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Influence of sulphites and modified atmosphere over the growth and diversity of spoiling and foodborne bacteria in poultry hamburgers

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Abstract: Poultry meat is the most consumed worldwide due to its low fat content, sensory qualities, and affordability. However, its rapid spoilage, especially when minced for products like hamburgers, is a challenge. Strategies such as sulphite addition or modified atmosphere packaging (MAP) can help control spoilage and microbial growth. This study evaluated both approaches by analyzing bacterial development in poultry hamburgers through total viable counts and MALDI-TOF identification. The addition of 5 mg/kg sulphites had a limited effect, whereas increasing CO_2 levels in packaging significantly extended shelf life by reducing bacterial growth rates and prolonging lag phases. The most affected bacteria were aerobic mesophilic and psychrotrophic bacteria, as well as *Brochothrix thermosphacta*. *Carnobacterium* spp. dominated the aerobic mesophilic group, while *Enterobacter* spp. was prevalent in *Enterobacteriaceae* and aerobic mesophilic isolates, highlighting its role in spoilage. *Hafnia alvei* was also relevant in the final spoilage stages. These results suggest the importance of these bacteria in poultry hamburger decay and demonstrate that MAP is an effective method to delay spoilage.

Keywords: meat products; bacterial identification; MALDI-TOF; decay

1. Introduction

Current trends in food consumption are evolving towards healthier but tasty products. New food products developed are following novel trends that bet both for the production of improved formulations, respectful with human health and environmental care, at the time they observe manufacturers´ interests. When talking about meat foodstuffs, there are a wide range of products at consumers´ disposal, not only unprocessed meat such as traditional steaks or whole chickens, but also processed meat products such as sausages or hamburgers, whose launching usually involves customers´ preferences consideration [1].

Hamburgers stand out between meat products as one of the most widely accepted between consumers of different age groups [2]. They have conventionally been formulated with bovine meat [3]. Nevertheless, reasons such as higher bovine meat production costs compared to other species, nutritional profiles of bovine meat (commonly characterized by high fat content and tough digestibility of proteins [4], or even affective preferences related to smell and taste [5], have pushed the meat industry to create new products, similar to the already existing ones, but with improvements in some of their characteristics. Indeed, a good example of improved meat products are newly formulated poultry hamburgers [6-8], entering the scene as a groundbreaking meat product that covers most of the previously cited benefits.

Thus, poultry meat has appeared as a good alternative for the formulation of hamburgers. Besides those reasons, poultry meat production has a lower environmental impact compared to other species such as bovine or pork [9], its nutritional profile considering, for instance, fat and protein content, is really attractive [10,11], it displays a high digestibility [12]; and its smell and taste is more palatable, even bearable for people that is not keen on eating meat. All these considerations make poultry meat one of the main consumed in Europe [13] and even worldwide [14]. Indeed, the last predictions published by the European Commission [13] and the Food and Agriculture Organization of the United Nations [14] estimate an increase in poultry meat consumption in the next decade.

Nevertheless, poultry meat also poses some disadvantages, as it is more perishable than meat obtained from other species. For instance, some studies describe shelf lives of 5-7 days in chicken breast preserved in cold conditions [15,16] or turkey meat [17,18], mainly due to the growth of spoiling microbiota [19], linked to modifications on their typical characteristics that make them unacceptable by consumers [20]. By contrast, longer shelf lives have been described for beef, that shows important signs of spoilage between 7 and 14 days of preservation in similar conditions [21], even minced [22]. As muscle is a sterile matrix, it can be easily inferred that microorganisms involved in poultry meat spoilage reach muscle during slaughter, skinning, evisceration and subsequent processes [23,24]. This risk is even higher when meat products are made from minced meat, risk exacerbated by the great concentration of nutrients and higher availability of water [25]. Hence, poultry hamburgers comply all the requirements previously cited to boost microbial growth, and although most of the microorganisms are only implied in meat product decay, poultry hamburgers have been also involved in several reported outbreaks [26-28].

Regarding worrisome foodborne pathogens in hamburgers, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytgenes* are of major concern. These microorganisms mainly reach hamburgers during the production process, for instance, while mincing or mixing meat, and can cause serious illnesses. In the last data published by the European Food Safety Agency corresponding to 2022 [29], *Salmonella* spp. was the second causative agent, behind those unknown, while *Campylobacter* spp. was the fourth. On its behalf, *L. monocytogenes* was the main causative agent of deceases, causing the 45% of all the deaths linked to foodborne outbreaks. Regarding vehicles, meat and meat products were the second largest group involved in foodborne outbreaks, being *Salmonella* spp. and *Campylobacter* spp. the most common causal agents. Although an accurate team-temperature cooking combination, as 70 °C / 2 min leads to a 6 logarithm reduction of one of the most heat resistant vegetative foodborne bacterium, *L. monocytogenes* [30], undercooking enables its survival, so that the study of their presence in raw products is of extreme importance to reduce the risk of their intake.

Although sanitary issues are of main relevance, as spoilage bacteria are responsible of the shortening in the shelf life of animal derived products, they are responsible of significant economic losses not only for meat products producers but also for consumers, as well as substantial rates of food waste [31-33]. Some of the most important microorganisms reported to be involved in meat decay are *Enterobacteriaceae* and *Pseudomonas* spp. this last one important to consider due to its ability to grow at low temperature forming biofilms [34], and able to, eventually, cause foodborne outbreaks. Additionally, there are some others that have not traditionally attracted so much attention [35] but are currently in the top of the line, such as *Brochothrix thermosphacta* [36,37].

In order to prevent microbial growth, there are several techniques that can be applied to food products. Nevertheless, it should be taken into consideration that meat is a raw product that can suffer from strong alterations when subjected to some physical treatments such as traditional heating [38], pulsed electric fields [39,40], ultrasonication [41] or high hydrostatic pressure [42]. To avoid changes related to these processes, apart from maintenance in cold or even freezing conditions (universal recommendations for extending raw meat products shelf life), some other procedures are combined to increase the preservation effect, such as the modification of the packaging atmosphere or the addition of certain additives. The modification in the gas concentration contained in the package has proved to be extremely effective postponing the spoilage of meat products, as the reduction in oxygen concentration and its exchange for inert gases is linked to a reduction in the growth rates of the aerobic populations of spoilage microbiota present in meat products [43]. Several studies describe increases in shelf life of poultry meat products when modified atmosphere is used in packaging. For instance, Chouliara *et al*. [44] found an extension of refrigerated chicken breast shelf live from 5-6 to 11-12 days

With the aim of extending shelf-lives of meat products, one of the most common additives added are sulfites. Although they may pose specific drawbacks over consumers´ helalth such as hypersensitivity, allergic diseases, vitamin deficiency, and dysbiotic events of gut and oral microbiota [45], their use in combination with other barriers such as refrigeration allows the use of a limited amount of sulfites, harmless to human health, with good results [46].

Hence, the aim of this study was to evaluate the impact of modified atmosphere and addition of sulfites over the evolution of the microbiota present in poultry hamburgers and their implications over shelf life.

2. Materials and Methods

2.1. Experimental Design

Commercial hamburgers were provided by a local enterprise. Hamburgers were formulated with chicken (64%) and turkey (12%) meat and presented a 7.9% of total fat content, a 3.9% of carbohydrates, a 16.6% of proteins and a 1.6% of salt. Hamburgers were divided in three different batches. Batch 1 was produced without the addition of sulphites and preserved with no modifications on the packaging atmosphere. In Batch 2, minced meat was added with a concentration of 5 mg/kg of sulphites during the formulation and production of hamburgers, maintaining the product without any modification in the packaging atmosphere during subsequent preservation in refrigeration. Batch 3 was not added with sulphites but was packaged with a modified atmosphere of N_2 enriched with a 20% of CO_2. Packaging was made of polyethylene (PET), that is an inert material that avoids transference between package and hamburgers, and between packaging atmosphere and the outer environment All the hamburgers were provided frozen, and freeze-preserved at -20 °C until used.

2.2. Sampling and Sample Preparation

In order to study the evolution of the microbiota, hamburgers were frozen immediately after production, defrosted at day 0 and maintained in refrigeration during the study. They were sampled each 2 days from days 0 to 16. Defrosting was done overnight maintaining hamburgers at 5 °C. Shelf life of poultry hamburgers was set by the manufacturer in 8-10 days, although an extension in the period studied beyond the shelf life was proposed in order to better establish the characteristics of the microbiota development. Sample preparation was done by mixing 25 g of hamburger and 225 mL of sterile buffered peptone water at 0.1% (Oxoid LTD, Basingstoke, United Kingdom), for 5 min at 230 r.p.m. on a Stomacher 400C (Cole-Parmer, Illinois, United States of America). After filtration, juice obtained was collected on sterile tubes. Serial dilutions were made on sterile buffered peptone water to adjust microbial concentrations to accurate counts to be sow in Petri plates by the pour plating technique. Each sampling day, 2 hamburgers per condition were analyzed, and 2 aliquots per hamburger were studied.

2.3. Bacterial Isolation

For the study of the microbiota evolution, the next bacterial groups were studied as spoilage bacteria and process hygiene criterion: total aerobic mesophilic microorganisms, psychrotrophic microorganisms, *Enterobacteriaceae* and *Brochothrix thermosphacta.* These microbial groups were investigated each 2 days between days 0 and 16, both included. Additionally, *Salmonella* spp, *L. monocytogenes* and *Campylobacter* spp were investigated as food safety criteria. In this case, only days

0, 8 and 16 were analyzed, as these bacteria pose a food safety risk and they should not be present in hamburgers. Also Pseudomonas spp. was studied matching these sampling pattern as an spoiling indicator.

2.3.1. Bacterial Culture Media

All the culture media and selective supplements used in this study were provided by Oxoid LTD.

For the total aerobic mesophilic and psychrotrophic bacteria growth, samples were pour plated and Tryptone Soy Agar supplemented with a 0.6% of Yeast Extract (TSA-YE) was used as growth media. Streptomycin Thallous Acetate Actidione (STAA) agar added with 7,5 g/ 100 mL of glycerol and selective suplement STAA SR0151E was used for the selective growth of *B. thermosphacta.* Again, samples were pour plated. Additionally, *Pseudomonas* spp. were studied by growing them in CFC Agar, prepared by mixing *Pseudomonas* Agar Base and selective supplement SR0103.

VRBG (Violet Red Bile Glucose) Agar was used for *Enterobacteriaceae* counting. Samples were pour plated, and after the addition of a first layer of agar, a second layer was added to get microaerophilic conditions. XLD Agar (Xylose Lisine Desoxycolate) was used for *Salmonella* spp. growth. The color of this medium changes when acidification occurs, and *Salmonella* colonies exhibit a characteristic red tone with a black center. L. monocytogenes was pour plated in Oxoid Chromogenic *Listeria* Agar (OCLA) supplemented with selective supplements SR0226E y SR0228E. Finally, Campylobacter spp. was grown in *Campylobacter* Blood-Free Selective Agar Base (CBFSA) supplemented with selective supplement SR0155E and microaerobic conditions. Microaerobic conditions were reached in small chambers by using the Campygen Oxoid™ kit (Thermo Scientific, Loughborough, United Kingdom). Hamburger samples were in all cases pour plated. Only in the case of *Enterobacteriaceae*, double layer technique was used: after sowing the sample, a second layer of agar was added to get microaerophilic conditions.

2.3.2. Bacterial Culture Conditions

Microbial culture conditions are presented in Table 1.

Table 1. Culture media and incubation conditions used for each microbial group, matching the requirements of the ISO standards.

Microbial group	**Medium**	**Temperature (°C)**	**Incubation time (days)**	**Atmosphere**	**ISO standards**
Mesophilic	TSA-YE	35	1	Aerobiosis	UNE-EN ISO 4833
Psychrotrophic	TSA-YE	7	7	Aerobiosis	UNE-EN ISO 4833
Enterobacteriaceae	VRBG	35	1	Aerobiosis	UNE-EN ISO 21528
B.thermosphacta	STAA	25	2	Aerobiosis	-
Pseudomonas spp.	CFC	20	2	Aerobiosis	-
Salmonella spp.	XLD	35	1	Aerobiosis	UNE-EN ISO 6579
L. monocytogenes	OCLA	10	1	Aerobiosis	UNE-EN ISO 11290
Campylobacter spp.	CBFSA	40	1	Microaerophilia	EN-ISO 10.272-2

2.3.2. Identification by MALDI-TOF

MALDI-TOF® Biotyper (Brüker, Massachusetts, United States of America) was used for bacterial identification. This equipment is intended for the characterization of bacteria by matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF/MS). Hence, this technology identifies bacteria based on mass spectra of cells or cellular components. One of the main advantages of this method compared with traditional identification methods is its speed of analysis, as it can identify bacteria in few minutes.

For this purpose, 215 isolates obtained from cultures in specific media and conditions, coming from hamburgers with and without sulphites and with preservation in unmodified and modified atmosphere were identified. For mesophilic aerobic bacteria, 5 colonies per plate and condition (hamburger without sulphites and no modifications in packaging atmosphere, hamburger with sulphites and no modifications in packaging atmosphere and hamburger without sulphites and added with a 20% CO2 in packaging atmosphere), matching days 0, 4, 8 and 16 were collected. In order to identify the microbiota, target days were submitted to MALDI-TOF. *Enterobacteriaceae* were identified in days 0, 4 and 16, as their involvement in food decay is of extreme importance in the first stages of food preservation, where it is commonly detected a fast exponential growth. Colonies growth in *L. monocytogenes* and *Salmonella* spp. isolation plates were identified by MALDI-TOF in days 0, 8 and 16 in order to have a balanced prospection of their eventual evolution in hamburgers, as they pose a safety risk and their appearance should be avoided. The same sampling was performed for the spoiling *Pseudomonas* spp. Colonies were collected directly from selective media Petri plates, and were kept frozen at -20°C on a cryoprotective solution consisting of 80% sterile peptone buffered water and 20% of glycerol. All the isolates were unfrozen and revitalized in TSA-YE prior to MALDI-TOF identification.

2.4. Data Representation, Modelling and Statistical Analysis

Bacterial evolution results were obtained from 2 replicates, 2 aliquots per replicate, and are presented as the mean value ± standard deviation. The PRISM® program was used for data processing and representation, as well as for statistical analysis via ANOVA (GraphPad Software, Inc., San Diego, CA, USA). Statistically significant differences were considered when $P < 0.05$.

3. Results and Discussion

3.1. Bacterial Counts

3.1.1. Aerobic Mesophilic Bacteria

Initial total viable counts (TVC) of aerobic mesophilic bacteria were of 5.63 ± 0.00 -5.81 ± 0.12 log CFU/g at day 0, and a comparable increase on the TVC of the three types of hamburgers lead to a final TVC of 9.59 ± 0.24 - 10.34 ± 0.02 log CFU/g at day 16, non-existing statistical differences between them ($P > 0.05$, Figure 1). Only lower TVC were documented in refrigerated hamburgers maintained in modified atmosphere conditions in days 8-14 of the shelf life of hamburgers, set by the manufacturers in 8 days. Henceforth, the increase in the CO_2 concentration present in the packaging could be an effective method to slow down bacterial development in poultry hamburgers, limiting their growth and side effects derived, even leading to the extension in shelf lives by optimizing the characteristics of the methodology. The increment in CO_2 concentration in the atmosphere used for packaging prolongs the lag phase of bacterial growth and decreases the growth rate during the logarithmic phase [47]. For instance, similar behavior has been assessed by Patsias *et al*. [48], that described an extension of more than 6 days by improving the modified atmosphere of precooked chicken product stored at 4 °C. Sulphites addition did not show any reduction on TVC in the period studied.

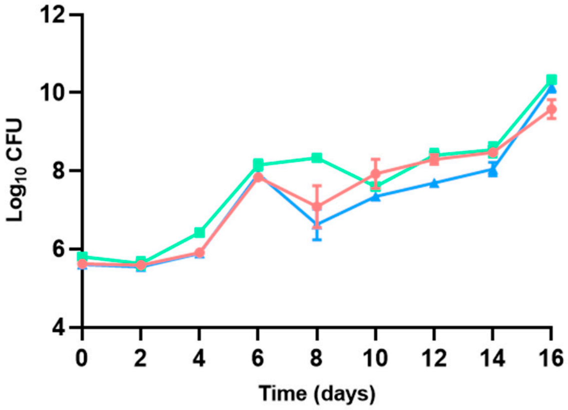

Figure 1. Aerobic mesophilic TVC described for poultry hamburguers without sulphites and no changes on the packaging atmosphere (●), with sulphite addition and no changes on the packaging atmosphere (■) and without sulphites but with modified atmosphere (▲).

Aerobic mesophilic bacteria comprise a scattered group of species present in meat and able to grow on aerobic conditions. It is commonly used as an indicator of food hygiene, and its determination is carried out in common surveillance plans developed in meat industries [49,50]. Although the bacterial growth increase detected at the end of the period studied avoided the modelling of the data, they arose an increment in poultry hamburgers shelf life when using sulphites or modified atmosphere enriched in CO2 packaging, being this last approach more fit-for-purpose.

3.1.2. Psychrotrophic Bacteria

Aerobic psychrotrophic bacteria were not detected up to 2-4 days of maintenance in refrigeration, moment that depended on the characteristics of the atmosphere used for packaging: whilst psychrotrophic bacterial growth began after 2 days of maintenance in refrigeration for hamburgers packaged without modifications in the atmosphere of the package, it started beyond 4 days for those packed with modified atmosphere (Figure 2). Whereas no statistically significant differences ($P > 0.05$) were found in psychrotrophic bacterial TVC in days 0 and 2, packaging on modified atmosphere showed a delay on the start of bacterial detection, together with lower TVC through all the period studied until day 16 ($P < 0.05$), where there were identified slightly lower TVC compared to mesophilic bacteria (8.98 ± 0.21 - 9.65 ± 0.16 log CFU/g), although no statistically significant differences were detected. Addition of sulphites did not show any delay on psychrotrohic bacterial development. As it was found with TVC of mesophilic bacteria, changes linked to modifications in the atmosphere included in the packaging pointed out to have an impact on bacterial development, hence being a suitable technique to be considered for shelf-life extension in poultry hamburgers. The same effect has been documented for beef patties [51] or ground beef [52] preserved in refrigeration and modified atmosphere.

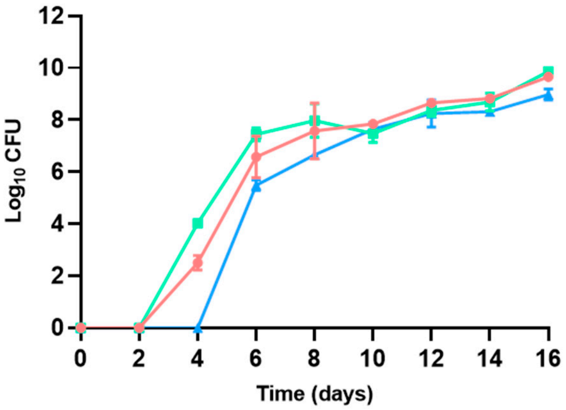

Figure 2. Aerobic psychrotrophic TVC described for poultry hamburgers without sulphites and no changes on the packaging atmosphere (●), with sulphite addition and no changes on the packaging atmosphere (■) and without sulphites but with modified atmosphere (▲).

Aerobic psychrotrophic bacteria comprises a group of bacteria able to grow on low temperature conditions. It is used in routine analyses performed, for instance, in the dairy sector [53], vegetable production [54], or in the meat industry [25] among others, and plays an important role in the monitoring of ready to eat products and some other refrigerated products, as small fluctuations in temperature allow significant reductions in the time to spoilage due to fast increases in psychrotrophic bacterial populations [31]. Data obtained show slight benefits on delaying and reducing the growth of psychrotrophic bacteria when modifying packaging atmosphere, event that might play an important role in hamburgers decay.

3.1.3. *Enterobacteriaceae*

In the present study, *Enterobacteriaceae* TVC were similar in the three conditions studied. There was described an increase from 3.19 ± 0.3 log CFU/g at day 0 up to 9.44 ± 0.39 log CFU/g in day 16, and no statistically significant differences were found between the three groups of hamburgers studied at any day tested ($P > 0.05$, Figure 3). This points out to a low efficiency of the amount of sulphites added or the modification in the gas concentration of the packaging atmosphere against this group of bacteria, facts that could be linked to a protective effect of hamburger components such as fat or proteins [55] or a low impact over oxygen availability decrease over Enterobacterales, as some of the microorganisms of this group are facultative anaerobic.

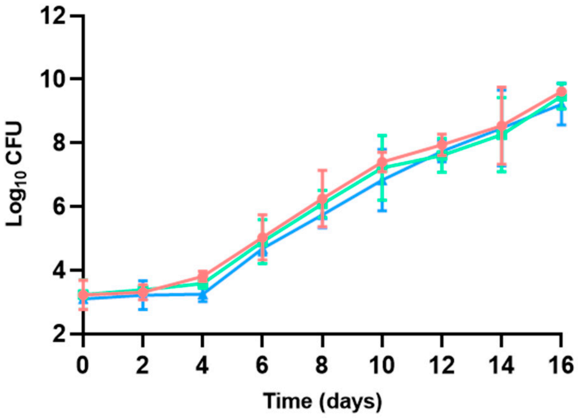

Figure 3. *Enterobacteriaceae* TVC described for poultry hamburgers without sulphites and no changes on the packaging atmosphere (●), with sulphite addition and no changes on the packaging atmosphere (■) and without sulphites but with modified atmosphere (▲).

Enterobacterales are usually used as an indicator of fecal cross contamination and general hygiene during the production process. Although high TVC at the beginning of the preservation period may point out to malpractices in the production process, they are commonly present in food products and their presence do not necessarily pose a safety risk for consumers, as they are usually found in some parts of the intestinal tract of mammals and some factors such as the specific microorganism and the host response play an important role on the pathogenesis [56]. Even though they pose a serious concern regarding the length of hamburgers shelf life, conditions tested in this study didn´t involve any important improvement.

3.1.4. *Brochothrix thermosphacta*

In this work, initial TVC of *B. thermosphacta* in the three groups of hamburgers were analogous ($P > 0.05$), enabling the calculation of joint initial TVC of 3.19 ± 0.3 log CFU/g, that increased up to 8.48 ± 0.36 log CFU/g on day 16 for those hamburgers without modifications in the packaging atmosphere and 7.56 ± 0.21 log CFU/g in those with modified atmosphere, both nether TVC that the ones described in the previously described bacterial groups ($P < 0.05$, Figure 4). Regardless the match on 0 and 16 days TVC, from day 4, B. thermospacta TVC were lower in hamburgers preserved in modified atmosphere, reaching 1.24 log CFU/g lower TVC at day 8 ($P < 0.05$) compared with unmodified atmosphere groups of hamburgers, reduction that was maintained during all the period investigated. This reduction on bacterial TVC points again towards a marked effect of the packaging atmosphere composition over bacterial development. In fact, although *B. thermosphacta* is a facultative anaerobic bacterium, its growth is markedly influenced by the availability of oxygen in the packaging atmosphere [57,58]. This finding reveals the reduction in the packaging atmosphere as a good strategy to delay the decay of poultry meat hamburgers, not so the addition of sulphites.

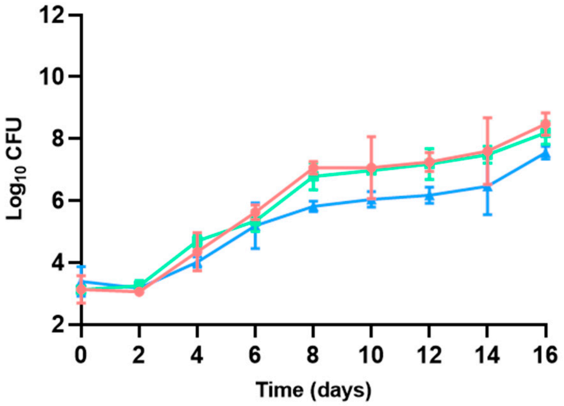

Figure 4. *B. thermosphacta* TVC described for poultry hamburgers without sulphites and no changes on the packaging atmosphere (●), with sulphite addition and no changes on the packaging atmosphere (■) and without sulphites but with modified atmosphere (▲).

B. thermosphacta is a microorganism from *Listeriaceae* family, able to grow at refrigeration temperatures, highly involved in meat spoilage, specifically on poultry meat spoilage [59,60]. It is widely spread by all the stages of the producing chain [31], and although it is facultative anaerobic, it can lead to a fastest spoilage of foodstuffs in aerobic conditions [61]. In meat products, it consumes glucose leading to smells characterized as cheese smell, associated with acetoine, diacetil and 3-

metilbutanol [62]. Results obtained in this study showed the efficiency of the modification of the packaging atmosphere to control the growth of *B. thermosphacta*, hence delaying hamburger decay.

3.1.5. *Pseudomonas* spp.

Although bacterial growth was detected in *Pseudomonas* isolation plates, colonies did not show the typical yellowish colour. Further MALDI-TOF identification was required to set the presence of this bacteria in poultry hamburgers.

3.1.6. *Salmonella* spp., *Listeria* spp. and *Campylobacter* spp.

The isolation of these three species was included in the study as an indicator of safety risk. Culture in OCLA agar for *Listeria* spp. pointed to the presence of two colonies of *L. monocytogenes* in one of the hamburgers at the beginning of the shelf-life study (day 0), based on their typical appearance in OCLA agar. Also *Salmonella* spp. was investigated, but no colonies with the typical appearance of *Salmonella* spp. in XLD agar were found. Likewise, no typical *Campylobacter* spp. colonies were found. These data were further confirmed by MALDI-TOF.

3.2. Bacterial Identification

Table S1 includes all the MALDI-TOF identifications performed, and Table 2, a brief summary of the identification results of aerobic mesophilic bacteria included in Table S1. All the identification results showed a good identification. Regarding spoiling microbiota corresponding to aerobic mesophilic bacteria, in hamburgers without sulphites and without modifications on their packaging atmosphere, the main identifications at day 0 corresponded to *Rothia nasimuirum* (30% of the isolates) and *Staphylococcus* spp. (30%), although *Macrococcus caseolyticus*, *Escherichia coli*, *Proteus mirabilis* and *Corynebacterium phoceense* were also identified. This microbiota was evolving towards a 60% of *P. mirabilis* and a 30% of *E. coli* at day 4, 50% of *Bacillus subtilis* and 50% of *Carnobacterium* spp. (mainly *C. divergens*) at day 8, increasing up to an 80% of *Carnobacterium* spp. at day 16.

Table 2. Summary of the identification results of aerobic mesophilic bacteria included in Table S1 and obtained by MALDI-TOF.

Sulphites	Atmophere	Day 0		Day 4		Day 8		Day 16	
		% Isolates	Identifcation	% Isolates	Identifcation	% Isolates	Identifcation	% Isolates	Identifcation
W/O*	Unmodified	30	*Rothia nasimurium*	60	*Proteus mirabilis*	50	*Carnobacterium* spp.	80	*Carnobacterium* spp.
		30	*Staphylococcus* spp.	30	*E.coli*	50	*B. subtilis*	10	*Leuconostoc mesenteroides*
		10	*Macrococcus caseolyticus*	10	*Staphylococcus simulans*			10	*Kurthia zopfii*
		10	*Escherichia coli*						
		10	*Proteus mirabilis*						
		10	*Corynebacterium phoceense*						
W*	Unmodified	70	Carnobacterium spp	50	*B. subtilis*	90	*Carnobacterium divergens*	100	*Carnobaterium* spp.
		20	*Staphylococcus* spp.	20	*Staphylococcus simulans*	10	*Proteus mirabilis*		
		10	*Enterococcus faecalis*	10	*Enterococcus faecalis*	10	*Proteus mirabilis*		
				10	*Pseudomonas lundensis*				
				10	*Carnobacterium divergens*				
W/O*	Modified	50	*Proteus mirabilis*	40	*Staphylococcus* spp.	50	*Carnobacterium* spp.	60	*Carnobacterium divergens*
		20	*Microbacterium liquefacens*	20	*Rothia nasimuirum*	20	*Bacillus* spp.	30	*Enterobacter* spp.
		10	*Carnobacterium maltaromaticum*	20	*Bacillus* spp.	20	*Staphylococcus* spp.	10	*Leuconostoc mesenteroides*
		10	*Rothia nasimurium*	20	*E. coli*	10	*E.coli*		
		10	*Escherichia coli*						

*W/O (Without): Hamburgers without sulphites. W (With): Hamburgers with 5 mg/kg of sulphites.

Regarding hamburgers with sulphites, a 70% of the isolates corresponded to *Carnobacterium* spp. on day 0, species that was the main identification through the shelf life of hamburgers, reaching a 90% of the identifications at day 8 and 100% at day 16. Other important species identified in this kind of hamburgers were *B. subtilis* and *Staphylococcus* spp. (50% and 20% of the identifications at day 4), and *Proteus* spp. (10% at day 8). Aerobic mesophilic TVC in hamburgers without sulphites and modified atmosphere started with a 50% of *Proteus mirabilis*, 20% of *Micobacterium liquefacens* and 10% of *Carnobacterium mataromaticum*, evolving towards a 40% of *Staphylococcus* spp., 20% *of Rothia nasimurium*, 20% of *Bacillus* spp. and 20% of *E. coli* on day 4; 50% of *Carnobacterium* spp., 20% of *Bacillus* spp. and 20% of *Staphylococcus* spp. on day 8 and 60% of *Carnobaterium* spp. and 30% of *Enterobacter* spp. on day 16.

Data obtained showed that addition of sulphites reduced bacterial diversity, so much so that in day 16 only *Carnobacterium* spp. was identified. A similar effect was obtained at the others conditions tested, as 60 and an 80 % of the isolates identified at day 16 in absence of sulphites with and without modifications in the packaging atmosphere were also *Carnobacterium* spp., mainly *C. divergens*, fact that points out to this species as one of the most relevant concerning spoilage of poultry hamburgers, regardless other protective techniques used. Predominance of *Carnobacterium* spp. during the last phases of shelf lives of poultry meat has been previously reported [37], and it is usually considered as one of the main genera involved in meat spoilage [63]. Hence, its increase through the period evaluated and predominance at the final stages of the study points ratify *Carnobcterium* spp. as one of the main causes of spoilage. This microorganism is likely to be involved in the unexpected increase in bacterial counts detected at the end of the period studied.

Likewise, *Proteus mirabilis* was highly isolated, mainly in the early days of the study. It is a common component of the normal intestinal microbiota of chicken [64], fact that enables its transfer to the slaughter line, hence its cross-contamination, especially in evisceration processes [65]. It is an opportunistic bacterium that can cause several diseases in humans, standing out urinary tract infections as the most prevalent infection [66]. Hence, it should be regarded, although it is commonly isolated from chicken meat [67,68]. Other pathogenic microorganisms as *Staphylococcus* spp. were quite prevalent, and although they are commonly isolated from chicken meat [69,70], they should also be kept under control, as species such as S. epidermis are so significant that have even been discussed as one of the main causes of hospital-acquired bacteremia [71]. Also *Enterobacter* spp. was relevant only at the final stages of the study in hamburgers with modified atmosphere, fact that could be related to its facultative anaerobic metabolism. Although it was less dominant, it should similarly be monitored as it is a frequent nosocomial infection [72].

Isolates corresponding to *Enterobacteriaceae* plates (Table 3) started with microbiota mainly typified as *Pseudomonas lundensis* (75%) and *Staphylococcus epidermi*s (25%), which reveals low rates of enterobacterales in hamburgers at the first stages of preservation after production under the conditions tested. The preservation of hamburgers without modified atmosphere led to an increase in enterobacterales, being only recovered *Enterobacter* spp (100% of the identifications) at day 16. The addition of sulphites led to a strong increase in bacterial diversity (40% of *Serratia liuqefacens*, 20% of *E. coli*, 20% of *Pseudomonas lundensis* and 20% of *Citrobacter freundii*) at day 4. At day 16, a 40% of the bacterial isolates in hamburgers with sulphites and no modifications on the packaging atmosphere corresponded to *Hafnia alvei*, and a 60% to *Enterobacter* spp. Whilst, the maintenance of hamburgers in modified atmosphere did not have an impact as marked as the addition of sulphites over bacterial diversity, as its addition reduced the. identificaction to an 80% of *E. coli* and a 20% of *Enterobacter* spp. at day 4, diversity even reduced in day 16, when only *Enterobacter* spp. was recovered.

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Table 3. Summary of the identification results of *Enterobacteriaceae* included in Table S1 and obtained by MALDI-TOF.

Sulphites	Atmophere	Day 0		Day 4		Day 16	
		% Isolates	Identifcation	% Isolates	Identifcation	% Isolates	Identifcation
W/O*	Unmodified	75	*Pseudomonas lundensis*			100	*Enterobacter* spp.
		25	*Staphylococcus epidermis*				
W*	Unmodified	75	*Pseudomonas lundensis*	40	*Serratia liquefaciens*	60	*Enterobacter* spp.
		25	*Staphylococcus epidermis*	20	*Pseudomonas lundensis*	40	*Hafnia alvei*
				20	*Citrobacter freundii*		
				20	*Escherichia coli*		
W/O*	Modified	75	*Pseudomonas lundensis*	80	*Escherichia coli*	100	*Enterobacter* spp.
		25	*Staphylococcus epidermis*	20	*Hafnia alvei*		

*W/O (Without): Hamburgers without sulphites. W (With): Hamburgers with 5 mg/kg of sulphites.

As it happened in aerobic mesophilic bacteria, *Enterobacter* spp appeared as an important bacterium in the last stages of the study. Indeed, it was the predominant species of Enterobacterales on day 16, so much so that it was the only species detected on day 16 in hamburgers without sulphites (being *Enterobacter kobei* the microorganism mainly detected). Only other microorganisms were detected in hamburgers with sulphites: a 40% of the isolates were identified as *Hafnia alvei* whereas a 60% of the isolates were *Enterobacter* spp., specifically 4 different species. *Hafnia alvei* is commonly found in minced meat products, and both species are associated with the appearance of putrid off odours and/or greening of the meat [36].

Also *Pseudomonas* spp. were identified in the first stages of maintenance. They were investigated in order to have a better view of one of the main psychrotrphic bacteria involved in meat-derived products spoilage (Table S1). Data obtained showed that the main bacteria identified pertained to *Pseudomonas* spp. (50% of the isolates, corresponding to *P. aeruginosa*, *P. putida* and *P. lundensis*), but also *Citrobacter freundii* (44%) and *Proteus mirabilis* (6%) were identified. *Pseudomonas* spp. is a ubiquitous genus in meat products, involved in meat spoilage at cold temperatures [73-75]. It has been documented to cause discolorations, off odors and slime formation [74,76], and its growth during meat storage has been associated to important sensory changes [77]. Together with *Citrobacter* spp. and *Proteus* spp, is some of the main microorganisms associated with meat spoilage [78].

Regarding bacteria used as safety risk indicators, culture in OCLA agar for Listeria spp. isolations pointed the presence of *L. monocytogenes* in one of the hamburgers at the beginning of the shelf-life study, firstly because of their typical appearance in OCLA agar (brilliant green colonies), and secondly, supported by MALDI-TOF identification (Table S1). Further analyses would be needed to establish the acceptability of the risk of these hamburgers consumption, as data of *L. monocytogenes* presence was linked to a unique product unit, and some other hamburgers from the same batch and/or enterprise should be analyzed in order to reject an ubiquitous and worrisome presence of *L. moncytogenes*, Hence, although *L. monocytogenes* was identified at the initial stages of hamburgers preservation, further studies would be required to consider it as a health risk and discard cross contamination events during hamburger manipulation during bacterial microbiota studies performance. Other bacteria also identified in this medium was *Pseudomonas* spp. and *Rothia nasimurium* situation previously documented for similar selective [79]. Both microorganisms are commonly related to meat spoilage [37].

Also *Salmonella* spp. was investigated (Table S1). No colonies showed the typical appearance of Salmonella spp. in XLD agar. Nevertheless, some of the isolates were identified by MALDI-TOF, finding that although at the beginning of the study there was a marked bacterial diversity, including identification of *Staphylococcus* spp., *Pseudomonas* spp., *Kocuria* spp. or *E. coli*, at the final stages of the study, biodiversity decreased and the main bacterium present in hamburgers without sulphites and no modifications on their atmosphere was *Staphylococcus* spp. and *Hafnia alvei* for those hamburgers with sulphites (exactly as it happened in *Enterobacteriaceae* identifications). The inclusion of modifications on the atmosphere increased bacterial diversity, identifying *Enterobacter* spp., *Carnobacterium* spp. and *Leuconostoc* spp., again pointing out to *Enterobacter* spp. as one of the main microorganisms involved on meat spoilage. No typical *Campylobacter* spp. colonies were found, neither sent for MALDI-TOF identification.

4. Conclusions

The study of the spoiling microbiota revealed differences over bacterial groups and the influence of the presence of sulphites / modified packaging atmosphere. While sulphites presented a minimal impact over bacterial growth, the increase in CO_2 concentration on the packaging atmosphere led to a generalized reduction in TVC of aerobic mesophilic and psychrotrophic bacteria and *B. thermosphacta*, not only through poultry hamburger shelf lives but also through extensions in this period. Although final TVC reached were comparable, these results point at the reduction in the available oxygen of the packaging atmosphere as an effective technique to slow down bacterial growth, fact that could set the basis to the lengthening of poultry shelf lives and even increase the quality of poultry hamburgers. Regarding bacterial identification, *Carnobacterium* spp. was the main species detected on aerobic mesophilic bacteria isolation plates, although it should be mentioned that bacterial diversity increased in hamburgers added with sulphites or maintained in modified atmosphere. On their behalf, the most identified bacteria in *Enterobacteriaceae* isolation plates were *Enterobacter* spp., fact strongly patent in the last days of the study, when bacterial diversity markedly decreased. As *Enterobacter* spp. was also highly identified on aerobic mesophilic plates, it seems to play an important role meat decay. *Hafnia alvei* was also important at the final stages of the study when sulphites were added, which could be related to a higher resistance when compared to other microorganisms. Although *L. monocytogenes* was identified at the beginning of hamburger preservation (day 0), no isolates were detected in subsequent days, implying irrelevance concerning food safety. All these findings suggest the implication and importance of bacteria such as *Carnobacterium* spp., *Enterobacter* spp. or even *Hafnia alvei* over spoilage, and the suitability of atmosphere modification for its control and the postponement of poultry hamburgers decay.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1; Table S1: Results from the identification of 215 isolates obtained from the culture in specific conditions of the flora of poultry hamburgers from days 0 to 16 during the preservation period.

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