2012). A total of 337 OTUs were obtained and 54 bacterial genera were identified. To evaluate the number of OTUs, 0.03 memberships in samples from 21-day old cheeses, i.e. samples at the end of the maturation time, Venn diagrams were constructed; the diagrams indicated specific and common OTUs of all producers. Relatively few OTUs were shared between all groups of mature cheese samples, ca. 5% of the 337 total OTUs detected. Chao's richness estimated similar values of shared OTUs in all cheese samples, which would represent the core components of the artisanal Pico cheese (Fig. 1). *Acinetobacter*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pantoea*, *Rothia*, *Staphylococcus*, *Streptococcus* and unclassified genera belonging to the Enterobacteriaceae family are included in those 17 shared OTUs (Table 3). Sequences that could not be assigned taxonomic affiliations at 97% level of similarity (i.e., 7.7% of all sequences) were labelled as "unclassified". More than 99% of unclassified sequences were assigned to the Enterobacteriales order.

Studies on other cheeses, using pyrosequencing to gain insight into their microbiota, have also led to the definition of a common core microbiota in several cheese-making units within the same broad geo- graphic area (De Filippis et al., 2014; Fuka et al., 2013; Quigley et al, 2012). The naturally-selected core microbiome is fundamental for the maturation process in artisanal, regional cheeses. Defining its OTU composition is an important step towards the stabilization of theses cheeses' production process, both in terms of product quality and in terms of their safety.

As in other dairy products, including artisanal cheeses (Aldrete-Tapia et al., 2014; Alegría et al., 2012; Fuka et al., 2013; Quigley et al., 2012), lactic acid bacteria (LAB) predominate in Pico cheese throughout its ripening. Lactococci represent the vast majority of sequences for all cheese-makers, including the industrial unit (68.8–87.2% of the reads) (Table 4). Lactococci contribute to the acidification of the curd and casein

proteolysis, pivotal processes in cheese-making (McSweeney, 2004). The metabolism of amino acids and fatty acids by these LAB are major contrib- utors to flavor development (Di Cagno et al., 2008; McSweeney, 2004; Skelin et al., 2012; Randazzo et al., 2006), while their antimicrobial activity enhances autolysis and helps shaping the microbial communities in cheese (Banks and Williams, 2004). The high proportion of enterobacterial sequences found among the common OTUs may be related to the non-limiting values of pH (4.9-5.3), aw (0.940-0.980) and NaCl concentration (0.12-1.97%, w/w) in Pico cheese throughout ripening (unpublished data). Enterobacteria are common in artisanal cheeses made from raw milk (Parente and Cogan, 2004). Their presence indicates poor hygiene during the manufacture process and high numbers of Enterobacteriaceae have been linked to the accumulation of undesir- able compounds such as biogenic amines (Maifreni et al., 2013) as well as to flavor and texture defects in cheese (Sheehan, 2011). *Lactobacillus*, *Leuconostoc*, *Pantoea*, *Rothia* and *Staphylococcus* (respectively 0,205%, 0.081, 0.060, 0.193 and 0.117% of the total number of reads) were part of the subdominant microbiota of all studied cheeses, i.e. they represented 0.01–1% of the total sequences. Lactobacilli and leuconostocs are part of the LAB in artisanal cheeses (e. g. Aldrete-Tapia et al., 2014; Quigley et al., 2012), contributing to their flavor and texture (Peláez and Requena, 2005). *Pantoea* has been found as part of the enterobacteri- al microbiota of raw milk (Lafarge et al., 2004) and artisanal cheeses (Fuka et al., 2012). Actinobacteria are represented in the common core of OTUs in Pico cheese by *Rothia*, an adventitious bacterium found in many cheeses (Giannino et al., 2009). *Staphylococcus* can be detrimental for cheese quality and safety. Often, coagulase-positive staphylococci are toxigenic and should not reach high numbers in cheese (Kousta et al., 2010). However, some coagulase-negative staphylococci may participate in flavor formation due to their lipolytic activity (Verdier-Metz et al., 2012). Cheeses from raw milk can harbor other pathogens, such as *Salmonella*, *Campylobacter*, and *Listeria monocytogenes* (Brooks et al., 2012). No sequences belonging to *Salmonella*, *Campylobacter*, or *L. monocytogenes* were identified in our samples.

Microbial rank abundance curves showed that Pico cheese's microbial communities are characterized by a few dominant taxa, and many low-abundance, highly-diverse taxa, this latter group integrating the "rare biosphere" (Fig. 2). Dominant, subdominant and rare biosphere microbiota were defined by observed abundances, i.e., 1%, 0.01–1% and b 0.01% of the total sequences respectively. Quigley et al (2012) have also revealed the same abundance pattern in artisanal Irish cheeses.

3.3. Comparison between cheese-makers and ripening times

Non-metric multidimensional scaling (nMDS), based on distances calculated using Yue & Clayton's measure of community dissimilarity, was used to ordinate the data according to producer and ripening time. Pairwise comparisons of the various samples according to ripening time indicated no significantly diverse communities between producers at the initial stage of ripening. There was significant separation between B and A, and between B and C ($p < 0.01$ in both cases) at the intermediate and final

ripening times when a 3% threshold was employed (Fig. 3). Bacterial diversity in mature cheeses (21 d ripening) related samples from cheese-makers A and C indicating similar community composition. Similarly, cheese-maker B ranked closer to industrial Pico cheese, although no significant differences were observed between industrial cheese and that by producer A.

Few trends in abundance patterns were observed in taxonomic groups along cheese ripening in all producers (Fig. 4), the most remarkable being the increasing number of sequences of the *Lactobacillus* genus and the decrease in *Acinetobacter* and *Stenotrophomonas* as ripening progresses. *Lactococcus* did not experiment notable variation in sequence abundance, but *Streptococcus* showed a clear increasing trend in two out of three batches. *Chryseobacterium* and *Bifidobacterium* decreased throughout the process although they were not detected in cheese-maker A. Our results showed a diverse bacterial community at early cheese maturation, with an increase in the number of phylotypes for at least up to two weeks followed by a decrease toward the end of ripening. In Mozzarella cheese, Ercolini et al. (2012) also described a pattern of higher community richness estimates at initial stages as compared with later stages of ripening.

To compare mature cheeses for differentially-abundant OTUs, Metastats methodology was run. Results indicated that six OTUs were differentially abundant between producers C and A, and between C and B (P b 0.008), and seven OTUs between producers A and B. OTUs accounting for the chief significant abundance differences between producers are summarized in Table 5.

Differences between the three cheese-making units were apparent at the phylum level (Table 4). Cheese-maker B had the highest pro- portion of Firmicutes sequences (93.6%) and cheese-maker A the lowest (80.9%). Conversely, proteobacterial sequences were highest in cheese-maker A (19.0%) and lowest in cheese-maker B (5.8%). At the genus level, lactococci were the only dominant LAB in the case of cheese-maker A. In cheese-maker C, dominant LAB included also *Streptococcus*, while in cheese-maker B both streptococci and enterococci were part of the dominant microbiota. The microbiota of industrial Pico cheese is composed exclusively by LAB (*Lactococcus*, *Enterococcus* and *Lactobacillus*) represented by one OTU each, reflecting not only the bacteria that were added as a starter culture, but also the influence of non-starter microorganisms. It is interesting to note the presence of lactobacilli as part of the dominant LAB in industrial cheese, since they are not part of the dominant microbiota of the artisanal cheeses. This may mean that the starter culture used at present does not reflect the microbiota associated with the traditional product. In the samples from cheese-maker A and the industrial unit, *Streptococcus* were subdominant, representing respectively 0.115 and 0.198% of the total reads for each cheese-making unit. *Enterococcus* were part of the subdominant microbiota of cheese-makers A and C only (respectively, 0.412 and 0.410% of the total reads for each unit). The LAB genera found in our study are known to have a central role in the development of the characteristic sensorial properties in artisanal cheeses (Peláez and Requena, 2005).

Proteobacterial sequences that were dominant in at least one of the cheese-makers

included *Pseudomonas* and *Marinomonas* in unit C (respectively 1.447 and 1.101% of the total sequences for this unit). In other units, *Pseudomonas* (0.052 and 0.039% of the sequences in cheese-makers A and B, respectively) and *Marinomonas* (0.039% of the sequences in cheesemaker A) were present as part of the subdominant microbiota. In some of the studied cheese-makers, the subdominant microbiota also included *Stenotrophomonas* (in all artisanal cheeses), *Aeromonas* (in cheese-makers A and B), *Chryseobacterium* (in cheese-makers B and C) *Flavobacterium* (only in cheese-maker C), *Elizabethkingia* (only in cheese-maker A) and *Vagococcus* (in the industrial unit only). Spoilage-related, psychrotrophic γ-proteobacterial sequences (*Pseudomonas*, *Stenotrophomonas* and *Aeromonas*) have been found in other artisanal cheeses (Masoud et al., 2011). *Marinomonas*,a halotolerant γ-proteobacterium, was present in artisanal cheeses (Coton et al., 2012), but no clear association between cheese spoilage and its presence was found in the literature. Halotolerant bacteria may be transferred to the cheeses by the salt (Ishikawa et al., 2007). Bacteroidetes that take part in the microbiota of some artisanal

Pico cheese-makers (*Elizabethkingia* in A, *Flavobacterium* in C and *Chryseobacterium* in B and C) are also present in other cheeses and may have a negative impact on product quality (Aldrete-Tapia et al., 2014; Collins et al., 2003; Montel et al., 2014). *Exiguobacterium* was part of the dominant microbiota in cheese-maker A (1.042%). It has recently been described as part of the natural cheese microbiota (Lusk et al., 2012). However, its presence in raw milk is well established (Hantsis-Zacharov and Halpern, 2007), and it has been added to experimental cheeses as part of anti-listerial consortia (Callon et al., 2011). *Macrococcus*, another member of the Firmicutes that is regarded as an adventitious bacterium in cheese production (Giannino et al., 2009), was part of the predominant microbiota in cheese-maker B and was subdominant in industrial cheese. It is interesting that sequences corresponding to *Bifidobacterium* genus have been found in samples from cheese-makers B (0.02%) and C (0.30%). *Bifidobacterium* has been recently described for the first time in traditional cheeses (Alegría et al., 2012); they have industrial potential as probiotics, but their presence could also indicate animal fecal contamination (Delcenserie et al., 2011).

3.4. Biodiversity in cheese environments

In conclusion, next-generation sequencing technology (pyrosequencing) enabled an in-depth view of the microbiota in Pico cheese, provided insight into the core microbiota of this traditional Azorean product, allowed resolution between cheese-making units, ripening times, and provided information on the presence of bacterial genera of relevance for safety. Our study showed that Firmicutes (*Lactococcus*, *Streptococcus* and *Enterococcus*) predominate in this artisanal cheese, leading us to conclude that Pico cheese manufacture is a *Lactococcus*-driven process. This microbiota is accompanied by *Lactobacillus* and some Gammaproteobacteria (Enterobacteriaceae, *Acinetobacter*, *Marinomonas* and *Pseudomonas*)., This information may provide the basis for further in-depth studies leading to the design of

starter/adjunct cultures specifically for Pico cheese manufacture. It also highlighted the main hygienic and safety issues that need to be adequately addressed. Although no *Salmonella* or *Listeria* sequences were identified in our samples, *Staphylococcus* was among the most abundant genera; since some species in this genus are enterotoxin producers, further studies should be carried out to establish their role upon the safety of this product. The presence of Gammaproteobacteria that might be detrimental for cheese quality (e.g. *Pseudomonas*) is another relevant topic. Our study provided basic information that may be crucial in devising strategies to improve safety, extend shelf-life, enhance quality and reinforce market value of Pico cheese, thus ultimately, contributing to the sustainability and preservation of this Azorean food specialty.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.09.031.

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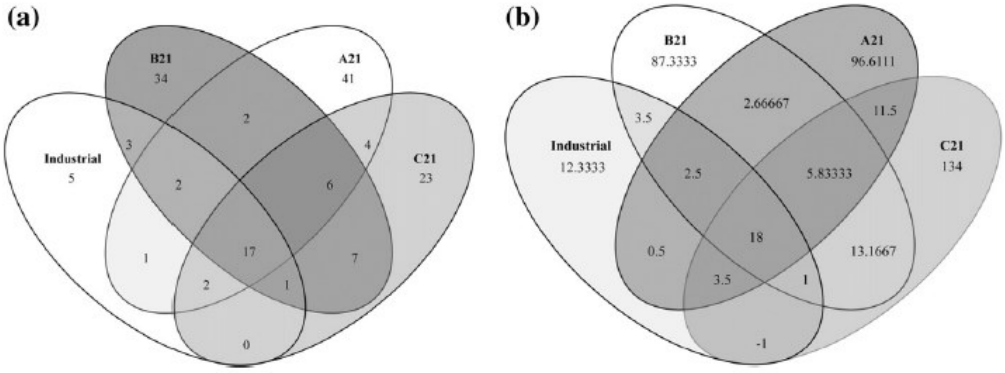


Fig. 1. Venn diagrams representing shared OTU diversity of bacteria between mature cheese samples (97% sequence similarity); (a) observed OTU (b) Chao1 richness estimator.

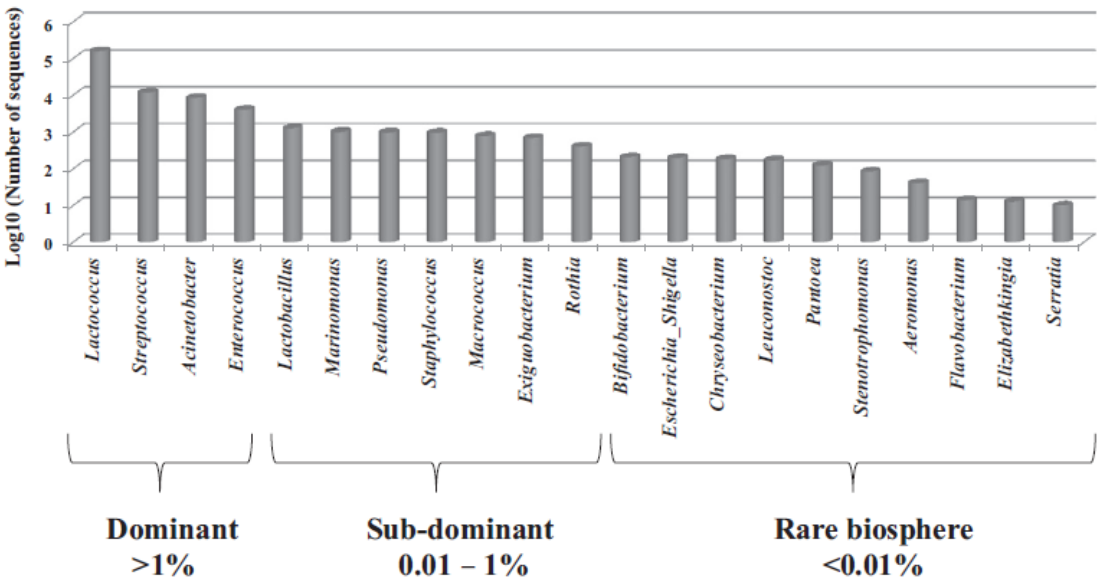


Fig. 2. Rank-abundance curve in Pico cheese. Dominant, subdominant and rare biosphere microbiota are represented by 1%, 0.01–1% and b0.01% of the total sequences. Taxa represented by less than 10 sequences were not taken into account.

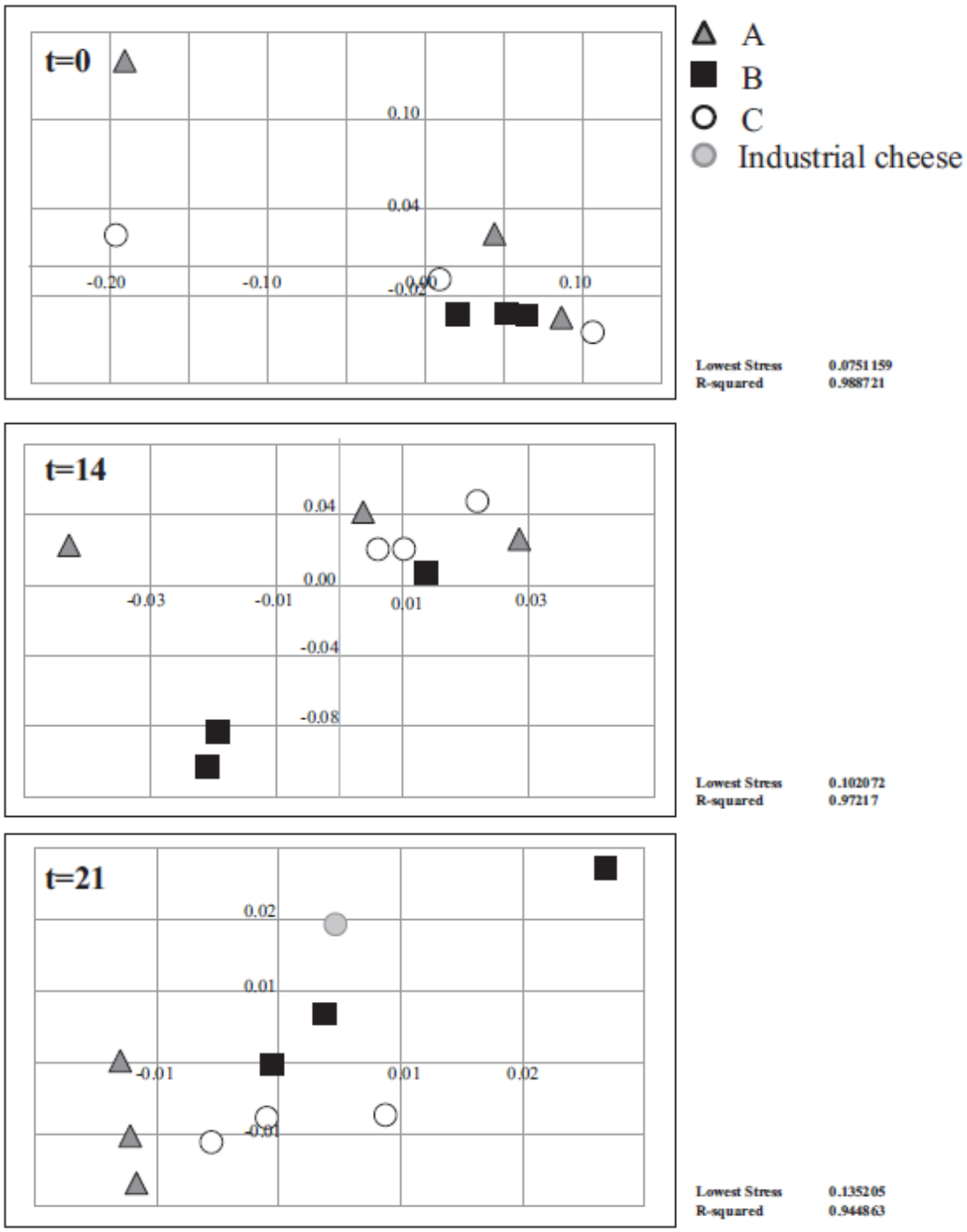
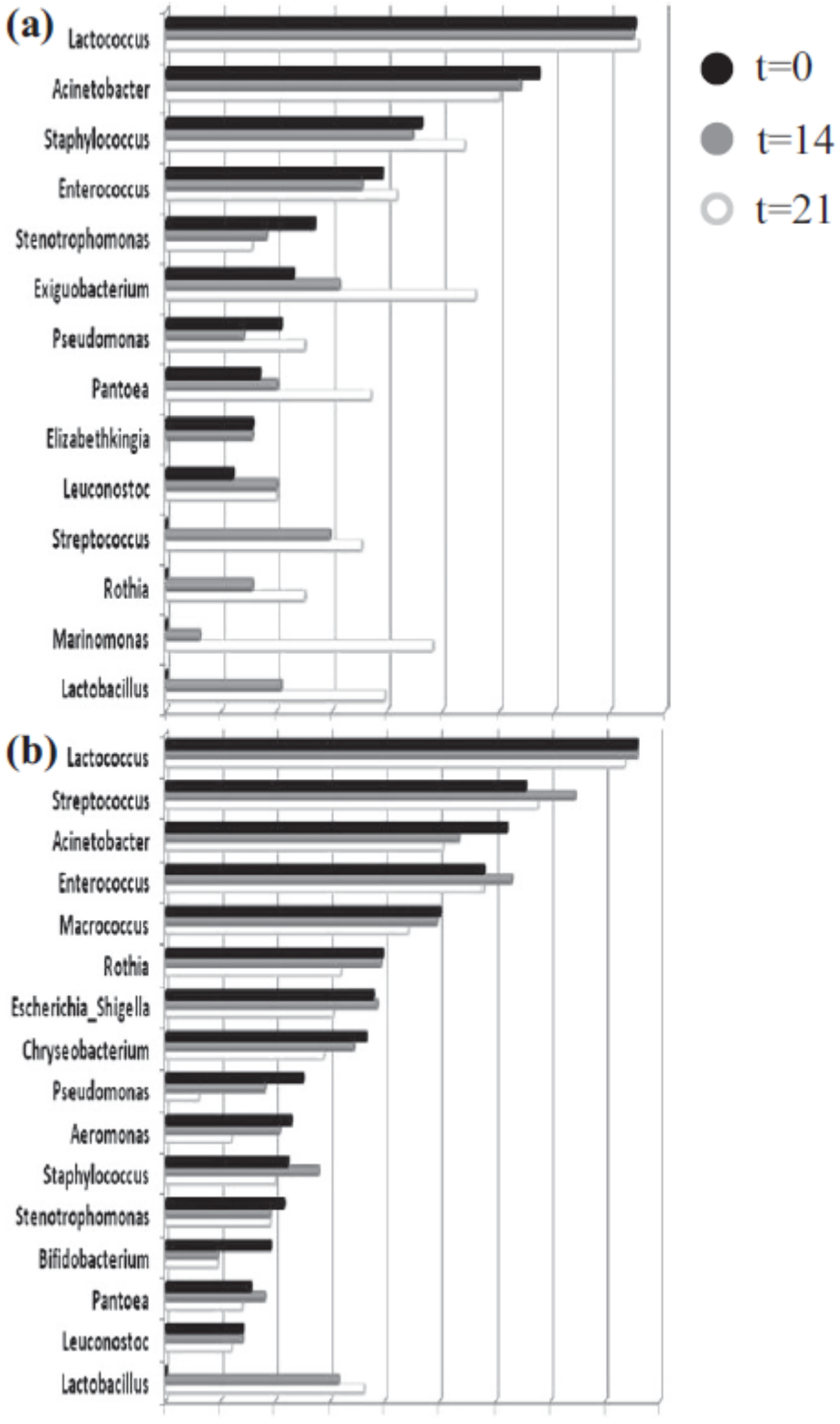


Fig. 3. Nonmetric multidimensional scaling plots (on Yue & Clayton measure of dissimilarity) depicting patterns of beta diversity for bacterial communities in Pico cheese. A phylip-formatted distance matrix that describes the dissimilarity (1-similarity) among groups was built. The Yue and Clayton index was used to calculate the dissimilarity values. The index ranges from 0 to 1, with 1 = complete similarity and 0 = complete dissimilarity, including species proportions of both the shared and non-shared species in each community. Points that are closer together on the ordination have communities that are more similar. AMOVA indicated that differences between bacterial communities mapped for the samples with 14 and 21 days of ripening were highly significant (P b 0.001), while

differences for cheese at the initial stage of maturation (day 0) were not significant (P N 0.05).


(a)
Lactococcus
Acinetobacter
Staphylococcus
Enterococcus
Stenotrophomonas
Exiguobacterium
Pseudomonas
Pantoea
Elizabethkingia
Leuconostoc
Streptococcus
Rothia
Marinomonas
Lactobacillus
t=0
t=14
t=21
(b)
Lactococcus
Streptococcus
Acinetobacter
Enterococcus
Macrococcus
Rothia
Escherichia_Shigella
Chryseobacterium
Pseudomonas
Aeromonas
Staphylococcus
Stenotrophomonas
Bifidobacterium
Pantoea
Leuconostoc
Lactobacillus

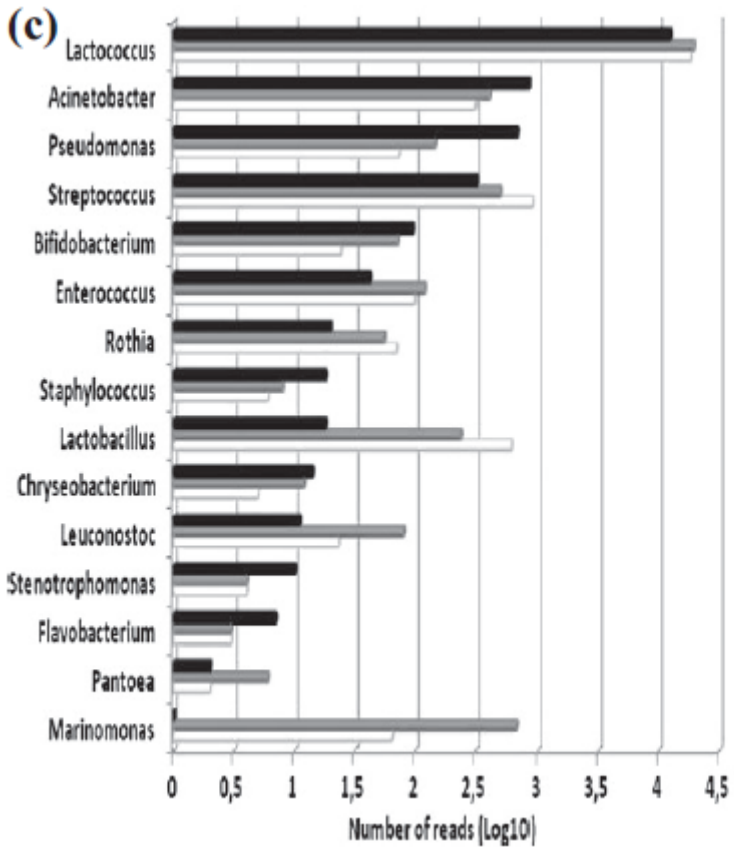


Fig. 4. Rank-abundance curve in Pico cheese according to ripening time; (a) at maturation day 0, (b) at maturation day 14, and (c) at maturation day 21.

Table 1

Summary of the characteristics of the pyrosequencing data, number of total tags sequenced by cheese-maker/maturation time and the observed richness, coverage and richness estimator at 97% similarity level. A, B, and C are the three artisanal cheese-makers, I represents the industrial cheese.

	Cheese-makers/maturation times									
Parameters	A/0	A/14	A/21	B/0	B/14	B/21	C/0	C/14	C/21	I/at retail
No. sequenced tags	22655	20791	22300	24603	28316	19673	17169	23981	22221	6053
Coverage	0.999	0.998	0.998	0.998	0.999	0.998	0.998	0.998	0.999	0.999
No. OTUs observed	62	65	75	84	88	72	77	96	60	31
Simpson	0.55	0.59	0.66	0.60	0.49	0.60	0.52	0.66	0.68	0.61
Shannon	1.141	1.052	0.972	1.140	1.337	1.110	1.286	1.002	0.921	0.920
Chao1	161.20	189.00	141.11	162.11	174.10	134.00	140.91	210.83	186.00	40.33
ACE	318.88	219.14	260.78	235.97	274.96	200.65	331.67	464.42	203.17	38.10

Table 2

AMOVA (a) and HOMOVA (b) comparisons of the batches constructed in this study from each cheese-maker. Libraries were considered significantly different (marked in bold) if the P-value was inferior to 0.0167

(a)

Column1	L1A0	L2A0	Column1	L1A14	L2A14	Column1	L1A21	L2A21
L2A0	**<0.0001**		**L2A14**	0.358		**L2A21**	0.588	
L3A0	**0.002**	**<0.0001**	**L3A14**	0.225	0.632	**L3A21**	0.020	0.045
Column1	**L1B0**	**L2B0**	Column1	**L1B14**	**L2B14**	Column1	**L1B21**	**L2B21**
L2B0	**<0.0001**		**L2B14**	**0.009**		**L2B21**	0.406	
L3B0	0.728	**<0.0001**	**L3B14**	0.023	0.175	**L3B21**	0.605	0.484
Column1	**L1C0**	**L2C0**	Column1	**L1C14**	**L2C14**	Column1	**L1C21**	**L2C21**
L2C0	**<0.0001**		**L2C14**	0.012		**L2C21**	0.046	
L3C0	**<0.0001**	0.023	**L3C14**	0.030	0.225	**L3C21**	0.145	0.389

(b)

Column1	**L1A0**	**L2A0**	Column1	**L1A14**	**L2A14**	Column1	**L1A21**	**L2A21**
L2A0	**0.0002**		**L2A14**	0.448		**L2A21**	0.959	
L3A0	0.162	0.038	**L3A14**	0.037	0.124	**L3A21**	0.559	0.539
Column1	**L1B0**	**L2B0**	Column1	**L1B14**	**L2B14**	Column1	**L1B21**	**L2B21**
L2B0	**<0.0001**		**L2B14**	0.059		**L2B21**	0.408	
L3B0	0.653	**<0.0001**	**L3B14**	**0.012**	0.520	**L3B21**	0.937	0.422
Column1	**L1C0**	**L2C0**	Column1	**L1C14**	**L2C14**	Column1	**L1C21**	**L2C21**
L2C0	**<0.0001**		**L2C14**	0.030		**L2C21**	0.198	
L3C0	**0.0001**	0.135	**L3C14**	0.094	0.132	**L3C21**	0.183	0.999

Table 3

Taxonomic affiliation and number of reads in the different ripening times per batch of the shared microbial consortia found in mature cheeses.

Phyla	Family	Genera	A0	A14	A21	B0	B14	B21	C0	C14	C21	Industrial
Firmicutes	Streptococcaceae	*Lactococcus*	16690	15880	18122	19027	19471	15118	12323	19355	18230	4660
Proteobacteria	Enterobacteriaceae	Unclassified	1353	1444	791	176	345	226	470	420	368	37
Proteobacteria	Moraxellaceae	*Acinetobacter*	796	436	156	12	9	2	15	3	7	1
Proteobacteria	Enterobacteriaceae	Unclassified	228	164	261	165	354	150	806	730	543	6
Proteobacteria	Enterobacteriaceae	Unclassified	212	27	25	3	5	2	358	391	307	1
Proteobacteria	Enterobacteriaceae	Unclassified	1412	1052	239	126	189	68	1067	665	543	9
Proteobacteria	Moraxellaceae	*Acinetobacter*	44	49	51	923	350	256	329	187	186	8
Firmicutes	Enterococcaceae	*Enterococcus*	51	21	48	427	697	349	24	101	71	44
Firmicutes	Streptococcaceae	*Lactococcus*	84	288	7	536	124	152	2	1	1	3
Firmicutes	Enterococcaceae	*Enterococcus*	39	38	74	346	691	421	18	19	27	501
Proteobacteria	Enterobacteriaceae	*Pantoea*	7	10	71	6	8	5	2	6	2	8
Firmicutes	Staphylococcaceae	*Staphylococcus*	21	26	114	13	25	10	17	8	6	4
Firmicutes	Streptococcaceae	*Streptococcus*	1	22	51	521	1482	735	298	480	827	12
Actinobacteria	Micrococcaceae	*Rothia*	1	6	18	96	92	40	20	55	70	4
Firmicutes	Leuconostocaceae	*Leuconostoc*	4	10	10	5	5	4	11	78	23	19
Firmicutes	Lactobacillaceae	*Lactobacillus*	0	6	27	0	18	7	3	87	123	146
Firmicutes	Lactobacillaceae	*Lactobacillus*	0	3	66	1	18	53	1	0	6	9

Table 4
Percentage of recovered reads from each taxon for each cheese-maker and maturation time.

Taxon	Cheese-maker				Maturation time (days)			Total
	A	B	C	I	0	14	21	
Firmicutes	80.91	93.62	83.57	98.78	81.35	86.87	90.69	86.68
Lactococcus	77.734	75.032	78.811	77.119	75.582	75.464	80.486	77.100
Streptococcus	0.137	13.197	2.716	9.119	3.391	7.958	5.281	5.748
Enterococcus	0.412	4.047	0.410	9.004	1.405	2.151	1.545	1.932
Lactobacillus	0.163	0.143	1.390	2.990	0.030	0.393	1.224	0.613
Staphylococcus	1.329	0.066	0.051	0.066	0.363	0.276	0.807	0.461
Macrococcus	0.000	1.079	0.000	0.083	0.497	0.408	0.257	0.379
Exiguobacterium	1.042	0.006	0.000	0.000	0.026	0.051	0.989	0.332
Leuconostoc	0.037	0.019	0.180	0.314	0.031	0.130	0.058	0.082
Granulicatella	0.000	0.008	0.000	0.000	0.002	0.006	0.002	0.003
Vagococcus	0.002	0.003	0.000	0.017	0.002	0.000	0.003	0.002
Zymophilus	0.002	0.000	0.000	0.000	0.002	0.000	0.000	0.001
Trichococcus	0.000	0.001	0.000	0.000	0.000	0.000	0.002	0.001
Clostridium IX	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.001
Bacillus	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.001
Brochothrix	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.001
Proteobacteria	19.02	5.81	15.81	1.16	18.15	12.70	8.99	12.91
Acinetobacter	7.316	2.850	2.470	0.149	6.761	3.318	2.591	4.069
Marinomonas	0.397	0.001	1.191	0.000	0.000	0.950	0.503	0.490
Pseudomonas	0.052	0.039	1.447	0.000	1.130	0.216	0.145	0.471
Escherichia–Shigella	0.000	0.271	0.002	0.000	0.121	0.118	0.053	0.095
Pantoea	0.134	0.026	0.016	0.132	0.023	0.033	0.122	0.060
Stenotrophomonas	0.055	0.041	0.028	0.000	0.068	0.029	0.030	0.040
Aeromonas	0.014	0.040	0.005	0.000	0.033	0.021	0.008	0.020
Serratia	0.009	0.001	0.005	0.000	0.002	0.012	0.000	0.005
Raoultella	0.002	0.001	0.005	0.000	0.000	0.004	0.003	0.002
Comamonas	0.002	0.003	0.002	0.000	0.003	0.001	0.002	0.002
Buttiauxella	0.003	0.000	0.003	0.000	0.003	0.001	0.002	0.002
Enhydrobacter	0.000	0.006	0.000	0.000	0.003	0.000	0.003	0.002
Delftia	0.000	0.000	0.006	0.000	0.002	0.004	0.000	0.002
Variovorax	0.002	0.000	0.003	0.000	0.005	0.000	0.000	0.001
Brevundimonas	0.000	0.003	0.002	0.000	0.005	0.000	0.000	0.001
Erwinia	0.000	0.000	0.005	0.000	0.003	0.001	0.002	0.001
Paracoccus	0.000	0.003	0.000	0.000	0.000	0.001	0.002	0.001
Shewanella	0.000	0.000	0.003	0.000	0.002	0.001	0.000	0.001
Psychrobacter	0.003	0.000	0.000	0.000	0.000	0.000	0.003	0.001
Pseudoalteromonas	0.003	0.000	0.000	0.000	0.000	0.000	0.003	0.001
Luteibacter	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.001
Trabulsiella	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.001
Kluyvera	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.001
Morganella	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.001
Sphingobium	0.000	0.000	0.005	0.000	0.000	0.000	0.005	0.001
Actinobacteria	0.04	0.34	0.53	0.07	0.35	0.32	0.25	0.30
Rothia	0.038	0.314	0.229	0.066	0.182	0.209	0.199	0.194
Bifidobacterium	0.000	0.021	0.301	0.000	0.161	0.103	0.042	0.099
Arthrobacter	0.002	0.000	0.000	0.000	0.000	0.000	0.002	0.001
Actinomyces	0.000	0.000	0.002	0.000	0.000	0.001	0.000	0.001
Bacteroidetes	0.03	0.22	0.08	0.00	0.016	0.11	0.07	0.11
Chryseobacterium	0.006	0.204	0.049	0.000	0.130	0.088	0.055	0.088
Flavobacterium	0.000	0.001	0.021	0.000	0.012	0.004	0.005	0.007
Elizabethkingia	0.020	0.000	0.000	0.000	0.009	0.008	0.002	0.006
Sphingobacterium	0.000	0.004	0.008	0.000	0.003	0.006	0.003	0.004
Kocuria	0.000	0.007	0.000	0.000	0.000	0.003	0.005	0.002
Pedobacter	0.000	0.000	0.002	0.000	0.000	0.001	0.000	0.001
Fluviicola	0.000	0.000	0.002	0.000	0.000	0.000	0.002	0.001
Nubsella	0.000	0.000	0.002	0.000	0.002	0.000	0.000	0.001
Sediminibacterium	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.001
Bacteroides	0.000	0.000	0.002	0.000	0.000	0.001	0.000	0.001
Unclassified	11.078	2.561	10.631	0.942	10.008	8.022	5.558	7.670

Table 5

Differentially-abundant OTUs among producers. Detection of differential abundance between samples was performed with Metastats.

OTU number	Number of total reads			Phyla	Sub-Phyla	Order	Family	Genera
	A21	B21	C21					
9	25	2	307	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Unclassified
12	51	256	186	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
13	2	1	38	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
29	1	28	4	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Chryseobacterium*
30	51	735	827	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*
34	12	1	27	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
45	27	7	123	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
64	0	165	0	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Macrococcus*
65	0	1291	71	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*
67	0	33	0	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia–Shigella*
68	0	22	1	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
87	0	42	0	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*
98	0	3	24	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*
116	1	0	491	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*
117	259	0	64	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oceanospirillaceae	*Marinomonas*