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Effects of bioprotective cultures on the microbial community during storage of Italian fresh filled pasta

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ABSTRACT

Filled pasta is a typical Italian product consisting in a thin dough stuffed with a filling containing dairy, meat or vegetable ingredients. When industrially produced, its microbial stability relies on thermal treatment, proper storage temperature and modified atmosphere packaging. Since these processes can strongly affect the traditional features of pasta (mainly flavor and texture), alternative strategies have been investigated. In this research milder heat treatments were applied and, to assure microbial quality and safety, they were combined with the addition of bioprotective cultures (*Lactobacillus rhamnosus* and *Lactobacillus paracasei*) in the filling of Ricotta based *Tortelloni*. Their effects on microbiological patterns during storage at 6°C was studied through culture dependent and independent methods and also the influence on organoleptic profile (through SPME-GC-MS and sensory evaluation) was assessed. The results demonstrated that bioprotective cultures had a relevant quantitative and qualitative effect on the microbiota of *Tortelloni* during storage: indeed, even if they were not dominant, their presence reduced the initial microbiota associated with raw materials and gave a competitive advantage to safer or organoleptically acceptable LAB species, such as leuconostocs. Although these LAB cultures influenced the aroma profile of filled pasta (increase of alcohols, esters and acids), the sensory evaluation confirmed the overall acceptability of the product. The addition of bioprotective cultures can be therefore a helpful strategy to reduce thermal treatments and better maintain the traditional textural and flavor characteristics of this product.

Key words: bioprotective cultures; filled pasta; shelf life; metagenomics; lactobacilli

1. INTRODUCTION

Filled pasta is a typical Italian product with different shapes and filling varying with the geographical area of production (Alexander, 2000). The pasta consists in a thin dough made with water, flour and usually eggs that is stuffed with a filling prepared with dairy, meat or vegetable ingredients (Marotta et al., 2018). The artisanal products are usually handmade and locally distributed at refrigerated temperature with a limited shelf life (4-5 days). On the contrary, shelf life of 60-90 days and more can be achieved for industrial production by applying thermal treatments, reducing the water activity (a_w) of the filling, packaging the pasta under modified atmosphere (MAP) and adopting appropriate storage temperature (Marotta et al., 2018; Zardetto & Dalla Rosa, 2015).

According to Italian legislation, "fresh pasta" (filled or not) must have a water content lower than 24% and a_w lower than 0.97 and higher than 0.92 (Decreto Repubblica Italiana, 2001) and it must be stored at temperature not higher than 4±2°C.

The microbial stability of this product relies in first instance on thermal treatments, which can be applied following two strategies. In the first case, only a treatment is carried out on the loose product with an injected steam belt pasteurizer. In the second case, this first treatment is followed by a further pasteurization in static chambers after packaging. In addition to the effects on microbial population, both these strategies definitely affect the mechanical and functional properties of the final products (Alampese, Casiraghi, & Rossi, 2008; Zardetto & Dalla Rosa, 2015). After packaging, the shelf life of filled pasta is strictly dependent on the ability of microorganisms survived to the thermal treatments to grow during storage, overcoming the hurdles determined by a_w, MAP composition and storage temperature (Sanguinetti et al., 2011, 2016).

In spite of the great diffusion of this typology of pasta and the increase of its consumption (ISMEA, 2018), there is a relatively scarce literature concerning its microbiological characteristics, mainly focused on the presence of *Enterobacteriaceae*, total mesophilic counts and moulds (Marotta et al., 2018; Ricci, Barone, & Petrella, 2017; Zardetto, 2005).

Tortelloni is a filled pasta produced in Emilia Romagna Region (Italy) since several centuries (Tanara, 1644) with a soft filling obtained mixing Ricotta and Parmesan cheese, eggs, salt, nutmeg and vegetables (beet, spinach or parsley, according to the zone). The productive process, including thermal treatments and a_w lowering, can have a strong impact on the "traditional" features of such type of pasta, inducing to adopt milder conditions to preserve regional traditions and peculiar characteristics of *Tortelloni*. In particular, in order to preserve its softness, often the a_w of the filling cannot be excessively lowered, with the consequent risk of rapid growth of spoiling microflora.

This aspect is in contrast with the shelf life expected for the commercialization of this product (45-60 days). For this reason, other hurdles to the microbial growth must be exploited to satisfy this need while maintaining the traditional organoleptic properties and assuring the hygienic quality of *Tortelloni*.

The use of bioprotective cultures is an interesting strategy proposed with the aims of reducing the risks associated with the growth of undesirable and pathogenic microorganisms and prolonging the shelf life of foods (Oliveira, Ferreira, Magalhães, & Teixeira, 2018). Biopreservation consists in the use of natural and selected microflora able to control or inhibit spoiling or pathogenic microorganisms by competition or production of specific antimicrobial molecules such as bacteriocins, organic acids, diacetyl, acetoin, etc. (Ghanbari, Jami, Domig, & Kneifel, 2014). Lactic acid bacteria (LAB) are ideal candidates for biopreservation due to their safe history in foods and their wide range of antimicrobial compound production (Cifuentes Bachmann & Leroy, 2015). Among the potential drawbacks of the use of bioprotective cultures there is the possibility of undesired sensory effects on the food organoleptic profile and the choice of LAB species must take into consideration a low or compatible impact on food flavor.

This work was aimed to evaluate the effects of the use of bioprotective cultures on the microbiological patterns of Ricotta based *Tortelloni* during production and storage. Filled pasta was produced in a small factory following the traditional recipe. In addition to traditional microbiological protocols, the microbial community profiling was performed through rDNA-targeting pyrosequencing to test the effects of two bioprotective cultures (*Lactobacillus rhamnosus* or a mixture of *L. rhamnosus* and *Lactobacillus paracasei*) on the spoilage microbiota during filled pasta storage. Further, the influence of the cultures on the organoleptic profile of the product was studied. Finally, a validation trial was carried out with the objective to optimize the use of this strategy for the stabilization of this filled pasta.

2. MATERIAL AND METHODS

2.1 Filled pasta production

Filled pasta samples were produced in a small factory located in Emilia Romagna Region (Italy). The *Tortelloni* (50% filling, thickness of pasta 0.7 mm) were obtained with a traditional recipe following the process reported in Figure 1. In particular, pasta was produced using durum wheat semolina (36% w/w) and soft wheat flour (36% w/w) added with pasteurized egg product (28% w/w). Filling was obtained by mixing Ricotta (70.5% w/w), Parmigiano Reggiano cheese (12% w/w), breadcrumbs (12% w/w), parsley (3.5 % w/w), salt (1.5% w/w) and nutmeg (0.5% w/w). The filling rested 18 hours at refrigerate temperature (4°C) and then underwent to the line production process.

The filling pH was about 5.5 $\pm$ 0.1 while its a_w was 0.960. After forming, *Tortelloni* were subjected to a heat treatment in one-line steam belt pasteurizer and a subsequent drying and cooling in a tunnel chamber. After cooling, the filled pasta was packed in polyamide/polypropylene (PA/PP) film under modified atmosphere (40% CO_2; 60% N_2). The film presented a water vapor permeability <5 gr/m²/24h (38°C, 90% R.H.) and an oxygen permeability <3 cc/m²/24h (23°C, 0% R.H.). After packaging, the samples were cooled and stored at 6°C for 30 days.

Two different trials were performed. During the first trial, three different samples were obtained: the control, produced without bioprotective cultures, and two samples inoculated with two different commercial bioprotective cultures available on the market, containing *Lactobacillus rhamnosus* (BC1) or a mixture of *L. rhamnosus* and *Lactobacillus paracasei* (BC2). After appropriate hydration according to the manufacturer's suggestions, these cultures were added (cell concentration of about 7 log CFU/g) in the filling during mixing. In the first trial, a mild pasteurization was carried out in a steam belt pasteurizer set for a treatment of 60°C for 3 min in the product inner part. In the second trial (validation), two samples were produced: the control and the sample with the bioprotective culture BC2 (inoculum level 7 log CFU/g), added as reported above. In this case, the thermal treatment was carried out at 70°C for 3 min in the product inner part. In both treatments, the reaching of target temperature was controlled using a data logger (S-Micro, Tecnosoft, Italy) inserted inside the product.

2.2 Microbial counts, pH and a_w analysis

Three replicates of each sample were examined at the different sampling times (before and after pasteurization and during the shelf life) for the enumeration of microbial population. 10 g of samples were 10-fold diluted with 90 ml of 0.9% (w/v) NaCl and homogenized in a Lab Blender Stomacher (Seward Medical, England) for 2 minutes. Decimal dilutions were performed and plated onto selective media: LAB were enumerated on MRS agar (Oxoid, Basingstoke, UK) incubated at 30°C for 48 h in anaerobic conditions, staphylococci were counted by surface-plating on Baird-Parker (added with egg yolk tellurite emulsion) (Oxoid) incubated at 30°C for 48 h, yeast and moulds were grown on Sabouraud Dextrose Agar (Oxoid) plates, added with 200 mg/l of chloramphenicol and incubated at 28°C for 72 h, while *Enterobacteriaceae* were enumerated by pour plating in Violet Red Bile Glucose agar (Oxoid) and incubating plates at 37°C for 24 h. The presence/absence of *Listeria monocytogenes* and *Salmonella* was evaluated according to the methods EN ISO 11290–1 and EN ISO 6579, respectively (2017).
The pH and a_w of Ricotta filled *Tortelloni* were monitored using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain) and an Aqualab CX3-TE (Labo-Scientifica, Parma, Italy), respectively.

2.3 Aroma profile analysis

For the analysis of volatile compounds, 3 g of samples were placed in 10-ml sterilized vials, added with 33 mg/kg of 4 methyl-2-pentanol (Sigma-Aldrich, Steinheim, Germany) as internal standard and sealed by PTFE/silicon septa. The samples were heated for 10 min at 45°C and the volatile molecules in the headspace were adsorbed with fused silica SPME fiber covered with 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) (Supelco, Steinheim, Germany) for 40 min. Adsorbed molecules were desorbed in the injector for 10 min with the same analytical conditions reported by Montanari et al. (2016), using a an Agilent

Hewlett–Packard 6890 GC gas-chromatograph and a 5970 MSD MS detector (Hewlett–Packard, Geneva, Switzerland) equipped with a CP-WAX 52CB (50 m X 0.32 mm X 1.2 µm) fused silica capillary column. Volatile peak identification was carried out by computer matching of mass spectral data with those of compounds included in NIST 2011 mass spectral library (Scientific Instrument Services, Ringoes, NJ, United States).

2.4 Sensory evaluation of cooked samples

Sensory evaluation was carried out using an affective test (Sanguinetti et al., 2016) involving 40 untrained consumers (age 22-60), among which 20 females and 20 males. Their acceptance of the sample proposed was based on an intensity scale from 0 to 7 (0, dislike; 7, extremely like) for selected attributes (odour intensity, colour, aroma intensity, salty, sourness, bitterness, cohesiveness, chewiness, overall acceptability). The evaluation was carried out in randomized and balanced order. The samples were served to the panelist after a 5 min immersion in boiling water (100°C) without salt added.

2.5 DNA extraction and Sequencing

Total genomic DNA was directly extracted from frozen *Tortelloni* samples by taking approximately 10 g of filled pasta. The samples were dissolved in 90 ml of physiological solution (0.9% NaCl) and were mechanically homogenized in stomacher for 4 minutes at 430 beats per minute. After decanting, 1 ml of the supernatant was collected and subjected to enzymatic treatment towards bacteria (lysozyme) and yeasts (lyticase) at 37°C for 1 h, followed by alkaline lysis with the addition of NaOH and SDS at a final concentration of 0.1 N and 1%, respectively. The extracted DNA was purified by chloroform:isoamyl alcohol 24:1 treatment and precipitated in 0.54 volumes of isopropanol. Finally, the purified DNA was resuspended in water and quantified using Qubit 4 Fluorimeter (ThermoFisher Scientific, Waltham, MA USA). The concentration of the DNA samples was normalized and the sequencing was carried out through Illumina MiSeq platform which generated 300 bp pair-end sequencing reads. The library for Illumina sequencing was generated from V3-V4 variable regions of ribosomal 16S rRNA in order to characterize the bacterial population of the samples.

2.6 Bioinformatics analysis

FASTQ sequence files from Illumina reads were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). A final quality assessment was performed on the remaining reads using the FASTQC quality control tool version 0.11.5.

The FASTQ sequences obtained were analyzed using DADA2 version 1.8 (Callahan et al., 2016) by R 3.5.1 environment. DADA2 implements a new quality-aware model of Illumina amplicon errors without constructing OTUs (Callahan et al., 2016). DADA2 was run as described in https://benjjneb.github.io/dada2/tutorial.html applying the following parameters: trimLeft equal to 30 and truncLen option set to 270 and 200 for the forward and reverse fastq files, respectively.

The taxonomic assignment was performed comparing the amplicon sequence variant (ASV) predicted from DADA2 against SILVA database (version 128, https://www.arb-silva.de/documentation/release-128/). ASVs belonging to taxa classified as external sample (Davis, Proctor, Holmes, Relman, & Callahan, 2018) contaminations were not included in the composition analysis for microbial population as well as for ASVs with low abundance setting a threshold of relative abundance equal to 0.5%.The assignment at specie level for the remaining ASVs was confirmed comparing the nucleotide sequence obtained from the Illumina sequencing with the 16S rRNA sequence of the available type strains included in the LTP database, which represents all bacterial and archaeal taxa with validly published names (Yilmaz et al., 2014).

2.7 Statistical analysis

Three independent samples were analysed for each sampling time, each of which analysed in triplicate. The data were statistically analysed using ANOVA. The Tukey critical difference test was performed to determine differences between samples ($p<0.05$). The presence of significative differences in the sensory test was tested using a non-parametric Mann-Whitney test ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1 Microbial counts

In the first experimental trial, a mild pasteurization was carried out with a double aim: *i)* to have a low impact on the characteristic of *Tortelloni* and *ii)* to have a limited effect on the viability of the microbial populations to better evidence the relationships between bioprotective cultures and wild spoilage microorganisms. Table 1 reports the results of the microbial counts before and immediately after the pasteurization and after 7, 15 and 30 days of storage at 6°C. The pH of the product was 5.7 and did not significantly change during storage in the control, while low reductions (5.5-5.6) were observed in the presence of bioprotective cultures (BC1 and BC2) after 30 days. The initial cell concentrations of fresh filled pasta before pasteurization were rather high, represented mainly by *Enterobacteriaceae* (more than 6 log CFU/g). Yeasts concentration was about 4 log CFU/g. Similar cell concentrations were found for staphylococci, but no coagulase positive colony was detected. Wild LAB in the control were 5.4 log CFU/g while in the samples added with bioprotective cultures reached the expected concentration (more than 7 log CFU/g).

After the mild pasteurization (corresponding to a treatment in the steam belt pasteurizer at 60°C for 3 min) yeasts were subjected to the more severe reduction, since in all the samples they were below the detection limit. *Enterobacteriaceae* and LAB decreased of about 2 log units while staphylococci counts were the less affected by heat (1 log unit reduction or less). These levels are high if compared with other industrial products and are more similar to data reported for artisanal fresh pasta (Ricci et al., 2017), due to the low thermal treatment. Marotta et al. (2018) found that 55% of Italian artisanal fresh filled pasta samples showed high *Enterobacteriaceae* counts (> 4 log CFU/g) while these microorganisms were below the detection limit in industrial samples. Higher thermal treatments (80°C for about 3 min) were able to decrease the concentration of this microbial group of at least 4 log units (Sanguinetti et al., 2011). In addition, Ricci et al. (2017) found a mean value of 4.3 log CFU/g of total mesophilic bacteria in packed industrial products and 5.5 log CFU/g in artisanal ones (unpacked). Chavez-Lopez, Vannini, Lanciotti, & Guerzoni (1998) described a prevalence of *Bacillus* spp. in industrial *Ravioli* with a meat-based filling after the thermal treatment. They suggested that shelf life depended also on the textural and micro-structural changes (particularly protein gelation) induced by the thermal treatment.

In the case of *Tortelloni*, the high survival rate determined a rapid multiplication of microorganisms during storage at 6°C. In particular, LAB counts reached 7 log CFU/g in the control after 15 days, while in the presence of bioprotective cultures the counts were about 8 log CFU/g at the same sampling time and further increased after 30 days.

In the control *Enterobacteriaceae* grew up to 5.9 log CFU/g, while in the samples added with bioprotective cultures their concentration significantly decreased during storage, particularly in the sample added with BC2. The concentration of coagulase negative staphylococci remained rather constant throughout the storage in the control, while the presence of BC1 and BC2 determined a significant decrease of the concentration of this microbial group. Yeasts were always below the detection limit in the presence of the cultures, while concentrations higher than 2 log CFU/g were observed in the control.

Pathogens (*Listeria monocytogenes* and *Salmonella*) were never detected in our samples. Mould growth was never observed in packages. Moulds can be responsible for the spoilage of fresh filled pasta but their growth can be controlled by using MAP packaging. According to Zardetto (2005), concentration of CO_2 up to 15% can stimulate the growth of *Penicillium* species, but higher concentrations rapidly inhibited their multiplication.

3.2 Study of microbial community composition through amplicon sequencing and metagenomics

Firmicutes were dominant in all the samples including the filled pasta before treatment (control). Immediately after production, in the samples without the addition of bioprotective cultures, *Streptococcus* and *Lactococcus* were the dominant genera (Figure 2). In particular, among streptococci, the species detected were *Streptococcus parauberis* (27.7% of ASVs); *Streptococcus uberis* group (11.4%), including the species *S. uberis*, *Streptococcus porcinus* and *Streptococcus pseudoporcinus*; *Streptococcus parasuis* (0.9%) and *Streptococcus salivarius* group (0.8%), including both subspecies of *S. salivarius* and *Streptococcus vestibularis*. Regarding lactococci, *Lactococcus lactis* dominated (18.5% of ASVs) followed by *Lactococcus raffinolactis* group (11.9%), which includes the species *Lc. raffinolactis* and *Lactococcus piscium* and *Lactococcus garviae* group (1.1%), including the species *Lc. garviae* and *Lactococcus formosensis*. Among lactobacilli, the homofermentative and thermophilic species *Lactobacillus delbrueckii* and the *Lactobacillus acidophilus* group, which includes the species *Lt. acidophilus* and *Lactobacillus helveticus*, were the most representative (5.0 and 4.9%, respectively), probably deriving from the whey used for Ricotta production. Among Gram positive, the sample without the addition of bioprotective cultures exhibited the presence of members of the species *Rothia endophytica* (belonging to *Micrococcaceae*) and belonging to the *Bacillus cereus* group (4.9 and 3.0% respectively). Gram negative bacteria were represented by *Hafnia* spp. (1.0%) and member of the genus *Acinetobacter*, which included *Acinetobacter guillouiae* group (0.7%) and *Acinetobacter johnsonii* group (4.1%). As expected, many of these species derive from dairy environment, including lactococci and lactobacilli. In particular, the relevant presence of the species *S. parauberis* and those belonging to the *S. uberis* group (*S. uberis*, *S. porcinus* and *S. pseudoporcinus*) can be related to the use of milk from cows affected by mastitis at some degree. In particular, the species *S. uberis* is implied in recurrent cow clinical mastitis (Jamali et al., 2018). Independently on the initial concentration, these species were able to multiply in the industrial Ricotta used for the filling.

After 15 days of storage in the samples without bioprotective cultures, members of the *S. uberis* group and of the species *S. parauberis* still accounted for a relevant proportion of the total population (3.7 and 20.9% of ASVs), together with *Lc. raffinolactis* group (7.8%). However, the species more represented was those of the *Leuconostoc mesenteroides* group (48.7 % of ASVs), including *Leuc. mesenteroides* and *Leuconostoc pseudomesenteroide*s, which were not detected immediately after pasteurization. The addition of bioprotective cultures changed the quantitative composition of the microbiota. *Leuc. mesenteroides* group remained the most relevant species but its relative presence greatly increased and reached 77.8% in the samples BC1 and 83.0% in the samples BC2. Streptococci and lactococci decreased significantly (especially *S. uberis* group and *S. parauberis*) while the presence of *Carnobacterium gallinarum* group ranged from 3.8% to 3.0% of ASVs in both samples. The detection of ASVs associated to the *Lactobacillus casei* group confirmed the presence of the bioprotective cultures, representing the 4.7 and 5.6% of the ASVs in BC1 and BC2, respectively. *L. casei* group is constituted by the phenotypically and genotypically closely related species *L. casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* (Huang *et al*., 2018). Even if the bioprotective cultures were not able to dominate the pasta microbiota and probably did not survive the thermal treatment, their addition to filling followed by an 18-hour adaptation before production changed significantly the ratios between microbial species during storage. Bacteriocin production is usually maximum in the mid exponential phase or at the end of growth phase (Beshkova & Frankova, 2012) and it is compatible with an accumulation in the overnight incubation of filling. In other words, heat stable bacteriocins can be present in the filling after production, independently of the viability of the cells responsible for their production, due to the thermal treatment. In addition, the inhibition of mastitis streptococci (*S. agalactiae*) by a bacteriocin producing *L. rhamnosus* strain has already been demonstrated by Ruíz et al. (2012).

3.3 Volatilome of filled pasta

The SPME-GC-MS aroma profile of filled pasta immediately after pasteurization and after 30 days of storage is reported in Table 2, where data are express as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). In general, ketones decreased during the storage in the presence of both bioprotective cultures. 2-pentanone, 2-hexanone and 2-heptanone decreased in the samples added with bioprotective cultures after storage, while they remained rather constant in the control. The presence of bioprotective cultures determined also an increase of 2,3-butanedione (diacetyl) and 3-hydroxy-2-butanone (acetoin) immediately after production, due to the overnight culture adaptation in fillings, but these molecules drastically decreased at the end of storage.

Aldehydes generally decreased during storage, especially where the bioprotective cultures were added. This diminution was mainly due to the reduction hexanal, more relevant in the samples BC1 and BC2, and 2-butenal.

Alcohols markedly increased in all the samples during storage, but their presence was almost doubled when bioprotective cultures were added. The main responsible for this change was ethanol, present in low amounts (about 10) in all the samples after production, and found after 30 days at level of 216 in the control and at values higher than 500 in the samples with the bioprotective cultures. Other alcohols increased their concentration, and namely 1-hexanol, 2-heptanol and 2-nonanol; the increase of these latter molecules can be related to the reduction of the corresponding aldehydes. Phenylethyl alcohol and 1-nonanol were found at the end of storage only in the samples with the bioprotective cultures.

Acids, present in similar amounts in all the conditions immediately after pasteurization, were represented by acetic, butanoic, hexanoic and octanoic acids. Acetic acid is produced by heterofermentative LAB as result of heterolactic pathway. However, it is also produced under nutritional stress conditions (scarcity or absence of fermentable sugars) through different pathways starting from pyruvate, which can derive from re-oxidation of lactate or from amino acid metabolism (Montanari et al., 2018; Zotta, Parente, & Ricciardi, 2017). During storage acids remained almost constant in the control, whereas they drastically increased in the samples BC1 and BC2.

A similar behavior characterized the esters, represented mainly by ethyl hexanoate, ethyl acetate, ethyl octanoate and ethyl butanoate. The esterase activity of LAB has been deeply described in dairy and enological environments (Sumby, Grbin, & Jiranek, 2010). Liu, Holland, & Crow (2003) demonstrated that

LAB esterases were active against short chain fatty acid mono- and diglycerides and not against triglycerides. Esterase activity was demonstrated also in leuconostocs by Pedersen, Ristagno, McSweeney, Vogensen, & Ardö (2013). All the molecules described have already been reported as component of dairy product flavor as result of microbial metabolism (Curioni & Bosset, 2002) and their presence can be, at some extent, compatible with a dairy-based filling. Leuconostocs were the dominant microbial component in *Tortelloni* samples, especially in the filled pasta containing protective cultures, and their role described in the aroma formation of dairy products is compatible with the volatile profile described for *Tortelloni* (Hemme & Foucaud-Scheunemann, 2004).

3.4 Validation

The effects of the bioprotective cultures on the spoilage profile and the shelf life of fresh filled pasta were validated in a second trial, applying a higher pasteurization treatment (70°C for 3 min) if compared with the first trial. However, this thermal profile was milder, if compared with those often adopted for this typology of filled pasta (Alampese et al., 2008), with the aim to preserve the characteristic of fresh *Tortelloni*. In this production, only the protective culture BC2 was used, due to its better inhibition effects against enterobacteria and staphylococci observed in the first trial. The results of microbial counts are reported in Table 3. Before pasteurization, LAB showed concentration of 5.7 and 7.0 log CFU/g in the control and in the samples added with the bioprotective culture, respectively. The thermal treatment decreased their presence at about 2 log CFU/g in both samples. However, LAB counts were higher in the samples containing BC2 during storage, reaching counts of about 7 log CFU/g after 30 days of storage at 6°C.
Enterobacteriaceae were below the detection level after the thermal treatment and no growth of this microbial group was observed during storage. By contrast, after 30 days lower counts of coagulase negative staphylococci were observed in the samples containing BC2 (4.2 log CFU/g *vs.* 5.8 log CFU/g). Also yeast concentration was lower in the presence of the culture BC2 (about 2.0 log CFU/g *vs.* 4.4 log CFU/g after 30 days).
The sensory analysis carried out on cooked *Tortelloni* immediately after production and after 30 days of storage evidenced the acceptability of both samples of pasta (Figure 3). After production, six of the attributes (colour, salty, sourness, bitterness, cohesiveness and chewiness), as well as the overall acceptability, did not result significantly different, while two (odour intensity and aroma intensity) received significantly higher scores in the filled pasta containing BC2, probably due to the activity of bioprotective culture during the 18 h resting of the filling. At the end of storage all the attributes showed no significant differences between the two samples (Figure 3).

4. CONCLUSIONS

The addition of bioprotective cultures to the filling of fresh filled pasta had a relevant quantitative and qualitative effect on the bacterial microbiota of the product during storage. Even if the added cultures were never dominant at the end of shelf life, their presence and/or activity during the overnight incubation of filling was sufficient to reduce the initial microbiota associated with raw materials and could drive the evolution of microbial community toward the predominance of safer or more organoleptically acceptable species, such as leuconostocs. Although the presence of these LAB cultures had some important effects on the aroma profile of filled pasta, the overall impact on the sensory attributes after cooking was not perceived by the panel, with the exception of more intense aroma and odour intensity immediately after production. This is attributable to the presence of molecules (such as acetoin and diacetyl) compatible with the Ricotta/cheese based filling of *Tortelloni*. The use of these cultures allowed the application of milder thermal treatments with the aim to better maintain the traditional textural and flavor characteristics of this kind of pasta combined with a higher a_w needed to preserve the softness of the filling.

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FIGURE CAPTIONS

Figure 1: Flow sheet of the Ricotta based *Tortelloni* production.

Figure 2: Microbial community composition at species level for *Tortelloni* samples by 16S rRNA gene V3–V4 region sequencing. The taxa were plotted for their relative abundance in the control sample immediately after production, control sample after 15 days, samples inoculated with *Lactobacillus rhamnosus* (BC1) or a mixture of *L. rhamnosus* and *Lactobacillus paracasei* (BC2) after 15 days of storage.

Figure 3: Sensory data of cooked samples produced during the validation test immediately after production and after 30 days of storage at 6°C. Sample C: control; sample BC2: sample added with a mixture of *L. rhamnosus* and *Lactobacillus paracasei*. For each attribute, the presence of an asterisk indicates significant differences between the two samples.

Table

Table 1: Microbial counts (log CFU/g) and pH values during production and storage of *Tortelloni* not inoculated (control) or inoculated with the bioprotective culture BC1 (*Lactobacillus rhamnosus*) or BC2 (*L. rhamnosus* and *Lactobacillus paracasei*). Results are the mean of three independent repetitions. For each microbial group significant differences between products at each sampling point are indicated by different capital letters.

Microbial group	Sample	Before pasteurization	After pasteurization*	T7	T15	T30
LAB	**Control**	5.43^{A}	3.83^{A}	4.03^{A}	7.08^{A}	7.14^{A}
	BC1	7.39^{B}	5.27^{B}	6.64^{B}	7.90^{B}	8.38^{B}
	BC2	7.28^{B}	5.31^{B}	6.56^{B}	8.03^{B}	8.55^{B}
Staphylococci	**Control**	3.87^{AB}	2.93	2.81^{A}	3.10	3.06^{A}
	BC1	4.12^{A}	3.30	3.77^{B}	3.46	3.10^{A}
	BC2	3.65^{B}	3.17	2.97^{A}	3.34	2.44^{B}
Yeasts	**Control**	3.96	<1**	<1	2.69^{A}	2.48^{A}
	BC1	4.07	<1	<1	$<1^{B}$	$<1^{B}$
	BC2	3.98	<1	<1	$<1^{B}$	$<1^{B}$
Enterobacteriaceae	**Control**	6.20	4.07^{A}	4.57	5.91^{A}	4.95^{A}
	BC1	6.39	4.52^{B}	4.21	3.29^{B}	2.57^{B}
	BC2	6.41	4.48^{B}	4.56	2.13^{C}	$<1^{C}$
pH	**Control**	5.70	5.88	5.77^{A}	5.85^{A}	5.74^{A}
	BC1	5.74	5.90	5.92^{B}	5.90^{A}	5.45^{B}
	BC2	5.70	5.94	5.98^{B}	5.61^{B}	5.58^{C}

* 60°C for 3 min in the product inner part

** Below detection limit (1 log CFU/g). In order to apply ANOVA these values have been numerically treated as 1.

Table 2: Volatile compounds detected by SPME-GC-MS in the samples immediately after pasteurization and after 30 days of storage at 6°C. Data are expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). For each sampling time, different capital letters represent statistically significant differences ($p<0.05$) between samples according to Tukey test of the two-way ANOVA.

Volatile compounds	**After pasteurization***			**After 30 days of storage**		
	Control	**BC1**	**BC2**	**Control**	**BC1**	**BC2**
Acetone	6.88^{A}	6.71^{A}	6.04^{A}	6.79^{A}	-^{**B}	-^{B}
2-butanone	4.64^{A}	3.15^{B}	2.22^{C}	2.81^{B}	-^{D}	-^{D}
2,3-butanedione	1.86^{A}	22.15^{B}	18.30^{B}	1.22^{A}	0.90^{A}	1.04^{A}
2-pentanone	26.83^{A}	27.03^{A}	26.90^{A}	19.07^{B}	2.00^{C}	2.64^{C}
2-hexanone	49.92^{A}	38.16^{B}	33.58^{B}	44.37^{A}	33.75^{B}	27.25^{B}
2-heptanone	89.79^{A}	98.90^{AB}	105.19^{B}	99.95^{AB}	64.09^{C}	89.66^{A}
2-octanone	1.89^{A}	1.87^{A}	2.24^{A}	1.96^{A}	1.48^{A}	1.81^{A}
3-hydroxy-2-butanone	3.94^{A}	13.12^{B}	11.17^{B}	-^{C}	-^{C}	-^{C}
2-nonanone	34.60^{A}	38.75^{A}	39.16^{A}	40.46^{A}	28.78^{B}	38.17^{A}
2-undecanone	5.57^{A}	5.80^{A}	5.92^{A}	6.81^{AB}	6.47^{A}	7.42^{B}
Total ketones	**225.92^{A}**	**255.64^{A}**	**250.72^{A}**	**223.44^{A}**	**137.47^{B}**	**167.98^{C}**
3-methyl - butanal	2.33^{A}	2.15^{A}	2.27^{A}	3.53^{B}	1.65^{A}	1.64^{A}
2-butenal	7.03^{A}	6.33^{A}	5.12^{A}	7.38^{A}	-^{B}	-^{B}
Hexanal	28.38^{A}	25.17^{A}	23.67^{A}	8.57^{B}	3.92^{C}	4.59^{C}
Nonanal	5.33^{AB}	6.99^{A}	6.55^{A}	6.29^{A}	4.53^{B}	6.83^{A}
Decanal	2.96^{A}	3.72^{A}	3.62^{A}	3.91^{A}	2.78^{A}	1.94^{B}
Benzaldehyde	4.94^{A}	6.17<^{B}	7.29^{B}	1.98^{C}	1.56^{C}	-^{D}
Benzenacetaldheyde	4.19^{A}	4.71^{A}	4.04^{A}	6.21^{B}	5.30^{AB}	6.32^{B}
Total aldehydes	**55.16^{A}**	**55.24^{A}**	**52.56^{A}**	**37.88^{B}**	**19.74^{C}**	**21.31^{C}**
Ethyl alcohol	10.17^{A}	8.33^{AB}	7.19^{B}	216.58^{C}	571.67^{D}	508.84^{D}
2-pentanol	5.83^{A}	6.54^{A}	6.96^{A}	10.27^{B}	8.86^{C}	9.39B^{C}
1-butanol	2.89^{A}	1.94^{A}	1.58^{A}	2.16^{A}	4.35^{B}	3.16^{AB}
1,2-cyclopentanediol, 3-methyl	1.71^{A}	1.80^{A}	2.90^{B}	1.33^{A}	2.80^{B}	2.68^{B}
1-pentanol	4.00^{A}	3.52^{A}	3.87^{A}	5.44^{B}	5.20^{B}	5.58^{B}
2-heptanol	7.88^{A}	8.15^{A}	8.03^{A}	8.42^{A}	21.78^{B}	21.63^{B}
3-penten-2-ol	-^{A}	2.33^{B}	2.60^{B}	0.93^{C}	0.77A^{C}	1.86B^{C}
1-hexanol	7.58^{A}	12.33^{B}	11.62^{B}	12.93^{B}	23.92^{C}	23.91^{C}
1-octen-3-ol	1.45^{A}	1.31^{A}	1.26^{A}	2.58^{B}	2.25^{B}	2.58^{B}
1-hexanol-2-ethyl	2.80^{A}	3.33^{A}	3.24^{A}	4.24^{B}	3.16^{A}	4.28^{B}
2-nonanol	2.20^{A}	2.50^{A}	2.47^{A}	2.62^{A}	9.19^{B}	8.99^{B}
1-nonanol	-^{A}	-^{A}	-^{A}	-^{A}	2.21^{B}	2.43^{B}
Phenylethyl alcohol	-^{A}	-^{A}	-^{A}	-^{A}	3.67^{B}	4.24^{B}
Total alcohols	**46.50^{A}**	**52.08^{A}**	**51.71^{A}**	**267.49^{B}**	**659.83^{C}**	**599.58^{C}**
Acetic acid, ethyl ester	2.81^{A}	1.83A^{B}	1.34^{A}	4.54C	46.22^{D}	57.09^{E}
Butanoic acid, ethyl ester	31.69^{A}	24.30^{B}	20.00^{B}	44.22^{C}	53.60^{D}	36.63^{AC}
Hexanoic acid, ethyl ester	52.63^{A}	58.12^{A}	66.93^{B}	66.96^{B}	120.58^{C}	97.81^{D}
Heptanoic acid, ethyl ester	1.33^{A}	1.19^{A}	1.30^{A}	1.81^{A}	2.49^{B}	2.58B^{A}
Octanoic acid, ethyl ester	7.45^{A}	6.06^{AB}	5.43^{B}	11.04^{C}	20.57^{D}	23.68^{D}
Decanoic acid ethyl ester	2.53^{A}	2.30^{A}	2.15^{A}	3.91^{B}	9.34^{C}	7.99^{C}
Pentanoic acid, ethyl ester	2.36^{A}	3.00^{A}	2.98^{A}	-^{B}	6.63^{C}	6.05^{C}
Total esters	**100.82^{A}**	**96.80^{A}**	**100.13^{A}**	**132.49^{B}**	**259.42^{C}**	**231.84^{C}**

Acetic acid	16.72^{A}	16.06^{A}	15.68^{A}	14.37^{A}	84.39^{B}	82.63^{B}
Butanoic acid	79.33^{A}	81.13^{A}	80.59^{A}	71.41^{A}	183.63^{B}	165.14^{B}
Hexanoic acid	95.57^{A}	100.27^{A}	104.90^{A}	81.54^{A}	249.19^{B}	229.06^{B}
Octanoic acid	28.00^{A}	38.41^{AB}	41.16^{B}	24.57^{A}	71.86^{C}	65.68^{C}
Total acids	**219.62^{AB}**	**235.87^{A}**	**242.33^{A}**	**191.89^{B}**	**589.07^{C}**	**542.51^{C}**

* 60°C for 3 min in the product inner part

** Not detected under the adopted conditions. For two-way ANOVA the samples under the detection limit were set as 0.

Table 3: Microbial counts (log CFU/g) and pH during production and storage of *Tortelloni* not inoculated (control) or inoculated with the bioprotective culture BC2 (*Lactobacillus rhamnosus* and *Lactobacillus paracasei*) during the validation test. Results are the mean of three independent repetitions. For each microbial group significant differences ($p < 0.05$) between the two products at each sampling point are indicated by the presence of an asterisk.

Microbial group	Sample	Before pasteurization	After pasteurization[1]	T7	T15	T30
LAB	**Control**	5.67*	2.44	1.95*	4.47*	6.70
	BC2	7.02	1.95	4.48	5.14	6.94
Staphylococci	**Control**	3.33	<1[2]	2.45*	3.70*	5.82*
	BC2	3.63	<1	<1	2.15	4.24
Yeasts	**Control**	2.80	1.32	1.33*	2.66*	4.37*
	BC2	2.41	1.51	<1	1.56	1.97
Enterobacteriaceae	**Control**	4.68	<1	<1	<1	<1
	BC2	4.51	<1	<1	<1	<1
pH	**Control**	5.39	5.38	5.73	5.71	5.64
	BC2	5.47	5.43	5.73	5.80	5.66

[1] 60°C for 3 min in the product inner part

[2] Below detection limit (1 log CFU/g). In order to apply ANOVA these values have been numerically treated as 1.

Figure 1

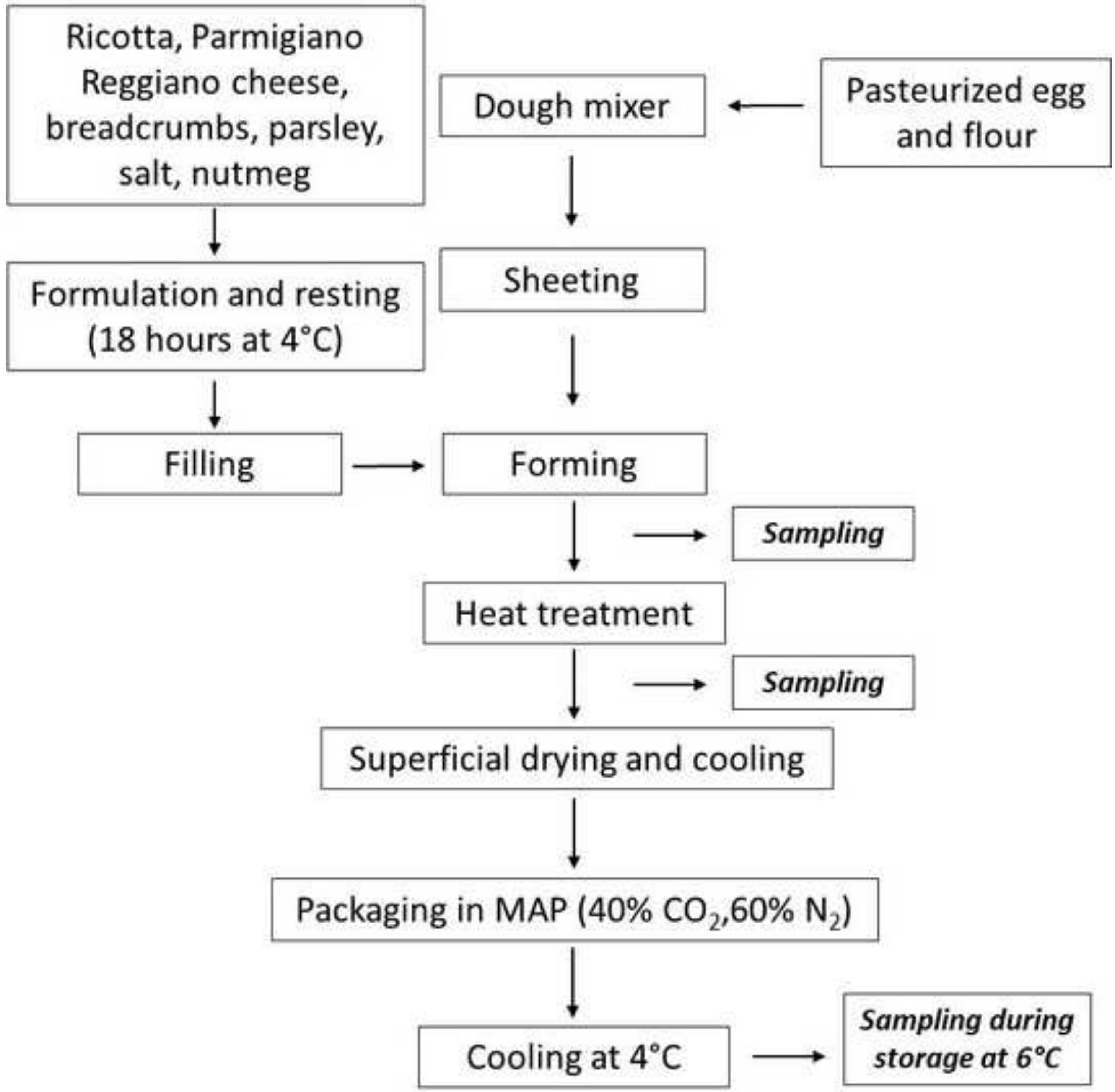

Ricotta, Parmigiano Reggiano cheese, breadcrumbs, parsley, salt, nutmeg
Dough mixer
Pasteurized egg and flour
Formulation and resting (18 hours at 4°C)
Sheeting
Filling
Forming
Sampling
Heat treatment
Sampling
Superficial drying and cooling
Packaging in MAP (40% CO_2,60% N_2)
Cooling at 4°C
Sampling during storage at 6°C

Figure 2

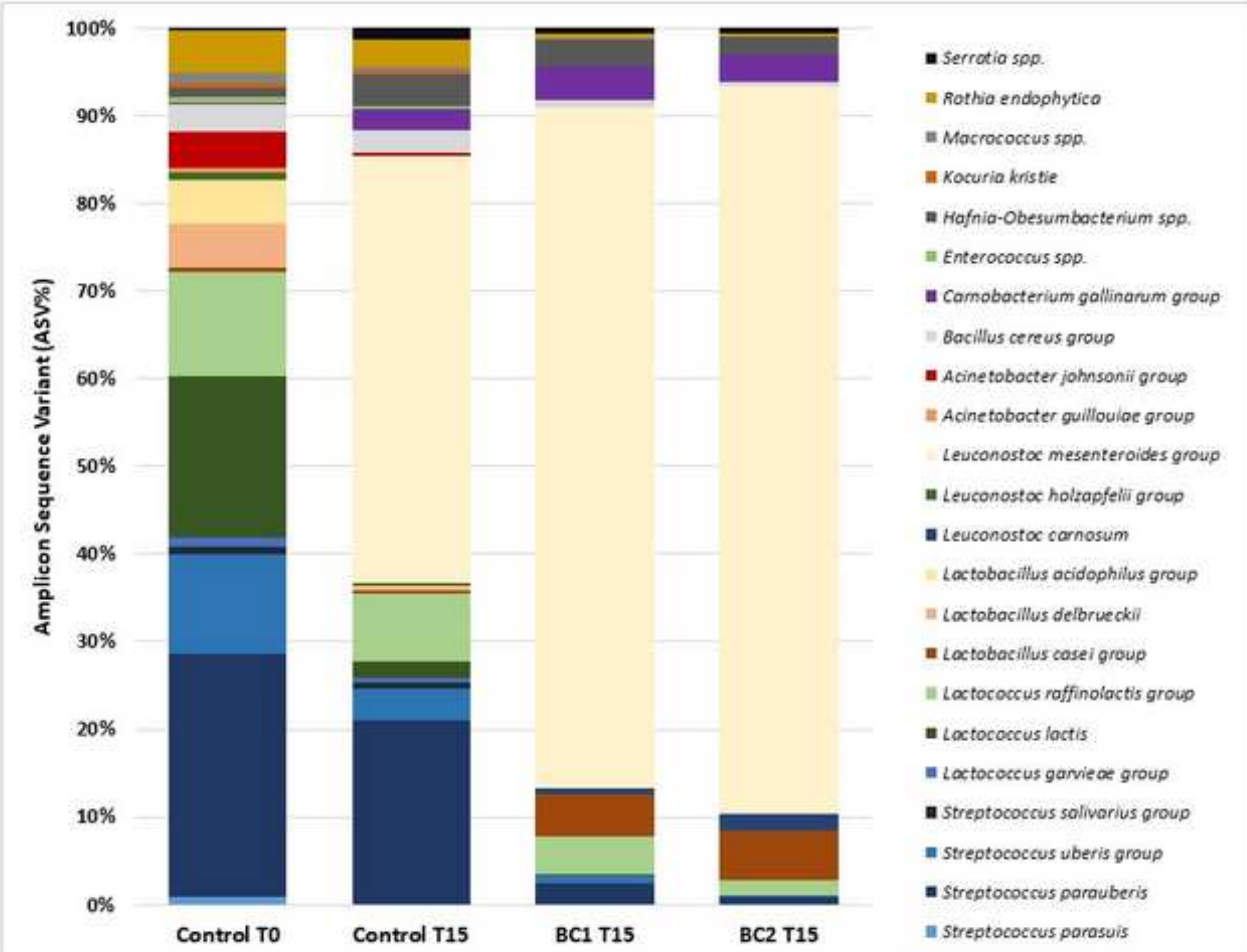
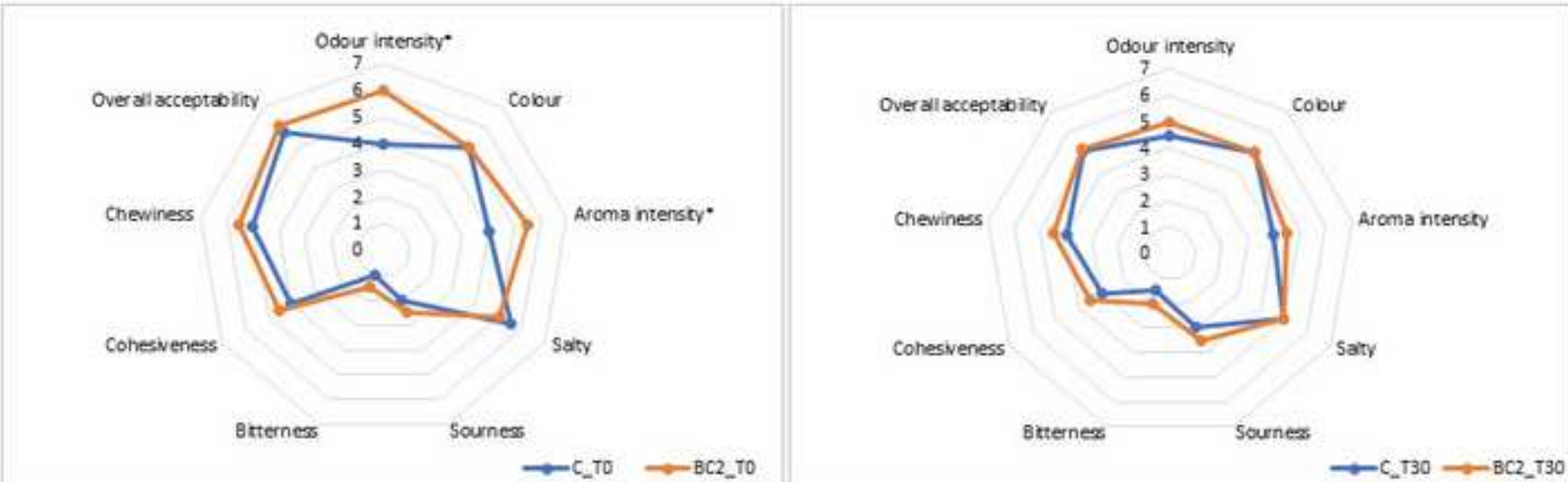

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Amplicon Sequence Variant (ASV%)
Control T0
Control T15
BC1 T15
BC2 T15
Serratia spp.
Rothia endophytica
Macrococcus spp.
Kocuria kristie
Hafnia-Obesumbacterium spp.
Enterococcus spp.
Carnobacterium gallinarum group
Bacillus cereus group
Acinetobacter johnsonii group
Acinetobacter guillouiae group
Leuconostoc mesenteroides group
Leuconostoc holzapfelii group
Leuconostoc carnosum
Lactobacillus acidophilus group
Lactobacillus delbrueckii
Lactobacillus casei group
Lactococcus raffinolactis group
Lactococcus lactis
Lactococcus garvieae group
Streptococcus salivarius group
Streptococcus uberis group
Streptococcus parauberis
Streptococcus parasuis

Figure 3



Odour intensity*
Colour
Aroma intensity*
Salty
Sourness
Bitterness
Cohesiveness
Chewiness
Overall acceptability
7
6
5
4
3
2
1
0
C_T0
BC2_T0
Odour intensity
Colour
Aroma intensity
Salty
Sourness
Bitterness
Cohesiveness
Chewiness
Overall acceptability
C_T30
BC2_T30