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MICROBIOLOGICAL QUALITY OF SOUS VIDE FISH MEAT TREATED WITH HONEY

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ABSTRACT

The research was aimed at studying the influence of sous vide heat treatment on the microbiological quality of fish meat marinated in honey sauce. The samples were vacuum packed and cooked at 55°C, 60°C, 65°C and 70°C for 15 minutes. The samples were first stored for 24 hours at 4°C and then stored for 7 days at 4°C. The microbiological quality of fish meat in honey sauce were examined. Microbiological indicators such as the total viable count (TVC), the number of coliform bacteria (CB) and the lactic acid bacteria (LAB) were determined. The microbiota was identified by mass spectrometry using a MALDI-TOF MS Biotyper (Bruker Daltonics, Germany). Microbial counts varied significantly depending on temperature and storage in honey sauce. TVC in raw fish meat was 2.89 log CFU.g^{-1} stored at 4°C. TVC in raw fish meat was 4.56 log CFU.g^{-1} after 7 days of storage at 4°C. TVC in sous vide fish meat samples without honey ranged from 1.62 to 3.02 log CFU.g^{-1}. TVC in sous vide fish meat samples with honey sauce was < 1 log CFU.g^{-1}. The presence of coliform bacteria was not detected in the samples. LAB in raw fish meat was 2.47 log CFU.g^{-1} in the sample stored at 4°C. LAB in raw fish meat was 2.77 log CFU.g-1 after 7 days of storage at 4°C. LAB in sous vide fish meat samples without honey ranged from < 1 to 1.33 log CFU.g^{-1}. LAB in sous vide fish meat samples with honey sauce ranged from < 1 to 2.66 log CFU.g^{-1}. Our results show, that in the samples that were stored in honey sauce, the number of microorganisms decreased and even no microorganisms were detected.

Keywords: fish meat, sous vide, honey, the total plate count, the number of coliform bacteria, lactic acid bacteria, MALDI-TOF MS Biotyper

INTRODUCTION

Sous vide processing is a well-known long-established French practice of food preparation and the term sous vide implies “under vacuum” in French (**Thathsarani *et al.*, 2022**). Cooking food in a water bath for an extended period of time—roughly seven to seventy-two hours—while it is wrapped in a plastic bag is known as sous vide cooking, sometimes known as low-temperature cooking or slow cooking (**Baldwin, 2012**). Many crucial results are obtained from the sous vide processing method, such as a decrease in chemicals and preservatives, a different strategy to reduce microbiological presence, and an increase in the shelf life of a product. (**Choi *et al.*, 2023**). Sous vide makes use of the low-temperature, long-time (LTLT) cooking method. The sous-vide may be cooked at any temperature between 65 and 95 °C. It is gaining popularity over traditional heat treatment because of the enhanced sensory quality and greater nutritional retention (boiling, roasting, frying and steaming) (**Ayub *et al.* 2019**). Unlike other techniques, sous vide uses uniform heat conduction during immersion to achieve a precise degree of doneness across the food product. Over the past ten years, the popularity of sous vide cooking has skyrocketed because to the availability of several cookers that are user-friendly and reasonably priced (**Hunt *et al.*, 2023**).
Fish and shellfish are among the foods that decay the fastest because of their high water activity and neutral pH, which promotes the growth of harmful bacteria and deterioration. The large concentrations of free amino acids in them that are susceptible to oxidation also reduce their shelf life. (**Coşansu *et al.*, 2022**). However, sous vide technology's vacuum packing prevents the oxidation and off-flavour that results from this. Fish prepared sous vide has a longer shelf life than fish prepared the old-fashioned way (**Zakrzewski *et al.*, 2023**). Compared to traditional cooking methods, sous-vide cooking has less detrimental impact on the nutritional and sensory qualities of seafood. However, since sous-vide cooking only involves a modest amount of heat, combining it with other precautions could increase the safety and quality of fish meat (**Coşansu *et al.*, 2022**).
Honey is defined as “the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature” (**Council directive, 2001**). Because of its distinct flavor, rich nutritional content, and many health advantages based on its chemical makeup, honey is considered a high-value food item that is often consumed. Globally, honey is produced and sold with notable variations in its biological significance and nutritional worth. Honey's antibacterial, anti-inflammatory, and antioxidant qualities are primarily linked to its functional and health-promoting qualities, and these qualities have been utilized in medicine (**Zhang *et al.*, 2021**). Honey is being added to a variety of foods, including sausages, beef patties, fish, fruits, and vegetables, to increase their bioactive qualities or to prolong their shelf life. It is also being used as a sugar alternative in chocolates, confectionary, and bakery goods (**Hakim *et al.*, 2019**). Since honey has a distinct flavor and exceptional health benefits, the worldwide honey business is focused on confirming its authenticity. Nevertheless, the honey industry has difficulties since the present quality standards and analytical techniques are still insufficiently efficient in determining and evaluating the authenticity of honey (**Tsagkaris *et al.*, 2021**).
The aim of the present study was to determine the change in the microbiological quality of raw fish meat treated with the sous vide method and stored in honey marinade.

MATERIAL AND METHODS

The aim of study was to evaluate the microbiological quality of raw fish meat after processing. The fresh fish was used in the experiment. The species of fish used for the analysis was large catfish (*Silurus glanis*). The fresh fish was purchased from a private seller. Before packaging and sous vide treatment, the fish was stored in a honey marinade for 24 hours. We performed microbiological analysis on samples of raw fish treated sous vide and on samples of raw fish stored in honey marinade and treated sous vide. The honey marinade was prepared from sunflower honey and sunflower oil. The microbiological parameters, we evaluated the total viable count (TVC), the number of coliform bacteria (CB) and lactic acid bacteria (LAB). All samples were then subjected to the identification of microorganisms using the MALDI TOF-MS Biotyper.
The microbiological analyzes were performed after 24 hours and after 7 days of storage of samples at temperature 4°C. A total of 18 samples in three replicates were analyzed

The ways of fish meat samples were listed in table 1.

Table 1 Ways of fish samples packaging

C	4 °C
R 55 °C 15'	R+M 55 °C 15'
R 60 °C 15'	R+M 60 °C 15'
R 65 °C 15'	R+M 65 °C 15'
R 70 °C 15'	R+M 70 °C 15'

Legend: C- control samples of raw fish without heat treatment and without the addition of honey, R - samples of fish meat treated by the sous vide method, R+M – samples of fish meat after application of honey and treated with the sous vide method

Plate dilution method

The plate dilution method was used to determine the number of colony forming units (CFU) in the samples. The first basic dilution (10^{-1}) was prepared by homogenizing 5 g of the sample with 45 ml of physiological solution and then we homogenized the solution for 30 minutes.
Dilution plating method was used to determine the microorganisms. For microorganism cultivation three types of cultivating mediums were used. Plate Count Agar (PCA, Oxoid, Basingstoke, UK) was used to cultivation of total viable counts and inoculated Petri dishes were incubated in a thermostat at 30 °C for 24 hours. Violet Red Bile Lactose Agar (VRBL, Oxoid, Basingstoke, UK) was used to cultivation of coliform bacteria, inoculated Petri dishes were incubated in a thermostat at 37 ° C for 24 to 48 hours. Rogosa and Sharpe agar (MRS, Oxoid, Basingstoke, UK) was used to cultivation of lactic acid bacteria. The inoculated Petri dishes were incubated in a thermostat with 5 % of CO_2 at 37 °C for 48-72 hours.

Recultivations of culture

The bacterial and yeasts colonies were subcultured on TSA agar (Tryptone Soya Agar, Oxoid, UK) for 18 –24 h prior to the identification. It is a universal, non-selective medium that provides nutrients for a wide range of microorganisms. It is used for a wide range of applications, including culture storage, cell counting or isolation of pure cultures. We poured 20 ml of TSA agar tempered to 45 ˚C +/- 1 °C into sterile Petri dishes. One colony of eight bacterial isolate was selected.

Identification of microorganisms using the MALDI-TOF MS Biotyper
Distilled water in the volume 300 µl was pipetted into the Eppendorf tubes. The biological material was taken from the culture dishes, transferred it to a test tube with water and mixed it well. Then we pipetted 900 µl of ethanol and mixed the contents of the tube well again. The samples were mixed using a centrifuge for two minutes. After centrifugation, we poured off the supernatant and centrifuged again. The remaining ethanol was removed and let the pellets dry for a few minutes. Formic acid (70 %) in the volume 50 µl was pipetted to the pellet and thoroughly mixed by pipetting and vortexing. After vortexing, 50 µl of acetonitrile was added and mixed thoroughly. The samples were centrifuged again at maximum speed for two minutes. After centrifugation, we pipetted 1 µl of the supernatant onto the plate and allowed it to dry. After drying, we covered the supernatant with 1 µl of the MALDI matrix solution. After drying, we inserted the plate into the device for identification.

Statistical analysis

Every analysis was carried out three times. The statistical variability of the data was handled with the aid of Microsoft Excel®. Analysis of variance (ANOVA) was used to evaluate the results.

RESULTS AND DISCUSSION

We investigated the microbiological quality of fish meat sous vide and the changes in the number of microbiological indicators after the use of honey marinade. A total of 18 samples were analyzed. The samples were examined after for 24 hours and after 7 days of storage at a temperature of 4°C.
From the microbiological quality, we determined the total viable count (TVC), the number of coliform bacteria (CB) and the lactic acid bacteria (LAB). We also focused on the identification of individual types of microorganisms using the MALDI-TOF MS Biotyper mass spectrometry device. We reported all results of microbiological indicators in log CFU.g^{-1}.

Determination of the total viable count (TVC)

TVC in raw fish meat was 2.89 log CFU.g^{-1} stored at 4°C. TPC in raw fish meat was 4.56 log CFU.g^{-1} after 7 days of storage at 4°C. TVC in sous vide fish meat samples without honey ranged from 1.62 to 3.02 log CFU.g^{-1}. TPC in sous vide fish meat samples with honey sauce was < 1 log CFU.g^{-1} (figure 1).

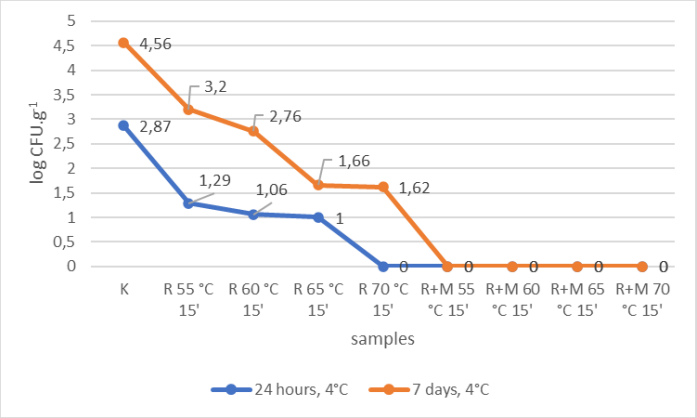

Figure 1 Average numbers (log CFU.g^{-1}) of TVC in samples of fish meat during 15 days of storage at 4 °C

The restriction of fast microbiological development, such as total mesophilic bacteria growth, for highly perishable foods is an important concern for food safety and consumer health in the food industry (**Çetinkaya *et al.*, 2021**). Because fish flesh has higher water activity and less connective tissue than the connective tissue of terrestrial animals, among other reasons, it is more perishable (**Petricorena, 2015**).
According to the worldwide standards for quality control, the lower microbiological counts might be explained by the honey's intrinsic qualities of lower pH, low moisture content, freshness, quality of processing, storage, and acceptable handling (**Laaroussi *et al.*, 2020**). Antimicrobial activity is regarded as one of the most significant benefits of honey for human health. It has previously been documented that stingless bee honey from different species and geographical areas has antibacterial action against a variety of diseases (**Avila *et al.*, 2018**).

Determination of the number of coliform bacteria (CB)

The presence of coliform bacteria was not detected in the samples.
According to definitions, coliform bacteria are rod-shaped, gram-negative, nonendospore-forming bacteria that digest lactose at 35–37°C and produce acid and gas as a by-product. Compared to other coliforms, faecal coliforms—of which E. coli makes up the great majority—are thought to be more closely linked to feces contamination from warm-blooded vertebrates Faecal coliforms are coliforms that produce gas within 48 hours at 44.5°C (instead of 35–37°C for coliform testing) by fermenting lactose in EC medium (EC stands for E. coli). The U.S. EPA regulation stipulates a zero limit for bacteria that cause coliform and faecal coliform (**Shen *et al.*, 2022**).
Overall, sulfite-reducing anaerobic bacteria, total coliforms, and Salmonella spp. were never detected in the analyzed samples (data not shown). **Russo et al. (2023)** analyzed the microbiological quality of different honey samples. They found out, that all analyzed samples fulfilled the European standard for all tested microbial groups in accordance with the current legislation on microbiological criteria applicable to foodstuffs (EC Regulation no. 2073/05). In honey samples from citrus (Z) and eucalyptus (E), Bacillus cereus was found, but the cell density eventually returned to within acceptable bounds as recommended by EC Regulation no. 2073/05. Only three samples contained bacteria beloínging to family Enterobacteriaceae.

Determination of the lactic acid bacteria

LAB in raw fish meat was 2.47 log CFU.g^{-1} stored at 4°C. TPC in raw fish meat was 2.77 log CFU.g^{-1} after 7 days of storage at 4°C. LAB in sous vide fish meat samples without honey ranged from < 1 to 1.33 log CFU.g^{-1}. TPC in sous vide fish meat samples with honey sauce ranged from < 1 to 2.66 log CFU.g^{-1} (figure 2).

One of the most widely used fermenting agents for meat products that have undergone fermentation is lactic acid bacteria (LAB), which is a general term for a class of gram-positive bacteria that lack budding and negative peroxidase. As of 2023, there are 25 genera, 589 species, and 58 subspecies of Lactobacillus. It can utilise carbohydrates to produce lactic acid and to produce antibacterial peptides and bacteriocins, which prevent the growth of pathogenic and spoilage bacteria. Moreover, LAB can encourage the breakdown of proteins, lipids, and carbohydrates and produce small molecules like peptides (**Xia *et al.*, 2023**). Lactic acid bacteria (LAB) are a diverse group of microorganisms that produce lactic acid as the primary end-product of the fermentation of carbohydrates (**Carr *et al.*, 2002**). The genus Weissella, which was previously categorized under the names Leuconostoc and Lactobacillus, is present in a range of meat products, such as processed and fermented meat products, fresh and vacuum-packed meats, and poultry (**Pringsulaka *et al.*, 2012**).

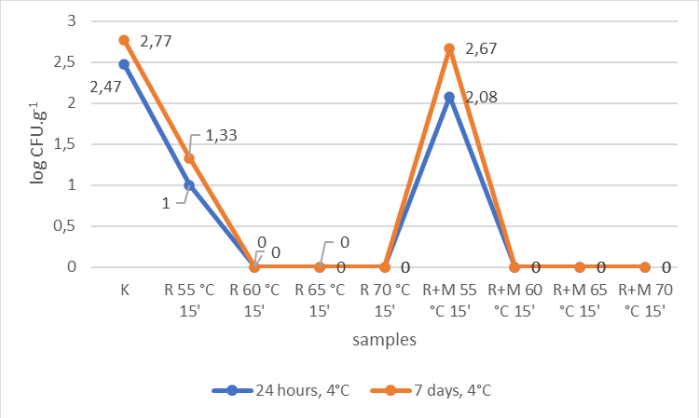

Figure 2 Average numbers (log CFU.g^{-1}) of LAB in samples of fish meat during 15 days of storage at 4 °C

Table 2 Isolated bacteria from samples of fish meat during 7 days of storage at 4 °C

Microorganism	**Sample**			
	C	**R 55 °C 15´**	**R 65 °C 15´**	**R+M 55 °C 15´**
Staphylococcus epidermidis	3	-	-	-
Staphylococcus lugdunensis				1
Staphylococcus warneri	1	1	-	2
Acinetobacter johnsonii	3	-	-	-
Chryseobacterium indologenes	1	-	-	-
Aeromonas popoffii	2	-	-	-
Aeromonas eucrenophila	1	-	-	-
Aeromonas media	1	-	-	-
Pseudomonas lundensis	-	8	-	-
Ralstonia pickettii	-	-	2	-
Rhizobium radiobacter	-	-	-	1
Kocuria rhizophila	-	-	-	6

Legend: C - raw fish without heat treatment and without the addition of honey, R - designation of samples with fish treated by the sous vide method, R+M – designation of samples with fish and honey treated with the sous vide method.

Table 3 Families of isolated microorganisms from fish samples

Microorganisms	**Family**
Staphylococcus epidermidis	Staphylococcaceae
Staphylococcus lugdunensis	
Staphylococcus warneri	
Acinetobacter johnsonii	Moraxellaceae
Chryseobacterium indologenes	Flavobacteriaceae
Aeromonas popoffii	
Aeromonas eucrenophila	
Aeromonas media	Aeromonadaceae
Pseudomonas lundensis	Pseudomonadaceae
Ralstonia pickettii	Burkholderiaceae
Rhizobium radiobacter	Rhizobiaceae
Kocuria rhizophila	Micrococcaceae

Table 3 presents the families of isolated microorganisms. The most frequently isolated microorganisms belong to the family Staphylococcaceae and Pseudomonadaceae. Other isolated microorganisms belong to families Micrococcaceae, Rhizobiaceae, Burkholderiaceae, Aeromonadaceae, Moraxellaceae and Flavobacteriaceae.

When chilled meat is packaged under aerobic conditions, the genus and species of Pseudomonas, Acinetobacter, Brochothrix, and Shewanella are typically thought to be the dominant communities. These organisms can cause slime, discoloration, and an off-odor, and the majority of them are psychrotrophic. Research on spoilage microorganisms currently ignores the heterogeneity among individual strains within a genus and instead concentrates on identifying the dominant spoilage bacteria, which is primarily determined by the quantity and abundance of bacteria in a given system (**Vasconcelos *et al.*, 2014**). About 36 species of the Gram-negative bacterium *Aeromonas* spp. are commonly linked to food, including *Aeromonas salmonicida*, *Aeromonas veronii*, *Aeromonas media*, *Aeromonas hydrophila*, and *Aeromonas caviae* (**Umutoni *et al.*, 2020**). Numerous species of Aeromonas can thrive in both raw and cooked food in a modified environment at cooled temperatures. Prior research has shown that *Aeromonas* isolates can cause fish and shrimp to produce more trimethylamine and total volatile basic nitrogen (TVBN), which can cause a strong odor and a loss of freshness (**Liu *et al.*, 2018**).

Four main ingredients in honey are primarily responsible for its antibacterial, anti-biofilm, and anti-quorum sensing properties: phenolic compounds, hydrogen peroxide, methylglyoxal, and bee defensin-1. Honey components affect the cell cycle and morphology of bacteria, changing their performance. To the best of our knowledge, this is the first review that lists every phenolic compound found in honey and describes how it might work against bacteria. Moreover, honey may serve as a possible delivery system for some beneficial lactic acid bacteria strains, including Bifidobacterium, Fructobacillus, and Lactobacillaceae, as well as Bacillus species (**Khataybeh *et al.*, 2023**).

Results of identification of microorganisms using MALDI-TOF MS Biotyper

Isolated bacteria and the number of the isolates are presented in Table 2. The most frequently isolated bacteria were *Pseudomonas ludensis* (8 isolates), followed by *Kocuria rhizophila* with 6 isolates and *Staphylococcus warneri* with 4 isolates (table 2).

Microbial activity and endogenous enzymes are the causes of fish spoilage (**Xie *et al.*, 2018**). Specific spoilage organisms (SSOs), which include *Pseudomonas* species, *Shewanella* species, *Acinetobacter* species, and *Aeromonas* species, are the main spoilage organisms responsible for the spoiling of seafood (**Carvalheira *et al.*, 2016**). *Ammonia, trimethylamine, and an off-odor are produced during the breakdown of nitrogenous materials by Pseudomonas and Acinetobacter species. Under ideal laboratory conditions, these investigations looked at one bacterial species' potential for spoiling and its volatile organic compounds* (**Parlapani *et al.*, 2019**). The genus Chryseobacterium is a member of the large family Flavobacteriaceae, which includes more than 120 species. It has been isolated from a variety of sources, including food, water, plant roots, sea sediments, flowers, sludge, and decomposing biomass found in mosquito midguts, bird feathers, cow's milk, raw meats, poultry, birds, and contaminated soils. The bacteria are widely dispersed, have been found in sick animals, and could potentially become a new threat to freshwater and marine fish populations (**Oh *et al.*, 2020**Staphylococcus warneri, Aeromonas salmonicida, and Aeromonas popoffii from the control group treated with EDTA, Staphylococcus hominis, and Staphylococcus epidermidis from meat treated with caraway essential oil (EO) were all isolated by **Kunová et al. (2017)**. **Kačániová et al. (2019)** identified 15 genera from meat treated with essential oils and in vacuum packaging, including Aeromonas, Aromatoleum, Buttiauxella, Clostridium, Enterobacter, Hafnia, Lactobacillus, Lysinobacillus, Pantotea, Pseudomonas, Rahnella, Raoultella, Serratia, Staphylococcus, and Yersinia

CONCLUSION

Raw fish meat supports the growth of microorganisms and is subject to spoilage. Storage and further processing can affect the microbiological quality of fish meat. Our study proves that the sous vide method is an effective way of preparing meat. Furthermore, we proved that fish meat stored in honey sauce has better microbiological parameters than fish meat without honey sauce. These microbiological results demonstrate the antimicrobial properties of honey and demonstrate that honey has self-cleaning and micro-organism-killing capabilities and improves food quality. We achieved the best results of microbiological parameters with samples of fish meat stored in honey sauce.

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