**UNIVERSITÀ DEGLI STUDI DI PADOVA**

*School of Agriculture and Veterinary Medicine*

*Department of Comparative Biomedicine and Food Science*

**Second Cycle Degree (MSc) in**

## Biotechnologies for Food Science

# The influence of food additives, *Staphylococcus carnosus*, and *Staphylococcus xylosus* bacteria on the color change and microbiological stability of pork meat.

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ACADEMIC YEAR 2024-2025

# ABSTRACT

Pork meat is highly perishable, with color and microbiological stability being critical factors for consumer acceptance. Traditional curing relies on synthetic additives such as sodium nitrite, but the growing demand for clean-label products has spurred interest in natural alternatives, including celery and acerola extracts and microbial starter cultures. This study evaluates the effects of these natural additives and *Staphylococcus* starter cultures (*S. carnosus* and *S. xylosus*) on the quality and safety of fermented pork sausages.

Two experimental phases were conducted: Phase 1 compared synthetic curing agents (nitrite, ascorbate, phosphate) with plant-based alternatives (celery and acerola extracts), while Phase 2 assessed the role of starter cultures in nitrate-to-nitrite conversion and color stabilization. Results showed that nitrite-based formulations achieved the highest redness (a* = 5.40), but natural alternatives—particularly celery extract (0.3%) combined with acerola (0.3%) and *S. carnosus*—provided comparable color stability (a* = 5.28). Microbial analysis revealed that *S. carnosus* effectively controlled total plate counts ($2.34 \times 10^7$ CFU/g) and enhanced nitrate conversion, whereas *S. xylosus* led to higher microbial loads ($9.61 \times 10^9$ CFU/g) without improving color retention. Texture analysis indicated that phosphate-containing samples were firmer (18.48 N), while natural formulations maintained tenderness comparable to controls (14.55–14.87 N). All samples were free of *Salmonella* spp. and *E. coli*, confirming microbiological safety.

These findings demonstrate that celery and acerola extracts, combined with *S. carnosus*, can serve as viable nitrite substitutes in clean-label sausages, achieving desirable color and safety attributes. However, further optimization is needed to ensure consistent microbial management and color development, particularly regarding spoilage flora. The results provide a foundation for optimizing natural curing systems that meet both consumer preferences and industry standards for quality and safety.



**Keywords:** pork sausages, clean-label meat, natural curing, celery extract, acerola extract, *Staphylococcus* starter cultures, color stability, food safety

# Contents

# 1. INTRODUCTION

## 1.1. Meat Quality and Consumer Expectations

Meat is a highly perishable food product that is susceptible to spoilage and microbial contamination, making preservation a critical aspect of meat processing. Color and microbiological stability are two key factors that influence consumer acceptance and marketability of meat products. Color, in particular, is a primary indicator of freshness and quality, with consumers often associating bright red hues with high-quality meat (Mancini & Hunt, 2005). As shown by Altmann et al. (2023), Pork color preferences vary globally, reflecting deep-rooted cultural perceptions - while some consumers prefer bright red hues, others associate this with lower quality or frozen meat. These variations present formulation challenges for global markets, as maintaining the desired color profile while ensuring microbiological safety during storage and distribution remains a significant industry challenge.

### 1.1.1 The Clean-Label Trend and the Use of Natural Ingredients in Meat Preservation

The clean-label movement reflects a growing consumer preference for minimally processed foods made with simple, natural ingredients. In meat products, this has led to increased use of plant-based ingredients that can provide preservation effects similar to traditional additives. Natural sources of nitrates, such as celery, spinach, and beetroot, have gained particular attention as alternatives to synthetic nitrites in cured meat production. These ingredients contain naturally occurring nitrates that, when properly processed, can be converted to nitrites and deliver comparable benefits for color stabilization and microbial safety (Sebranek et al., 2014).

The effectiveness of natural nitrate sources depends on several key factors. First, the concentration of nitrates varies significantly between different plant materials, requiring standardization to ensure consistent results (Sebranek et al., 2014). Second, the conversion of nitrates to functional nitrites relies on microbial activity during fermentation, making proper starter culture selection essential (Alahakoon et al., 2015). When used correctly, these natural ingredients can achieve the characteristic cured color and flavor while meeting clean-label demands (Pegg & Shahidi, 2000).

Beyond nitrite replacement, other natural ingredients are being explored for meat preservation. Plant extracts rich in polyphenols, such as rosemary and green tea, have shown potential as antioxidants to prevent lipid oxidation (Shah et al., 2014). Similarly, fermentation products like bacteriocins from lactic acid bacteria offer natural antimicrobial effects (Leroy et al., 2013). These approaches align with clean-label objectives while addressing the technical challenges of reducing synthetic additives in meat products.

Regulatory agencies have established guidelines for using natural preservatives in meat processing. The European Union permits vegetable nitrate sources (e.g., celery extract) under Regulation (EC) No 1333/2008, as amended by Regulation (EU) 2023/2108, with maximum residual nitrite levels set at 80 mg/kg (expressed as nitrite ion) for most cured meats, and up to 150 mg/kg for certain traditionally produced meats (European Food Safety Authority (EFSA) et al., 2019). This framework supports innovation in clean-label meat products while ensuring food safety standards are maintained. The development of effective natural preservation systems continues to be an active area of research as the food industry responds to evolving consumer preferences.

### 1.1.2 Pork as a Strategic Matrix for Clean-Label Innovation

Global meat consumption patterns indicate that pork remains a dominant component, accounting for approximately 36% of total meat intake worldwide (World Food and Agriculture – Statistical Yearbook 2023, 2023). Within this landscape, processed meat products, particularly those derived from pork such as sausages, are highly prevalent in the diets of developed markets. However, increasing consumer awareness regarding the potential health risks associated with conventional curing methods, especially the use of synthetic nitrites, has spurred significant demand for 'clean label' alternatives (T.-K. Kim, Yong, Choi, et al., 2021). Numerous epidemiological studies have established concerning links between regular processed meat consumption and increased disease risks. Specifically, a daily intake of 50g of nitrate/nitrite-cured meats has been associated with an 18% elevated risk of colorectal cancer (World Cancer Research Fund International, 2018). Beyond cancer, processed meat consumption has also been robustly linked to elevated risks of cardiovascular diseases and type 2 diabetes.

As shown in figure 1, these health concerns stem primarily from the formation of carcinogenic N-nitroso compounds (NOCs) when nitrites react with amines in meat under acidic stomach conditions or during high-temperature cooking (Bedale et al., 2023).

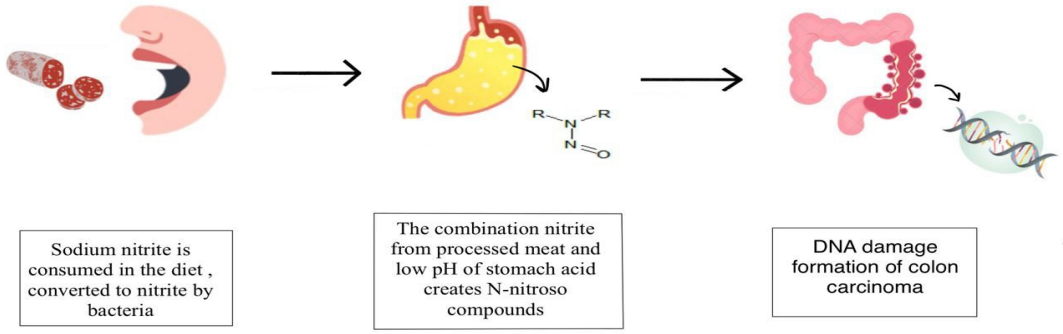


**Figure 1.** Formation of carcinogenic N-nitroso compounds and their link to colorectal cancer. This pathway illustrates how nitrites from processed meat, combined with acidic stomach conditions, form compounds that can damage DNA and promote colon carcinoma.

Driven by health concerns over synthetic nitrites and a desire for 'clean label' products, consumers increasingly seek 'uncured' meat alternatives using natural nitrate sources like celery powder. However, a critical caveat is that these plant-based nitrates are ultimately converted to the same bioactive nitrites within processed meats (Bowles et al., 2024), suggesting that more fundamental reformulation is necessary.

The biochemical challenges of pork processing further complicate clean-label innovation. Pork's high polyunsaturated fat content (35-50% of total lipids) makes it particularly susceptible to oxidation, especially in comminuted products like sausages where processing increases surface area exposure (Zhou et al., 2021). At the same time, pork's myoglobin chemistry shows unique responsiveness to natural curing systems, making it an ideal testing ground for alternative preservation methods. Emerging solutions focus on combining vegetable nitrate sources with

specific starter cultures, or utilizing novel pigment technologies, to develop safer products that maintain quality while addressing consumer health concerns.

### 1.1.3. Pork Meat Quality Attributes

Pork meat quality is a critical factor influencing consumer acceptance and marketability. Key attributes include color, texture, flavor, and microbiological safety. Color is one of the most important visual indicators of freshness, primarily determined by the state of myoglobin in the meat. Myoglobin can exist in three forms: deoxymyoglobin (purple-red), oxymyoglobin (bright red), and metmyoglobin (brown), with the latter indicating spoilage (Suman & Joseph, 2013). Recent studies demonstrate that retail pork maintains optimal color for only 2-3 days under refrigerated display, with surface discoloration progressing faster in pork compared to beef due to more rapid myoglobin oxidation, higher postmortem pH variability and greater susceptibility to protein denaturation at low (T. W. Kim et al., 2016 ; Gagaoua et al., 2023).

Texture development in pork involves complex postmortem biochemical changes, where calpain-mediated proteolysis of myofibrillar proteins determines ultimate tenderness (Bhat et al., 2018). The interplay between intramuscular fat content (typically 2-5% in commercial pork) and protein degradation creates the characteristic mouthfeel, with shear force values below 45 N being preferred by consumers (Van Oeckel et al., 1999). However, modern lean genetics have increased the incidence of pale, soft, exudative (PSE) meat, characterized by rapid pH decline, protein denaturation, and reduced water-holding capacity due to low ultimate pH (<5.4) and high carcass temperatures (Suliga et al., 2022).

Microbiological safety remains a critical concern in pork production, with pathogens like *Salmonella* spp., *Listeria monocytogenes*, and *Yersinia enterocolitica* posing significant risks due to their ability to colonize pork muscle tissue and processing environments (Baer et al., 2013). These pathogens demonstrate particular resilience, with *L. monocytogenes* capable of growth at refrigeration temperatures (Saldivar et al., 2018) and *Salmonella* exhibiting resistance to common processing interventions (Punchihewage-Don et al., 2024), creating unique food safety challenges that require targeted mitigation strategies. Recent advances in intervention technologies, including

novel packaging systems and non-thermal processing methods, show promise for enhancing safety while maintaining other quality attributes.

## 1.2 Meat Color

Meat color is one of the most critical factors influencing consumer purchasing decisions, often overriding other quality attributes like freshness or palatability (Purslow et al., 2020). The color of meat is primarily determined by the quantity, composition, and transformations of muscle pigments, particularly myoglobin and hemoglobin, while minor pigments (e.g., cytochrome C, carotenoids) play negligible roles (Suman & Joseph, 2013). Key factors affecting color intensity include the animal's species, breed, age, sex, diet, muscle type, and physical activity. The final color of cured meat products depends fundamentally on the myoglobin content of the raw meat, with species like beef and lamb naturally containing higher myoglobin concentrations that produce deeper red cured colors, while poultry and fish with lower myoglobin levels yield paler pink hues after processing (Han et al., 2024). Notably, myoglobin content is higher in pasture-raised animals, older livestock, and heavily exercised muscles, though visual color assessment can be misleading , for instance, PSE (pale, soft, exudative) pork appears lighter due to protein denaturation and fluid expulsion at low postmortem pH, despite retaining normal myoglobin content (Suliga et al., 2022).

### 1.2.1. Myoglobin Structure and Function

Myoglobin (Mb) is a 14-18 kDa water-soluble globin protein that serves as the primary oxygen carrier in muscle cells and the key determinant of meat color (Luchini et al., 2025). Its structure consists of a single polypeptide chain arranged in eight α-helical segments that form a hydrophobic pocket for the heme prosthetic group. This three-dimensional configuration positions hydrophilic amino acids on the protein surface while maintaining a hydrophobic interior that protects the heme cofactor - an iron-centered porphyrin complex (four pyrrole rings coordinating a central iron atom)(Suman & Joseph, 2013).

The redox state of Mb's iron center governs meat coloration through three interconvertible forms: (1) purple deoxymyoglobin ($Fe^{2+}$) in reduced state, (2) bright red oxymyoglobin ($Fe^{2+}$-$O_2$) when oxygenated, and (3) brown metmyoglobin ($Fe^{3+}$) in the oxidized form (Figure 2**)** (Suman & Joseph, 2013). The metmyoglobin (MetMb) percentage serves as a critical freshness indicator, with established quality thresholds: <20% MetMb maintains desirable bright red coloration; 20-30% causes noticeable darkening; 30-50% produces unappealing reddish-brown hues; and >70% results in complete brown discoloration signaling advanced spoilage (Han et al., 2024).

This oxidative conversion from $Fe^{2+}$ to $Fe^{3+}$ occurs progressively during storage, with kinetics influenced by environmental and processing factors. The dynamic equilibrium between Mb's three states continuously shifts post-mortem, making MetMb accumulation a reliable quantitative metric for both freshness assessment and consumer acceptance prediction.

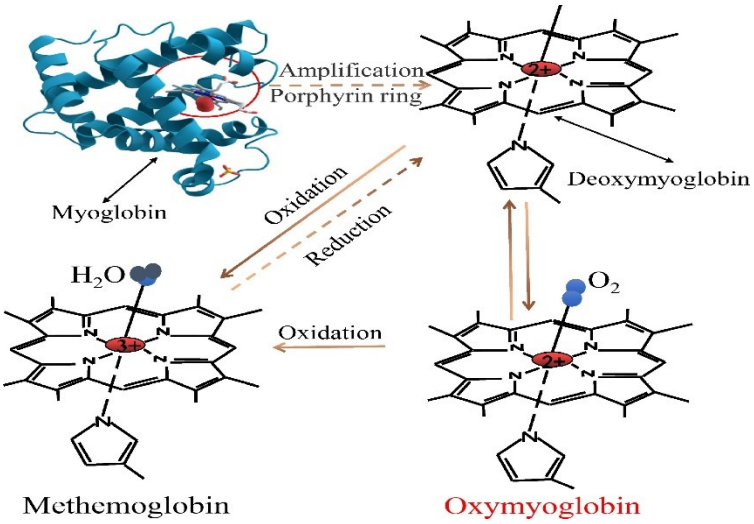


**Figure 2.** Structural changes of myoglobin (Han et al., 2024).

### 1.2.2. Color Changes in Meat: Mechanisms and Factors

The color of fresh and cured meats is primarily determined by the biochemical state of myoglobin, which undergoes dynamic changes in response to oxygen, pH, and processing conditions. In fresh meat, exposure to oxygen converts purple-red deoxymyoglobin ($Fe^{2+}$) into bright red oxymyoglobin ($Fe^{2+}$-$O_2$), the form most appealing to consumers. However, prolonged oxygen exposure leads to oxidation, forming brown metmyoglobin ($Fe^{3+}$), which is often perceived as a sign of spoilage (Suman & Joseph, 2013). Factors such as high storage temperatures, low pH, and lipid oxidation accelerate this process (Bekhit et al., 2013). Additionally, cooking induces structural changes in myoglobin; at high temperatures (>60°C), the denatured protein forms gray-brown hemichromes, whereas slower, low-temperature cooking may partially retain redness through hemochrome formation (Suman & Joseph, 2013). During meat curing, nitrite ($NO_2^-$) is reduced to nitric oxide (NO) which binds to myoglobin's heme iron ($Fe^{2+}$) to form stable pink nitrosylmyoglobin (MbFe(II)NO), the characteristic pigment of cured meats (Han et al., 2024).

Meat color stability is further influenced by packaging and storage conditions. Vacuum packaging maintains a deoxygenated state, preserving the purple-red hue of deoxymyoglobin, whereas high-oxygen modified atmosphere packaging (MAP) enhances surface redness but accelerates internal oxidation (Holman et al., 2018). Dietary α-tocopherol (vitamin E) supplementation in livestock more effectively stabilizes meat color than postmortem addition by integrating into muscle membranes during growth, where it simultaneously inhibits lipid and myoglobin oxidation at threshold concentrations (Faustman et al., 2010). Understanding these mechanisms is essential for optimizing meat quality, as color remains one of the most critical factors affecting consumer purchasing decisions. By controlling oxygen levels, temperature, and additives, the meat industry can enhance shelf life and maintain desirable visual appeal.

## 1.3. The Evolution and Mechanisms of Meat Curing

Originally, meat curing relied solely on nitrates (saltpetre), with the process depending entirely on the action of naturally occurring microorganisms. These denitrifying bacteria, which constitute the typical microflora of meat, play a crucial role in converting nitrate to nitrite - the active curing agent (Sánchez Mainar et al., 2017). Without sufficient quantities of these specific microbes, the desired cured color cannot develop properly (Toldrá, 2007). To ensure consistent reduction of

nitrate to nitrite, modern curing practices often incorporate starter cultures containing these essential microorganisms (Terns et al., 2011). Interestingly, such cultures are sometimes used even in nitrite-based curing to enhance color stability of the final product (Terns et al., 2011). This understanding of microbial conversion led to a significant advancement in curing technology - the direct use of nitrites instead of nitrates (Sebranek & Bacus, 2007).

In cured meats, the characteristic bright red color arises from the reaction of nitric oxide (NO) - generated from nitrite in acidic conditions - with myoglobin or metmyoglobin, forming nitrosylmyoglobin (Figure 3) (Han et al., 2024). This pigment is heat-stable; upon cooking, it transforms into nitrosylhemochrome, the pink-red compound seen in products like ham and sausages (Suman & Joseph, 2013). However, color stability depends on storage conditions - exposure to light and oxygen can degrade pigments, leading to fading (Shakil et al., 2022). Beyond color, nitrite contributes to cured meat quality by enhancing aroma, inhibiting microbial growth, and preventing fat oxidation (Jin et al., 2018). The curing process is highly pH and temperature dependent: lower pH accelerates nitrosylation, while higher pH improves pigment stability (Han et al., 2024). Yet, maintaining low temperatures during curing is crucial to avoid microbial spoilage, even if it slows the reaction. Balancing these factors is key to achieving optimal color and safety in cured meat products.

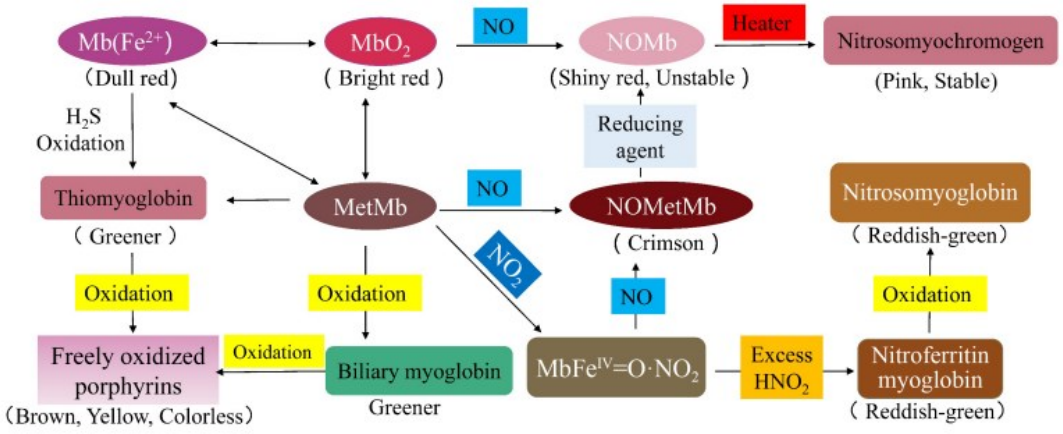


**Figure 3.** Structural change pathway of myoglobin in cured meat (Han et al., 2024).

## 1.4. Food Additives in Meat Curing

### 1.4.1. Sodium Chloride (NaCl)

Sodium chloride serves as the foundational component in meat curing, playing multiple critical roles in both preservation and quality development. As a primary curing agent, salt induces structural changes in muscle proteins through its ionic strength, causing swelling of myofibrils and enhancing water-holding capacity. This protein modification leads to improved texture and juiciness in the final product (T.-K. Kim, Yong, Jung, et al., 2021). Beyond its technological functions, salt exerts significant antimicrobial effects by reducing water activity (aw), effectively inhibiting common spoilage microorganisms like Pseudomonas spp. (Taormina, 2010). The chloride anion additionally activates endogenous proteolytic enzymes, contributing to flavor development through generation of peptides and free amino acids during the curing process (Koohmaraie et al., 2002). In production practice, salt concentrations typically range from 2% to 4.5% depending on product type, with careful balance required to avoid excessive hardness in dry-cured products (Yin et al., 2023). Despite its functional benefits, high salt intake (9–12 g/day in many diets) is linked to hypertension and cardiovascular diseases, prompting the WHO to recommend a reduction to ≤5 g/day (Cluff et al., 2016, WHO,2012).

### 1.4.2. Curing Salt (NaCl/$NaNO_2$ Mixture)

Curing salt, typically a mixture of sodium chloride (NaCl) and sodium nitrite ($NaNO_2$), plays a crucial role in meat processing by enhancing flavor, color, and safety. The nitrite component reacts with myoglobin in meat to form nitroso-hemochrome, giving cured meats their characteristic pinkish-red hue (Di Nunzio et al., 2022). Additionally, nitrite acts as a potent antimicrobial agent, inhibiting pathogens like *Clostridium botulinum* and *Listeria monocytogenes* (García-Díez & Saraiva, 2021), while its antioxidant properties prevent lipid oxidation and rancidity (Pisoschi et al., 2021). Despite these benefits, nitrite's potential to form carcinogenic nitrosamines during high-temperature cooking has raised health concerns (Shakil et al., 2022).

To mitigate risks, natural alternatives such as nitrate-rich leafy greens (e.g., spinach, celery, and parsley) and plant extracts (e.g., barberry and beetroot) are being explored (Yong et al., 2021;

Munekata et al., 2021). For instance, celery powder and Swiss chard powder effectively replicate nitrite's functions in sausages, improving color stability and shelf life (Restrepo Molina et al., 2019). Similarly, barberry extract (90 ppm) combined with reduced nitrite levels (30–60 ppm) enhances antioxidant activity while maintaining sensory quality (Serdaroğlu et al., 2023). However, no single alternative yet matches nitrite's multifunctional role, underscoring the need for further research into cost-effective, natural substitutes (Dissanayake et al., 2024).

### 1.4.3. Sodium Tripolyphosphate (STPP, E451i)

Sodium tripolyphosphate serves as a crucial processing aid in cured meat production, primarily functioning to improve water retention and texture (McGee et al., 2003). Through its alkaline nature, STPP induces a pH shift that promotes protein solubilization and swelling of myofibrillar structures (Nguyen Huynh Bach Son Long, 2011). This mechanism significantly enhances the water-holding capacity of meat proteins, particularly important in cooked products where moisture retention directly impacts yield and sensory quality. In comminuted products like sausages, phosphates contribute to emulsion stability by improving fat binding and preventing cook-out losses during thermal processing (Glorieux et al., 2017). The optimal combination of 1.8% salt and 0.3% STPP has been identified as the most effective for achieving superior texture, including increased hardness, gumminess, and chewiness, while maintaining cohesiveness (Choi & Chin, 2020). Additionally, STPP contributes to color stability, with higher salt and phosphate levels increasing redness (a*) and yellowness (b*) without affecting lightness (L*) (Choi & Chin, 2020).

### 1.4.4. Sodium Ascorbate (E301)

Sodium ascorbate (E301) is a key additive in cured and fermented meat products such as sausages, where it plays an essential role in enhancing color development and oxidative stability. It acts as a reducing agent that accelerates the conversion of nitrite-derived metmyoglobin to the stable pink nitrosylmyoglobin pigment, improving the characteristic cured meat color (Vossen et al., 2012). Additionally, sodium ascorbate functions as a potent antioxidant by scavenging free radicals, thereby inhibiting lipid oxidation and extending shelf life (Berardo et al., 2016). However, under certain conditions, especially in the presence of metal ions, it can also exhibit pro-oxidant effects (Villaverde et al., 2014).

Beyond color and oxidation control, sodium ascorbate contributes to food safety by reducing the formation of carcinogenic nitrosamines through its interaction with nitrite (Izumi et al., 1989). Its combined antimicrobial and antioxidant properties make it indispensable in modern meat processing. Studies have shown that appropriate supplementation of sodium ascorbate improves sensory attributes and shelf life in fermented sausages and other processed meats, particularly when nitrite levels are reduced for consumer safety concerns (Vossen et al., 2012).

### 1.4.5. Celery Extract

Celery extract has emerged as the predominant natural alternative to synthetic nitrites in meat processing, driven by consumer demand for clean-label products and organic market growth (T.-K. Kim, Yong, Choi, et al., 2021). Health concerns regarding nitrosamine formation and consumer preference for natural ingredients have prompted the development of vegetable-based alternatives, with celery becoming the industry standard due to its high nitrate content (2-3%) and neutral sensory profile (Sebranek et al., 2014). Modern applications utilize either traditional nitrate-rich celery powder requiring microbial conversion by starter cultures like *Staphylococcus carnosus*, or pre-converted concentrates containing 10,000-15,000 mg/kg nitrite, which eliminate the need for fermentation steps (Sindelar et al., 2010).

The technological implementation of celery extract presents both opportunities and challenges. While enabling "no-nitrate-or-nitrite-added" labeling claims, the natural curing process demonstrates variable efficiency in nitrate-to-nitrite conversion and typically achieves lower residual nitrite levels (100-200 ppm) compared to conventional curing (Sebranek et al., 2014). Furthermore, the alkaline nature of celery concentrates (pH 8.5-10) may elevate product pH by 0.3 units, potentially reducing the antimicrobial efficacy of nitrite against pathogens like *Listeria monocytogenes* (Myers, 2012). Research indicates that the complex matrix of celery-derived compounds (proteins, carbohydrates, and minerals) may interact differently with meat systems compared to purified nitrite salts, affecting both curing reactions and microbial inhibition (Djeri, 2010).

Despite these limitations, celery extract remains commercially viable when properly standardized and supplemented with starter cultures, offering comparable safety and quality attributes to conventional curing at typical usage levels of 0.2-0.4% (Sindelar et al., 2007).

### 1.4.6. Acerola Extract

Acerola (Malpighia emarginata) extract serves as an effective natural alternative to synthetic ascorbates in meat products, primarily enhancing color stability, shelf-life, and safety (Van Buren et al., 2024). It's widely cultivated in Brazil, particularly valued for its exceptionally high vitamin C content, which is at least 10 times greater than citrus fruits, along with other bioactive compounds including anthocyanins, phenolic compounds, and carotenoids that contribute to its antioxidant, anti-inflammatory, and antimicrobial properties (Hoang et al., 2022; Ruiz-Torralba et al., 2018).

Research on beef products has shown that incorporation of 0.15% (w/w) acerola extract can extend shelf life by at least three days, improve lipid stability, maintain better color characteristics, and reduce rancid flavor intensity without negatively impacting microbial counts (Realini et al., 2015). For pork products, the combination of acerola residue extract with ultrasound treatment during marination has been shown to improve quality characteristics by reducing hardness and chewiness while increasing myofibrillar fragmentation and enhancing the supply of sarcoplasmic calcium to proteolytic enzymes (Araújo et al., 2022).

Additional studies have demonstrated that topical application of acerola cherry powder in combination with rosemary extract can effectively maintain redness and delay oxidation processes in frozen-thawed beef cuts (Van Buren et al., 2024). Acerola extract exhibits strong antimicrobial activity in meat systems, effectively inhibiting foodborne pathogens like *Listeria monocytogenes* and *Salmonella* (Santos et al., 2023) while also suppressing spoilage bacteria, making it a promising natural preservative for meat products (Hoelscher et al., 2024). Da Costa Lima et al., (2022) confirmed that acerola by-product extracts significantly reduce *Escherichia coli* counts by up to 99.9%, demonstrating potent antimicrobial activity against this common foodborne pathogen. These findings reinforce the potential of acerola extracts as effective natural preservatives to enhance the microbial safety of meat products.

## 1.5. Nitrite Use in Cured Meats: Risks and Regulations

Nitrites play an important role in cured meats, serving as critical preservatives against pathogens like *Clostridium botulinum* while creating the characteristic color and flavor consumers expect (Sebranek & Bacus, 2007). However, since the International Agency for Research on Cancer (IARC) classified processed meats as carcinogenic in 2018 due to potential nitrosamine formation from nitrites (International Agency for Research on Cancer et al., 2018), consumer perceptions have shifted toward reduced-additive alternatives, despite limited awareness of specific health risks (Bedale et al., 2023). While vegetable-based substitutes like celery powder offer solutions, their inconsistent nitrate content and weaker antimicrobial efficacy pose technical challenges for manufacturers striving to balance safety, regulatory compliance and sensory quality (T.-K. Kim, Yong, Choi, et al., 2021; Sebranek & Bacus, 2007).

The European Union's most recent regulatory update, Commission Regulation (EU) 2023/2108, significantly restricts the use of nitrite and nitrate additives in cured meat products (European Commission, 2023). The regulation sets maximum permitted levels at 55–80 ppm (as $NO_2^-$ ion) for sodium nitrite (E250) and potassium nitrite (E249), and 90 ppm (as $NO_3^-$ ion) for sodium nitrate (E251) and potassium nitrate (E252) (European Commission, 2023; FoodTimes, 2023). These changes follow the European Food Safety Authority's (EFSA) 2017 reevaluation of nitrosamine risks and reflect increasing consumer and policy demands for reduced additive use in food processing (EFSA, 2017).

Consequently, the meat industry faces the challenge of innovating to produce "clean-label" products that satisfy consumer preferences for traditional cured meat attributes while ensuring microbial safety and regulatory compliance (Dissanayake, 2024).

## 1.6. Role of Starter Cultures in Meat Fermentation

### 1.6.1. Fundamental Roles of Starter Cultures in Meat Fermentation

Starter cultures serve as essential drivers of quality and safety in fermented meat production, with carefully selected strains of lactic acid bacteria (LAB) and coagulase-negative *staphylococci* (CNS) performing distinct yet complementary functions (García-Díez & Saraiva, 2021). The industrial application of defined starter cultures has become standard practice to ensure process

control, product standardization, and biosafety (Ravyts et al., 2012). LAB, predominantly *Lactobacillus sakei*, initiate rapid acidification of the meat batter, while CNS, particularly *Staphylococcus xylosus* and *S. carnosus*, contribute to color development and flavor formation (Leroy et al., 2006).

*S. xylosus,* commonly dominant in artisanal fermentations, excels in proteolytic and lipolytic conversions, generating key aroma compounds from branched-chain amino acids (leucine, isoleucine, valine) and free fatty acids (Ravyts et al., 2010). In contrast, *S. carnosus*, though rarely found in spontaneous fermentations, has been domesticated for industrial use due to its exceptional technological properties, including nitrate/nitrite reduction, carbohydrate degradation, and catalase activity (Sánchez Mainar et al., 2017). Its compact genome lacks virulence factors, making it particularly suitable for food applications.

The aroma profile of fermented sausages can be precisely modulated through starter culture selection and process parameters. Fast-ripened sausages benefit from increased inoculum levels of *staphylococci* that enhance methyl-branched aldehyde production, while slow-ripened products develop more complex flavor profiles with varying levels of methyl-branched acids, diacetyl, and ethyl esters depending on inoculation density. Additional factors including nitrate, nitrite, ascorbate, and environmental conditions further influence the generation of volatile compounds (Leroy et al., 2006).

### 1.6.2. Microbiological Stability in Meat Products

While modern starter cultures and controlled fermentation processes have significantly improved the safety of fermented meats, microbial risks persist - particularly in artisanal and short-ripened products. Recent studies confirm that despite the antimicrobial effects of acidification and competitive exclusion, pathogens like *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* remain concerns in fermented sausage production (Baer et al., 2013; Laranjo et al., 2017). Notably, *L. monocytogenes* is especially problematic due to its prevalence in raw meat reported contamination rates exceeding 60% in some Greek-style sausage productions and its severe implications for public health (Saldivar et al., 2018).

Advances in functional starter cultures, particularly bacteriocin-producing strains such as *Lactobacillus sakei* and *Lactococcus lactis*, have demonstrated enhanced antimicrobial activity against these pathogens, offering a promising approach to improve safety without compromising product quality (García-Díez & Saraiva, 2021; Laranjo et al., 2017). However, variability in product formulations, ripening conditions, and initial microbial loads necessitates tailored strategies to ensure consistent microbiological stability. This is especially critical under stringent regulatory frameworks like the U.S. zero-tolerance policy for *L. monocytogenes* in ready-to-eat meat products (Taormina, 2010; U.S. FDA, 2020).

# 2. Aim of the Project

This thesis aims to investigate the impact of food additives and starter cultures on the quality and safety of fermented pork sausages. Using a combination of physicochemical and microbiological analyses, we will evaluate color stability, microbial inhibition, texture properties, and pH changes in sausage samples treated with both natural (celery extract, acerola extract) and synthetic (sodium nitrite, sodium ascorbate) curing agents, with and without inoculation by *Staphylococcus carnosus* and *Staphylococcus xylosus*. The research focuses on comparing treatment effects through instrumental color measurements, Warner-Bratzler Shear Force analysis, microbial counts, and pH monitoring to understand how different curing approaches influence sausage quality parameters. Special attention will be given to the role of starter cultures in nitrate-to-nitrite conversion and their subsequent effects on color development and microbiological safety during the fermentation process. Understanding these interactions will contribute to developing optimized, clean-label strategies for sausage production that maintain product quality while meeting consumer demands for reduced synthetic additives.

# 3. Materials and Methods

## 3.1. Sample Preparation

The study comprised two distinct experimental phases. Phase 1 evaluated eight sausage formulations using either synthetic curing agents or natural nitrate without microbial inoculation. Phase 2 investigated modified formulations incorporating starter cultures (*Staphylococcus carnosus* and *S. xylosus* at $10^6$ CFU/g), with adjusted curing agent concentrations to account for microbial nitrate reductase activity.

### 3.1.1. Phase 1: Pork Sausage Preparation with Control Formulations

The pork sausage samples were prepared using fresh ground pork with a lean-to-fat ratio of 80:20, divided into eight experimental batches with different combinations of curing agents and additives as specified in Table 1. The formulations included varying percentages of salt, curing salt (99.4% NaCl and 0.6% $NaNO_2$), sodium tripolyphosphate (E451i), sodium ascorbate (E301), celery extract, and acerola extract, with total curing agents ranging from 3.15 to 4.55 g per batch. For each batch, 125 g of ground pork was thoroughly mixed with 50 g of curing agents and additives using a planetary mixer for 5 minutes at 4°C. The mixtures were then stuffed into 32-mm artificial casings to form sausages weighing 175 ± 5 g each, with four replicates prepared per batch, totaling 32 sausages. The sausages were cooked in a water bath at 72°C for 20 minutes, with the core temperature verified to reach 68°C using a thermometer. After thermal processing, all sausages were vacuum-sealed stored at 4°C for up to 72 hours until analysis.

| composition | Variants (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 |
| Salt | 1.80 | | | | | 1.80 | 1.80 | 1.80 |
| Curing salt | | 1.80 | 1.80 | 1.80 | 1.80 | | | |
| Sodium tripoliphospates | | | 0.70 | | 0.70 | | | |
| E451i | | | | | | | | |
| Sodium askorbate (E301) | 0.50 | | | 0.70 | 0.70 | | | |
| Celery extract | | | | | | 0.30 | 0.30 | 0.50 |
| Acerola | | | | | | 0.20 | 0.30 | 0.30 |

**Table 1.** phase1: pork sausage composition defined per batch.

### 3.1.2. Phase 2: Starter Culture-Modified Formulations

Four additional batches were prepared according to the formulations specified in Table 2, maintaining the same base preparation and processing methods as Phase 1 (125 g 80:20 lean-to-fat pork, 50 g curing agents, 72°C/20 min thermal processing). The modified formulations incorporated either 0.001% *Staphylococcus carnosus* (Batches 1 and 3) or 0.002% *S. xylosus* (Batches 2 and 4) while adjusting other curing components as detailed in Table 2. All sausages were similarly stuffed into 32-mm casings (175 ± 5 g), vacuum-sealed after cooking, and stored at 4°C for analysis within 72 hours.

| composition | Variants (%) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| salt | 1.80 | 1.80 | 1.80 | 1.80 |
| Sodium tripoliphospates (E451i) | 0.70 | 0.70 | 0.70 | 0.70 |
| Acerola | 0.30 | 0.30 | 0.30 | 0.30 |
| celery | 0.30 | 0.30 | - | - |
| bacteria | 0.001 | 0.002 | 0.001 | 0.002 |

**Table 2.** phase2: pork sausage composition defined per batch.

## 3.2. Color measurement

Surface color of pork sausages was evaluated using a Spectro-pen spectrophotometer following the CIELAB color system (CIE 1976). The instrument was calibrated with a white reference tile prior to analysis. Three random locations on each sausage surface were measured to account for color heterogeneity, with the L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) values recorded and averaged. Measurements were conducted 30 minutes after sample removal from refrigeration (4°C). The total color difference (ΔE*) was calculated according to equation below (Ferro et al., 2021), providing a quantitative measure of color variation between samples.

$$\Delta E = \sqrt{(a_0 - a)^2 + (b_0 - b)^2 + (L_0 - L)^2}$$

## 3.3. Texture Analysis

Texture of the pork sausages was analyzed using a Texture Analyzer TA-XT2i (Stable Micro Systems, UK) fitted with a Warner-Bratzler (WB) shear blade, a 50 kg load cell, and controlled by Exponent software. Post-thermal processing, sausages were cooled to room temperature (20°C) and cut into cylindrical cores (0.5-inch diameter, 0.5-inch height). Six cores per sausage were sheared perpendicular to the long axis at a head speed of 0.5 m/min (~8.33 mm/s). Two parameters were recorded: maximum cutting force (Firmness, N), reflecting sausage hardness, and cutting work (Toughness, N·sec), calculated as the area under the force curve. Results were averaged across the six measurements per sample. The analyzer was calibrated before each session, and shear force values were recorded for statistical analysis to evaluate the effect of additive formulations on sausage texture.

## 3.4. pH Measurement

A portable pH meter with a knife probe electrode (EPC-50) was used for non-invasive pH measurements of meat samples. The electrode was inserted into five points on each sample. The device was calibrated with pH 4.00 and 6.88 buffer solutions at 20 ◦C.

## 3.5. Microbiological Analysis: Total Plate Count (TPC)

The microbiological quality of sausage samples was evaluated using total plate count (TPC) according to standard methods. For each sample, 10 g of homogenized sausage was aseptically mixed with 90 mL of sterile peptone water (0.1% w/v) and stomached at 260 rpm for 2 minutes to create a uniform suspension. Serial decimal dilutions ($10^{-1}$ to $10^{-6}$) were prepared in peptone water, and 100 µL aliquots of appropriate dilutions were spread-plated in duplicate onto Plate Count Agar (PCA). Plates were incubated aerobically at 30°C for 72±3 hours, after which colonies were enumerated using an automated colony counter. Afterward, TPC was calculated using the formula below:

$$\mathbf{TPC} = \frac{\sum \mathbf{CFU}}{\mathbf{n_1} \times \mathbf{V_1} \times \mathbf{d}}$$

Where ΣCFU is the total number of colonies counted across all valid plates, n is the number of plates counted at the selected dilution, V is the volume plated (0.1 mL), and d is the dilution factor of the counted plates.

## 3.6. Detection and Enumeration of *Staphylococcus xylosus*

The detection and quantification of *Staphylococcus xylosus* in pork sausage samples were performed using a selective culture-based method followed by biochemical confirmation. For sample preparation, 10 g of homogenized sausage were aseptically weighed and transferred into a sterile stomacher bag containing 90 mL of 0.1% (w/v) peptone water. The mixture was homogenized at 260 rpm for 2 minutes to obtain a uniform suspension. Serial decimal dilutions ($10^{-1}$ to $10^{-8}$) were prepared in sterile peptone water to accommodate the expected microbial load range ($10^2$–$10^9$ CFU/g).

From each dilution, 100 µL aliquots were spread-plated in duplicate onto Mannitol Salt Agar (MSA), a selective medium containing 7.5% NaCl and phenol red indicator. The plates were incubated aerobically at 37°C for 24 hours to allow for colony development. Following incubation, presumptive *S. xylosus* colonies were identified by their characteristic pink-to-red coloration, indicating non-mannitol fermentation. Colonies within the countable range (25–250 CFU/plate) were enumerated using an automated colony counter, with manual verification when necessary. Results were expressed as colony-forming units per gram (CFU/g) and $\log_{10}$-transformed for statistical analysis.

To confirm species identity, representative colonies were subjected to biochemical testing. Catalase activity was assessed using 3% hydrogen peroxide, with bubble formation indicating a positive result. Coagulase activity was tested using rabbit plasma, with *S. xylosus* exhibiting a negative reaction. Final identification was performed using the API Staph system, which evaluates 20 enzymatic and metabolic reactions to generate a species-specific biochemical profile. Method validation included positive (*S. xylosus* ATCC 29971) and negative (sterile peptone water) controls, with all procedures conducted under aseptic conditions in a laminar flow cabinet to

prevent contamination. The detection limit of the method was established at $10^2$ CFU/g, ensuring sensitivity across the observed microbial load range in the samples.

## 3.7. Detection of *Salmonella spp*. and *E. coli*

For pathogen detection, two validated rapid methods were employed:

*E. coli quantification* was performed using Hygiena's MicroSnap® test, a bioluminogenic assay detecting β-glucuronidase activity characteristic of *E. coli*. 25g sausage samples were homogenized in 225 mL of phosphate buffer, enriched for 6-8 hours at 37°C, then analyzed via enzymatic reaction in the Detection Device. Luminescence (measured in RLUs using an EnSURE® Touch luminometer) was converted to CFU/g through a matrix-specific calibration curve, providing results within 8 hours with sensitivity to $10^1$ CFU/g.

*Salmonella* screening utilized Hygiena's InSite™ Salmonella (AOAC-RI Certified), an integrated swab-based system combining enrichment and detection. Environmental surfaces (100 $cm^2$ areas) were sampled using the pre-moistened foam swab, which was then activated and incubated at 37±1°C for 24-48 hours. Presumptive positives were indicated by a color change from purple to yellow, with confirmation through culture isolation on XLD agar and biochemical testing (TSI, LIA). The closed-system design achieved 98% correlation with ISO 6579-1 while reducing processing time by 72 hours.

## 3.8. Statistical Analysis

Statistical analyses were performed using SPSS Statistics 27 (IBM Corp.). For color parameters (L*, a*, b* values), one-way ANOVA with Tukey's HSD post-hoc test was used for multiple comparisons between sample groups. Microbiological counts were log10-transformed and analyzed using one-way ANOVA with Tukey's HSD post-hoc test to compare treatment effects. Pathogen detection results (*Salmonella* and *E. coli* presence/absence in 25g samples) were evaluated descriptively. All tests were conducted at a 95% confidence level ($\alpha = 0.05$), with data presented as mean ± standard deviation.

# 4. RESULTS AND DISCUSSION

This chapter presents experimental findings on the efficacy of synthetic and natural curing systems in pork sausages, expanding upon prior research on clean-label meat preservation. The study systematically evaluated how different additive combinations ranging from conventional nitrite-based cures to plant-derived alternatives affect color stability, microbial safety, and physicochemical properties. Statistical analysis identified significant differences among treatments, with particular attention to the interplay between nitrate sources, antioxidants, and starter cultures. While synthetic curing agents are well-documented for their preservation capacity, their natural counterparts face challenges in achieving comparable color and safety profiles. This work addresses that gap by quantifying the performance of celery extract and acerola as nitrite substitutes while assessing their synergies with *Staphylococcus* starter cultures.

## 4.1. phase one: Control Formulations (With and Without Natural Additives)

### 4.1.1. Formulation Strategy and Experimental Design

This study developed eight distinct sausage formulations (S1-S8) to systematically evaluate the effects of synthetic and natural curing agents on color stability and technological properties. The experimental design progressed from basic to complex systems (Figure 4), beginning with control formulations: S1 (1.8% salt + 0.5% sodium ascorbate) established baseline antioxidant effects without nitrites, while S2 (1.8% curing salt) isolated nitrite's role in myoglobin stabilization. Building on this foundation, S3 (S2 + 0.7% sodium tripolyphosphate) assessed phosphate-mediated water retention, and S4 (S2 + 0.7% sodium ascorbate) tested nitrite-ascorbate synergy. The synthetic system culminated with S5 (S2 + 0.7% ascorbate + 0.7% phosphate) as an industry-standard benchmark. Transitioning to clean-label alternatives, S6-S8 progressively replaced synthetic additives with natural options - S6 (1.8% salt + 0.3% celery extract + 0.2% acerola) introduced basic plant-based curing, S7 increased acerola to 0.3% to optimize nitrate reduction, and S8 (0.5% celery + 0.3% acerola) represented a high-potency natural system. This gradient approach enabled direct comparison of nitrite versus nitrate curing efficiency, antioxidant synergies, and the technological performance of phosphates in clean-label applications, while maintaining constant salt content (1.8%) across all batches for controlled evaluation.

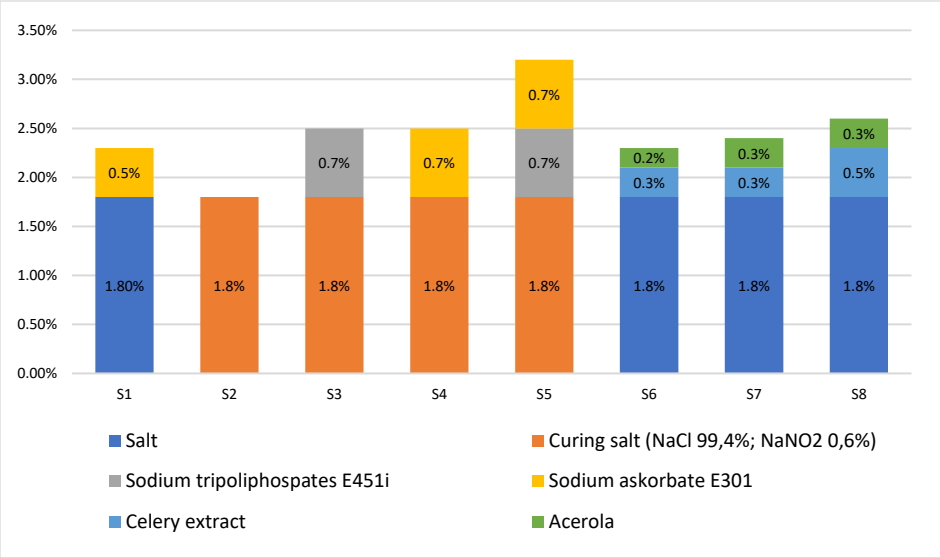


**Figure 4.** Additive composition (%) of the eight sausage treatment formulations (S1–S8) in phase one.

### 4.1.2. Color Stability of Cured Sausages

The color parameters (L*, a*, b*) revealed significant variations ($p < 0.05$) across formulations, reflecting distinct curing efficiencies. Samples with synthetic nitrite (S3–S5) exhibited markedly higher redness (a = 4.74–5.40) compared to the control (S1: a* = 1.64), confirming nitrite's role in nitrosylmyoglobin formation (Figure 5). Notably, S4 (curing salt + sodium ascorbate) achieved the highest a* value (5.40 ± 0.12), demonstrating ascorbate's synergistic enhancement of nitrite-derived pigmentation. These findings are in line with Sindelar et al (2007) and Sebranek & Bacus (2007), who reported that nitrite is crucial for the formation of the characteristic pink-red color in cured meats, while natural alternatives like celery extract can provide comparable redness, especially when nitrate content is high even in the absence of starter cultures, as seen in the present study.

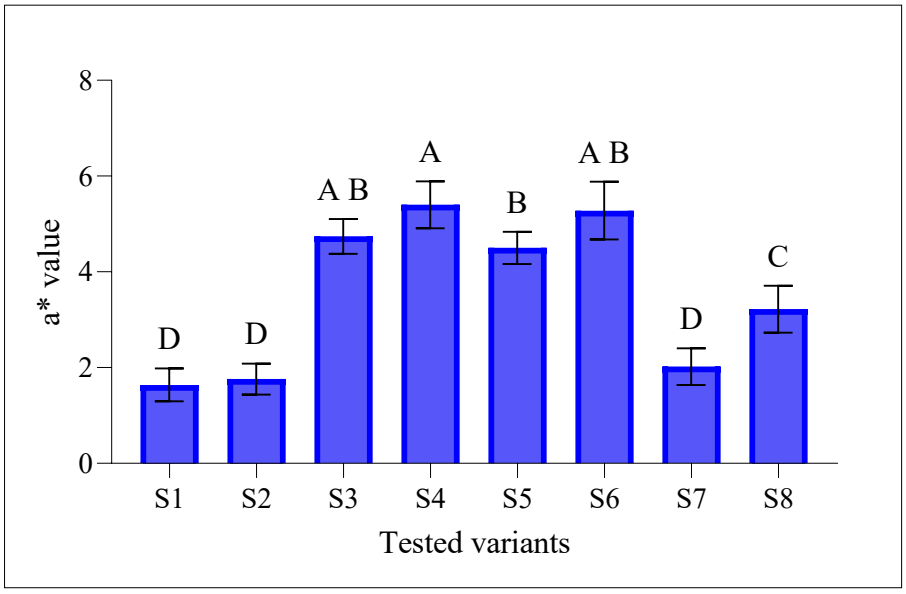


**Figure 5.** Redness (a*) of meat samples depending on the variant tested.

Lightness (L*) ranged from 61.98 (S3) to 66.92 (S2), with phosphate-containing batches (S3, S5) showing lower L* values (Figure 6), likely due to protein-water interactions altering surface reflectance. Natural formulations (S6–S8) displayed intermediate a* values (2.02–5.28), with S6 (0.3% celery + 0.2% acerola) approaching synthetic systems (a* = 5.28), suggesting efficient nitrate-to-nitrite conversion. However, S7's sharp a* decline (2.02) implied insufficient reductants for sustained curing at lower celery/acerola ratios (Figure 5). These results are consistent with previous findings, where the addition of nitrite and phosphate led to decreased lightness due to enhanced pigment stabilization and water retention (Terns et al., 2011; (Suman & Joseph, 2013).

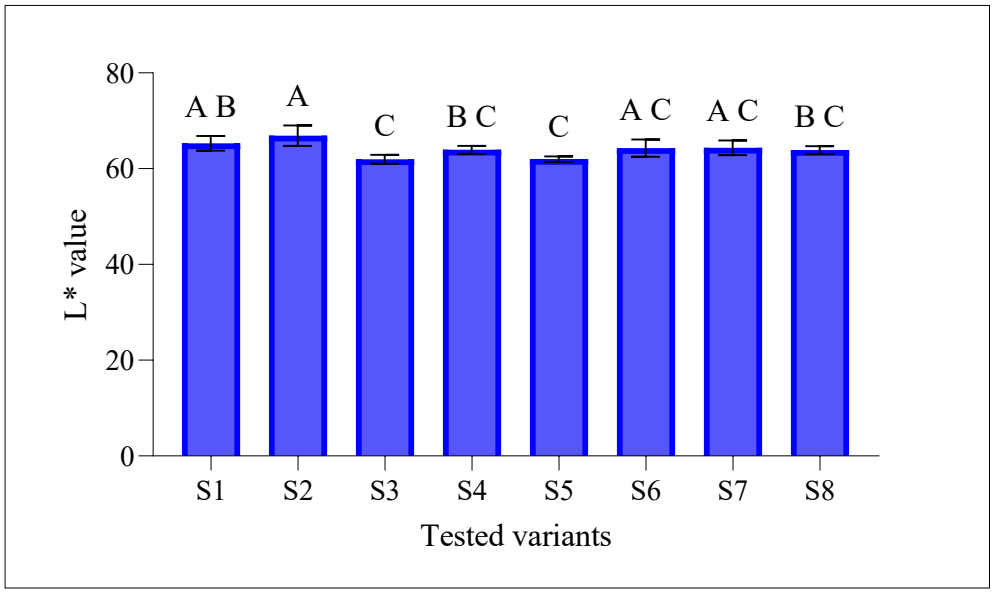


**Figure 6.** Lightness (L*) of meat samples depending on the variant tested.

Yellowness (b*) correlated with antioxidant content, peaking in ascorbate-rich S7 (12.26) and S1 (11.88), while nitrite-dominated batches (S3–S5) showed reduced b* (5.10–7.24), reflecting nitrite's suppression of lipid oxidation-derived yellow pigments (Figure 7). This trend is consistent with the literature, which suggests that phosphate and nitrite reduce b* values by stabilizing myoglobin and minimizing non-enzymatic browning reactions (Terns et al., 2011).

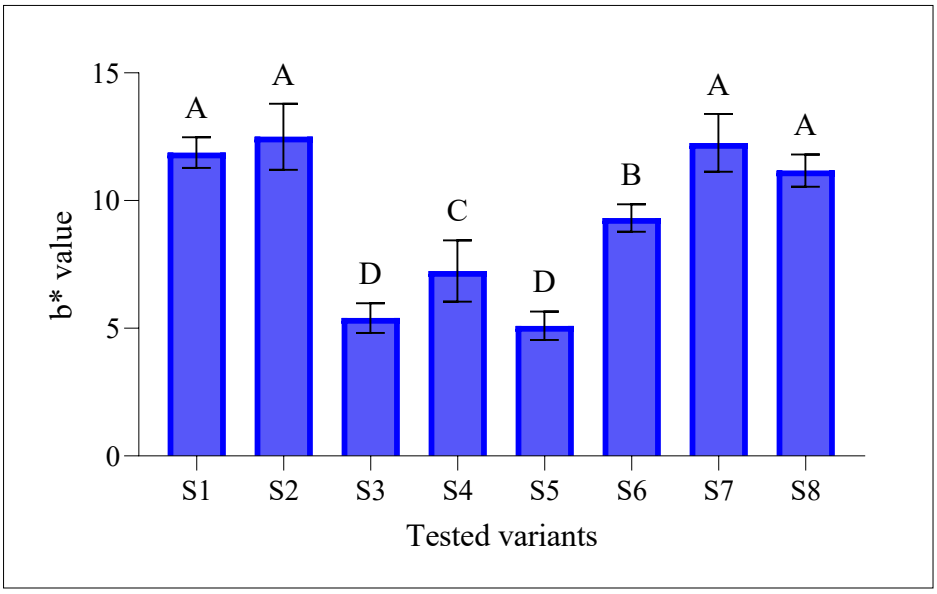


**Figure 7.**Yellowness (b*) of meat samples depending on the variant tested.

Overall, the Phase 1 results show that while the highest redness was achieved in the phosphate/ascorbate sample (S4), the celery extract formulation (S6) also produced a pronounced cured color, supporting the potential of plant-based alternatives for clean-label sausage production. However, as noted by (Alahakoon et al., 2015), the absence of starter cultures may limit the full conversion of nitrate to nitrite, potentially affecting long-term color stability. These findings highlight the importance of formulation strategy and ingredient selection.

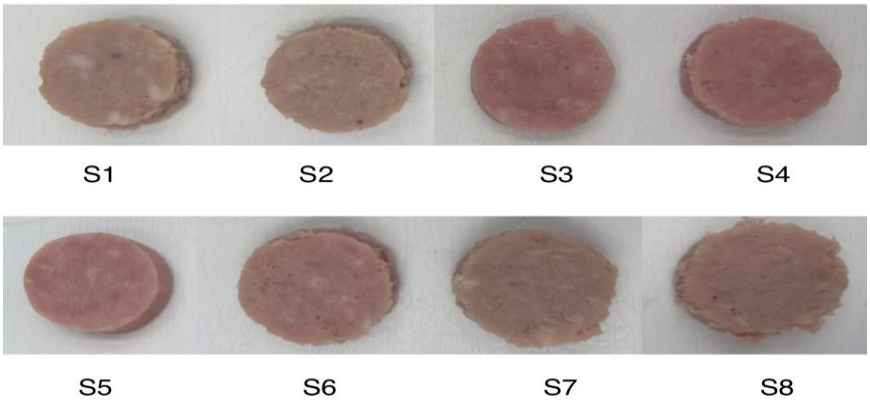


**Figure 8.** Visual appearance of cured pork sausages with different additive formulations after 24 hours of storage.

### 4.1.3. Texture Profile Analysis

Texture profile analysis using the Warner-Bratzler shear force method revealed significant differences in tenderness among the various sausage formulations, as confirmed by one-way ANOVA ($F = 35.80$, $p < 0.001$), specified in Table 3. The analysis demonstrated that phosphate-containing samples (S3 and S5) exhibited the highest shear force values (18.33 N and 18.48 N, respectively), indicating a firmer texture compared to phosphate-free variants, which ranged from 14.55 to 14.87 N. The salt-only control (S1) and natural additive formulations with celery and acerola extracts (S6–S8) displayed similar tenderness, with no statistically significant differences observed between these groups according to the post-hoc Tukey HSD test (Figure 9). These findings confirm the well-established role of phosphates in enhancing protein cross-linking and water retention, resulting in increased resistance to cutting, as also reported by (Alahakoon et al., 2015). Notably, the use of clean-label alternatives such as celery and acerola extracts did not adversely affect the texture profile, achieving shear force values comparable to the control and within the preferred consumer range for pork sausages. Compared to values reported in the literature, where Warner-Bratzler shear force for pork sausages typically ranges from 15 to 25 N depending on formulation and processing conditions (Pietrasik & Gaudette, 2014), the results of this study fall within the lower end of the spectrum, likely reflecting the specific muscle cuts and processing methods employed. Overall, these results demonstrate that natural additive

formulations can maintain desirable tenderness in clean-label pork sausages, supporting their viability as alternatives to traditional phosphate-containing products.

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 124.1931 | 7 | 17.74188 | 35.80299 | 2.77792E-15 | 2.249024 |
| Within Groups | 19.82167 | 40 | 0.495542 | | | |
| Total | 144.0148 | 47 | | | | |

**Table 3.** ANOVA Results for Warner-Bratzler shear force Analysis.

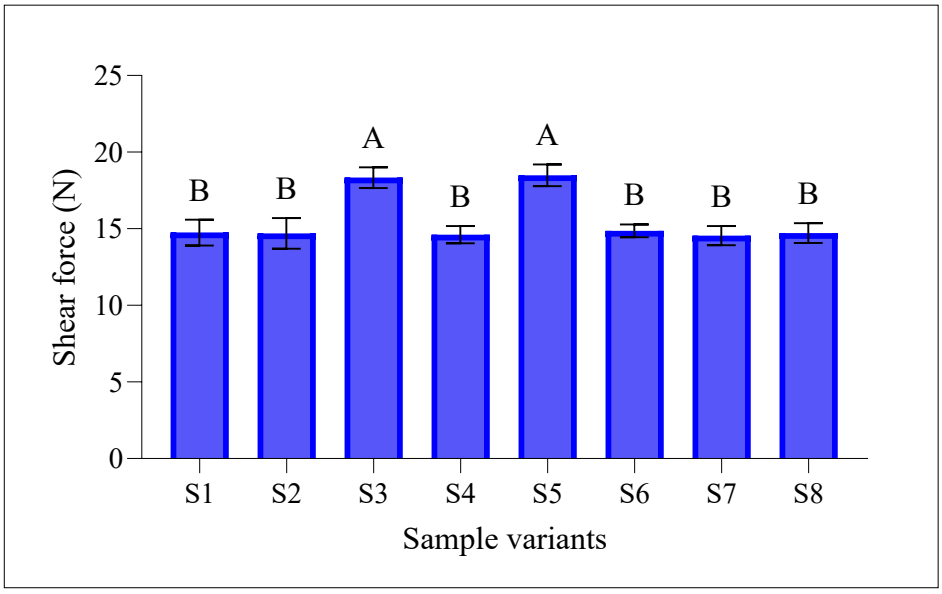


**Figure 9.** Warner-Bratzler shear force of meat samples depending on the variant tested.

The observed inverse correlation between shear force and lightness (L*) values ($r = -0.82$, $p < 0.01$) indicates that lighter-colored pork sausages exhibited significantly greater tenderness, likely

due to reduced protein aggregation. This finding aligns with established meat science principles demonstrating that higher L* values often correlate with improved water-holding capacity and less dense protein networks (Mancini & Hunt, 2005). Overall, these results demonstrate that natural additive formulations can maintain desirable tenderness in clean-label pork sausages, supporting their viability as alternatives to traditional phosphate-containing products.

| Variants | Attributes | | | | |
|---|---|---|---|---|---|
| | L* | a* | b* | Ph | WB |
| S1 | 65.32 | 1.64 | 11.88 | 5.56 | 14.75 |
| S2 | 66.92 | 1.76 | 12.5 | 5.64 | 14.70 |
| S3 | 61.98 | 4.74 | 5.40 | 5.83 | 18.33 |
| S4 | 63.94 | 5.40 | 7.24 | 5.62 | 14.61 |
| S5 | 62.00 | 4.50 | 5.10 | 5.88 | 18.48 |
| S6 | 64.30 | 5.28 | 9.32 | 5.66 | 14.86 |
| S7 | 64.38 | 2.02 | 12.26 | 5.72 | 14.55 |
| S8 | 63.90 | 3.22 | 11.18 | 5.70 | 14.71 |

**Table 4**. Means values of color parameters, pH and WB Shear force in pork meat, influenced by formulation type.

#### 4.1.4. PH Profile and Functional Implications

The pH measurements across formulations (5.56–5.88) revealed significant variation ($p < 0.05$) reflecting additive-specific biochemical interactions (Figure 10). Samples with sodium tripolyphosphate (S3: 5.83; S5: 5.88) exhibited the highest pH values, consistent with STPP's alkaline properties and its role in elevating meat pH to enhance water-binding capacity. Notably, the natural additive formulations (S6-S8: 5.66–5.72) maintained pH levels closer to the control (S1: 5.56) than synthetic counterparts, suggesting celery and acerola extracts minimally disrupt natural meat acidity. The inverse correlation between pH and Warner-Bratzler shear force ($r = -0.79$, $p < 0.01$) indicates that higher pH formulations (S3/S5) developed firmer textures through protein swelling and gel network formation. However, the narrow pH range ($\Delta 0.32$) across all samples implies that observed textural differences were primarily driven by phosphate-mediated

protein modifications rather than bulk pH shifts. These results demonstrate that clean-label formulations can achieve pH stability comparable to conventional curing systems while avoiding alkaline pH extremes that may impact flavor perception.

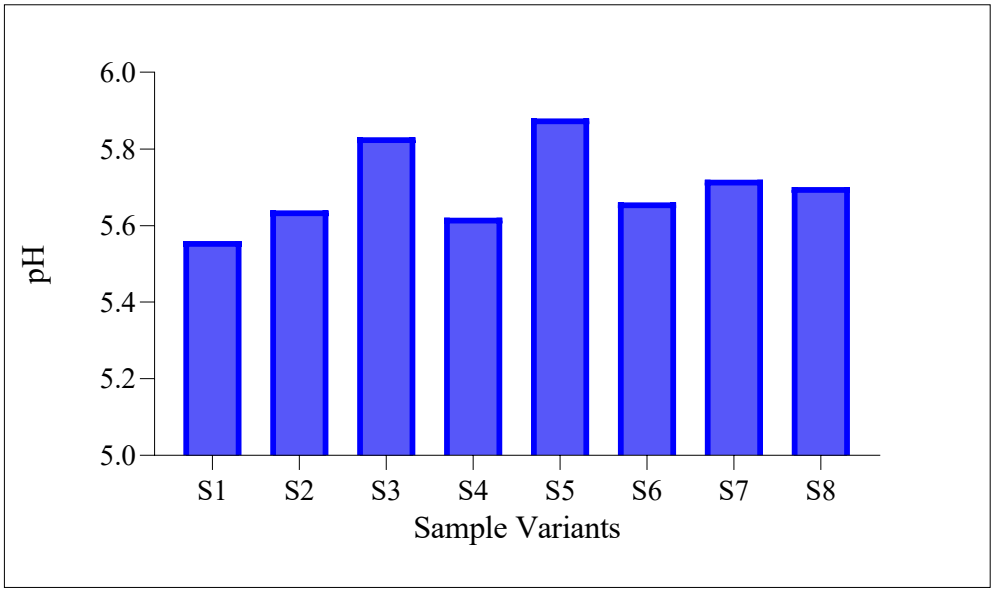


**Figure 10.** pH of meat samples depending on the variant tested.

## 4.2. Phase Two: Starter Culture-Modified Formulations

Four experimental batches were designed to assess the impact of starter cultures on color stability in cured sausages, building upon Phase 1 formulations. All samples contained a standardized base of 1.80% salt, 0.70% sodium tripolyphosphate (E451i), and 0.30% acerola extract to ensure consistent curing and antioxidant activity. Samples 1 and 2 incorporated 0.30% celery extract as a natural nitrate source, while Samples 3 and 4 omitted celery to isolate microbial effects. The formulations were differentiated by specific starter culture inoculations: Samples 1 and 3 received *Staphylococcus carnosus* (0.001%), while Samples 2 and 4 were inoculated with *S. xylosus* (0.002%). This 2×2 factorial design (with/without celery × *carnosus/xylosus*) enabled systematic evaluation of nitrate conversion efficiency and strain-specific pigment stabilization capabilities during the 24-hour exposure period. The fixed phosphate and acerola concentrations

controlled for texture and oxidative stability variables, allowing direct comparison of microbial contributions to color parameters (Figure 11).

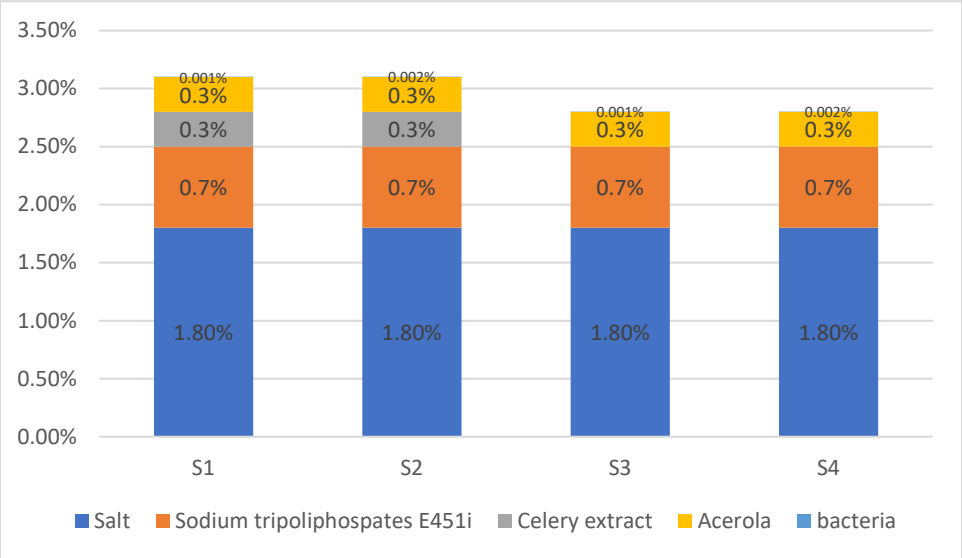


**Figure 11.** Additive composition (%) of the eight sausage treatment formulations (S1–S8) in phase two.

### 4.2.1. Color Stability Analysis with Starter Cultures

The comparative evaluation of post-cooking versus 24h-exposed samples revealed distinct patterns in color degradation influenced by starter culture selection and formulation (Figure 12). Immediately after processing, Sample 1 (*S. carnosus* with celery) exhibited optimal redness (a* 3.77), demonstrating the synergistic effect of microbial nitrate reduction combined with plant-derived nitrates. However, after 24h exposure, all samples showed significant ($p<0.05$) decreases in lightness (ΔL* avg. -8.3) and redness (Δa* avg. -0.8), with Sample 3 (*S. carnosus* without celery) displaying the highest L* retention (54.58 vs initial 63.47), suggesting this strain's superior oxidative protection independent of nitrate conversion. Notably, the *S. xylosus*-inoculated samples (2 & 4) showed more pronounced a* value declines (Δa* -0.44 and -0.27 respectively), indicating potential strain-specific limitations in nitrosylmyoglobin stabilization during storage. The stability of yellowness (b) across all formulations (Δb <1.5) confirmed the effectiveness of 0.3% acerola in maintaining oxidative stability regardless of microbial treatment. These results highlight the critical balance required between nitrate availability (celery), microbial selection, and antioxidant systems for sustained color performance in clean-label cured meats.

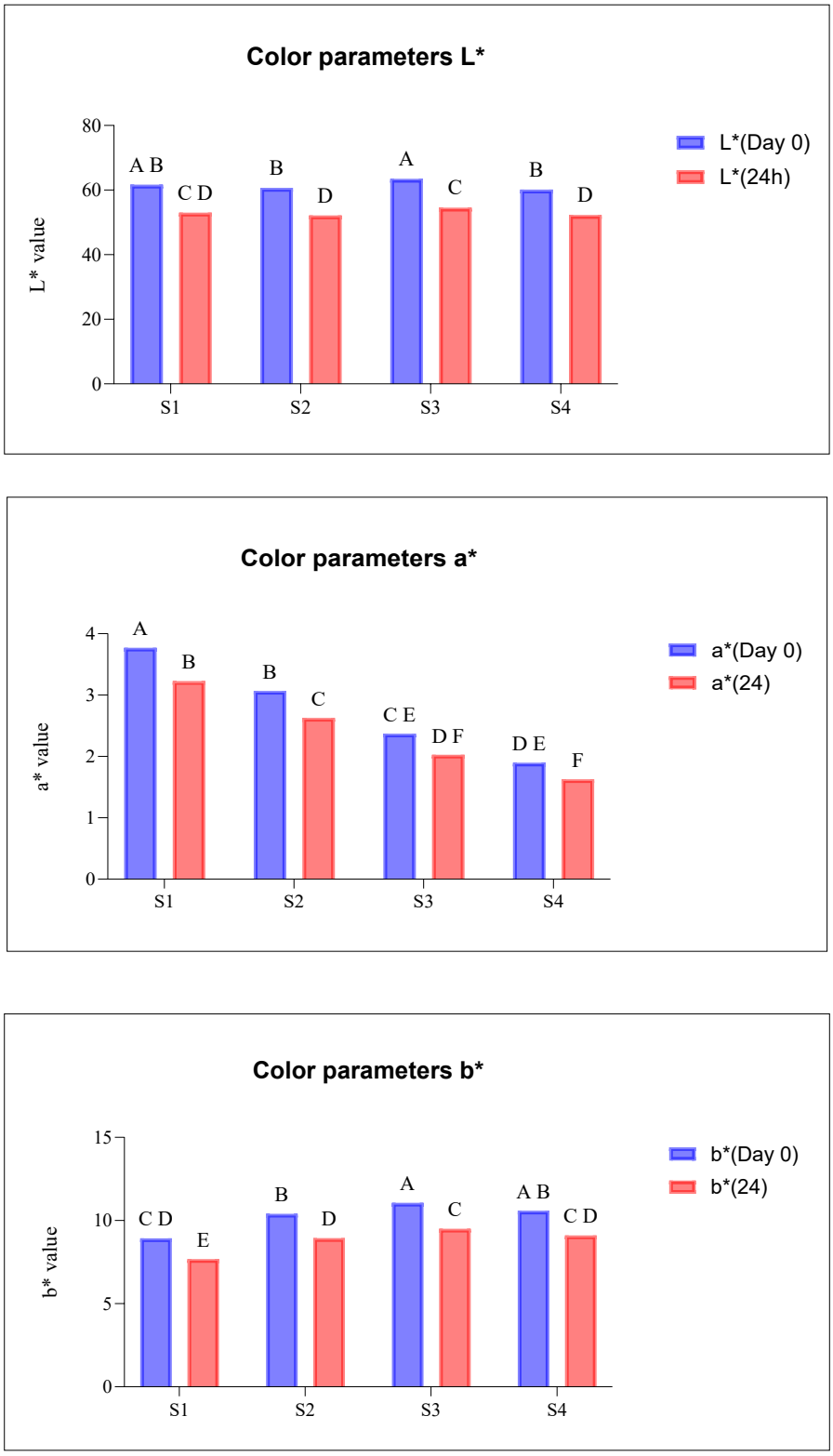


**Figure 12.** Color parameters (L*, a*, b*) of meat samples depending on the variant tested.

### 4.2.2. Microbial analysis

The use of *Staphylococcus carnosus* and *S. xylosus* as starter cultures, combined with celery extract as a natural nitrate source, was evaluated across four formulations (S1–S4). The findings highlight the distinct impacts of these interventions on Total Plate Count (TPC), *S. xylosus* populations, pathogen safety, and color attributes (L*, a*, b*, ΔE), underscoring the challenges and opportunities in replacing synthetic nitrites with natural alternatives.

The microbial analysis revealed significant differences in TPC among the formulations, ranging from 6.27 log CFU/g in S3 (*S. carnosus*, no celery) to 9.61 log CFU/g in S4 (*S. xylosus*, no celery). Samples inoculated with *S. xylosus* (S2 and S4) exhibited 2–3 log higher TPC than those with *S. carnosus* (S1 and S3), a difference attributable to the higher inoculum level of *S. xylosus* (0.002% vs. 0.001%) and its robust growth in the sausage matrix. This high microbial load, particularly in S4 (9.61 log CFU/g), raises concerns about shelf-life stability in clean-label formulations, as excessive microbial activity can accelerate spoilage and sensory deterioration. In contrast, *S. carnosus* demonstrated superior microbial control, with S3 achieving the lowest TPC, suggesting competitive exclusion or reduced metabolic activity compared to *S. xylosus*. This makes *S. carnosus* a promising candidate for clean-label sausages where microbial stability is a priority.

| Variants | Attributes | | | | | | |
|---|---|---|---|---|---|---|---|
| | L* | a* | b* | ΔE | TPC | *Staphylococcus Xylosus* | pathogens(25g) |
| S1 | 53.03 | 3.23 | 7.68 | 8.75 | 2.34*10^7 | 1.73*10^4 | absent |
| S2 | 52.17 | 2.63 | 8.94 | 8.64 | 3.67810^9 | 4.62*10^7 | absent |
| S3 | 54.58 | 2.03 | 9.51 | 9.03 | 1.85*10^6 | 2.48*10^5 | absent |
| S4 | 52.28 | 1.63 | 9.11 | 8.03 | 4.12*10^9 | 5.6*10^9 | absent |

**Table 5**. Color stability, microbial load, and safety attributes of cured pork sausages with different starter cultures after 24h storage.

*S. xylosus* enumeration, conducted using Mannitol Salt Agar with biochemical confirmation, further clarified the microbial dynamics. Counts ranged from 4.24 log CFU/g (S1) to 9.75 log CFU/g (S4), with S2 and S4 (*S. xylosus*-inoculated) showing significantly higher populations than S1 and S3 *(S. carnosus*-inoculated). The low *S. xylosus* counts in S1 and S3 likely reflect residual or environmental populations, as these samples were not inoculated with *S. xylosus*. The strong correlation between TPC and *S. xylosus* counts ($r = 0.96$, $p < 0.05$) in S2 and S4 indicates that *S. xylosus* dominated the microbial flora, consistent with its role as a starter culture. However, the slightly lower *S. xylosus* count in S2 (7.66 log CFU/g) compared to S4 (9.75 log CFU/g) suggests a minor inhibitory effect of celery extract, possibly due to its alkaline pH (8.5–10) or nitrate interactions affecting microbial metabolism. This effect was not sufficient to reduce TPC significantly, indicating that celery's antimicrobial potential is limited under these conditions.

The absence of *E. coli* and *Salmonella* in all samples confirms the microbiological safety of the formulations, a critical requirement for clean-label products. The combined antimicrobial effects of 1.80% salt, 0.30% acerola extract, and thermal processing (72°C for 20 minutes) effectively eliminated pathogens, as validated by the sensitive MicroSnap® and InSite™ detection methods. This safety profile supports the feasibility of replacing synthetic preservatives with natural alternatives, provided microbial growth is managed to ensure shelf-life stability.

Color stability, a key quality attribute for consumer acceptance, was closely linked to microbial dynamics and formulation components. Sample S1 (*S. carnosus* with celery) achieved the highest redness ($a^* = 3.23$) and lowest yellowness ($b^* = 7.68$), indicating effective nitrate-to-nitrite conversion by *S. carnosus* and synergy with celery's nitrate content. This formulation closely mimicked the color profile of synthetic nitrite-cured meats, highlighting its potential for clean-label applications. In contrast, S4 (*S. xylosus*, no celery) exhibited the lowest redness ($a^* = 1.63$), underscoring the necessity of celery for color development and the limited efficacy of *S. xylosus* in stabilizing nitrosylmyoglobin. The inverse correlation between TPC and lightness ($r = -0.81$, $p < 0.05$) suggests that high microbial loads in S2 and S4 ($L^* = 52.17$ and 52.28, respectively) contributed to surface darkening, likely through oxidative metabolism generating reactive oxygen species. However, S4's low total color difference ($\Delta E = 8.03$) despite its high microbial load (9.61 log CFU/g) suggests that sodium tripolyphosphate (STPP, 0.70%) played a protective role,

possibly by enhancing protein-water interactions or acting as an antioxidant to mitigate color degradation.

The interplay between microbial activity and color stability reveals trade-offs in clean-label sausage production. While *S. carnosus* with celery (S1) offers a balanced approach, achieving desirable color and moderate microbial growth, *S. xylosus* formulations (S2 and S4) pose challenges due to excessive microbial proliferation. The high *S. xylosus* counts in S4 did not enhance color stability, as evidenced by its low a* value, suggesting that *S. xylosus*'s metabolic activity may prioritize growth over nitrate reduction. The consistent presence of 0.30% acerola extract across all samples effectively minimized lipid oxidation, as indicated by stable b* values (7.68–9.51), with S1 showing the best performance. STPP's role in S4's low ΔE further highlights its importance in clean-label formulations, though its synthetic nature may conflict with strict natural labeling requirements (Figure 13).

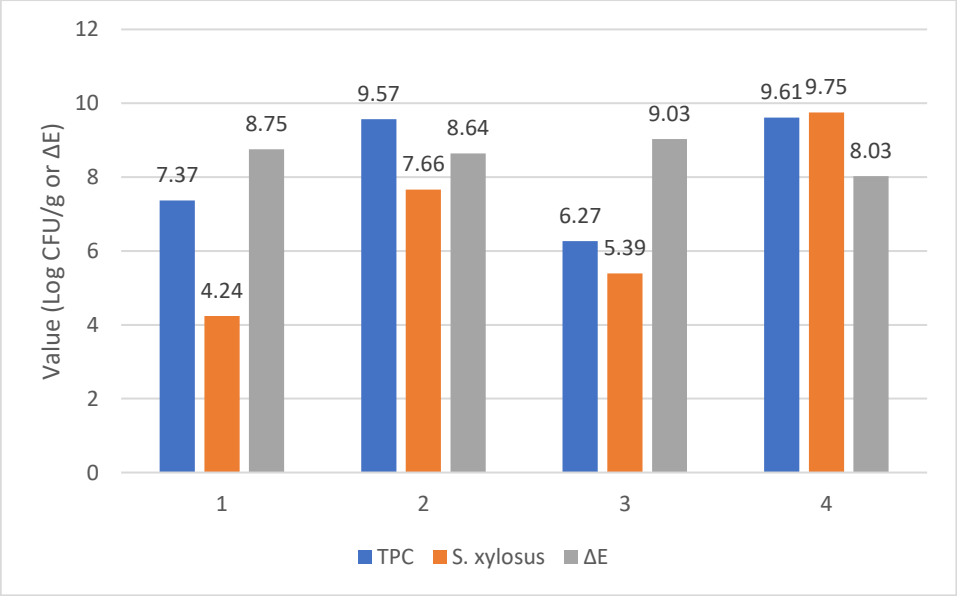


**Figure 13.** S.xylosus , TPC and Color Change After 24h Storage of meat samples.

# 5. Conclusion

This study provides valuable insights into the potential of natural additives and starter cultures as alternatives to synthetic nitrites in fermented pork sausages. The results demonstrate that while traditional nitrite-based curing remains highly effective for color development and microbial stability, clean-label alternatives - particularly celery extract combined with acerola and *Staphylococcus carnosus* - can achieve comparable quality attributes.

Key findings show that *S. carnosus* was more effective than *S. xylosus* in nitrate-to-nitrite conversion and maintaining color stability, while also demonstrating better control over microbial growth. The combination of 0.3% celery extract and 0.3% acerola extract produced sausages with redness values approaching those of nitrite-cured products, suggesting these natural ingredients can partially replace synthetic additives without compromising visual appeal. Texture analysis revealed that while phosphates provided superior firmness, natural formulations maintained acceptable tenderness, indicating they could satisfy consumer expectations for mouthfeel.

From a food safety perspective, all formulations - whether synthetic or natural - effectively prevented pathogen growth, with no detection of *Salmonella spp*. or *E. coli* in any samples. This confirms that clean-label approaches can meet basic safety requirements when properly formulated. However, challenges remain in achieving consistent results with natural curing systems, particularly regarding microbial load management and long-term color stability. Future research should focus on optimizing starter culture combinations and fermentation conditions to improve the reliability of natural curing processes. Additionally, sensory evaluation studies would help determine consumer acceptance of these clean-label products.

In conclusion, this work demonstrates that natural alternatives show promise for reducing reliance on synthetic nitrites in meat processing. By combining plant-based nitrate sources with carefully selected starter cultures, manufacturers can develop safer, more natural products that align with current consumer trends while maintaining the quality standards expected of fermented meat products. Further refinement of these techniques could facilitate wider adoption of clean-label strategies in the meat industry.

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