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Diversity of mesophilic and psychrophilic proteolytic bacteria in different minced meat samples

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Abstract

Since minced meat provides a large contact surface, it is a food in which the total bacterial load and the enzymatic profiles of the species that make up this load should be addressed, even in cases where it does not contain pathogens. Therefore, this study aimed to investigate the effect of freezing, one of the most commonly used storage methods for minced meat, on total and proteolytic bacterial loads. To simulate the actual storage process, individuals belonging mainly to the genera *Bacillus, Serratia, Pseudomonas, Citrobacter, Acinetobacter*, and *Escherichia* were isolated from the samples examined directly and after 15 days of freezing, without any process that would cause a change in the microbial load. 71.11% of these isolates exhibited valine and cystine aminopeptidase activity, while 37.77 and 33.33% exhibited trypsin and α-chymotrypsin activity, respectively. In addition, the results show that cryopreservation allows psychrophilic proteolytic bacteria to increase their numbers in meat.

Keywords: cryopreservation; mesophilic bacteria; minced meat; proteolytic enzymes; psychrophilic bacteria; spoilage

Introduction

Meat and meat products are the primary protein sources consumed by people worldwide. However, these protein sources are not only nutritious for humans, but owing to their high protein, carbohydrates, lipids, minerals, and water contents, they are also a suitable environment for the reproduction of microorganisms (Adame *et al*., 2024; Elbarbary *et al*., 2024). Although the inner parts of animal meat are considered sterile before slaughter under normal conditions, they may be contaminated with microorganisms during butchering, transportation, or marketing phases, as the meat comes into contact with other surfaces (Al-Obaidi *et al*., 2021; Atasever and Atasever, 2014).

In addition to the cross-contamination during the processing of carcasses, factors such as temperature of the storage environment and chemical factors such as moisture, water activity (aw), and pH of the meat, inadequate hygiene of processing machines, and inadequate packaging and storage conditions also cause an increase in the deterioration of meat and meat products. Many bacteria such as lactic acid bacteria (LAB), *Pseudomonas* spp., *Acinetobacter* spp., *Aeromonas* spp., *Brochothrix thermosphacta*, *Flavobacterium* spp., *Psychrobacter* spp., *Moraxella* spp., and Enterobacteriaceae, when colonized on meat and meat products, secrete metabolites that adversely affect the quality of meat (appearance, texture, color and taste, of course) and make it unfit for consumption (Casaburi *et al*., 2015; Doulgeraki *et al*., 2012; Ercolini *et al*., 2006; Marcelli *et al*., 2024).

Microorganisms reproduce on meat and initiate the degradation process with their extracellular metabolites. In this enzymatic event, factors such as pH, temperature, or

oxygen content, which vary depending on the structural properties of the meat and storage conditions, may cause differences in the process or duration of meat spoilage. In other words, meat spoilage process is influenced by bacterial populations and environmental conditions (Ercolini *et al*., 2009; Shi *et al*., 2024). Contaminated meats, whether visibly spoiled or in the early stages of deterioration, present significant risks to public health. According to the World Health Organization (WHO), approximately 1 in every 10 people falls ill yearly due to consuming food contaminated with bacteria, viruses, or parasites (WHO, 2023). Although these data are based on pathogen-induced mortality, it is crucial not to ignore the microbial activity responsible for meat spoilage. Although bacteria and their enzymes can be deliberately introduced into meat processing during the resting process or in the production of fermented meat products, uncontrolled growth always brings the danger of spoilage. The main criteria for meat to be considered spoiled are sensory symptoms such as loss of texture, integrity, discoloration, mucus formation, undesirable flavors, and the increase in the microbial load. Although it also depends on the type and source of animal, meat with a colony-forming unit per square centimeter (CFU/cm^2) value above 5×10^6 to 10^8 is considered spoiled. Microbial load makes a very sharp distinction possible in meat. Population growth is so effective that cultures such as LAB added to ensure the fermentation process, especially in processed meats, are the main factors in the souring and spoilage of the product after the expiration date or shortly after the package is opened (Smith *et al*., 2024; Uhlig *et al*., 2024).

It is a fact that there are significant differences in bacterial populations between fresh meat and meat that has been stored in the freezer for an extended period. Species of mesophilic bacteria that grow best in the range of 25–42°C can survive at higher temperatures (Christian *et al*., 2009). Psychrophilic bacteria, conversely, are defined as microorganisms that develop between 0 and 20°C. These microorganisms have adapted to live in colder environments compared to mesophiles. Therefore, meat products contaminated with psychrophilic microorganisms can spoil even if stored in the refrigerator or freezer (Oh and Lee, 2024). These bacteria contribute to meat spoilage through the action of extracellular enzymes, including proteolytic enzymes. Proteases, or proteolytic enzymes, hydrolyze proteins into smaller structures and originate from plants, humans, bacteria, or fungi. Proteases can be categorized as serine or cysteine proteases based on their catalytic characteristics and amino acid active sites (Mótyán *et al*., 2013).

"Cig kofte" is a traditional Turkish dish made from raw ground meat and spices. It is a popular delicacy in Turkey and has been studied by food scientists to determine its nutritional value and the potential effects of eating raw meat. However, uncooked minced meat, consumed directly or after being stored in the refrigerator or the freezer, causes many microbiological risks due to pathogens or flora responsible for spoilage (Aslan *et al*., 2012). Due to the psychrophilic flora components, storing these meats in cold environments causes their deterioration with microbial activities. Consequently, it is essential to examine foods with an increased surface area, such as ground meat, that can be consumed raw, for pathogens and other bacteria that may disrupt their structure and integrity (Beynon & Bond, 2001). For this reason, we aimed to examine the minced meat samples from different butchers in Istanbul, which are suitable for use in the cig kofte. This examination was directed to explore the mesophilic and psychrophilic bacteria present in the meat samples and their proteolytic enzyme production capacity.

Material and Methods

Collection of samples

Within the scope of our study, 15 different beef mince samples were collected from butchers in different districts of Istanbul in 2015. Minced meat samples were prepared fresh from a piece of meat on the day of the study and divided into portions of 25 g under aseptic conditions immediately after being brought to the laboratory within a cold chain. One portion was used immediately for microbiological analysis, while the others were stored in their original packaging in the deep freezer at −20°C and studied after 15 days.

Culturable bacteria counts and isolation

For the isolation of mesophilic and psychrophilic proteolytic bacteria, 25 g of minced meat samples were homogenized in 225 mL of peptone water using a masticator (IUL- Instruments, Barcelona). After serial dilution in sterile physiological saline, 100 µL of the obtained homogenates were spread on Plate Count Agar (PCA) (Enzymatic digest of casein 5 $g.L^{-1}$, yeast extract 2.5 $g.L^{-1}$, glucose 1 $g.L^{-1}$, agar 15 $g.L^{-1}$; Final pH 7.0 ± 0.2 at 25°C) and Skim Milk Agar (SM-A) (Skim milk powder 28 $g.L^{-1}$, casein enzymic hydrolysate 5 $g.L^{-1}$, yeast extract 2.5 $g.L^{-1}$, dextrose 1 $g.L^{-1}$, agar 15 $g.L^{-1}$; Final pH 7.0 ± 0.2 at 25°C) media. Petri dishes were incubated at 20°C for 72 h and at 37°C for 24 h to isolate mesophilic proteolytic bacteria, and at 4°C for 10 days and at 10°C for 6 days to isolate psychrophilic bacteria. At the end of the incubation periods, the total number of bacteria in the Petri dishes and the number of bacteria showing proteolytic activity were recorded (Rodriguez *et al*., 2003). Colonies growing on

SM-A were meticulously evaluated based on the formation of transparent zones around them. The total population was determined by counting all growing colonies, while the colonies surrounded by a clear zone on the SM-A medium represent the proteolytic population. Therefore, the proteolytic bacteria isolated in this study are the results of a rigorous and thorough research process. A total of 108 proteolytic bacteria with different colony morphology and forming a zone on SM-A selected from the isolation process were purified on nutrient agar medium, examined their morphological and microscopic features, and stored as stock cultures at –86°C in 20% glycerin solution. Fresh cultures prepared from these stock cultures were used in our subsequent experiments.

Phylogenetic analysis of isolates

Bacterial suspensions prepared from a 24-hour fresh bacterial culture were centrifuged at 15,000 rpm to precipitate bacterial cells, and the supernatants were removed. DNA isolation was conducted following the kit manufacturer's instructions (Invitrogen PureLink™ Pro 96 Genomic DNA Kit). The 16S rRNA gene regions of the isolated DNAs were amplified within 25 µL of PCR mixtures containing 1 µL template DNA, 1 µL 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1 µL 1492R (5'-GGTTACCTTGTTACGACTT-3') primers, 0.25 µL MyTaq polymerase (Bioline, USA), 5 µL 5x buffer, following this protocol: initial denaturation at 94°C for 3 min, and 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2-minute cycles (Uludag *et al.*, 2023). PCR products were analyzed by Sanger sequencing. OQ381126-OQ381234 are the access codes of bacterial species placed on the GenBank sequence library after BLAST analysis based on 16S rRNA gene sequences obtained from isolated bacteria.

Upon obtaining the sequences, we conducted a detailed analysis using Mega 11 to construct a cladogram illustrating the phylogenetic classification of the isolates. We calculated evolutionary distances utilizing the p-distance method, and the results were presented as the count of base differences per site. To maintain precision, we eliminated ambiguous positions from each pair of sequences using the pairwise deletion option. Our final dataset comprised 1700 positions.

Determination of enzyme profiles

To determine the enzyme profiles of isolates, representative bacterial strains were selected according to different criteria. For this purpose, one representative strain of each distinct microscopic morphology (Gram positive, Gram negative, cocci, coccobacilli, bacilli, etc.) was selected from each minced meat sample, both directly and after freezing. The enzyme profiles of representative strains were investigated by the API ZYM test system (bioMerieux, Basingstoke, Hants). The test strips loaded with 1.5–2×10^9 cfu.ml^{-1} bacteria suspension were incubated at 37°C in a humid environment for 4 h. After the incubation period, the enzymatic activity of isolates was recorded by observing the color changes.

Results and Discussion

In our research, we studied different minced meat samples (K1-K15) taken from various butchers in Istanbul. We aimed to determine the culturable proteolytic bacterial diversity of this meat product, which can also be consumed raw, and the effect of freeze storage protocol on this bacterial diversity. Initially, this research aimed to investigate the impact of storing minced meat in deep freeze, on the proteolytic bacterial population in this type of meat product. A total of 108 mesophilic or psychrophilic proteolytic bacteria were isolated from directly processed or frozen minced meat samples. The microscopic examination of the isolates determined that 64.67% of the isolates were gram-negative rods, 17.96% were gram-positive rods, and 17.36% were gram-positive coccobacillus. When analyzed on a sample basis, it was determined that the highest gram-negative isolates were obtained from samples K4 and K8 (87.5%), followed by samples K3 and K5 (83.33%), samples K9 (80%), and samples K1 and K6 (75%). Additionally, the highest isolation rate of the gram-positive rods was 33% from the samples K10 and K11, while the highest rate of gram-positive cocci was isolated from K13 sample (36%).

Considering the number of bacterial colonies growing on the PCA medium, it is seen that incubation temperature or cryopreservation is not an important factor. However, these factors are essential parameters regarding the isolated bacterial species (Figures 1 and 4). On the other hand, it was found that the ratio of proteolytic bacteria to the total population varied according to the sample and the total bacteria colonizing it (Figure 2). Incubation temperature was also found to be another important factor. For example, the ratio of the proteolytic bacteria population to the total population observed in the K1 sample was not affected by the freezing process as the incubation temperature decreased. A similar situation was observed in K5 and K12 samples. When we examined the K3 sample, the dominance of the proteolytic population was noted, and there was no difference depending on the incubation temperature. Unlike these, it was determined that there was a psychrophilic population in K10 and K11 samples, and the dominant species had proteolytic activity.

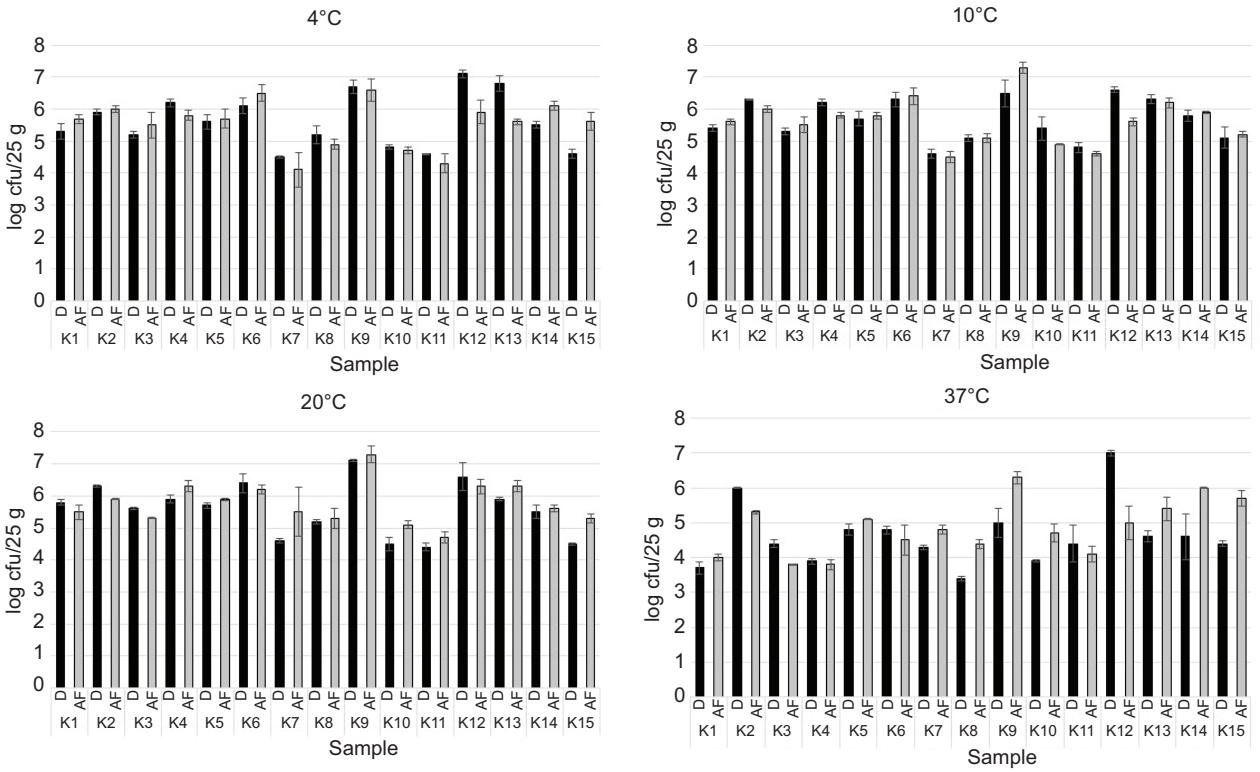

Figure 1. Bacterial counts on PCA medium (D: The sample studied directly; AF: The sample studied after 15-day freezing period).

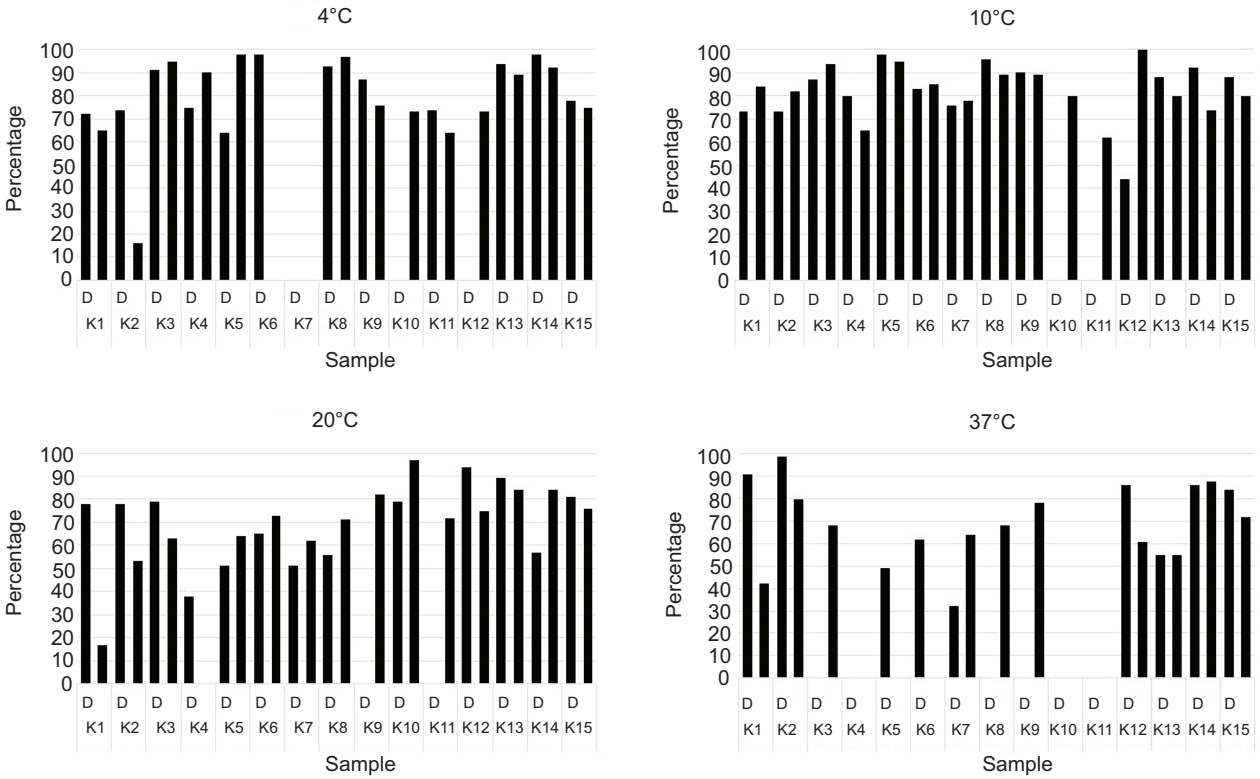

Figure 2. Percentage of colonies showing proteolytic activity on SM-A (D: The sample studied directly; AF: The sample studied after a 15-day freezing period).

Despite its initial sterility, meat is prone to contamination with various microorganisms throughout the processing stages, from slaughter to butchering and beyond (Ray, 2004). The bacterial density in minced meat samples is substantial, ranging from approximately 10^4–10^5 microorganisms per gram. The mishandling or improper cooking of meat with such high bacterial loads can lead to foodborne illnesses, underscoring the critical importance of proper storage and cooking techniques. Due to being a suitable environment for the reproduction of microorganisms, with nutritional richness and amount of water, meats are one of the most perishable foods. Researchers have found that when meat products are frozen, the water molecules in the meat transform into ice crystals while the bacteria on the meat become inactive, also known as dormant, due to the alteration in the environment. During the thawing of the meat, these bacteria become active again and begin to reproduce on the meat. As the process of thawing is slower compared to freezing, it creates a favorable environment for microbial growth. Consequently, the initial number of bacteria increases rapidly (Leygonie *et al*., 2012). Improper handling of meat products is a severe public health risk that should not be taken lightly. Furthermore, this can rapidly increase microbial populations, leading to meat spoilage and the growth of pathogenic microorganisms. Therefore, adhering to proper hygiene and storage conditions is essential to prevent such risks (Marcelli *et al*. 2024; Oh and Lee, 2024; Smith *et al*., 2024; Uhlig *et al*., 2024).

Meat spoilage can occur due to various factors, such as microbial population growth, enzymatic autolysis, and oxidation incidents. The hydrolysis of macromolecules such as protein, carbohydrates, and lipids in meat and meat products into smaller molecules causes meat to odor and spoil (Iulietto *et al*., 2015). The storage temperature of meat significantly affects the lag phase duration of the flora bacteria, proliferation rates, and number of viable cells. Therefore, the storage temperature of meat plays a crucial role in the speed at which it deteriorates (Doulgeraki *et al*., 2012).

The results we obtained from the minced meat samples, in which we aimed to determine whether the storage of meat products in the deep freezer causes differences in the culturable bacterial flora, are compatible with other studies (Abd El-Rahman and Ahmed, 1988; Ahmed *et al*., 1998; Doulgeraki *et al*., 2012; Ercolini *et al*., 2009; Leygonie *et al*., 2012). For example, considering the results of incubation at 10°C, we found that the number of psychrophilic proteolytic bacteria increased in the K1, K2, K3, K6, K9, K10, and K15 minced meat samples after 15 days of freezing compared to the directly studied samples when we considered numerical distributions rather than bacterial percentages (Table 1). Conversely, the count of psychrophilic proteolytic bacteria at 4°C that emerged from freezing increased in only K4, K5, and K15 minced meat samples. In addition, the results obtained by directly studying K2 and K12 samples indicate that raw mince will exhibit an intense presence of mesophilic and psychrophilic proteolytic bacteria. When we identified the isolates, we observed that the samples studied directly and those studied after freezing differed in bacterial diversity, with the freezing protocol increasing the number of bacteria. The results suggest that freeze storage may favor psychrophile proteolytic bacteria depending on the bacterial diversity. It is important to note that freezing does not necessarily mean that some bacterial species are metabolically dormant. The most important question is what metabolic activities will be exhibited by proteolytic bacteria both during freezing and after thawing and how these will affect the quality of the meat. Psychrotrophic bacteria are the primary cause for the decay of meat and meat products. These bacteria secrete many vital hydrolytic enzymes in cold environments, sustaining metabolic activities that enable them to thrive (Oh and Lee, 2024). It is crucial to prevent these bacteria from contaminating frozen products.

The most frequently isolated bacterial species from minced meat samples belong to the genus *Bacillus,* with a rate of approximately 30%. Besides *Bacillus* species, isolates belonging to *Serratia, Pseudomonas, Citrobacter,* and *Escherichia* species were frequently detected. On the other hand, *Proteus* sp., *Hafnia* sp., *Mammaliicoccus* sp., *Microbacterium* sp., and *Staphylococcus* sp. genera were rarely identified (Figures 3 and 4). The proteolytic nature of many of these detected bacteria and their role in meat spoilage is well-documented in the literature (Oh and Lee, 2024; Smith *et al*., 2024; Uhlig *et al*., 2024). These bacteria, some of which are psychrotrophic, prove that the most preferred freezing technique will be inadequate for storing meat in many cases.

It has been observed that there are differences in both bacterial population and diversity between the minced meat samples before and after the freezing procedure. We have identified a range of bacterial species present in frozen minced meat samples, such as *Hafnia sp., Mammaliicoccus vitulinus, Bacillus albus, Bacillus altitudinis, Bacillus proteolyticus, Serratia quinivorans, Pseudomonas versuta, Microbacterium foliorum, Microbacterium oxydans, Microbacterium sp.,* and *Staphylococcus saprophyticus*. Several isolates were identified as mesophilic bacteria, including *Hafnia sp.* and *Microbacterium sp.* are found at 37°C, and *M. vitulinus, B. albus, B. altitudinis, S. quinivorans, P. versuta*, and *S. saprophyticus* are found at 20°C. Additionally, *B. proteolyticus, M. foliorum*, and *M.oxydans* isolated at 10°C and *S. saprophyticus* isolated at 4°C were classified as psychrophilic bacteria. On the other hand, upon analyzing the study results, it was apparent that some species were

Table 1. Proteolytic bacterial counts on SM-A (log cfu/25 g).

Sample	37°C		20°C		10°C		4°C	
	Direct	After freezing	Direct	After freezing	Direct	After freezing	Direct	After freezing
K1	1.7 ± 0.1	1.4 ± 0.1	4.5 ± 0.3	0.9 ± 0.13	4.0 ± 0.0	4.7 ± 0.1	3.9 ± 0.0	3.6 ± 0.2
K2	5.8 ± 0.0	4.2 ± 0.1	5.1 ± 0.1	3.1 ± 0.1	4.5 ± 0.3	4.8 ± 0.2	4.1 ± 0.1	0. 9 ± 0.1
K3	0.0 ± 0.0	2.6 ± 0.0	4.0 ± 0.0	3.1 ± 0.1	4. 7 ± 0.2	5.0 ± 0.0	4.9 ± 0.0	4.5 ± 0.5
K4	0.0 ± 0.0	0.0 ± 0.0	2.4 ± 0.1	0.0 ± 0.0	4.6 ± 0.2	3.7 ± 0.1	4.6 ± 0.1	5.3 ± 0.3
K5	2.3 ± 0.1	0.0 ± 0.0	3 ± 0.0	3.7 ± 0.1	5.5 ± 0.2	5.4 ± 0.1	3.5 ± 0.0	5.8 ± 0.0
K6	2.9 ± 0.2	0 ± 0	4.2 ± 0.2	4.7 ± 0.2	5.4 ± 0.6	5.5 ± 0.2	6.1 ± 0.1	0.0 ± 0.0
K7	1.3 ± 0.0	2.7 ± 0.6	2.4 ± 0.1	3.2 ± 0.1	3.8 ± 0.2	3.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
K8	0.0 ± 0.0	2.8 ± 0.3	2.5 ± 0.4	4.3 ± 0.1	4.6 ± 0.1	4.6 ± 0.2	5.0 ± 0.1	4.0 ± 0.02
K9	0.0 ± 0.0	4.5 ± 0.09	0.0 ± 0.0	6.0 ± 0.0	5.8 ± 0.2	6.5 ± 0.0	6.0 ± 0.0	5.2 ± 0.1
K10	0.0 ± 0.0	0.0 ± 0.0	3.4 ± 0.1	4.4 ± 0.2	0.0 ± 0.0	4.0 ± 0.1	0 ± 0	3.3 ± 0.1
K11	4.4 ± 0.5	4.1 ± 0.2	4.8 ± 0.2	4.7 ± 0.1	0 ± 0	0 ± 0	0 ± 0	3.3 ± 0.2
K12	7.0 ± 0.1	5.0 ± 0.5	6.6 ± 0.1	5.7 ± 0.1	6.0 ± 0.2	2.8 ± 0.0	6.2 ± 0.2	4.7 ± 0.0
K13	4.6 ± 0.2	5.4 ± 0.3	6.3 ± 0.1	6.2 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	5.7 ± 0.5	5.4 ± 0.3
K14	4.6 ± 0.7	6.0 ± 0.0	5.8 ± 0.2	5.9 ± 0.0	3.4 ± 0.1	4.5 ± 0.1	3 ± 0.0	4.7 ± 0.2
K15	4.4 ± 0.1	5.7 ± 0.2	5.1 ± 0.3	5.2 ±0.1	3.5 ± 0.3	3.5 ± 0.1	3.9 ± 0.1	4.3 ± 0.1

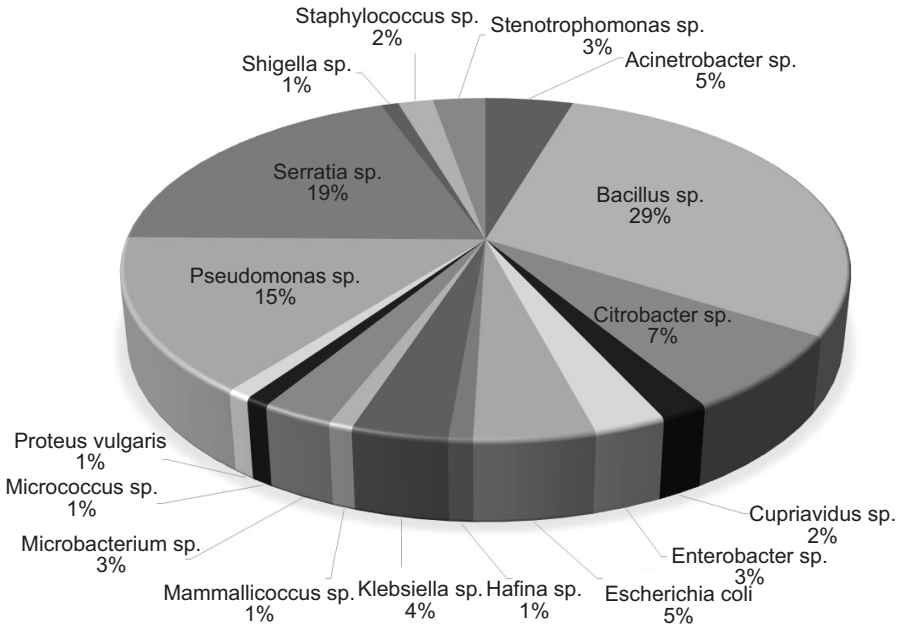

Figure 3. Percentages of the isolated bacterial genera.

only detectable in the directly studied samples. These included psychrophilic isolates such as *Bacillus safensis, S. marcescens, S. plymuthica, P. weihenstephanensis, P. azotoformans, S. maltophilia, Cupriavidus* sp., *M. aloeverae*, *C. europaeus, C. arsenatis, P. vulgaris*, and *P. libanensis*, as well as mesophilic isolates such as *Bacillus proteolyticus, Bacillus velezensis, K. aerogenes, K.oxytoca, S. maltophilia, Stenotrophomonas* sp., *Enterobacter mori, Enterobacter* sp., *Shigella flexneri*, and *K. pneumoniae*.

Psychrophilic microorganisms commonly found in meat products encompass various genera, including *Acinetobacter, Pseudomonas, Brochothrix, Flavobacterium, Psychrobacter, Moraxella, Staphylococcus, Micrococcus, Clostridium,* LAB, and some members of the *Enterobacteriaceae* family (Casaburi *et al.*, 2015). Additionally, meat products may be contaminated by psychrotrophic species such as *Leuconostoc, Clostridium laramie*, some coliforms, *Serratia,*

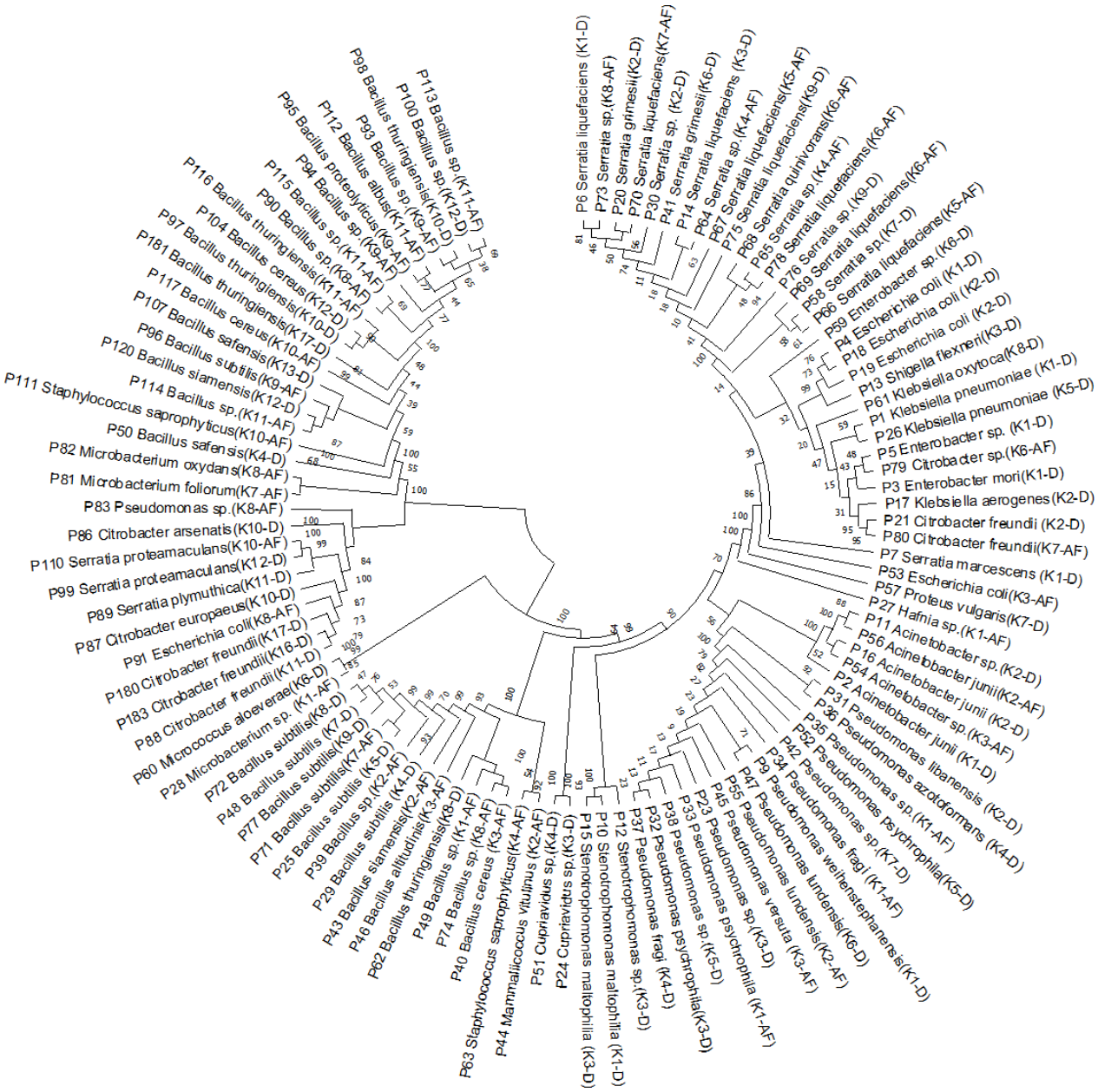

Figure 4. Cladogram of the phylogenetic classification of isolates. The species isolation sample codes are listed in parentheses. The codes in front of the species name are isolate codes.

Alteromonas, Achromobacter, Alcaligenes, Aeromonas, and *Proteus* can contaminate meat products. *Listeria monocytogenes* and *Yersinia enterocolitica*, which are psychrotrophic pathogens, are also found in meat and meat products (Ray, 2004). Furthermore, the isolates of our study, including *A. junii, Pseudomonas sp.,* and *E. mori,* align with these psychrophilic bacterial species.

Our results revealed that freezing minced meat samples affects the culturable bacterial diversity. While *Klebsiella* sp., *Enterobacter* sp., *Stenotrophomonas* sp., *Cupriavidus* sp., *Shigella sp. Micrococcus* sp. and *Proteus* sp. were detected in the directly studied minced meat samples, these strains were not found in the frozen minced meat samples. In contrast, *Microbacterium* sp., *Hafnia* sp., *Mammaliicoccus* sp., and *Staphylococcus* sp. were found only in frozen mincemeat samples and could not be detected in the directly studied minced meat samples (Table 2).

In 1998, Ahmed *et al*. studied the presence of proteolytic and lipolytic bacteria in frozen hamburgers and ground meat. Their findings revealed the existence of proteolytic

psychrophilic bacteria with an average of 6×10^4 in minced meat samples. In their study, *Micrococcus luteus, Micrococcus roseus, Bacillus brevis, Aeromonas* spp., *Bacillus pumilus, Acinetobacter, Bacillus pulvifaciens, Pseudomonas* spp., and *Bacillus polymyxa* proteolytic psychrophiles were isolated. Our study determined the proteolytic mesophilic bacterial counts to be 2.3×10^4–3.5×10^6 at 37°C and 2×10^4–3.5×10^7 at 20°C. We determined the proteolytic psychrophilic bacterial counts to be 2×10^4–5×10^7 at 10°C and 10^4–9×10^6 at 4°C. Our findings corroborate with those of Ahmed *et al*., which showed that minced meats are heavily contaminated with mesophilic and psychrophilic proteolytic bacteria.

In a study run by Abd El-Rahman and Ahmed (1988), it was discovered that various bacteria, including *Acinetobacter*, *A. hydrophilia*, *Enterobacter liquefaciens*, *E. coli, Micrococci, Moraxella spp., Pseudomonas*, and *Proteus spp.*, as well as proteolytic psychrophiles such as *B. cereus, B. cereus* var. *mycoides, B. subtilis, B. megaterium, E. coli, Lactobacillus, Proteus spp*., and *Pseudomonas aeruginosa*, were isolated from hamburger, raw sausage, raw milk, and soft cheese. In addition, some of the *Bacillus* strains (such as *B. cereus*, *B. cereus* var *mycoides*, *B. circulans*, *B. coagulans*, *B. polymyxa*) were isolated from mesophilic and some (such as *B. subtilis*, and *B. stearothermophilus*) from psychrophilic temperatures. The obtained psychrophilic and mesophilic bacteria with proteolytic properties overlap with the bacteria we isolated in our study.

Studies on microbial spoilage of fish have revealed that mostly gram-negative fermentative bacteria and psychrotolerant gram-negative bacteria (such as *Pseudomonas* spp. and *Shewanella* spp.) were responsible for the spoilage. It is known that different bacterial species release different compounds because of their metabolism, and these compounds are responsible for fish spoilage. For example, ketones, aldehydes, esters, and non-HS sulfides formed by *Pseudomonas* spp. results in meat spoilage. In addition, studies have shown that *P. putrifaciens, P. fluorescens*, and fluorescent *Pseudomonas* have high spoilage activity. It has been determined that *Moraxella, Acinetobacter*, and *Alcaligenes* bacteria have moderate spoilage activity, and *Aerobacter, Lactobacillus, Flavobacterium, Micrococcus, Bacillus, and Staphylococcus* bacteria show low spoilage activity under specific conditions (Ghaly *et al*., 2010).

In our study, according to the protease activities of representative strains, it was determined that all 45 representative strains had leucine aminopeptidase enzyme, 32 of them (71.11%) had valine aminopeptidase and cystine aminopeptidase enzymes, 17 of them (37.77%) had trypsin, and 15 of them (33.33%) had α-chymotrypsin. All protease enzymes (leucine, valine, cysteine aminopeptidase, trypsin, and α-chymotrypsin) were detected in 11 (%24.44) strains. All isolates were determined to have alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, and Naphthol-AS-BI-phosphohydrolase enzymes. While 1/3 of the isolates (15 strains) had lipase (C4) and α-mannosidase enzymes, 13 of the selected isolates (29%) had α-galactosidase, β-galactosidase, β-glucuronidase, and α-fucosidase enzymes. Additionally, 10 of the isolates (22%) had N-acetyl-β-glucosaminidase, 9 (20%) had β-glucosidase, and 6 (13%) had α-glucosidase enzymes.

It is known that bacteria with proteolytic properties are more crucial than others in food spoilage. Some proteolytic bacteria, such as *Pseudomonas*, break down protein-structured tissues, such as muscles, through their proteolytic enzymes when food nutrients decrease. In this way, they cause the degradation of food by reproducing faster than nonproteolytic ones. It has been confirmed that *Pseudomonas* species are among the most commonly occurring gram-negative bacteria in meat and meat products, and this observation aligns with the findings of the study conducted by Wickramasinghe *et al*. (2019). Some *Pseudomonas* species belonging to this group, such as *P. fragi, P. weihenstephanensis, P. azotoformans, P. versuta, P. psychrophila*, and *P. libanensis* are some of the isolates examined in terms of enzymatic activity. It has been determined that some of these isolates have strong activity in terms of some proteolytic enzymes such as leucine aminopeptidase, valine aminopeptidase, and cystine aminopeptidase.

To determine proteolytic activity, media containing casein, the milk protein, can be used (Grob, 1946). Bacteria that grow in the casein medium, which has an opaque appearance in Petri dishes, hydrolyze this milk protein and form a transparent hydrolysis zone around their colonies. As part of our study, we selected isolated colonies based on this distinct feature. However, proteases should not be thought of only as enzymes that cause meat spoilage. Proteolytic enzymes have been used for industrial purposes for many years. For example, proteases are frequently used in the detergent and leather industry, as well as in studies using proteases as therapeutic agents (Li *et al*., 2013). Several types of proteases obtained from microbial sources may be necessary for different industries. Bacterial M-protease is used as a second-generation protease in the detergent industry because of its high alkaline activity. Moreover, microbial proteases are used in the removal of turbidity in beer production, in the production of biscuits, cookies, crackers, or pastries, in the production of silk and the leather processing industry, in the purification of non-protein products from animal or vegetable extracts, in the formation of protein concentrates, and in the recovery of silver. While recombinant chymosin, which emerged in the 1980s, is used in cheese making, *Bacillus* alkaline serine

Table 2. Incidence of isolated bacterial species and isolation samples.

Bacterial genus	Bacterial species	Incidence (%)	Isolated sample
Bacillus	*Bacillus* sp.	9.17	K12 (D); K1, K2, K8, K9, K11 (AF)
	B. subtilis	6.42	K4, K5, K7, K8, K9 (D); K7, K9 (AF)
	B. thuringiensis	4.59	K8, K10, K17 (D); K11 (AF)
	B. cereus	2.75	K2 (D); K3, K10 (AF)
	B. safensis	1.83	K4, K13 (D)
	B. siamensis	1.83	K12 (D); K2 (AF)
	B. albus	0.92	K11 (AF)
	B.altitudinis	0.92	K3 (AF)
	B. proteolyticus	0.92	K9 (AF)
Serratia	*S. liquefaciens*	7.34	K1, K3, K9 (D); K5, K6, K7 (AF)
	Serratia sp.	5.50	K2, K7, K9 (D); K4, K8 (AF)
	S. grimesii	1.83	K2, K6 (D)
	S. proteamaculans	1.83	K12 (D); K10 (AF)
	S. marcescens	0.92	K1 (D)
	S. plymuthica	0.92	K11 (D)
	S. quinivorans	0.92	K6 (AF)
Pseudomonas	*Pseudomonas* sp.	4.59	K3, K5, K7 (D); K1, K8 (AF)
	P. psychrophila	2.75	K3, K5 (D); K1 (AF)
	P. fragi	1.83	K4 (D); K1 (AF)
	P. lundensis	1.83	K6 (D); K2 (AF)
	P. azotoformans	0.92	K4 (D)
	P. weihenstephanensis	0.92	K1 (D)
	P. libanensis	0.92	K2 (D)
	P. versuta	0.92	K3 (AF)
Citrobacter	*C.freundii*	4.59	K2, K11, K14, K15 (D); K7 (AF)
	C. europaeus	0.92	K10 (D)
	C. arsenatis	0.92	K10 (D)
	Citrobacter sp.	0.92	K6 (AF)
Klebsiella	*K. pneumoniae*	1.83	K1, K5 (D)
	K. aerogenes	0.92	K2 (D)
	K. oxytoca	0.92	K8 (D)
Escherichia	*E. coli*	4.59	K1, K2 (D); K3, K8 (AF)
Stenotrophomonas	*S. maltophilia*	1.83	K1, K3 (D)
	Stenotrophomonas sp.	0.92	K3 (D)
Microbacterium	*M. foliorum*	0.92	K7 (AF)
	M. oxydans	0.92	K8 (AF)
	Microbacterium sp.	0.92	K1 (AF)
Enterobacter	*Enterobacter sp.*	1.83	K1 (D)
	E. mori	0.92	K1, K6 (D)
Cupriavidus	*Cupriavidus sp.*	1.83	K3, K4 (D)
Staphylococcus	*S. saprophyticus*	1.83	K4, K10 (AF)
Shigella	*S. flexneri*	0,92	K3 (D)
Micrococcus	*M. aloeverae*	0.92	K6 (D)
Proteus	*P. vulgaris*	0.92	K7 (D)
Hafnia	*Hafnia sp.*	0.92	K1 (AF)
Mammaliicoccus	*M. vitulinus*	0.92	K2 (AF)

D: directly studied sample; AF: samples studied after the freezing at −20°C.

proteases or neutral proteases from *B. licheniformis* are frequently used to prepare protein hydrolysates (Ward, 2011).

As a result, although meat is a sterile food product, it is contaminated with many microorganisms due to processing conditions. While contaminated meat carries essential risks to human health, this situation is caused primarily by storage conditions. Pathogenic organisms and various mesophilic and psychrophilic bacteria can grow in meat that is not stored under appropriate conditions, which reduces its nutritional value and accelerates spoilage. These bacteria, especially those with proteolytic enzymes, are much more critical in meat spoilage. Considering that meat and meat products are some of the most intensely consumed animal sources in the world, the majority of the human population is affected by the consumption of spoiled meat products leading to substantial health problems. As the consumption of spoiled meat can lead to serious health problems, including death, it is essential that meat is processed using correct and hygienic methods and that storage conditions are also effective against the micro-organisms responsible for spoilage. To avoid this situation, all processes should be done carefully to prevent or minimize microbiological contamination from the area where the butchering is stored.

Conclusions

Our study presents essential evidence that nonpathogenic bacteria flora of ground meat constitutes a substantial risk to public health. This pivotal ingredient in numerous culinary traditions, notably in preparing uncooked dishes such as çiğ meatballs, requires meticulous handling to mitigate potential hazards. While it is established that meat is initially sterile, improper transportation and inadequate storage conditions can lead to the proliferation of various microorganisms, thus posing significant threats to consumer safety. The study delves into the effects of freezing, a widely used preservation technique, on the distribution and diversity of proteolytic bacteria in ground meat. We observed significant shifts in bacterial populations post-freezing, indicating a profound change in microbial diversity. The increased presence of psychrophilic and mesophilic proteolytic bacteria in frozen ground meat can compromise its nutritional integrity, making it unsuitable for consumption.

Moreover, the specific proteolytic enzymes produced by bacteria such as Pseudomonas species underscore their crucial role in meat product spoilage. As the culinary field continues to explore the diverse applications of meat, strict adherence to hygiene and storage protocols becomes vital to ensure the safety and quality of dishes involving raw or minimally processed meat. The standards are not merely advisable; they underpin the integrity of culinary practices and the broader public health domain. A comprehensive understanding of the role of bacteria in meat products is crucial for ensuring proper processing, mitigating the incidence of foodborne illnesses, and maintaining equilibrium in the food supply chain, particularly in the context of an ever-increasing global population.

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Author Contributions

The article has been meticulously crafted to convey the author's original ideas and insights. Extensive research and detailed analysis were conducted during this process, enabling the independent development of the content. Every aspect of this article has been created without external assistance.

Conflicts of Interest

I hereby declare that I have no personal, financial, or professional conflicts of interest that could influence the findings or opinions presented in this article.

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