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Temporal and spatial differences in microbial composition during the manufacture of a Continental-type cheese

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Abstract

We sought to determine if the time, within a production day, that a cheese is manufactured has an influence on the microbial community present within that cheese. To facilitate this, 16S rRNA amplicon sequencing was used to elucidate the microbial community dynamics of brine salted Continental-type cheese in cheeses produced early and late in the production day. Differences in microbial composition of the core and rind of the cheese were also investigated.

Throughout ripening, it was apparent that late production day cheeses had a more diverse microbial population than their early day equivalents. Spatial variation between the cheese core and rind was also noted in that cheese rinds were found to initially have a more diverse microbial population but thereafter the opposite was the case. Interestingly, the genera *Thermus*, *Pseudoalteromonas* and *Bifidobacterium*, not routinely associated with a Continental-type cheese produced from pasteurised milk were detected. The significance, if any, of the presence of these genera will require further attention. Ultimately, the use of high throughput sequencing has facilitated a novel and detailed analysis of the temporal and spatial distribution of microbes in this complex cheese system and established that the period during a production cycle at which a cheese is manufactured can influence its microbial composition.

Introduction

Commercial cheeses produced with defined starter/adjunct strains often suffer from variations in cheese flavour profile and microbial content (1). This is thought to be primarily due to batch variations in milk quality and storage time as well as manufacturing practices (2) and the adventitious microbial populations present (3, 4). Indeed, in the latter case, aroma and taste defects, along with biogenic amine formation, mineral deposition (calcium lactate) issues and irregular gas formation are common defects associated with a variety of microorganisms (5).

Analysis of the bacterial composition of cheese has traditionally involved the use of culture based techniques which, while effective for quantifying common starter/non-starter bacteria as well as certain spoilage bacteria (*Clostridium*, *Staphylococus*), do not always accurately reflect the total microbiota present (6, 7). PCR based molecular profiling techniques targeting either particular populations or select taxonomic communities are also routinely used and have been extensively reviewed (8-10). PCR based methods cannot, however, provide comprehensive coverage of total microbial populations.

The advent of high throughput next generation sequencing (NGS) has advanced the field of microbial ecology by providing a powerful means of analysing dominant and sub-dominant populations and their dynamics in highly complex ecosystems (2). NGS has been applied extensively to a variety of environments including the sea (11), soil (12) as well as the gut (13). More recently, NGS of bacterial 16S rRNA amplicons has been used to characterise the microbial communities of a variety of fermented foods and beverages (14-20), as well as of raw milk and raw milk cheeses (21-26). Indeed, this approach has led to identification of a number of genera previously not associated with cheese ecosystems (*Prevotella, Helcococcus*) or with particular cheese types

(*Arthrobacter* in goat's milk cheese). Microbial content has also been shown to vary with milk source, processing (raw or pasteurised) and addition of various ingredients (27). Ultimately, NGS platforms offer significantly increased detection sensitivity over more traditional molecular methods with respect to the study of bacterial communities (2, 26, 28, 29). NGS based approaches have also been used to profile communities present in production facilities providing a unique insight into possible microbial reservoirs important for cheese sensory characteristics or for identifying potential biofilm forming genera (2).

Both culture and molecular based approaches have been used to better understand the spatial distribution of microbes in cheese. Microbial composition varies throughout the cheese block due to several factors including salt, moisture, pH and the availability of oxygen (30). The effect of salt is particularly important in brine-salted cheese varieties as salt migrates to the core of the cheese over the ripening process, affecting moisture levels and microbial growth (31). To date the majority of studies examining the spatial distribution of microbial populations in cheese have relied on two methods. One involves non-destructive fluorescent microscopy, based on production of a gel cassette system (32) or via cryosectioning, followed by fluorescence in situ hybridization (FISH) using rRNA targeted probes (33, 34). The second involves destructive sampling of selected regions of cheese followed by an assessment of the microbiota by culture-dependent and/or independent methods (3, 30, 35-37). More recently an NGS approach was used by Wolfe *et al*. to reveal both the microbial composition and functional potential of 137 cheese rind communities. In this case, 16S rDNA and Internal Transcribed Spacer (ITS) amplicon sequencing allowed for characterisation of microbial communities while 'shotgun' metagenomics permitted an in-depth analysis of pathways involved in flavour formation (38).

In this study, 16S rRNA amplicon sequencing was used to describe, from both a spatial and a temporal perspective, the microbiota present in a brine-salted continental-type cheese produced within a single production day. This study builds on results from a previous study which reported a significant interaction between time of day of manufacture and stage of ripening on mean viable counts of Non Starter Lactic Acid Bacteria (NSLAB) ($p< 0.04$), with cheeses ($n=42$), produced late (in comparison to those produced early or middle in the day of manufacture) having significantly higher mean viable NSLAB counts (39). We assess if production of the cheese early or later during the daily cheese-making cycle impacts on the subsequent development of its bacterial community, investigate how these populations change throughout the ripening process and examine variance in microbial spatial distribution between the cheese core and rind. In each case noteworthy variations in the microbial composition, resulting from differences in production phase, stage of ripening or the part of the cheese being studied, are apparent.

Materials and Methods

Cheese Production, Sampling and Nucleic Acid Extraction

Four blocks of a semi-hard brine salted Continental-type cheese produced from pasteurised milk were sourced, one day post production. The blocks were produced in a single production day, from separate vats and corresponded to early day (morning sampling; [ED], n=2) and late day (afternoon sampling; [LD], n=2) production with 6-8 hours separating ED and LD manufacture. Furthermore, two blocks were received from each respective vat. Cheeses were produced based on a Swiss-type model using the thermophilic starters *Streptococcus thermophilus* and *Lactobacillus helveticus. Propionibacterium freudenreichii* was added as an adjunct. Post production, cheeses were subjected to ripening at 10°C for 10 days prior to hot-room ripening (20°C) from day 10 to day 40. Cheeses were then stored at 6°C for the remainder of ripening.

Each individual block was sampled aseptically, using a cheese trier, at 4 stages; 1 day post production (TP1), 10 days post production (TP2), 40 days post production (TP3) and after maturation at 64 days post production (TP4). Internal (core) and external (rind/1cm segment) regions of the cheese, at each time point, were also sampled. 1g of cheese was homogenised in 9ml of a 2% tri-sodium citrate buffer (VWR, Dublin, Ireland). Enzymatic lysis treatment on homogenised cheese samples was conducted prior to DNA extraction and included treatment with lysozyme (1mg/ml), mutanolysin (50U/ml) and proteinase K (800µg/ml) and incubation for 1 hour at 55°C as per Quigley *et al.* (40). DNA was extracted using the PowerFood Microbial DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, USA). Grated samples from cheeses were analysed for salt (41), moisture (42) and pH (43) at TP4.

PCR amplification of the microbial 16S rRNA gene. Extracted DNA was amplified using universal primers targeting the V4 region of the bacterial 16S gene (239nt) (4, 44). Primers, predicted to bind to 94.6% of all bacterial 16S genes, consisted of a forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). Primers also included a 19-mer sequence (GCCTGCCAGCCCGCTCAG) at the 5' end to allow emulsion based clonal amplification for the 454-Pyrosequencing system. Identification of individual sequences from the pooled samples was achieved by incorporating molecular identifier tags between the primer sequence and the adaptamer.

PCR reactions were carried out in triplicate and contained 25µl BioMix Red Master Mix (Bioline, London, UK), 1µl of each primer (200 nmol l^{-1}), 5µl of the DNA template (standardised to 100ng DNA/sample) and nuclease free water to a final volume of 50µl. PCR amplification was carried out using a G-Storm Thermal Cycler (Gene Technologies, UK). Amplification consisted of an initial denaturation at 94°C for 10 minutes followed by 40 cycles of; denaturation at 94° for 1 minute, annealing at 52°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final elongation step at 72°C for 2 minutes. PCR amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, UK). DNA quantity was assessed using the Quant-It Picogreen dsDNA reagent (Invitrogen, USA) in accordance with manufacturer's guidelines and in conjunction with the NanoDrop 3300 Fluorospectrometer (Thermo-Fisher Scientific, Wilmington, USA).

Furthermore, DNA was standardised to equi-molar concentrations prior to library preparation and sequencing.

High-throughput sequencing and bioinformatic analysis. 16S rRNA amplicons from the V4 region were sequenced on a Roche 454 FLX platform (Roche Diagnostics Ltd, West Sussex, UK) as previously described (17, 44) and according to protocols. Reads were quality filtered using the RDP sequencing pipeline (45). Reads with low quality scores (below 40), short lengths (less than 150bp), and reads lacking exact matches with respect to primer sequence were discarded. Reads were clustered, aligned and chimeras removed also within QIIME (46). All assigned OTUs were considered. A phylogenetic tree was generated using the FastTree software and subsequently alpha and beta diversities were calculated. Principle coordinate analysis (PCoA), measuring dissimilarities at phylogenetic differences based on weighted/unweighted Unifrac analysis were carried out using the QIIME suite of programs (46). Resultant PCoA plots were visualised with KiNG. Each trimmed FASTA sequence was assessed using the BLAST programme (47) against the SILVA 16S database (version 1.06). The resultant BLAST programme output was parsed using MEGAN (48). Bit scores were used for filtering the results prior to tree construction and summarization (absolute cut-off, BLAST bit score of 86, relative cut-off, 10% of top hit). Reads were deposited in the SRA database under the accession number PRJEB8181.

Results

α and β diversity of microbial populations in early and late day production cheeses. Blocks of a brine salted Continental-type cheese, manufactured early or late during a production cycle, were sampled at various stages throughout the ripening process. Post DNA extraction, amplicons corresponding to the V4 region of the bacterial 16S rRNA gene were generated by PCR. These amplicons were then subjected to NGS, generating 294,853 reads. This corresponded to 87,156 reads for TP1, 97,045 reads for TP2, 62,248 reads from TP3 and 48,404 reads from TP4 (full list of reads/individual sample and associated bar graphs located in Table S1/Figure S2). Species diversity (α-diversity) and richness were calculated for each time point as well as for time of manufacture (early/late day) and the location (core or rind) from which the samples were collected. These are presented in Table 1. Chao1 values, reflective of Operational Taxonomic Unit richness, ranged from 237.8 to 529.38, while the Shannon index, used to measure overall sample diversity, ranged from 2.51 to 3.82. Analysis of this data reveals that α-diversity decreases throughout the ripening process. Cheeses produced early in the production day had a less diverse microbiota than those produced late in the production day. Diversity appeared greatest in the rinds of the samples at TP1 whereas, for all subsequent time points, core populations were more diverse. These observations held true regardless of whether the samples were from ED or LD manufacture. Rarefaction curves, used to determine species richness from sampling, were calculated at 97% similarity. These revealed that bacterial diversity was well represented as samples are nearing parallel with the x-axis (Figure S1).

β diversity, based on the Unweighted UniFrac matrix, and represented in the form of a PCoA plot, was used to determine if samples grouped with respect to ripening point,

time of manufacture (early/late) and internal/external regions of the cheese (Figure 1A/B). Notably, samples from the same time point during the cheese ripening process generally grouped together, with data points from TP1/TP2 and TP3/TP4 also forming distinct clusters. In addition, samples clustered according to time of cheese production with those produced early in the production day clustering together and away from a more diffuse cluster of data points corresponding to samples from cheeses manufactured later in the production cycle (Fig. 1A). Core and rind samples also formed distinct clusters. The distinction between the core and rind populations was more apparent in samples manufactured later in the production cycle (Fig. 1B).

Cheese composition. Cheese pH, salt and S/M was determined at TP4 for both ED and LD cheeses. Results were similar with respect to pH (5.39 ED and 5.45 LD), salt (0.59% ED and 0.57% LD) and Salt/Moisture (1.55% ED and 1.51% LD).

High throughput sequencing reveals differences in microbial taxa between cheeses produced early and late in the production day. Phylogenetic assignment of high throughput sequence data revealed the presence of bacteria corresponding to 5 phyla; *Firmicutes, Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus* and *Actinobacteria.* As expected the *Firmicutes* dominated throughout the study representing 93.46 – 99.75% of reads in the ED samples. The percentages of the reads that corresponded with *Firmicutes* were lower in the LD samples and ranged from 72.26 – 85.56%. *Deinococcus-Thermus* was detected in both the ED and LD samples but at higher percentage populations in LD samples. Less dominant populations, corresponding to *Actinobacteria* and *Proteobacteria*, were also detected. *Proteobacteria* populations were highest at TP1 in both ED and LD samples.

At genus level, a number of differences were noted between cheese produced early and late in the production day (Fig. 2). *Lactobacillus* and *Streptococcus* populations

dominated in both ED and LD samples throughout the study. Percentage populations of *Lactobacillus* were similar in both ED and LD samples at TP1 (64.4% ED and 63.5% LD), thereafter it was noticed that populations were consistently higher in the ED samples. Proportions of *Streptococcus* were greater in the ED samples (31.1%) than the LD samples (18.3%), a trend that continued throughout the study. *Thermus* was detected in both ED and LD samples but at consistently greater proportions in the LD samples (0.1% – 5% in ED and 10.9% – 24.4% in LD).

Among the sub-dominant populations, there were a number of other notable observations. At TP1 and 2, *Acinetobacter* and *Pseudomonas* were detected exclusively in the ED samples while *Brevibacterium* and *Corynebacterium* were detected only in the LD samples at TP1. *Clostridium* was identified at TP2 in both ED and LD samples and was consistently detected throughout the remainder of the study. In all instances, *Clostridium* was present at higher proportions in ED samples. *Staphylococcus*, a genus commonly associated with food spoilage, was detected in both ED and LD samples at TP2 only. Of the other sub-dominant populations detected, *Vibrio*, *Lactococcus* and *Psychrobacter* were present in both ED and LD samples recurrently, while *Pseudoalteromonas* was present in ED and LD samples up until TP4. A full list of both dominant and subdominant genera present is located in Table S2.

Distribution of microbial communities present in the core and rind of a brine salted continental-type cheese. Although the majority of genera detected in this study were localised in both the core and rind of the cheese sampled (Fig. 3 and 4), differences in proportions were noted. This is most obvious when examining populations corresponding to the genus *Lactobacillus* which were consistently higher in the core of the cheeses than in the rind throughout the ripening process. In contrast,

Streptococcus populations were consistently higher in the respective rinds than in the core. *Thermus* populations were also noticeably higher in the rinds than the core. This difference was particularly apparent in the LD samples (i.e. the samples in which *Thermus* levels were highest). Populations including *Lactococcus*, *Vibrio* and *Psychrobacter* were consistently detected in both the core and the rind throughout the ripening process. Similarly *Pseudomonas* and *Pseudoalteromonas* were identified in the core and rind at initial ripening stages but not at TP4. Of the other subdominant populations, *Clostridium*, present in TP2, 3 and 4, was only detected in the respective cheese cores. Similarly, *Ruminococcaceae Incertae Sedis, Bifidobacterium* and *Arthrobacter* were sporadically detected in core regions only. *Brevibacterium and Corynebacterium,* genera commonly associated with surface ripened cheeses, were located in the rind as were *Staphylococcus* and *Weisella*. A full list of both dominant and subdominant genera present is located in Table S3.

Discussion

In this study, NGS of 16S rRNA amplicons provided a detailed insight into the microbiota present in a brine salted continental-type cheese produced with thermophilic starter bacteria. As expected, bacterial diversity was found to decrease throughout the ripening process. Interestingly, bacterial diversity in late production day cheeses were determined to be greater than those produced early in the production day. Differences in microbial populations present in the respective cores and rinds were noted while several genera not usually associated with cheese produced from pasteurised milk were also detected.

Microbial diversity (α diversity) was greatest at TP1 (1d post production) in both early and late production day samples. While diversity may seem low in comparison to gut or soil communities (12, 49), it is comparable to that seen in studies of similar cheese types (27). Cheeses that were produced later during the initial manufacturing day ultimately had a more diverse microbial population than their early day equivalents. This trend persisted throughout ripening demonstrating, for the first time, that the time of day at which production occurs impacts on the microbiota present not only in the final product but throughout ripening. Greater diversity in terms of microbial populations present in LD cheeses may be due to accumulating microbial load during the manufacturing process or as a result of longer milk storage times. The significance of this phenomenon with respect to cheese quality will be the focus of further investigations.

Prior studies have described differences in the spatial distribution of microbial communities between the rind and core of several cheeses produced from both raw and pasteurised milk. Variation is likely due to the abiotic characteristics of the cheese including O_2, pH, salt, a_w, redox potential and temperature fluctuations (30, 50). In

26 this study greater initial diversity in the rind may be due to the high cook temperatures
27 associated with some continental-type cheeses. Dependent on block size, cheese cores
28 may hold higher temperatures longer than the rind, consequently reducing microbial
29 growth. Increased diversity in the rind, at TP1, may also be due to the presence of
30 halophiles (*Vibrio*, *Pseudoalteromonas*) associated with the salting process. Aerobic
31 and aerotolerant microbes, including *Streptococcus, Pseudoalteromonas*,
32 *Psychrobacter*, *Vibrio*, and *Brevibacterium*, were detected more often and at greater
33 percentage populations in the cheese rind than in the core. This is likely due to the
34 oxygen concentration present at/near the surface of the cheese in contrast to the more
35 anaerobic core (35). Prior studies have shown that Gram-positive LAB are more
36 likely to be distributed in the core than the rind of smear ripened and Swiss-type
37 cheeses (Comté, Morbier, Langres) (3). In agreement, we observed consistently
38 higher proportions of *Lactobacillus* in the core than the rind, throughout ripening
39 possibly due to their preference for a micro-anaerobic environment. In contrast,
40 *Streptococcus*, present in both the core and rinds throughout ripening, were found at
41 higher percentages in the rind. In samples from TPs 2 – 4, the cores of both ED and
42 LD cheeses had higher microbial diversity than the rinds. This difference was
43 particularly evident in the late production day samples. Reduced diversity in the rind
44 may be due to several factors including substrate competition, availability of O_2 as
45 well as pH/salt micro-gradients (50). Aerobic staphylococci were also identified in the
46 rinds of both early and late day samples at TP2 in agreement with Maher and Murphy,
47 who described rinds of smear ripened cheeses as providing conditions that are
48 complimentary for the survival of spoilage microbes (51).
49 Gram-negative bacteria were detected throughout this study, many of which would
50 not generally be associated with a commercial cheese produced from pasteurised

51 milk. *Thermus* was detected throughout ripening and at higher percentage populations
52 in the late day samples (10.9% at TP1 up to 24.4% at TP4). The presence of *Thermus*
53 was confirmed by subsequent PCR using *Thermus* specific primers (data not shown).
54 This aerobic, marine associated thermophilic and heterotrophic genus was originally
55 isolated from alkaline hot springs in Yellowstone National Park (52, 53). As *Thermus*
56 has previously been identified in two separate hot water systems, it is conceivable that
57 this bacterium was introduced *via* a water source (53, 54). No negative health effects
58 have been reported from with consumption of these cheeses but further studies will be
59 required to assess the effect of *Thermus* on cheese quality. Other Gram-negative
60 genera detected include *Pseudomonas*, *Pseudoalteromonas*, *Psychrobacter*, *Vibrio*
61 and *Flavobacterium*. *Vibrio* and *Pseudoalteromonas* are marine-associated, halophilic
62 genera and therefore may have gained access to the cheese *via* the brining process.
63 While it is not yet clear what the significance of the presence of these populations is,
64 particularly at the levels present in the cheese, they may play a role in ripening (38,
65 50, 55). Psychrotrophic bacteria including *Psychrobacter* and *Pseudomonas* have
66 previously been isolated from a variety of cheeses as well as raw milk and are
67 particularly adapted to low temperature milk storage conditions (50, 56, 57).
68 Many genera more commonly associated with artisanal and surface ripened cheeses
69 were detected. *Brevibacterium* and *Corynebacterium* were identified immediately
70 post-production and are associated with flavour and colour development in smear
71 ripened cheese (22, 58-60). *Arthrobacter, Weissella* and *Acinetobacter*, previously
72 isolated from a variety of artisanal cheeses, were also identified, although their impact
73 on cheese quality is unknown (61-68). The significance of the presence of gut
74 associated genera, including *Bifidobacterium* and *Ruminococcaceae Incertae Sedis*, is
75 also unclear.

76 *Clostridium* was consistently identified in all time points aside from TP1. The
77 percentages of clostridia present, with respect to early production day samples,
78 increased throughout ripening to 3.1% in TP4 ED cheeses. While the presence of
79 *Clostridium* is a particular issue due its association with late gas production in various
80 cheeses (5), in this instance no defects were noted at the time of sampling. Finally,
81 *Propionibacterium* populations were not detected despite their addition as adjuncts.
82 Further investigation of this revealed that *Propionibacterium* species are one of the
83 very few species that are not successfully amplified by the degenerate primers used in
84 this study.

85 In conclusion, the use of high throughput amplicon sequencing to profile the
86 microbiota present in a brine-salted, continental-type cheese has revealed distinct
87 differences in bacterial diversity, throughout ripening, between cheeses produced
88 early and late in the production day. As mentioned, the differences between ED and
89 LD cheeses may be due to increased microbial load and/or increased milk storage
90 time between production runs and therefore adapting these practices may allow for a
91 more microbiologically consistent product. Spatial variation due to environmental
92 factors present in the core and rind was also described in this study. Furthermore, the
93 presence of genera that would usually not be traditionally associated with this cheese
94 type (*Thermus*, *Bifidobacterium*, *Ruminococcaceae Incertae Sedis, Psychrobacter,*
95 *Pseudoalteromonas*) were described. The significance of the presence of these genera
96 requires further investigation.

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343 Figure Legends:

344 Figure 1: Principal Coordinate analysis of the β diversity (unweighted Unifrac) of
345 cheese samples. (A) Co-ordinates reflect early and late day samples and are colour
346 coded to reflect the ripening phase of the cheese (B) The same data is depicted but in
347 this instance core and rind samples are distinguished.

348

349 Figure 2: Relative abundance of bacteria at genus level for a Continental-type cheese
350 produced early and late (ED and LD) in the production day. Results depicted are mean
351 values of reads generated from individual core/rind samples from each respective
352 cheese block.

353

354 Figure 3: Relative abundance of bacteria at genus level for each TP according to
355 sample location (Core/Rind). Data presented are mean values of respective reads from
356 individual cheese samples.

357

358 Figure 4: Venn diagram depicting spatial differences in microbial composition at each
359 time point. Genera located in the intersecting region were detected in both the core
360 and the rind while those on the periphery were detected exclusively in the core/rind.

361

362 Table Legends

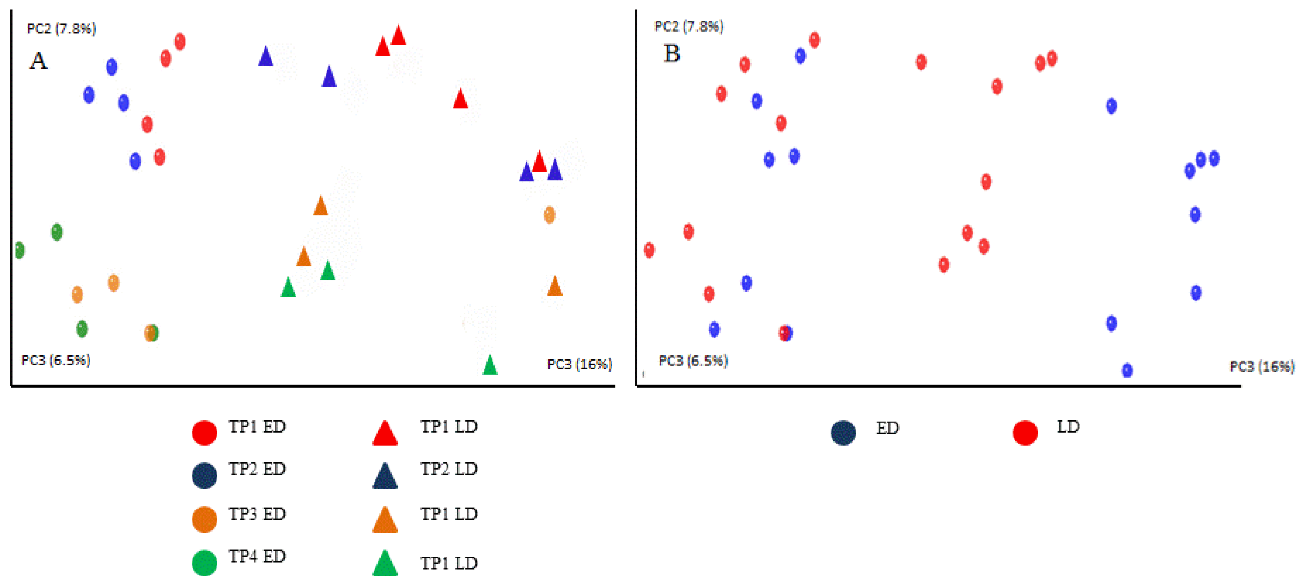
363 Table 1: Alpha diversity of continental-type cheeses segregated according to time of
364 production day (Early day [ED] and Late day [LD]) and spatial distribution
365 (Core/Rind)

366

367

368 List of Figures:

369 **Figure 1:**



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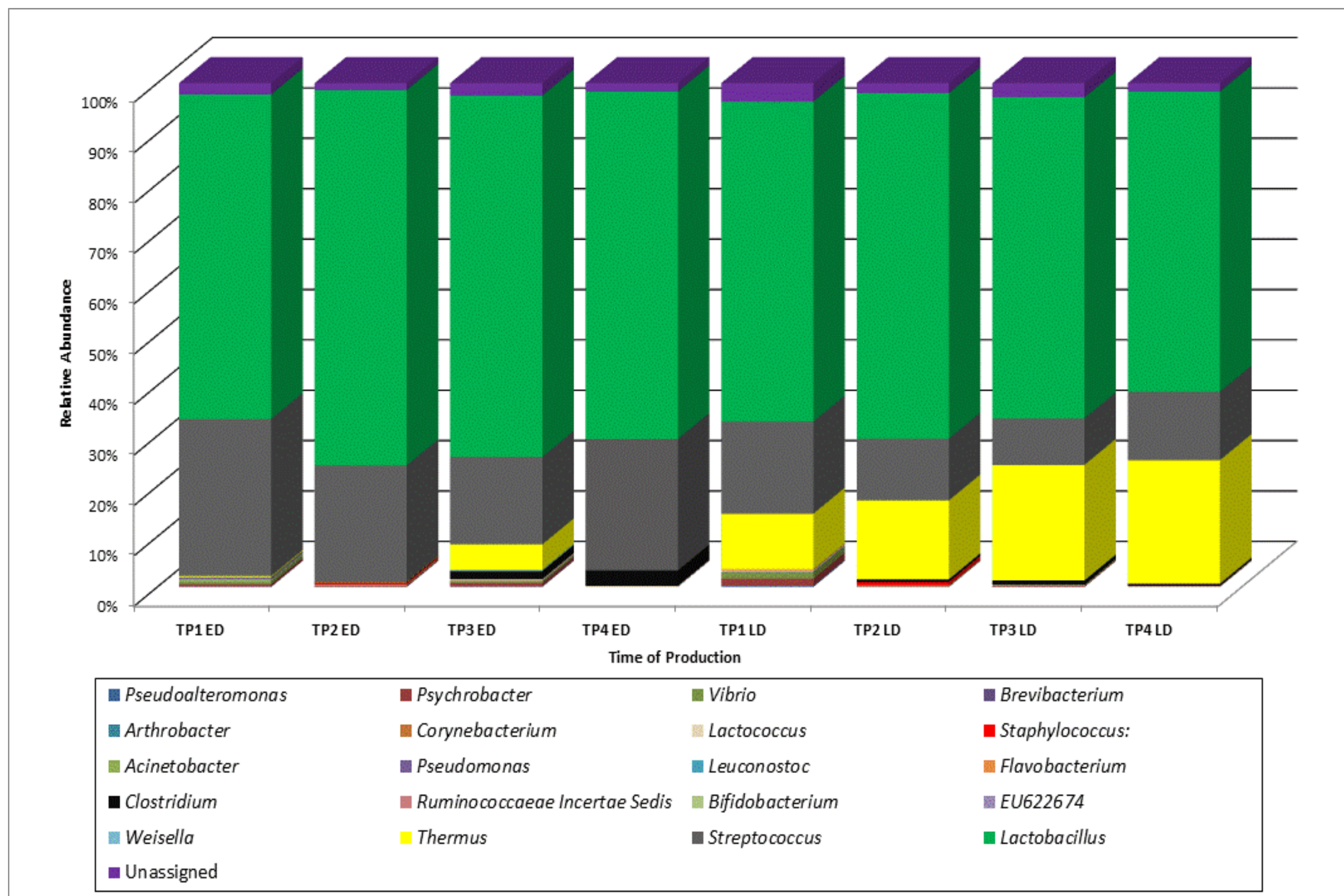
377 

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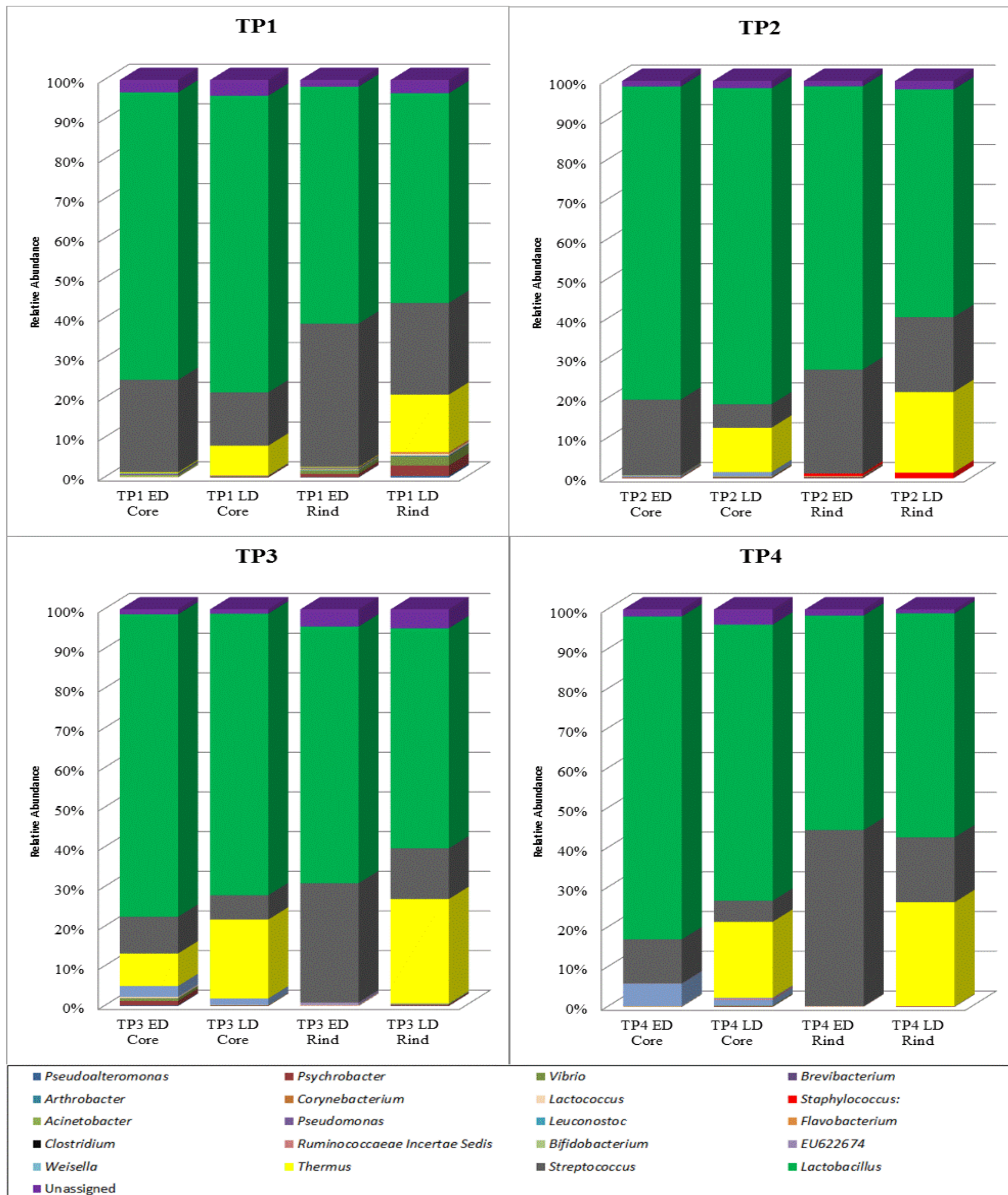

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388 individual cheese samples and were standardised to equi-molar concentrations prior to

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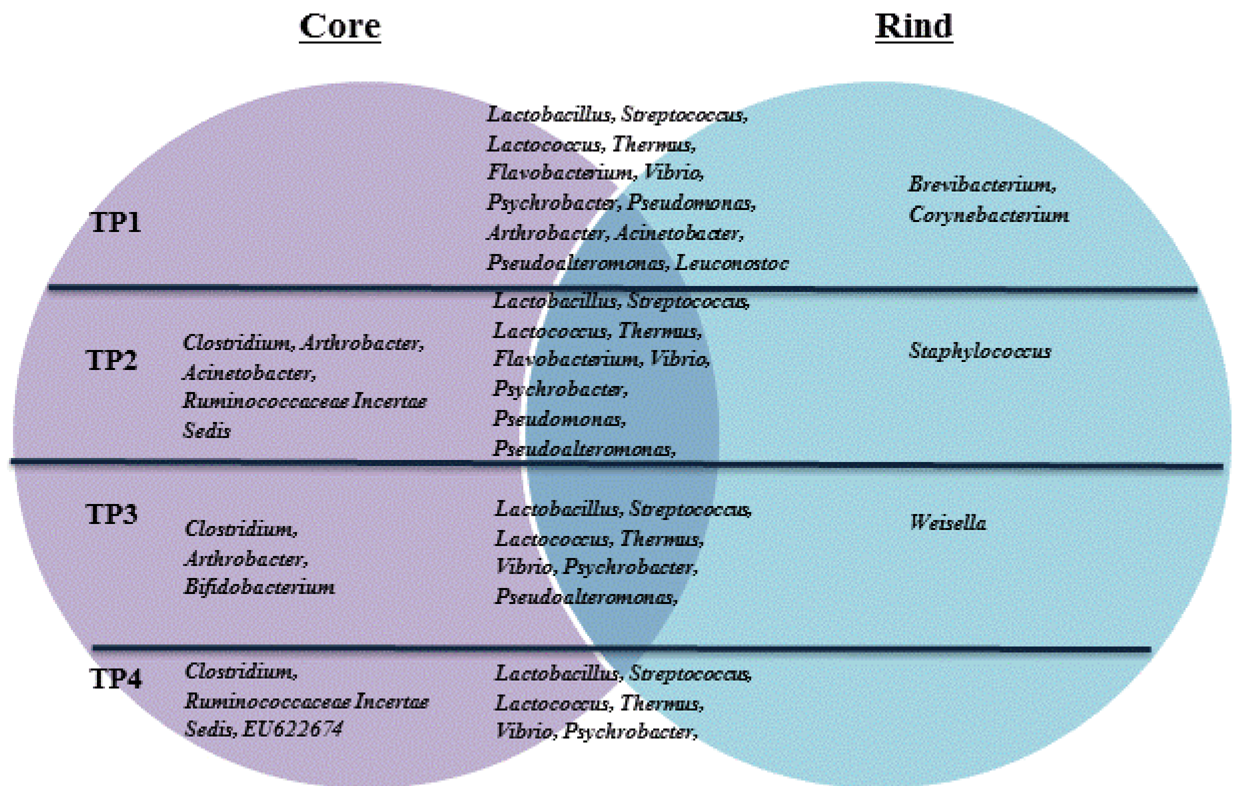

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393 **Figure 4:** Venn diagram depicting spatial differences in microbial composition at
394 each time point. Genera located in the intersecting region were detected in both the
395 core and the rind while those on the periphery were detected exclusively in the
396 core/rind

397 **Table 1:**

Early and Late Day Production	Chao1	Simpson	Shannon Index	Phylogenetic Diversity	Observed OTUs
Early Day Production					
TP1 ED	401.77	0.69	2.88	13.25	222.50
TP2 ED	328.80	0.65	2.62	11.13	198.25
TP3 ED	345.91	0.73	3.17	12.19	210.00
TP4 ED	304.11	0.66	2.63	9.72	165.25
Late Day Production					
TP1 LD	523.31	0.80	3.56	16.15	310.25
TP2 LD	478.63	0.75	3.29	14.58	292.75
TP3 LD	397.96	0.82	3.60	12.69	236.75
TP4 LD	357.94	0.78	3.34	12.46	215.33
Core and Rind					
Early Day Production					
TP1 Core	372.24	0.67	2.80	12.18	194.00
TP2 Core	294.59	0.62	2.51	11.37	182.00
TP3 Core	417.14	0.72	3.16	13.00	238.50
TP4 Core	370.37	0.61	2.56	11.65	183.50
TP1 Rind	431.30	0.70	2.96	14.32	251.00
TP2 Rind	363.00	0.67	2.72	10.89	214.50
TP3 Rind	274.69	0.75	3.18	11.37	181.50
TP4 Rind	237.84	0.71	2.70	7.78	147.00
Late Day Production					
TP1 Core	517.23	0.80	3.52	14.80	290.00
TP2 Core	471.02	0.75	3.32	14.86	295.00
TP3 Core	412.17	0.83	3.60	12.71	244.50
TP4 Core	405.02	0.83	3.82	15.40	241.00
TP1 Rind	529.38	0.79	3.60	17.51	330.50
TP2 Rind	486.25	0.76	3.26	14.29	290.50
TP3 Rind	383.75	0.81	3.60	12.67	229.00
TP4 Rind	334.40	0.76	3.10	10.99	202.50

398 **Table 1:** Alpha diversity of continental-type cheeses segregated according to time of

399 production day (Early day [ED] and Late day [LD]) and spatial distribution

400 (Core/Rind)

401