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Changes in the microbiota and colour of pork meat batter during refrigerated storage in relation to the application of High Hydrostatic Pressure

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ABSTRACT

The aim of this work was to study the effect of High Hydrostatic Pressures (HHP) on microbial degradation pattern and colour stability of pork meat batters used to produce fresh sausages. Besides treatments at 300 or 600 MPa for 5 min, pH (5.8 *vs* 5.4) and nitrite addition (100 *vs.* 0 mg/kg) were considered as variables. A not treated control was also monitored. The different samples were stored at 4 °C and periodically analysed for their microbiota and colour. At the end of shelf life (TMC > 6.5 log CFU/g) a metagenomic analysis was also carried out to better clarify the effect of the treatments on microbial spoilage patterns. The results showed that the treatment at 300 MPa slightly affected initial cell load, but slowdown growth kinetics, especially at low pH, while 600 MPa strongly reduced microbial spoilage. The application of HHP was able to prolong shelf life of meat batters, from about two weeks (control) to 40 and 80 days in the case of treatments at 300 and 600 MPa, respectively. Metagenomic analysis revealed different degradation patterns in relation to tested variables: untreated samples showed the prevalence of *Brochothrix* spp., while the application of HHP determined the presence also of *Carnobacterium*, *Bacillus*, and *Staphylococcus*, depending on the treatment and the meat batter pH. Concerning colour, differences were mainly observed in redness (a*) and were due to the presence of nitrite. These findings suggest that HHP can be a feasible strategy to stabilize fresh sausages, although further studies are needed to assess their safety.

1. Introduction

Fresh unfermented sausages are the result of mixing lean and fatty minced meat, usually of pork origin, with a mincing degree which may differ according to local recipes (Seman et al., 2018). In Italy, NaCl (2.5%–3.5%) and spices (mainly black pepper, but also fennel, garlic, etc.) are generally added and the meat mixture is stuffed into natural casings (sheep or pig) and must be cooked for safety reasons before consumption. Due to the high pH (approx. 5.8) and high a_w (0.97 or more), the product must be stored at 0–4°C and has a short shelf life (of approximately 10–15 days). In many cases, refrigeration is coupled with the use of modified atmosphere packaging (Cocolin et al., 2004; Raimondi et al., 2018; Tremonte et al., 2005). The main causes of degradation of these products depend on the growth of the microorganisms present in the raw materials, with a consequent production of off-odour and off-flavour, on colour changes determined by the myoglobin status, and on oxidative reactions due to the high fat content (Hugo & Hugo, 2015).

Therefore, the first factor determining the shelf life of fresh sausages is certainly the initial microbial load characterizing the meat batter, which can be highly variable, with values ranging from 4 to 6 log CFU/g (Cocolin et al., 2004; Kamdem et al., 2007; Nuvoloni et al., 2012). In addition, these contaminants may include pathogenic species that, although the product is cooked before consumption, may result in cross-contamination during preparation, even at domestic level (De Cesare et al., 2007).

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Among those responsible for degradation, microbial groups with psychrotrophic attitudes are often prevalent: *Brochothrix*, *Carnobacterium*, *Serratia*, and *Yersinia*. Heterofermentative lactic acid bacteria (LAB) can also cause alteration (*Leuconostoc, Weissella*) together with some lactobacilli (*Latilactobacillus sakei, Latilactobacillus graminis, Latilactibacillus curvatus*) (Benson et al., 2014; Cocolin et al., 2004; Fougy et al., 2016; Raimondi et al., 2018).

From a commercial point of view, the perishability of fresh sausages and the resulting reduced shelf life are factors limiting the market expansion of these products. Therefore, much research has been directed towards finding possibilities for delaying microbial growth and chemical processes that alter the saleability of the product. The application of treatments that reduce the microbial population is often a suitable strategy to enhance safety and extend the shelf life of food products (dos Santos Rocha et al., 2022). Given the impossibility of heat-treating the fresh sausages, the use of non-thermal treatments may prove decisive. Among the latter, the use of High Hydrostatic Pressures (HHP) is particularly interesting. During this treatment, after packaging in suitable material (resistant to pressure), the food is subjected to hydrostatic pressure of several hundred MPa (200–600 MPa) at room or chilled temperature for a time usually not longer than 5 min (Aganovich et al., 2021). The death of microbial cells due to HHP is a multifactorial event caused by the accumulation of damages which can involve cell molecules (*i.e.*, protein/enzyme denaturation), structures (membrane fluidity modification, ribosome disruption, effects on cell wall) and DNA (Rendueles et al., 2011; Simonin et al., 2012). For these reasons, the cell death kinetics are not usually linear. In general, spores are highly more resistant than vegetative cells and, among the latter, Gram-negative bacteria are more susceptible than Gram-positive ones (Syed et al., 2016). While the main process parameters affecting the microbial deactivation are the pressure applied and the time of the treatment, among intrinsic factors the effects of pH, a_w and food composition have been studied. Nevertheless, HHP-processed foods cannot usually be considered sterilized and must be stored under refrigeration to maintain their sensory characteristics and microbiological stability (Rendueles et al., 2011). The antimicrobial efficacy of HHP has been demonstrated in several types of animal-derived products, such as sliced fermented sausages (Possas et al., 2019; Ramírez et al., 2022), whole fermented sausages (Rubio et al., 2018) and meat batter before fermentation (Omer et al., 2015). However, concerning fresh sausages, the only HHP application, to our knowledge, is focused on the reduction of *Listeria monocytogenes* at 414 MPa for 60 min (Murano et al., 1999).

Despite its negative effect on the colour, also meat products have shown good application potential (Simonin et al., 2012; Zhou et al., 2010). Indeed, the colour of meat depends in the first instance on the status of myoglobin, which contains a heme ring with a central iron atom. The valence of iron and the ligand present are responsible for meat colour (Mancini & Hunt, 2005). The application of HHP can cause partial denaturation (in relation to the entity of treatment) of this molecule causing discoloration related to myoglobin denaturation and oxidation of ferrous to ferric myoglobin (Carlez et al., 1995). These negative effects can be attenuated by adding chemical treatment with salts commonly used for cured meat, such as nitrite and ascorbate (Giménez et al., 2015), even if the colour is modified with respect to the untreated meat (Rubio et al., 2007). In addition, the use of nitrite for improving the colour after HHP treatment, can have an important microbial consequence. In fact, the products are generally treated under vacuum, and the survival of spores of psychrotropic *Clostridium botulinum*, which can pose serious concerns, can be avoided using nitrite.

The aim of this work was to evaluate the microbial degradation pattern in meat batters used to produce fresh pork sausages, packed under vacuum and treated with HHP at 300 and 600 MPa for 5 min. The meat batters were also differentiated in terms of pH value (5.4 and 5.8) and for the presence (100 mg/kg) or absence of nitrite. The study was focused on the evaluation of the growth of the different microbial groups during storage at 4°C, compared with untreated products. A metagenomic analysis was carried out in the different meat batters at the end of storage to deeply define the effect of the treatments on the microbial spoilage patterns. In addition, the meat colour was monitored overtime as it represents an important feature of this type of product for assuring the consumers' acceptability.

2. Materials and methods

2.1. Raw pork meat batter preparation and HHP treatment

Four meat batters with different pH values (5.4 and 5.8) and with (N) or without (F) added nitrate were produced in an industrial plant (CLAI, Cooperativa Lavoratori Agricoli Imolesi, Imola, Italy). Fresh pork meat and adhering subcutaneous fat (40 kg) from hot deboning of the shoulders was obtained and used after 24 h post-mortem for mixture preparation. The ratio of lean meat/fat was approx. 80:20. The raw meat was coarsely ground (4–5 mm) at 0°C and mixed with NaCl 2.2% w/w, grounded black pepper 0.1% w/w and ascorbate 0.02% w/w. This batter was divided into two batches: the first was maintained at its natural pH (5.8) while, in the second batch, the pH was lowered at 5.4 using L-lactic acid (80% solution, Enologica VASON, San Pietro in Cariano, Verona, Italy). The two batters with different pH were further divided into two batches, one of which added with $NaNO_2$ at a concentration of 0.01% (Europrodotti Food Ingredients, Concorezzo, MB, Italy).

Meat batter samples (approx. 100 g) were vacuum packaged and stored under refrigeration conditions (4°C) overnight. The samples obtained by each meat batter were respectively split into three sub-batches for HHP treatment and, after 24 h, two of them were treated at 300 MPa or 600 MPa for 5 min. The last sub-batch was considered a control treatment (NT), not processed with HHP.

High pressure treatment on the under vacuum meat batters was carried out using an industrial plant AVURE AV-50X (HPP Italia, Traversetolo, PR, Italy). The process parameters were set to obtain treatments at 300 and 600 MPa for 5 min, at an initial temperature of 4°C. The treatment resulted in an increase in temperature of 10°C and 16°C for the samples treated at 300 MPa and 600 MPa, respectively.

The experimental plan is shown in Fig. 1. All the samples were stored at 4 °C and monitored for different storage periods on the basis of their microbial cell load. They were analysed for different times, depending on the treatment applied: 18 days for not treated samples, 43 for batter treated at 300 MPa, 90 days for samples treated at 600 MPa. Time zero (0 days) pertains to samples analysed immediately after the batter production before HHP treatment, while Time 1 (1 day) refers to the samples analysed immediately after the HHP treatment.

2.2. Microbiological analyses

For microbial analyses, approx. 10 g of the sample were transferred into a sterile stomacher bag, mixed with 90 ml of 0.9% (w/v) NaCl sterile solution and homogenized in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. Appropriate decimal dilutions were prepared and plated onto selective media to detect: total mesophilic counts (TMC) onto Plate Count Agar (PCA); lactic acid bacteria (LAB) by using de Man-Rogosa-Sharpe (MRS) agar, added with 200 mg/l of cycloheximide; coagulase-negative cocci (CNC) enumerated onto Mannitol Salt Agar (MSA). All these media were incubated at 30 °C and the microbial populations were enumerated after 48 h. Moreover, Enterobacteriaceae were detected onto Violet Red Bile Glucose Agar (VRBGA) after 24 h at 37°C. All media were provided by Oxoid (Basingstoke, UK). Each analysis was performed in triplicate.

2.3. Determination of pH

The evolution of pH during processing and storage was measured using a pH-meter HD2105.2 (Delta Ohm, Padova, Italy) by weighing 10 g of each meat batter at each sampling time, adding an equal amount of

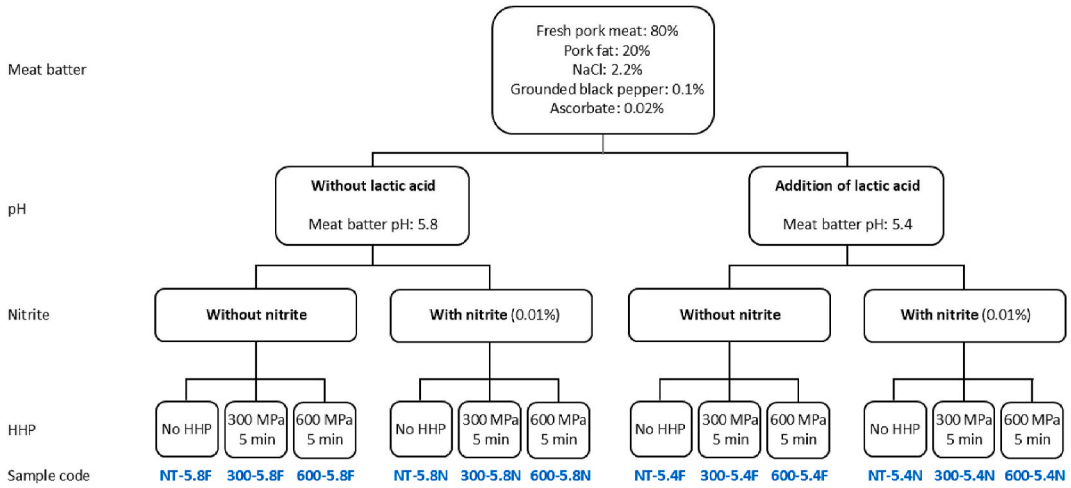

Fig. 1. Experimental plan with sample codes for each condition tested.

distilled water and then homogenizing in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. The analysis was performed in triplicate.

2.4. Metagenomic analysis

Total genomic DNA was extracted from approx. 2 g of frozen samples collected at the initial time (meat mixture) and at the end of storage through a lysozyme treatment at 37°C for 1 h. Subsequently, samples were subjected to mechanical lysis with glass beads through TissueLyser II (Qiagen, Germantown, USA) with a frequency of 30 Hz for 1 min, followed by proteinase K treatment for 30 min at 60°C. Then, the extracted DNA was purified using a DNeasy mericon Food Kit (Qiagen) and resuspended in TrisHCl 10 mmol/l to be quantified with a Qubit 4 Fluorimeter (ThermoFisher Scientific, Waltham, USA).

Subsequently, two amplification steps were carried out through Illumina 16 S Metagenomic Sequencing Library Preparation protocol to obtain libraries. In particular, an initial 35 cycle PCR amplification using 16S rDNA V3-V4 specific PCR primers (16 S-341 F 5′-CCTACGGGNBGCASCAG -3′ and 16S-805 R 5′- GACTACNVGGGTATC-TAATCC -3′) was performed, followed by a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131- 1001/FC-131-1002). Libraries were sequenced on NovaSeq instrument (Illumina, San Diego, USA) using 300 bp paired-end mode. Base calling, demultiplexing and adapter masking were carried out through Illumina BCL Convert v3.9.3 (https://emea.support.illumina.com/). The taxonomic assignment of the reads was determined by Kraken 2 (Wood et al., 2019). Subsequently, Bracken was run on the Kraken 2 output files for the estimation of the relative abundance for each taxon identified (Lu et al., 2017), producing reports suitable for visualization with Pavian (Breitwieser & Salzberg, 2020). Data were expressed as relative reads percentage (%) with respect to the total reads generated by the sequencing.

2.5. Colour measurement

The colour (CIE L* = lightness, a* = redness, and b* = yellowness) was determined through a reflectance colorimeter (mod. Chroma Meter CR-400, Minolta, Milan, Italy) equipped with an illuminant source (C) and previously calibrated with a reference colour standard ceramic tile (King et al., 2023). For each sample, ten measurements were carried out due to the uneven colour of the meat mixture and the presence of fat particles.

Differences in colour (L*a*b*) at the same sampling time were assessed using the One-way ANOVA. Mean differences were separated using Tukey's HSD post hoc test at a significance level of 0.05.

3. Results

3.1. Microbiological counts

Total mesophilic bacteria, lactic acid bacteria, enterobacteria and staphylococci were considered in microbiological counts. The results obtained for the meat batters stored at 4°C are reported in Figs. 2–4.

Among the factors limiting the shelf life of foods, including fresh meat products, microbial growth is one of the most relevant. High microbial counts may lead to the production of off-flavours, off-odours, pH changes, production of slime and exudates as well as discolorations. In general, it is impossible to exactly define the microbial threshold after which these changes are perceivable by consumers, but it is usually accepted that a cell concentration of 7 log CFU/g is a limit after which the product can be considered spoiled (McMeekin et al., 2006; McMeekin & Ross, 1996). This is particularly true for meat, a substrate poor in carbohydrates and rich in amino acids, where the use of nutrients easily brings to the production of end products with high sensory impact (Odeyemi et al., 2020). For this reason, in this work a threshold of 6.5 log CFU/g was chosen as an indicator for the shelf life length.

In the meat batter, the initial TMC was approx. 4 log CFU/g. In all the samples not treated with HHP (NT), counts higher than 6.5 log CFU/g were observed between 15 and 18 days of storage (Fig. 2a). The batter with pH 5.8 without nitrite (NT-5.8 F) showed a more rapid growth kinetic if compared to the others. In any case, all the not treated samples after 18 days can be considered spoiled.

The application of a treatment at 300 MPa caused a limited reduction of the initial TMC (about 0.5 log unit) but determined a relevant slow-down of the growth kinetics (Fig. 2b). In general, considering the threshold concentration of 6.5 log CFU/g, the shelf life was considerably incremented, and a storage period of 43 days was considered. The sample 300-5.8 F was characterized by the fastest growth rate among the samples treated at 300 MPa. This sample reached a concentration of TMC of 6.5 log CFU/g after about 25 days, which increased up to approx. 8 log CFU/g at the end of the storage period (43 days). The batter with the same pH but containing nitrite (300-5.8 N) was characterized by a similar total final concentration but with a lower growth rate (the reaching of the threshold was between 30 and 35 days of storage). The acidified batter (pH 5.4) showed a lower TMC after 43 days of storage. In particular, the presence of nitrite in these latter samples allowed to maintain the microbial concentration below 5 log CFU/g throughout the storage time, while, in the absence of nitrite, the TMC was about 6 log CFU/g.

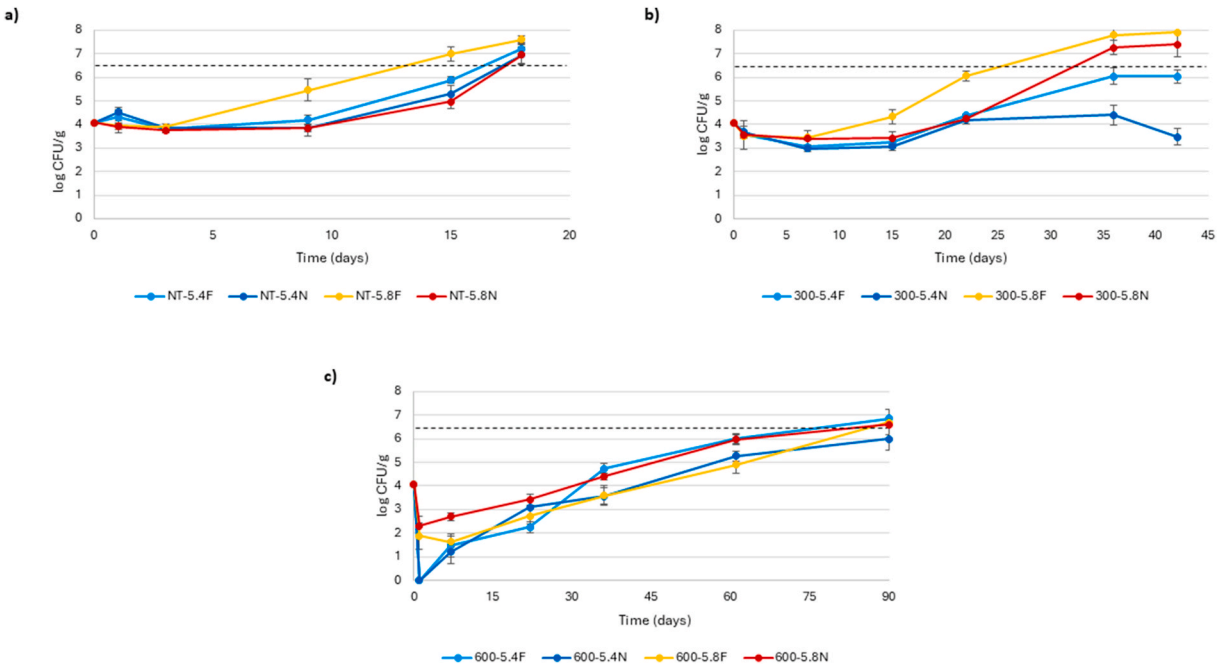

Fig. 2. Total mesophilic counts (TMC) during storage of meat batters. The data reported are relative to the not treated samples (a) and to the samples treated at 300 (b) and 600 (c) MPa. The dotted line represents the threshold used as shelf life indicator (6.5 log CFU/g).

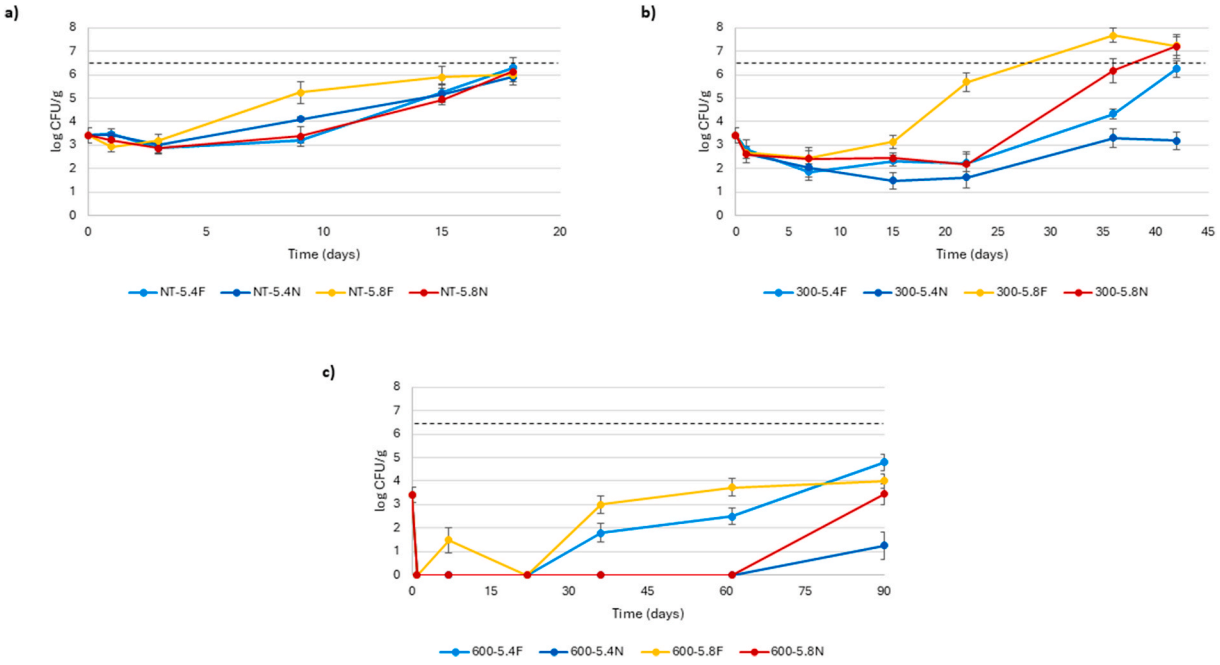

Fig. 3. Lactic acid bacteria (LAB) during storage of meat batters. The data reported are relative to the not treated samples (a) and to the samples treated at 300 (b) and 600 (c) MPa. The dotted line represents the threshold used as shelf life indicator (6.5 log CFU/g).

The application of 600 MPa treatment determined a reduction of TMC of approx. 2 log units in the batter at pH 5.8, while in the acidified batter (pH 5.4) the counts were below the detection limit (<1 log CFU/g), independently of the presence of nitrite (Fig. 2c). In general, a relevant increase of the shelf life was obtained. The samples were stored for 90 days, after which concentrations slightly higher than 6.5 log CFU/g were observed, except for the sample 600-5.4 N, in which counts lower than this threshold were found also at the end of the storage. In the other conditions, the threshold level was reached after about 65, 70 and 80 days of storage in the samples 600-5.4 F, 600-5.8 N and 600-5.8 F, respectively.

Concerning LAB (Fig. 3), the initial counts on MRS were approx. 3.4 log CFU/g. In the samples not treated with HHP, their behaviour was similar to TMC, but their final concentrations were lower. In all samples, LAB reached counts of approx. 6 log CFU/g at the end of the storage period (18 days) (Fig. 3a). In the batter treated at 300 MPa (Fig. 3b), the samples in which pH was not modified presented a more rapid growth of this microbial group, with the sample 300-5.8 F reaching a concentration of almost 7 log CFU/g after about 30 days of storage. The sample 300-5.8 N reached similar cell loads after about 40 days from the

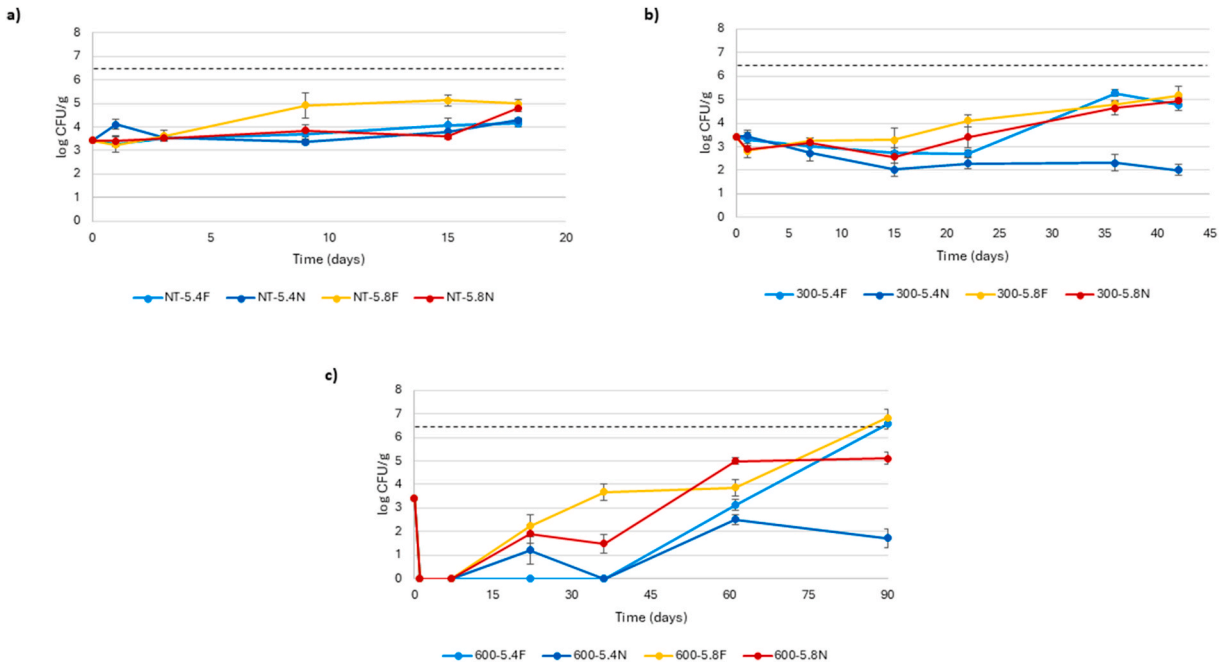

Fig. 4. Staphylococci counts during storage of meat batters. The data reported are relative to the not treated samples (a) and to the samples treated at 300 (b) and 600 (c) MPa. The dotted line represents the threshold used as shelf life indicator (6.5 log CFU/g).

production. On the contrary, in the samples acidified at pH 5.4 (300-5.4 N and 300-5.4 F), the threshold of 6.5 log CFU/g was never achieved. Immediately after the treatment at 600 MPa the LAB counts were below the detection limit. A cell recovery was observed after 20 or 60 days of storage in the samples without or with nitrites, respectively. Anyway, in all the samples the LAB counts never exceeded 5 log CFU/g during the whole storage (Fig. 3c).

The results of staphylococci counts, which presented a concentration of about 3.4 log CFU/g in the meat batter before HHP treatments, are reported in Fig. 4. This microbial population did not present relevant increases in NT and 300 MPa samples (Fig. 4a and b). In the samples treated at 600 MPa, after 90 days of storage staphylococci increased to approx. 7 log CFU/g in both samples without nitrite (600-5.8 F and 600-5.4 F) (Fig. 4c).

Enterobacteria (data not shown) were always found al low concentrations (below 2 log CFU/g) in all the samples, except for the not treated meat batter at pH 5.8 without nitrite, in which they reached counts of about 4 log CFU/g.

Concerning pH, the initial values of the different meat batters showed only slight increases (approx. 0.2 units) immediately after the HHP treatment (data not shown). However, during the following storage at 4 °C, no significant changes were observed independently of the level of spoilage of the samples.

3.2. *Metagenomic analysis*

Metagenomic analyses were carried out to highlight the microbiological profile of meat batters immediately after preparation and at the

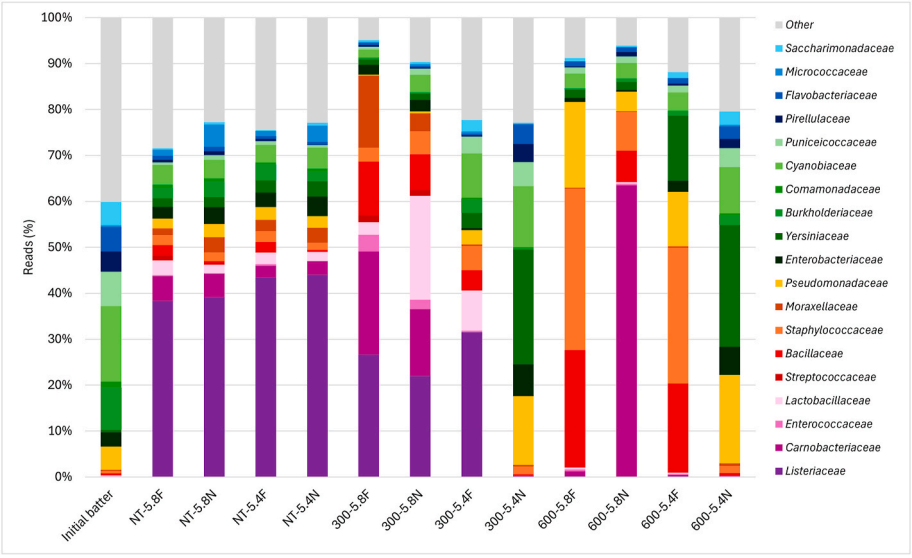

Fig. 5. Relative percentage of reads attributed to bacteria Families in the meat batter before High Hydrostatic Pressures (HHP) treatments and in the HHP treated samples at the end of storage (18, 42 and 90 days for NT, 300 MPa and 600 MPa treatments, respectively).

end of storage in relation to the conditions applied. Fig. 5 shows the results expressed as the relative percentage of reads, grouped at Family level, detected in the initial meat batter and in all the samples (NT, 300 MPa and 600 MPa) at the end of storage (18, 42 and 90 days, respectively). In Table S1 the percentages of Genera attributable to the main Families are also reported.

The untreated meat batter immediately after stuffing was characterized by a low initial contamination, as revealed by the microbial counts reported above (Figs. 2–4). In this sample the higher reads were attributable to *Cyanobiaceae* (16.5%), *Burkolderiaceae* (9.2%), *Puniceicoccaceae* (7.5%), *Flavobacteriaceae* (5.3%), *Saccharimonadaceae* (5.1%) and Pseudomonadaceae (5.1%). Firmicutes represented only 6.4% of total reads and mainly belonged to the Class Clostridia (3.6%) and Bacilli (2.8%), the latter including *Bacillales* (0.8%) and *Lactobacillales* (1.0%). A low prevalence (0.5%) of reads attributable to *Staphylococcaceae* was also evidenced.

In samples not treated with HHP (NT) after the 18 days storage (in which all the samples presented TMC higher than 7 log CFU/g) the percentage of *Cyanobiaceae*, dominant in the initial meat batter, decreased always below 5%, while Firmicutes represented the dominant Phylum (ranging from 50.0% to 57.1%). The Family *Listeriaceae* presented the higher percentage (from 38.3% to 44.0%) and all the reads attributed to this Family belonged to the Genus *Brochothrix* (Table S1). Among the remaining Firmicutes, lower amounts of *Lactobacillaceae*, *Carnobacteriaceae* and *Staphylococcaceae* were found. Among Proteobacteria, *Yersiniaceae*, mainly represented by *Serratia* spp., increased their proportion up to 2.0%–3.5%.

Concerning the batters treated at 300 MPa, both the samples at pH 5.8 presented TMC higher than 7 log CFU/g after 43 days (Fig. 2b). In these samples, the reads were mainly attributed to Firmicutes (> 70%) (Fig. 5). Reads attributable to *Listeriaceae* (particularly *Brochothrix* spp.) were again characterized by high percentages (approx. 44%), together with important concentrations of *Carnobacteriaceae* (22.5% in the sample 300-5.8 F and 14.6% in the sample 300-5.8 N), *Lactobacillaceae* (2.7% and 22.6%; in the latter case mainly represented by *Leuconostoc* spp.) and *Bacillaceae* (11.7% and 7.8%). Smaller percentages of *Enterococcaceae* (3.6% and 2.1%) and *Staphylococcaceae* (2.9% and 4.9%) were also detected. Among Gram negative bacteria, a high concentration of reads (15.7%) attributable to *Moraxellaceae* (and, in particular, *Psychrobacter* spp.) were observed in the samples at pH 5.8 without nitrite (300-5.8 N). The meat batters subjected to the same HHP treatment at pH 5.4 presented different microbial patterns. In these samples, however, the TMC values were always lower than the threshold chosen as shelf life indicator (6.5 log CFU/g) (Fig. 2b). *Cyanobiaceae* still constituted a relevant percentage of reads (9.7%) in the sample 300-5.4 F, characterized by the presence of approx. 50% of Firmicutes, represented by *Listeriaceae* (31.5%), *i.e. Brochothrix* spp., *Lactobacillaceae* (8.7%, mainly *Leuconostoc* spp.) and *Staphylococcaceae* (5.2%). Regarding the sample 300-5.4 N, the proportion of *Cyanobiaceae*, which can be considered an indirect index of the growth of other bacteria, remained rather high (13.3%) and the reads attributable to Firmicutes were less than 4%. In these samples, the most relevant Families were *Pseudomonadaceae* (14.9%) and *Yersiniaceae* (24.9%).

After 90 days, the samples at pH 5.8 treated at 600 MPa presented TMC concentrations close to 7 log CFU/g and Firmicutes accounted for 65.6% (600-5.8 F) and 80.7% (600-5.8 N) of reads in the meat batters without or with nitrite, respectively. *Staphylococcaceae* (in particular *Staphylococcus* spp.) were the dominant Family (35.1%) in the sample 600-5.8 F, followed by *Bacillaceae* (25.5%). Among Gram negative bacteria, *Pseudomonadaceae* (*Pseudomonas* spp.) accounted for 18.7% of reads. The presence of nitrite (600-5.8 N) changed this spoilage pattern and the 63.5% of reads were attributable to *Carnobacteriaceae* (*Carnobacterium* spp.) and only 8.4% to *Staphylococcaceae*, while *Pseudomonadaceae* were less than 5%. The 600-5.4 F samples also presented TMC value higher than the threshold considered as spoilage indicator and the metagenomic results were similar to those observed in sample 600-5.8 F. In fact, *Staphylococcaceae* (29.5%) and *Bacillaceae* (19.2%) were the most relevant Families among Firmicutes, together with a high proportion of Proteobacteria, such as *Pseudomonadaceae* (11.8%) and *Yersiniaceae* (14.1%). The sample 600-5.4 N after 90 days had microbial counts lower than 6.5 log CFU/g and Firmicutes were characterized by reads percentage lower than 5%. In this case, the most relevant percentage of reads was attributable to *Pseudomonadaceae* (19.2%), *Yersiniaceae* (26.5%) and *Cyanobiaceae* (10.1%).

The differences in the distribution of Genera observed in relation to the treatments are summarized in the heat map reported in Fig. 6. The untreated batters presented a high similarity in their spoilage profile, with prevalence of *Brochothrix* spp. The presence of the same Genus, together with *Carnobacterium* spp., determined a similar behaviour in the batters at pH 5.4 treated at 300 MPa, while *Bacillus* spp. And especially *Staphylococcus* spp. Grouped the products treated at 600 MPa in the absence of nitrite. The *Carnobacterium* spp. high percentage was responsible for the diversity, with respect to the other meat batters treated at 600 MPa, of the sample at pH 5.8 added with nitrite (600-5.8 N).

3.3. Colour

The colour of the meat batters before and immediately after HHP treatments is shown in Fig. 7. Compared with the not treated samples, in the absence of nitrite, the batters exhibited an unpleasant brownish colour, while the presence of nitrite conferred a more acceptable pink colour. In addition, also pH influenced the appearance of the samples.

The outcomes concerning the evaluation of the CIE colour parameters on the meat batters exposed to different HHP treatments and then stored at refrigerated temperature are shown in Figs. 8–10.

Concerning lightness (L*), only marginal differences among the meat batters exposed to different HHP treatments were observed (Fig. 8). No differences were evidenced at different storage times for both NT and samples treated at 300 MPa, with the only exception being the values assessed after 3 and 7 days, 7 respectively, which were significantly higher in the samples formulated without the addition of nitrite (NT-5.8 F, NT-5.4 F, 300-5.8 F and 300-5.4 F), regardless of their pH. As for samples treated at 600 MPa, significant differences were found only immediately after HHP, when the samples 600-5.8 F exhibited remarkably higher L* values in comparison with those measured when nitrites were added (600-5.4 N and 600-5.8 N).

As for redness (a*), overall, the effect exerted by the inclusion of nitrites seemed to be more relevant than that ascribable to HHP treatment or pH (Fig. 9). In fact, meat batters formulated with the addition of nitrite (5.4 N and 5.8 N) showed remarkably higher values if compared with the other sausages. This trend was confirmed for each sampling time and regardless of the pressure level tested (0, 300, or 600 MPa).

As for yellowness (b*), meat batters formulated without the addition of nitrites and having low pH (5.4 F) exhibited the highest values regardless of the pressure level applied (either 300 or 600 MPa) (Fig. 10).

4. Discussion

The initial microbial load of the meat batter was relatively low (approx. 4 log CFU/g) but still comparable with those found by other authors in similar products (Cocolin et al., 2004; Raimondi et al., 2018). Among the microbial groups revealed by metagenomic analysis, the presence of high proportions of *Cyanobiaceae* (mainly *Synechococcus* spp. And *Prochlorococcus* spp.) and *Puniceicoccaceae* could be related to contamination of raw materials, in particular salt, given that the natural habitat of these microorganisms is water, including marine and brackish water (Paix et al., 2020; Yoon, 2014). In any case, these microorganisms were not able to grow in meat and in food environment, and probably their contribution to metagenomic derives from residual DNA of cells already dead. The other main Families found were usually ubiquitous

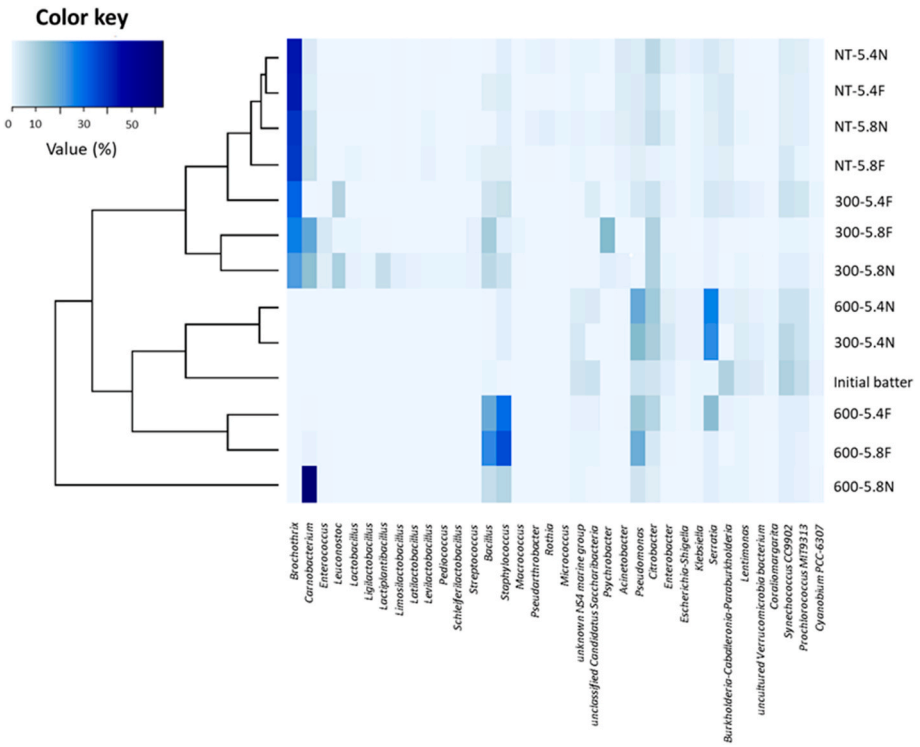

Fig. 6. Heatmap relative to the percentage of the Genera attributable to the main Families in the meat batter before High Hydrostatic Pressures (HHP) treatments and in the HHP treated samples at the end of storage (18, 42 and 90 days for NT, 300 MPa and 600 MPa treatments, respectively).

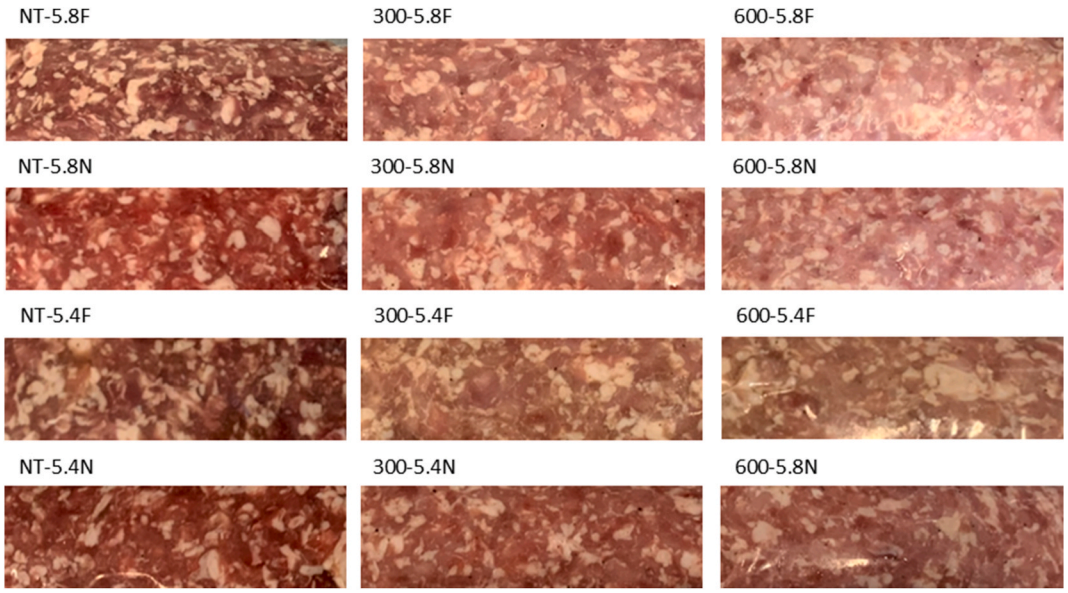

Fig. 7. Appearance of meat batters before and immediately after High Hydrostatic Pressures (HHP) treatments.

bacteria whose provenience can be meat, ingredients and industrial plant.

The growth kinetics showed that all the samples not pressurized were spoiled after 18 days of storage at 4 °C, even if the samples at pH 5.8 without nitrite showed higher growth rate. According to metagenomic analyses carried out at the end of storage, the Genus more represented in these samples was *Brochothrix*. Also, *Carnobacterium* was found, though at much more low proportions. Both Genera did not or poorly and slowly grow in MRS (Casaburi et al., 2011; Stackebrandt & Jones, 2006) and this aspect justify the differences observed between TMC and MRS counts (approx. 1 log unit). In fact, *Lactobacillaceae* accounted only for about 2–3% of reads. These data confirm the qualitative results obtained by Raimondi et al. (2018) in similar sausages in which nitrite, however, was not added. *Brochothrix* and *Carnobacterium* are well adapted for the colonization of meat environments and are characterized by psychrotrophic aptitudes (Leisner et al., 2007; Mohsina et al., 2020; Wong et al., 2023).

In general, the application of a pressure of 300 MPa caused a small reduction of the initial microbial counts (less than 1 log cycle), confirming the observation of Duranton et al. (2012) in pork meat. However, a slow increase was observed during storage, but the counts found in the initial meat batters were reached only after 20 days. This allowed

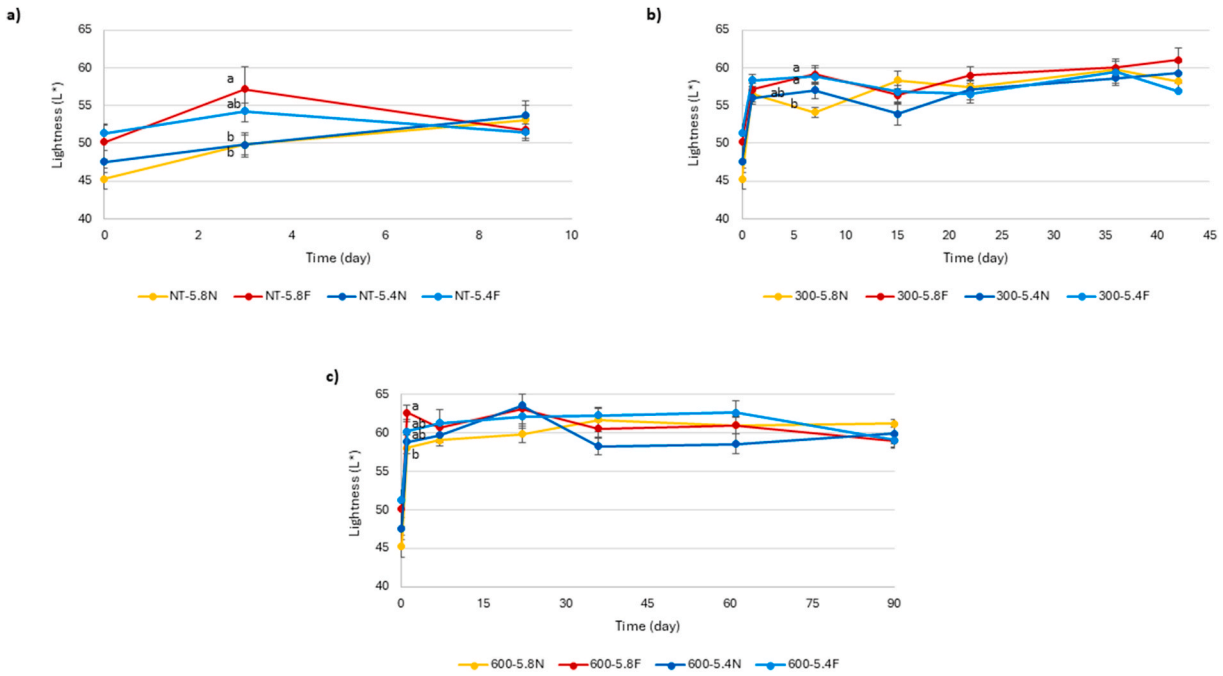

Fig. 8. Evolution of lightness (L*) during storage of meat batters. The data reported are relative to the not treated samples (a) and to the samples treated at 300 (b) and 600 (c) MPa. Means within the same sampling time not sharing the same letters are significantly different (a,b: $P \leq 0.05$; One-way ANOVA, Tukey's HSD).

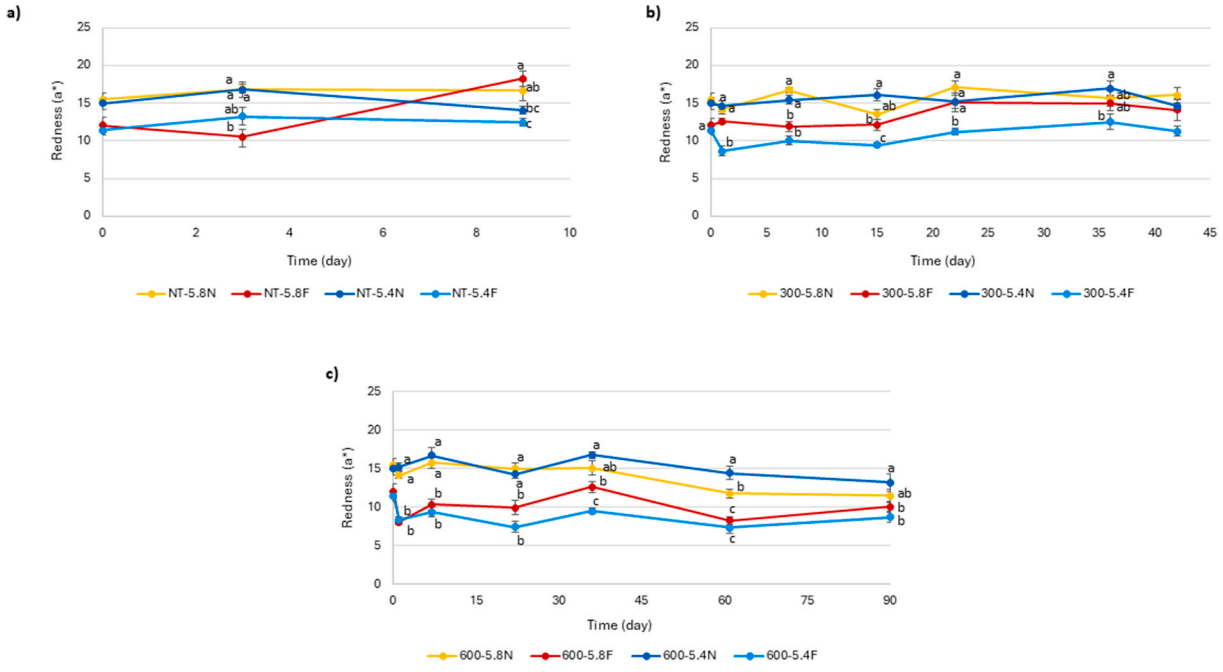

Fig. 9. Evolution of redness (a*) during storage of meat batters. The data reported are relative to the not treated samples (a) and to the samples treated at 300 (b) and 600 (c) MPa. Means within the same sampling time not sharing the same letters are significantly different (a–c: $P \leq 0.05$; One-way ANOVA, Tukey's HSD).

a considerably prolongation of sausage shelf life. The effects of pressure on microbial viability are determined by several factors. Cell membrane fluidity and functionality seems to be one of the main targets of microbial damage, together with ribosome dissociation (Aganovic et al., 2021; Simonin et al., 2012). Gram negative bacteria are considered more susceptible than Gram positive ones (Rendueles et al., 2011). Thus, it is not surprising that the percentage of reads attributed to Firmicutes was higher in pressurized batters, independently of the pressure applied. On the other hand, after 43 days of storage, the samples at pH 5.4 presented slower spoilage kinetics. In addition, the presence of nitrite kept the microbial counts below the spoilage limit.

As expected, the treatment at 600 MPa had a more relevant effect on the initial microbial counts. Interestingly, this effect was more pronounced in the batter at pH 5.4. On the other hand, the information that microbial susceptibility to pressure increases with the decrease of pH has been reported by several authors (Alpas et al., 2000; Syed et al., 2016). *Listeriaceae* (namely *Brochothrix* spp.), which were dominant in the not treated samples, were responsible for the higher percentage of reads in spoiled sausages treated at 300 MPa (Komora et al., 2023). Moreover, in these samples the presence of *Carnobacteriaceae* and

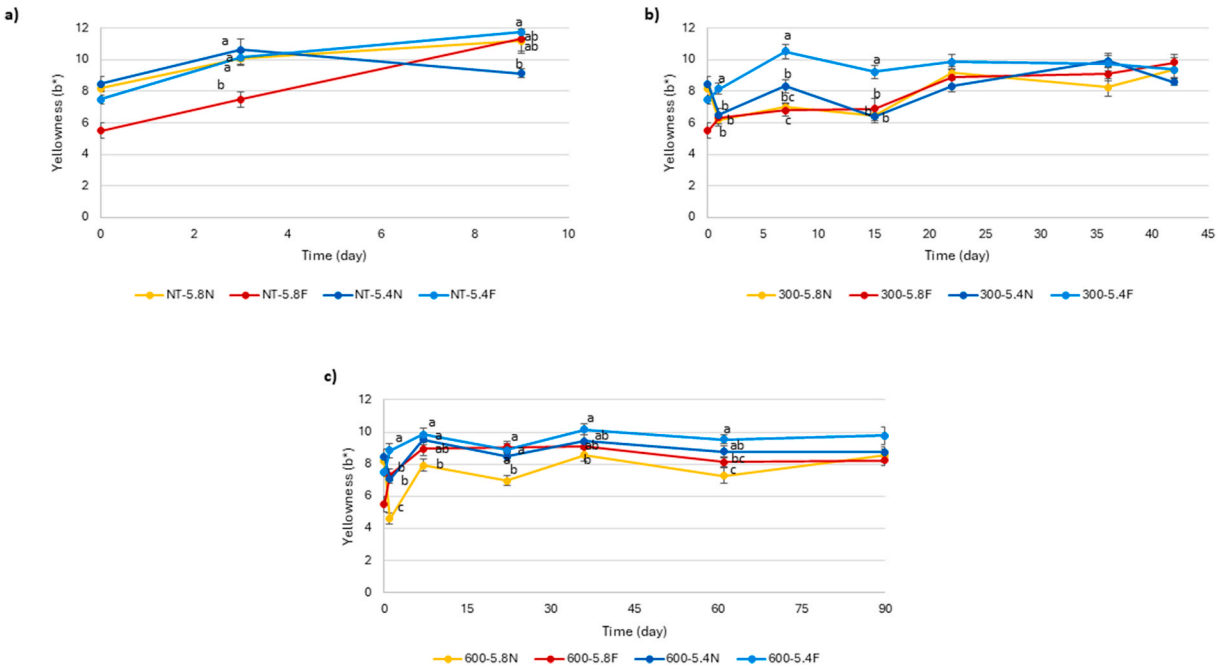

Fig. 10. Evolution of yellowness (b*) during storage of meat batters. The data reported are relative to the not treated samples (a) and to the samples treated at 300 (b) and 600 (c) MPa. Means within the same sampling time not sharing the same letters are significantly different (a–c: $P \leq 0.05$; One-way ANOVA, Tukey's HSD).

Lactobacillaceae was higher. In addition, *Listeriaceae* were absent in the samples pressurized at 600 MPa, indicating a higher sensitivity of this Family to pressure.

The effective cell damage or inactivation due to pressurization depends also on parameters such as the medium composition. It has been demonstrated that the presence of high fat concentration and low a_w have a protective effect on cell barosensitivity (Rubio et al., 2007; Ruiz-Capillas et al., 2007). In this case, while the a_w was high (around 0.97), the batters presented relevant fat presence (20%). In addition, also pH may play an important role. The sausages at pH 5.8 without nitrite showed the faster growth dynamics. Moreover, the TMC counts in the batters at pH 5.4 were rather low at the end of storage, in particular in the presence of nitrite. *Brochothrix* spp., *Carnobacterium* spp. And lactobacilli (mainly *Leuconostoc* spp.) were able to survive to the treatment and grow during storage, due to their psychrotrophic aptitudes. Nevertheless, the presence of nitrite limited their growth in the samples at pH 5.4. The interactive effects between nitrite and pressure have been discussed by Duranton et al. (2012) in pork meat. They found an effect of the presence of nitrite on LAB, especially in the first days after pressure treatment. Then, a considerable recovery was observed and no particular differences in the counts were detected at the end of storage.

The HHP treatment of meat batters determined undesired changes in meat colour attributable to irreversible myoglobin denaturation (Fernández et al., 2007). In particular, the reason of this grey/brown discoloration in the sample without nitrite could be due to the oxidation of ferrous myoglobin to ferric metmyoglobin (Bak et al., 2019; Jung et al., 2003). The addition of nitrite improved the colour after treatment, but it was different with respect to not treated samples, being characterized by a pink note, as already described in beef meat by Rubio et al. (2007).

The negative effects of pressure in the batters without nitrite mainly interested the decrease of meat redness (a*), a phenomenon already described by Bolumar et al. (2021). This decrease did not occur in the samples containing nitrite. The way in which the use of nitrite in cured meats affects the colour is well known. The NO chemically formed from nitrite can bind with a histidine residue of myoglobin, forming nitrosomyoglobin (Cava et al., 2024; Hammes, 2012). The finding of this work may be ascribed to the development of nitrosomyoglobin that is stabilized by HHP treatment (Bak et al., 2019) ultimately leading to a stable bright red colour (Honikel, 2008; Rubio et al., 2007). Indeed, if compared to both the native and oxymyoglobin, nitrosomyoglobin is more resistant to the oxidative modifications induced by HHP (Rubio et al., 2007). The increase of L* values in the treated samples without nitrite is in agreement with Giménez et al. (2015). In the sample containing nitrite, this whitening effect (described also by Bak et al., 2019) was reduced, and this outcome may be of relevant practical importance. Indeed, colour variation (*i.e.,* increase of lightness) is one of the main consequences associated to the application of HHP treatment (Campus, 2010). Therefore, reducing the colour fading due to HHP application through the addition of nitrite in meat batter formulation may represent a promising strategy to minimize the negative impact of such processing treatment. An increase of L* (together with a* decrease) has already been observed for ham (Andres et al., 2006). Also, the results concerning yellowness (b*) confirm the observations obtained by Fernández-López et al. (2004) and can be related to the development of metmyoglobin occurring at a higher extent under more acidic conditions.

5. Conclusion

The application of HHP has proven to be an effective method to prolong the shelf life of meat batters used to produce fresh sausages. Indeed, compared to a shelf life of about two weeks in the untreated product, the application of HHP can extend this value to around 40 and 80 days in the case of treatments at 300 and 600 MPa, respectively. This fact is due not only to the quantitative reduction of the microbial population initially present, but also to the differentiated effects that the treatment has on the different components of the microbiome. This was confirmed by the different degradation patterns highlighted through metagenomic analysis. The other two variables taken into consideration, namely pH and addition of nitrite, also contributed to this differentiation. In particular nitrite, besides its inhibiting action against any sporogenous anaerobic pathogens present (clostridia), plays also an important role in the formation of a colour that is more appreciable to the consumer. In other words, these first tests point to the HHP treatment as an important tool for stabilizing fresh sausages, although further trials are needed to assess their safety and further improve the

characteristics of the final product.

CRediT authorship contribution statement

Chiara Angelucci: Investigation, Formal analysis. **Federica Barbieri:** Investigation, Formal analysis. **Francesca Soglia:** Formal analysis. **Massimiliano Petracci:** Writing – review & editing, Supervision. **Chiara Montanari:** Writing – review & editing, Conceptualization. **Gabriele Gardini:** Formal analysis. **Fausto Gardini:** Writing – original draft, Conceptualization. **Giulia Tabanelli:** Supervision, Conceptualization.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.105419.

Data availability

Data will be made available on request.

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