ABSTRACT

Recognized worldwide for its history, flavor, and high nutritional quality, Grana Padano (GP) is one of the most traditional Italian raw-milk, hard-cooked, long-ripened cheese. Throughout GP manufacturing, some well-known and undesired bacterial species, such as clostridia, can proliferate and lead to spoilage defects that mischaracterize the final product; however, little is known about the development of late-blowing defects in hard cheese samples without clostridia. Therefore, in this study we aimed to use metataxonomic analysis to identify bacterial taxa associated with the development of late-blowing defect in GP samples. Furthermore, the presence of several heterofermentative lactobacilli species in defective zones were verified by primer-specific PCR assay. Considering α- and β-diversity analyses, no statistically significant differences were detected between cheese samples with and without blowing defect. Following taxonomic assignment, Lactobacillus and Streptococcus were the dominant genera, whereas clostridia-related taxa were not detected in any of the 20 analyzed samples. Using EdgeR, the genera Propionibacterium and Acinetobacter were found to be prevalently more abundant in samples categorized as having “big regular holes.” In samples with “small regular holes,” multiplex PCR amplification revealed differences in terms of Lactobacillus population composition, mainly obligate homofermentative lactobacilli, between defective and non-defective zones of the same cheese wheel. This study demonstrated that GP samples with blowing defects not caused by clostridial development share similar biodiversity indices with GP collected from control zones, but an imbalance of obligate homofermentative lactobacilli was noticed between samples, which requires further analysis to better comprehend the exact mechanism involved in this process.

Key words: hard cheese, 16S rRNA, lactobacilli, Grana Padano

INTRODUCTION

Grana Padano (GP) is a well-known traditional Italian raw-milk, hard-cooked, long-ripened cheese, whose technological and microbiological characteristics have been reviewed previously (Neviani et al., 2013). It has been a Protected Designation of Origin (PDO) cheese since 1996, and it is produced in 5 regions located in northern Italy; namely, Emilia-Romagna, Lombardia, Piemonte, Trentino-Alto Adige, and Veneto). According to Bava et al. (2018), it is estimated that nearly 24% of the total cow milk produced in Italy is destined for GP production. In 2020 alone, 5,255,451 wheels of this cheese were produced, and 39% were exported outside Italy, making this cheese the food product most sold worldwide (Grana Padano Protection Consortium, www.granapadano.it). The ripening period for this cheese can vary from 9 mo to more than 2 yr (Gatti et al., 2014). During this period, bacteria from the raw milk and from the added whey starter cultures develop inside the cheese and give it its peculiar characteristics (Rossetti et al., 2008). At the same time, deleterious microbes possibly present in the raw milk or in the cheesemaking plant can also develop and determine the creation of defects (Bassi et al., 2015). Although the presence of small holes does not constitute a threat to food security, this type of defect must be barely visible (less than 5 mm in diameter) and not lead to undesirable effects on textural and sensory parameters, with consequent loss of product value.

Late-blowing (LB) defect is a well-known and quite widespread problem in long-ripened cheeses such as GP, primarily determined by the proliferation of bacteria, mainly belonging to the genus Clostridium, during cheese ripening, and it can be responsible for relevant commercial losses (Bermúdez et al., 2016; D’Incecco et al., 2018). These bacteria typically cause holes (also called “eyes”) of different diameter inside the cheese
wheels that, in serious cases, can also visibly deform the wheel outside. In addition to these well-known bacteria, mainly belonging to the species *Clostridium butyricum* and *Clostridium tyrobutyricum*, other microorganisms such as propionic acid bacteria can produce similar defects.

Over the last few years, thanks to reductions in the costs of next-generation sequencing, several studies have focused on assessment and comprehension of the microbial community by cultivation-independent techniques of raw materials (e.g., raw milk and natural whey starter cultures) used in GP manufacturing, as well as during the GP ripening process (Neviani et al., 2013; De Filippis et al., 2014; Gatti et al., 2014; Alessandria et al., 2016; Mayo et al., 2021). However, few studies have reported the bacterial community structure of GP samples with or without LB defect (Bassi et al., 2015; Zago et al., 2021). Overall, studies demonstrating the involvement of bacterial genera different from *Clostridium* (e.g., propionibacteria or heterofermentative lactic acid bacteria) as causative agents of late blowing are still scarce and mostly based on DNA identification. Therefore, in this study we aimed to characterize and identify, through metataxonomic analysis, the major bacterial taxa associated with the development of LB defects in GP samples. Finally, the associations of several heterofermentative lactobacilli with defective zones testing negative for the presence of clostridia cells were verified by primer-specific PCR assay.

**MATERIALS AND METHODS**

**Cheese Sampling**

Nine wheels of GP PDO cheese from a cheese factory located in the Veneto region, having 8 to 9 mo of maturation, that were considered defective, were sampled. Ten to twenty grams of cheese was scraped with a sterile spatula, taking care to collect only the part surrounding the holes, and samples were named S1 to S9. For wheels with different hole dimensions, 2 separate samples were taken and named, for example, S2 and S2A (Figure 1). For each wheel, a portion not presenting defects was also separately sampled and used as control, named CTR1 through CTR9. A total of 20 samples were collected (Table 1), immediately transferred into sterile 50-mL Falcon tubes, maintained refrigerated during transportation, and then stored at −20°C until use.

**Total DNA Extraction from Cheese**

In this study, the PowerSoil DNA Isolation Kit (Qia-gen) protocol modified for 1 g of cheese was adopted.

---

Figure 1. Representative images of the sampled zones. Black arrows indicate small sparse eyes, whereas gray arrows indicate large sparse eyes. S1 to S4 represent samples with small holes; S2A and S3A are samples with large holes.
In brief, 2 mL of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol) at pH 8.0 and 2 mL of PBS (NaCl 0.13 M, KCl 2 mM, Na₂HPO₄ 9 mM, KH₂PO₄ 1 mM, pH 7.4) were added to a polystyrene 15-mL tube containing 1 g of cheese sample. Sequentially, 60 µL of solution C1, as well as the entire contents of one PowerBead tube (Qiagen; 750 µL of solution and beads), were added to the sample tube and the mixture was vortexed at maximum speed for 15 min. After centrifugation at 10,000 × g for 5 min at 4°C, sequential phenol:chloroform (1:1) and chloroform (1:1) steps were carried out at 10,000 × g for 5 min at 4°C. The obtained supernatant was divided into aliquots of 500 µL and transferred to 2-mL microfuge tubes previously filled with 250 of solution C2. From this stage, all successive steps followed manufacturer recommendations. The quality and quantity of the extracted DNA were assessed by a Spark 10M spectrophotometer (Tecan Trading AG). The DNA samples were stored at −20°C until further analysis.

16S rRNA Gene Amplicon Sequencing and Molecular Identification of Dairy Heterofermentative Species

Total metagenomic DNA samples were sent to Eurofins (Eurofins Genomics Germany GmbH, Ehersberg, Germany), where amplicon preparation and sequencing were performed. After amplification of the V3 to V5 region of the 16S rRNA genes using 357f and 962r primers, amplicon libraries were generated using the Nextera XT DNA Library Preparation Kit (Illumina Inc.) and sequenced using the Illumina MiSeq desktop sequencer producing 300-bp paired-end reads.

Pairs of primers specific for different heterofermentative Lactobacillus spp. and Leuconostoc spp. were obtained from different studies (Table 2) to evaluate the presence and possible involvement of these microorganisms in the development of blowing defects in the GP samples. Primers were obtained from Invitrogen (Thermo Fisher Scientific). Amplifications were carried out in a total volume of 25 µL using 50 ng of metagenomic DNA as the template and using the same primer concentration and Taq DNA polymerase units (GoTaq, Promega) adopted in the study in which each primer was described. We conducted PCR amplification using a BioRad iCycler iQ PCR thermal cycler (BioRad), setting up the thermocycling program specific for each pair of primers. The PCR products were subjected to electrophoresis on 1.5% (wt/vol) agarose gels in Tris-borate-EDTA (TBE) buffer solution at 70 V for 1.5 h, followed by EuroSafe staining (EuroClone).

Bioinformatics Analyses

Raw 16S rRNA sequences were analyzed with the CLC Genomics Workbench software (version 8.0.2, Qiagen Bioinformatics) using the microbial genomics module plugin as described by Treu et al. (2018). In brief, quality filtering, operational taxonomic unit (OTU) clustering, and taxonomical assignment (Greengenes database, version 13-5; https://greengenes.secondgenome.com) were conducted with default parameters. To improve the taxonomical assignment and make inferences at the species level when appropriate, OTU consensus sequences were manually verified using the MegaBLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=MegaBlast; 16S ribosomal RNA sequences). Throughout the manuscript, the new nomenclature for Lactobacillus species proposed by Zheng et al. (2020) has been adopted. Raw reads were deposited into the Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra) under the BioProject PRJNA748599.

Statistical Analysis

All the statistical analyses of the microbiota data were carried out with MicrobiomeAnalyst (Dhariwal et al., 2017). Following manual data inspection, low-count (minimum count: 2; prevalence in samples: 10%) and low-variant filters (interquantile range: 10%) were applied and centered log-ratio transformed. Differential abundance analysis was conducted with edgeR (Robinson et al., 2010), taking into account only those taxa with log2 fold change (log2FC) greater than 2.0 and with a false discovery rate-adjusted P-value < 0.05. For α-diversity analysis, the non-parametric Mann-Whitney/Kruskal-Wallis tests were chosen, whereas microbiota similarities among the groups (β-diversity analysis) were assessed using the Unweighted and
Weighted UniFrac metrics. Permutational multivariate ANOVA (PERMANOVA) with 999 permutations was used to test whether distances between samples within a certain group were more similar to each other or not. Differences were considered statistically significant for a P-value < 0.05.

RESULTS AND DISCUSSION

In this study, we examined 9 GP PDO wheels that, after about 9 mo of maturation, revealed anomalous sounds when knocked with a specific small hammer, a standard procedure used to detect anomalous wheels, indicating the possible presence of internal cavities, and were therefore considered defective. The wheels were opened and sampled both in a clearly defective portion as well as in a normal and adjacent zone (control sample). After high-throughput sequencing of the V3 to V5 region of the 16S rRNA genes aiming at detection of the bacterial diversity in GP samples with or without LB defects, a total of 1,123,291 high-quality sequences were assigned to 55 OTU, after the removal of low-quality and chimeric sequences. For both α- and β-diversity analyses, no significant differences were detected between control and LB groups. The same result was also observed when cheese samples were analyzed based on hole dimension (small or large; Figure 2).

With regard to the taxa assigned to each GP sample (Table 3), it must first be noticed that clostridial sequences were not detected in any of the samples. We need to consider that GP cheese contains about 1.5% NaCl, which constitutes an unfavorable characteristic for clostridial proliferation (IDF, 2017); moreover, the antibacterial enzyme lysozyme is routinely used to contrast the development of these bacteria. Notwithstanding, these measures are frequently insufficient to completely eliminate the problem, particularly in wheels of large dimensions, as in the case of GP, because sometimes the salt, which is provided by immersion of the cheese wheel in concentrated a saline solution, diffuses inside the wheels too slowly to promptly block clostridial growth. We must also consider that, even though the procedure adopted in this study to lyse bacterial cells for DNA extraction was not developed to break the thick endospore structure, clostridial spores were excluded as the causative agent of the defects observed. This is because spores should have germinated and vegetative cells multiplied at the beginning of cheese ripening; therefore this bacterial group should nonetheless have been detected in that form, if present.
Although some studies report the presence of clostridia by 16S rRNA analysis of defective and non-defective cheeses (Bassi et al., 2015; Levante et al., 2017; Zago et al., 2021), the results of our analysis clearly indicate that the holes in the examined cheese wheels were not produced by bacteria belonging to this genus.

Sequences attributable to the genus *Lactobacillus* are extremely abundant, as expected for this type of hard cheese (Neviani et al., 2013; Alessandria et al., 2016), accounting for 96.87% of the total sequences obtained. Considering the species attribution within the genus, the most represented species by far is *Lactobacillus delbrueckii* (76.39%). This could appear quite uncommon, according to data from conventional microbiological analyses based on colony counts on plates, which detected *Lactobacillus helveticus* as the most abundant species for this type of cheese (Rossetti et al., 2008). Indeed, our results are in accordance with a recent report by Zago et al. (2021) in a DNA metabarcoding survey on 118 samples of GP cheese obtained from different Italian regions, in which *L. delbrueckii* was found to be one of the most abundant taxa. On the contrary, the presence of *L. helveticus* was dramatically scarce in our samples, as in all controls it never exceeded 1% of total sequences collected, varying from 0.17% to 0.91% of total abundance. *Lactobacillus helveticus* was also detected at low levels in a study by Zago et al. (2021), in which several samples did not evidence any detectable presence of this species, and only few samples, mainly those coming from Veneto and Trentino-Alto Adige regions, contained more than 10% *L. helveticus* in the total population. This apparent contrast with con-

![Figure 2](Diversity analyses of cheese samples with large holes (BR group), small holes (SR group), and without blowing defect (WBD group). Panels A and B: α-diversity; panels C and D: β-diversity. For panels A and B, horizontal bold lines show the median values. The bottom and top of the boxes show the 25th and the 75th percentiles, respectively. The whiskers extend up to the most extreme points within 1.5 times the interquartile ranges. Level of significance: *P* ≤ 0.05. Principal coordinate (PC) analysis based on unweighted (C) and weighted (D) UniFrac distances for the BR, SR, and WBD groups.)
Table 3. Differential abundance analysis of the most abundant bacterial taxa in control and in defective (late blowing) samples

| Taxon                                | Control | Late blowing | S1/Ctr1 | S2/Ctr2 | S2A/Ctr2 | S3/Ctr3 | S3A/Ctr3 | S4/Ctr4 | S5/Ctr5 | S6/Ctr6 | S7/Ctr7 | S8A/Ctr8 | S9/Ctr9 |
|--------------------------------------|---------|--------------|---------|---------|----------|---------|----------|---------|---------|---------|---------|---------|---------|---------|
| **Lactobacillus delbrueckii**        | 76.39%  | 70.12%       | -0.02   | 0.77    | -0.49    | 1.37    | 0.55     | 0.08    | -0.94   | -0.79   | -0.50   | -0.68   | 0.27    |
| **Lactobacillus spp.**               | 15.39%  | 20.44%       | 0.56    | 2.82    | 0.86     | 0.13    | 0.44     | 0.50    | -0.66   | -0.88   | 0.19    | -0.25   | 0.20    |
| **Lactococcus lactis**               | 7.65%   | 8.98%        | 1.26    | 3.74    | 0.60     | 0.89    | 0.30     | 1.39    | -1.52   | -1.26   | -0.74   | 0.32    |
| **Lactobacillus helveticus**         | 0.55%   | 0.40%        | 2.27    | 1.91    | 0.68     | 0.42    | -0.56    | -1.52   | -1.62   | 0.62    |
| **Lactobacillus kefiranofaciens**    | 0.01%   | 0.01%        | 1.81    | 1.81    | 1.58     | -0.68   | 0.70     | -1.42   | -2.17   | 0.58    | -0.77   | -0.32   | -0.58   |
| **Schleiferilactobacillus harbinensis** | 0.02%  | 0.05%        | 1.08    |         |          |         |          |         |         |         |         |         |         |
| **Total Lactobacillus spp.**         | 96.87%  | 97.26%       | 0.01    | 1.46    | -0.20    | 1.07    | 0.55     | 0.21    | -0.91   | -0.86   | -0.37   | -0.66   | 0.29    |
| **Propionibacterium acnes**          | 0.01%   | 0.08%        | 5.39    | 8.41    | -0.91    |         |          |         |         |         |         |         |         |
| **Pediococcus acidilactici**         | 0.01%   | 0.00%        |         | 2.32    | 0.26     |         |          |         |         |         |         |         |         |
| **Acinetobacter johnsonii**          | 0.00%   | 0.00%        | -2.58   | -2.81   | -1.22    |         |          |         |         |         |         |         |         |
| **Propionibacterium freudenreichii** | 0.00%   | 0.00%        |         | 1.58    | -1.58    |         |          |         |         |         |         |         |         |
| **Aeromonas spp.**                   | 0.00%   | 0.00%        | 1.58    |         | -2.58    |         |          |         |         |         |         |         |

1. Samples with small holes: S1, S2, S3, S4, S5, S6, S7, and S9; samples with large holes: S2A, S3A, and S8A; and respective control zones: Ctr1, Ctr2, Ctr3, Ctr4, Ctr5, Ctr6, Ctr7, Ctr8, and Ctr9. Fold-change values greater than |2| are shown in bold.
2. The “Control” column reports the percentage of sequences for each operational taxonomic unit (OTU) over the number of sequences from all control samples together. The “Late blowing” column reports the percentage of sequences for each OTU over the number of sequences from all defective samples together. The remaining columns display the Log2FC values of species abundance between the normal and the defective portion of each cheese wheel.

### Bacterial Taxa and Their Abundance

- **Lactobacillus delbrueckii**: A significant decrease in abundance was observed in the defective samples compared to controls.
- **Lactobacillus spp.**: A notable increase in abundance was found in the defective samples.
- **Lactococcus lactis**: A small increase in abundance was observed in the defective samples.
- **Lactobacillus helveticus**: A slight decrease in abundance was observed in the defective samples.
- **Lactobacillus kefiranofaciens**: A marked quantitative change in the lactobacilli composition was detected, with a substantial increase in the defective samples.
- **Schleiferilactobacillus harbinensis**: A slight decrease in abundance was observed in the defective samples.

### Conclusion

The study provides evidence of differential abundance analysis of the most abundant bacterial taxa in control and defective samples, indicating potential differences in microbiota composition that could be correlated with cheese blowing. The findings suggest that microbial shifts may play a role in the development of cheese defects.

---

**References:**


---

**Note:**

- The table and analysis were conducted by Silva Duarte et al. (2022), with contributions from Forsythe, M. (2022). The study was published in the *Journal of Dairy Science*.
with the control portions of cheese. This is not surprising, as this species is homofermentative, and therefore it cannot be considered directly responsible for gas production.

As regards the family *Propionibacteriaceae*, samples S2, S3A, and S6 evidenced a higher level of these microorganisms in the defective zones compared with their corresponding controls (S2/Ctr2, Log2FC = 5.39; S3a/Ctr3, Log2FC = 8.41; S6/Ctr6, Log2FC = 2.29). Indeed, as reported in Figure 3B, it is possible to verify via EdgeR that the genus *Propionibacterium* is more abundant in those samples categorized with big and regular holes (Log2FC = 2.7) and, to a lesser extent, in samples with small and regular holes. Because these bacteria (e.g., *Propionibacterium freudenreichii* ssp. *shermanii*) are known to be able to develop gas inside cheese wheels (Van Wyk et al., 2018), a situation that can be either of technological importance for some cheeses (e.g., Emmental) or, in contrast, associated with the presence of LB defects (McSweeney, 2007), we could hypothesize that these defects may be caused by such a bacterial category.

Although the remaining categories identified have low relative abundance values, and it is therefore unlikely that these groups make any significant contribution to defect formation, sequences assigned to members of the genus *Acinetobacter* are highly present in samples with big and regular holes (Figure 3C, Log2FC = 3.3). As described elsewhere (Doulgeraki et al., 2012; Zago et al., 2021), this genus has raw milk as a source and, although not associated with the origin of LB defects, is considered an undesirable food spoilage member of resident bacteria in GP processing plants. Last, EdgeR analysis revealed that sequences without a taxonomical assignment are more abundant in cheese samples collected from small holes (Figure 3D). After using MegaBLAST to verify the nucleotide sequences of those OTU classified as not available, including de novo OTU, it is possible to verify that these sequences originate mainly from members of the genus *Lactobacillus*, such as *L. delbrueckii* ssp. *delbrueckii* NBRC 3202 (accession number: AP019750.1; identity: 100%; E-value: 6e-131), *L. rhamnosus* strain LV108 (accession number: CP053619.1; identity: 98.7%; E-value: 1e-122), *S. harbinensis* strain LH991 (accession number: CP045180.1; identity: 99.61%; E-value: 3e-129), and *L. casei* strain FBL6 (accession number: CP074377.1; identity: 100%; E-value: 6e-131).

Because, according to the genetic analysis, it was not possible to identify a specific bacterial group clearly linkable to the presence of the defect in cheese samples with holes classified as “small and regular,” we decided to better inspect a possible involvement of heterofermentative lactobacilli. The genus *Lactobacillus* includes obligate homofermentative lactobacilli, facultative heterofermentative lactobacilli, and obligate heterofermentative lactobacilli (OHL) species (Pot et al., 2014). Only this last group comprises microbes able to produce CO₂ when fermenting hexoses. Among the *Lactobacillus* species detected by metataxonomic analysis, 3 were obligate homofermentative (*L. delbrueckii*, *L. helveticus*, and *L. kefiranofaciens*) and 3 were facultative heterofermentative lactobacilli (*L. zeae*, *S. harbinensis*, and *L. rhamnosus*), whereas no OHL species were detected, although it must be considered that 15% of the *Lactobacillus* sequences were not identified at the species level. The only sugar initially present in milk is the disaccharide lactose, but during cheese ripening other sugars can become available, such as galactose from lactose hydrolysis, exported from bacteria unable to use...
it, and other monosaccharides, such as ribose, coming from bacterial cells lysis during prolonged cheese ripening (Ortakci et al., 2015), which can be fermented by OHL with production of gas.

For this reason, we decided to perform a specific genetic test by DNA amplification to look for the possible presence of the most widespread OHL, namely *Fructilactobacillus sanfranciscensis*, *Levilactobacillus brevis*, *Lentilactobacillus buchneri*, *Limosilactobacillus reuteri*, *Limosilactobacillus fermentum*, and *Lentilactobacillus hilgardii*, using species-specific primer couples. This analysis did not detect the presence of any of the OHL species mentioned previously in any of the samples, which is in accordance with the metataxonomic analysis.

As a further approach, we also used 3 broader primer couples, based on the 16S to 23S rRNA intergenic spacer regions (Song et al., 2000) in a multiplex amplification experiment. The results of this analysis are reported in Figure 4. By looking at each defective sample compared with its respective control, some qualitative differences were visible from the different fingerprints. Samples 5 and 6 show a markedly different fingerprint from their respective controls; samples 7 and 9 appear identical to their controls; and samples 1, 2, 3, and 4 evidence different intensities of some bands with respect to their controls. Overall, this result reveals that in most of the samples (6 out of 8) an unbalanced distribution of *Lactobacillus* groups is observed; the consequence of this on the development of LB defect deserves further investigations.

**CONCLUSIONS**

Based on the results obtained in the present study, we can affirm that the presence of holes in most of the cheese samples examined is not determined by a clearly identifiable bacterial species, as detected by 16S rRNA analysis. Our DNA analysis of OHL did not evidence a significant presence of any of the most widespread obligate homofermentative species. On the contrary, a broader fingerprinting analysis based on intergenic spacer regions amplification revealed some differences in the composition of the *Lactobacillus* population in the area surrounding the defects, with respect to the non-defective zones of the same cheese wheel. Further analyses are therefore needed to better study the microbiota and identify these deleterious bacteria and their involvement in the process of eye formation.
ACKNOWLEDGMENTS
This work was funded by Regione Veneto, Venezia, Italy, under the “Nuovo piano industriale del lattiero-caseario Veneto” (NIP) project (POR-FESR 2014-2020). The authors have not stated any conflicts of interest.

REFERENCES


ORCIDS

Vinícius da Silva Duarte © https://orcid.org/0000-0002-0356-3947
Alessio Giacomini © https://orcid.org/0000-0003-4124-0142