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Influence of meat type and shelf life on the microbiota of fresh chicken and pork sausages

Influência do tipo de carne e do tempo de conservação na microbiota de linguiças frescais de frango e suína

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Fresh sausages are an affordable source of protein, but concerns about foodborne diseases are significant in ensuring their safety. This study aimed to assess the impact of meat type on the microbiota, shelf life, and regulatory compliance of fresh sausages, sold in bulk and from different brands. A total of 40 sausages were analyzed - 20 made from pork (PS) and 20 from chicken (CS). Half of them were analyzed immediately after being collected and the other half were stored at 4°C and tested on the eighth day. Both pork and chicken sausages exhibited samples that did not meet regulatory standards, with chicken sausages posing a higher health risk due to the presence of *Salmonella* spp. Nitrite was detected in all sausage samples, regardless of the meat type. Of these, 20% of the chicken samples and 10% of the pork samples exhibited nitrite concentrations near to the maximum limit established by law. On day 1, chicken sausages showed lower populations of indicator and spoilage microorganisms but posed a higher risk due to the potential presence of *Salmonella spp.* and higher counts of molds and yeasts. On the eighth day, PS showed an increase in staphylococci, fungi, and yeasts, posing a health risk. The type of meat used in the production of fresh sausages and the length of refrigeration storage influence their microbiological quality. Furthermore, it is likely that the activity of microbial lipolytic enzymes is more prevalent in CS on day 8, and in PS on days 1 and 8.
Keywords: quality, legislation, nitrite.

Os embutidos frescos são uma fonte de proteína acessível, mas as preocupações com as doenças de origem alimentar são importantes para garantir a sua segurança. Este estudo teve como objetivo avaliar o impacto do tipo de carne na microbiota, no prazo de validade e na conformidade das linguiças frescais comercializas a granel e de diferentes marcas. Foram analisadas 40 amostras - 20 de carne suína (LS) e 20 de frango (LF). Metade delas foi analisada imediatamente após a aquisição e a outra metade após o oitavo dia (4°C). Tanto as LS como as LF apresentaram amostras que não cumpriam as normas regulamentares, sendo que as de frango representam um maior risco para a saúde devido à presença de *Salmonella* spp. O nitrito foi detectado em todas as amostras, independentemente do tipo de carne. Destas, 20% das LF e 10% das LS apresentavam concentrações de nitritos próximas do limite máximo estabelecido por lei. No dia 1, as LF apresentavam populações mais baixas de microrganismos indicadores e deteriorantes, mas representavam um risco mais elevado devido à presença de *Salmonella* spp., e contagens mais elevadas de bolores e leveduras. No oitavo dia, LS apresentou aumento de estafilococos, fungos e leveduras, representando risco à saúde. O tipo de carne utilizada na produção de linguiças frescas e o tempo de armazenamento refrigerado influenciam na qualidade microbiológica. Além disso, é provável que a atividade das enzimas lipolíticas microbianas seja mais prevalecente nas LF no dia 8, já nas LS nos dias 1 e 8.
Palavras-chave: qualidade, legislação, nitritos.

1. INTRODUCTION

Due to its wide acceptance by consumers and for being a cost-effective protein option, sausage stands out among Brazilian processed meat products, accounting for 14.9 % of total consumption in 2022 [1]. According to the heat treatment, sausages are classified as fresh, cooked, and smoked. Fresh sausages, which do not undergo the curing or smoking process, can be made with meat from different species, such as pork and chicken, as well as fatty tissues and other ingredients. They are encased in natural or artificial wrapping and stored in cold rooms [2].

The technological process for producing sausages requires a series of handling steps, which increases the chances of contamination by pathogenic and/or spoilage microorganisms, compromising their microbiological quality, safety, and shelf life [3]. Sausages are also characterized by their high fat content, which can reach up to 30% [2]. This high fat content can favor lipid oxidation and the action of spoilage microbial lipolytic enzymes, which compromises the sensory quality of the food. [4, 5].

Meat has been an important source of foodborne diseases among the different foods worldwide [6]. In Brazil, between 2014 and 2023, the annual average of reported Food and Waterborne Outbreaks (FWBO) was 763. During this period, a total of 110,614 people became ill, resulting in 12,346 hospitalizations and 121 deaths [7].

Using nitrates and nitrites as inhibitors of the multiplication of pathogenic microorganisms is an alternative to guarantee their microbiological safety and, in addition, to add color and flavor to these foods. In Brazil, although the legislation allows a maximum of 0.015 g of nitrite to be added to 100 g of meat [8], this is not always respected. Adding nitrite in quantities above the permissible limit can cause methemoglobinemia, with a consequent reduction in oxygen transport. In addition, carcinogenic N-nitroso compounds, such as nitrosamines, can be formed both in food and in the human body [9].

Given the importance of fresh sausages as a source of affordable protein and the public health concerns about the occurrence of foodborne diseases, the influence of the type of meat on the microbiota and shelf life of fresh sausages was analyzed. In addition, the conformity of these foods with current legislation was evaluated to identify safer products for consumption.

2. MATERIAL AND METHODS

This study was carried out between November 2023 and May 2024, in the city of Londrina (Latitude 23°18′37″ S, Longitude: 51°09′46″ O). Londrina is an important point of reference and commerce in the North of the State of Paraná, as it is the second largest city in the state [10].

2.1 Selection of grocery store

Initially, a search was carried out on the Google platform using the search term "Grocery Stores in the Municipality of Londrina", in which 65 sites were located. Next, to verify the sale of bulk meat sausages in these stores, phone contact was attempted, with a response rate of 66 %. In the end, 40 (61 % of the total) confirmed the sale of the products in question.

These 40 grocery stores were organized according to the regions of the city in which they are located (North, South, East, West and Central) and, from there, the selection of establishments for sample collection was carried out by drawing lots for 2 establishments in each of the 5 regions of the city, giving a total of 10 stores as the target of the study. If the same supermarket chain was drawn in more than one region, the establishment was replaced by another in a subsequent draw.

2.2 Sample collection and preparation

A total of 40 fresh sausages (400 g/each) sold in bulk were purchased in the selected markets, 20 of which were made from pork (PS) and 20 from chicken (CS). All samples were from different batches, had an inspection service seal, and represented eight different commercial brands. The

temperature of the refrigerated shelves from which the samples were collected in the grocery stores varied between 2 and 4 ºC. Samples were kept in an isothermal box with recyclable ice (7 °C) and sent immediately to the Food Laboratory at Unopar - Londrina.

Each sample was aseptically fractionated into two portions of 200 g (01 and 02), totaling 40 samples of CS and 40 of PS. The “01 portions” were analyzed on the same day as collection, and the “02 portions” were stored individually in sterile packaging at 4 °C for 8 days. This temperature was based on the maximum storage temperature of the sausages in the establishments visited. The storage time followed the same criterion, since it was identified during the visits that, after the sausages had been fractioned by the establishment, they had an average shelf life of 8 days.

2.3 Microbiological analysis

After the decimal dilutions of the samples, quality indicator and pathogenic microorganisms were analyzed using Petrifilm™ plates (3M), under the following conditions: mesophilic aerobes (35 ºC/ 48 h), total coliforms and *Escherichia coli* (35 ºC/ 48 h), Molds and Yeasts (25 °C / 5 days), *Staphylococcus aureus* (35 ºC/ 24 h, followed by 35 ºC/3 h with Dnase confirmatory disk) and *Salmonella* spp. (41,5 °C /24 h). All analyses were carried out in duplicate, according to the manufacturer's instructions and the results were presented in CFU/g of sausage.

To test for spoilage microorganisms, psychrotrophic populations were determined on Plate Count Agar (Himedia, Mumbai, India) incubated at 7 °C for 10 days, and lipolytic psychrotrophs on Tributyrin Agar Base supplemented with 1% tributyrin (FDO81-5VL) incubated at 21 °C for 72 h [11].

The microbiological standards for *E. coli*, mesophilic aerobes and *Salmonella* ssp. followed the guidelines of Normative Instruction No. 161 of the Ministry of Health, National Health Surveillance Agency. Although not covered by current legislation, the research also included the determination of the population of important meat spoilage agents (Yeasts and Molds, Psychrotrophs and Lipolytic Psychrotrophs) and *Staphylococcus* spp., a microorganism of public health importance associated with several cases of food poisoning [7].

2.4 Nitrite analysis

The qualitative determination of nitrites in meat was carried out using the Griess-Ilosvay reaction [12]. Portions of 15 g of the samples were macerated with 30 mL of distilled water and filtered through sterile gauze. To a total of 10 mL of the filtrate was added 1 mL of sulphanilic acid solution (1 + 4). The mixture was stirred in a vortex for 10 seconds, followed by 1 mL of alpha-naphthylamine hydrochloride and further stirring for 10 seconds. The presence of nitrite was confirmed by the formation of a pink color, the intensity of which varied according to the amount of nitrite present. For the positive control, which served as a standard for comparison, a sample was prepared by adding 0.015 g of nitrite to 100 g of meat (chicken and pork), which corresponds to the maximum amount permitted by Brazilian regulations [8].

2.5 Statistical analysis

The influence of shelf life (1 and 8 days) on the microbiological counts of the same group (fresh chicken or pork sausages) and the variation in microbiological counts between the groups (fresh chicken and pork sausages) on the same day of storage (1 or 8 days) were evaluated using the Mann-Whitney test. These analyses were carried out at a significance level of 5 % using the Statistica 13.0 program.

3. RESULTS AND DISCUSSION

3.1 Compliance with microbiological legislation

The microbiological analyses required by Brazilian regulations [13] for fresh sausages are tests for quality indicators (mesophilic aerobes and *E. coli*) and *Salmonella* spp, an important foodborne pathogen. Considering the populations of mesophilic aerobes, the sausages analyzed in this study complied with the legislation (Table 1), indicating adequate sanitary quality of the food and general processing and storage conditions [14] within the expected standards. Regarding *E. coli*, 10 % of the pork sausage samples showed values above those permitted by current legislation on day 1, but the same sample came into compliance in the analysis on day 8 (Table 1). The reduction in contamination on day 8 suggests the occurrence of microbial antagonism. During this process, competition for nutrients and/or the production of inhibitory compounds by other microorganisms, such as lactic acid bacteria (which are part of the natural microbiota of meat), suppressed the *E. coli* population [15].

The presence of *Escherichia coli* in food is indicative of fecal contamination and suggests the probable presence of other enteric pathogens. In addition, some strains of *E. coli* are pathogenic and can lead to food or waterborne outbreaks (FWBO). According to the Brazilian Ministry of Health, between 2014 and 2023, the most common etiological agent identified in FWBO was *E. coli*, present in 34.8 % of them. A FWBO is characterized when two or more people presenting similar clinical symptoms and have in common the consumption of contaminated food or water [7].

Table 1. Non-compliance rate (%) (Brazil, 2022) of fresh pork and chicken sausages available in grocery stores in the city of Londrina, PR, between November 2023 and May 2024.

	Non-compliance rate				**Compliance values***	
Sausage type	Chicken[#]		Pork[#]		Chicken	Pork
Day of analysis	1	8	1	8		
E. coli	0 %	0 %	10 %	0 %	5x 10^3 CFU/g	10^3 CFU/g
Mesophilic Aerobes	0 %	0 %	0 %	0 %	10^6 CFU/g	10^6 CFU/g
***Salmonella* spp.**	10 %	10 %	0 %	0 %	Absent	Absent

* Normative Instruction No. 161 of the Ministry of Health, National Health Surveillance Agency [13]. [#] 10 samples (duplicate analysis/sample)

The observation that 10 % of chicken sausages were non-compliant for *Salmonella* contamination aligns with the significant risks posed by this pathogen in chicken products. *Salmonella* can persist in raw chicken products due to contamination from processing equipment, carcasses, or poor sanitation practices. While temperatures below 7 °C inhibit most microbial growth, *Salmonella* can remain present, albeit at lower levels, originating from sources like the viscera or cross-contamination during processing [16]. According to the Brazilian Ministry of Health [7], *Salmonella* spp. is the third (9.6 %) most frequent etiological agent associated with FWBO in the country. This pathogen is responsible for salmonellosis or enterocolitis, which is an infection caused by ingesting water or food contaminated with animal and human feces. In most cases, the disease is self-limiting, but it can develop into systemic infections and can also be transmitted by chronic carriers [17]. Between 2014 and 2023, chicken was the third type of meat most associated with FBWO in Brazil, while processed meat products ranked fourth. In the same period, homes were the main site of FWBO (34 %) in the country, which highlights the importance of cross-contamination and the population's lack of knowledge about food safety [7].

Improper thermal processing, post-contamination, cross-contamination or consumption of raw sausages can lead to outbreaks of foodborne illness caused by thermosensitive microorganisms such as *Salmonella* spp. and *E. coli.* Food safety guidelines therefore require heat treatment to ensure that the entire product reaches a minimum temperature of 70 °C. According to food safety

directives, heat treatment must ensure that all parts of the food reach a temperature of at least 70 ºC to eliminate these bacteria. However, lower temperatures may be used in heat treatment if their combination with time is sufficient to ensure the hygienic and sanitary quality of the food [18, 19]. Thus, both types of sausages presented samples that did not comply with the legislation, although the greatest health risk may be caused by chicken sausages, in which the presence of *Salmonella* spp. was observed.

The presence of nitrite was detected in all the sausage samples, both pork and chicken. However, the intensity of the color reaction, which varies according to the amount of nitrite present, was not uniform. Only 20% of chicken samples and 10% of pork samples exhibited a colorimetric reaction that corresponded to the maximum legal limit for nitrite (0.015 g of nitrite in 100 g of meat) [8]. This result indicates that, although nitrite was present in all samples, most of them probably contained a concentration below the maximum limit, resulting in a less intense color reaction. Nevertheless, it is crucial to consider the potential health implications associated with nitrite consumption. According to the World Health Organization (WHO), the acceptable daily intake (ADI) of nitrite, based on the risk of methemoglobinemia, is 0.07 mg/kg of body weight, which is equivalent to 4.2 mg (0.0042 g) of nitrite per day for a 60 kg adult [20]. Nitrite, as well as being a preservative, adds color and flavor to food when it is reduced to nitric oxide. This component reacts with the myoglobin present in the meat, forming the typical color of cured products [21]. The presence of nitrite in the diet has historically been linked to a high risk of developing cancer in different sites due to its ability to transform into highly carcinogenic N-nitrosamines. So, caution is recommended in the intake of nitrites and nitrates from processed foods [22].

3.2 Influence of the type of meat on the microbiota of fresh sausages

The higher populations of indicator and spoilage microorganisms in pork sausages compared to chicken ones, on the first day of analysis (Table 1), indicate poor sanitary practices and lead to spoilage of these foods and possible safety problems. Factors such as sub-optimal processing temperatures and inadequate handling also aggravate the risk of spoilage. Zhao et al. (2022) [23] observed a greater diversity of bacterial species in spoiled pork stored at 10°C compared to that kept at lower temperatures. Temperature is an important parameter for controlling microorganisms in food and its variation during the production and transportation processes, which was not monitored in this study, may have contributed to the microbiological differences between the two types of sausages on day 1.

An exception to this pattern on the first day of analysis was observed about yeasts and molds, the population of which was 5.6 times higher in chicken sausages than in pork ones (Table 2). Meat products can be contaminated by fungi during the transportation, storage and handling of meat, with yeasts becoming an important deteriorating psychrotrophic microbiota when these foods are stored at 10 °C. These results indicate health risks, as the presence of fungi in food is associated with the production of mycotoxins, particularly aflatoxin, fumonisin and deoxynivaenol. Once synthesized, mycotoxins are difficult to degrade due to their physical and chemical stability. Aflatoxins are hepatotoxic in humans and can range from acute to chronic, with the development of cirrhosis, and may result in liver cancer [24].

Regarding the *Staphylococcus* spp. population, as the end of the shelf life approached (day 8), the pork sausages had the highest population of these microorganisms compared to the chicken sausages. Staphylococci can inhabit the skin and mucous membranes of humans and animals. *S. aureus* stands out in food because it has a series of virulence factors, such as resistance to antimicrobials, production of heat-resistant enterotoxins, and the ability to form biofilms [25]. According to the Brazilian Ministry of Health, *Staphylococcus* spp. is the second most identified etiological agent, associated with 9.7 % of WBDO cases [7]. In Brazil, the maximum accepted standard for coagulase-positive staphylococci in meat products is 10^3 CFU/g [14]. In this study, the *Staphylococcus* counts were lower than this value (Table 2) and below the population required to synthesize enterotoxins, which means a low risk of food poisoning [26].

Even so, on the eighth day at 4 °C, chicken sausages had the highest populations of total coliforms, molds and yeasts, aerobic mesophiles, and total psychrotrophs. This results in reduced product safety and quality at the end of the storage period. Meat spoilage causes huge economic losses and numerous food recalls [27] and can be caused by biological agents such as microorganisms (bacteria, yeasts, and molds), and by the action of enzymes in meat, such as lipases and proteases [28].

3.3 Influence of the day of analysis on the microbiota of fresh sausages

Considering the microbiota of each type of sausage independently, storage time was associated ($p < 0.05$) with an increase in the population of all indicator and spoilage microorganisms for fresh chicken sausage (Table 2), except for *E. coli*, whose population decreased by 59.5 % after 8 days. Refrigeration effectively slows the growth of *Escherichia coli* due to its inability to multiply efficiently at lower temperatures. The optimal growth range for *E. coli* is between 35 °C and 42 °C, with minimal activity below 7 °C [29].

Total and lipolytic psychrotroph populations showed significant increases, of 7.1 and 7.7 times in samples aged 8 days, respectively, compared to those on day 1. This result indicated that the storage time/temperature binomial favored bacterial multiplication, leading to probable sensory defects and reduced shelf life. The development of sensory spoilage is related to microbial consumption of meat nutrients, such as sugars and free amino acids, and the release of undesired volatile metabolites [30].

In the pork sausages, there was an increase in the population ($p < 0.05$) of mesophilic aerobes, molds and, yeasts, and staphylococci after 8 days of storage. The spoilage microorganisms (total and lipolytic psychrotrophs) maintained their initial population ($p > 0.05$), and the total coliform and *E. coli* counts were reduced to 28.3 % and 3.9 % of the initial value, respectively (Table 2).

Table 2 - Populations of quality indicator microorganisms (CFU/g) (mean and standard deviation) present in fresh chicken and pork sausages available in supermarkets in the city of Londrina, PR, between November 2023 and May 2024.

Quality indicator	**Fresh Chicken Sausage**		**Fresh Pork Sausage**	
(CFU/g)[#]	**Day 1**	**Day 8**	**Day 1**	**Day 8**
Total coliforms	$2.2 \times 10^2 \pm 0.21 \times 10^2$ ^{b,B}	$8.1 \times 10^2 \pm 0.21 \times 10^2$ ^{a,A}	$1.0 \times 10^3 \pm 0.018 \times 10^3$ ^{a,A}	$2.9 \times 10^2 \pm 0.36 \times 10^2$ ^{b,B}
Escherichia coli	$2.1 \times 10 \pm 2.5 \times 10$ ^{b,A}	$1.2 \times 10 \pm 3.0 \times 10$ ^{a,B}	$3.2 \times 10^2 \pm 0.78 \times 10^2$ ^{a,A}	$1.2 \times 10 \pm 3.0 \times 10$ ^{a,B}
Yeasts and Molds	$2.6 \times 10^3 \pm 0.052 \times 10^3$ ^{a,B}	$1.7 \times 10^4 \pm 0.0017 \times 10^4$ ^{a,A}	$4.8 \times 10^2 \pm 0.31 \times 10^2$ ^{b,B}	$2.8 \times 10^3 \pm 0.061 \times 10^3$ ^{b,A}
***Staphylococcus* spp.**	$2.1 \times 10^2 \pm 0.46 \times 10^2$ ^{b,B}	$4.6 \times 10^2 \pm 0.10 \times 10^2$ ^{b,A}	$2.4 \times 10^2 \pm 0.22 \times 10^2$ ^{a,B}	$6.9 \times 10^2 \pm 0.15 \times 10^2$ ^{a,A}
Aerobic Mesophiles	$1.6 \times 10^4 \pm 0.001 \times 10^4$ ^{b,B}	$1.0 \times 10^5 \pm 0.00011 \times 10^5$ ^{a,A}	$1.7 \times 10^4 \pm 0.0040 \times 10^4$ ^{a,B}	$2.1 \times 10^4 \pm 0.0027 \times 10^4$ ^{b,A}
Psychrotrophs	$3.9 \times 10^4 \pm 0.0024 \times 10^4$ ^{b,B}	$2.8 \times 10^5 \pm 0.00022 \times 10^5$ ^{a,A}	$1.3 \times 10^5 \pm 0.00013 \times 10^5$ ^{a,A}	$1.6 \times 10^5 \pm 0.00027 \times 10^5$ ^{b,A}
Lipolytic Psychrotrophs	$1.9 \times 10^4 \pm 0.0015 \times 10^4$ ^{b,B}	$1.4 \times 10^5 \pm 0.00012 \times 10^5$ ^{a,A}	$6.2 \times 10^4 \pm 0.0082 \times 10^4$ ^{a,A}	$5.8 \times 10^4 \pm 0.007 \times 10^4$ ^{b,A}

^{A,B} Different superscript capital letters on the same row represent significant differences in microbiological populations between days 1 and 8 of analysis within each group (fresh chicken sausage and fresh pork sausage), using the Mann-Whitney test ($p < 0.05$).

^{a,b} Different lowercase letters superscripted on the same row represent significant differences in the populations of each microorganism between the groups (fresh chicken sausage and fresh pork sausage) on the same day of analysis, using the Mann-Whitney test ($p < 0.05$).

$^{\#}$ average values of 10 samples (duplicate analysis/sample) .

After storage, various intrinsic and extrinsic factors affect the process of microbial meat spoilage, including oxygen demand, pH, temperature, and competing organisms. The diversity of these ecophysiological factors affects the dynamics of microbial growth, including microbial succession and microbiota composition [31]. As the two types of sausage were under the same storage conditions, the pH (a parameter not measured in this study) and the higher initial population of bacteria in the pork sausages (Table 1) may have been the factors that contributed to the final population of this product.

Thus, observing the influence of storage time on the microbiota of fresh sausages, those produced with chicken meat, despite having lower counts of indicator and spoilage microorganisms on day 1, are the ones that pose the greatest risk. This is because they may contain *Salmonella*, as well as high populations of molds and yeasts. In relation to pork sausage, storage time (8 days) resulted in an increase in microorganisms that pose a health risk, like staphylococci, molds, and yeasts. Therefore, storage time negatively impacted the microbiological quality of both types of sausage. The high prevalence of lipolytic microorganisms suggests a high potential for spoilage due to enzymatic action during the 8 days of storage for pork sausages and at the end of this period for chicken sausages.

4. CONCLUSION

The meat used in the production of fresh sausages and the length of time they are stored under refrigeration influence their microbiological quality. Disease-causing microorganisms such *Staphylococcus* spp. and *Salmonella* spp. are present in this type of food, showing that keeping sausages under refrigeration and cooking them properly are essential to avoid foodborne outbreaks.

In addition, the presence of deteriorating microorganisms in high populations, especially psychrotrophs, points to the need for immediate consumption or keeping sausages frozen to inhibit the increase in the number of these bacteria, which can multiply at refrigeration temperatures. Greater attention should be paid to chicken sausages, which showed the highest number of samples compatible with the maximum permitted limit of nitrite in their composition.

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