Elsevier Editorial System(tm) for Food Microbiology
Manuscript Draft

Manuscript Number:

Title: Understanding the bacterial communities of hard cheese with blowing defect

Article Type: Research Paper

Keywords: Grana Padano cheese; blowing defect; Clostridium; Lactobacillus; Illumina-based sequencing; lysozyme.

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Abstract: The environment of hard cheese encourages bacterial synergies and competitions along the ripening process, which might lead in defects such as clostridial blowing. In this study, Denaturing Gradient Gel Electrophoresis (DGGE), a quantitative Clostridium tyrobutyricum PCR and next-generation Illumina-based sequencing of 16S rRNA gene were applied to study 83 Grana Padano spoiled samples. The aim was to investigate the community of clostridia involved in spoilage, the ecological relationships with the other members of the cheese microbiota, and the effect of lysozyme. Three main genera were dominant in the analysed cheeses, Lactobacillus, Streptococcus and Clostridium, and allowed an assignment at the species level of 94.3% of 4,477,326 high quality sequences. C. tyrobutyricum and C. butyricum were the most prevalent clostridia. Hierarchical clustering based on the abundance of bacterial genera, revealed three main clusters: one characterized by the highest proportion of Clostridium, a second where Lactobacillus was predominant and the last, dominated by S. thermophilus. Ecological relationships among species were found: cheeses characterized by an high abundance of S. thermophilus and Lactobacillus rhamnosus were spoiled by C. tyrobutyricum while, when L. delbrueckii was the most abundant Lactobacillus, C. butyricum was the dominant spoiling species. Lysozyme also shaped the bacterial community, reducing C. tyrobutyricum in favour of C. butyricum. Moreover, this preservative increased the proportion of L. delbrueckii and obligate heterofermentative lactobacilli and lowered L. helveticus and non-starter species, such as L. rhamnosus and L. casei.

Cover Letter

PIACENZA-CREMONA

Istituto di Microbiologia

UNIVERSITÀ
CATTOLICA
del Sacro Cuore

16 March 2015

Dear prof. M.L. Tortorello,

please find attached the manuscript "Understanding the bacterial communities of hard cheese with blowing defect" by Daniela Bassi, Edoardo Puglisi and Pier Sandro Cocconcelli submitted for possible publication in Food Microbiology.
In this work, an Illumina-based technology for the analysis of 16S rRNA has been used for the first time to determine in 83 cheese samples with blowing defect the community of clostridia involved in spoilage and the ecological relationships with the other members of the cheese microbiota. This new methodology based on 16S rDNA amplification together with high-throughput sequencing technology (HTS) made possible to assign at the species level the 94.3% of the achieved sequences. Moreover, this technique, together with a genus-specific *Clostridium* Cluster I PCR-DGGE and a qPCR on *C. tyrobutyricum* allowed investigating the main agents of spoiling defect, the effect of lysozyme as preservative and the ecological relationships among species.
We believe that this work, due to high number of samples and the three different methodological approaches (qPCR, DGGE and NGS) improves actual knowledge on *Clostridium* species ecology in spoiled hard cheese and might help a better understanding of interactions among bacterial species during anomalous fermentation processes of cheeses.

Your sincerely

Prof. Pier Sandro Cocconcelli
Istituto di Microbiologia
Facoltà di Agraria - Università Cattolica del Sacro Cuore

Highlights

- A study of 83 Grana Padano cheese samples with blowing defect
- Community of clostridia involved in spoilage have been studied using DGGE and qPCR
- A NGS approach to analyse the ecological relationships among members of the cheese microbiota
- *C. tyrobutyricum* and *C. butyricum* were the most prevalent clostridia
- Lysozyme shaped the bacterial community

Understanding the bacterial communities of hard cheese with blowing defect

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Abstract

The environment of hard cheese encourages bacterial synergies and competitions along the ripening process, which might lead in defects such as clostridial blowing. In this study, Denaturing Gradient Gel Electrophoresis (DGGE), a quantitative *Clostridium tyrobutyricum* PCR and next-generation Illumina-based sequencing of 16S rRNA gene were applied to study 83 Grana Padano spoiled samples. The aim was to investigate the community of clostridia involved in spoilage, the ecological relationships with the other members of the cheese microbiota, and the effect of lysozyme. Three main genera were dominant in the analysed cheeses, *Lactobacillus, Streptococcus* and *Clostridium*, and allowed an assignment at the species level of 94.3% of 4,477,326 high quality sequences. *C. tyrobutyricum* and *C. butyricum* were the most prevalent clostridia. Hierarchical clustering based on the abundance of bacterial genera, revealed three main clusters: one characterized by the highest proportion of *Clostridium*, a second where *Lactobacillus* was predominant and the last, dominated by *S. thermophilus.* Ecological relationships among species were found: cheeses characterized by an high abundance of *S. thermophilus* and *Lactobacillus rhamnosu*s were spoiled by *C. tyrobutyricum* while, when *L. delbrueckii* was the most abundant *Lactobacillus, C. butyricum* was the dominant spoiling species. Lysozyme also shaped the bacterial community, reducing *C. tyrobutyricum* in favour of *C. butyricum*. Moreover, this preservative increased the proportion of *L. delbrueckii* and obligate heterofermentative lactobacilli and lowered *L. helveticus* and non-starter species, such as *L. rhamnosus* and *L. casei.*

Key words: Grana Padano cheese, blowing defect, *Clostridium*, *Lactobacillus*, Illumina-based sequencing, lysozyme.

1. Introduction

Long ripening times characteristic of hard cheeses production create the favourable conditions for microbial communities able to survive along the dairy process. This could result in beneficial effects or, alternatively, in spoilage defects that damage the final product. Late blowing is one of the most frequent problems still affecting hard cheese production in dairy factories. Spore-forming clostridia are considered the main agents of such damages (Coulon et al., 1991; Guericke, 1993; Ingham et al., 1998; Klijn et al., 1995; Vissers, 2007) thanks to their spores surviving attitude to heat treatment and their added capacity to germinate during cheese ripening. Different clostridial species have been associated to spoiling of hard cheeses, firstly *Clostridium tyrobutyricum* as the main agent (Bergère and Sivelä, 1990; Klijn et al., 1995; Le Bourhis et al., 2007b; Nishihara et al., 2014) followed by *Clostridium sporogenes, Clostridium butyricum, Clostridium beijerinckii,* and less frequently *Clostridium cochlearium, Clostridium perfringens, Clostridium septicum* (Le Bourhis et al., 2007b; Lycken and Borch, 2006; Reindl et al., 2014). All these microorganisms, alone or in association, have been related during time to the blowing problem, but few data are available about their dynamic changes in the cheese shape and their relationships all along the ripening period.

To reduce losses connected to clostridia spoilage, preservatives such as nitrate and lysozyme, which consistently modify the cheese environment in which microorganisms survive and multiply, are added to milk during hard cheese manufacture (Lodi and Stadhouders, 1990; Stadhouders, 1990). The use of nitrate in milk to prevent late blowing defect was also common in hard cheese-making, particularly in Emmental production (Devoyod, 1975; Korenekova et al., 2000), but its employ was banned after the European Food Safety Authority (EFSA) proposed to reduce levels of nitrosamines in food products (EFSA, 2010). In Italy, Grana Padano (GP) hard cheese is produced from raw cow's milk added with natural whey starter cultures and protected from clostridia spoilage by lysozyme addition. Therefore, the use of this preservative may be responsible of different

bacterial dynamics in cheese, depending also on dairy conditions and on microbial milk contamination.

The main bacterial populations associated to the cheese alteration process have been previously studied with the use of cultivation-dependent techniques and PCR-based approaches, but on a small number of samples (Cocolin et al., 2004; Garde et al., 2011; Le Bourhis et al., 2007b). The new methodologies based on 16S rDNA amplification together with high-throughput sequencing technology (HTS) have the advantage to determine, with a high resolution power, the bacterial communities present in food environments: published examples include cheese (De Filippis et al., 2014; Ercolini et al., 2012; Masoud et al., 2011; Masoud et al., 2012), seafood (Kiyohara et al., 2012; Koyanagi et al., 2011; Roh et al., 2010) and dry fermented sausages (Połka et al., 2015). The recent development in Illumina technology, coupled with multiplexing approaches, allows analysing up two 16S rRNA hypervariable regions in thousands sequences per sample, an approach that was shown to gain an almost complete coverage of the bacterial communities of fermented foods, with correct taxonomical assignment at the species level for more than 95% of the analysed reads (Połka et al., 2015). However, these approaches have not been yet applied for the study of microbial communities involved in late blowing spoilage of hard cheese.

The aim of the present work was to assess the microbial communities of spoiled hard cheese using new high-throughput sequencing technologies (HTS) associated to quantitative and qualitative cultivation-independent techniques. Respectively, a PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) approach to obtain a qualitative characterization of clostridia heterogeneity, a TaqMan qPCR on single *C. tyrobutyricum* species and a HTS approach based on Illumina MiSeq sequencing of the V3-V4 16S rRNA of total bacteria were applied to a total of 91 Grana Padano cheese samples from nine production facilities, with or without the addition of lysozyme and with blowing defects appearance at different ripening times. Information gathered from this study could be useful to assess the effect of lysozyme as a preservative and to measure the

effects of ripening time on the evolution of clostridia and their relationships with other bacterial species present in the cheese paste.

2. Materials and methods

2.1. Cheese sampling

Eighty-three samples of hard cheese, with anomalous pastry defects and cavities were collected from the Grana Padano cheese production area in Northern Italy. The samples had different ripening times corresponding to the appearance of defect and ranging from 1 to 23 months. In addition, eight hard cheeses without defect were added as negative controls. A total of 56 cheese samples were produced with the addition of lysozyme, while 35 samples were negative to the additive. Collected cheese portions were 50 cm large and 20-30 cm high and were cut directly from the entire cheese shapes; for each cheese, a representative sample was grounded and suddenly frozen at -40°C in our laboratory until processing.

For all analyses reported in the paper, samples were labelled according to this legend: the first two letters followed by a three numbers code indicate the production area, the following number indicates the ripening time in months and "+" or "-" refer to the use or not of lysozyme.

2.2.Bacterial strains and genomic DNA isolation

Reference type and isolated clostridial and non-clostridial strains used in this study are listed in table 1. For DNA extraction, 1 ml of culture was collected and centrifuged at 8,000g for 5 min. Genomic DNA was isolated from the pelleted cells using the Nucleospin Tissue DNA Isolation Kit (Macherey Nagel, Germany) and DNA was verified on a 1% agarose gel containing SYBR®YSafe (Invitrogen Corporation Life technologies).

2.3.DNA extraction from cheese samples

Total bacterial DNA was extracted from 50 g of grounded cheese for each sample; samples were homogenized in 125-µm filter stomacher bags (Biochek, Foster City, Calif.) with 50 ml of distilled water. The filtered homogenates were collected and centrifuged at 5,000 rpm for 10 minutes at 4°C to obtain pellets successively processed using the bead-technology based FastDNA® SPIN kit and the FastPrep® Instrument (Qbiogene, Inc., CA) according to the protocol previously described (Bassi et al., 2013a). DNA was then finally suspended in 100 µL of DES solution for further applications. All extracted nucleic acids were examined at a 0.8% electrophoresis agarose gel, and quantified using the picogreen method of the Quant-iTTM HS ds-DNA assay kit (Invitrogen, Paisley, UK) in combination with the QuBitTM fluorometer.

2.4.16S-based Clostridium *cluster-I specific PCR and DGGE analysis*

The PCR-DGGE strategy was based on a first amplification of a 235 bp fragment specific for the order of *Clostridiales* in the V3-V4 region of the 16S rRNA gene, using primers DGGECl2 f (5'-GCGGCGTGCCTAATACATGC-3') and P4 r (5'- ATCTACGCATTTCACCGCTAC-3'). Primers were designed after aligning more than 150 16S rDNA sequences among representatives of the genera *Clostridium, Ruminococcus, Lactobacillus, Bacillus, Streptococcus, Leuconostoc,* and *Enterococcus* that could frequently be present in dairy products in order to avoid cross-reactions. PCR amplifications were performed in a final volume of 25 µl, which included 12.5 µl of 2X MasterMix PCR (Promega), 0.4 µM primers, and 3 µl of genomic DNA. Template DNA was generally diluted 10-fold and 100-fold to minimize PCR inhibitors. Reactions were heated to 95°C for 5 min and cycled at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Cycles were repeated 35 times for all samples. Finally, 5 µl of each PCR product was used for visualization on a 2% agarose gel containing SYBR® Safe (Invitrogen Corporation Life technologies). Negative (without DNA) and positive (with DNA from reference strains) controls were included in each amplification run. 24 *Clostridium* strains, and 30 non-clostridial species (*Bacillus, Lactobacillus, Leuconostoc, Listeria, Staphylococcus, Enterococcus, Streptococcus, Ruminococcus*) were selected and used for

144 the sensitivity test of the PCR protocol (Table 1). A nested PCR on the first DNA template was then
145 performed using primers DGGE2Cl f clamped at 5' with a 33bp GC sequence (5'-
146 CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCC) and P4 r. The reaction was performed in
147 a 25 µl volume, with 12.5 µl of 2X MasterMix PCR (Promega), 0.1 µM of each primer and 1 µl of
148 DNA obtained in the previous PCR reaction. The amplification cycle was the same of the first PCR
149 reaction except for the annealing temperature of 63°C and the extension performed at 72 °C for 1
150 min. Denaturing gradient gel electrophoresis (DGGE) was performed using an INGENY phorU-2
151 (Ingeny International BV, Netherlands) DGGE system. A portion of each PCR template was loaded
152 on a 8% (w/v) acrylamide gel containing a 45-65% (w/v) denaturant gradient of urea and
153 formamide and electrophoresed at 90 V, 60°C for 16 h in 1X TAE buffer. Together with samples,
154 amplicons of the V3-V4 region of different *Clostridium* reference strains were loaded on the gel as
155 a ladder. Denaturing gradient gels were stained with 1X SYBR Green I (Roche, Milan, Italy) for 15
156 min and analysed under UV illumination. Selected DGGE bands were punched from the gel and
157 transferred in 50 µl of sterile water to let them diffuse overnight at 4°C. Only products migrating as
158 a single band, were PCR amplified with the original primer without GC-clamp, purified and sent to
159 a commercial sequencing facility (BMR Genomics, Padova, Italy) for sequencing. The sequences
160 were blasted in RDP database (http://rdp.cme.msu.edu) and in the GenBank using the NCBI
161 BLAST program (Altschul et al., 1997).
162 Banding patterns of DGGE profiles were analysed with Fingerprinting II software (Bio-Rad
163 Laboratories, Richmond, CA, USA) using the unweight-pair group method with averages
164 (UPGMA) for the generation of dendrograms.
165
166 ***2.5.Real-time PCR analysis for* C. tyrobutyricum *enumeration***
167 The optimised TaqMan qPCR protocol for *C. tyrobutyricum* enumeration previously described
168 (Bassi et al., 2013b) was applied to all cheese samples. Reactions were performed with a
169 LightCycler® 480 Real-Time PCR System (Roche Diagnostics Corporation) using a LightCycler®

480 Probes Master kit. The analysis of variance using Tukey method and 95% confidence was applied with R software v 3.1.1 in order to demonstrate significant differences respectively between the effect of lysozyme and the ripening period with *C. tyrobutyricum* counts obtained in real-time PCR. Data were considered significant with a *P*-value < 0.05.

2.6.Illumina 16S rRNA next generation sequencing

A high-throughput sequencing approach was applied on 40 samples selected among the total 91 in order to have 20 lysozyme negative samples and 20 lysozyme positive with different ripening time and clostridia composition. Two control samples without late blowing defect were also included. The bacterial V3-V4 16S rRNA region was amplified with the primer pairs 343F (5'-TACGGRAGGCAGCAG-3') and 802R (5'-TACNVGGGTWTCTAATCC-3') using the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific, Inc. Waltham, MA, USA). PCR reactions were performed in 25 μL using 12.5 μL of Phusion Master Mix, 0.5 μL of each primer, 0.1 ng of template DNA and nucleases-free PCR grade water. In order to allow a simultaneous analysis of several samples in the same sequencing run, each sample was tagged by adding to the forward primer a nine nucleic acids extension at its 5' end, where the first seven bases served as sample index for multiplexing and the next two as linker bases designed in order to not match any bacterial sequence entry at these position in the Ribosomal Database Project (RDP). This approach was adopted in order to minimize potential biases possibly introduced by the indexed primer extension. These biases were further addressed by adopting a two-step PCR program as implemented in Berry et al. (Berry et al., 2011), with a first step in which untagged primers amplify the template DNA for 23 cycles, and a second step where 1 μL of product of the first PCR is used as a template for 10 final cycles using the barcoded primers. For both steps the PCR conditions used were: initial 4 min at 94°C, cycles made up of 30'' of denaturation at 94 °C, 30'' of primers annealing at 50 °C and 30'' of primers elongation at 72 °C, followed by a final elongation step of 10 min at 72 °C. The PCR products of the 2nd step for all samples were multiplexed in a single

196 pool in equimolar amounts on the basis of the QuBit quantification data. The PCR products pool
197 was then purified using the solid phase reverse immobilization (SPRI) method of the Agencourt®
198 AMPure® XP kit (Beckman Coulter, Italy, Milano) and sequenced at Fasteris SA (Geneva,
199 Switzerland). The TruSeq™rDNA sample preparation kit (Illumina Inc., San Diego, CA) was
200 applied for the amplicon library preparation, while the sequencing reaction was performed with a
201 MiSeq Illumina instrument (Illumina Inc, San Diego, CA) with V3 chemistry, generating 300 bp
202 paired-end reads.
203
204 ***2.7.Sequences data preparation and analyses***
205 The amplicons pool generated as described in the previous section was sequenced by Fasteris SA
206 (Geneva, Switzerland) using the MiSeq Control Soft. V2.3.0.3, the RTA v1.18.42.0 and the
207 CASAVA v1.8.2 for base calling and Illumina barcode demultiplexing. Since most of the V3-V4
208 amplicons regions are shorter than 500 bp (Vasileiadis et al., 2012) and the sequencing was
209 performed with 300bp x2 paired-reads, the "PANDAseq" script with the restrictions of at least 30
210 bp of overlap between read pairs and two mismatches allowed was applied in order to reconstruct
211 the full amplicons for the majority of sequences. Assembled sequences were then demultiplexed
212 according to the sample indexes and primers using the fastx-toolkit
213 (http://hannonlab.cshl.edu/fastx_toolkit/).
214 Sequences were then analysed with Mothur v.1.33.0 (Schloss et al., 2009) in order to
215 remove: (i) sequences with large homopolymers (>10), (ii) sequences that aligned outside the
216 targeted V3-V4 region, (iii) chimeric amplicons and (iv) sequences that the Bayesian classifier
217 identified as not belonging to Bacteria by using the ribosomal database project (RDP) training set
218 with a 80% bootstrap cutoff.
219 Downstream sequence analyses were then performed using both the operational taxonomic
220 unit (OTU) and the taxonomy based approach. The OTU and the taxonomy matrices were obtained
221 using Mothur v.1.33.0 (Schloss et al., 2009), while all the other statistical analyses were performed

in R v 3.1.1. In the OTU approach, sequences were fist aligned against the SILVA database bacterial reference alignment (Pruesse et al., 2007) using the NAST algorithm and a kmer search approach (Schloss, 2010). The resulting aligned sequences were then hierarchically clustered into 3 % distance defined OTUs using the average linkage algorithm. If not stated differently, OTUs having a sum of their overall abundance across all samples of less than 0.1% were grouped together in a one "rare" OTU group. For the taxonomy based analyses, sequenced were hierarchically classified in different taxa using the Greengenes database. A manual amendment of the database was carried out by retrieving on RDP and adding to the database all species level sequences available of type strains (1700 taxonomically annotated sequences in total) belonging to genera that were frequently found in the samples after a first taxonomical analysis of sequences.

The composition of PCR products in OTUs and taxonomical groups were used for estimating the associated α- and β- diversity of the analysed samples for the two previously mentioned approaches. Calculated α-diversity indices included the inverse Simpson's index (D) (Heip et al., 2001) and the observed richness (S), while the Good's coverage estimate was calculated for assessing the percentage of captured diversity by the devoted sequencing effort (Good, 1953).

2.8.Accession number and data availability.

Raw sequences in read-pairs format were submitted to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA - Bioproject PRJNA277133).

3. Results

3.1. Development of a genus specific PCR for DGGE analysis of Clostridium *cluster I species*

A genus specific PCR for the amplification of variable region of 16S rRNA gene was developed in order to analyse the community of clostridia in spoiled cheeses and to identify the species involved in late blowing defect. The choice of the *Clostridium* specific primers was based on the analysis of the 16S rRNA gene sequence of the order *Clostridiales* and other genera included in the Ribosomal Database Project (RDP-II) (Cole et al., 2005). This analysis revealed that members of the *Clostridium* cluster I, differently from related taxonomical units of *Firmicutes*, presented a deletion of about 17bp (positions 583-600) + 15 bp (positions 604-618) in the V3 region of *16S rRNA* gene. This allowed to design DGGECl2 (position 480-500 bp) and P4 (position 694-715 bp) oligonucleotides targeted to the sequence upstream and downstream the deletion. *In silico* analysis indicated that the DGGECl2-P4 primer couple, is able to amplify almost uniquely 16S rRNA gene from *Clostridium* species. To confirm the specificity of the method, 24 strains belonging to the *Clostridium* cluster I and other 30 strains belonging to *Firmicutes* were tested. The PCR protocol allowed amplification of DNA from clostridial strains but not from strains of other genera. Upon DNA amplification with DGGECl2 and P4 primers, each *Clostridium* reference strains used produced a single DGGE band and these bands constituted a normalization ladder.

3.2.Detection of clostridial populations in cheese samples by PCR-DGGE analysis

Putative *Clostridium* cluster I-specific amplicons have been obtained for 78 out of 91 (86%) cheese samples. Single band migration patterns were observed for the clostridia type strains used as a marker, thus allowing the identification in cheese samples of *C. butyricum*, *C. sporogenes*, *C. tyrobutyricum*, *C. perfringens* and *C. septicum* as the most commonly isolated species. Differently, only one of the eight cheese samples without defects and positive to lysozyme used as negative control, showed a band related to *C. butyricum* in the DGGE analysis. The most indicative bands for each sample were excised from the acrylamide gel and sequenced. These showed a high

homology (>99%) with 16S rRNA genes from *Clostridium* species present in databases. Clostridia distribution in cheese samples with blowing defect was mainly affected by the presence or absence of lysozyme in cheeses (Table 2). Rare correlations have been found with the ripening times that varied from 1 to 28 months from the appearance of defect. In general, *C. tyrobutyricum* was the species most frequently found in the total samples, followed by *C. butyricum, C. sporogenes, C. perfringens* and *C. septicum.* In the 56 samples (50 spoiled cheeses and 6 negative controls) where lysozyme was added to milk, we found most frequently *C. butyricum* (50% of the samples) followed by *C. tyrobutyricum* (30%), *C. perfringens* (23%), *C. sporogenes* (21%) and *C. septicum* (5%). On the contrary, in the 35 cheeses (33 spoiled cheeses and 2 negative controls) made without lysozyme the higher clostridial contamination was represented by *C. tyrobutyricum* (77%) that was found to be the dominant species in the majority of samples and, in most cases, it was the only detected species. In addition, *C. butyricum* contamination was observed in 8 samples (22%), *C. sporogenes* in 6 samples (17%), *C. perfringens* in 3 samples (8%) and *C. septicum* in only 2 samples (5%). In all analysed samples, *C. sporogenes* was never found alone but it appeared in constant association with *C. butyricum* or *C. tyrobutyricum*. Interestingly, mutual exclusive dominance of *C. butyricum* or *C. tyrobutyricum* was observed in all but four of the samples, independently from the ripening time.

3.3.Quantitative analysis of C. tyrobutyricum

Since from PCR-DGGE analysis, *C. tyrobutyricum* appeared to be the dominant species in most of the samples, real-time quantitative PCR was applied for its enumeration in cheeses with late blowing defect (Bassi et al., 2013b). This method was applied on the 91 cheese samples and, 60% of them showed positivity to *C. tyrobutyricum*; in cheese samples manufactured with lysozyme, 45% were positive to *C.* tyrobutyricum with counts ranging from 2.0 to 9.2 log CFU/50 g. A higher prevalence of this species was observed in cheese without lysozyme (83% of samples) where only 6 samples were negative and counts varying from 2.0 to 8.9 log CFU/50 g (table 2). These

observations were confirmed by the statistical analysis of variance that revealed a significant effect of lysozyme (P<0.01) on the *C. tyrobutyricum* prevalence. No significant effect of ripening time on *C. tyrobutyricum* counts was observed.

3.4.NGS assessment of bacterial communities in Grana Padano cheese

To obtain a deeper view of the microbiota of spoiled cheese, 20 samples with lysozyme and 20 without lysozyme (19 spoiled and one control cheeses in both cases), were randomly selected, the DNA extracted and the V3-V4 region of 16S rRNA genes amplified using universal primers for *Bacteria*. The sequencing of amplicons pooled in equimolar amounts from the analysed samples resulted in 7,539,211 paired-end sequence reads, reduced to 5,045,205 after assembly and demultiplexing on the basis of sample indexes, with an average of 126,130 sequences per sample. The 4.9% of these sequences was discarded because of quality issues (length, presence of homopolymers or ambiguous bases), 0.6% did not align on the V3-V4 targeted region and 1.8% were identified as chimeras. After these screening steps, 4,477,326 sequences were retained and used for further analyses. The taxonomical identification was carried out by comparing the obtained sequences with those included in the GreenGenes database, containing 202,421 aligned reference sequences. The database was manually curated and amended, in order to increase the representation of reference sequences belonging to the genera mostly represented in the cheese samples after a first taxonomical assignment. A total of 1700 type strain 16S full-length sequences were thus downloaded from RDP and added to the database. Alignment to this improved database showed that among 4,477,326 high quality filtered sequences, 97.2% were correctly classified at the genus level and 94.3% at the species level. The number of sequences per samples was downscaled to 32,566 sequences per sample, in order to avoid biases related to α-diversity and β-diversity estimations when analysing and comparing samples having unequal size (Gihring et al., 2012; Lundin et al., 2012). A number of richness and diversity indexes were calculated for all samples from OUT data, and results are reported in Supplementary table S1. No significant differences according to ripening

time, lysozyme or defect were found for all analysed indexes (data not shown). The Good's coverage showed an average value per sample of 98.2% (standard deviation 0.2%), thus indicating that the even with the reduction of sequences to downscale all samples at 32,566 sequences, most of the bacterial diversity in the cheese samples was still covered. The observed richness (i.e., the total number of OTUs) ranged between 720 and 907 OTUs, with 25 OTUs covering the 99.9% of the total bacterial communities. Chao richness index had an average value of 2901, with 291 of standard deviation, while the evenness, as estimated by Shannon index was 1.78 ± 0.3.

The eight most abundant bacterial genera found in the analysed cheese samples with and without lysozyme, which account for 95% of the total bacterial populations, are reported in Figure 1, together with a hierarchical clustering of samples according to the average linkage algorithm. Eight bacterial genera in total, namely *Lactobacillus* (65.3%), *Streptococcus* (14.4%), *Clostridium* (9.54%), *Brevibacterium* (1.5%), *Enterococcus* (0.97%), *Staphylococcus* (0.96%), *Acinetobacter* (0.77%) and *Chryseobacterium* (0.5%), were present at 5% or higher percentages at least in one of the analysed samples. Consistently with cultivation based studies in cheese (Monfredini et al., 2012; Pogačić et al., 2013), most of the sequence of phylum *Firmicutes* belonged to the *Lactobacillus*, which covered in 19 out of 40 samples more than 90% of the total diversity. Sequences assigned to *Clostridium* were detected in all samples, although in different amounts. Specifically this genus covered almost 40% of the detected bacterial diversity in the samples VI616_3-, TN303_6-, BS634_1-, PC544_3+, PC544_7+. The two control samples without late blowing defect (TN303_7- and MN425_6+) contains DNA from clostridia, although in low amount. In general, samples clustering, as depicted in Figure 1, was not affected by either cheese factory, lysozyme use, ripening time or blowing defect. Only the sample CN911_11- presented a complete different bacterial community, as it was dominated by two species *Brevibacterium aureum* and *Staphylococcus equorum*. Hierarchical clustering based on the abundance of bacterial genera, revealed three main clusters (fig. 1). Cluster A, composed by 13 samples, was characterized by the highest proportion of sequences assigned to the *Clostridium* genus and a relevant presence of *Streptococcus* sequences,

ascribed to *S. thermophilus*. The genus *Lactobacillus* was predominant in the 20 samples of cluster B, a cluster that included also the two negative controls TN303_7- and MN425_6+. This cluster was also characterized by a low number of clostridial sequences. In Cluster C, composed by three samples, more than 50% of the sequences were assigned to *S. thermophilus*. Interestingly, comparing the data achieved by quantitative detection of *C. tyrobutyricum* with the results of this cluster analysis, a correlation between the abundance of *S. thermophilus* and the *C. tyrobutyricum* counts was observed. In the samples characterized by a relevant presence of *S. thermophilus* (cluster A and C), the average value of *C. tyrobutyricum* achieved by real time PCR in cluster C was 6.5 log cells/g, while in cluster A the mean count was 3.4 log cells/g.

A detailed analysis on relative abundances of species was then carried out on the two most relevant genera, *Lactobacillus* (Figure 2) and *Clostridium* (Figure 3), being the first the responsible for primary fermentation and ripening process, and the second involved in the insurgences of defects in hard cheese. The diversity of the *Lactobacillus* population in the analysed samples was captured by data achieved with NGS: DNA from *L. helveticus* and *L. delbrueckii*, the dominant species in the primary fermentation of Grana Padano cheese (Neviani et al., 2009; Zago et al., 2011), which reached the highest levels in the first days or ripening and then rapidly declined, were detected in all samples. Different species of non-starter lactic acid bacteria (NSLAB), mainly obligate and facultative heterofermentative species, such as *L. rhamnosus, L. casei, L. fermentum*, and the obligate heterofermentative *L. buchneri*, were also detected in all analysed samples. Hierarchical clustering of samples according to the average linkage algorithm identified three main clusters on the basis of the *Lactobacillus* species relative abundance (Fig. 2): in cluster Lb-A, *L. delbrueckii* was the predominant species, cluster Lb-B was characterized by a relevance of *L. rhamnosus*, while *L. casei* was highly represented in cluster Lb-C.

Metastats models were employed to assess if the use of lysozyme had significant effects on the relative proportions of *Lactobacillus* (Figure 4a) and *Clostridium* species (Figure 4b). Cheese samples with lysozyme presented significantly higher proportion of *L. delbrueckii, L. parabuchneri*

and *L. parafarraginis* and significantly lower levels of *L. buchneri,* and *L. paracasei*, when compared with samples without the additive. Moreover, lower numbers of *L. helveticus* and *L. rhamnosus* were found in cheese with the lytic enzyme.

Seven species of clostridia, namely *C. sporogenes, C. butyricum, C. disporicum, C. perfringens, C. difficile, C. sordelii* and *C. tyrobutyricum* were found in the defected cheeses and, among them, the most abundant were *C. butyricum* and *C. tyrobutyricum*. When hierarchical cluster analysis (Fig. 3) was applied to the clostridial community, three clusters were defined manly of the ratio between the two species *C. butyricum* and *C. tyrobutyricum*. Thus, *C. butyricum* was highly predominant *in* cluster Cl-A, cluster Cl-B contained samples characterized by similar amounts of sequences assigned to *C. butyricum* and *C. tyrobutyricum,* while cluster in Cl-C, more than 80% of the analysed sequences were identified as *C. tyrobutyricum.* Other clostridial species identified in the analysed cheese samples were detected in lower percentages. Consistently with the data achieved using the DGGE analysis, the presence of lysozyme in the cheese paste significantly influenced the relative presence of *C. butyricum* and *C. tyrobutyricum,* as shown by the Metastats analysis reported in fig 4b. In the absence of this lytic enzyme, *C. tyrobutyricum* dominated the clostridial community, accounting for more than 50% of the DNA sequences assigned to this genus. When lysozyme was added as preservative, a shift in the dominance was observed and *C. butyricum* was the most present species.

4. Discussion

A crucial step for the reduction of cheese blowing defects caused by clostridia is to investigate the community of these anaerobic sporeformers cause of spoilage and their ecological relationships with the other members of the cheese microbiota (Doyle et al., 2015). Many factors may affect together or independently the ripening process of Grana Padano cheese, such as the dairy technology, the use of autochthonous starters and additives, the environmental parameters (Bittante et al., 2011; Neviani and Gatti, 2013); moreover, in the case of Grana Padano cheese, its size

(approximately 40 kg) and long ripening times (up to 24 months) make difficult the understanding of bacterial dynamics, including those of clostridia, in a so complex food matrix. In general the quality of cheese is mostly dependent on the LABs fermentations, that influence its sensorial and aromatic features (Steele et al., 2013); therefore, the understanding of interactions, still lacking, between these last and clostridia might be of help to better clarify the blowing defect.

In order to provide an updated insight of the major clostridial species and the other microbial populations affecting the cheese environment, 83 GP cheese samples removed from the ripening process, due to the presence of the typical signs, eyes, fractures and blowing, were studied using DNA-based approaches. Moreover, eight cheeses without defects were considered in the study as negative controls. The majority of cheeses were produced using egg-white lysozyme as food additive; this muramidase is intentionally added to milk to inhibit clostridia in the outgrowth step after germination, with the aim of preventing the late blowing defects of hard cheese (Wasserfall and Teuber, 1979). The culture-independent analysis was carried out using three different approaches, a *Clostridium* cluster-I specific DGGE, a quantitative qPCR enumeration of *C. tyrobutyricum* and a next-generation Illumina-based sequencing on 16S rRNA gene, which provided firstly a qualitative and then a quantitative picture of the entire bacterial community of spoiled cheese.

The number of OTUs determined in the cheese samples was high, with an average of 804 (Table S1). Other studies relying on NGS analyses for cheese revealed maximum OTU values of 192 for Mozzarella (Ercolini et al., 2012), of 73 OTUs for Parmigiano Reggiano and 64 for Grana Padano (De Filippis et al., 2014). This difference in outcome may be explained by the fact that our study was based on Illumina sequencing technology, while the ones here cited on 454 pyrosequencing technology, which has a lower throughput. The high coverage values that we obtained, together with a an assignment at the species level of 94.3% of all analysed sequences, prove that Illumina NGS of 16S amplicons is a reliable approach for a thorough analyses of cheese bacterial communities, as already demonstrated for fermented meats (Połka et al., 2015) and

426 beverages (Bokulich et al., 2012). It must also be stressed out that, among all the OTUs retrieved,
427 just a fraction of maximum 26 OTUs covers the 99.9% of the total bacterial community. This result
428 points to a major microbiome constituted by a few selected groups (Figure 1), and a complex rare
429 microbiome whose possible role in ripening and final cheese characteristics still needs to be
430 addressed. The diversity and richness indexes calculated for our samples where instead in
431 agreement with published reports (De Filippis et al., 2014; Ercolini et al., 2012).

432 The advantage of the PCR-DGGE approach selectively amplifying *Clostridiales* members
433 was the possibility to analyse almost exclusively species belonging to the cluster I of the
434 *Clostridium* genus, reducing the interference of other bacteria, such as lactic acid bacteria, which
435 dominate the microbial communities of dairy products. *Clostridium* cluster-I is a wide taxonomic
436 group which contains all the clostridial species identified as agents of cheese spoilage, such as *C.*
437 *beijerinckii, C. butyricum, C. sporogenes, C. tyrobutyricum* (Cocolin et al., 2004; Ingham et al.,
438 1998; Le Bourhis et al., 2007b). Moreover, this approach reduced the need of additional steps, such
439 as pasteurization and selective medium enrichment, previously applied to the study of late blowing
440 in hard cheese (Cocolin et al., 2004), which may introduce biases in the analysis of the clostridial
441 communities. Results obtained with *Clostridium* cluster I-specific PCR-DGGE analysis provided a
442 qualitative picture of the dominant clostridia biodiversity and suggested that clostridia, in case of
443 blowing defect, are nearly ubiquitous members of the GP cheese ecosystem and are strictly related
444 to the spoilage event. In general, outputs of HTS data regarding clostridial communities were in
445 accordance with those obtained by PCR-DGGE analysis even targeting different 16S hyper-variable
446 regions and with a major resolution and quantitative power for the NGS approach. The major
447 performance of NGS as compared to PCR-DGGE was recently proven for Italian dry-fermented
448 sausages (Połka et al., 2015) and for Oscypek, a traditional Polish smoked cheese (Alegría et al.,
449 2012). Analyses of NGS data defined three main clusters, the first (Cl-A) characterized by the
450 dominant presence of *C. butyricum*, the second (Cl-B) by a co-presence of *C. butyricum* and *C.*
451 *tyrobutyricum* and the third (Cl-C) by a higher prevalence of *C. tyrobutyricum* (Fig. 3). These data

indicated that late blowing defect in this hard cheese was primarily caused by this two butyric spore formers. Moreover, although the two analytical methods were not totally comparable, the quantitative data obtained from qPCR on *C. tyrobutyricum* were in accordance with the results of NGS analysis. Thus, samples belonging to the dominant *C. tyrobutyricum* cluster (fig. 3, Cl-C) presented an average value of 7.45 Log target genome/50g, higher than the other two clusters, corresponding to 4.40 Log target genome/50g and 2.86 Log target genome/50g for Cl-A and Cl-B respectively.

Clostridium cluster I-specific PCR-DGGE data demonstrated that *C. tyrobutyricum*, previously described as the main responsible of hard cheese spoilage (Klijn et al., 1995; Le Bourhis et al., 2005), was the most frequent species, hosted in the 50% of total samples. According to the literature, *C. tyrobutyricum* is commonly associated with late blowing defects appearing in cheese after 6-8 months of ripening (Klijn et al., 1995; Le Bourhis et al., 2005), while *C. butyricum* is most frequently involved in early blowing (Bottazzi, 2001). However, in our samples we found no significant correlations between the ripening time and the prevalence of a particular *Clostridium* species. It has been demonstrated (Le Bourhis et al., 2007a) that the presence of other clostridia, such as *C. sporogenes* and *C. beijerinckii*, synergistically stimulate the germination and growth of *C. tyrobutyricum* in the early stages of Emmental type cheese. Differently, our analysis of Grana Padano cheese samples showed that that the simultaneous presence of *C. sporogenes* and *C. tyrobutyricum* did not induce an early insurgence of the defect.

Lysozyme, rather, seemed to influence bacterial distribution both in terms of *Clostridium* and *Lactobacillus*, the two most abundant genera, together with *Streptococcus*, found in the analysed cheeses. Among clostridia, *C. tyrobutyricum* was negatively affected by lysozyme, as demonstrated by DGGE, NGS data and by quantitative results obtained with species-specific TaqMan real-time method (Bassi et al., 2013b). In particular, the quantitative data achieved by real-time PCR, which indicated a significant higher number of *C. tyrobutyricum* in absence than in presence of lysozyme (4.6 Log target genome/50g, vs 2.5 Log target genome/50g) (Table 2), were confirmed by the

Metastats modelling of NGS data at genus level (Figure 4b). Recent experiments in milk and RCM medium proved that lysozyme was particularly effective in limiting *C. tyrobutyricum* cells and spores growth respect to other clostridia (Ávila et al., 2014). Otherwise, *C. butyricum* resulted the most prevalent species when lysozyme was added to milk, as shown by DGGE data (Table 2) and confirmed by the relative abundance analysis of 16S rRNA gene sequence (Figure 3). This observation confirmed the *in vitro* data (Ávila et al., 2014) of high resistance of *C. butyricum* to this additive. *C. butyricum* has been rarely associated to late blowing defects in hard cheese using cultivation based approaches and non-quantitative PCR methods: Kljin et al. (1995) did not detect this species in samples of Gouda cheese by PCR, while Cocolin et al. (2004) found this species in only 2 out of 17 samples by DGGE. Our data, based on both genus specific DGGE and NGS approaches revealed that *C. butyricum* is a common component of the clostridial population involved in late blowing of hard cheese. *C. sporogenes,* which was detected in approximately 20% of the analysed samples, always associated to *C. butyricum* or *C. tyrobutyricum* and present in low amounts, seemed to be independent on lysozyme addition in both DGGE and NGS analyses. In the same way, Garde et al. (2011) demonstrated that in spoiled Manchego cheese samples with lysozyme *C. sporogenes* was the dominant species. This observation reflects a diversity in the clostridia community of different hard cheeses. Among the other clostridia species detected in lower amounts, *C. perfringens* too was more prevalent in cheese samples with lysozyme. The presence of this species does not rise safety concern; indeed, food-borne disease caused by this pathogenic bacterium is related to the ingestion of 10^8 vegetative cells of the enterotoxin producing strains and only a limited fraction of strains produce enterotoxins. Moreover, no cases of outbreaks have been described from cheese and this organism is widely present in a variety of foodstuffs (EFSA, 2005). Shifts in the *Lactobacillus* community were also observed in the presence of lysozyme for both bacterial species of the primary lactose fermentation and NSLABs. An increase in the proportion of *L. delbrueckii* and obligate heterofermentative NSLABs was detected in the

503 presence of added muramidase, while in its absence *L. helveticus* and other NSLABs, such as *L.*
504 *rhamnosus*, *L. casei* and *L. buchneri*, were more abundant (Figure 4a).

505 When the quantitative NGS data of lactic acid bacteria, *Lactobacillus* and *Streptococcus*, and
506 of clostridia were analysed by hierarchical clustering, several ecological relationships were found.
507 Most of the samples from cluster Cl-C, characterized by a high abundance of *C. tyrobutyricum*,
508 showed also the dominance of *S. thermophilus* and *L. rhamnosu*s. These data were confirmed by the
509 qPCR analysis of *C. tyrobutyricum* that demonstrated higher amounts of this species in all samples
510 of this cluster. A possible relationship between *S. thermophilus* and *C. tyrobutyricum* could be
511 explained by the effect on germination produced by L(+) lactate, the stereoisomer of lactate
512 produced in cheese. This end product of lactose metabolism by *S. thermophilus* alone or in
513 association with amino acids, such as alanine, is the most effective germinant for *C. tyrobutyricum*
514 (Bassi et al., 2009) and other clostridia such as *C. botulinum* (Peck et al., 2011). *L. rhamnosus*, was
515 found as dominant species in samples of cluster Cl-C, in accordance to other study that showed in
516 GP an increase in number over ripening up to the sixth month (Pogačić et al., 2013). Thus, this L(+)
517 lactate producer might further stimulate the germination of *C. tyrobutyricum.* Differently from other
518 clostridia detected in cheese, *C. tyrobutyricum* is able to use lactate as energy source. This species
519 metabolises both D(-) and L(+) racemic forms of lactic acid, although D(-) lactate acid is more
520 rapidly dissimilated to butyric acid when cells grow in RCM medium (Huchet et al., 1997). These
521 authors suggested that the use of *Lactobacillus* producing L(+) in cheese fermentation may reduce
522 the growth rate of *C. tyrobutyricum* and the spoilage of Emmentaler cheese. Differently our data
523 indicate that *C. tyrobutyricum* is most abundant when there is a dominance of *S. thermophilus* and
524 *L. rhamnosu*s, two L(+) lactate producers. The key enzymes of this metabolism are NAD
525 independent lactate dehydrogenases, which convert lactate to pyruvate. The analysis of the *C.*
526 *tyrobutyricum* genomes (Bassi et al., 2013c; Jiang et al., 2013) revealed the presence of genes
527 coding for both D and L lactate dehydrogenases and of a L-lactate permease. It is still not clear if
528 the ratio between the two racemic forms affect the vegetative cycle of *C. tyrobutyricum* in cheese.

All the samples dominated by *C. butyricum* (cluster Cl-A) were also clustered in Lb-A, where *L. delbrueckii* was the most abundant *Lactobacillus*. Based on the energetic metabolism, it cannot be explained why the presence of *L. delbrueckii* relates to a higher prevalence of *C. butyricum.*

It is now well recognised that bacteria interact in the food communities, influencing the final quality of fermented products. Our data indicated that in hard cheese presenting the typical signs of late blowing, the spoiling clostridial species coexisted with the LAB populations and often they did not dominate in quantitative terms the cheese microbiota. Some preliminary hints deriving from the NGS analysis of the bacterial community indicated a correlation between the inhabitant LAB, both natural starters and NSLAB, and the prevalence of different species of clostridia. Moreover, our study showed that the use of lysozyme, added to affect spore germination and their vegetative cell outgrowth, shaped the species composition of the cheese bacterial communities of both LAB and butyric clostridia. Further studies aimed to analyse the interactions LAB-clostridia might provide in the future a deeper knowledge of the ecological relationships in cheese microbiota and new approaches for the reduction of hard cheese spoilage.

Acknowledgements

The research was supported by grants from the Ministero delle politiche agricole, alimentari e forestali (MIPAAF) national project “Filigrana” D.M. 25741/7303/11, Consorzio per la Tutela del Formaggio Grana Padano, Italy and Regione Lombardia ERSAF national project “GPL-free” D.11/08/2011 n° 7656. The authors are grateful to Sotirios Vasileiadis for the designing of the primer-linker-index constructs and for the setup of several data analysis methods used for the elaboration of the Illumina sequences.

Figure Legends

Table 1. Specificity of the *Clostridiales* PCR amplification using a range of clostridial and non-clostridial species.

Table 2. Data obtained from *Clostridium* cluster I-specific PCR-DGGE analysis and qPCR for *C. tyrobutyricum* enumeration in cheese samples with and without added lysozyme.

Figure 1. Hierarchical clustering of classified sequences using the average linkage algorithm at genus classification level for taxa participating with ≥ 5 % in at least one sample. Taxa with lower participations were added to the "other" sequence group. Three main clusters have been defined (A, B and C).

Figure 2. Hierarchical clustering of classified sequences using the average linkage algorithm at the species classification level limited to sequences belonging to *Lactobacillus* genus. Taxa with participations lower than 1% were added to the "other" sequence group. Three main clusters have been defined with Lb-A, Lb-B and Lb-C.

Figure 3. Hierarchical clustering of classified sequences using the average linkage algorithm at the species classification level limited to sequences belonging to *Clostridium* genus. Taxa with participations lower than 1% were added to the "other" sequence group. Three main clusters have been defined with Cl-A, Cl-B and Cl-C.

Figure 4. Metastats model assessing the effects of lysozyme on the relative abundances of *Lactobacillus* (a) and *Clostridium* sequences (b). Genera showing significant differences are highlighted with an asterisk.

Table S1. Number of sequences per sample, coverage and diversity indexes calculated on the 1,204,942 high quality sequences analyzed in the casing samples.

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736
737
738

Table 1

Bacterial strains	Isolate number[a]	Source	*Clostridiales*-specific PCR amplification
Bacillus cereus	UC9027	Cheese	negative
Bacillus coagulans	UC9021	Cheese	negative
Bacillus coagulans	UC9021	GP Cheese	negative
Bacillus sp.	UC9024	Cheese	negative
Bacillus subtilis	UC9028	Cheese	negative
Bacillus thuringensis/cereus	UC8553	GP cheese	negative
Clostridium cochlearium	*DSM1285T*	Type strain	positive
Clostridium acetobutylicum	*LMG5710T*	Type strain	positive
Clostridium botulinum	*UC9031*	Animal feed	positive
Clostridium butyricum	DSM10702^T	Type strain	positive
Clostridium butyricum	UC9041	Cheese	positive
Clostridium butyricum	UC9045	GP cheese	positive
Clostridium perfringens	UC9042	Silage	positive
Clostridium perfringens	UC9043	Silage	positive
Clostridium sporogenes	ATCC7955^T	Type strain	positive
Clostridium sporogenes	UC9015	Milk	positive
Clostridium tyrobutyricum	DSM2637^T	Type strain	positive
Clostridium tyrobutyricum	UC7086	GP Cheese	positive
Clostridium tyrobutyricum	ATCC25755^T	Type strain	positive
Clostridium tyrobutyricum	UC9030	GP Cheese	positive
Clostridium tyrobutyricum	UC9036	GP Cheese	positive
Clostridium tyrobutyricum	UC9037	GP Cheese	positive
Clostridium tyrobutyricum	UC9014	GP Cheese	positive
Clostridium tyrobutyricum	UC9044	Milk	positive
Clostridium tyrobutyricum	UC8981	Milk	positive
Clostridium tyrobutyricum	UC8984	Ricotta cheese	positive
Clostridium tyrobutyricum	UC8988	Ricotta cheese	positive
Clostridium tyrobutyricum	UC9008	GP cheese surface	positive
Clostridium tyrobutyricum	UC9038	GP cheese	positive

Clostridium tyrobutyricum	UC9040	GP cheese	positive
Enterococcus faecalis	UC8823	Sausage	negative
Enterococcus faecium	UC8821	Sausage	negative
Enterococcus faecium	UC9029	Cheese	negative
Lactobacillus acidophilus	UC10110	Cheese	negative
Lactobacillus curvatus	UC8266	Taleggio cheese	negative
Lactobacillus helveticus	UC8900	Cheese	negative
Lactobacillus helveticus	UC10206	GP cheese	negative
Lactobacillus plantarum	UC10119	Cheese	negative
Lactobacillus rhamnosus	UC8509	GP cheese	negative
Lactobacillus rhamnosus	UC8514	GP cheese	negative
Lactobacillus sakei	UC8705	Sausage	negative
Lactobacillus sakei	UC8168	Casera cheese	negative
Leuconostoc mesenteroides	UC8133	Scimudin Cheese	negative
Leuconostoc mesenteroides	UC8182	Casera cheese	negative
Listeria monocytogenes	UC8159	Gorgonzola cheese	negative
Ruminococcus flavefaciens	ATCC19208 ^{T}	Type strain	negative
Ruminococcus gnavus	ATCC29149 ^{T}	Type strain	negative
Staphylococcus equorum	UC8190	Scimudin cheese	negative
Staphylococcus xylosus	UC8449	Salami	negative
Stapylococcus saprophyticus	UC8555	GP cheese	negative
Streptococcus macedonicus	UC9022	Cheese	negative
Streptococcus thermophilus	UC10185	GP cheese	negative
Streptococcus thermophilus	UC8547	milk	negative
Streptococcus thermophilus	UC10166	GP cheese	negative

[a] UC, Università Cattolica del Sacro Cuore culture collection; ATCC, American Type Culture Collection; DSM, German Collection of Microorganisms.

Table 2

LYSOZYME POSITIVE SAMPLES						
Sample	***C. butyricum***	***C. sporogenes***	***C. perfringens***	***C. septicum***	***C. tyrobutyricum***	**TaqMan Log target genome/50g *C. tyr***
BS603_28+						0.00
BS607_7+	+	+				0.00
BS607_8+	+	+	+			0.00
BS619_10+a	+					0.00
BS619_10+b			+			0.00
BS619_10+c			+			0.00
BS623_8+a	+					0.00
BS623_8+b	+					0.00
BS628_2+a	+	+	+			0.00
BS628_2+b	+	+				0.00
MN427_7+	+			+	+	0.00
MN437_6+	+	+			+	0.00
MN449_4+						0.00
MN449_7+	+	+	+			0.00
MN460_5+					+	0.00
MN460_8+	+	+	+			0.00
MN460_9+		+				0.00
MN481_11+a			+			0.00
MN481_11+b						0.00
MN481_14+					+	0.00
PC508_7+	+					0.00
PC540_5+						0.00
PC540_6+						0.00
PC544_7+	+					0.00
PC544_8+	+					0.00
BS627_5+						0.00
CN903_6+						0.00
MN425_6+						0.00
MN443_7+						0.00
PC521_6+	+					0.00
PC544_5+						0.00
MN449_5+			+		+	2.00
MN449_8+			+		+	2.04
BS628_6+			+		+	3.70
MN460_8+		+			+	4.49
PC508_8+	+					4.56
BG506_4+	+					4.60
PC508_11+	+		+			4.87
PC544_7+	+		+			4.94
PC544_6+	+			+		4.96
MN437_5+	+	+			+	4.98
BS634_1+	+	+			+	5.00
PC544_3+	+					5.11

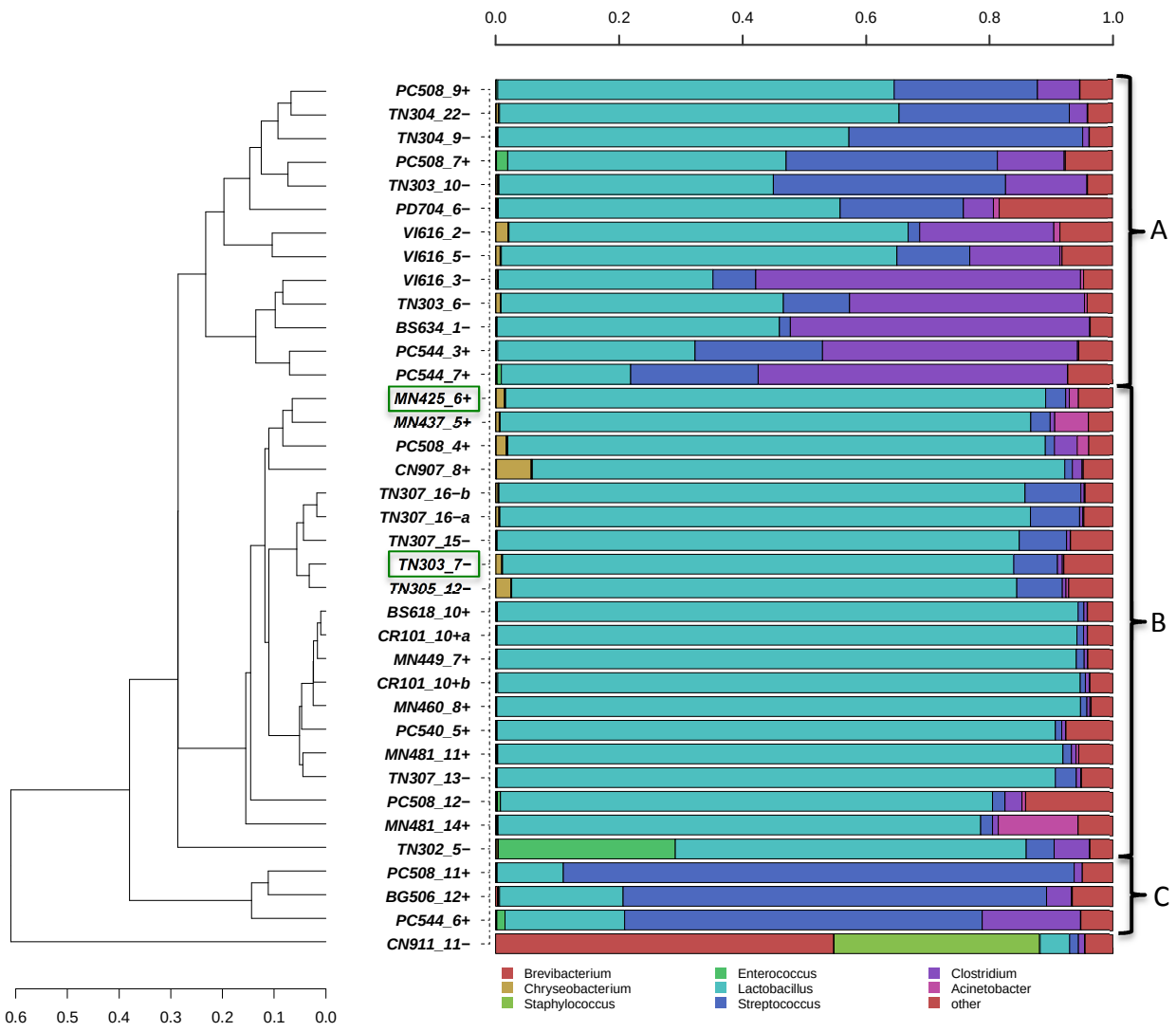
VI618_8+						5.15
BG506_12+	+					5.38
BS618_10+a	+					5.43
BS618_10+b				+		5.48
CR101_10+b			+		+	5.66
PC508_7+	+	+				5.88
CR101_10+a	+					6.54
PC508_9+	+					6.54
PC508_4+a					+	7.98
CN907_9+					+	8.00
CN907_8+					+	8.15
CN907_7+					+	8.56
PC508_4+b					+	8.92

LYSOZYME NEGATIVE SAMPLES

Sample	***C. butyricum***	***C. sporogenes***	***C. perfringens***	***C. septicum***	***C. tyrobutyricum***	**TaqMan Log target genome /50g *C. tyr***
TN302_4-					+	0.00
TN302_6-					+	0.00
TN305_12-	+				+	0.00
TN307_16-						0.00
TN303_7-						0.00
TN305_6-						0.00
MN460_8-			+		+	2.04
BS628_9-	+	+				2.39
BS654_8-					+	2.52
BS634_1-	+					2.97
BS611_9-	+	+			+	3.11
BS654_7-			+		+	3.18
BS654_6-					+	3.51
TN307_13-					+	4.28
TN305_12-						4.46
TN307_15-	+	+			+	4.49
TN307_16-	+	+				4.80
TN307_13-	+				+	4.81
TN302_5-					+	4.91
TN305_11-a		+			+	4.93
TN305_11-b					+	5.38
PC508_12-	+				+	5.46
TN304_9-					+	6.52
VI616_5-					+	6.71
PD704_6-					+	6.72
TN303_5-					+	6.72
VI616_8-					+	7.11
TN303_10-					+	7.23
TN303_6-					+	7.40
VI616_4-					+	7.40
TN304_22-				+	+	7.51
VI625_10-					+	7.62

VI616_3-	+			+	8.23
CN911_11-		+		+	8.28
VI616_2-			+	+	9.20

Figure 1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TN303_10−
PD704_6−
VI616_2−
VI616_5−
VI616_3−
TN303_6−
BS634_1−
PC544_3+
PC544_7+
MN425_6+
MN437_5+
PC508_4+
CN907_8+
TN307_16−b
TN307_16−a
TN307_15−
TN303_7−
TN305_12−
BS618_10+
CR101_10+a
MN449_7+
CR101_10+b
MN460_8+
PC540_5+
MN481_11+
TN307_13−
PC508_12−
MN481_14+
TN302_5−
PC508_11+
BG506_12+
PC544_6+
CN911_11−
A
B
C
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0.5
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0.3
0.2
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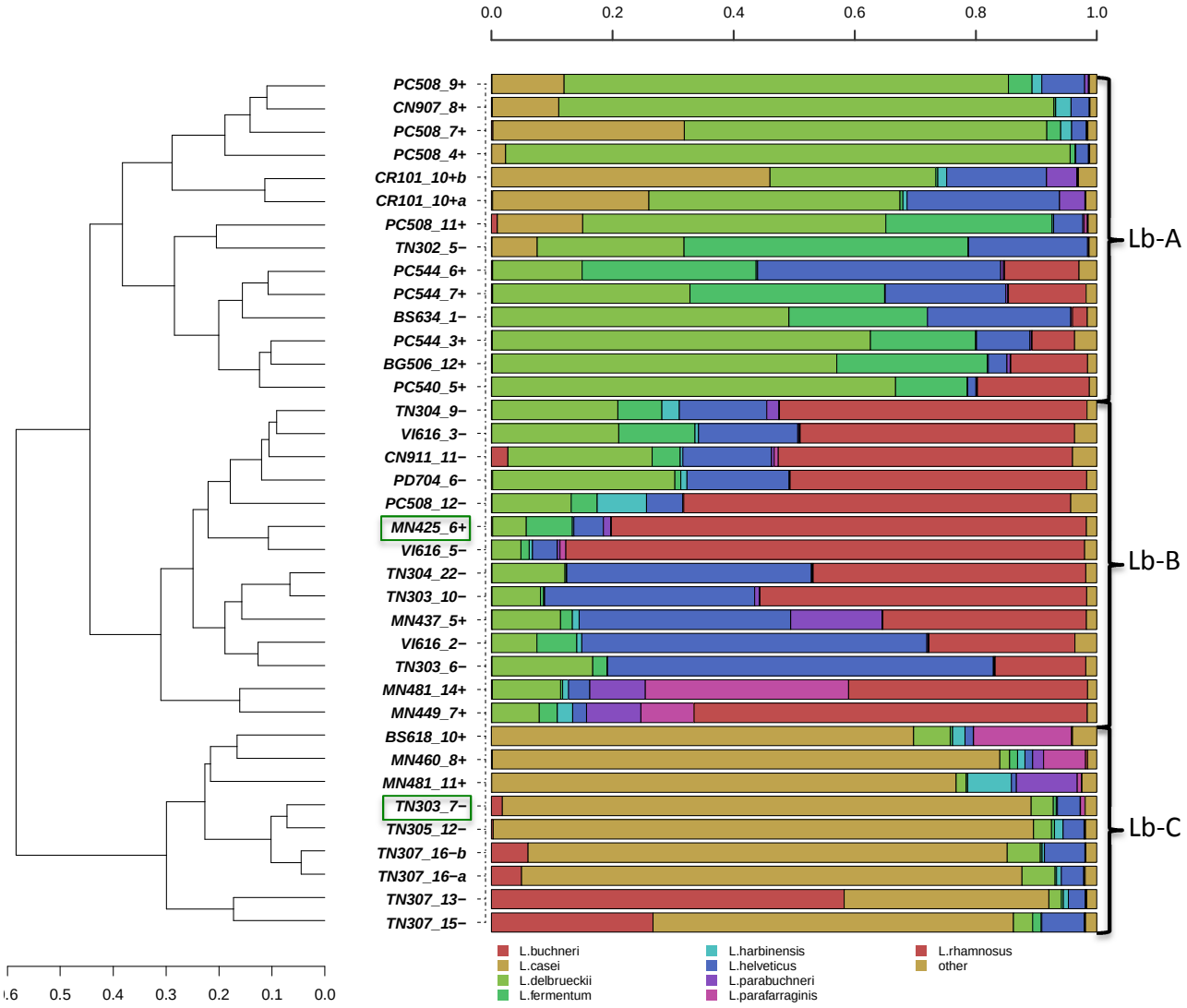
Chryseobacterium
Staphylococcus
Enterococcus
Lactobacillus
Streptococcus
Clostridium
Acinetobacter
other

Figure 2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CR101_10+b
CR101_10+a
PC508_11+
TN302_5−
PC544_6+
PC544_7+
BS634_1−
PC544_3+
BG506_12+
PC540_5+
TN304_9−
VI616_3−
CN911_11−
PD704_6−
PC508_12−
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VI616_5−
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BS618_10+
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MN481_11+
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TN305_12−
TN307_16−b
TN307_16−a
TN307_13−
TN307_15−
Lb-A
Lb-B
Lb-C
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0.5
0.4
0.3
0.2
0.1
0.0
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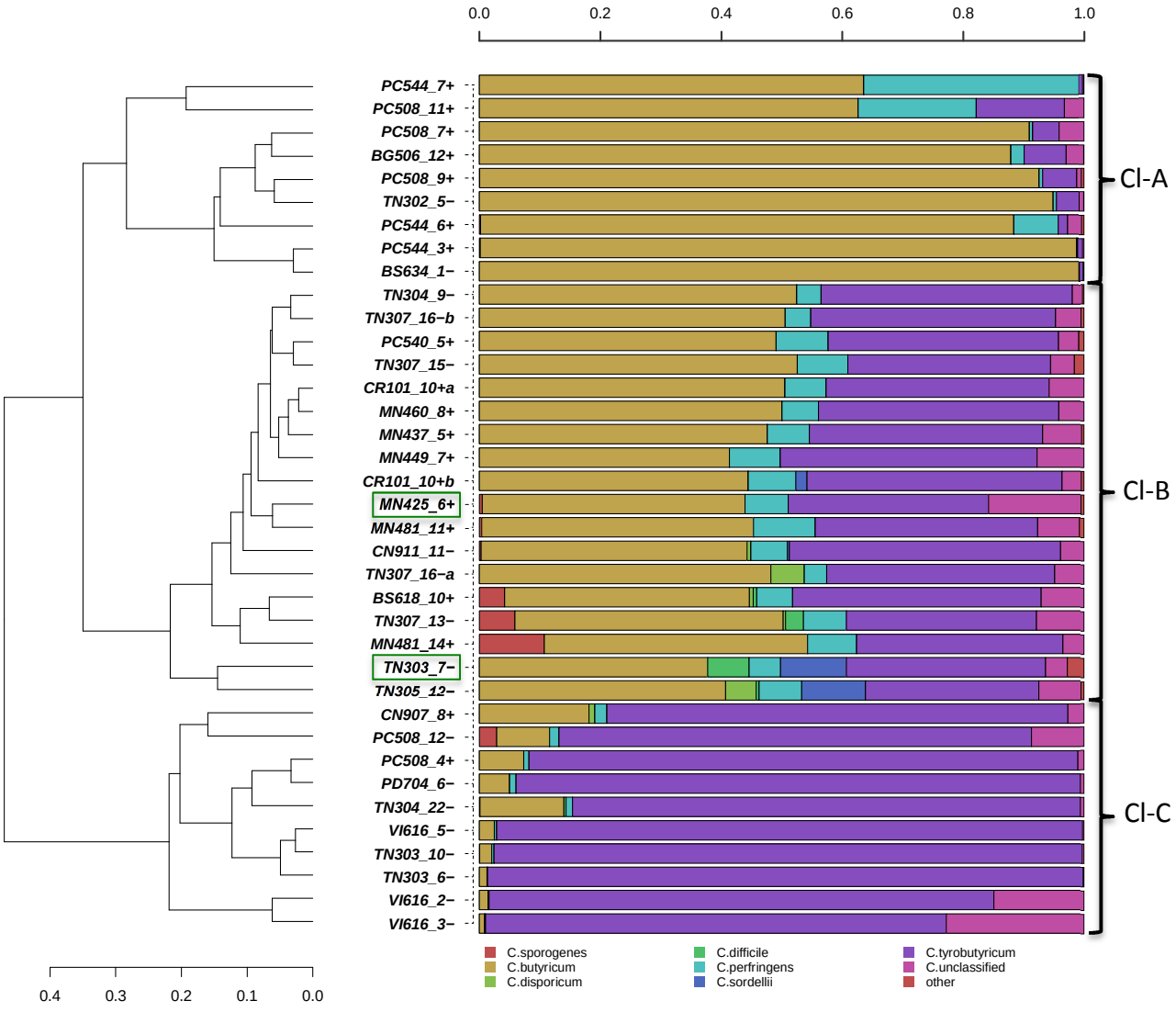
L.casei
L.delbrueckii
L.fermentum
L.harbinensis
L.helveticus
L.parabuchneri
L.parafarraginis
L.rhamnosus
other

Figure 3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VI616_5−
TN303_10−
TN303_6−
VI616_2−
VI616_3−
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Cl-B
Cl-C
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0.2
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C.difficile
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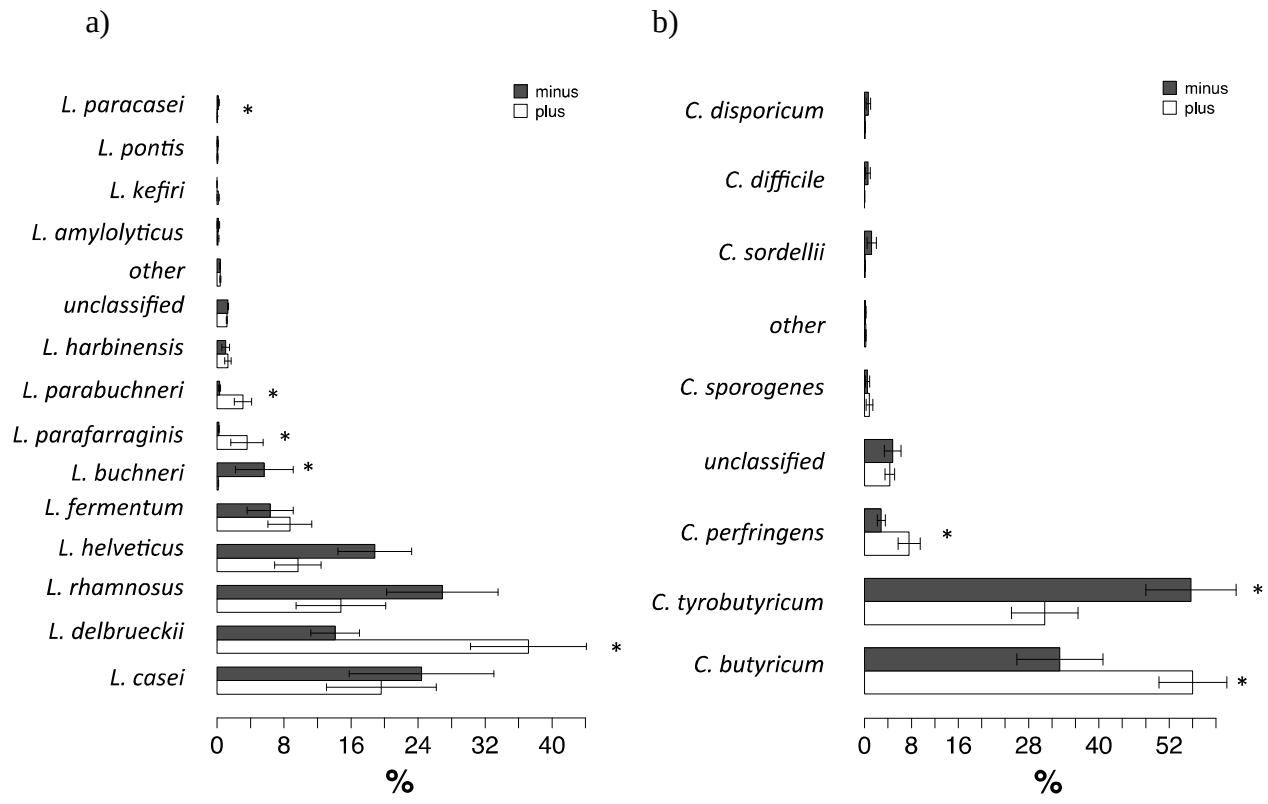
C.sordellii
C.tyrobutyricum
C.unclassified
other

Figure 4


a)
b)
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plus
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L. kefiri
L. amylolyticus
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unclassified
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L. parabuchneri
L. parafarraginis
L. buchneri
L. fermentum
L. helveticus
L. rhamnosus
L. delbrueckii
L. casei
0 8 16 24 32 40
%
C. disporicum
C. difficile
C. sordellii
other
C. sporogenes
unclassified
C. perfringens
C. tyrobutyricum
C. butyricum
0 8 16 28 40 52
%

Supplementary Table S1

Sample	Nseqs	coverage	Sobs	Simpson eveness	inverted Simpson	Chao	Shannon eveness	np Shannon
PC_minus_12	32566	0,981	801	0,00405	3,24	3261,46	0,301	2,121
TN_minus_5	32566	0,983	731	0,00610	4,46	2556,60	0,295	2,040
TN_minus_6	32566	0,980	850	0,00399	3,39	3415,43	0,261	1,876
TN_minus_10	32566	0,981	822	0,00487	4,01	2837,37	0,272	1,940
TN_minus_12	32566	0,982	813	0,00218	1,78	2446,64	0,206	1,496
TN_minus_16	32566	0,982	766	0,00242	1,85	2503,11	0,202	1,454
TN_minus_16bis	32566	0,982	778	0,00261	2,03	2892,12	0,211	1,523
TN_minus_15	32566	0,982	781	0,00388	3,03	2920,29	0,258	1,826
TN_minus_13	32566	0,981	847	0,00305	2,59	3006,50	0,233	1,685
PD_minus_6	32566	0,981	837	0,00642	5,37	3049,76	0,363	2,540
VI_minus_5	32566	0,980	859	0,00333	2,86	3053,62	0,272	1,954
VI_minus_3	32566	0,980	873	0,00512	4,47	3103,10	0,312	2,228
VI_minus_2	32566	0,980	837	0,00496	4,15	3346,28	0,329	2,319
CN_minus_11	32566	0,979	907	0,00269	2,44	3007,01	0,218	1,612
TN_minus_22	32566	0,982	785	0,00454	3,56	2659,68	0,254	1,800
TN_minus_9	32566	0,983	739	0,00506	3,74	2770,15	0,272	1,896
BS_minus_1	32566	0,982	770	0,00365	2,81	2653,57	0,228	1,624
TN_minus_7	32566	0,981	827	0,00220	1,82	3279,57	0,221	1,600
MN_plus_7	32566	0,980	820	0,00289	2,37	3056,53	0,228	1,651
MN_plus_8	32566	0,982	751	0,00201	1,51	3214,89	0,158	1,162
CR_plus_10	32566	0,982	782	0,00273	2,13	3162,28	0,200	1,448
CR_plus_10bis	32566	0,982	766	0,00347	2,66	2764,93	0,224	1,597
BS_plus_10	32566	0,979	905	0,00229	2,08	3015,07	0,204	1,523
MN_plus_11	32566	0,980	882	0,00216	1,91	3118,82	0,210	1,552
MN_plus_14	32566	0,981	807	0,00513	4,14	2872,61	0,295	2,085
BG_plus_12	32566	0,980	868	0,00365	3,16	3291,67	0,276	1,987
PC_plus_11	32566	0,980	847	0,00210	1,78	3222,31	0,194	1,435
PC_plus_7	32566	0,981	791	0,00560	4,43	3106,78	0,302	2,124
CN_plus_8	32566	0,982	751	0,00239	1,79	2931,65	0,203	1,457
PC_plus_4	32566	0,983	720	0,00197	1,42	2361,51	0,158	1,152
PC_plus_9	32566	0,983	734	0,00406	2,98	2393,16	0,260	1,822
PC_plus_5	32566	0,983	755	0,00309	2,33	2396,38	0,220	1,565
PC_plus_7	32566	0,982	772	0,00728	5,62	2653,03	0,332	2,308
PC_plus_6	32566	0,981	798	0,00464	3,70	2909,51	0,295	2,078
PC_plus_3	32566	0,981	834	0,00478	3,99	2577,45	0,293	2,082
MN_plus_5	32566	0,982	785	0,00468	3,68	2691,56	0,279	1,968
MN_plus_6	32566	0,982	790	0,00252	1,99	2851,50	0,232	1,657