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Original scientific paper

# Detection of celery and mustard food allergens in foods of animal origin in Serbia for the period 2021–2023

*Vesna V. Janković[1], Branko Velebit[1], Radmila Mitrović[1], Brankica Lakićević[1], Lazar Milojević[1], Dunja Mišić[1] and Slaven Grbić[2]*

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ABSTRACT

Food Allergy is a growing global public health concern. The presence of undeclared allergenic ingredients or the presence of traces of allergens due to contamination during food processing poses a great health risk to sensitised individuals. Celery and mustard belong to the group of 14 basic food allergens, and thus, they are potentially hazardous. Therefore, the objective of this mini-review is to evaluate the presence of celery and mustard in foods of animal origin in Serbia for the period 2021–2023. The present study provides a summary of qualitative detection of specific DNA sequences by real time PCR techniques conducted on 179 retail products of animal origin, i.e., coarse-ground cooked sausages, cooked sausages with meat pieces, fermented sausages, smoked products, chicken meat, dairy and meat alternatives, quick-frozen dough products and snacks. Celery DNA (the mannitol dehydrogenase gene region was used for specific celery identification in samples) was detected in 15 samples, while mustard DNA was detected in 14 samples.

## 1. Introduction

Food allergies are becoming a major global health concern and a serious food safety issue for both private and public health systems (*Muraro et al*., 2022). Consumers must be provided accurate and understandable information regarding the allergenic profile of foods (*Codex Alimentarius Commission FAO/WHO,* 2020).

The term food allergy is used to refer to an immune response directed toward foods (*Sampath et al*. 2021). Food allergies can cause a wide range of symptoms on the skin and in the gastrointestinal and respiratory systems, and can result in anaphylactic shock, which can be fatal *(Renz et al*., 2018). The symptoms usually appear quickly, a few minutes after the triggering food is consumed, and in severe cases, they might result in a deadly reaction (*Ho, Wong, & Chang*, 2014). Over the past three decades, food allergies have become more common in both developed and developing nations. It is believed that they impact up to 8% of young children and 2–3% of adults in Western countries (i.e., Europe, North America, and Australia) (*Sicherer & Sampson*, 2014, *Sampath et al*., 2021). Since it was considered to be an unusual condition until recently, there is insufficient information available in other geographical locations (*EFSA*, 2014; *Loh & Tang*, 2018). The primary approach to controlling food allergies is to avoid or stay clear of foods that trigger an allergic reaction. Patients with food allergies rely on precise information from the allergy statement provided in the ingredient list to prevent adverse effects. Effective avoidance is a complex issue that affects patients and their families, public health authorities, the food sector and governments (*Gargano et al*., 2024).

In Serbia, the *Rulebook on declaring, labeling and advertising of food* (2017–2024) and the *Rulebook on the health safety of dietary foods* (2017–2024) provide allergen legislation considering 14 food ingredients

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that can cause allergic reactions or intolerance, method of declaration, and the recommended method for detection (gluten) when this is used as an ingredient in manufactured foods. Precautionary allergen labels (PAL), sometimes referred to as "may contain" labels, are additional labels that customers with food allergies can encounter on product packaging. The allergens that could be present in the product as a result of accidental cross-contamination during food manufacturing are, by definition, the subject of these precautionary labels (*Food Standards Agency*, 2021). PAL are currently unregulated by law and are optional in most countries, including Serbia (*Popov-Raljić et al*., 2022). Because there is no legislation governing when and how to use PAL, their current use—or misuse—is unclear.

Among allergenic substances whose presence in food must be indicated on the label are celery (*Apium graveolens*) and mustard (*Sinapis alba*). These two minor food ingredients are attracting increasing attention because of their popularity as seasoning material worldwide. Celery is an important member of the *Apiaceae* family that is cultivated worldwide. Major identified celery antigens are Api g 1 and Api g 7. Even small amounts of celery can immediately lead to allergic reactions in sensitive individuals. According to the German Federal Institute for Risk Assessment (VITAL, 2019) the eliciting dose 'ED01' and 'ED05' values of 0.05 mg and 1.3 mg protein, respectively, are now the new reference doses for celery. For mustard, 0.05 mg protein has now been derived as a reference dose for 'ED01', and 0.4 mg protein for 'ED05'. The mustard plant belongs to the *Cruciferae* (*Brassicaceae*) family that includes other vegetables, such as radish, rutabaga, cabbage, broccoli, turnip, watercress, horseradish, castor oil plant and rapeseed. Mustard contains three main cultivated species: *Sinapis alba* (yellow mustard), *Brassica nigra* (black mustard) and *Brassica juncea* (oriental mustard). Considering *Sinapis alba*, the main allergens are sin a1, sin a 2, sin a 3 and sin a 4 (*Tanno et al*., 2023).

## 2. Materials and Methods

A total of 179 food products of animal origin were randomly selected from retail food stores in Serbia. Sampled products were digitally recorded and assigned to one of eight product categories: coarse-ground cooked sausages, cooked sausages with meat pieces, fermented sausages, smoked products, chicken meat, dairy and meat alternatives, quick-frozen dough and snacks. The product name, manufacturer, country of origin, product ingredients list, and any information regarding substances or products causing allergies or intolerances outlined in Annex II of the relevant European regulation (*Regulation (EU) No 1169/2011*, 2011) were among the information gathered from food products.

Additionally, all selected products were analysed using validated methods for the food allergens, celery and mustard. Analysis were carried out according to *SRPS EN ISO 15634-2:2019* (2019) and *SRPS EN ISO 5:2023* (2023), with results being expressed as < or >10 mg/kg. DNA from the food products was extracted using a protocol with cetyltrimethylammonium bromide (CTAB). The DNA was quantified by spectrophotometry using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The applied CTAB extraction method resulted in DNAs with a OD260 nm/OD2800 nm ratio of 1.8 to 2.0 from all samples, indicating the high quality of the extracted DNA.

Extracted DNA materials were used as templates for amplification, identification, and qualitative detection using real-time PCR. Real-time PCR assays were performed with Aria MX (Agilent Technologies). The program included an initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and primer annealing and elongation at 60 °C for 1 min. Real-time PCR detection of celery was based on a 101 bp (base pair) sequence from mannitol dehydrogenase gene (GenBank acc. no. AF067082) from celery (*Apium graveolens*) and for mustard (*Sinapis alba*) by determining the gene MADS-D.

## 3. Results and Discussion

Of the products analysed, 114 of 179 (63.69%) were declared as causing allergies in the ingredient lists. The most frequently listed allergen was soya (27.37 %)(Figure 1). PAL statements were provided for 67 (37.43%) of the food products (Figure 2).

Results for the food allergens in the 179 foods of animal origin are presented in Table 1.

Celery DNA (the mannitol dehydrogenase gene region was used for specific celery identification in samples) was detected in 15 samples, while mustard DNA was detected in 14 samples. Of the 15 samples in which celery DNA was detected, celery was listed on the declaration (list of ingredients) of four products, while celery was mentioned in the PAL statement of 10 products. However, one product (a breakfast grain) in which celery was found did not have this allergen included in the list of ingredients, nor did it have a PAL statement for celery.

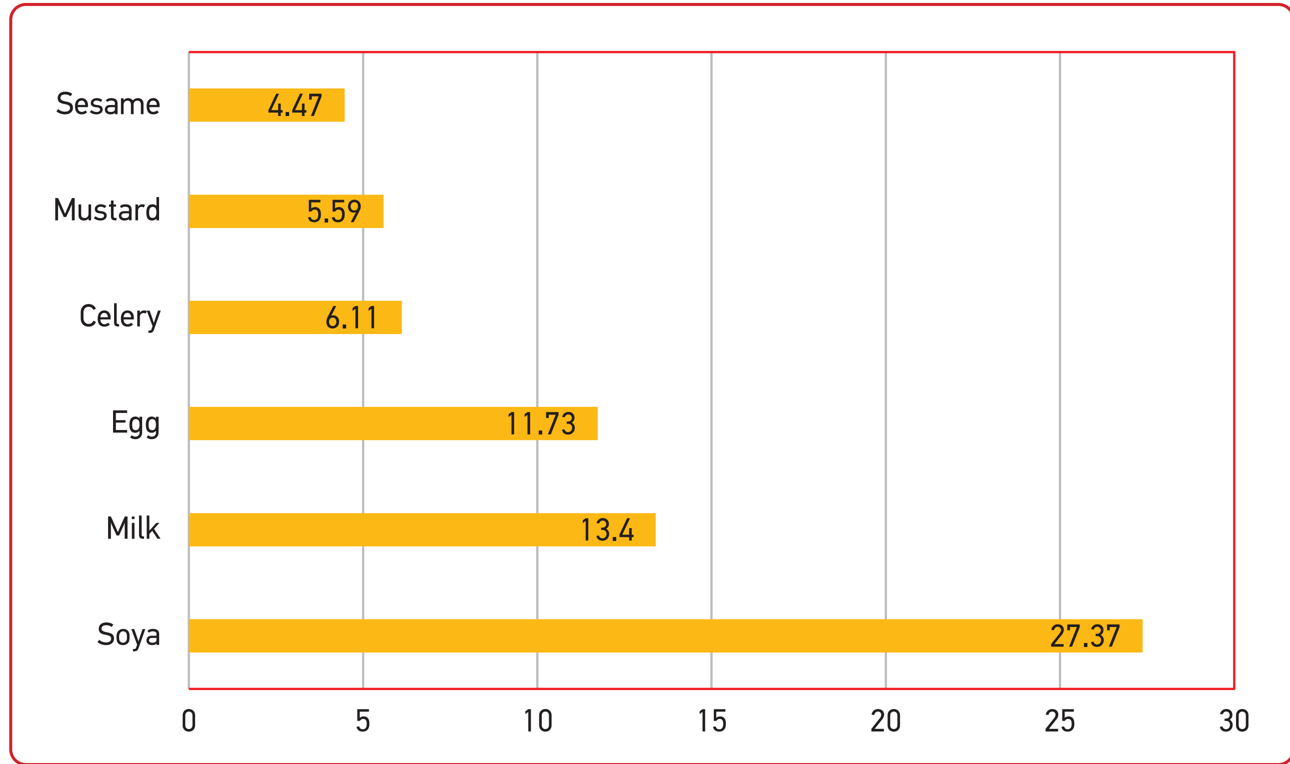


**Figure 1.** Prevalence of food allergens provided on 179 food labels from foods of animal origin randomly sourced from retail outlets in Serbia

Mustard was detected in seven food products that included mustard in the declaration (list of ingredients), while in five food products, the allergen mustard was mentioned in the PAL statement. In the case of two food products in which mustard DNA was detected, the allergen was not mentioned at all. The obtained results were in accordance with those of other studies (*Vipa et al*., 2021; *James et al*., 2024).

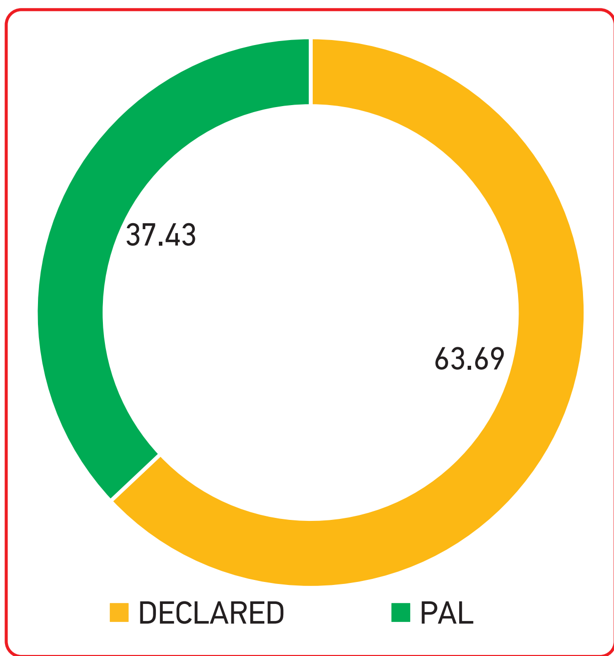


**Figure 2.** Percentage of sampled foods with food allergen information in food declaration ingredient lists (declared) and PAL statements (PAL)

This study demonstrated how ineffective PAL currently is for people with food allergies. The absence of established reference doses has led to uneven PAL application by the food industry and to uneven levels of contamination that trigger enforcement officers to instigate a food withdrawal, according to a summary of the opinions of all the major stakeholders (including clinicians, patients, the food industry and regulators). The real risk of allergic consumers developing an allergic reaction is not well correlated with the use of PAL or the presence of allergens in foods. As a result, consumers are less likely to trust food labels and to make wise decisions.

In the context of assessing results for qualitative allergen detection, voluntary incidental trace allergen labelling (VITAL) can be instrumental in risk assessment and labelling decisions. VITAL could be applied for:

1. Thresholds for risk assessment: VITAL establishes action levels for allergen presence, meaning detected allergens can be categorised by risk threshold.

**Table 1.** Presence of mustard and celery food allergens in foods of animal origin

| PRODUCT TYPE | NUMBER OF SAMPLES | CELERY > 10 mg/kg | MUSTARD > 10 mg/kg |
|---|---|---|---|
| COARSE-GROUND COOKED SAUSAGES | 25 | 4 | 4 |
| COOKED SAUSAGES WITH MEAT PIECES | 30 | 5 | 5 |
| FERMENTED SAUSAGES | 21 | < 10 mg/kg | < 10 mg/kg |
| SMOKED PRODUCTS | 16 | < 10 mg/kg | < 10 mg/kg |
| CHICKEN MEAT | 12 | < 10 mg/kg | < 10 mg/kg |
| DAIRY AND MEAT ALTERNATIVES | 28 | 3 | 3 |
| QUICK-FROZEN DOUGH | 18 | < 10 mg/kg | 1 |
| SNACKS | 29 | 3 | 1 |

2. Qualitative detection interpretation: Since PCR can qualitatively detect DNA from specific allergens, a presence/absence result is obtained, rather than a quantified result. VITAL guidelines for actionable risk can help in the interpretation of these qualitative results. If PCR indicates the presence of a particular allergen, VITAL's thresholds can inform whether this result should lead to labelling based on the typical serving size or consumption pattern.

## 4. Conclusion

This study is the first to identify the risk that unintentional inclusion of celery or mustard in food products both with and without PAL poses to Serbian consumers who are sensitive to these ingredients. It is apparent from the study that there are no explicit standards for food manufacturers using PAL. Also, the use of VITAL should improve risk management in order to develop risk-based labelling strategies (a trace level below VITAL's Action Level would mean labelling is unnecessary, while higher levels, such as those above Action Level 2, indicate that precautionary labelling is advised). In summary, VITAL can guide interpretation, risk categorisation, and labelling decisions based on PCR detection of allergens, enhancing the practical applicability of PCR results in food safety and allergen management. Prospective novel strategies might assist in resolving the present problems with food labelling for those with severe food allergies. To help food allergic consumers in Serbia, the best practices include standardising detection assays, certifying reference materials, defining acceptable risk levels for food allergies, and harmonising labelling actions.

# Utvrđivanje prisustva alergena celera i slačice u hrani životinjskog porekla u Srbiji za period 2021–2023 godine

*Vesna V. Janković, Branko Velebit, Radmila Mitrović, Brankica Lakićević, Lazar Milojević, Dunja Mišić i Slaven Grbić*

INFORMACIJE O RADU

*Ključne reči:*
Celer
Slačica
PCR u realnom vremenu
Alergeni u hrani
Bezbednost hrane

APSTRAKT

Alergija na hranu (AH) je predstavlja ozbiljan globalni problem za javno zdravlje. Prisustvo nedeklarisanih alergenih sastojaka ili njihovo prisustvo u tragovima usled kontaminacije tokom prerade hrane predstavlja veliki zdravstveni rizik. AH predstavljaju značajan zdravstveni problem širom sveta i potrošači bi trebalo da imaju pouzdane podatke o prisustvu alergena. Celer i slačica spadaju u grupu od 14 osnovnih alergena u hrani i stoga su potencijalno opasni. Cilj ovog pregleda je procena prisustva celera i slačice u hrani životinjskog porekla u Srbiji za period 2021–2023. godine Ova studija pruža rezime kvalitativne detekcije specifičnih DNK sekvenci PCR tehnikom u realnom vremenu sprovedenom na 179 maloprodajna proizvoda, uključujući grubo mlevene kuvane kobasice, kuvane kobasice sa komadima mesa, fermentisane kobasice, dimljene proizvode, pileće meso, mlečne proizvode i alternative za meso, grickalice i brzo zamrznuti proizvodi od testa.

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# Effect of vacuum packaging on microbial and sensory quality indicators of cold-smoked freshwater fish

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A R T I C L E I N F O

*Keywords:*
Common carp (*Cyprinus carpio*)
Bighead carp (*Hypophthalmichthys nobilis*)
Shelf-life
Aerobic plate count
Lactic acid bacteria

A B S T R A C T

The aim of this research was to monitor the effect of vacuum packaging on selected microbial and sensory parameters of cold-smoked common carp (*Cyprinus carpio*) and cold-smoked bighead carp (*Hypophthalmichthys nobilis*) fillets stored at 3±0.5 °C, and to determine the shelf-life of the products. Fillets were analysed on days 1, 7, 10,12, 14, 15 and 16. The APCs were significantly higher ($p < 0.05$) in common carp than in bighead carp from storage day 12. At the end of study, the APC in both species of fish had not reached 7 logcfu/g. No significant differences ($p > 0.05$) were determined between the PBCs in common carp and bighead carp during the whole period of storage. In cold-smoked bighead carp, the lactobacilli group was dominant at the end of the storage period. According to sensory assessment, it was concluded that vacuum-packaged cold-smoked common carp remained acceptable for up to 15 days of storage, while vacuum-packaged cold-smoked bighead carp remained unchanged until the end of the study (16 days).

## 1. Introduction

Various preservation techniques, like freezing, drying, salting and smoking, are primarily used to minimize post-harvest fish losses (*Sakyi et al.*, 2019). Among these techniques, fish smoking is one of the oldest and most popular methods, appreciated for the distinct smoky flavour and color it imparts. Additionally, smoked fish is often considered "ready-to-eat," as it can be added directly to meals either whole or in powdered form (*Steiner-Asiedu et al.*, 1991).

Smoking is a traditional preservation method primarily valued for its sensory benefits, such as enhanced taste and color, particularly in minimally processed products with reduced salt content to appeal to consumer preferences. The main preservation mechanisms of smoking include lowering water activity levels through drying, and the antimicrobial and antioxidant properties of smoke components (*Gomez-Guillen et al.*, 2009). Smoke consists of various compounds, including aldehydes, ketones, alcohols, acids, hydrocarbons, esters, phenols and ethers (*Guillen & Errecalde*, 2002). These compounds are deposited on the fish's surface and gradually penetrate into the muscle. Research has shown that phenols in smoke can slow the growth of spoilage microorganisms and inhibit *Listeria monocytogenes* in smoked fish (*Montero et al.*, 2007). The main advantages of cold smoking fish are enhanced flavour, extended shelf-life, and the preserved nutritional and textural qualities of fish, making it a popular method in both traditional and modern fish processing.

The quality of smoked fish is influenced by several factors, including: (i) *Fish freshness*. The quality of the raw fish is one of the most critical factors (*Sikorski and Kolodziejska*, 2002). Fish that are fresh and free from spoilage will produce a superior

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smoked product. Degradation of proteins and fats in old or poorly handled fish can lead to off-flavours and undesirable textures in the final product. (ii) *Smoking method*. The choice between hot and cold smoking significantly affects the texture, flavour and microbial stability of the fish. Hot smoking involves cooking the fish while imparting a smoky flavour, while cold smoking mainly adds flavour without fully cooking the product. Both methods require precise control of time and temperature to ensure safety and quality (*Sikorski and Kolodziejska*, 2002). (iii) *Type of wood used*. The type of wood used for smoking greatly influences the flavour profile of the fish. Woods like oak, hickory, alder, or fruitwoods, such as apple and cherry, impart distinct smoky notes. The choice of wood should match the desired flavour characteristics of the final product (*Puke and Galoburda*, 2020). (iv) *Smoking duration and temperature*. Proper control of smoking time and temperature is essential. For hot smoking, maintaining temperatures above 70 °C ensures complete coagulation of proteins and elimination of harmful microorganisms. Cold smoking typically occurs at much lower temperatures, and the duration is longer to allow adequate flavour absorption without cooking the fish (*Puke and Galoburda*, 2020). (v) *Brining process*. Before smoking, fish is often brined to enhance flavour, improve texture and extend shelf-life. The salt concentration and duration of the brining process can affect both the taste and texture of the smoked fish. Over-brining can lead to an overly salty product, while under-brining could result in poor preservation. (vi) *Moisture content*. Proper drying before and after smoking is crucial to achieving the desired texture and microbial stability. Excessive moisture can lead to a soggy product with reduced shelf-life, while overly dry fish can be tough and unappetizing. (vii) *Packaging and storage*. The packaging method, such as vacuum sealing, and storage conditions, particularly temperature control, play a key role in maintaining the quality and extending the shelf-life of smoked fish. Inadequate packaging can result in oxidation, loss of flavour and microbial growth (*Sikorski and Kolodziejska*, 2002). (viii) *Microbial number*. The microbial safety of smoked fish is critical, especially for cold-smoked products, which do not reach high enough temperatures to reduce all potential pathogens. Proper handling, hygiene and storage practices are necessary to minimize contamination and ensure the product's safety. By carefully managing these factors, producers can create a high-quality smoked fish product with excellent flavour, texture and safety characteristics.

Modern consumers looking for high-quality foods that preserve the sensory characteristics and nutritional value of the raw materials used in their production, while also meeting strict safety standards. This demand is largely fulfilled by vacuum or modified atmosphere packaging. In Serbia, most wild-caught and farmed fish are sold for human consumption either fresh or frozen. However, there has been a significant rise in the popularity of smoked fish products.

Cyprinid species, such as common carp, bighead carp and grass carp, are the most commonly farmed in Serbia. This study aimed to monitor changes in selected microbial and sensory parameters in vacuum-packaged cold-smoked fillets of common carp (*Cyprinus carpio*) and bighead carp (*Hypophthalmichthys nobilis*) during storage at 3±0.5 °C, with the goal of determining the products' shelf-life.

## 2. Materials and Methods

### *2.1 Fish preparation*

Eight common carp and eight bighead carp of 2.50±0.30 kg and 2.70±0.50 mean live weight, respectively, were obtained from a fishpond where semi-intensive rearing system was used. The fish were processed at a freshwater fish processing plant using a standard processing procedure (killing by electrocution, descaling, evisceration and filleting). Two fillets from each carp were prepared, and each fillet was divided into four portions, i.e., a total of eight portions were obtained from one fish. After primary treatment, fish portions were washed and soaked in brine for 24 h, then pressed and laid on the grid in chambers for an hour at 20 ºC. Smoking was performed in an automated smokehouse at the temperature of 28 ºC for 8 h.

The sixty-three portions of cold-smoked common carp and the sixty-three portions of cold-smoked bighead carp were vacuum packaged using a Variovac machine (Variovac Primus, Zarrentin, Germany), and a polyethylene-polyamide film (Suomen Union Verpackungs, Helsinki, Finland) with an oxygen permeability of 29–45 ml $O_2$ /m$^2$/24 h/atm (23 °C, 50% relative humidi ty, RH) and a water vapour permeability of 10–15 g/m$^2$/24Xh (38 °C, 90% RH) (1atm=101 325 Pa). All fish portions were stored at the temperature of 3±0.5 °C, and on days 1, 7, 10, 12, 14, 15 and 16 of storage, microbial and sensory testing was performed.

*2.2. Microbial analyses*

Fish fillets (25g) were homogenized in 225 ml of MRD (Oxoid, Great Britain) in a stomacher (AES; Comburg, France) for 90 sec. Serial dilutions (10-fold) of fish homogenate were spread onto the surface of the appropriate dried media in Petri dishes for enumeration of: aerobic plate count (APC) and psychrotrophic bacteria count (PBC) in plate count agar (PCA, Merck, Germany) incubated at 30 °C for 3 days and at 4 °C for 5 days, respectively; lactic acid bacteria (LAB) on de Man Rogosa Sharpe (MRS) agar (Oxoid, Great Britain) incubated at 30 °C for 2 days under microaerophilic conditions; total yeast and mould count (TYMC) on Dichloran Rose-Bengal chloramphenicol agar (DRBC) (Merck, Germany) incubated at 25 °C for 5 days. All plates were prepared in duplicate and examined visually for typical colony types and morphological characteristics associated with each growth medium. Microbial counts were expressed as logarithms of the number of colony-forming units per gram (logcfu/g).

*2.3. Sensory evaluation*

The sensory evaluation was performed by six trained panellists. The fish portions were considered for overall acceptability, with regard to odour, flesh color and texture using 1-5 intensity scale, with 5 corresponding to the most liked portion and 1 corresponding to the least liked portion. The product was defined as unacceptable if it achieved a score of less than 2 points recorded by at least of 50% of the judges. Fish from each test group was evaluated throughout the storage period on each sampling day.

*2.4. Statistics*

The mean values and standard deviations were calculated by using column statistics with the processing of six values for each analyzed group. Significant differences between groups were calculated by using one-way ANOVA. When a significant F was found, additional post-hoc tests with Tukey's adjustment were performed. Differences were considered as significant when p-value was $\leq 0.05$. All analyses were performed using the program Microsoft Office Excel (2016).

## 3. Results and Discussion

The initial low numbers of micro-organisms in common carp and bighead carp (Table 1) suggest that brining and washing as well as smoking process reduced the number of bacteria. At the beginning of our study, the APCs in both groups of fish were the highest compared to other examined micro-organisms. During the storage period, an increase in the APC was observed in both groups of fish. From storage day 12, a significantly higher ($p < 0.05$) APC was detected in common carp than in bighead carp.

**Table 1.** Aerobic plate count (APC), psychrotrophic bacteria count (PBC), lactic acid bacteria (LAB) and total yeast and mould count (TYMC) expressed as log cfu/g (mean ± SD) of cold-smoked common carp fillets and cold-smoked bighead carp fillets during the storage period

| Parameter | Samples | Days of storage | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 7 | 10 | 12 | 14 | 15 | 16 |
| APC | Common carp | $3.28±0.27^a$ | $3.65±0.45^a$ | $3.80±0.30^a$ | $4.50±0.14^a$ | $4.55±0.44^a$ | $4.83±0.42^a$ | $5.06±0.56^a$ |
| | Bighead carp | $3.06±0.43^a$ | $3.40±0.47^a$ | $3.43±0.77^a$ | $3.82±0.52^b$ | $3.85±0.22^b$ | $3.93±0.37^b$ | $4.09±0.64^b$ |
| PBC | Common carp | $0.56±0.50^a$ | $2.10±0.27^a$ | $2.31±0.33^a$ | $2.53±0.22^a$ | $2.56±0.26^a$ | $2.76±0.31^a$ | $2.82±0.28^a$ |
| | Bighead carp | $0.50±0.07^a$ | $2.00±0.16^a$ | $2.34±0.27^a$ | $2.59±0.23^a$ | $2.60±0.22^a$ | $2.72±0.19^a$ | $2.94±0.32^a$ |
| LAB | Common carp | $0.85±0.25^a$ | $2.14±0.40^a$ | $2.85±0.58^a$ | $3.16±0.20^a$ | $3.57±0.30^a$ | $4.30±0.24^a$ | $4.52±0.32^a$ |
| | Bighead carp | $0.69±0.23^a$ | $2.27±0.23^a$ | $3.41±0.34^b$ | $3.88±0.32^b$ | $4.25±0.26^b$ | $4.99±0.55^b$ | $5.45±0.37^b$ |
| TYMC | Common carp | $0.64±0.50^a$ | $1.91±0.62^a$ | $2.60±0.23^a$ | $3.34±0.27^a$ | $3.32±0.23^a$ | $3.40±0.47^a$ | $2.68±0.12^a$ |
| | Bighead carp | $0.82±0.13^a$ | $2.32±0.54^b$ | $2.52±0.20^a$ | $3.35±0.24^a$ | $3.40±0.35^a$ | $3.70±0.11^a$ | $3.67±0.53^b$ |

**Legend:** Same lowercase letters in a column indicate no significant differences (p>0.05)

During the storage, vacuum packaging influenced microbial growth, and the APCs in both groups of fish did not reach 7 logcfu/g, which likely was reflected in sensory changes of products. Our research showed there was not a good correlation between the APCs of cold-smoked fish and overall sensory acceptability (see below). The low number of aerobic Gram-negative bacteria can be explained by increase of carbon dioxide levels in the gaseous phase inside the packaging due to bacterial metabolism and the gas' bacteriostatic effect (*Radetić et al*., 2007). On the other hand, *Olafsdottir et al.* (2005) reported that APC numbers were from 7 to 8 logcfu/g at the end of the shelf-life of vacuum packaged cold-smoked salmon. They suggested there was good corelation between APC numbers and sensory evaluation of overall acceptability.

Between the PBCs of common carp and bighead carp, no significant differences ($p > 0.05$) were determined during the whole period of storage. During the first seven days of storage, and among the examined microorganisms, the PBC showed the most intensive growth in both fish groups. The reason for that could be the storage temperature, which was very close to the optimal temperature for growth of these micro-organisms. After that period of time, PBC in both fish groups remained quite stable until the end of the study.

In our study, the LAB numbers increased during the storage in both groups of fish. During the storage, LAB numbers determined in bighead carp were significantly ($p < 0.05$) higher compared to LAB numbers determined in common carp. At the end of the storage period, LAB was the dominant microbiota in cold-smoked bighead carp. The dominance of these bacteria has been previously reported in vacuum packaged lightly preserved fish products stored at chilled temperature. In three species of vacuum packaged cold-smoked fish, *Gomez-Guillen et al.* (2009) reported that LAB was the dominant group of micro-organisms. *Truelstrup Hansen et al.* (1998) found the same in vacuum packaged cold-smoked salmon. The ability of these bacteria to grow rapidly under anaerobic conditions at low temperatures, as well as their tolerance to $CO_2$, could be the reason for dominance of these facultative anaerobic micro-organisms. Although LAB can easily cause spoilage of cold-smoked products (*Lyhs et al.* 1998), it is considered that these micro-organisms in vacuum-packed food contribute to prolonged shelf-life by inhibiting the growth of other bacteria through creating lactic acid and bacteriocins (*Gram and Dalgaard*, 2002). This characteristic of LAB can be a reason for the lower APC and better shelf-life of cold-smoked bighead carp than common carp in our study.

Fungal growth (mainly yeasts) in vacuum-packed cold-smoked fish is quite common, which was confirmed by our study. Intensive growth of TYMC was recorded in our cold-smoked bighead carp compared to in the cold-smoked common carp. According to *Gonzales-Rodriguez et al.* (2002), fungus counts were higher in smoked trout fillets than in smoked salmon, although differences were not significant ($p>0.05$). In *Leroi et al.* (1998), the TYMC in cold-smoked salmon remained low during whole storage period. *Cakli*

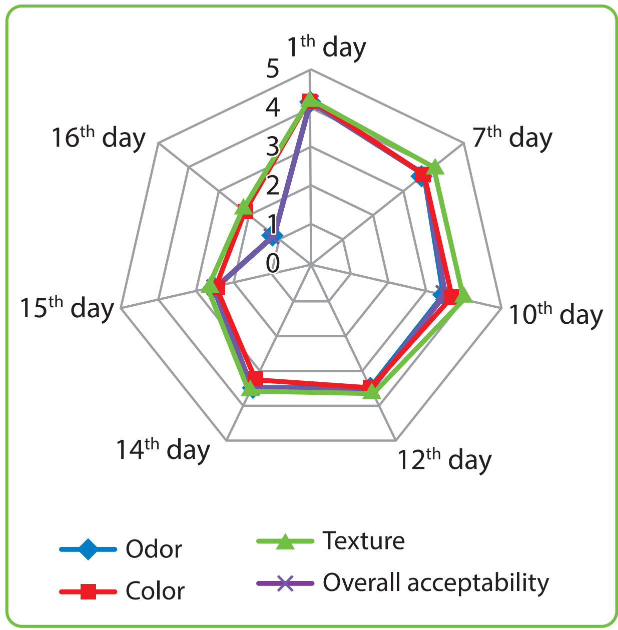


**Figure 1.** Sensory evaluation of cold-smoked common carp fillets during the storagecategories

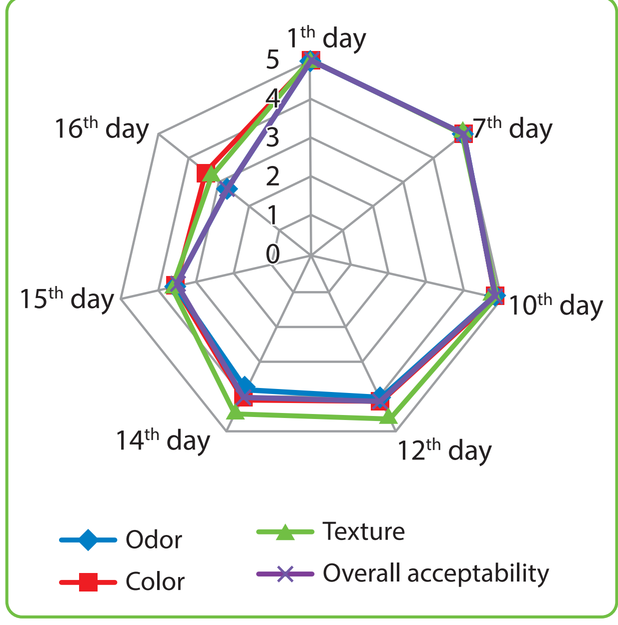


**Figure 2.** Sensory evaluation of cold-smoked bighead carp fillets during the storage

*et al.* (2006) did not detect the presence of TYMC in hot-smoked trout packaged in vacuum or in modified atmosphere. They concluded that the growth of yeasts and moulds is influenced by the strong effect of smoke and the temperature during hot-smoke fish processing. The spoilage caused by the growth of fungi is sometimes manifested by an unpleasant smell and taste, but more often, is evident in a change in the appearance of the product which is characterized by surface pigmentation and slime. Since in our research, relatively low numbers of TYMC were recorded, we concluded that TYMC did not cause spoilage of common carp fillets.

The results of the sensory evaluation of cold-smoked common carp fillets and cold-smoked bighead carp fillets are presented in Figs 1 and 2. At the beginning of the storage period, color, flesh texture, odour and overall acceptability were evaluated with very high scores in both groups of fish. During the storage, the average grades decreased for sensory parameters of both fish species. As the results show, all estimated sensory characteristics of common carp received significantly lower ($p < 0.05$) scores on day 15. The odour of fermentation in common carp, detected on day 16, caused the odour score to be lower than the acceptability limit of 2. On the last day of storage, reduced intensity of the pink-cream colouring of common carp muscle was observed, as was softened texture and surface slime. Metabolic activities of microorganisms at the end of storage period could be a reason for the unpleasant fermentation odour, while the changes in texture could be a consequence of the activity of autolytic enzymes, given that the temperature during the cold smoking never exceeded 18 °C , so their inactivation did not occur (*Truelstrup Hansen et al*., 1995). In contrast with common carp, all examined sensory characteristics of bighead carp fillets were within the acceptability level during the study.

## 4. Conclusion

Cold-smoked freshwater fish packaged in vacuum longer retains its desirable sensory characteristics during cold storage, and the growth of examined micro-organisms are slowed down under these conditions. Based primarily on sensory results, it was concluded that vacuum-packaged cold-smoked common carp remained acceptable up to 15 days of storage, while vacuum-packaged cold-smoked bighead carp remained unchanged until the end of the study (16 days). These results provide useful information about the storage of cold-smoked freshwater fish under vacuum conditions, which could be useful for processor and retailer level.

# Uticaj vakuum pakovanja na mikrobiološke i senzorne parametre kvaliteta hladno dimljene slatkovodne ribe

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INFORMACIJE O RADU

*Ključne reči:*
Šaran (*Cyprinus carpio*)
Tolstolobik (*Hypophthalmichthys nobilis*)
Održivost
Ukupan broj bakterija
Bakterije mlečne kiseline

APSTRAKT

Cilj ovog istraživanja bio je da se ispitaju promene odabranih mikrobioloških i senzornih svojstava hladno dimljenih fileta šarana (*Cyprinus carpio*) i hladno dimljenih fileta tolstolobika (*Hypophthalmichthys nobilis*) pakovanih u vakuum koji su čuvani na temperaturi od 3 ± 0.5 °C, kao i da se odredi održivost proizvoda. Ispitivanja su rađena 1, 7, 10, 12, 14, 15 i 16 dana. Od dvanaestog dana eksperimenta, ukupan broj bakterija hladno dimljenih fileta šarana bio je statistički značajno veći ($P < 0.05$). Ukupan broj bakterija kod obe vrste dimljene ribe nije dostigao vrednost 7 logcfu/g. Tokom celog perioda ispitivanja nije utvrđena statistički značajna razlika ($p > 0.05$) između ukupnog broja psihrotrofnih bakterija. Bakterije mlečne kiseline kod hladno dimljenih fileta tolstolobika bile su dominantna microflora na kraju ispitivanja. Na osnovu promene senzorskih svojstava može se zaključiti da su hladno dimljeni fileti šarana pakovani u vakuumu bili prihvatljivi 15 dana, dok su hladno dimljeni fileti tolstolobika pakovani u vakuumu bili prihvatljivi 16 dana.

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*Original scientific paper*

# Effect of pomegranate (*Punica granatum*) fruit molasses as a natural marinade on the microbiological quality and shelf life of refrigerated chicken fillet

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A R T I C L E   I N F O

*Keywords:*
Pomegranate
Chicken meat
Food Quality
Shelf life
Spoilage
Natural preservatives

A B S T R A C T

Pomegranate molasses (PM) could be suitable as a marination ingredient in Mediterranean cuisine. This study is, thus, aimed at investigating the effects of PM on the microbial characteristics of chicken breast fillets. For this purpose, PM marinades were prepared at three different concentrations for the marination of chicken breast fillets, which were assigned to three treatment groups: T1 (immersed in 0.5% v/w PM), T2 (immersed in 1.0% v/w PM) and T3 (immersed in 1.5% v/w PM). Chicken breast fillets were marinated for 2 hours and then aerobically stored at 4°C for 15 days. Non-marinated fillets were used as the control. Levels of aerobic bacteria, psychrotrophic bacteria, coliforms, and lactic acid bacteria were determined to evaluate the evolution of spoilage. The results revealed that the growth rate of the microbial populations during storage at 4°C decreased with the increasing concentration of PM. The groups of aerobic, psychrotropic, and lactic acid bacteria may be continuously increased on each sampling day, with bacteria numbers on the control and T1 fillets surpassing those on fillets exposed to the other treatments ($p < 0.05$) from day 3 until day 15, when sampling stopped. All PM treatments had significantly decreased coliform counts ($p < 0.05$) than did the control group. At 4°C, the shelf life of PM-marinated chicken breast fillets was significantly extended compared to the controls, achieving up to 6, 9, and 12 days for T1, T2, and T3, respectively, as evaluated by microbiological analyses. The findings of this study suggest that pomegranate molasses could be used as an ingredient to improve the microbiocidal quality of marinade or as a sole marinade, both of which uses could prolong the shelf life of chicken breast fillets.

## 1. Introduction

Chicken meat is considered a desirable nutrient source for human health as it contains many polyunsaturated fatty acids, low lipid levels, and minerals. Also, it is low cost and has favorable organoleptic attributes, resulting in increased production and consumption of chicken meat in recent decades (*Zhang et al.*, 2021). However, chicken meat has a limited shelf life depending on various factors such as pre-slaughter handling, initial bacterial load, processing technology, pH, chemical composition and water activity of muscle, residual blood, carcass temperature, and storage and transport conditions (*Özünlü & Ergezer*, 2022).

In recent years, there has been growing demand for the use of natural additives and preservatives, rather than synthetic ones, in the food industry, as it has been claimed that synthetic additives and preservatives have hazardous and cancerous effects on human

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health (*Lemay et al*., 2002). Because of consumer awareness of the hazards of synthetic additives, people seek out foods devoid of them, increasing demand for natural preservatives that perform the same function as synthetic ones (*Kaderides et al*., 2021).

Pomegranate (*Punica granatum*) is cultivated in a wide range of areas, such as in the Mediterranean and South Asian regions. In recent years, pomegranate fruit production and consumption have increased due to the fruit's healthy beneficial effects, as it is used in treating inflammatory and digestive diseases (*El-Said et al*., 2014). Pomegranate molasses is derived from pomegranate juice, which is rich in total phenolic and flavonoid compounds and has become one of the most important additives that is becoming more applicable in different foods and widely distributed in various international markets in all countries, especially in the Middle East (*Nasser et al.*, 2017). There is increasing consumer interest in the nutritional value of pomegranate molasses, as it contains bioactive compounds, such as anthocyanins, ellagic acid derivatives, hydrolyzable tannins, and other phenolic compounds, that have anti-inflammatory and anti-tumor effects. Also, it gives the food a sweet taste and flavor (*Faour-Klingbeil & Todd*, 2018).

To the best of our knowledge, there are limited studies and literature on the role of pomegranate molasses in improving chicken meat shelf life, unlike other pomegranate by-products such as peel extract or juice. Therefore, this study was designed to examine the effect of marinating chicken breast muscle fillets in traditional pomegranate molasses in its pure form and at different concentrations; microbial levels were measured at three-day intervals and for the shelf life of the chicken breast fillets.

## 2. Materials and methods

### *2.1. Ethical approval*

The study was conducted after the research proposal was approved by the Care and Use Committee Research Ethics, Faculty of Veterinary Medicine, Benha University (BUFVTM, 15/10/23), Egypt.

### *2.2. Preparation of pomegranate molasses*

High-purity pomegranate molasses was purchased from the local market of El-Gharbia governorate, Egypt, in July 2022 and diluted in water to reach concentrations of 0.5%, 1% and 1.5%.

### *2.3. Chicken fillet treatment and storage*

Chicken fillets were purchased from the local market of El-Gharbia governorate, Egypt and transferred to the laboratory in cold conditions. The chicken fillets were divided into four parts and transferred into four containers. To marinate fillets, they were soaked in a pomegranate molasses/water solution containing 0% (CO), 0.5% (T1), 1% (T2), or 1.5% (T3) molasses for two hours. The marinated fillets were placed in sterile bags and kept at 4°C, and fillet microbiological evaluation was conducted at three-day intervals.

### *2.4. Microbiological Analysis*

#### 2.4.1. Sample preparation

The chicken breast fillet samples for microbiological analyses were prepared by placing 10 g of excised fillet into 90 ml of peptone water (0.1%), mixing in a sterile bag, and homogenizing with a Stomacher (Stomacher® 400 Circulator, Seward, UK) at 200 rpm/min for 1 minute. Then, other decimal dilutions were prepared from this dilution in tubes containing peptone water.

#### 2.4.2. Microbiological analysis

Bacterial counts were performed using plate count agar (C# CM0325, OXOID, UK) for aerobic plate count (APC); the inoculated plates were incubated at 30±1 °C for 72±3 h (ISO, 2013). The psychotropic plate count (PPC) was performed using plate count agar (C# CM0325, OXOID, UK), and the inoculated plates were incubated at 7 °C for 10 days (ISO, 2019). The lactic acid bacteria count (LAB) was performed using De Man-Rogosa Sharp Agar (MRS) (C# NCM0190A, NEOGEN®, USA). The incubation conditions were 30±1 °C for 72±3 h for LAB (ISO, 1998). Violet red bile agar (VRBA) (C# CM0485, Oxoid, UK) plates were used for total coliform count (TCC), and the inoculated plates were incubated at 30±1 °C for 72±3 h (ISO, 2006). All counts were expressed as $\log_{10}$CFU/g and were performed in duplicate.

### *2.5. Microbial reduction calculation*

Using the average values of these counts, the microbial reduction percentages were calculated according to the following formula in relation to the control: Microbial reduction percentage (%) = (control CFU − test CFU)/control CFU) × 100.

In addition, the logarithmic scale reduction factor ($log_{10}$) was calculated using the formula RF = $Log_{10}$ (A) − $Log_{10}$ (B), where A is the number of colonies recovered from the unexposed (control) and B is the number of colonies recovered from the exposed (test) from T1 to T3 (*Mascarenhas et al.*, 2022).

### *2.6. Statistical Analyses*

The collected data were exposed to one-way Analysis of Variance (ANOVA) using SPSS (version 20; IBM, Chicago, IL, USA) followed by Tukey's multiple comparison tests (Tukey, 1953) to compare the differences between dietary treatments, where significant differences were observed ($p \leq 0.05$).

## 3. Results

The effects of marinating chicken breast meat fillets in PM at different concentrations and storing them at refrigeration temperature were studied. To assess the progression of spoilage and shelf-life extension, APC, PPC, TCC, and LAB counts of the chicken breast meat fillets were measured. (Figures 1–4).

The initial APC in all treated chicken fillet groups ranged from 4.7 to 4.3 $log_{10}$CFU/g at the beginning of the storage period. During the 4°C storage period, the APCs of control and PM-treated chicken fillet groups increased. The control fillet group reached the limit of acceptability on day 6, as the APC was 6.02 $log_{10}$CFU/g, which is the point that indicates the spoilage of chicken meat (red dash-dot line in Figure 1A). However, APCs in the treated fillets increased more slowly than those in the control fillets. As the PM concentration increased, there was a significant slowdown in the growth and a relative reduction of the APC ($p < 0.05$). T3 had the greatest reduction in APC compared to the control, as it exceeded the spoilage limit of 6.5 $log_{10}$CFU/g after 15 days of storage (Figure 1A). The mean APC reduction percent due to marinating fillets in PM was significantly lower ($p<0.05$) than the control. This reduction trend continued on days 6, 9, 12, and even day 15, as T3 APC reduction percent was significantly ($p < 0.05$) greater than any other treatments at those times (Figure 1B).

The PPC bacteria increased in all fillet groups during the period of storage. The maximum PPC increase was recorded in the control group, going from 4.5 $log_{10}$CFU/g at initial storage to 7.7 $log_{10}$CFU/g at final storage, increasing by 3.2 log cycles during the shelf life. On the other hand, the groups marinated in PM showed lower growth of PPC during the storage life as compared to the control, as recorded on day 15: 7.5 $log_{10}$CFU/g,7.2 $log_{10}$CFU/g, and 7.0 $log_{10}$CFU/g in T1, T2, and T3, respectively (Figure 2A). In this study, PPC on T3 fillets did not exceed 6 $log_{10}$CFU/g until the 12th day; this level was determined at the start of the study as the PPC threshold for non-consumable products (Figure 2A). The mean PPC reduction percent due to marinating fillets in PM was significantly greater ($p < 0.05$) than in the control. This reduction pattern began on day 0 and continued through sampling on days 6, 9, 12, and 15, as T3 PPC reduction percent was substantially ($p < 0.05$) greater than reductions for any other treatment on those days,

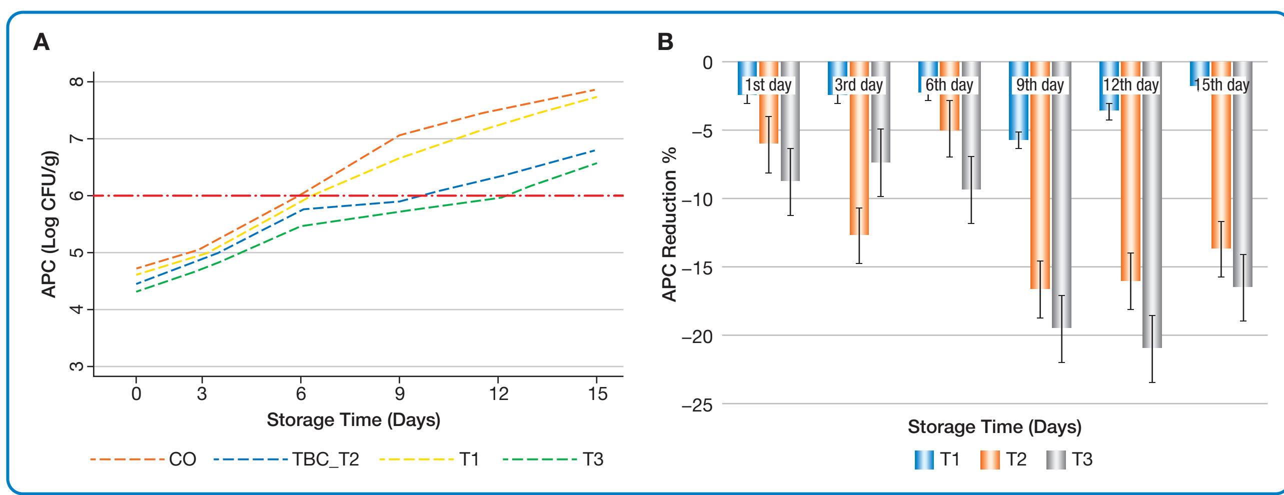


**Figure 1.** (A): Evolution of APC ($log_{10}$ CFU/g) counts of control (CO) and pomegranate molasses-marinated chicken breast fillet groups; T1 (0.5% v/w), T2 (1% v/w) and T3 (1.5% v/w), stored at 4 °C, (B) Microbial reduction percentage of APC

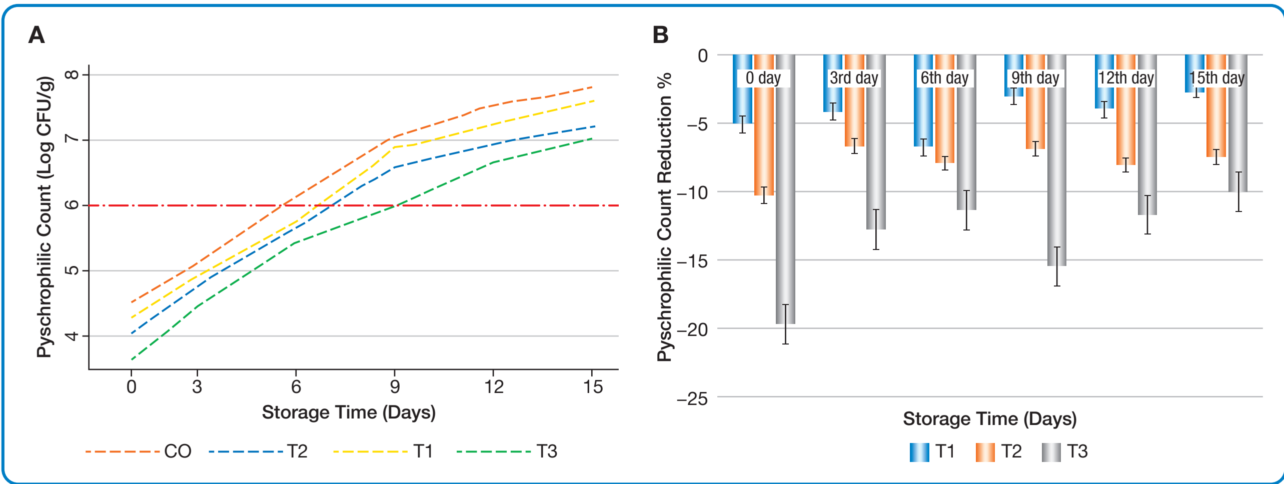


**Figure 2.** (A): Evolution of PPC ($log_{10}$ CFU/g) counts of control (CO) and pomegranate molasses-marinated chicken breast fillet groups; T1 (0.5% v/w), T2 (1% v/w) and T3 (1.5% v/w) stored at 4 °C, (B): Microbial reduction percentage of PPC

with the best reduction in T3 recorded on day 0 and generally declining with time (Figure 2B).

The LAB counts on day 0 ranged from 3.8 $log_{10}$ CFU/g in T3 to 4.5 $log_{10}$ CFU/g in the control group. During the storage period, the growth of LAB was greatest on the control chicken fillet group (LAB numbers reached 7.5 $log_{10}$ CFU/g) followed by T1 (7.2 $log_{10}$ CFU/g), then T2 (6.4 $log_{10}$ CFU/g), and T3 (6.0 $log_{10}$ CFU/g) on day 15 of storage (Figure 3A). The control chicken fillets exceeded the deterioration limit (6 $log_{10}$ CFU/g) on day 6, while the T2 and T3 groups exceeded the limit in 12 and 15 days, respectively. T3 group's LAB reduction percent was substantially ($p < 0.05$) greater than seen in any other treatment group on the same respective days, with the best reduction seen on day 15 (Figure 3B).

Results of the TCC microbial analysis are presented in Figure 4. The TCC was countable on day 0 in the control fillet group, being 2.6 $log_{10}$ CFU/g, but TTCs remained below the limit of detection in the other treatment groups until day 3 of storage (Figure 4A). Throughout the storage, the TCC in the control group increased until it reached 5.6 $log_{10}$ CFU/g at the end of storage. In contrast, the TCC in the PM-treated chicken fillets began to be detected from day 3 in the T1 and T2 fillet groups, with counts of 2.8 $log_{10}$ CFU/g and 2.6 $log_{10}$ CFU/g, respectively, until TCC reached around 5 $log_{10}$ CFU/g in both groups after 15 days of storage. In the T3 fillet group, the TCC remained below the limit of detection until day 6, when the count was 2.6 $log_{10}$ CFU/g, and increased to 4.3 $log_{10}$ CFU/g at the end of storage (Figure 4A). T3 fillet group's TCC

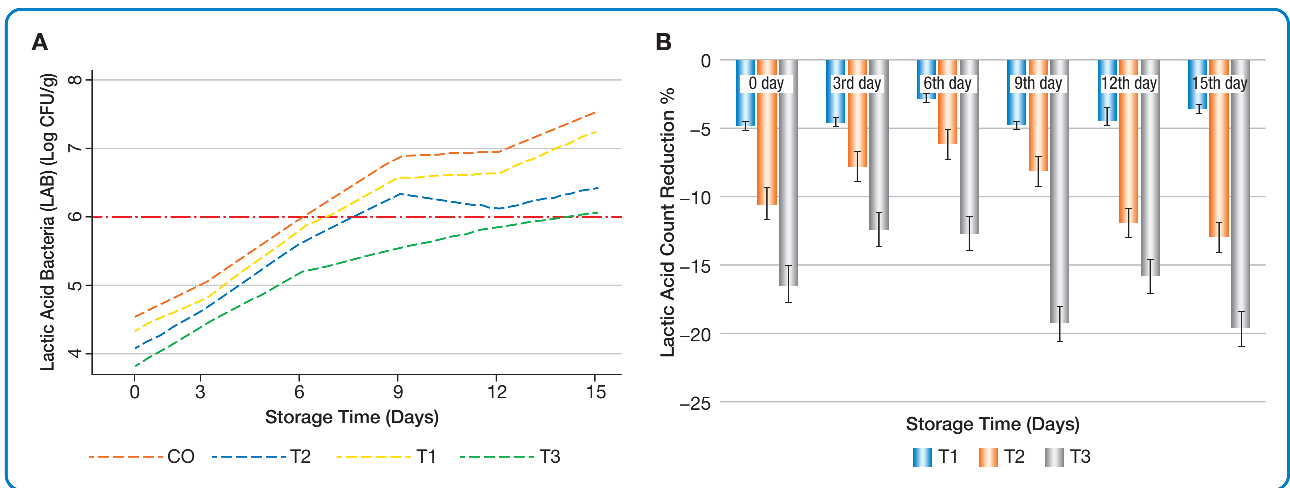


**Figure 3.** (A): Evolution of LAB ($log_{10}$ CFU/g) counts of control (CO) and pomegranate molasses-marinated chicken breast fillet groups; T1 (0.5% v/w), T2 (1% v/w) and T3 (1.5% v/w) stored at 4 °C, (B): Microbial reduction percentage of LAB

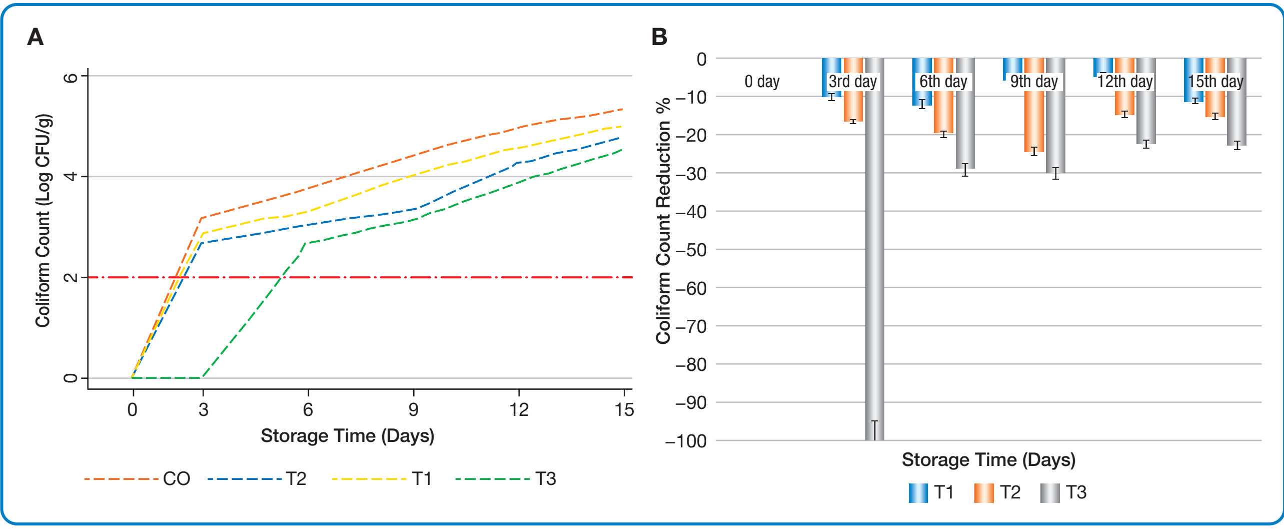


**Figure 4.** Evolution of TCC ($log_{10}$ CFU/g) counts of control (CO) and pomegranate molasses-marinated chicken breast fillet groups; T1 (0.5% v/w), T2 (1% v/w) and T3 (1.5% v/w) stored at 4°C

reduction percentage was significantly ($p < 0.05$) greater than in any other fillet group on the same respective day, with the greatest reduction on day 3 (Figure 4B).

## 4. Discussion

Bacteria are one of the main agents that cause fresh meat to lose its quality, as these products are more likely to be spoiled with microorganisms if they are not properly handled and preserved in good condition; therefore, nowadays, there is an increase in the use of preservatives with antimicrobial properties (*Pires et al.*, 2022). Microbiological assessment, including bacterial determination, is an important key in evaluating the safety and quality of marinated chicken meat, as it is quick, inexpensive, and very accurate in identifying bacteria (*Augustyńska-Prejsnar et al.*, 2023).

Pomegranate fruit has polyphenolic compounds that inhibit the microbial growth that is responsible for food deterioration and food-borne illness (*Çam et al.*, 2014). Many phytochemical compounds in pomegranate have antimicrobial activity, such as ellagic acid and larger hydrolyzable tannins, such as punicalagin (*Ferrazzano et al.*, 2017). The pomegranate fruit and its derivatives possess antibacterial activity against both Gram-negative and -positive bacteria due to the broad spectrum of bioactive compounds with antimicrobial characteristics (like polyphenol, ellagic and tannins) in the fruit (*Gullon et al.*, 2016). In this study, PM at high concentrations in marinade was found to be able to lengthen the shelf life of the chicken fillets, agreeing with (*Zhuang et al.*, 2019a), who said that treating big head carp fillet with pomegranate peel extract increased the shelf life by 1–2 days over that of the control. The shelf life of chicken meat can be determined by the total aerobic count, with the recommended limit in chilled chicken meat being 6 $log_{10}$ CFU/g (NFSA, 2021). In this study, the APC of the control group was 4.7 $log_{10}$ CFU/g initially, which agrees with other results (*Bazargani-Gilani et al.*, 2015a; *Vaithiyanathan et al.*, 2011), which reported initial APCs were 4.5 $log_{10}$ CFU/g and 4.8 $log_{10}$ CFU/g for chicken meat. Microbial increases in chicken meat during storage at refrigerated temperature result from deterioration of the physicochemical characteristics of meat (*Sujiwo et al.*, 2018).

The APC in our T3 group was 6.5 $log_{10}$ CFU/g at the end of storage; this agreed with *Rahnemoon et al.* (2021), who observed that their coated chicken meat with nano-encapsulated pomegranate peel extract reached the same limit after 14 days. Also, the APCs in shrimp dipped in 1 g/100 mL pomegranate extract (5.99 $log_{10}$ CFU/g) and 2 g/100 mL pomegranate extract (5.96 $log_{10}$ CFU/g) were lower than in the control (*Basiri et al.*, 2015). However, there was no significant difference between the treatment groups in that study. In addition, *Ghimire et al.* (2022) found a significant difference between APCs in the control and their pomegranate peel extract-incorporated ground buffalo meat. However, there was no significant difference in APCs between the 1% and 1.5% pomegranate peel extract-incorporated buffalo meat. Finally, pomegranate juice and extract had an antibacterial effect, confirmed by minimum APCs on frozen burgers (*Shahamirian et al.*, 2019).

The current results revealed that our T2 and T3 chicken fillet groups exceeded the upper permissible limit for acceptability, which was an APC of 7.0 $log_{10}$

CFU/g, on days 12 and 15 of storage, respectively. We speculate this extension of the shelf life was due to the antimicrobial action of the condensed compound of pomegranate, especially tannins and protein perceptible compound (*Bazargani-Gilani et al*., 2015a). The APCs of chicken meat products enclosed with pomegranate rind extract increased during the storage life. However, APCs on the pomegranate-treated products were lower than those of the control on all sampling days (Bazargani-Gilani et al., 2015a), likely because of the phenolic compounds and other components of pomegranate rind extract, which are reported to have antimicrobial properties against many microorganisms in meat products (*Dua et al*., 2016). Pomegranate peel extract in buffalo meat has been reported to prolong the shelf life and the meat's quality for up to an 8-day storage period (*Rasuli et al*., 2021). In another study, the total aerobic count was significantly lower in Frankfurter containing pomegranate juice concentrate and rind powder than in the control, and this indicates the pomegranate juice concentrate and rind powder is a wealthy source of phytochemical and phenolic compounds that provide antibacterial activity against a wide range of microorganisms (*Firuzi et al*., 2019).

Psychrotrophic bacteria are the most prevalent bacteria on refrigerated chicken by-products. They are the microorganisms of choice to detect the true microbial loads of chicken products and provide better detection of issues regarding product temperature (*Cortez-Vega et al*., 2012). The current study's results agreed with *Özünlü and Ergezer* (2022), who stated that the psychrophilic counts gradually increased with storage time and product unacceptability for human consumption, while it is considered chicken breast meat is spoiled at a PPC level of 6.0 $\log_{10}$ CFU/g. The smaller increase of PPC we found in PM-marinated chicken fillets (compared with the control PPC) complied with the result obtained by *Bazargani-Gilani et al*. (2015b), who found that the highest count in chicken meat was recorded in the control group rather than in other treatments that contained pomegranate juice; the pomegranate phenolic compound inhibited the psychrotrophic bacteria under the chill storage conditions. Moreover, treatments with 1 and 2 g/100 mL pomegranate extract resulted in decreasing PPC in shrimps compared to the control (*Basiri et al*., 2015). The psychrotrophic and thermophilic counts of chicken meat patties treated with pomegranate by-products and their extracts were significantly lower than the control group's counts (*Sharma & Yadav*, 2020). Similarly, the PPC remained lower in the meat sample with pomegranate juice than in the untreated control from 14 to 28 days of storage (*Vaithiyanathan et al.*, 2011). Those authors reported that the significant concentration of detectable phenolic compounds and condensed tannins in pomegranate juice provided antibacterial action in samples treated with the juice through protein binding or enzyme inhibition (*Vaithiyanathan et al*., 2011).

The LAB is one group of multiple bacteria genera associated with the spoilage of chicken meat during the storage period (*Pellissery et al*., 2019; *Zhuang et al*., 2019b). In the current study, the LAB level on the fresh control was 3.8 $\log_{10}$ CFU/g on day 0, but 7.5 $\log_{10}$ CFU/g on the final storage day (day 15), which was slightly lower than the LAB counts reported by Bazargani-Gilani et al. (2015b) and (*Fratianni et al*., 2010). The slower increase of the LAB observed in this study on T2 and T3 groups (compared with the control and T1) was in line with the results of *Bazargani-Gilani et al.* (2015b) and *Basiri et al*. (2015), who reported that pomegranate juice has a lowering effect on the LAB on chicken compared to the control during storage. T3 produced the lowest LAB count, indicating that marinating chicken fillets in PM influences LAB growth, particularly as the concentration increases.

The low TCC level observed in this study was due to coliforms being not primarily present in the freshly slaughtered carcasses and having a lower count than mesophilic bacteria. The presence of coliforms indicates direct or indirect contamination with a high degree of contamination (*Fliss et al.*, 1991). In fact, coliforms on chicken carcasses can be connected with the absence of hygiene or sanitary conditions during slaughter and processing (*De Moura Oliveira et al*., 2005). Low counts of these microorganisms can indicate good sanitary conditions of the chicken breast meat (*Özünlü & Ergezer*, 2022). In our study, the absence of coliforms on PM-marinated chicken fillets in the initial storage stage could have been due to the low pH of pomegranate, considered an inhibitory factor that can limit the growth of bacteria. The direct bactericidal action of organic acids results from a pH decrease within bacterial cells (*Raftari et al*., 2009). The TCC on the control in our study was 2.6 $\log_{10}$ CFU/g on day 0, which was lower than reported by *Bhoir et al.*, (2019), who found that TCCs in untreated samples were 4.22 $\log_{10}$ CFU/g on day 0.

The slowing of coliform growth observed in this study with PM marination agreed with the result (*Dakheli*, 2020) when pomegranate waste extract caused significant minimization in the number of coliforms on poultry carcasses compared with the controls. The increase in the concentration of the pomegranate extract resulted in a significant decrease in coliforms in the treated poultry carcass groups *(Dakheli*, 2020). *Kanatt et al*. (2010) reported that fecal coliforms were

not detected in any chicken lollipop samples containing 0.1% and 0.5% pomegranate extract during 12 days of storage, and also reported that 1% pomegranate extract increased the shelf life of chicken by two weeks due to the antimicrobial action of phenolic compounds in the plant extract. Our study's results were also in agreement with *El-Nashi et al.* (2015), who reported that TCCs decreased during storage of beef sausages treated with different concentrations of pomegranate peel powder compared with control.

The polyphenolic compounds (flavonoids, tannins) from plant components like pomegranate fruit by-products have antibacterial properties. These secondary metabolites inhibit bacteria by forming complexes with proteins and sulfhydryl groups that make them unavailable for the microorganisms (*Indices et al*., 2021). The possible mechanism of the antimicrobial effect of the pomegranate extract might be related to their phenolic compounds, as these can bind to substrates such as minerals, vitamins, and carbohydrates, making them inaccessible to microorganisms; phenolic compounds can also denature enzymes. Furthermore, phenols can disturb the structure and function of the cell membrane (*Essid et al*., 2020).

## 5. Conclusion

In conclusion, marination in pomegranate molasses effectively delayed chicken breast fillet spoilage. Microbial analysis showed that pomegranate molasses, a natural product, can control the microbial growth in chicken breast muscle fillets marinated in pomegranate molasses (1.5% v/w) and stored under refrigeration at 4 °C. The treated breast meat fillets were microbiologically acceptable for 12 days of refrigerated storage. The limited microbial growth that occurred suggests that poultry meat processors could utilize these findings to replace chemical preservatives in ready-to-cook poultry products without lowering quality and shelf life, and without decreasing consumer acceptance of the products. Thus, using pomegranate-based marinades could lead to convenient and upgraded ready-to-cook products.

# Uticaj voćne melase nara (*Punica granatum*) kao prirodne marinade na mikrobiološki kvalitet i rok trajanja rashlađenog pilećeg filea

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I N F O R M A C I J E O R A D U

*Keywords:*
Nar
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Kvalitet hrane
Rok trajanja
Kvarenje
Prirodni konzervansi

A P S T R A K T

Melasa od nara (PM) bi mogla biti pogodna kao sastojak za mariniranje u mediteranskoj kuhinji. Cilj ove studije je bio usmeren na istraživanje uticaja PM na mikrobne karakteristike fileta pilećih prsa. U tu svrhu pripremljene su PM marinade u tri različite koncentracije za mariniranje fileta pilećih prsa, koje su raspoređene u tri grupe tretmana: T1 (filei uronjeni u 0,5% v/w PM), T2 (filei uronjeni u 1,0% v/w PM) i T3 (filei uronjeni u 1,5% v/w PM). Fileti pilećih prsa su marinirani 2 sata, a zatim aerobno čuvani na 4 °C 15 dana. Kao kontrola korišćeni su nemarinirani fileti. Određeni su nivoi aerobnih bakterija, psihrotrofnih bakterija, koliformnih bakterija i bakterija mlečne kiseline da bi se procenila evolucija kvarenja. Rezultati su otkrili da se stopa rasta mikrobnih populacija tokom skladištenja na 4 °C smanjuje sa povećanjem koncentracije PM. Grupe aerobnih, psihrotropnih i bakterija mlečne kiseline mogu se kontinuirano povećavati svakog dana uzorkovanja, pri čemu broj bakterija u kontrolnoj grupi filea, kao i kod T1 grupe fileta nadmašuje one na filetima izloženim drugim tretmanima ($p < 0{,}05$), od 3. do 15. dana, kada je uzorkovanje prestalo. Svi tretmani PM su imali značajno smanjen broj koliformnih bakterija ($p < 0{,}05$) nego u kontrolnoj grupi. Na 4°C, rok trajanja fileta pilećih prsa mariniranih u PM je značajno produžen u poređenju sa kontrolom, dostižući do 6, 9, odnosno 12 dana za T1, T2 i T3, kako je procenjeno mikrobiološkim analizama. Nalazi ove studije sugerišu da bi melasa od nara mogla da se koristi kao sastojak za poboljšanje mikrobiocidnog kvaliteta marinade ili kao jedina marinada, a obe upotrebe bi mogle da produže rok trajanja fileta pilećih prsa.

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# *In vitro* evaluation of *Hydrilla verticillata* extract as a natural preservative for chicken meat

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A R T I C L E I N F O

*Keywords:*
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A B S T R A C T

The intent of this research is to study the preservative effect of ethanolic extract of *Hydrilla verticillata* enriched with chitosan coating on the meat sample to increase the shelf life of the palatable product. The chemical, microbiological, sensory, and nutritional analysis such as pH, the amount of lipid peroxidation, total bacterial count, protein, and fat content of the fresh chicken meat coated with ethanolic extract of *H. verticillata* and chitosan were performed for 21 days. The results of the experiment show that *H. verticillata* extract enriched with chitosan coating met the need to act as a preservative by degrading the growth of microorganisms, maintaining the pH, and also by increasing the nutritional values.

## 1. Introduction

Natural preservatives are compounds found in natural sources which is used for long term preservation of organoleptic qualities (color, flavour, taste, smell, freshness), prevention of rapid degradation and to prolong the shelf life of food (*Bondi et al.,* 2017). The natural preservatives are witnessing higher demand owing to their acceptance in processing by relevant regulatory agencies coupled with rising health consciousness among customers. Chicken meat is a predominant dietary protein source and the treatment to preserve its quality plays a vital role. Treatment procedures may follow physical, chemical and biological processes. The physical process includes dehydration, freeze-drying methods etc., and chemical process where synthetics are added such as benzoates, nitrites, etc. The biopreservation process includes the addition of enzymes and plant extracts which have natural antioxidants that promote health benefits. Moreover, consumer's preference for natural food preservatives and concern regarding the safety of synthetic preservatives urged the food industry to look for natural alternatives (*Esmaeili et al.,* 2021).

*Hydrilla verticillata* is a submerged herbal medicinal aquatic plant that contains more of beneficial compounds which possess both antimicrobial and antioxidant properties (*Pal & Nimse,* 2006). The presence of the phytochemical in *H. verticillata* namely phytol may act as a natural preservative as it has both the antimicrobial and antioxidant activities that play an integral role in acting as a natural preservative. The phenol and flavonoids content in it also contributes to the high antioxidant capacity. Due to its essential properties and presence of vital bioactive compounds, the ethanolic extract of *H. verticillata* can be used as a preservative (*Byju et al.,* 2013).

Chitosan is a natural, biodegradable, biorenewable and non-toxic substance that has been considered for applications in the food industry (*Sinha et al.,* 2022). This is due to its physicochemical properties, film forming and barrier properties against pathogenic microbes, anti-microbial and anti-fungal activities *(Xing et al., 2016)*. Application of chitosan

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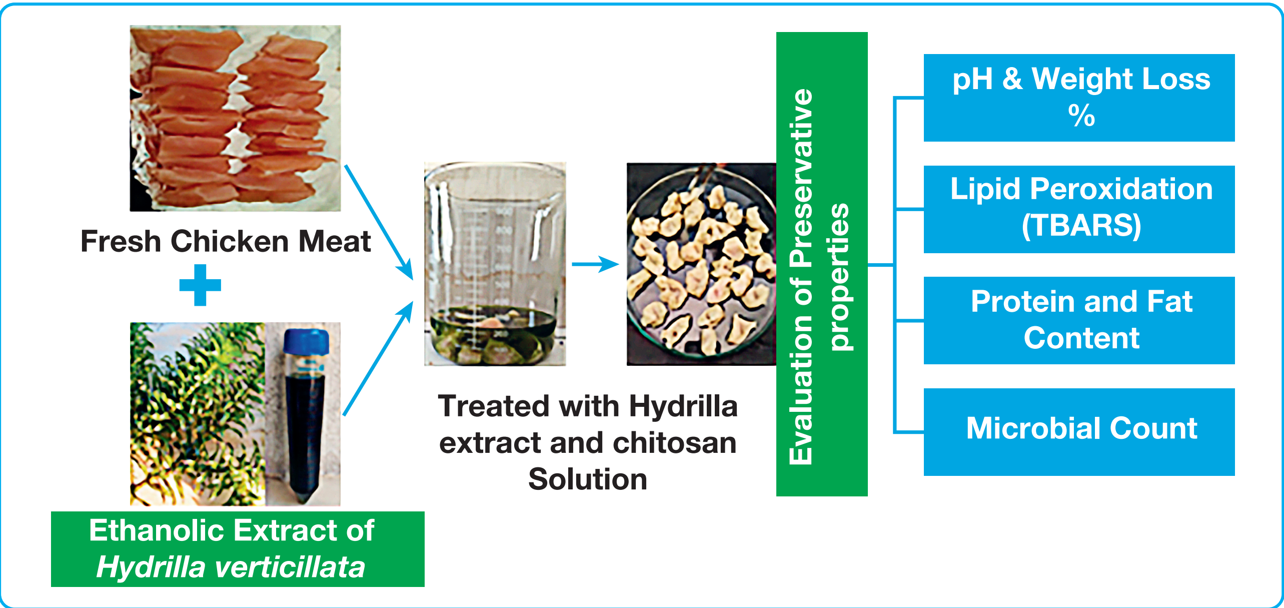


**Figure 1.** Comprehensive Overview of the Synergistic Preservative Effects of Hydrilla verticillata and Chitosan on Chicken Meat

to meat will be a barrier to water vapour that will reduce the moisture content and weight loss, maintain the color of the meat, retard the lipid peroxidation and increase the shelf life and storage quality. When chitosan is combined with the plant extract, it additionally enriches the preservative action. The 1% chitosan is most commonly preferred as it effectively inhibits the growth of bacteria, putrefaction, reduces the thiobarbituric acid **(**TBA) value and increases anti-oxidation *(Shafiei & Mostaghim, 2022)*. Hence, the experiment involved the application of extract derived from *H. verticillata* along with chitosan to analyze the preservative effect on the chicken meat.

## 2. Materials and Methods

*Preparation of ethanolic extract of Hydrilla verticillata*

The preparation of ethanolic extract of *H. verticillata* was done as described in our previous article (*Prabha et al.,* 2019).

*Preparation of 1% chitosan solution*

4 g of chitosan was dissolved in 4 mL of glacial acetic acid and it was stirred in the magnetic stirrer until it is completely dissolved for about 10 min at 50 °C and then 150 mL of distilled water was added to the mixture and stirred again until it is mixed properly. Then the solution was made up to 300 mL with distilled water.

*Preparation of chicken samples*

Fresh chicken breast meat was brought from the market and cut into small pieces each weighing approximately 15 g and divided into three groups namely:

- Group I – Control
- Group II – Treated with hydrilla extract
- Group III – Treated with hydrilla extract and chitosan

The group I chicken pieces were dipped in distilled water for 1.5 min, the group II chicken pieces were dipped in extract solution of *H. verticillata* for 10 min and the group III chicken pieces were first treated with extract solution for 10 min and then with 1% chitosan solution for 1.5 min. Finally, all the groups were stored at refrigerated condition at 4 °C.

*Evaluation of preservative properties*

Chemical, microbiological, nutritional and sensory analysis are important in evaluation of preservation properties. The data is presented as the mean ± standard deviation of the mean (SDM ) calculated over a period of 21 days.

*Measurement of pH*

The pH for all the groups of the sample was done by homogenizing 0.5 g of sample with 5 mL of distilled water for 1 min. The homogenized samples were kept at room temperature for 10 min and then pH was determined using the pH meter and the values were recorded *(Karthik et al., 2021)*.

### 2.1 Analysis of lipid oxidation

*Determination of thiobarbituric acid reactive substances (TBARS)*

4 g of sample was blended with 8 mL of trichloroacetic acid (5 mg/100 mL) and 8 mL of 0.5% butylated hydroxy toluene (BHT) with the help of mortar and pestle and then the solution was filtered through Whatmann 4 filter paper. 5 mL of filtrate was added with 5 mL of thiobarbituric acid and they were heated in the boiling water bath for 30 min and measured at 532 nm *(Pandi et al., 2022)*. They are expressed as mg malonaldihyde/kg meat.

*Analysis of weight loss %*

Weight loss was calculated by initially weighing the weight of the sample meat of all groups and then the final weight for the consecutive days *(Adu et al., 2019)*.

The weight loss in percentage was calculated using the formula given below:

$$\text{Weight loss (\%)} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Final Weight}} \times 100$$

### 2.2 Nutritional analysis

*Determination of protein content*

The protein content of all the groups of sample was determined by Lowry's method. The concentration of protein can be determined using colorimeter at 670 nm (*Karthik et al.,* 2021).

*Determination of fat content*

The fat content of different batches of sample was determined by Liebermann-Burchard method *(Adu et al., 2019)*.

### 2.3 Microbiological analysis

*Pour plating technique for counting the total bacterial co unt*

1.1 g of sample from each group was homogenized with 10 mL of 0.1% sterile peptone water. From this homogenate, 1 mL was added to 9 mL of 0.1% sterile peptone water and the appropriate serial dilutions were carried out. Two dilutions of $10^{-2}$ and $10^{-3}$ were taken and 1 mL from each dilution was first placed into the plate and then the melted plate count agar was poured over it and then the plate was incubated at 37 °C and the numbers of colonies were calculated every day for a period of 21 days (*Mehdizadeh & Mojaddar Langroodi,* 2019).

### 2.4 Sensory analysis

It was performed by evaluation of the acceptability (total sensory evaluation score) as a composite of odor, color and appearance using a nine-point hedonic scale. The scale points were: excellent, 9; very good, 8; good, 7; acceptable, 6; poor (first off odor, off-taste development) < 6; a score of 6 would be taken as the lower limit of acceptability. The sample would be defined as unacceptable after development of first off-odor or off-taste.

### 2.5 Statistical analysis

All data expression is done using mean ± standard deviation. Significant results were reported by one-way analysis of variance follow-up test by Tukey's HSD post hoc multiple comparisons with the help of GraphPad Prism Scientific Software. $p<0.05$ is considered as statistically significant.

## 3. Results and Discussion

One of the main purposes of food industry is to optimize the preservation technologies of perishable foods to reach a final product with optimal quality.

As pH is the measurement of acidity, it will affect the water holding capacity and the color of the meat, which in turn will influence the overall quality of the meat. So maintaining the pH value of the meat is very important. The pH values of three groups of samples were determined by using a pH meter. The comparative results of three groups are depicted in Figure 2. The untreated control meat was spoiled after one week, but the hydrilla and hydrilla with chitosan treated meat samples retained pH 6.11 and 5.56 respectively after three weeks. It was observed that the only 10–13% changes in pH on the coated meat during the storage period. In this study, the pH values of the treated samples were significantly ($p<0.05$) lower than the control group during the storage period. This is mainly due to the phytochemicals present in the *H. verticillata* extract and acidic properties of chitosan solution and it prevents the microbial growth on the surface of the samples, which is due to the antimicrobial property (*Eldaly et al.,* 2018). During storage, there is a gradual increase in pH values in both Experimental groups II and III, primarily due to the activity of endogenous enzymes, bacterial metabolites, and

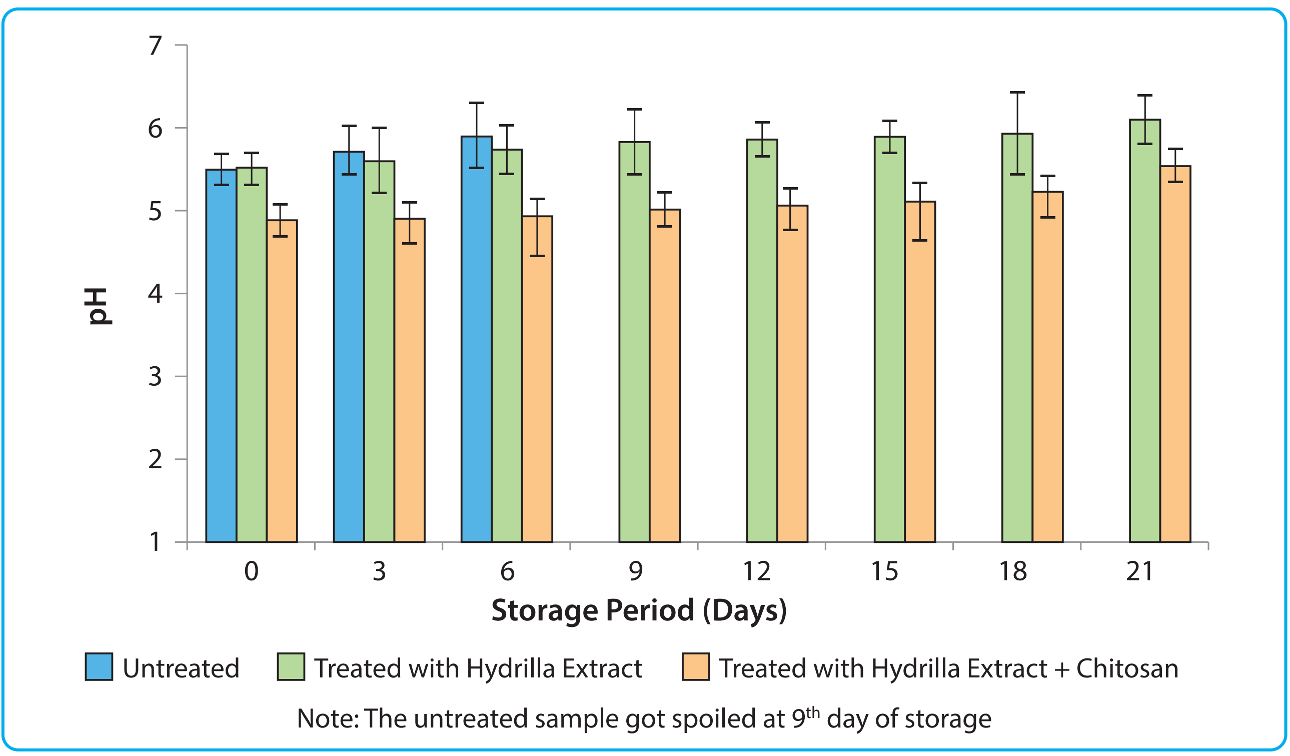


**Figure 2.** Effect of *Hydrilla verticillata* extract and chitosan coating on pH

the production of volatile organic compounds such as ammonia (*Alam et al.,* 2018). Notably, a significant difference was observed between these two groups, attributed to the presence of chitosan in Experimental group III. Chitosan plays a crucial role in mitigating pH fluctuations in meat samples by buffering the pH, inhibiting microbial growth, forming a protective film, and chelating metal ions. These mechanisms collectively contribute to the preservation of meat quality (*Thambiliyagodage et al.,* 2023).

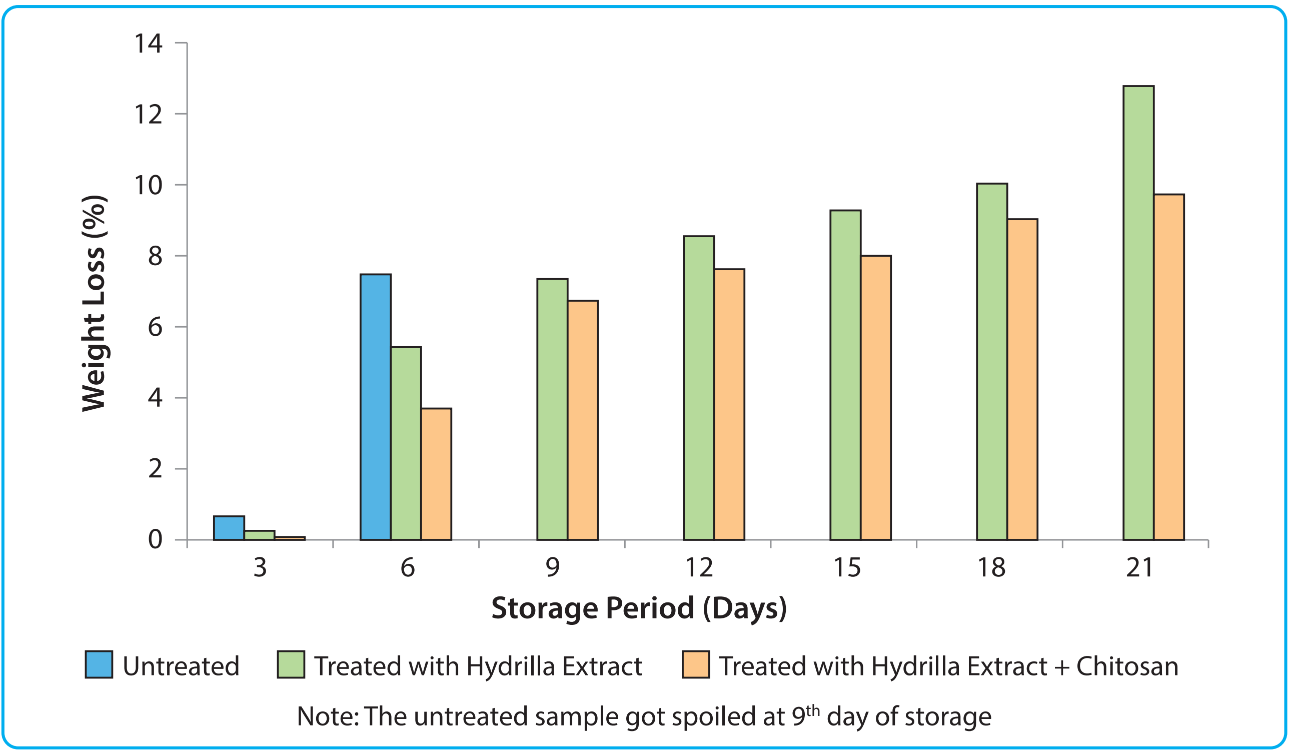


**Figure 3.** Effect of *Hydrilla verticillata* extract and chitosan coating on Weight loss %

Weight loss in meat occurs due to dehydration of meat during storage under refrigerated conditions. The main reason for dehydration is due to the fact that surface of the meat is exposed to mass transfer exchange (evaporation) with the environment. The weight loss percentages for three groups of sample were represented in the Figure 3. The study revealed that around 10% weight loss is observed on 9th day for untreated meat, however the same percentage of weight loss is observed on 18th and 21st day for hydrilla and hydrilla with chitosan coated meat. The coated samples showed a significantly lower weight loss than the uncoated sample meats. This is because the *H. verticillata* extract and the chitosan coating retained the moisture content by maintaining the weight which is mainly required to manage the quality of meat (*Mehdizadeh & Mojaddar Langroodi*, 2019).

Lipid peroxidation is a main factor that limits the shelf life of muscle foods and it is measured in terms of TBARS. The concentrations of TBARS formed due to lipid peroxidation in the chicken samples were found out using the standard calibration curve and expressed as mg/kg. As seen in this experiment, initially there was no significant difference in the TBARS values, but upon storage, there was rapid increase in TBARS in the uncoated sample as compared to the coated samples (Figure 4). Lipid peroxidation generally involves the degradation of polyunsaturated fatty acids and the production of secondary decomposition products, including carbonyls and hydrocarbon compounds. The oxidative stability of meat depends on the balance of anti-oxidants and the composition of oxidizable substrates, including PUFAs, cholesterol, proteins, and pigments (*Pereira & Vicente,* 2013). The antioxidants present in the hydrilla plays effective role in the inhibition of lipid peroxidation by donating hydrogen and helps to form the hydroxyl groups (*Jonaidi Jafari et al.,* 2018). Moreover, the scavenging activity of chitosan enhance the antioxidant activity of the formulated preservative (Ngo & Kim, 2014). The observed results revealed that *H. verticillata* extract and chitosan coating inhibited the lipid oxidation in all the meat samples during the storage.

The concentrations of protein in the three groups of sample were found out using the standard calibration curve and the concentration is expressed as mg/ml. The results of the experiment are represented in Figure 5. It was observed that the minor change in protein content 17% and 13% on the hydrilla and hydrilla with chitosan coated meat. The untreated meat samples could be stored under refrigeration for 6 days but the hydrilla extract treated samples could be stored for 21 days by retaining near normal protein content of the meat. The antimicrobial activity can help prevent protein degradation

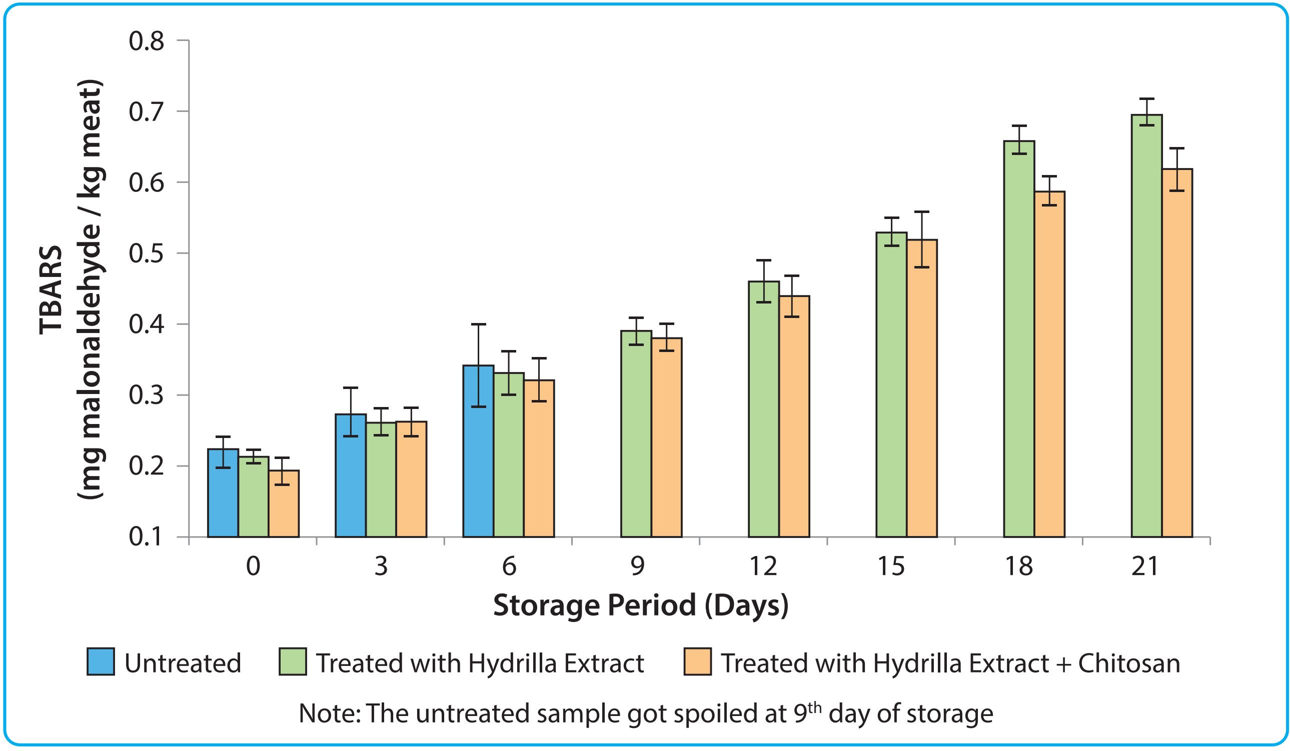


**Figure 4.** Effect of *Hydrilla verticillata* extract and chitosan coating on TBARS

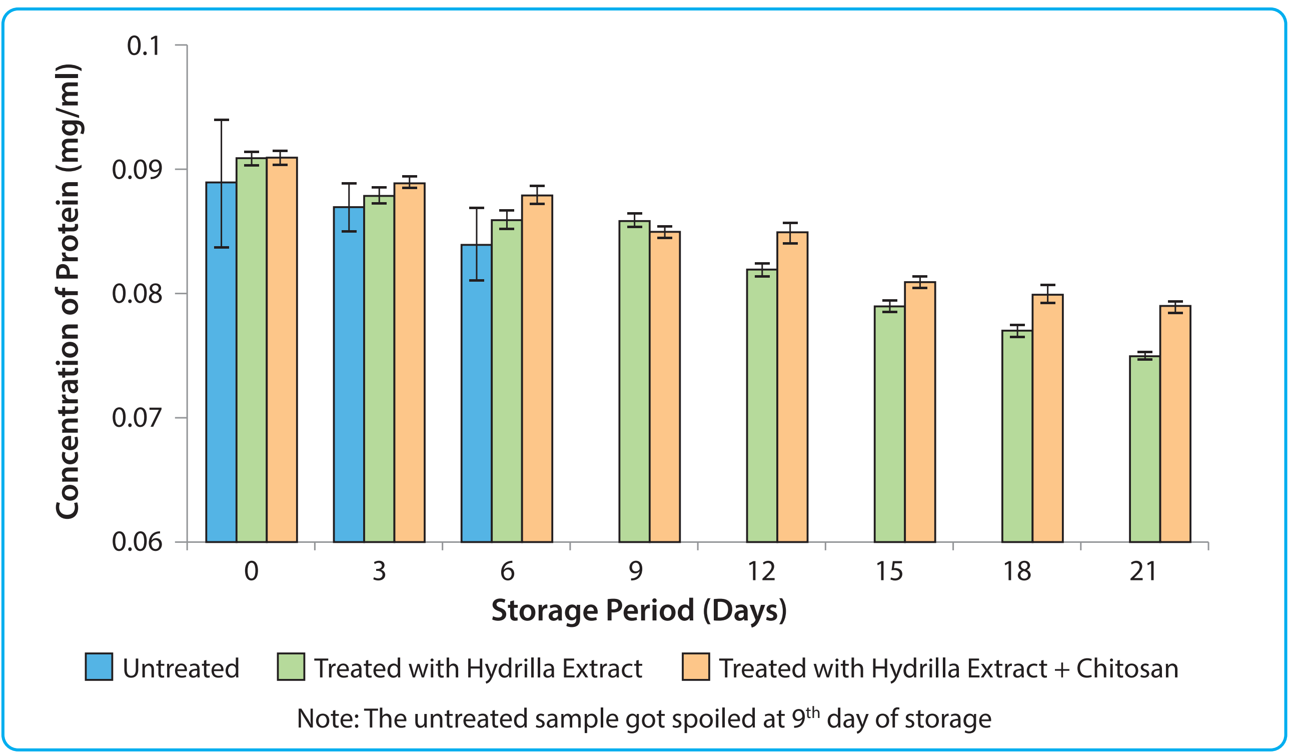


**Figure 5.** Effect of *Hydrilla verticillata* extract and chitosan coating on Protein content

caused by microbial spoilage, which can lead to nutrient loss. The protease inhibitors present in the hydrilla extract can help preserve the protein content of meat products by inhibiting proteolytic enzymes (*Olvera-Aguirre et al.,* 2023).

The concentrations of fat in the three groups of sample were found out using the standard calibration curve and the concentration is expressed as mg/mL. The results are represented in Figure 6. There is no change in the fat content upto 6 days for treated and

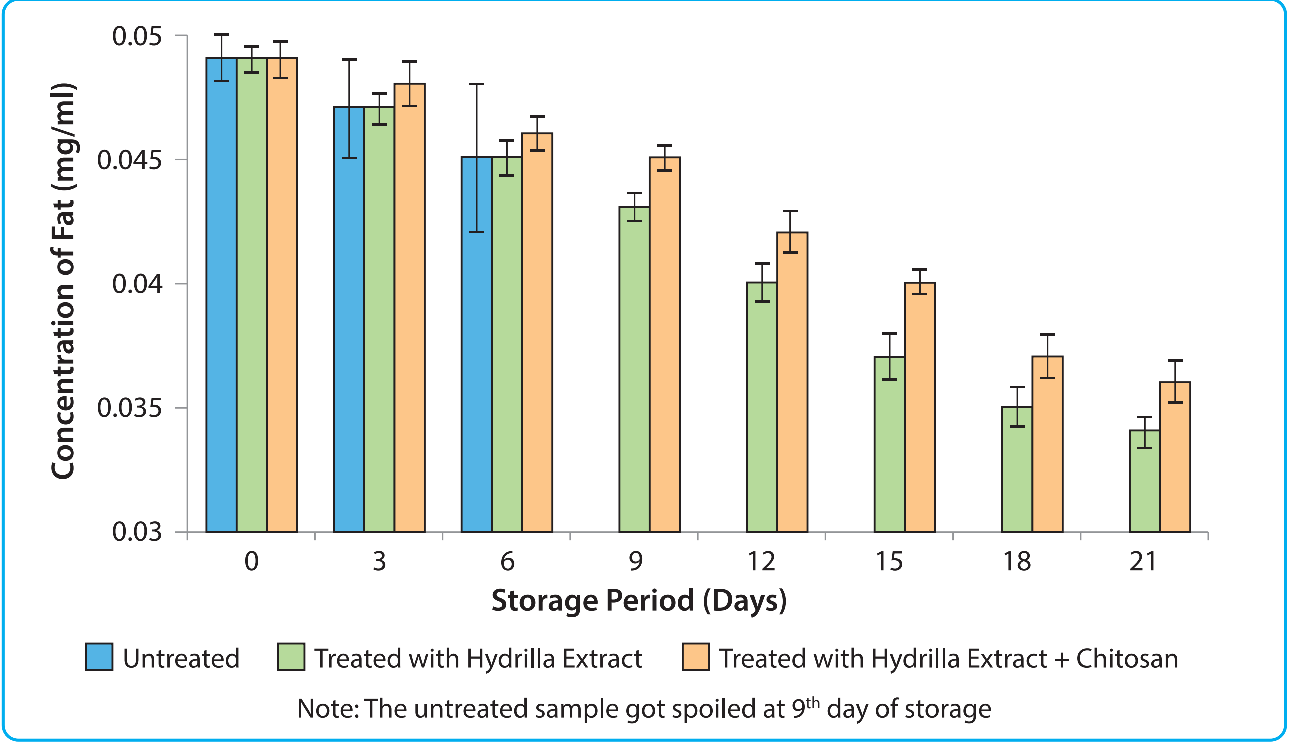


**Figure 6.** Effect of *Hydrilla verticillata* extract and chitosan coating on Fat content

untreated meat samples, but afterwards slight change in in fat content is observed on the coated samples whereas the uncoated samples are spoiled. The results revealed that 75% of the fat is retained during the storage period 21 days on the hydrilla and hydrilla with chitosan coated meat. A variety of things happen during the processing and storage of preserved meat that can affect its nutritional content. The nutrient loss of meat is a major concern during the refrigerated storage. Meat is a valuable source of protein, iron, vitamin $B_{12}$ as well as other B complex vitamins, zinc, selenium and the fat content (*Bustabad,* 1999). The major source of nutrient in chicken meat is protein and fat content. It is observed that the application of *H. verticillata* extract and chitosan coating maintained the protein and fat content at a constant rate without major loss. The hydrilla extract is rich in antioxidants, such as polyphenols, flavonoids, and vitamins. The antioxidants help combat oxidative reactions that can lead to nutrient degradation in meat products. By scavenging free radicals and inhibiting lipid and protein oxidation, plant extracts can help preserve the nutritional content of vitamins, minerals, and amino acids in meat (*Petcu et al.,* 2023).

The ethanolic extract of *H. verticillata* contains phytol as one of its chemical constituents, along with other compounds like chlorophyll, carotenoids, polyphenols, and more. Some studies have shown that phytol can scavenge free radicals and protect cells from oxidative damage. Phytol has also shown antimicrobial activity against a range of microorganisms, including bacteria, fungi, and some parasites. Its antimicrobial effects are attributed to its ability to disrupt microbial cell membranes and interfere with their growth and replication. This makes phytol a potential candidate for the development of antimicrobial agents or as a natural preservative in food products. Ethyl palmitate and ethyl linolenate, the chemical compounds that can be used as a preservative in various food products and found naturally in the hydrilla extract. These esters can function as an antioxidant and helps prevent the oxidation of fats and oils in food and products. Oxidation of fats can lead to off-flavors, rancidity, and a decrease in product quality. By inhibiting lipid oxidation, ethyl palmitate can extend the shelf life of meat and meat products (*Lin & Long,* 2023). Hence, the hydrilla extract may be used as natural antioxidants, antimicrobial agents, or ingredients in dietary supplements.

The total bacterial count was determined using the pour plate technique and it is expressed as logarithm of colony forming units (log CFU/g). The results of the comparative study are displayed in Figure 7. Even though the slight increase in bacterial count is observed on the coated meat, it could be eliminated during cooking process. *H. verticillata* has been documented for its broad antimicrobial activity

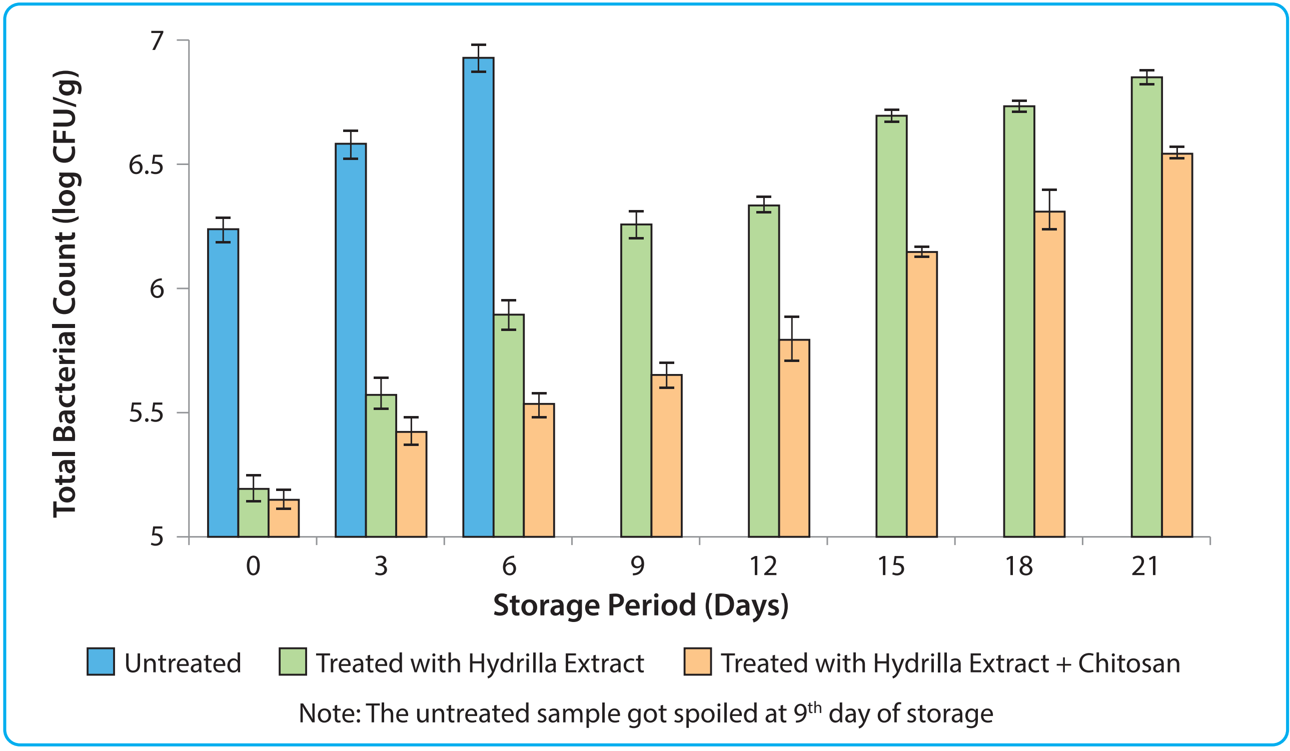


**Figure 7.** Effect of *Hydrilla verticillata* extract and chitosan coating on total bacterial count

against bacteria and fungi. The antibacterial action of plant extract is related to prevent cell division, causing the changes to membrane phospholipid and fatty acid value and prohibits RNA and DNA synthesis. Furthermore, chitosan as a coating solution acts as an oxygen barrier around the bacterial cell and thus prevents the growth of aerobic bacteria and the antimicrobial property of chitosan is associated with its unique polycationic property, which interrupts the microbial cell membrane (*Pereira & Vicente,* 2013). The results indicated that plant extract along with chitosan coating decreased the bacterial population in the treatment. If the plant extract is applied alone it showed a low antibacterial impacts, but when the plant extract and the chitosan is applied together, it leads to stability of the antibacterial properties for a significant period. This is due to the phenomenon that allowing plant extract to hydrolyze the peptidoglycan layer surrounding the cytoplasmic membrane of bacteria, increasing the antibacterial effect of chitosan (*Darmadji & Izumimoto,* 1994). The data revealed that the coated sample meats led to a significant reduction in total bacterial count over the time of storage period. This reduction in microbial count is due to the antimicrobial effect of *H. verticillata* extract and chitosan (1%) on the spoilage bacteria . The distinction between Experimental groups II and III is primarily due to the presence of chitosan in Experimental group III. Chitosan effectively inhibits bacterial growth through several mechanisms, including disrupting the bacterial cell membrane, blocking nutrient absorption, inducing osmotic imbalance, chelating essential metal ions, interfering with DNA and RNA synthesis, and generating oxidative stress. These combined actions render chitosan a potent antimicrobial agent (*Ardean et al.,* 2021).

The results (Table 1) indicated that the hydrilla and hydrilla with chitosan coated meat have excellent sensory characteristics for three days, followed by good for another 3–4 days, and then reached the acceptable limit. However, the untreated sample reached the poor sensory characteristics at the 6$^{th}$ day storage period. The role of sensory evaluation is to provide valid and reliable information for consumer acceptability. The sensory scores of the samples were not affected by *H. verticillata* extract and chitosan as it does not produce any off-flavors, color and the appearance of the sample were not objectionable and either of which could potentially lead to rejection of products by the consumer. Whereas, the uncoated sample meats produced off-flavor during the 9$^{th}$ day of storage which indicates the effects of *H. verticillata* and chitosan on preserving the sensory characteristics of chicken meat. According to sensory evaluation, the shelf life of the meat samples is determined to be 6 days for the untreated samples and 21 days for those treated with hydrilla extract. Additionally, the group treated with chitosan was able to maintain good quality for 15 days, while the hydrilla extract group maintained good quality for 12 days.

## 4. Conclusion

The *H. verticillata* being a weed plant with profound uses has also resulted to act as a potent natural preservative. Since chicken meat is largely consumed by many the nature of the chicken meat is difficult to maintain as days pass by. In this case, the extract from *H. verticillata* is used in retaining the raw nature, taste, intrinsic factors, color, and the texture of the chicken meat for many days. The re-

**Table 1.** Sensory characteristics of meat sample during chilled storage

| Storage period (Days) | Untreated | Treated with Hydrilla Extract | Treated with Hydrilla Extract + Chitosan |
|---|---|---|---|
| 0 | 9 | 9 | 9 |
| 3 | 7 | 9 | 9 |
| 6 | 5 | 8 | 9 |
| 9 | NA | 8 | 8 |
| 12 | NA | 8 | 8 |
| 15 | NA | 7 | 8 |
| 18 | NA | 7 | 7 |
| 21 | NA | 7 | 7 |

**Legend:** NA-Not applicable (The sample got spoiled at 9$^{th}$ day of storage)

sults of the comparative study represented that the preservative effect of *H. verticillata* extract enriched with chitosan coating sustained the quality of chicken meat under 4 °C by maintaining the pH, protein, fat, TBARS, microbial growth and also the sensory characteristics such as color, odor and appearance during the storage period of 21 days. The antioxidant present in the extract prevents the free radicals and the antimicrobial activity against the microbes ensure the preservative properties of the hydrilla extract. These results suggest that *H. verticillata* extract along with the chitosan (1%) coating can be applied as natural preservative to the meat products in the food industry to preserve quality and extend the shelf life .

# In vitro evaluacija ekstrakta Hydrilla verticillata kao prirodnog konzervansa za pileće meso

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INFORMACIJE O RADU

*Ključne reči:*
Antioksidativno dejstvo
Antimikrobno dejstvo
Konzervirajuće dejstvo
Vodena biljka
Rok trajanja

APSTRAKT

Cilj istraživanja je da se ispita efekat konzervacije etanolnog ekstrakta Hydrilla verticillata obogaćenog hitozanom na uzorku mesa kako bi se produžio rok trajanja proizvoda. Hemijske, mikrobiološke, senzorne i nutritivne analize kao što su pH, količina peroksidacije lipida, ukupan broj bakterija, sadržaj proteina i masti u svežem pilećem mesu koje je obloženo etanolnim ekstraktom H. verticillata i hitozanom, su rađene u periodu od 21 dan. Rezultati eksperimenta pokazuju da je ekstrakt H. verticillata obogaćen hitozanskom prevlakom delovao kao konzervans na degradaciju rasta mikroorganizama, održavanje pH, kao i povećanje nutritivnih vrednosti.

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*Original Scientific Paper*

# Biofilm production and acetic acid sensitivity of *Staphylococcus aureus* and *Escherichia coli* isolated from poultry slaughterhouse environment, broiler carcasses and offal in Algeria

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ABSTRACT

The environment of poultry slaughterhouses, broiler carcasses and offal can act as reservoirs and spread various zoonotic bacterial pathogens such as *Staphylococcus aureus* and *Escherichia coli*. The objectives of this study were to determine the prevalence of *S. aureus* and *E. coli* in broiler carcasses and offal, and the environment of poultry slaughterhouses, and to evaluate the capacity for biofilm formation and sensitivity to acetic acid of certain bacterial isolates. A total of 210 samples were taken from different parts of the carcasses (wings, thighs and breasts) and offal (livers and hearts) of broiler chickens, and 19 environmental samples were collected from various compartments of poultry slaughterhouses (walls, floors and equipment) to determine the prevalence of *S. aureus* and *E. coli*. Fourteen *S. aureus* strains and 14 *E. coli* strains isolated from broiler products, as well as 14 *S. aureus* strains and 14 *E. coli* strains isolated from the environment of poultry slaughterhouses, were specifically selected to evaluate their ability to form biofilms. The tube and the tissue culture plate methods were used to evaluate biofilm forming capacity, while the minimum inhibitory concentration (MIC) of acetic acid on these bacterial isolates was determined by the agar dilution method. The total quantities of biofilm produced by the different categories of bacterial strains were compared by statistical analysis. The prevalences of *S. aureus* and *E. coli* were 100% in broiler carcass and offal samples, while in environmental samples, the prevalence of *E. coli* was 94.73% and that of *S. aureus* was 78.94%. Using the tube method, 35.71% of *S. aureus* strains demonstrated strong biofilm production, 50% demonstrated moderate production and 14.28% demonstrated weak production. No strain was categorized as non-biofilm producing. Similarly, for *E. coli* strains, 32.14% had strong biofilm production, 21.42% moderate production, and 46.42% weak production, with no strain being non-biofilm producing. Using the tissue culture plate method, 39.28% of *S. aureus* strains had moderate biofilm production, while 60.71% showed weak production. No isolates were identified as having strong production or being non-biofilm producers. For *E. coli* strains, 14.28% showed strong biofilm production, 39.28% moderate production, and 46.42% weak production, with no isolate being categorized as a non-biofilm producer. The two methods made it possible to detect biofilm production by all studied bacterial isolates. The tube method revealed a higher rate of isolates with strong biofilm production (33.92%) compared to the tissue culture plate method (7.14%). In contrast, the tube method recorded a lower rate of isolates exhibiting moderate biofilm production (35.71%) compared to the tissue culture plate method (39.28%).

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Similarly, the tube method showed a lower rate of isolates with weak biofilm production (30.35%) compared to the tissue culture plate method (53.57%). Regarding measures of total biofilm produced, environmental bacteria presented a not significantly higher value (total optical density (OD)=12.45) than did bacteria isolated from broilers (total OD=11.83). Likewise, the total quantity of biofilm produced by all 14 *E. coli* (total OD=12.78) was numerically but not significantly higher than that produced by all *S. aureus* isolates (total OD=11.5). Among the isolates from broilers, the 14 *E. coli* strains produced a numerically not significantly higher amount of biofilm (total OD=6.76) than the 14 *S. aureus* strains (total OD=5.07). The minimum inhibitory concentration (MIC) of acetic acid was ≤0.08% for all bacterial isolates, except for two *S. aureus* isolates, for which the minimum inhibitory concentration was 0.16%. In conclusion, *S. aureus* and *E. coli* are frequently present in the environment of poultry slaughterhouses and in broiler products. All bacterial isolates demonstrated an ability to form biofilms. These bacteria were very sensitive to acetic acid, which is therefore considered an ideal agent for disinfection of the poultry slaughterhouses environment and decontamination of broiler carcasses.

## 1. **Introduction**

Broiler flocks are an essential food source in Algeria, but they can also serve as disease reservoirs and spread various zoonotic bacteria. Among these zoonotic bacteria, *Staphylococcus aureus* and *Escherichia coli* is particularly known as major pathogens capable of causing infections in animals and humans. In addition, these bacteria are responsible for recurrent foodborne infections in meat and poultry products in Algeria, resulting in significant economic losses. Food poisoning caused by *S. aureus*, *E. coli*, *Salmonella enterica subsp.* Enteritidis and *S.* Typhimurium, mainly linked to the consumption of contaminated chicken meat, is one of the main public health problems in developing countries (*Mead*, 2004; *Antunes et al.*, 2016; *Bortolaia et al.*, 2016). Despite efforts by the poultry industry to reduce foodborne illnesses associated with chicken products, they remain one of the main culprits associated with foodborne illnesses in Algeria.

*S. aureus* and *E. coli* have the ability to form biofilms that protect them against hostile conditions such as temperature variations, limitations or deprivation of nutrients, as well as dehydration *(Idrees et al.*, 2021). The formation of biofilm reduces bacterial susceptibility to antimicrobial agents and the host's immune defences, thus making infections difficult to eliminate. Additionally, upon infection, dispersal of biofilm cells can lead to spread to secondary sites and worsen infection (*Lister and Horswill*, 2014). Consequently, biofilm formation by *S. aureus* on medical devices and host tissues (*Lister and Horswill*, 2014), as well as by *E. coli* in the urinary tract (*Ballén et al.*, 2022), can lead to chronic infections. These chronic biofilm-related infections often lead to a significant increase in morbidity and mortality (*Moormeier and Bayles*, 2017).

Additionally, *S. aureus* and *E. coli* are capable of forming biofilms on poultry processing surfaces, which can lead to cross-contamination of slaughtered broiler carcasses and offal. It should be noted that the knife blade used for neck cutting could be a potential source of cross-contamination during slaughter (*Mead et al.*, 1994). Moreover, pathogenic bacteria demonstrate the ability to survive in hot water, thus increasing the risk of transmission of viable microorganisms between carcasses during scalding (*Henry et al.*, 2012). The plucking phase is emerging as a significant source of cross-contamination (*Morar et al.*, 2014), favoured by the surface of the rubber fingers that facilitates the transfer of bacteria to the carcasses (*Fries*, 2002). Evisceration requires special attention due to its high potential for bacterial cross-contamination, with faecal contamination from this step being one of the main concerns in poultry processing (*Brizio and Prentice*, 2015). When inspectors manually handle carcasses, there is an accidental risk of increasing cross-contamination between carcasses (*Oosterom et al.*, 1983). Furthermore, *Lillard* (1990) reported that the cooling tank is a major site of cross-contamination between carcasses.

The presence of biofilms in the poultry slaughterhouse environment is a major problem due to the link between biofilms and the survival and pathogenicity of bacteria (*Ducková et al.*, 2023), as is the ability of biofilm bacteria to persistently contaminate carcasses, offal of slaughtered broilers and even poultry slaughterhouse workers. Biofilm formation appears to play a key role in many foods poisoning cases, particularly those involving contaminated broilers from the poultry slaughterhouse environment, where *S. aureus* and *E. coli* are frequently implicated (*Mead*, 2004; *Bortolaia et al.*, 2016). Biofilm formation in poultry slaughterhouses

compromises the effectiveness of cleaning, disinfection and decontamination of slaughtered broiler carcasses (*Ducková et al.*, 2023), posing a serious threat to the white meat industry. This threat can be avoided by the application of a good manufacturing practice (GMP) program that is mainly based on the exclusion and elimination of unwanted and foreign materials, as well as the inhibition and destruction of undesirable microorganisms (*de Oliveira et al.*, 2016).

With regard to the decontamination of broiler carcasses, several methods have been developed to reduce the levels of bacterial contamination. Currently, most methods focus on washing and sanitizing procedures with agents like hot water, chlorine, short-chain organic acids, quaternary ammonium and sodium hypochlorite (*Dickson and Anderson*, 1992). Alternative processes, such as gamma irradiation and the use of cold water, are also effective (*Dickson and Anderson*, 1992).

The hazard analysis and critical control point (HACCP) system guarantees regular monitoring of the entire chicken processing procedure, optimizes hygiene control, checks control parameters and records the results, ensures compliance with hygiene legislation, raises awareness of personnel to food safety requirements, and establishes uniform operational standards throughout the industry. However, it does not completely resolve the drawback of microbiological risks associated with processing operations, which are often difficult to control effectively. To overcome this gap, the HACCP system must be put in place after the implementation of good hygienic practice (GHP), GMP and sanitation standard operating procedure (SSOP) programs. GHP/GMP/SSOP are operational prerequisite programs (oPRPs) used for the analysis and control of the facility and its environment, personnel, the cleaning and disinfection process, equipment and utensils, as well as storage and distribution (*de Oliveira et al.*, 2016). GHP/GMP/SSOP programs are based on the exclusion and elimination of unwanted and foreign materials, with the inhibition and destruction of pathogenic microorganisms. The integration of GHP/GMP/SSOP programs followed by the HACCP system enables process hygiene requirements and impacts on meat safety, thus ensuring control of foodborne diseases (*de Oliveira et al.*, 2016). The decontamination of carcasses could also be added as a food safety management choice, usually when batches of high-risk broilers from farms with a low level of biosecurity are destined for slaughter; decontamination of such animals should contribute to the reduction of foodborne infections in humans (*Dinçer and Baysal*, 2004).

Different interventions have been put in place to effectively reduce the bacterial load on broiler carcasses. The interventions are classified as either physical or chemical interventions, the latter including the use of organic acids (*Loretz et al.*, 2010). Organic acids are weak acids, most of which have no defined limits in terms of acceptable daily intake for humans. The antimicrobial activity of organic acids relies on two main mechanisms: cytoplasmic acidification with subsequent uncoupling of energy production and regulation, and accumulation of the dissociated acid anion to toxic levels. It is likely that the interaction of these mechanisms leads to the inhibition of microbes (*Mani-López et al.*, 2012). For many years, organic acids have been successfully used for the decontamination of beef, pork and poultry products against various bacteria (*Mani-López et al.*, 2012). Table 1 shows some studies on organic acids used for decontamination of broiler carcasses. They have proven to be safe, simple, effective and economical meat decontamination agents, highly recommended on a large scale (*Raftari et al.*, 2009). The use of acetic acid is a well-known method for the decontamination of poultry carcasses and offal, as well as for the disinfection of poultry slaughterhouses. This is an efficient and commonly used approach in the industry (*Idrees et al.*, 2021).

The emergence of multi-drug resistant bacteria contaminating the environment of poultry slaughterhouses and broiler carcasses in Algeria, such as *methicillin-resistant S. aureus* (MRSA) (*Bounar-Kechih et al.*, 2018) and extended-spectrum β-lactamase-producing ***E.** coli* (*Aberkane et al.*, 2023), has led to the search for solutions to eliminate these bacteria, in particular by using other molecules with antibacterial activity. The use of organic acids, such as acetic acid, could solve this problem (*Nkosi et al.*, 2021). Therefore, it is important to assess the ability of these bacteria to form biofilm and their sensitivity to acetic acid, in order to develop effective disinfection and decontamination strategies.

Numerous studies have demonstrated the antimicrobial effectiveness of acetic acid against *S. aureus* and *E. coli* present in broiler meat (*Abdul Wahid*, 2008; *Bin Jasass*, 2008; *Sakhare et al.*, 1999). However, research on acetic acid's effectiveness against strains isolated from the poultry slaughterhouse environment is limited. In addition, no study has yet been carried out in Algeria to assess acetic acid's effectiveness against bacteria isolated from carcasses, offal of broiler chickens or the environment of local poultry slaughterhouses.

**Table 1.** Organic acids used for decontamination of broiler carcasses

| Organic acids | Application of organic acids | Antibacterial effectiveness of organic acids | References |
|---|---|---|---|
| Citric acid ($C_6H_8O_7$) | Cloacal washing of broiler carcasses with citric acid (5% and 10%, w/v). | Reduction in the number of psychrophilic or mesophilic bacteria on carcasses, of 0.88 $log_{10}$ CFU $cm^2$ and 0.56 $log_{10}$ CFU $cm^2$ for both concentrations respectively. | *Meredith et al*., 2013 |
| | Treatment of previously inoculated chicken breast pieces by vacuum-infusion with 150.0 mM citric acid. | Reduction of *S. Typhimurium* counts to almost undetectable levels on day 6 of storage (100 CFU/g) and to undetectable levels after day 9 of storage at 4 °C. | *Over et al*., 2009 |
| Lactic acid ($C_3H_6O_3$) | Cloacal washing of broiler carcasses with lactic acid (5%, v/v). | Reduction in the number of *Campylobacter* on carcasses by 0.66 $log_{10}$ CFU $cm^2$. | *Meredith et al*., 2013 |
| | Washing broiler carcasses with lactic acid (1% and 3%, v/v). | Reduction in the number of aerobic mesophilic bacteria, coliforms and *E. coli* on carcasses, of 1.259 log CFU, 1.685 log CFU, 2.023 log CFU and 2.502 log CFU, 3.876 log CFU, 3.820 log CFU compared to the control samples, for both concentrations respectively.<br>Total elimination of *Salmonella* with both concentrations. | *Halil & Abdurrahman Üsame*, 2000 |
| Propionic acid ($C_3H_6O_2$) | Immersing freshly inoculated chicken thighs in a propionic acid solution (1% and 2%, v/v). | Reduction in the number of *L. monocytogenes* of 2.72 log CFU on the thighs compared to the controls, with the 2% concentration, after 3 days of storage. | *González-Fandos & Herrera*, 2013a |
| Succinic acid ($C_4H_6O_4$) | Immersion of broiler breasts in 80 mL of a *Salmonella* cocktail at $10^7$ CFU/mL for 2 min, then transferred into sterile beakers containing 250 mL of succinic acid (2% and 5%, v/v) for 5 min. | Reduction in *Salmonella* counts from 1.27 to 1.47 log CFU/g and from 2.00 to 3.20 log CFU/g on breasts compared to controls, with both concentrations respectively. | *Radkowski et al*., 2018 |
| Malic acid ($C_4H_6O_5$) | Soaking freshly inoculated chicken thighs in a malic acid solution (1% and 2%, v/v) for 5 min. | Reduction at 4 °C in the number of *L. monocytogenes* of approximately 1.66 log CFU on the thighs compared to controls, with the 2% concentration | *González-Fandos & Herrera*, 2013b |
| | Soaking broiler chicken thighs previously inoculated in a malic acid solution (1% and 2%, v/v). | Reduction in the number of *C. jejuni* by 1.18 log CFU on the thighs compared to controls, with the 2% concentration. | *González-Fandos & Maya*, 2015 |
| Tartaric acid ($C_4H_6O_6$) | Application of tartaric acid (0.5% and 1%, v/v) to broiler breast skin previously inoculated, under simulated scald (50°C for 2 min). | Reduction in the number of *S.* Typhimurium on the skin by 2.64 and 1.23 log CFU log CFU for both concentrations respectively. | *Tamblyn & Conner*, 1997b |
| | Vacuum-infusion of chicken breast pieces previously inoculated, in tartaric acid (150 mM). | Reduction of *S.* Typhimurium counts on meat to almost undetectable levels by the 6th day of storage (100 CFU/g) and to undetectable levels after the 9th day of storage at 4°C. | *Over et al*., 2009 |

**Legend:** CFU – colony-forming unit; mM – millimolar

Consequently, our study aimed to determine the prevalence of *S. aureus* and *E. coli* in the poultry slaughterhouse environment and in the carcasses and offal of broiler chickens in Algeria, to evaluate, using two distinct methods, the capacity of selected bacterial isolates to form biofilms, and to determine the isolates' sensitivity to acetic acid by determining the concentration minimal inhibitory (MIC).

## 2. Materials and Methods

### *2.1 Sampling and detection of S. aureus and E. coli*

Sampling was carried out in poultry slaughterhouses located in Algeria. In total, 210 samples were taken from different parts of broiler carcasses and offal, from 14 farms. For each farm, 15 samples were collected, including 3 wings, 3 thighs, 3 breasts, 3 livers and 3 hearts. This sampling procedure was carried out in the drying room. In addition, 19 environmental samples were taken from various compartments of poultry slaughterhouses, including 3 walls (scalding and plucking room, evisceration and washing room, and conditioning room), 6 floors (reception room, stunning and bleeding room, scalding and plucking room, evisceration and washing room, drying room and conditioning room), as well as 10 pieces of equipment (bleeding knife, scalding tank, plucking machine, finisher fingers, head remover, evisceration knife, leg cutter, recovery cart, worker hand and recovery table).

The swabs were subjected to bacteriological analysis using Chapman agar (BIOKAR®, France) and Hektoen agar (BIOKAR®, France) for the isolation of *S. aureus* and *E. coli* strains, respectively. The bacterial strains were identified using standard microbiological tests and biochemical tests using API Staph strips (BioMérieux®, France) for *S. aureus* and API 20E strips (BioMérieux®, France) for *E. coli*.

### *2.2 Bacterial strain selection*

Fourteen strains each of *S. aureus* and *E. coli*, isolated from broiler products, were carefully chosen to explore their ability to form biofilm. Each broiler farm was represented by one strain each of *S. aureus* and *E. coli*. Another 14 strains each of *S. aureus* and *E. coli*, isolated from different sources in the poultry slaughterhouses environment, were also specifically selected to evaluate their ability to form biofilm. The selected bacterial isolates were stored at −80 °C in tryptic soy broth (TSB) (BIOKAR®, France) containing 20% (V/V) glycerol for subsequent analyses. Before each experiment, the bacterial isolates were

**Table 2.** Origin of selected bacterial strains used in the study

| Bacterial strain number | Environment | | Broiler | |
|---|---|---|---|---|
| | *S. aureus* | *E. coli* | *S. aureus* | *E. coli* |
| **1** | Wall (scalding and plucking room) | Wall (scalding and plucking room) | Wing | Wing |
| **2** | Wall (evisceration and washing room) | Wall (evisceration and washing room) | Wing | Wing |
| **3** | Wall (conditioning room) | Wall (conditioning room) | Thigh | Thigh |
| **4** | Floor (reception room) | Floor (reception room) | Thigh | Thigh |
| **5** | Floor (scalding and plucking room) | Floor (scalding and plucking room) | Breast | Breast |
| **6** | Floor (evisceration and washing room) | Floor (evisceration and washing room) | Breast | Breast |
| **7** | Floor (drying room) | Floor (drying room) | Breast | Breast |
| **8** | Bleeding knife | Head remover | Liver | Liver |
| **9** | Scalding tank | Scalding tank | Liver | Liver |
| **10** | Plucking machine | Plucking machine | Liver | Liver |
| **11** | Evisceration knife | Evisceration knife | Liver | Liver |
| **12** | Leg cutter | Leg cutter | Heart | Heart |
| **13** | Recovery cart | Recovery cart | Heart | Heart |
| **14** | Recovery table | Recovery table | Heart | Heart |

thawed and subcultured on nutrient agar (BIOKAR®, France). Table 2 shows the origin of the selected bacterial strains for the biofilm study.

### *2.3 Qualitative detection of biofilm by the tube method*

All selected isolates of *S. aureus* and *E. coli* were evaluated for their ability to form a biofilm using the tube method, as described by *Christensen et al.* (1982), which allows qualitative detection of the biofilm. A single colony of isolate was inoculated into test tubes containing 3 ml of tryptic soy broth supplemented with 1% glucose (TSBG) (BIOKAR®, France). The tubes were then incubated at 37 °C for 48 h. A negative control containing only TSBG (BIOKAR®, France) without bacterial inoculum was also included. After incubation, the tubes were decanted, washed with phosphate-buffered saline (pH 7.2) and dried. Then, the tubes were stained with a solution of gentian violet (0.1%) for 15 min, followed by rinsing with distilled water. The tubes were dried in an inverted position. Assessment of biofilm formation was performed visually and results were scored according to control strains. The formation of a biofilm was considered positive when a visible film was observed covering the wall and the bottom of the tube. The biofilm-producing capacity of the different isolates was classified, according to the intensity of the biofilm's violet color, as none, weak, moderate or strong (*Hassan et al.*, 2011). Each experiment was performed in triplicate and repeated three times.

### *2.4 Quantitative assay of biofilm formation by the tissue culture plate method*

This test was performed using the method of *Christensen et al.* (1985), which allows the quantitative detection of biofilm formation. Isolated bacteria from fresh agar plates were inoculated into 10 mL of tryptic soy broth supplemented with 1% glucose (TSBG) (BIOKAR®, France). The broths were incubated overnight at 37 °C. Using a flat-bottomed polystyrene 96-well tissue culture plate (3 wells for each strain), each well was filled with 20 µL of the previous night's culture (equivalent to 0.5 McFarland standard) and then topped up with 180 µl of sterile TSBG medium (BIOKAR®, France). Wells inoculated with sterile TSBG medium (BIOKAR®, France) were used as a negative control. After aerobic incubation for 24 h at 37 °C, the contents of each well were removed by gentle tapping, then the wells were carefully washed three times with 0.2 mL of phosphate-buffered saline (pH 7.2) to eliminate detached bacteria. Then, each well was filled with 200 µL of methanol 99% to fix the adherent bacteria for 15 min. The plates were decanted, left to dry, then stained for 7 min with 0.2 mL of crystal violet (0.1%). Excess dye was rinsed off with tap water. After the plates were air-dried, the dye bound to adherent cells was resolubilized with 160 µL of ethanol per well. The optical density (OD) of each well was measured at 630 nm using a microplate reader (Mindray MR-96A®). Absorbance values were measured twice: before the ethanol addition, then after the ethanol addition. According to the absorbance values, the adhesion ability of each bacterial isolate was classified into four categories: none, weak, moderate and strong. The cut-off absorbance value (optical density (ODc)) was taken as three standard deviations (SD) above the mean OD of the negative control. Each experiment was performed in triplicate and repeated three times. The interpretation of biofilm production (formation) was carried out according to the criteria of *Stepanovic et al.* (2007). Table 3 shows the classification of bacterial cell adhesion and biofilm formation in the tissue culture plates.

### *2.5 Determination of the minimum inhibitory concentration (MIC) of acetic acid*

The minimum inhibitory concentration (MIC) of acetic acid was determined against all selected bacterial isolates of *S. aureus* and *E. coli* using the agar dilution method based on the guidelines of the Clinical and Laboratory Standards Institute

**Table 3.** Classification of bacterial cell adhesion and biofilm formation in the tissue culture plate method

| Average value of OD | Adhesion | Biofilm formation |
|---|---|---|
| **OD ≤ Odc** | None | None |
| **ODc < OD ≤ 2ODc** | Weak | Weak |
| **2 ODc < OD ≤ 4ODc** | Moderate | Moderate |
| **4 ODc < OD** | Strong | Strong |

**Legend:** OD – optical density; ODc – cut-off absorbance value of optical density

(*CLSI*, 2018) with the use of Muller-Hinton (MH) agar (BIOKAR®, France). Acetic acid was incorporated into MH agar plates at the following concentrations: 2.5%, 1.25%, 0.63%, 0.31%, 0.16% and 0.08% (v/v). Then, a standardized bacteria suspension (adjusted to 0.5 McFarland standard) containing a concentration of $5 \times 10^8$ CFU $mL^{-1}$ was prepared. This standardized bacteria suspension was diluted to approximately $10^7$ CFU $mL^{-1}$, and 2 µL of this dilution were spotted at several points onto MH agar plates with acetic acid, so each spot contained approximately $10^4$ CFU. An agar plate without antibacterial agent was used as a control. After aerobic incubation at 37 °C for 24 h, the agar plates were visually examined to assess growth. The growth of the isolate indicates that it is resistant to the acetic acid concentration incorporated into the MH agar.

### *2.6 Statistical analysis*

To quantify biofilm formation using the tissue culture plate method, experiments were independently repeated three times, with three replicate of plate wells for each bacterial strain. In order to compare the total quantities of biofilm produced by the different categories of bacterial strains, a statistical analysis was carried out using the IBM SPSS Statistics V28 software. A one-way analysis of variance (ANOVA) test, followed by a t-test paired two sample for means, was used to assess differences in biofilm mass. A value of $p \leq 0.05$ was considered statistically significant.

## 3. Results

### *3.1 Bacterial isolates*

All samples of broiler carcasses and offal tested positive for the presence of *S. aureus* and *E. coli*, thus establishing a prevalence of 100% for these two bacteria. Regarding environmental samples, *E. coli* was detected in 18 out of 19 samples, with a prevalence of 94.73%, while *S. aureus* was identified in 15 out of 19 samples, showing a prevalence of 78.94 %. In detail, the three samples from the walls revealed the presence of three strains each of *S. aureus* and *E. coli*. For the six soil samples, five strains of *S. aureus* and six strains of *E. coli* were detected, while in the ten equipment samples, seven strains of *S. aureus* and nine strains of *E. coli* were identified.

### *3.2 Qualitative test for the detection of biofilm with the tube method*

Based on the results of the qualitative biofilm tube test, *S. aureus* and *E. coli* isolates were classified according to their ability to produce biofilm in TSBG medium. For *S. aureus*, of the tested 28 isolates, 10 (35.71%) showed strong biofilm production, 14 (50%) showed moderate production, 4 (14.28%) showed weak production, and no isolate was a non-biofilm producer. As for *E. coli*, of the tested 28 isolates, 9 (32.14%) showed strong biofilm production, 6 (21.42%) showed moderate production,

**Table 4.** Biofilm production capacity of bacteria assessed by the tube method

| Bacteria | Origin | Biofilm production capacity | | | |
|---|---|---|---|---|---|
| | | None | Weak | Moderate | Strong |
| *S. aureus* | Environment 14 isolates | 0/14 (0%) | 4/14 (28.57%) | 8/14 (57.14%) | 2/14 (14.28%) |
| | Broiler 14 isolates | 0/14 (0%) | 0/14 (0%) | 6/14 (42.85%) | 8/14 (57.14%) |
| | Total 28 isolates | 0/28 (0%) | 4/28 (14.28%) | 14/28 (50%) | 10/28 (35.71%) |
| *E. coli* | Environment 14 isolates | 0/14 (0%) | 13/14 (92.85%) | 1/14 (7.14%) | 0/14 (0%) |
| | Broiler 14 isolates | 0/14 (0%) | 0/14 (0%) | 5/14 (35.71%) | 9/14 (64.28%) |
| | Total 28 isolates | 0/28 (0%) | 13/28 (46.42%) | 6/28 (21.42%) | 9/28 (32.14%) |

**Table 5.** Biofilm production capacity of bacteria assessed by the tissue culture plate method

| Bacteria | Origin | Biofilm production capacity | | | |
|---|---|---|---|---|---|
| | | None | Weak | Moderate | Strong |
| *S. aureus* | Environment 14 isolates | 0/14 (0%) | 6/14 (42.85%) | 8/14 (57.14%) | 0/14 (0%) |
| | Broiler 14 isolates | 0/14 (0%) | 11/14 (78.57%) | 3/14 (21.42%) | 0/14 (0%) |
| | Total 28 isolates | 0/28 (0%) | 17/28 (60.71%) | 11/28 (39.28%) | 0/28 (0%) |
| *E. coli* | Environment 14 isolates | 0/14 (0%) | 7/14 (50%) | 6/14 (42.85%) | 1/14 (7.14%) |
| | Broiler 14 isolates | 0/14 (0%) | 6/14 (42.85%) | 5/14 (35.71%) | 3/14 (21.42%) |
| | Total 28 isolates | 0/28 (0%) | 13/28 (46.42%) | 11/28 (39.28%) | 4/28 (14.28%) |

13 (46.42%) showed weak production, and no isolate was a non-biofilm producer. The complete results of the biofilm-producing capacity of all bacterial isolates by the tube method are shown in Table 4.

### 3.3 Quantitative assay of biofilm formation with the method of tissue culture plate method

The bacterial isolates were classified according to the results obtained with the tissue culture plate method. For *S. aureus*, of the 28 tested isolates, 11 (39.28%) showed moderate biofilm production, 17 (60.71%) showed weak production, and no isolates were classified as having strong production or not producing biofilm. Regarding *E. coli*, of the 28 tested isolates, 4 (14.28%) showed strong biofilm production, 11 (39.28%) showed moderate production, 13 (46.42%) showed weak production, and no isolates were classified as non-biofilm producing. The complete results of the biofilm-producing capacity of all bacterial isolates by the tissue culture plate method are presented in Table 5.

### 3.4 Comparison of detection methods

Both methods detected biofilm production by all bacterial isolates, but with differences in the amounts of biofilm produced. The number of isolates with strong biofilm production was higher with the tube method 19/56 (33.92%) compared to the tissue culture plate method, which detected only 4/56 (7.14%). In contrast, the number of isolates with moderate biofilm production was lower with the tube method 20/56 (35.71%) compared to the tissue culture plate method, which identified 22/56 (39.28%). Similarly, the number of isolates with weak biofilm production was lower with the tube method 17/56 (30.35%) compared to the tissue culture plate method which

**Table 6.** Comparative screening of *S. aureus* and *E. coli* isolates producing biofilm by the tube and tissue culture plate methods

| Classification of biofilm production | Number of isolates (%) according to biofilm formation | |
|---|---|---|
| | Tube (qualitative method) | Tissue culture plate (quantitative method) |
| Strong | 19/56 (33.92%) | 4/56 (7.14%) |
| Moderate | 20/56 (35.71%) | 22/56 (39.28%) |
| Weak | 17/56 (30.35%) | 30/56 (53.57%) |
| None | 0/56 (0%) | 0/56 (0%) |

revealed 30/56 (53.57%). Table 6 presents a comparison of types of biofilm produced by *S. aureus* and *E. coli* isolates as assessed by the tube and tissue culture plate methods.

### *3.5 Comparison of biofilm production by different categories of bacteria*

Overall, the total amount of biofilm produced by environmental bacteria (total OD=12.45) was higher than that produced by bacteria isolated from broilers (total OD=11.83), but this difference was not statistically significant (p>0.05). Similarly, the total amount of biofilm produced by all *E. coli* isolates (total OD=12.78) was higher than that produced by all *S. aureus* isolates (total OD=11.5), but this difference was also not statistically significant (p>0.05).

Regarding the comparison of the total amounts of biofilm produced by the different categories of bacteria, the 14 strains of *E. coli* isolated from broilers produced the greatest amount of biofilm (total OD=6.76), followed by the 14 strains of *S. aureus* isolated from the environment (total OD=6.43), the 14 strains of *E. coli* isolated from the environment (total OD=6.02) and finally the 14 strains of *S. aureus* isolated from broilers (total OD=5.07). However, none of these differences were statistically significant (p>0.05). Table 7 shows the OD of biofilm produced by each bacterial strain and by the different categories of bacteria.

### *3.6 Minimum inhibitory concentration (MIC) of acetic acid*

The study of the minimum inhibitory concentration (MIC) of acetic acid on all *S. aureus* and *E. coli* isolates revealed that all isolates were susceptible to all tested concentrations, with the exception of two isolates of *S. aureus* isolated from broiler livers which were resistant at the concentration of 0.08%.

**Table 7.** Optical density of biofilm produced by each bacterial strain and by different categories of bacteria

| **Bacterial strain number** | **Environment** | | **Broiler** | |
|---|---|---|---|---|
| | ***S. aureus*** | ***E. coli*** | ***S. aureus*** | ***E. coli*** |
| **1** | 0.57466667 | 0.51666667 | 0.356 | 0.86433333 |
| **2** | 0.53933333 | 0.89833333 | 0.252 | 0.83566667 |
| **3** | 0.21466667 | 0.45733333 | 0.80366667 | 0.77166667 |
| **4** | 0.27166667 | 0.33766667 | 0.35233333 | 0.93633333 |
| **5** | 0.43666667 | 0.306 | 0.592 | 0.47533333 |
| **6** | 0.66433333 | 0.347 | 0.29833333 | 0.22433333 |
| **7** | 0.30833333 | 0.39566667 | 0.232 | 0.24133333 |
| **8** | 0.30366667 | 0.335 | 0.37866667 | 0.46966667 |
| **9** | 0.60033333 | 0.43466667 | 0.26233333 | 0.47466667 |
| **10** | 0.31833333 | 0.431 | 0.428 | 0.49433333 |
| **11** | 0.60266667 | 0.44566667 | 0.27133333 | 0.16966667 |
| **12** | 0.73933333 | 0.35233333 | 0.28366667 | 0.26433333 |
| **13** | 0.56433333 | 0.34366667 | 0.354 | 0.24033333 |
| **14** | 0.29233333 | 0.42433333 | 0.211 | 0.29833333 |
| **Total** | 6.43066666 | 6.02533334 | 5.07533333 | 6.76033332 |
| | 12.456 | | 11.83566665 | |

**Table 8.** Minimum inhibitory concentration of acetic acid on each bacterial strain

| Bacterial strain number | Environment | | Broiler | |
|---|---|---|---|---|
| | *S. aureus* | *E. coli* | *S. aureus* | *E. coli* |
| **1** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **2** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **3** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **4** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **5** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **6** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **7** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **8** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **9** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **10** | ≤0.08% | ≤0.08% | 0.16% | ≤0.08% |
| **11** | ≤0.08% | ≤0.08% | 0.16% | ≤0.08% |
| **12** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **13** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |
| **14** | ≤0.08% | ≤0.08% | ≤0.08% | ≤0.08% |

Thus, for all *E. coli* isolates, the MIC of acetic acid was ≤0.08%. For 26 isolates of *S. aureus*, the MIC of acetic acid was also ≤0.08%, while for the other two strains of *S. aureus* (isolated from broiler livers), the MIC of acetic acid was 0.16%. Table 8 shows the MIC of acetic acid for each bacterial strain.

## 4. Discussion

*S. aureus* is the most pathogenic species of the genus *Staphylococcus*. It is very common in the commensal state; it colonizes the skin, the digestive tract and the nasal cavities of humans and warm-blooded animals. However, it can become pathogenic and be responsible for localized suppurative infections, life-threatening infections and food poisoning in humans. It can survive in the external environment and it can be found in poultry slaughterhouses, which amplifies transmission phenomena. Also, it is considered a notorious pathogen due to its antibiotic resistance and phenotypic adaptability, as a result of its ability to develop biofilms.

In our study, all *S. aureus* isolates produced biofilm, which is consistent with results from other studies. For example, *Bernier-Lachance et al.* (2020) reported that all 15 MRSA from chicken meat were able to form biofilms. Moreover, in the study conducted by *Igbinosa et al.* (2023), the biofilm-producing capacity of 110 MRSA strains isolated from poultry meat was assessed. The results revealed that 27 (24.55%) were weak biofilm producers, 18 (16.36%) were moderate biofilm producers and 39 (35.45%) were strong biofilm producers. Previous studies, such as those by *Knobloch et al.* (2002) and *Rewatkar and Wadher* (2013), also reported that the biofilm-forming capacity in *S. aureus* varies depending on the detection method used.

*E. coli* naturally occurs in the digestive tract of humans and warm-blooded animals, most often without causing any disease. It is a so-called commensal bacterium normally present in the intestinal microbiota. While the majority of *E. coli* strains are harmless, some have acquired virulence factors that make them pathogenic and capable of causing severe food poisoning in humans, especially in young children and the elderly. They can be found in the form of biofilm in the poultry slaughterhouse environment, once it has been soiled by poultry droppings. Contaminated feathers constitute an important means of introducing *E. coli* into the poultry slaughterhouse environment (*Rigby et al.*, 1980). *E. coli* contamination of the poultry slaughterhouse environment can also result from splashes and aerosols generated when washing carcasses (*Sofos et al.*, 2013).

According to our results, all *E. coli* isolates produced biofilm, which is in agreement with the results of the study conducted by *Crecencio et al.* (2020). That study evaluated the biofilm-forming capacity of 88 *E. coli* strains isolated from chilled raw chicken meat cuts. Their results revealed that 70.44% of the strains were able to form biofilms (moderate to strong), of which 31 strains were strong biofilm producers. Several other studies have also reported the ability of avian pathogenic *E. coli* (APEC) and avian faecal *E. coli* (AFEC) strains to form biofilm. For example, studies by *Al-Marri et al.* (2021), *Crecencio et al*. (2020) and *Skyberg et al.* (2007) confirmed this biofilm-producing capacity in these bacteria.

The ability of *E. coli* to produce biofilm varies depending on the experimental conditions. *Cremet et al.* (2013) pointed out that the detection rate of biofilm-producing strains differs depending on the method used, and that biofilm production is influenced by atmospheric and nutrient factors. Additionally, *Reisner et al.* (2006) reported the impact of environmental and genetic factors on biofilm formation. *Skyberg et al.* (2007) also noted that the ability to form biofilm differed depending on the pathotype of *E. coli* and nutrient conditions. According to *Oosterik et al.* (2014), biofilm formation by APEC strains is affected by serogroup and surface material. It is also important to emphasize that measured biofilm formation by *E. coli* depends on the method used, the specific strain and is strongly modulated by the culture conditions, as indicated by *Naves et al.* (2008).

These studies revealed significant differences in the biofilm-forming ability between various strains of *S. aureus* and *E. coli*, whether from chicken meat or other sources, which confirms our results. Our study employed two distinct methods to evaluate biofilm production capacity, namely the tube and the tissue culture plate methods. Both methods revealed the propensity of all selected bacterial strains to produce biofilms. In accordance with *Hassan et al.* (2011), the tissue culture plate method was more effective than the tube method for analysing biofilm production capacity, as demonstrated in the study of 110 clinical isolates. Furthermore, the study of *Knobloch et al.* (2002) established a significant correlation between the tube method and the tissue culture plate method for strong biofilm-producing strains, based on the analysis of 128 *S. aureus* isolates.

In our study, the minimum inhibitory concentration (MIC) of acetic acid was less than or equal to 0.08% for all bacterial isolates, except for two isolates of *S. aureus* which had an MIC of 0.16%. These results are encouraging and satisfactory, in comparison with other studies. For example, *Fraise et al.* (2013) reported that acetic acid was effective at dilutions as low as 0.166% against various bacterial pathogens. Similarly, in the study by *Amrutha et al.* (2017), the MIC of acetic acid was 1.5% for *E. coli* and 1% for *Salmonella* spp. Another study by *Ouattara et al.* (1997) showed that concentrations of acetic acid ranging from 0.1% to 1% (w/v) completely inhibited the growth of several common bacteria implicated in meat spoilage. These results reinforce the effectiveness of acetic acid as an inhibitory agent against pathogenic bacteria.

Several studies have demonstrated the antibacterial effectiveness of spraying broiler carcasses with acetic acid (Table 9). In addition, *Bin Jasass* (2008) reported that portions of chicken previously immersed in a suspension of *E. coli* were soaked in different concentrations of acetic acid (0.5%, 1% and 1.5%). Those authors revealed a reduction in the total number of *E. coli* of 0.7, 1.1 and 1.4 log CFU $cm^{-2}$, respectively, on the surface of soaked chicken meat. These studies thus confirm the effectiveness of this antimicrobial agent, acetic acid, in reducing bacterial contamination on chicken carcasses without altering their appearance (*Abdul Wahid*, 2008).

The effectiveness of acetic acid against other bacterial species known to be causative agents of food poisoning has been confirmed by several studies. For example, *Zhao and Doyle* (2006) demonstrated that acetic acid concentrations of 0.5%, 1%, 1.5% and 2% reduced *C. jejuni* counts by 0.5 log CFU/ml in 2 min in a suspension at 48 °C, and that a concentration of 2% reduced *C. jejuni* counts by 1.4 log CFU/g for up to 45 s on chicken wings at 48 °C. In addition, the study by *Tamblyn and Conner* (1997a) revealed that acetic acid concentrations of 0.5%, 1%, 2%, 4% and 6% exhibited bactericidal activity against *S.* Typhimurium on the skin of poultry. Acetic acid treatment was applied during simulated cooling (0 °C for 60 min), post-treatment immersion (23 °C for 15 s) or scalding (50 °C for 2 min). This bactericidal activity was dependent on the concentration and the method of application. *Salmonella*, whether firmly or loosely attached to the skin of poultry, demonstrated superior resistance to acetic acid compared to freely-suspended *Salmonella*. Notably, a concentration of 4% acetic acid was needed to eliminate approximately 2 log levels of *S.* Typhimurium attached to the skin of broilers.

The effectiveness of acetic acid against bacteria responsible for food poisoning can be influenced by several factors. *Oh et al*. (2009) observed a significant increase in resistance to acetic acid (400 mM)

in *E. coli* O157:H7 isolates from various sources, as the temperature decreased to 15 °C, for a given pH. No significant differences ($p \geq 0.05$) were observed between the various strains. All strains of *E. coli* O157:H7 showed reductions of between 1.8 to 4.5 log levels at pH 3.3 and 30 °C after 25 minutes. Anaerobic incubation was the most protective condition for all strains of *E. coli* O157:H7, compared to other atmosphere conditions. Furthermore, *McKellar and Knight* (1999) reported the effectiveness of acetic acid on 19 strains of enterohemorrhagic *E. coli*, isolated from humans and food, after 24 h. Outbreak strains showed significantly greater survival ($p \leq 0.05$) upon acid treatment than did strains isolated from fermented foods, high pH, or animal or human isolates. Significant differences ($p \leq 0.05$) were observed between serotypes as well as between O157:H7 and other serotypes after 3 or 6 h of exposure to acetic acid. In another study conducted by *Lee and Kang* (2009), various combinations between three factors, namely heat (55 °C), acetic acid (0.25%, v/v) and salt (3%, w/v), were tested and compared to individual treatments to eliminate *E. coli* O157:H7 in laboratory media. On combining salt with heat, no significant further reduction of *E. coli* O157:H7 was measured (there was no additive effect over the effect of heat alone). However, the combination of acid and heat resulted in a more significant reduction in *E. coli* O157:H7 (synergistic effect). When salt was combined with acid treatment, the salt provided protection against the acid treatment (antagonistic effect), thus resulting in less reduction of *E. coli* O157:H7 in the combined treatment compared to the individual acid treatment.

Acetic acid has long been known to be used as an antiseptic, disinfectant and food preservative due to its antimicrobial potential. The proper use of acetic acid in broiler processing can help minimize the risk of food poisoning. However, exposure of *S. aureus* and *E. coli* to acetic acid could result in resistance gene acquisition and the development of resistance, which is problematic due to limited broiler disinfection options. Biofilm formation complicates disinfection of acetic acid-resistant bacteria, as biofilms are a favourable environment for the exchange of these resistance determinants. The use of acetic acid in poultry slaughterhouses can lead to a reduction in the microbial load in the environment, on broiler carcasses and in offal, but this approach must not be seen as a replacement for the proper hygiene management when slaughtering broilers. Therefore, prerequisite

**Table 9.** Antibacterial and antimicrobial effectiveness of spraying broiler carcasses with acetic acid ($C_2H_4O_2$)

| Treatment method | Evaluation parameters | Treatment without acetic acid | Treatment with acetic acid | References |
|---|---|---|---|---|
| **Spray wash** broiler carcasses with acetic acid (0.5%), after scalding. | Total Plate Count (log CFU $cm^{-2}$) | 4.02 ± 0.26 | 3.71 ± 0.19 | *Sakhare et al*. (1999) |
| | Yeast and Mold (log CFU $cm^{-2}$) | 1.98 ± 0.08 | 1.02 ± 0.10 | |
| | *S. aureus* (log CFU $cm^{-2}$) | 1.51 ± 0.07 | 1.14 ± 0.09 | |
| | Coliforms (MPN $cm^{-2}$) | 0.17 ± 0.05 | 0.10 ± 0.03 | |
| **Spray wash** broiler carcasses with acetic acid (0.5%), after defeathering. | Total Plate Count (log CFU $cm^{-2}$) | 4.07 ± 0.28 | 3.79 ± 0.26 | *Sakhare et al*. (1999) |
| | Yeast and Mold (log CFU $cm^{-2}$) | 2.19 ± 0.13 | 1.47 ± 0.11 | |
| | *S. aureus* (log CFU $cm^{-2}$) | 1.71 ± 0.05 | 1.10 ± 0.07 | |
| | Coliforms (MPN $cm^{-2}$) | 2.53 ± 0.11 | 1.71 ± 0.08 | |
| **Spray wash** broiler carcasses with acetic acid (0.5%), after evisceration. | Total Plate Count (log CFU $cm^{-2}$) | 3.36 ± 0.07 | 3.10 ± 0.21 | *Sakhare et al*. (1999) |
| | Yeast and Mold (log CFU $cm^{-2}$) | 1.86 ± 0.09 | 1.00 ± 0.10 | |
| | *S. aureus* (log CFU $cm^{-2}$) | 1.96 ± 0.07 | 0.91 ± 0.10 | |
| | Coliforms (MPN $cm^{-2}$) | 2.03 ± 0.08 | 1.51 ± 0.09 | |
| Spraying broiler carcasses with acetic acid (1%). | Mesophilic Bacteria (CFU/$cm^2$) | $27.47 \times 10^3$ | $10.50 \times 10^3$ | *Abdul Wahid* (2008) |
| | Coliforms (CFU/$cm^2$) | $2.71 \times 10^3$ | $1.03 \times 10^3$ | |
| | *E. coli* (CFU/$cm^2$) | $4.41 \times 10^2$ | $7.5 \times 10^1$ | |
| | *S. aureus* (CFU/$cm^2$) | $2.74 \times 10^2$ | $1.05 \times 10^2$ | |

**Legend:** CFU – colony-forming unit; MPN – most probable number

programs, such as GHP/GMP/SSOP, must be established before the implementation of the HACCP system that more closely controls the risks to human health, as well as the prevention of modifications of foodstuffs by means of control practices in all stages of white meat production (*de Oliveira et al.*, 2016).

## 5. Conclusion

The importance and impact of this study lie in its innovative character in Algeria, being the first to explore the capacity of biofilm formation by *S. aureus* and *E. coli* contaminating the poultry slaughterhouse environment, broiler carcasses and offal, while evaluating the microorganisms' sensitivity to acetic acid.

Contamination by *S. aureus* and *E. coli* of poultry slaughterhouses and broilers at slaughter raises serious concerns for public health. Of particular concern is that these bacteria species have the ability to form biofilms that protect/harbour pathogenic strains. The persistence of biofilm-forming bacteria throughout the chicken processing chain greatly increases the risk of contamination of broiler meat and offal. It is imperative to establish GMP and SSOP programs followed by the HACCP system, which should help reduce the presence of *S. aureus* and *E. coli* such that less biofilm is formed in the poultry slaughterhouse environment. In turn, the chicken meat produced will then carry lower levels of contamination with these two pathogenic microorganisms and so should be safer from the public health point of view. This will minimize the risk of dissemination of these bacteria and their associated genes. At the same time, it is essential to understand the mechanisms involved in the formation of biofilm by these bacteria in order to develop new strategies to effectively eliminate the biofilm. Concerted efforts in these areas will help ensure food safety and protect consumer health.

The high level of susceptibility of the bacteria isolated in our study to acetic acid suggests that it is suitable for use in poultry slaughterhouses to disinfect the environment and decontaminate broiler carcasses and offal effectively, as a method of choice for food safety management.

# Proizvodnja biofilma i osetljivost na sirćetnu kiselinu *Staphilococcus aureus* i *Escherichia coli* izolovanih u klanicama za živinu, na trupovima brojlera i iznutricama u Alžiru

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INFORMACIJE O RADU

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APSTRAKT

Klanice za živinu, trupovi brojlera i iznutrice mogu delovati kao rezervoari i širiti različite zoonotične bakterijske patogene kao što su *Staphilococcus aureus* i *Escherichia coli*. Cilj ovog istraživanja je da se utvrdi prevalencija *Staphilococcus aureus* i *Escherichia coli* na trupovima brojlera i iznutricama, kao i u klanicama za živinu, uz procenu kapaciteta za formiranje biofilma i osetljivosti na sirćetnu kiselinu pojedinih bakterijskih izolata. Uzeto je ukupno 210 uzoraka sa različitih delova trupova (krila, karabatak i grudi) i iznutrica (jetra i srca) pilića brojlera, a 19 uzoraka životne sredine je prikupljeno iz različitih odeljenja klanica živine (zidovi, podovi i oprema) da se utvrdi prevalencija *Staphilococcus aureus* i *Escherichia coli*. Za procenu sposobnosti da formiraju biofilmove, posebno je odabrano 14 sojeva *Staphilococcus aureus* i 14 sojeva *Escherichia coli*, izolovanih iz proizvoda brojlera, kao i 14 sojeva *Staphilococcus aureus* i 14 sojeva *Escherichia coli* izolovanih iz okruženja klanica. Za procenu kapaciteta formiranja biofilma korišćena je metoda epruvete i metoda ploče za kulturu tkiva, dok je minimalna inhibitorna koncentracija (MIC- minimum inhibitory concentration) sirćetne kiseline na ovim bakterijskim izolatima određena metodom razblaživanja agarom. Ukupne količine biofilma proizvedenih od strane različitih kategorija bakterijskih sojeva upoređene su statističkom analizom. Prevalencija *Staphilococcus aureus* i *Escherichia coli* bila je 100% u uzorcima trupova brojlera i iznutrica, dok je u uzorcima iz klanice prevalencija *Escherichia coli* bila 94,73%, a *Staphilococcus aureus* 78,94%.

Metodom epruvete, procena sojeva *Staphilococcus aureus* je pokazala da je 35,71% pokazalo visoku proizvodnju biofilma, dok je 50% pokazalo umerenu produkciju, a 14,28% nisku proizvodnju. Nijedan soj nije kategorisan kao ne-biofilm. Slično, za sojeve *Escherichia coli*, rezultati su pokazali da je 32,14% imalo visoku proizvodnju biofilma, 21,42% umerenu proizvodnju i 46,42% nisku proizvodnju, pri čemu nijedan soj ne proizvodi biofilm. Metodom ploče za kulturu tkiva, procena sojeva *Staphilococcus aureus* je pokazala da je 39,28% imalo umerenu produkciju biofilma, dok je 60,71% pokazalo nisku produkciju. Nisu identifikovani izolati koji imaju visoku proizvodnju ili nisu proizvođači biofilma. Za sojeve *Escherichia coli*, 14,28% je pokazalo visoku proizvodnju biofilma, 39,28% umerenu proizvodnju i 46,42% nisku proizvodnju, pri čemu nijedan izolat nije kategorisan kao proizvođač koji nije biofilm. Dve korišćene metode omogućile su otkrivanje proizvodnje biofilma kod svih proučavanih bakterijskih izolata. Metoda epruvete je pokazala veću stopu izolata sa visokom produkcijom biofilma (33,92%) u poređenju sa metodom ploče za kulturu tkiva (7,14%). Nasuprot tome, metoda epruvete je zabeležila nižu stopu izolata koji pokazuju umerenu proizvodnju biofilma (35,71%) u poređenju sa metodom ploče za kulturu tkiva (39,28%). Slično, metoda epruvete je pokazala nižu stopu izolata sa niskom produkcijom biofilma (30,35%) u poređenju sa metodom ploče za kulturu tkiva (53,57%). U pogledu ukupne količine proizvedenog biofilma, bakterije životne sredine su imale veću vrednost (ukupni OD=12,45) u poređenju sa bakterijama izolovanim iz brojlera (ukupni OD=11,83), pri čemu razlika nije značajna ($p>0,05$). Isto tako, ukupna količina biofilma proizvedenog kod svih izolata *Escherichia coli* (ukupni OD=12,78) bila je veća od one koju proizvode svi izolati *Staphilococcus aureus* (ukupni OD=11,5), bez značajne razlike ($p>0,05$). Među izolatima brojlera, 14 sojeva *Escherichia coli* imalo je najveću količinu biofilma (ukupni OD=6,76), dok je 14 sojeva *Staphilococcus aureus* imalo najmanju količinu (ukupno OD=5,07), sa neznatnom razlikom ($p> 0,05$). Minimalna inhibitorna koncentracija (MIC) sirćetne kiseline bila je ≤0,08% za sve bakterijske izolate, osim za dva izolata *Staphilococcus aureus*, za koja je minimalna inhibitorna koncentracija bila 0,16%. Zaključno, *Staphilococcus aureus* i *Escherichia coli* su često prisutni u okruženju klanica živine i u proizvodima od brojlera. Svi bakterijski izolati su pokazali sposobnost formiranja biofilma. Utvrđeno je da su ove bakterije veoma osetljive na sirćetnu kiselinu, koja se stoga smatra idealnim sredstvom za dezinfekciju okruženja u klanicama živine i dekontaminaciju trupova brojlera.

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*Original Scientific Paper*

# Evaluation of food additive analyses based on five years of food safety and quality controls

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ABSTRACT

Analyses of eight groups of additives in food and animal feed for nearly five years were included in this research. Food samples were grouped according to EU directive 1333/2008 and national regulation 53/2018 into 18 food categories. A total of 4539 samples was analysed, of which the most numerous categories were meat and dairy products, with 2833 (62.4%) and 649 (14.3%) samples, respectively, and a total of 8203 analyses. Over 90% of all analyses were determinations of food colourants, inorganic anions and preservatives & sweeteners, accounting for 3478 (42.4%), 2937 (35.8%) and 1122 (13.7%) of the analyses, respectively. The least common were tartaric and fumaric acid determinations, and the food categories with the lowest numbers of analyses were: food supplements (rarest), fats and oils and fat and oil emulsions (second rarest), and sugars, syrups, honey and table-top sweeteners (third rarest). The analyses of additives are unevenly represented in food and animal feed and it is necessary to balance and harmonise them with legislative requirements. Adequate control of food additives is an important part of the regulatory requirements and can only be fulfilled by continuous monitoring of additive use in food and animal feed.

## 1. Introduction

Regulation (EC) No 1333/2008 of the European Parliament and of the Council describe food additives as "substances that are not normally consumed as food itself but are added to food intentionally for a (certain) technological purpose" (*European Union*, 2008). They have various roles in food preparation and are commonly used to improve some quality attributes, from acceptability to the safety of food, as well as prolong shelf-life of food commodities etc. Current food industry practices and manufacturing would not be possible without the use of food additives.

On the other hand, animal feed additives are defined by Regulation (EC) No 1831/2003 as "substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the functions", such as to: favourably affect the characteristics of feed and animal products; change/enhance the colour of ornamental fish and birds; positively impact animal production, performance or welfare, particularly by affecting the gastrointestinal flora or digestibility of feeding stuffs; mitigate the environmental consequences of animal production; satisfy the nutritional needs of animals; have a coccidiostatic or histomonostatic effect (*European Union*, 2003).

Both regulations set the terms of the categories of additives, permitted amounts and authorise additive usage in particular food and feed. Setting such

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conditions requires the development of a reliable methodology for determining the correct amounts of additives in food and animal feed. The existing techniques of chemical analysis of food, especially based on chromatography (*Bajcic et al.*, 2021, *Petronijevic et al.*, 2021, *Petronijevic et al.*, 2023), more or less successfully satisfy this requirement. The greatest problems are determining the content of additives that are naturally present in a particular form in the raw materials in the food and animal feed industry, because it is often impossible to determine to what extent they originate from the raw material, and how much comes from the additive itself (*Petronijevic et al.*, 2023).

At the end of the last century, risk assessment of food and animal feed additive usage became especially relevant due to the general increase in consumption of packaged and processed foods rich in additives. Possible connections of chronic consumption of food additives to adverse effects on human and animal health are described (*Polak et al*., 2018; *Bajcic et al*., 2018). In order to accurately estimate food additives' impacts on health based on the results of new scientific research, the European Union (EU) set up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 (*European Union*, 2008) under the jurisdiction of the European Food Safety Authority (*European Union*, 2010).

National regulation in Serbia on food additives (*Serbia*, 2018) is mostly harmonised with EU legislation, and usage of additives in animal feed is authorised by the Serbian Regulation on animal feed quality (*Serbia*, 2017; this regulation refers to the *Official Gazette of the Republic of Serbia 4/2010, 113/2012, 27/2014, 25/2015, 39/2016*). Therefore, continuous monitoring of the use of additives in food and animal feed is not a matter of good will but a legal obligation that must be systematically implemented and controlled at the state level. The results presented in this research are the consequence of the implementation of monitoring of particular additive groups in food and feed produced in or imported into Serbia. The data provides the possibility to determine the type of additives and their quantity introduced through the diet, individually or in total, as well as what kind of products have a greater impact on the increased intake of additives. Consequently, these data are significant for reliable risk assessments of additive consumption in human and animal nutrition.

## 2. Materials and Methods

### *2.1 Chemicals*

All standard chemicals and reagents were purchased from Merck KgaA, Darmstadt, Germany. Ultrapure water, ≥18 MΩ, was obtained from ELGA Ultrapure (LabWater, Lane End, High Wycombe, UK).

#### *Samples*

Food and feed samples were part of regular control of quality and safety parameters, obtained from retail, producers and importers.

#### *Sample preparation*

Solid food and feed products were ground and homogenised prior to analysis. Depending on the applied determination technique and the type of additive, the samples were prepared according to appropriate procedures.

### *2.2 Methods*

#### *Antioxidants*

An in-house, validated method of high-performance liquid chromatography with UV/VIS detection via photodiode array (HPLC-PDA) was used for the determination of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHT), and propyl-, octyl- and dodecyl-gallate. The chromatographic system was an Alliance 2695 separation module with photodiode array detector 2996 (Waters, Milford, Massachusetts, USA). Antioxidants were extracted from the samples with methanol and centrifuged, and after filtration, the supernatants were submitted for analysis. Identification of each analyte was based on retention time (RT) and UV/VIS spectra.

#### *Food colourants*

Determination of 13 synthetic food dyes was performed in accordance to the reference method (*ISO*, 2021): Tartrazine, E 102, Sunset yellow FCF, E 110, Azorubine, E 122, Amaranth, E 123, Ponceau 4R, E 124, Erythrosine, E 127, Red 2G, E 128, Allura Red AC, E 129, Patent Blue V, E 131, Indigotine, E 132, Brilliant Blue FCF, E 133, Green S, E 142 and Brilliant Black BN, E 151. An identical chromatographic system as for antioxidant determination was used.

Carminic acid, E 120, was determined by liquid chromatography with mass spectrometry (LC-MS/MS) on a triple quadrupole mass spectrometer, LCMS-8050 CL (Shimadzu Corporation, Japan). Preparation of sample for analysis included extraction of colourants in acidified ethanol, centrifugation and filtration. MS detection was in MRM mode, and 491.1 to 446.75 transition was used for quantification.

*Fumaric acid*

Fumaric acid analysis was carried out by IFU method Nr. 72 (*IFU*, 1998).

*Hydrosoluble vitamins*

Analyses of vitamins C, B2 and B6 were performed in accordance with reference methods (*ISO*, 2018; *SRPS EN*, 2014; *SRPS EN*, 2008b).

*Inorganic anions*

For determination of inorganic anion additives (mainly phosphoric acid and mono-, di-, tri- and polyphosphates, nitrites and nitrates, and sulphites) in food and animal feed, IC with conductometric detection was used. The IC system consisted of an 858 Professional Sample Processor, 930 Compact IC Flex with Oven/SeS/PP, and Conductivity Detector, (Metrohm AG, Herisau, Switzerland). The separation column was Metrosep A Supp 7 250/4.0 (Metrohm), and separation of anions was achieved by a mobile phase gradient in accordance with the original method provided by manufacturer (*Metrohm*, 2019).

*Liposoluble vitamins*

Vitamins A and E were determined by reference methods (*SRPS EN ISO*, 2011; *SRPS EN ISO*, 2008) based on HPLC.

*Preservatives*

Determination of sorbate and benzoate additives was according to the procedure described in the reference method (*SRPS EN*, 2008a). The chromatographic system was the same as was used for determination of antioxidants and artificial colourants.

*Tartaric acid*

The reference method (*SRPS EN*, 2008c) was applied for determination of tartaric acid.

*2.3 Statistics*

Food samples were strictly categorised into 18 groups according to food categories in EU directive 1333/2008 (*European Union*, 2008) and national regulation 53/2018 (*Serbia*, 2018). The meat category refers not only to raw meat, but also to meat products and all other products covered by this category, including meat casings, etc.

MS Office 2016 Excel software was applied for data preparation. Contingency analysis of categorical data was performed in JMP Statistical Discovery 10 (SAS Institute Inc. NC, USA https://www.jmp.com).

## 3. Results and Discussion

Analyses of eight important groups of additives in food and animal feed for almost five years were included in this research. A total of 4539 food/feed samples was analysed, of which 224 (5%) were animal feed. The most numerous categories were meat products and dairy products and analogues, with

**Table 1.** Number of samples per food category

| Food categories | Samples |
|---|---|
| Additives | 36 |
| Animal feed | 224 |
| Bakery wares | 34 |
| Beverages | 105 |
| Cereals and cereal products | 10 |
| Compound food | 15 |
| Confectionery | 42 |
| Dairy products and analogues | 649 |
| Edible ices | 62 |
| Eggs and egg products | 21 |
| Fats and oils and fat and oil emulsions | 2 |
| Fish and fishery products | 124 |
| Food supplements | 1 |
| Fruits and vegetables | 284 |
| Meat | 2833 |
| Ready-to-eat savouries and snacks | 25 |
| Salts, spices, soups, sauces, salads and protein products | 69 |
| Sugars, syrups, honey and table-top sweeteners | 3 |
| **Total** | **4539** |

**Table 2.** Contingency analysis of additive group by food category

| Food category | Antioxidants | Food colourants | Fumaric acid | Hydrosoluble vitamins | Inorganic anions | Liposoluble vitamins | Preservatives & sweeteners | Tartaric acid | Total |
|---|---|---|---|---|---|---|---|---|---|
| Additives | | 87 | | | | 10 | 10 | | 107 |
| Animal feed | 95 | 39 | | 90 | 22 | 403 | 1 | | 650 |
| Bakery wares | | 17 | | | 43 | | 19 | | 79 |
| Beverages | | 4 | 3 | 18 | 214 | 1 | 9 | 1 | 250 |
| Cereals and cereal products | | | | | 11 | | 5 | | 16 |
| Compound food | | | | | 14 | | 6 | | 20 |
| Confectionery | | 97 | 5 | 1 | 1 | | 36 | | 140 |
| Dairy products and analogues | | 338 | 4 | | 488 | | 547 | | 1377 |
| Edible ices | | 169 | | | 51 | | 17 | | 237 |
| Eggs and egg products | | 11 | | | 8 | | 26 | | 45 |
| Fats and oils and fat and oil emulsions | | 27 | | | | | | | 27 |
| Fish and fishery products | | 205 | | 5 | 123 | | 27 | | 360 |
| Food supplements | | | | | 2 | | | | 2 |
| Fruits and vegetables | | 67 | | 2 | 477 | | 116 | | 662 |
| Meat | | 2318 | | 3 | 1346 | 3 | 267 | 2 | 3939 |
| Ready-to-eat savouries and snacks | | 9 | | | 17 | | 12 | | 38 |
| Salts, spices, soups, sauces, salads and protein products | 20 | 90 | | | 115 | | 24 | | 249 |
| Sugars, syrups, honey and table-top sweeteners | | | | | 5 | | | | 5 |
| **Total** | **115** | **3478** | **12** | **119** | **2937** | **417** | **1122** | **3** | **8203** |

2833 (62.4%) and 649 (14.3%) samples, respectively (Table 1). Fewer analyses were conducted on fruits and vegetables, animal feed, fish and fishery products and beverages (Table 1).

As shown in Table 1, some food categories had only a few requests for additive analysis (< 10 samples per year) in the research period. This group included food categories that are widely consumed (bakery wares, confectionery, cereals and snacks) or mainly imported or exported (additives, egg products, supplements) as raw materials for use in the food industry. Therefore, with respect to their demand and presence on the market, the lack of extensive control of additive content in these food categories is surprising. This is especially the case considering that some of those categories are highly processed foods with significant quantities of one or more additives.

Table 2 presents a comprehensive overview of the results, showing individual numbers and totals of analyses by food categories and in each additive group. In almost five years, 8203 analyses were performed. The most common analyses were determinations of food colourants, inorganic anions and preservatives & sweeteners, accounting for 3478 (42.4%), 2937 (35.8%) and 1122 (13.7%) of analyses, respectively. In fact, 91.9% of all analyses were for these additives. On the other hand, the least common analyses performed were determinations of tartaric and fumaric acids, 3 and 12 times, respectively. Analyses of hydrosoluble and liposoluble vitamins, as additives in food and feed samples, made up less than 10% of all determinations.

Following the nature of the obtained data, since they consisted of a large number of results that could be classified into several categories and groups based on frequency, contingency analysis was chosen. The uneven number of analyses per sample and the large disparity in the number of samples per food category was the main obstacle in presenting and interpreting results. Hence, to enable their distinct presentation, results had to be divided into two groups based on the number of additive analyses performed in the correspondent food categories. One group consisted of the most common determinations: food colourants, inorganic anions, vitamins, preservatives & sweeteners and antioxidants in the following food categories: additives; animal feed; bakery wares; beverages; confectionery; dairy products and analogues; edible ices; fish and fishery products; fruits and vegetables; meat; and salts, spices, soups, sauces, salads and protein products. A second group contained less frequent analyses of fumaric acid, tartaric acid, food colourants, inorganic anions and preservatives & sweeteners in the following food categories: beverages; cereals and cereal products; compound food; confectionery; dairy products and analogues; eggs and egg products; fats and oils and fat and oil emulsions; food supplements; meat; ready-to-eat savouries and snacks; and sugars, syrups, honey and table-top sweeteners. Visual representations of additive analyses by food category for each of the groups are given in Figures 1 (main, common analyses) and 2 (infrequent analyses).

Determinations of food colourants, inorganic anions and preservatives & sweeteners accounted for most of the analyses performed in the main (commonly analysed) food categories. This is certainly a consequence of legal requirements, because additives from these three groups are permitted and regulated in most food categories. However, the category of animal feed differed, as determinations of liposoluble vitamins, followed by antioxidants and hydrosoluble vitamins were more common, mainly due to the specific requirements of the corresponding regulations. An equally significant contribution was requests from animal feed manufacturers to control and validate the composition and quality of their products. However, analysis of permitted preservatives in feed was performed only once in the 5-year period, compared to other food categories where this is one of the most common determinations.

The type and number of analyses from the category of additives as raw materials for the food and animal feed industries (the additives category in the tables and Figure 1) is primarily a consequence of import controls. No conclusion can be made or generalised because of the relatively small number of samples analysed (36), but results can be considered indicative.

Figure 2 shows the results for food categories that either had few samples or few additive analyses. The results presented for this group should be taken with caution due to the small numbers of determinations and samples, and so could be regarded as inconclusive. The only unmistakable conclusion is that these food categories should be given greater importance regarding their additive content analysis, both in terms of the number of samples and the types of additives. For example, according to official statistical data (*Serbia*, 2023), the import of animal and vegetable oils and fats in Serbia over a 5-year period (2018–2022) was worth US$394 million. A significant part of that was frying oils and fats for fast-food restaurants and the confectionery industry. Considering the amounts of fast-food, fried food and confectionery products that are now consumed, especially

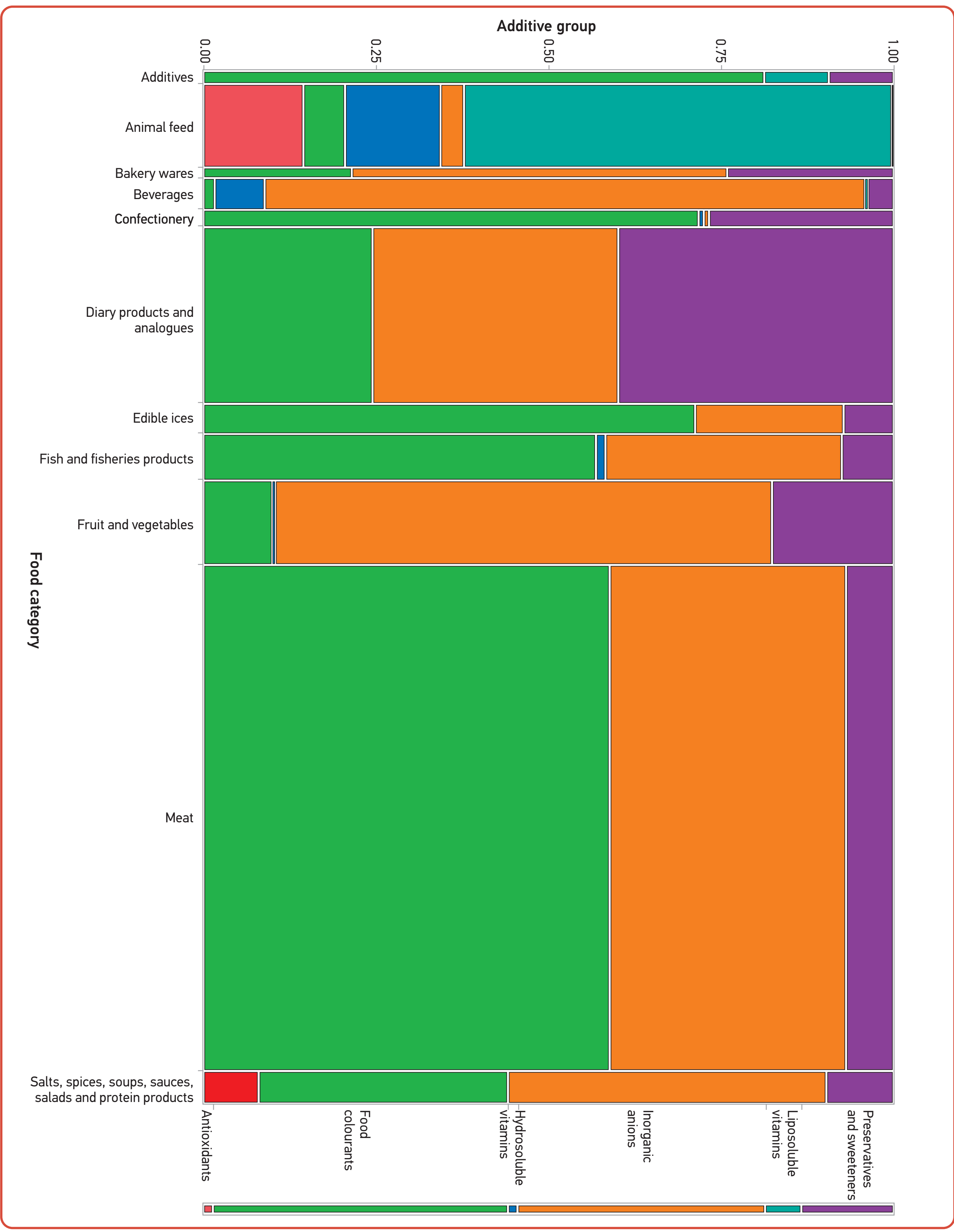


**Figure 1.** Graphic representation of the food categories vs. main analyses. The X-axis represents the relative ratios of the number of samples by food category, and the Y-axis shows the relative ratios of the food additive analyses within each food category. The blocks depicted show the relative proportions of the performed analyses within the entire population. Each additive determination is marked with a different colour. The side bar shows the overall ratios of food additive analyses within the group of more commonly analysed food and feed products.

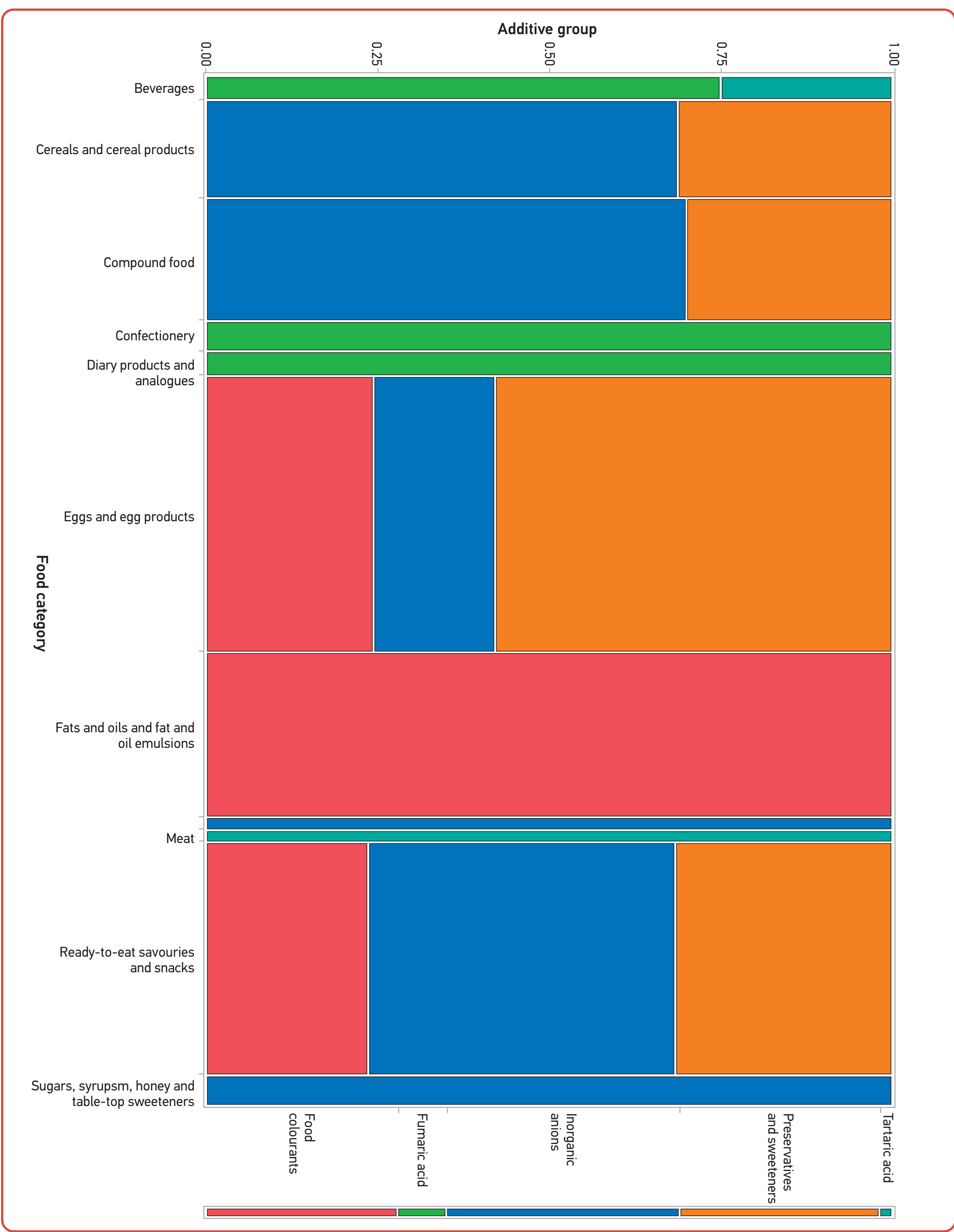


**Figure 2.** Graphic representation of the food categories vs. infrequent analyses. The X-axis represents the relative ratios of the number of samples by food category, and the Y-axis shows the relative ratios of the food additive analyses within each food category. The blocks depicted show the relative proportions of the performed analyses within the entire population. Each additive determination is marked with a different colour. The side bar shows the overall ratios of food additive analyses within the group of infrequent analyses.

by the young population, it is unnecessary to underline the relevance of determining the chemical safety of imported fats and oils, which includes the analysis of additives. Consequences and implications of inadequate control, along with other health issues related to fast food and confectionery consumption, could have great and long-term adverse impacts on public health.

## 4. Conclusion

Food additives have gained a lot of attention in recent decades. On the one hand, they have become an irreplaceable factor in food production today, but on the other hand, their use is, from time to time and justifiably or not, associated with controversies regarding their adverse impact on human or animal health. In addition, a negative side of the food additives can be their use to mask food frauds and adulterations.

The processing of the results of 5-year additive analyses in food and animal feed at the national level showed that the control of additives is carried out regularly in some food categories, while in others it is not. Also, in some cases, in the categories in which regular control is performed, analyses of all relevant additives are not included.

From the results, it can be concluded that the most common determinations were for food colourants, inorganic anions, preservatives & sweeteners, which made up almost 92% of the analyses performed. Among the food categories, the largest number of analyses were for meat, while four times fewer analyses were conducted for dairy products and analogues, followed by fruits and vegetables, animal feed, fish and fishery products and beverages.

In animal feed, the main determinations were for liposoluble vitamins, followed by antioxidants and hydrosoluble vitamins. Analysis of permitted preservatives in feed was performed only once in the observed period.

In conclusion, the results indicate that the control of additives in food and animal feed is uneven. Whatever the reasons for this situation, it is necessary to balance the control of additives in some food categories, and harmonise them to legislative requirements, deriving the assessment from the needs of the national market, the import of raw materials and the export of food products. Adequate control of food additives is an important part of the fulfilment of the legal regulation requirements that ensure better quality and safer food.

# Procena učestalosti analize prehrambenih aditiva na osnovu petogodišnje kontrole bezbednosti i kvaliteta hrane

*Radivoj Petronijević, Srđan Stefanović, Čaba Silađi, Aleksandar Bajčić, Jelena Ćirić, Danijela Vranić i Danka Spirić*

INFORMACIJE O RADU

*Ključne reči:*
Hrana
Prehrambeni aditivi
Hrana za životinje
Prehrambene boje
Vitamini
Konzervansi
Antioksidansi
Kvalitet hrane
Bezbednost hrane

APSTRAKT

Istraživanje je obuhvatalo analize nekoliko grupa aditiva u hrani i hrani za životinje u toku 5 godina. Uzorci su grupisani prema kategorijama hrane definisanim u EU direktivi 1333/2008 i Pravilniku o prehrambenim aditivima, Službeni glasnik br. 53/2018. Analizirano je ukupno 4539 uzoraka, od kojih su najbrojnije grupe bile meso, 2833 (62,4%), i mlečni proizvodi, 649 (14,3%) uzoraka, sa ukupno 8203 izvršene analize. Preko 90% svih analiza odnosilo se na određivanje boja, anjona i konzervanasa, 3478 (42,4%), 2937 (35,8%) i 1122 (13,7%) analize, redom. Najmanje učestale analize su bile određivanje sadržaja vinske i fumarne kiseline, a najmanji broj uzoraka bio je u tri kategorije namirnica: dodaci ishrani, masti i ulja i emulzije masti i ulja i šećeri, sirupi, med i stolni zaslađivači. Analize aditiva su neravnomerno zastupljene u hrani i hrani za životinje i potrebno ih je izbalansirati i uskladiti sa zakonskom regulativom. Adekvatna kontrola aditiva u hrani je važan deo ispunjavanja regulatornih zahteva kontinuiranim praćenjem upotrebe aditiva u hrani i hrani za životinje.

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**ISO (2021)**. Meat and meat products – Detection and determination of colouring agents. *ISO 13496:2021*.

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# Assessment of carcass contamination in a slaughterhouse in the governorate of Blida, Algeria

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A R T I C L E  I N F O

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A B S T R A C T

The initial contamination of meat occurs during slaughter procedures. The objective of this study was to evaluate the hygiene in a slaughter establishment by evaluating the surface contamination of the carcasses and the level of hygiene of the workforce, the environment and the equipment. Altogether, 122 samples were taken, (50 from carcasses and 72 from personnel, equipment and environment). The state of cleanliness of animals was assessed for 125 sheep and 150 cattle. Bacteriological analyses conducted were the enumeration of total coliforms, thermo-tolerant coliforms and Escherichia coli and the detection of Salmonella.

The carcasses were significantly contaminated with bacterial hygiene indicators and there were no significant differences (P> 0.05) in contamination levels between the carcass species, or between the days of the weeks or the microbial groups enumerated. Evaluation of animals' cleanliness showed that 68% of the examined sheep were dirty or very dirty, and 91.33% of the cattle were lightly soiled or dirty. Examination of the contamination of personnel, equipment and the environment between the start and the end of the week did not reveal a significant difference (P> 0.05). In order to minimize the contamination of carcasses at the slaughterhouse level, it is recommended to apply good hygiene practices.

## 1. Introduction

Ensuring food safety at all levels of the food production chain has become a fundamental priority for the food industry. Meat is an excellent source of animal protein, but in addition to the requirements for its nutritional and taste qualities, health quality is essential. Meat is a highly perishable foodstuff the hygienic quality of which depends on the one hand on contamination during slaughter and cutting operations and on the other hand on the development and growth of contaminating microbiota during cooling, storage and distribution ([*Dennaï et al.,* 2001; *El Hadef El Okki et al.,* 2005; *Salifou et al.,* 2013).

The veterinary controls in force at the slaughterhouse level provide some guarantee of the meat's hygienic status. The controls focus more on animal health compliance that results in healthful meat for consumption, i.e detection of animal diseases that can be transmitted to humans (*Sadoud,* 2017). Studies have shown that it is the microbial hazards present primarily in healthy animals that are the greatest source of risk to human health, such as *Salmonella enteritidis*, *Campylobacter jejuni*, *Eschirichia. coli*, *Clostridium perfringens*, *Yersinia enterocolitica* and *Listeria monocytogenes* (*FOA,* 2006).

In fact, surface contamination of meat mainly takes place at the slaughterhouse despite efforts made by veterinary services to ensure safe meat (*Sadoud,* 2017). This contamination is, therefore, not desirable, but inevitable. From the point of view

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of legislation, carcasses of slaughter animals are not subject to food safety criteria at slaughterhouse level, i.e., to criteria for which the thresholds must imperatively be respected to place the product in the market. However, the evaluation of surface contamination of carcasses reflects the level of hygiene of the processes and allows corrective actions.

The objective of this study is to assess and determine the surface contamination of the carcasses and the potential sources of contamination in one facility, in order to evaluate the level of hygiene of the slaughterhouse and the cleanliness of live animals.

## 2. Materials and Methods

### *2.1 Samples*

One hundred and twenty-two samples were taken as follows, 50 from carcasses (25 cattle and 25 sheep); 20 from personnel hands, 20 from personnel clothing (shirts); 16 from knives, 16 from axes and 08 from walls (building).

The visual assessment of the state of cleanliness of animals before slaughter concerned 125 sheep and 150 cattle.

### *2.3 Sampling*

Five carcasses of each species were examined per day each week. Every week, one day of the five days of slaughterhouse exploitation, was dedicated to collecting samples. The choice of carcasses was made randomly.

- Surface swab samples were taken from the surface of freshly slaughtered carcasses declared fit for consumption after health inspection and before the start of drying. The non-destructive method (swabbing) was carried out using abrasive household sponges with a dimension of 5 cm ×5 cm, or 25 $cm^2$.
- Two zones were swab sampled per half-carcass (sides and shoulder), i.e., 4 per carcass, making a total area of 400 $cm^2$ delimited by a plastic template.
- The sponges from each carcass were placed in the same identified sterile Stomacher bag, supplemented with 100 ml of buffered peptone water. The bag was then hermetically sealed, and placed in a cooler.

Samples from hands, shirts, knives, axes and walls were taken at the beginning and end of the week, using the double swab method. This entailed a first swab within the template using a cotton tipped swab stick soaked in buffered peptone water followed by a second dry swab within the delimited surfaces. The two swabs were placed aseptically in the same tube with buffered peptone water. The samples were stored in a cooler and sent to the laboratory.

Visual assessment of the state of cleanliness of the animals before slaughter concerned 125 sheep and 150 cattle. Classification of the cattle was based on a grid of four cleanliness classes, A to D (*Bastien et al.,* 2006). The grading of sheep was based on a grid developed on the model of the grid for large cattle but adapted for sheep It was made up of four classes A to D but took into account dry and wet soiling (*Evrat-Georgel,* 2013). The state of humidity was assessed by palpation of the sheep's fleece.

### *2.4 Method of microbiological analysis*

Upon receipt at the laboratory, each sample was homogenized in a stomacher for ten minutes. The resulting suspension was directly and aseptically poured into an identified sterile vial, this was the stock suspension.

From the stock suspension, a series of decimal dilutions ($10^{-1}$ to $10^{-6}$) was carried out in buffered peptone water (IPA®).

### *2.5 Enumeration of total coliforms and thermotolerant coliforms*

The coliform count was carried out by deep-seeding into Petrie dishes containing crystal violet and neutral red agar (VRBL). The dishes were incubated separately, one at 30 °C for 24 hours for the enumeration of total coliforms, and the other at 44 °C for 24 hours for the enumeration of thermotolerant coliforms.

### *2.6 Enumeration of Escherichia coli*

The Petrie dishes positive for thermotolerant coliforms at the level of two successive dilutions were retained. A determined number of five characteristic colonies on each of the selected dishes were subcultured, with a view to making a biochemical identification of pure cultures.

### *2.7 Salmonella detection*

After preparing the decimal dilutions, the remainder of the stock suspension was incubated at 37 °C for 24 hours for pre-enrichment. Enrichment was performed by adding 1mL of pre-enrichment in Selenite

Broth and incubating 37 °C for 24 h. Isolation on Hektoen agar, incubate at 37 °C for 24 h.

After purification of the isolates, we performed biochemical identification with the Api $20^E$ gallery.

*2.8 Statistical analysis*

Statistical analyses of data were performed using SPSS version 21 software. Results were subjected to analysis of variance (ANOVA) for multiple comparison tests. The level (of p <0.05) was considered the significant

## 3. Results

*Evaluation of carcass microbiological quality*

The results of the enumeration of bacteria indicative of hygiene on bovine and ovine carcasses are shown in Table 1.

The week of sample collection had no significant effect (P> 0.05) on the contamination of bovine or ovine carcasses.

For each microbial group enumerated, only non-significant differences (P> 0.05) were noted between bovine carcasses and ovine carcasses, except for thermo-tolerant coliforms during the third and fifth week, which did differ significantly (Table 1).

*3.1 Assessment of the state of cleanliness of animals*

The results of the assessment of the state of cleanliness of sheep and cattle are reported in Table 2.

The majority of sheep (68%) slaughtered were classes C or D (dirty and very dirty, respectively); while about. 91% of the cattle examined were classes B or C (lightly soiled and dirty, respectively).

*3.2 Evaluation of the hygiene of the workforce, the equipment and the environment*

The results of the enumeration of bacteria indicative of personnel hygiene (hands and shirts), equipment surfaces (knives, axes) and the environment (walls) of the slaughterhouse are shown in Table 3.

**Table 1.** Means (± standard deviation) of microbial loads in bovine and ovine carcasses over five weeks

| | W1 | W2 | W3 | W4 | W5 |
|---|---|---|---|---|---|
| | | | **Total coliforms** | | |
| BC | $4.713 \pm 1.234^{aA}$ | $4.717 \pm 1.139^{aA}$ | $5.056 \pm 0.452^{aA}$ | $5.448 \pm 0.461^{aA}$ | $5.382 \pm 0.824^{aA}$ |
| OC | $5.049 \pm 0.470^{aA}$ | $5.353 \pm 0.990^{aA}$ | $5.363 \pm 0.600^{aA}$ | $4.889 \pm 0.506^{aA}$ | $5.718 \pm 0.476^{aA}$ |
| | | | **Thermotolerant coliforms** | | |
| BC | $4.533 \pm 1.580^{aA}$ | $4.584 \pm 1.393^{aA}$ | $3.791 \pm 0.567^{aA}$ | $4.582 \pm 1.180^{aA}$ | $4.234 \pm 0.728^{Aa}$ |
| OC | $4.172 \pm 0.450^{aA}$ | $3.941 \pm 0.989^{aA}$ | $5.123 \pm 0.802^{aB}$ | $4.332 \pm 0.800^{aA}$ | $5.289 \pm 0.586^{aB}$ |
| | | | ***Escherichia. coli*** | | |
| BC | $3.376 \pm 0.973^{aA}$ | $4.038 \pm 0.962^{aA}$ | $3.411 \pm 0.790^{aA}$ | $4.000 \pm 0.862^{aA}$ | $3.577 \pm 0.685^{aA}$ |
| OC | $3.592 \pm 0.690^{aA}$ | $3.122 \pm 0.624^{aA}$ | $4.126 \pm 0.571^{aA}$ | $3.780 \pm 0.512^{aA}$ | $4.134 \pm 0.605^{aA}$ |

**Legend:** Values are in log CFU/$cm^2$; OC: ovine carcasses, BC: bovine carcasses, W: weeks.

For each microbial group, values followed by a different lowercase letter within the same row are significantly different (P <0.05) and values followed by a different uppercase letter within a row. The same column is significantly different (P <0.05).

**Table 2.** Assessment of the state of cleanliness of the animals presented for slaughter

| | | Class A | Class B | Class C | Class D |
|---|---|---|---|---|---|
| Sheep presented for slaughter (n=125) | **n** | 15 | 25 | 46 | 39 |
| | **%** | 12% | 20% | 36.8% | 31.2% |
| Cattle presented for slaughter (n=150) | **n** | 10 | 112 | 26 | 02 |
| | **%** | 6.66% | 74.66% | 17.33% | 1.33% |

**Table 3.** Means (± standard deviation) of the microbial loads of the personal (hands, shirts), equipment (knives, axes) and environment (wall) of the slaughterhouse. Values are in log CFU/cm$^2$

| | First dayof week(Sunday) | Last day of week(Thursday) |
|---|---|---|
| | **Personnel hands** | |
| Total coliforms | 5.098±0.065[a] | 4.930±0.565[a] |
| Thermotolerant coliforms | 4.635±0.392[a] | 4.381±0.756[a] |
| *Escherichia. coli* | 3.700±0.398[a] | 3.757±0.637[a] |
| | **Shirts** | |
| Total coliforms | 3.487±0.353[a] | 3.866±0.642[a] |
| Thermotolerant coliforms | 3.011±0.259[a] | 3.158±0.414[a] |
| *Escherichia. coli* | 2.478±0.610[a] | 2.647±0.466[a] |
| | **Knives** | |
| Total coliforms | 4.583±0.434[a] | 4.623±0.393[a] |
| Thermotolerant coliforms | 3.792±0.199[a] | 3.605±0.316[a] |
| *Escherichia. coli* | 2.752±0.131[a] | 2.783±0.147[a] |
| | **Axes** | |
| Total coliforms | 4.482±0.417[a] | 4.702±0.180[a] |
| Thermotolerant coliforms | 3.559±0.384[a] | 3.811±0.258[a] |
| *Escherichia. coli* | 2.857±0.098[a] | 2.684±0.151[a] |
| | **Walls** | |
| Total coliforms | 4.286±0.151[a] | 2.840±0.976[a] |
| Thermotolerant coliforms | 4.119±0.231[a] | 2.418±0.570[b] |
| *Escherichia. coli* | 2.974±0.273[a] | 1.342±0.542[b] |

For each microbial group, values followed by a different lowercase letter within the same row are significantly different (P <0.05).

Between the first and the last working days (respectively Sunday and Thursday) contamination of the personnel (hands of the personnel and shirts), equipment surfaces the material (knives and axes) and the environment (slaughterhouse walls) did not differ significantly (P> 0.05), except for walls, for these, significant differences (P <0.05) were recorded for thermo-tolerant coliforms and *Escherichia. coli*.

## 4. Discussion

Ensuring food safety at all levels of the production chain has become a fundamental priority for the agro-food industries. Currently food hygiene is based on risk analysis. For meat hygiene, slaughter is considered the stage where the greatest opportunities for contamination exist (*Hammoudi et al.,* 2013) and so the slaughterhouse is a strategic point of intervention for the protection of human health. Strict monitoring of good slaughter hygiene practices is essential in preventing microbial contamination of carcasses. In some countries, slaughter animal carcasses are not subject to criteria for which thresholds must be met, but rather they are subject to process hygiene indicator criteria, the exceeding of which does not require withdrawal measures but corrective actions relating to process hygiene (*OJEU,* 2005). In Algeria; meat inspection at slaughter establishment level is based on visual examination, palpation and compulsory incision of specified organs in order to exclude from consumption meat that would present a danger to the consumer. However, despite the efforts made by the veterinary services to ensure safe meat, hygienic conditions remain far from optimal and the surface contamination of carcasses is significant (*Nouichi and Taha Mossadak,* 2009 ; *Harhoura et al.,* 2012; *Hammoudi et al.,* 2013; *Benaissa et al.,* 2014).

In the absence of Algerian legislation for process hygiene criteria, we referred to the European Union standards which recommend the enumeration of *Enterobacteriaceae* with a lower limit m of

1,5 log CFU/cm$^2$ and an upper limit M of 2,5 log CFU/cm$^2$ (*OJEU,* 2005). Poor surface quality (in terms of hygiene) of sheep and bovine carcasses has been reported by several studies at the national level (*Nouichi and Taha Mossadak,* 2009 ; *Harhoura et al.,* 2012; *Hammoudi et al.,* 2013; *Bennadji et al.,* 2013; *Benaissa et al.,* 2014). According to *Doulgeraki et al.* (2012), the bacterial spoilage of meat depends on the initial number of microorganisms, the time / temperature combination of storage conditions and the physico-chemical properties of the meat. Contamination occurs mainly as a result of poor hygienic and handling conditions in slaughterhouses (*Schlegelová et al.,* 2004).

This lack of hygiene was highlighted by the current study that showed there was no significantly measurable difference in hygiene between the days of sampling. Earlier *Bennadji et al* (2013), showed that hygiene was sufficient on Saturday and Sunday, acceptable on Monday and insufficient on the last three days of the week. The sufficiently hygienic situation as we recorded during (Saturday and Sunday) appeared to be the result of the efficient cleaning carried out at the end of the week.

The results also showed there was no significant difference between the contamination of sheep and bovine carcasses. This was probably due to the slaughtering process for sheep and cattle at the slaughter establishment visited where we noted that the slaughter and the start of skinning took place on the floor for both species. Operators manually tear off the skin. This practice forces them to simultaneously touch the fleece and the carcass. This finding is supported by the study by *Sadoud* (2017) in the Chelf region who reported that slaughter takes place in fixed stations, so the animal is bled, skinned and eviscerated in the same place. In addition, slaughterhouses are, most of the time overcrowded, which promotes contamination. In the study by *Bakhtiary et al* (2016) in Iran where Halal slaughter is carried out reported the bacterial diversity of environmental samples in the sheep slaughter line was higher than that of cattle, probably due to manual slaughter of sheep being practiced on the ground and transmissible contamination via fleece from one animal to another was transmissible. In the cattle slaughter line, all slaughter processes were carried out on a production line with vertical rail dressing and automatic skin removers (*Bakhtiary et al.,*2016). Contamination of carcasses with *Escherichia. coli* can be of concern. Although these bacteria are commensal to the gastrointestinal tract of many animals, some strains that can be very pathogenic including Shiga-toxin producing *E. coli* STEC. The transmission of these pathogens to humans occurs mainly through the ingestion of food including meat contaminated with digestive contents or bovine feces (*Chaucheyras-Durand et al.,*2016).

Contamination of carcasses can also be explained by contamination of the animals themselves, i.e. the skin, which is often soiled with various dirt, mud or feces can be a source of contamination. In the present study, cleanliness assessment of sheep showed the majority of the animals were classes C or D (dirty and very dirty, respectively); while the cattle were mostly classes B or C (slightly soiled and dirty, respectively). According to the *FAO* (2006), sheep fleeces can bring large amounts of dirt and feces into the slaughterhouse. Contamination of sheep carcasses cannot be avoided when the fleece is very dirty. Likewise for bovine carcasses, the skin is a source of contamination. According to *Xianqin et al* (2015) and *Dickson and Acuff* (2017), minimizing skin contamination or decontaminating the skin could reduce subsequent contamination of the carcass.

Skinning and evisceration are the two most influential steps that can contaminate carcasses and equipment with intestinal bacteria (*Lerma et al.,* 2013).

This study confirms the probable participation of personnel, surfaces and the slaughter environment in the final bacterial load of the carcass. During our presence on the site, we noted during the slaughter some anomalies which can be implicated in general hygiene faults and carcass contamination. We noticed that the staff did not wear appropriate work clothes. The clothes they wore were neither washed nor changed during the entire period of our study. Indeed, hands, hair, beards, and aprons can harbour many microorganisms which can pass very easily to the surface of carcasses by direct contact or by splashes (*Labadie*, 1999). At the bleeding level, the operator slaughters the first animal, wipes the blade of the knife used on the fleece of the slaughtered animal, and repeats the same gestures to bleed each animal without rinsing his hands or the knife used. In fact, in most of our slaughterhouses, the equipment (knives and axes) is just rinsed at the end of the day (*Benaissa et al.,* 2014). According to *Labadie* (1999), hooks, storage bins and all equipment (knives, saws, cleavers) that come into contact with meat are soiled by microorganisms. It is essential to remember the fact that each contact brings additional contamination.

The presence of blood, and fat from meat waste on the ground and on the walls contributes to the contamination of carcasses. This state of affairs was reported by *Benaissa et al* (2014) where poorly designed wall coverings with crevices and cracks that were difficult to clean were nests for microorganisms.

It is very likely that all these unconventional behaviours and the poor hygiene of the environment contributed to the poor hygienic quality found in the carcasses.

## 5. Conclusion

In order to guarantee meat safety and thus protect consumer health, it is imperative to control the food from barn to table. The slaughterhouse is one of the major critical points in the meat product production chain, and is where biological risks are probably the most worrying. However, the application of good practices and general hygiene can considerably limit microbial contamination of carcasses.

# Procena kontaminacije trupova u klanici u pokrajini Blida, Alžir

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INFORMACIJE O RADU

*Ključne reči:*
Goveda
Životna sredina
Higijena
Klanje
Ovce

APSTRAKT

Inicijalna kontaminacija mesa se dešava tokom postupka klanja. Cilj ovog istraživanja je bio da se proceni higijena u objektu za klanje proverom površinske kontaminacije trupova i nivoa higijene zaposlenih, životne sredine i opreme. Ukupno su uzeta 122 uzorka (50 sa trupova i 72 od osoblja, opreme i životne sredine). Stanje čistoće životinja procenjeno je za 125 ovaca i 150 goveda. Bakteriološke analize su uključivale broj ukupnih koliforma, termotolerantnih koliforma i Escherichia coli i otkrivanje salmonele.

Trupovi su bili značajno kontaminirani indikatorima higijene bakterija i nije bilo značajnih razlika (P>0,05) u nivoima kontaminacije između vrsta trupova, niti između dana u sedmici ili popisanih grupa mikroba. Procena čistoće životinja pokazala je da je 68% ispitanih ovaca bilo prljavo ili veoma prljavo, a 91,33% goveda je bilo slabo zaprljano ili prljavo. Ispitivanje kontaminacije osoblja, opreme i životne sredine između početka i kraja nedelje nije otkrilo značajnu razliku (P>0,05). Da bi se kontaminacija trupova na nivou klanice svela na minimum, preporučuje se primena dobre higijenske prakse.

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# On-farm welfare conditions of dairy donkeys: A case study in Northern Serbia

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ABSTRACT

The use of donkey milk in human nutrition and the cosmetic industry has led to increased interest in donkey breeding and, consequentially, the intensification of dairy donkey farms, particularly in Europe. Despite the expanding number of farms and greater milk production, there is still no consensus on the management and welfare conditions of donkeys. Therefore, this study aimed to assess and compare the welfare, health and housing conditions of dairy donkeys on five farms in Northern Serbia. The welfare of dairy donkeys on the five farms (A, B, C, D and E) was assessed using the Animal Welfare Indicator (AWIN) protocol for donkeys, represented by four principles (Good Feeding, Good Housing, Good Health and Appropriate Behaviour). Body condition scores were the highest on farms C and D. In addition, none of the examined dairy donkeys on farm D showed any signs of the examined health indicators (except for nasal discharge and hoof neglect) or inappropriate behaviour. Furthermore, the lowest percentage of nasal discharge and hoof neglect was recorded in dairy donkeys on farm D. In contrast, the highest frequency of alopecia, skin lesions, unhealthy hair coat, faecal soiling and hoof neglect was recorded in dairy donkeys on farm A. In conclusion, welfare conditions on farm A were rated as the most unacceptable, while the welfare conditions on farm D were rated as the most acceptable.

## 1. Introduction

Interest in donkey breeding has grown rapidly in recent years, mainly due to the use of donkey milk in human nutrition and the cosmetic industry (*Raspa et al.,* 2019; *Čobanović et al*., 2023). The importance of donkey milk is reflected in its composition, as it is most similar to human breast milk, which qualifies it as an ideal food for infants who have no possibility of being breastfed . Additionally, the hypoallergenic feature of donkey milk provides a quality substitution for children prone to multiple allergies (e.g. allergy to cow milk, hydrolysed cow milk protein, soy, goat milk) (*Dai et al*., 2018; *Martini et al*., 2021). The growing interest in donkey milk has led to the intensification of its production, as well as popularisation of dairy donkey farms (*Valle et al*., 2017). In Europe, the production systems for donkey farms vary from semi-extensive to semi-intensive. When it comes to Serbia, all donkey farms belong to the extensive system type.

However, despite the increasing number of donkey farms and greater milk production, there is still no consensus regarding the management and welfare aspects of these farms (including dairy farms) (*Dai et al*., 2018; *Dalla Costa et al*., 2021). At the end of 2017, the guidelines *Dairy donkeys: good practice principles for sustainable donkey milk production* were compiled, containing suggestions for the proper management of

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dairy donkeys (*Dai et al*., 2019). Despite that, these guidelines are still not widespread enough. The reason for the lack of consensus lies in the fact that very little information is available regarding the proper and adequate care and management of these animals (*Dai et al*., 2017). Additionally, given the different production requirements under which donkeys are kept, including milk and meat production, as well as their involvement in labour and being treated as pets and therapy animals, there are different viewpoints and definitions of donkey welfare (*McLean and Navas Gonzalez*, 2018; *Davis*, 2019). At the level of the European Union, protocols for the evaluation of the welfare of equids (including donkeys) have been developed and proposed in the last few years. The Animal Welfare Indicator (AWIN) assessments protocol for donkeys is based on four Welfare Quality principles and their welfare criteria (*AWIN*, 2015).

In Serbia, the most abundant breed of donkey is the Balkan donkey, which is an autochthonous breed, highly important for the preservation of Serbia's genetic resources. Balkan donkeys are typically medium-sized, with males averaging around 100 cm at the withers and females around 95 cm. Males can weigh up to 250 kg and females up to 200 kg. Their coat colour varies from grey, dark-grey, brown, to chestnut, with most individuals having a darker stripe along the back and a distinct cross pattern on the withers (*Trailović et al.*, 2011; *Stanišić et al.*, 2017). Known for their resilience, they can thrive in harsh environments with minimal care, enduring poor-quality forage, rough terrain and variable weather (*Trailović et al.*, 2011). However, this adaptability can make it difficult to assess their health, as they can often hide signs of distress even in severe conditions (*Deng et al.*, 2021).

Due to the fact that a central database of donkeys in Serbia is still lacking, monitoring the health and welfare of these dairy animals is especially difficult. Therefore, the aim of this study was to determine and compare the welfare, health status and housing conditions of dairy donkeys on five farms in Northern Serbia.

## 2. Materials and Methods

Ethical approval: No ethical approval was obtained because this study did not involve laboratory animals and only involved non-invasive procedures.

This study included a total of 329 dairy donkeys that originated from five different farms in Northern Serbia, visited in March and April 2022. Of the five included farms, one (Farm A) was located in Srem region (n=103), three farms (Farm B, Farm C and Farm D) were located in Bačka region (n=19; n=17; n=30, respectively), and one farm (Farm E) was located in Mačva region (n=160). As a sample for welfare assessment, a minimum of 25% of the total number of donkeys (only lactating individuals) from each farm was evaluated.

The assessment of donkeys was performed according to the AWIN welfare assessment protocol for donkeys (*AWIN*, 2015). The welfare indicators included four principles – Good Feeding, Good Housing, Good Health and Appropriate Behaviour – and twelve criteria, described by the *Welfare Quality*® (2009). The Good Feeding principle was assessed by evaluating the Body Condition Score (BCS), estimation of dehydration by skin tent test and by evaluating water availability. BCS is a standardised method used to monitor the health and productivity of donkeys by assessing their body fat, and it was the only indicator within the Appropriate Nutrition welfare criteria (*AWIN*, 2015). Evaluation of BCS included visual assessment and palpation, and based on the AWIN protocol, was determined using a 5-point scale (score 1 – poor, score 2 – moderate, score 3 – ideal, score 4 – fat, score 5 – obese) (*Burden*, 2012). Assessment of water availability included evaluation of the presence and type of water points, and their functionality and cleanliness. The Good Housing principle was assessed by recording the presence of bedding and its quality, shelter dimensions, and by evaluating the animals for signs of thermal stress. The Good Health principle was assessed by evaluating the condition of hair coat, and by recording the evidence of integument alterations, swollen joints, lameness, prolapses, faecal soiling, discharges (ocular, nasal, genital), abnormal breathing, cheek abnormalities, hoof neglect and hot branding. In individuals with detected discharge, its character was assessed (consistency, transparency, colour and presence of blood). The Appropriate Behaviour principle was assessed by recording the social interaction of donkeys, evidence of stereotypies and by testing the human-animal relationship, which included the Avoidance Distance (AD) test, Walk Down the Side test and Tail Tuck test.

Statistical analysis of the results was conducted using the software GraphPad Prism version 9.5.1 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Differences between farms in most examined welfare indicators (except for BSC) were evaluated using the Chi-squared test. Significant differences for BSC between farms were assessed using the one-way analysis of variance

(ANOVA) and post hoc pairwise comparisons using Tukey's test (mean value and standard deviation). In all cases, significance was fixed at the level of $p \leq 0.05$.

## 3. Results

### *Good Feeding and Good Housing principles*

The results for Good Feeding and Good Housing principles recorded on five dairy donkey farms in Northern Serbia are displayed in Table 1. The BCSs recorded on farms C and D ($p<0.0001$) were higher than those of the other farms. None of the examined donkeys on the five farms showed signs of dehydration (they were all negative on the skin tent test) or thermal stress.

On all visited farms, the water points were troughs. Troughs were dirty on farms A, C and E, partly dirty on farm B, while on farm D, the troughs were clean . No bedding was recorded on any of the assessed farms, while the surroundings where the donkeys were kept were dirty on farms A, C and E, partly dirty on farm B, and clean on farm D. All of the assessed farms provided shelter for their dairy donkeys. The shelter area provided per donkey by the assessed farms were 2.43 $m^2$, 2.11 $m^2$, 11.76 $m^2$, 16.67 $m^2$ and 7.50 $m^2$ (farms A, B, C, D and E, respectively).

### *Good Health principles*

The results for Good Health principles recorded on five dairy donkey farms in Northern Serbia are presented in Table 2. Integument alterations were recorded in donkeys from all of the examined farms, except farm D. The highest frequencies of integument alterations were recorded in donkeys from farm A, where alopecia (90.00%) ($p<0.0001$) and skin lesions (30.00%) ($p=0.0009$) were the most

**Table 1.** The results for Good Feeding and Good Housing principles recorded on five farms in Northern Serbia

| | Farm A | Farm B | Farm C | Farm D | Farm E | Chi-square | df | p-value |
|---|---|---|---|---|---|---|---|---|
| Number of animals (per farm) | 103 | 19 | 17 | 30 | 160 | | | |
| Sample size (per farm) | 40 | 15 | 15 | 20 | 40 | | | |
| *Good feeding* | | | | | | | | |
| Body condition score | $2.25 \pm 0.25^a$ | $2.50 \pm 0.42^a$ | $2.83 \pm 0.24^b$ | $3.00 \pm 0.00^b$ | $2.44 \pm 0.40^a$ | - | - | <0.0001 |
| Dehydrated animals (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Type of water point | Trough | Trough | Trough | Trough | Trough | - | - | - |
| Water point cleanliness | Dirty | Partially clean | Dirty | Clean | Dirty | - | - | - |
| *Good housing* | | | | | | | | |
| Signs of thermal stress (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Shelter | Yes | Yes | Yes | Yes | Yes | - | - | - |
| Shelter area ($m^2$/donkey) | 2.43 | 2.11 | 11.76 | 16.67 | 7.50 | | | |
| Bedding | No | No | No | No | No | - | - | - |
| Surrounding cleanliness | Dirty | Relatively dirty | Dirty | Clean | Dirty | - | - | - |

**Note:** Significant differences (except for BSC) between farms were evaluated using the Chi-squared test. Significant differences for BSC between farms were evaluated using the ANOVA test and post hoc pairwise comparisons using Tukey's test. Different letters in the same row indicate a significant difference at $p \leq 0.05$ $^{(a–b)}$.

**Table 2.** The results for Good Health principles recorded on five farms in Northern Serbia

| | Farm A | Farm B | Farm C | Farm D | Farm E | Chi-square | df | p-value |
|---|---|---|---|---|---|---|---|---|
| Number of animals (per farm) | 103 | 19 | 17 | 30 | 160 | | | |
| Sample size (per farm) | 40 | 15 | 15 | 20 | 40 | | | |
| *Good health* | | | | | | | | |
| Alopecia (%) | 90.00$^{a}$ | 0.00$^{b}$ | 40.00$^{c}$ | 0.00$^{b}$ | 30.00$^{c}$ | 65.76 | 4 | <0.0001 |
| Skin lesions (%) | 30.00$^{a}$ | 0.00$^{b}$ | 0.00$^{b}$ | 0.00$^{b}$ | 10.00$^{b}$ | 18.82 | 4 | 0.0009 |
| Swelling of hoof and coronet area (%) | 0.00$^{a}$ | 20.00$^{b}$ | 0.00$^{a}$ | 0.00$^{a}$ | 0.00$^{a}$ | 23.54 | 4 | <0.0001 |
| Swollen joints (%) | 0.00$^{a}$ | 20.00$^{b}$ | 20.00$^{b}$ | 0.00$^{a}$ | 0.00$^{a}$ | 20.97 | 4 | 0.0003 |
| Unhealthy hair coat (%) | 100.00$^{a}$ | 0.00$^{b}$ | 0.00$^{b}$ | 0.00$^{b}$ | 0.00$^{b}$ | 130.0 | 4 | <0.0001 |
| Lameness (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Prolapse (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Faecal soiling (%) | 90.00$^{a}$ | 20.00$^{b}$ | 20.00$^{b}$ | 0.00$^{c}$ | 0.00$^{c}$ | 91.59 | 4 | <0.0001 |
| Ocular discharge (%) | 50.00$^{a}$ | 20.00$^{b}$ | 20.00$^{b}$ | 0.00$^{c}$ | 90.00$^{d}$ | 56.24 | 4 | <0.0001 |
| Nasal discharge (%) | 50.00$^{a}$ | 0.00$^{b}$ | 20.00$^{c}$ | 10.00$^{bc}$ | 50.00$^{a}$ | 23.08 | 4 | 0.0001 |
| Genital discharge (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Cheek abnormalities (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Abnormal breathing (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Hoof neglect (%) | 90.00$^{a}$ | 40.00$^{b}$ | 60.00$^{b}$ | 10.00$^{c}$ | 20.00$^{c}$ | 53.71 | 4 | <0.0001 |
| Hot branding (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |

**Note:** Significant differences between farms were evaluated using the Chi-squared test. Different letters in the same row indicate a significant difference at p≤0.05 $^{(a–d)}$.

common findings. The highest frequency of swelling of the hoof and coronet area was recorded on farm B (20.00%, p<0.0001). Swollen joints were observed in donkeys on farms B and C, with a frequency of 20.00% (*p*=0.0003). Hair coat was healthy in most of the assessed donkeys, with the exception of farm A donkeys, where all examined individuals (100.00%) had poor quality hair coat (p<0.0001). None of the examined donkeys from the five farms showed signs of lameness, prolapse, genital discharge, dyspnoea, cheek abnormalities or hot branding.

The highest percentage of faecal soiling (p<0.0001) was detected in donkeys from farm A, followed by farms B and C, while it was absent in donkeys from farms D and E. Ocular and/or nasal discharges were observed in donkeys from all assessed farms, with the highest frequency on farm E, where 90.00% of individuals had ocular discharge (p<0.0001) and 50.00% had nasal discharge (p=0.0001). In all cases, the discharge was watery, transparent, colourless and blood-free. On each visited farm, most of the assessed donkeys showed some signs of hoof neglect, with the highest frequency (90.00%) on farm A (p<0.0001).

### *Appropriate Behaviour principles*

The results for Appropriate Behaviour principles recorded on the five dairy donkey farms in Northern Serbia are presented in Table 3. All of the assessed donkeys (100.00%) from five farms had social contact with other conspecifics. The human-animal relationship tests showed that most (93.08%) of the donkeys had a positive relationship with humans. The only exception was noted among donkeys on farm C, with 40.00% of animals showing avoidance behaviour (p<0.0001) and 20.00% having a negative reaction to the walk down the side test (p<0.0001). None of the examined donkeys on the five farms showed signs of fear (negative tail tuck test) and stereotypies.

**Table 3.** The results for Appropriate Behaviour principles recorded on five farms in Northern Serbia

| | Farm A | Farm B | Farm C | Farm D | Farm E | Chi-square | Df | p-value |
|---|---|---|---|---|---|---|---|---|
| Number of animals (per farm) | 103 | 19 | 17 | 30 | 160 | | | |
| Sample size (per farm) | 40 | 15 | 15 | 20 | 40 | | | |
| Social contact (%) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | - | - | - |
| Stereotypies (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |
| Avoidance behaviour (%) | 0.00[a] | 0.00[a] | 40.00[b] | 0.00[a] | 0.00[a] | 48.23 | 4 | <0.0001 |
| Negative walking down the side (%) | 0.00[a] | 0.00[a] | 20.00[b] | 0.00[a] | 0.00[a] | 23.54 | 4 | <0.0001 |
| Tail tuck (%) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | - | - |

**Note:** Significant differences between farms were evaluated using the Chi-squared test. Different letters in the same row indicate a significant difference at $p \leq 0.05$ [(a–b)].

## 4. Discussion

This study assessed the welfare conditions of dairy donkey farms in Northern Serbia, and using the AWIN Welfare Protocol, the observed animals were evaluated according to several welfare criteria.

Determination of BCS answers whether the donkey's energy requirements have been fulfilled. BCS can vary depending on the several factors, like season, food availability, physical activity, reproductive condition, dental problems, parasitic infections, diseases etc. (*AWIN*, 2015). In the present study, most of the dairy donkeys had a BCS between 2.0 and 3.0, except on farm A, where the BCSs were between 2.0 and 2.5. The obtained results indicate that dairy donkeys on these farms are more likely to be slightly thin rather than obese, which is consistent with the results reported by *Dai et al*. (2018). This can also be attributed to the fact that donkeys, during the first few months of lactation, can lose body weight, despite being on a balanced diet (*Cruz et al*., 2021). For this reason, BCS is an important parameter in dairy donkeys, and it is recommended a BCS between 3.5 and 4.0 is reached before foaling (*Raspa et al*., 2019).

Based on the AWIN protocol (2015), the shelter area per donkey was satisfactory on farms C, D and E, and higher than the recommended 7 $m^2$ per animal (height at the withers between 120 and 148 cm). In contrast, the space allowance per donkey in the shelter area was unsatisfactory on farms A and B, indicating inappropriate housing conditions on these farms (*AWIN*, 2015). All of the visited farms were equipped with troughs as water points. The trough and water were clean only on farm E, while on the other farms, the troughs were either dirty or partly dirty. Dirty water is one of the reasons donkeys may refuse to drink, and if insufficient water is intaken, gastrointestinal problems, such as constipation and colic, can result (*Smith and Burden*, 2013; *Raspa et al*., 2019). The donkey's natural adaptations to survive in poor environmental conditions are possibly the reasons why the provision of water is often neglected in the literature (*Pearson*, 2005; *Deng et al*., 2021). Although donkeys have lower water needs than other domesticated animals, lactating donkeys need twice as much water compared to non-producing donkeys (*Raspa et al*., 2019; *Farias et al*., 2021). Despite the fact that the results of this investigation showed non-ideal conditions of some water points, the dairy donkeys on the observed farms did not show signs of dehydration (skin tent tests were negative). Considering this, it can be assumed that these dairy donkeys did not refuse to drink water even when it was supplied from dirty troughs. Nonetheless, adequate water intake is an important welfare parameter, and owners should be educated about donkeys' water needs (*Dai et al*., 2016).

Integument alterations were one of the most common welfare problems found in this study, while various factors can lead to these pathologies: equipment used on animals, the type and intensity of the work performed by animals, trauma/injuries, diseases etc. (*Cruz et al*., 2021). The changes noted in this study included hairless patches (alopecia), scabs, skin lesions, wounds and swellings. Alopecia was the most frequently observed integument alteration, with the highest occurrence on farm A. The possible reasons for this condition could be ectoparasites, fungal diseases or other conditions that cause pruritus (*Dai et al*., 2016). Among other

integument changes, skin lesions were observed in donkeys from farms A and E, while swelling in the hoof area was seen in individuals from farm B. The fact that the farms did not have bedding for the animals could have contributed to the observed skin lesions, given that there is literature data on the connection between the lack of bedding and the occurrence of skin lesions (*Dalla Costa et al*., 2014; *Dai et al*., 2018). Also, the mutual contact of animals is an additional risk factor for the occurrence of skin lesions (*Dalla Costa et al*., 2014).

Joint swelling was observed in dairy donkeys from farms B and C, in about 20% of the animals. This swelling occurs due to an increase of fluid in the tissue surrounding the joint (*AWIN*, 2015). As an extremely painful condition for the individual, joint swelling can potentially indicate the presence of arthritis, injury, infection or broken bones (*AWIN*, 2015).

Hair coat was evaluated as healthy in all assessed donkeys, except on farm A, where 100% of individuals had unhealthy hair coat. Hair coat condition indicates the hair coat health, as well as the health status of the animal in general, considering that the coat loses its quality if the individual is sick or has poor nutrition (*AWIN*, 2015; *Cruz et al*., 2021).

The presence of faecal residues on hind limbs was observed in dairy donkeys from farms A, B and C, with the highest frequency on farm A (90.00%). When faecal soiling is detected, it is an indicator of diarrhoea, which mostly suggests a clinical condition (*Dalla Costa et al*., 2014).

Ocular and nasal discharges were found in the donkeys on all evaluated farms, with different frequencies. Depending on its characteristics, discharge from the nostrils and/or eyes can be a symptom of a specific localised or generalised disease. Based on the character of discharge, and the fact that the assessment of well-being on these farms was carried out in the period of transition from winter to spring and there were still notable temperature variations during the day, it can be assumed that the discharge is not indicative of a specific disease, but a consequence of the individuals being in the cold. Nonetheless, when nasal and/or ocular discharge is observed, it is advised that a more detailed physical examination is carried out (*Cruz et al*., 2021; *Mshelia et al*., 2023).

Within the framework of this research, some degree of hoof neglect was seen in most dairy donkeys on the visited farms, with the highest frequency on farm A. The condition of the hooves is one of the leading welfare problems of this animal species (*Dai et al*., 2018). Adequate hoof care implies regular and proper hoof trimming, as well as a number of other factors that consequently affect the hoof condition, such as a balanced diet and adequate pens on the farm (floors, fences, gates and corridors) (*Raspa et al*., 2019). The problem of neglected hooves is, consequently, related to many other conditions and problems interfering with health and well-being of donkeys (*Dai et al*., 2018; *Thiemann and Poore*, 2019). The pain and stress that donkeys experience due to overgrown or improperly trimmed hooves lead to more frequent and longer periods of lying down, lack of movement and reduced food intake. This may be the reason why the dairy donkeys from farm A, where the highest frequency of hoof neglect was observed (90.00%), had the lowest BCSs, compared to individuals from other farms. On the other hand, improper care of a donkey's hooves can cause extremely painful conditions for the animal, including lameness, laminitis and chronic hoof disease (*Dai et al*., 2016; *Dai et al*., 2018; *Raspa et al*., 2019). It is important to note that the education of owners and keepers on adequate hoof care procedures of dairy donkeys is immensely important for the prevention of these conditions.

Based on the behavioural observations in this study, all of the assessed dairy donkeys had positive human-animal relationships, except those on farm C, where avoidance behaviour (40.00%) and negative reaction to the walking down the side test (i.e., negative reaction to the observer's movement) (20.00%) were noted. Human-animal relationship tests can show the quality of the relationship between the animals and humans. The welfare of donkeys is directly influenced by the way they perceive and engage with humans (*AWIN*, 2015). The presence of avoidance behaviour and negative reaction to the observer's movement could indicate mistreatment by the animal's owner (or carer) and, therefore, a state of poor welfare. However, whether the tests were conducted by a person unfamiliar to the animal, to whom the donkeys were not adapted, should be taken into account (*Mshelia et al*., 2023). This may explain the negative results obtained in this research, and not necessarily indicate mistreatment by the animals' owner. Nonetheless, the assessment of human-donkey relationship as an indicator of welfare is highly significant, and it is essential to highlight the importance of educating owners regarding the proper treatment of their donkeys (*Cruz et al*., 2021).

## 5. Conclusion

The results of this study showed different housing and welfare conditions between five dairy donkey farms in Northern Serbia. The greatest differences were observed in terms of integument alterations and hoof condition. The significance of these parameters lies in their connection with potential health disorders and pain, which can seriously compromise on-farm donkey welfare. The poorest welfare conditions were observed on farm A, shown by the highest frequency of alopecia, skin lesions, unhealthy hair coat, faecal soiling and hoof neglect. On the other hand, the most acceptable welfare conditions were observed on farm D, whereby none of the examined dairy donkeys showed any signs of poor health (except the lowest frequencies of nasal discharge and hoof neglect among the five farms) or inappropriate behaviour.

# Uslovi dobrobiti muznih magarica na farmi: prikaz slučaja u severnoj Srbiji

*Marija Kovandžić, Štefan Pintarič, Jasna Đorđević, Tijana Ledina, Radoslava Savić Radovanović i Nikola Čobanović*

INFORMACIJE O RADU

*Ključne reči:*
Muzne magarice
Zdravlje
Mleko
Dobrobit
Balkanski magarac

APSTRAKT

Upotreba mleka magarica u ishrani ljudi i kozmetičkoj industriji je dovela do povećanog interesovanja za uzgoj magaraca i posledično, do intenziviranja farmi muznih magarica, posebno u Evropi. Uprkos povećanju broja farmi i proizvodnje mleka, još uvek ne postoji konsenzus u pogledu menadžmenta i uslova dobrobiti magaraca. Cilj ovog rada je procena i poređenje dobrobiti, zdravlja i uslova smeštaja muznih magarica na pet farmi u severnoj Srbiji. Dobrobit muznih magarica sa pet farmi (A, B, C, D i E) procenjena je upotrebom AWIN protokola za magarce, predstavljenog kroz četiri principa (dobra ishrana, dobar smeštaj, dobro zdravlje i adekvatno ponašanje). Ocena telesne kondicije je bila najviša na farmama C i D. Pored toga, nijedna od ispitanih muznih magarica sa farme D nije pokazala znake loše dobrobiti u okviru ispitanih zdravstvenih parametara (osim nosnog iscetka i zanemarenih kopita), kao ni znake neadekvatnog ponašanja. Takođe, najmanji procenat nosnog iscetka i zanemarenih kopita je zabeležen kod muznih magarica sa farme D. Nasuprot tome, najveća učestalost alopecije, lezija kože, nezdravog dlačnog pokrivača, zaprljanosti fecesom i zanemarenih kopita zabeležena je kod muznih magarica na farmi A. U zaključku, uslovi dobrobiti na farmi A ocenjeni su kao najneprihvatljiviji, dok su uslovi dobrobiti na farmi D ocenjeni kao najprihvatljiviji.

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**Note:** In Meat technology 64(1) 2023 in the published article „Development, characterization and investigation of antimicrobial and antioxidant potential of sodium caseinate-based edible films infused with Berberis pseudumbellata fruit extract, and effects of the films on the quality of raw ground beef during refrigeration" by Habiba Shah, Shakeel Ahmed, Faizah Urooj, Sidra Zaheer and Nilofer Fatimi Safdar Figure 2 has been inadvertently duplicated in place of Figure 3.

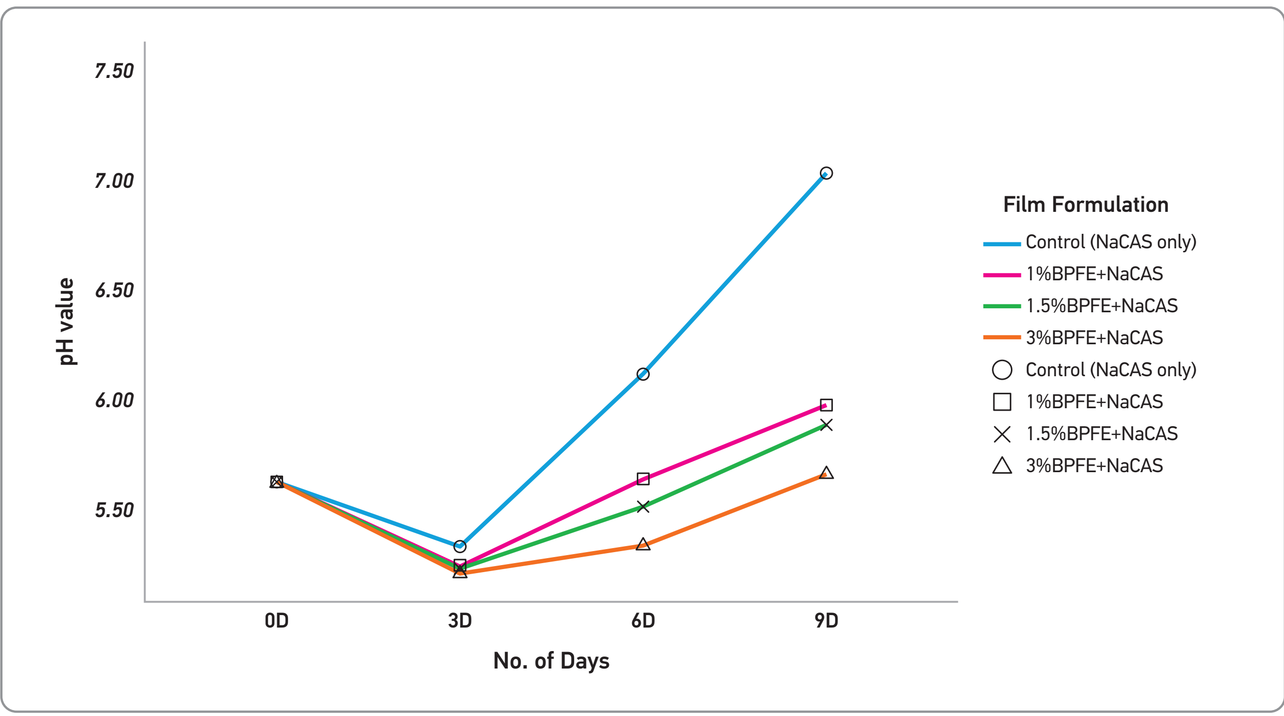


**Figure 2.** The pH of ground beef covered with NaCAS films with/without *Berberis pseudumbellata* fruit extract (infused/non-infused) during storage at 7°C for 9 days.

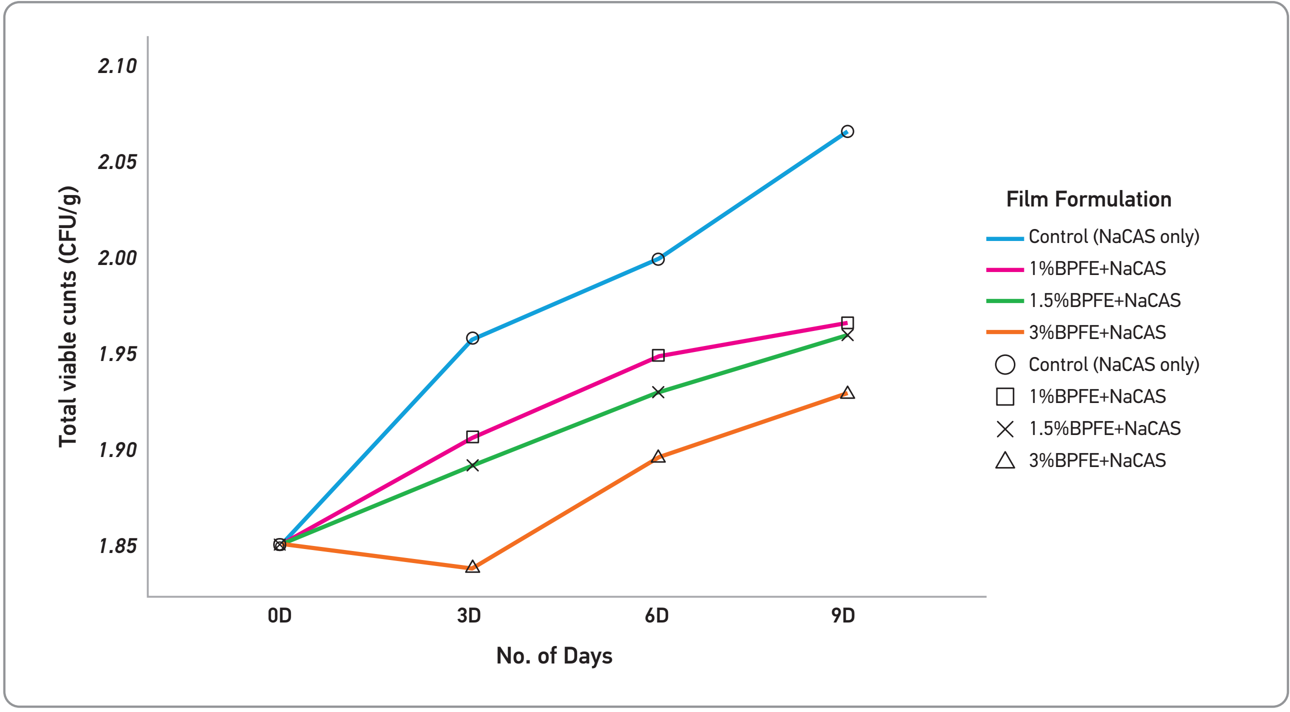


**Figure 3.** Total viable counts (TVC) of ground beef covered with NaCAS films with/without *Berberis pseudumbellata* fruit extract (infused/non-infused) during storage at 7°C for 9 days.

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