UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

EVALUATION OF THE PRESENCE OF LISTERIA MONOCYTOGENES IN BOVINE CARCASSES AT A SLAUGHTERHOUSE

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RESUMO

Avaliação da presença de *Listeria monocytogenes* em carcaças de bovinos num matadouro

Uma das doenças de origem alimentar mais graves na UE é a listeriose humana, apresentando uma elevada taxa de hospitalização e mortalidade, especialmente em populações de alto risco. A prevalência de *Listeria monocytogenes* em ambientes de produção de gado variou entre 24% e quase 50%, o que sugere uma elevada probabilidade de contaminação pela entrada destes animais nos matadouros, contribuindo significativamente para a contaminação cruzada de carcaças com *Listeria monocytogenes*. O objetivo do nosso trabalho foi avaliar a frequência de *Listeria monocytogenes* em carcaças de bovinos num matadouro, comparando os resultados da amostragem *on-line* do matadouro com aqueles obtidos em carcaças após arrefecimento. A amostragem foi realizada em diferentes dias de abate de acordo com as diretrizes da ISO 17604:2015.

Os resultados mostram que nos 72 animais amostrados, 30,6% eram portadores de *Listeria* spp., dos quais 20,8% pertenciam a *Listeria monocytogenes*, indicando uma taxa de prevalência preocupante. Um total de 143 colónias suspeitas foram detetadas nas amostras, sugerindo potenciais falhas nas medidas de controlo. *Listeria monocytogenes* foi encontrada em 20 amostras positivas provenientes de armazenamento em refrigeração e uma ao longo da linha de abate, destacando-se vulnerabilidades de contaminação em toda a cadeia de processamento. A análise revelou enormes diferenças de prevalência entre a área frigorífica (aproximadamente 35,9%) e a linha de abate (aproximadamente 3,03%). Os testes de sensibilidade aos antibióticos em seis isolados de *Listeria monocytogenes* mostraram sensibilidade a vários antibióticos, mas observou-se resistência à Ciprofloxacina e à Tetraciclina, enfatizando a importância da seleção precisa dos antibióticos. As conclusões sublinham a necessidade de medidas abrangentes de monitorização e controlo nos matadouros, especialmente no armazenamento frigorífico, para garantir a segurança dos alimentos e a saúde pública.

Palavras-chave: *Listeria monocytogenes*, bovinos, matadouro, segurança dos alimentos

ABSTRACT

Evaluation of the presence of *Listeria monocytogenes* in bovine carcasses at a slaughterhouse

One of the most serious foodborne illnesses in the EU is human listeriosis, which has a high hospitalization and fatality rate, particularly in high-risk populations. The prevalence of *Listeria monocytogenes* in cattle farm environments ranged from 24% to almost 50%, which suggests a high risk of contamination from entering animals in slaughterhouses contributing significantly to the cross-contamination of carcasses with *Listeria monocytogenes*. The aim of our work was to assess the frequency of *Listeria monocytogenes* in bovine carcasses at a slaughterhouse, comparing results from slaughter house online sampling with those obtain in carcasses after cooling. The sampling was performed in different days of slaughter according to the guidelines of the ISO 17604:2015.

The results show that among the 72 animals sampled, 30.6% were carriers of Listeria which 20.8% of that belonged to *Listeria monocytogenes*, indicating a concerning prevalence rate. A total of 143 suspicious colonies were detected in samples, suggesting potential lapses in control measures. *Listeria monocytogenes* was found in 20 positive samples from meat cold storage and one along the slaughter line, highlighting contamination vulnerabilities throughout the processing chain. The analysis revealed a significant difference in prevalence between the cold storage area (approximately 35.9%) and the slaughter line (approximately 3.03%). Antibiotic sensitivity tests on six *Listeria monocytogenes* strains showed sensitivity to several antibiotics but resistance to Ciprofloxacin and Tetracycline, emphasizing the importance of accurate antibiotic selection. The findings underscore the need for comprehensive monitoring and control measures in slaughterhouses, especially in cold storage, to ensure food safety and public health.

Keywords: *Listeria monocytogenes*, bovine, slaughterhouse, food safety

RESUMO ALARGADO

Avaliação da presença de *Listeria monocytogenes* em carcaças de bovinos num matadouro

Uma das doenças de origem alimentar mais graves na União Europeia é a listeriose humana, apresentando uma elevada taxa de hospitalização e mortalidade, especialmente em populações de alto risco. O Painel dos Riscos Biológicos (BIOHAZ) da Autoridade Europeia para a Segurança dos Alimentos (EFSA) identificou a *Listeria monocytogenes* como um dos principais agentes patogénicos transmitido por alimentos e o seu potencial impacto na saúde humana. Esta bactéria pode levar a duas formas distintas de listeriose: a não invasiva e a invasiva. A listeriose não invasiva geralmente ocorre em indivíduos imunocomprometidos enquanto a listeriose invasiva afeta indivíduos de alto risco como grávidas, idosos e também imunocomprometidos. A listeriose tornou-se assim uma preocupação de saúde pública devido à sua gravidade e a numerosos surtos ligados à contaminação de alimentos por *Listeria monocytogenes*. A ocorrência de listeriose humana aumentou notavelmente em vários países, e esse aumento é atribuído principalmente à mudança de hábitos alimentares, com o aumento de alimentos prontos a consumir, como a carne bovina processada, que foram identificados como uma importante fonte de transmissão de *Listeria monocytogenes*. A contaminação pode ocorrer a partir de matérias-primas e durante as etapas de processamento de alimentos, afetando a prevalência e concentração de *Listeria monocytogenes* no produto final. As carcaças dos animais infetadas têm sido vistas como uma fonte significativa de agentes patogénicos no produto final. Esta prevalência de *Listeria monocytogenes* em ambientes de produção de gado variou entre 24% e quase 50%, o que sugere uma elevada probabilidade de contaminação pela entrada destes animais nos matadouros, contribuindo significativamente para a contaminação cruzada de carcaças com *Listeria monocytogenes*. Grande parte da informação de vigilância relativa à *Listeria monocytogenes* em animais e na sua alimentação é o resultado de programas de monitorização não normalizados em diferentes Estados-Membros. Não existem obrigações de comunicação de informações para esta matéria. As informações relativas à presença de *Listeria monocytogenes* nos alimentos para animais só são recolhidas no âmbito de investigações clínicas realizadas em animais de criação. Como resultado, os dados sobre *Listeria monocytogenes* na alimentação animal são raramente acessíveis. Portanto, é essencial implementar medidas preventivas para controlar a *Listeria monocytogenes*, em contextos agrícolas, bem como a prevenção e o controle da listeriose em animais utilizados para a produção e processamento de alimentos são passos cruciais para garantir a saúde humana e animal. Uma das medidas preventivas contra a *Listeria monocytogenes* é a utilização de agentes antimicrobianos e bacteriófagos. Outras medidas preventivas que

podem ser implementadas para controlar o crescimento de *Listeria monocytogenes* nos alimentos, incluem boas práticas de fabrico (BPF), como saneamento, higiene e controlo de temperatura adequados, bem como o uso de tecnologia de barreiras, que envolve a combinação de várias medidas preventivas para controlar eficazmente a contaminação por *Listeria monocytogenes* em produtos cárneos.

O objetivo do presente trabalho foi avaliar a frequência de *Listeria monocytogenes* em carcaças de bovinos num matadouro, comparando os resultados da amostragem *on-line* do matadouro com aqueles obtidos em carcaças após arrefecimento. A amostragem das carcaças de bovinos (*n*=72) foi realizada entre janeiro de 2023 e maio de 2023, em diferentes dias de abate, de acordo com as recomendações da ISO 17604:2015. A recolha das amostras foi feita nas carcaças em posição suspensa, tanto das patas traseiras individuais (esquerda e direita), do lombo, das patas dianteiras e do pescoço de cada meia carcaça. A amostragem destas áreas permite uma avaliação exaustiva da presença de *Listeria monocytogenes* nas carcaças. Cada carcaça foi esfregada com gaze estéril (previamente preparada) utilizando um delimitador de área de 100 cm^2, para obter uma área total amostrada de 1.000 cm^2. Após a recolha, todas as amostras foram transportadas para o Laboratório de Tecnologia Alimentar da Faculdade de Medicina Veterinária, Universidade de Lisboa, em condições de refrigeração (3 ± 2 ºC) e analisadas no mesmo dia. A deteção de *Listeria monocytogenes* foi realizada de acordo com a norma ISO 11290-1:2017. Após inoculação no meio Agar *Listeria* Ottaviani e Agosti (ALOA, BioMérieux, France) e incubação durante 24 h a 37°C, foram selecionadas colónias características de *Listeria* de cor azul e inoculadas em Agar Tríptico de Soja (TSA, Scharlau, Espanha) e novamente incubadas a 37°C durante 24 h. Em seguida, as placas foram observadas para identificar características indicativas de *Listeria* spp., como forma, tamanho e cor da colónia. Várias colónias características foram então selecionadas, purificadas e preservadas. Após extração do DNA, utilizando o método Chelex 100 adaptado de Talon et al. (2007), mediu-se a concentração e pureza do DNA extraído usando o NanoDrop One (ThermoFisher, EUA). As razões de pureza utilizadas foram A260/A280 e A260/A230. De seguida, procedeu-se à identificação de *Listeria monocytogenes* pela técnica de PCR (*polymerase chain reaction*), segundo o protocolo de Simon et al. (1996) usando como controlo positivo *Listeria monocytogenes* CECT 934, e eletroforese em gel de agarose. Após a identificação de *Listeria* spp e *Listeria monocytogenes*, seis isolados foram selecionados para estudar o perfil de resistência aos antibióticos. Os isolados de *Listeria monocytogenes* foram caracterizados quanto à sua resistência aos antibióticos, nomeadamente Meropenem, Penicilina, Ampicilina, Ciprofloxacina, Linezolida, Eritromicina, Sulfametoxazol/Trimetoprim, Gentamicina e Tetraciclina.

Os resultados mostram que nos 72 animais amostrados, 30,6% eram portadores de *Listeria* spp., dos quais 20,8% pertenciam a *Listeria monocytogenes*, indicando uma taxa de prevalência preocupante. Um total de 143 colónias suspeitas foram detetadas nas amostras, sugerindo potenciais falhas nas medidas de controlo. A *Listeria monocytogenes* foi encontrada em 20 amostras positivas provenientes de armazenamento em refrigeração e uma ao longo da linha de abate, o que mostra que para além da vulnerabilidade de contaminação em toda a cadeia de processamento, estas bactérias também conseguem sobreviver a temperaturas muito baixas comparativamente a outros microrganismos. A análise revelou enormes diferenças de prevalência entre a área frigorífica (aproximadamente 35,9%) e a linha de abate (aproximadamente 3,03%). De acordo com os resultados do antibiograma, os testes de sensibilidade aos antibióticos em seis isolados de *Listeria monocytogenes* mostraram sensibilidade a vários antibióticos, mas observou-se resistência à Ciprofloxacina e à Tetraciclina, enfatizando a importância da seleção precisa dos antibióticos. Como conclusão, é fundamental a adoção de medidas abrangentes de monitorização e de controlo específicas nos matadouros, especialmente no armazenamento frigorífico, para garantir a segurança dos alimentos e a saúde pública.

Palavras-chave: *Listeria monocytogenes*, bovinos, matadouro, segurança dos alimentos

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1. Introduction

Listeria monocytogenes is a facultatively anaerobic bacteria that is thought to be widespread since it is a common foodborne disease and the bacteria can be a component of the fecal microflora of many mammals. It is possible to separate it from a variety of environmental sources, including farm surroundings, water, sewage, plants, animal feeds, silage, and soil (Ferreira et al., 2018). According to the report of European Centre for Disease Prevention and Control (ECDC, 2021), one of the most serious foodborne illnesses in the EU is human listeriosis, which has a high hospitalization and fatality rate, particularly in high-risk populations. Currently, it has a low incidence rate (less than 1 per 100,000 EU citizens), however, since EU surveillance began in 2008, a considerable rising trend has been seen. According to EFSA and ECDC (2022), *Listeria monocytogenes* infections are most frequently recorded in adults over 64 years old, and particularly in those over 84 years old. As the population of Europe ages, more cases of *Listeria monocytogenes* can be anticipated. This pathogen can form biofilms and can live in settings that are extremely acidic, salty, and cold (Borucki et al., 2003). Therefore, it's crucial to create new methods to prevent *Listeria monocytogenes* contamination and maintain food safety.

According to other research, the prevalence of *Listeria monocytogenes* in cattle farm environments ranged from 24% to almost 50%, which suggests a high risk of contamination from Animals entering the slaughterhouses (Nightingale et al. 2004). Infected carcasses have been seen as a significant source of pathogens in the final meat product (Nastasijevic et al., 2017). In particular, bovine hides are linked to the cross-contamination of carcasses with *L. monocytogenes* (Wieczorek et al., 2012).

There have been a number of *Listeria monocytogenes* outbreaks in meat in recent years in Europe. In Europe, there were 2,480 confirmed cases of listeriosis in 2017, with a case fatality rate of 16.6%, according to a 2018 publication in Eurosurveillance. According to the report, "meat and meat products are the most common food categories associated with human cases" (Goulet et al., 2018).

This dissertation is divided into two parts: state-of-the-art and experimental work. The work results are presented with a discussion and final conclusions.

2.State of the art

In the past decade, there have been numerous newly identified species within the Listeria genus, resulting in a total of 30 distinct species (LPSN, 2023). Some of these recent species were discovered in natural environments and decaying matter, while others were found in food and food processing settings. Among all the Listeria species, *Listeria monocytogenes* is strongly the most significant species in terms of its impact on human health. Following it is *Listeria. ivanovii*, which is exceptionally rare in food. The primary mode of exposure to these bacteria, for both animals and humans, is through oral ingestion. In fact, it is estimated that a staggering 99% of all human cases of listeriosis are linked to food consumption (EFSA, 2018).

According to Matle et al. (2020), *Listeria monocytogenes*, originally discovered in 1910 as *Bacillus hepatis* by Hülphers in Sweden and later as *Bacterium monocytogenes* by Murray in the United Kingdom in 1926, was also isolated by Pirie in South Africa in 1927 and initially named *Listerella hepatolytica*. However, it wasn't until 1940 that the current name, *Listeria monocytogenes*, was established. In 1929, Nyfeldt first isolated *Listeria monocytogenes* in humans, while Gill described its impact on sheep as circling diseases. At that time, it was primarily associated with sporadic human infections and mainly linked to individuals working with diseased animals. In the 1980s, increased interest in *Listeria monocytogenes* appeared following outbreaks such as the Vacherin Mont d'Or in Switzerland (1983–1987) and improperly pasteurized milk in the United States (1983). These incidents sparked awareness of the pathogen among food manufacturers. Since then, *Listeria monocytogenes* outbreaks have been associated with various contaminated foods, including dairy, meat, seafood, and vegetables.

Listeria monocytogenes can be found in a wide range of sources, both living and non-living, including the environment and various food items. Contamination can occur from raw materials and during the food processing stages, affecting the prevalence and concentration of *Listeria monocytogenes* in the final product. In terms of consumer exposure, the contamination of raw materials is of utmost concern, especially when producing minimally processed foods, and when the processing conditions are inadequate in eliminating the bacterium. (EFSA, 2018).

2.1. *Listeria monocytogenes*: a hazard in food

Listeria monocytogenes can cause significant sickness in people, especially in elderly people, pregnant women, and people with compromised immune systems. It is a disease that is spread by food and is present in many foods, including meat and dairy products. The presence of *Listeria monocytogenes* in meat products is a concern for public health officials, as it can lead to outbreaks of listeriosis. To reduce the risk of infection, it is important to handle and cook meat properly. Consumers should always wash their hands before and after handling raw meat, and

should cook meat to the appropriate temperature to kill any bacteria present namely the Safety Indicators *Salmonella* and *Listeria monocytogenes* (WHO, 2018).

In 2020, the European Union encountered a notable occurrence of *Listeria monocytogenes* infections, as reported in the European Centre for Disease Prevention and Control's One Health 2020 Zoonoses Report (EFSA, 2020). This bacterium led to 1,876 confirmed cases in 27 Member States, resulting in 780 hospitalizations and 167 unfortunate fatalities within the EU. It emerged as the fifth most commonly recorded zoonosis affecting humans in the region.

Listeriosis is not well understood, and although mortality rates have risen among individuals over 65, the overall incidence has remained stable. Recent molecular typing advances reveal previously unknown outbreaks of this foodborne illness. Listeriosis presents as occasional cases, with varying incubation periods, and the focus is now on managing localized infections and those linked to prosthetic devices. This disease is a significant concern for the food industry, leading several countries to enact laws to mitigate its spread (Lepe, 2020).

The notification rate of *Listeria monocytogenes* within the EU stood at 0.42 per 100,000 population, marking a reduction of 7.1% when compared to 2019's rate (0.46 per 100,000 population) or 14.2% when considering the rate without data from the United Kingdom from 2019 (0.49 per 100,000 population). Although a decline in cases was noted at the EU level in 2020, primarily attributed to the COVID-19 pandemic's influence, the overall trend in listeriosis from 2016 to 2020 did not exhibit any statistically significant increases or decreases (EFSA, 2022).

The overall case fatality rate within the EU remained high at 13.0%, albeit a decrease from the rates observed in 2019 (17.6%) and 2018 (13.6%). Consequently, listeriosis remains a severe foodborne disease under EU surveillance. Notably, *Listeria monocytogenes* infections were predominantly reported among individuals aged 'over 64 years,' with a higher incidence in the 'over 84 years' age group (EFSA, 2022). Furthermore, in 2020, *Listeria monocytogenes* was identified as the causative agent behind 16 foodborne outbreaks across the EU, involving seven member states and resulting in 120 cases of illness, 83 hospitalizations, and 17 fatalities. Nine of these outbreaks had strong supporting evidence, while eight had weaker evidence. The most implicated food sources in the strongly supported listeriosis outbreaks were 'fish and fish products,' 'other or mixed meat and products thereof,' and 'cheese' (EFSA, 2022).

Regarding food safety efforts, twenty-four Member States reported a total of 136,346 samples from various 'ready-to-eat food' categories at the retail or processing stages in 2020. This marked a 37.6% decrease in reported sampling efforts compared to 2019. The prevalence of *Listeria monocytogenes* varied across different 'ready-to-eat' food categories and sampling stages, indicating the foreseeable contamination rate. At the retail level, the proportion of single

samples testing positive for *Listeria monocytogenes*, as conducted by competent authorities, remained consistently low across all 'ready-to-eat' food categories covered by Regulation (EC) No 2073/2005, ranging from 0.0% for five out of eleven categories to 1.3% and 1.4% for 'ready-to-eat' fishery products and ready-to-eat fish, respectively (EFSA, 2021). However, at the processing stage, the proportion of single samples testing positive for *Listeria monocytogenes*, again administered by competent authorities, was consistently higher compared to the retail level across all 'ready-to-eat' food categories. 'Ready-to-eat' fishery products (3.8%) and 'ready-to-eat' fish (3.5%) exhibited the highest proportions at processing, followed by products of meat origin other than fermented sausages (2.2%). In terms of primary production, the percentage of positive units was notably low (1.0%) in cattle, the most frequently sampled animal species in the EU. The limited data reported by Member States reflected the absence of harmonized EU regulations at the primary production level (EFSA, 2021).

In the European Union, the European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) has identified *Listeria monocytogenes* as a significant foodborne pathogen. The panel has conducted numerous risk assessments on *Listeria monocytogenes* contamination of ready-to-eat foods and its potential impact on human health.

Listeriosis has become a significant public health concern due to its severity and numerous outbreaks linked to *Listeria monocytogenes* contamination of food (Todd and Notermans, 2011). The global incidence rate of listeriosis is estimated to range from 0.1 to 10 cases per million people per year, but it varies widely among continents and countries (WHO, 2018). Todd and Notermans (2011) note that the majority of listeriosis cases occur in industrialized nations, particularly in Europe, Canada, the United States, and, to a lesser extent, Australia, and New Zealand. In contrast, underdeveloped nations have reported only a limited number of cases, possibly due to different dietary and drinking habits, host susceptibilities, or a lack of diagnostic and informational resources (Rocourt et al., 2000). Most human listeriosis cases are sporadic and are often associated with ready-to-eat foods that facilitate the growth and spread of *Listeria monocytogenes* throughout the food supply chain (Okutani et al., 2004; Todd and Notermans, 2011; Lambertz et al., 2012). Despite increased knowledge gained from outbreak investigations, the actual number of sporadic cases is believed to be significantly higher than reported figures, potentially even twice as high (Mead et al., 1999).

In 2016, 28 member states of the European Union (EU) reported 2536 cases of invasive listeriosis, with an incidence rate of 0.47 per 100,000 people, representing a 9.3% increase from the previous year. Over the past decade, there has been a noticeable and significant rise in the incidence rate of invasive listeriosis (EFSA, 2017).

When compared to other food-borne illnesses, such as Salmonella, this incidence rate is still low, but it shows a serious clinical state with a high fatality rate of 16.2% in 1524 cases of

infection in 2016 (EFSA, 2017). The EFSA research states that of the diseases examined, listeriosis has the greatest risk of hospitalization. Information was provided on the number of hospitalization cases for the 17 Member States in 2016, with hospitalizations accounting for 97.7% of reported cases (EFSA, 2017). According to EFSA's latest report on zoonoses and foodborne outbreaks in the EU, there were 2,549 confirmed cases of listeriosis in 2019, resulting in 229 deaths. Most cases were reported in people over 64 years old. The report also noted that ready-to-eat foods were the most common source of *Listeria monocytogenes* infections. In the year 2020, 14 member states provided information regarding ready-to-eat bovine meat items. In total, *Listeria monocytogenes* was found in approximately 7.4% of the 856 units that were tested across the European Union. It's worth noting that out of the 63 positive outcomes observed, a significant portion of them (44) originated from a single investigation that was reported by the Netherlands (EFSA, 2021).

The probability of *Listeria monocytogenes* contamination in ready-to-eat foods can be mitigated through proper food handling and processing practices. This includes maintaining strict hygiene standards during production, storage, and distribution, as well as implementing effective monitoring and control measures (EFSA, 2018).

According to a report by the EFSA, Portugal had the highest number of reported cases of listeriosis in 2019 among European Union (EU) countries. The report also stated that ready-to-eat products, such as cooked meat and smoked fish, were the most common sources of listeriosis outbreaks in the EU. In Portugal, the National Authority for Food and Economic Safety (ASAE) is responsible for ensuring food safety and has implemented measures to prevent the spread of *Listeria monocytogenes* in meat products. These measures include regular inspections of food establishments and testing of food samples (EFSA, 2018). *Listeria monocytogenes* poses a risk to Portuguese meat products, with several listeriosis cases being reported yearly. To stop the spread of this harmful pathogen and guarantee the safety of meat products, both consumers and governmental organizations must act (EFSA, 2018).

The bacterium *Listeria monocytogenes* possesses several traits that enable it to grow in a variety of environments. *Listeria monocytogenes*, in contrast to many other bacteria that cause foodborne illness, is resistant to many techniques used to control microbial growth, including cold temperatures, low water activity (aw), and low pH (Mena et al., 2004). This bacterium can be isolated from a wide range of sources, including soil, freshwater, effluents, food, human faeces, animals, and especially decomposing plant matter, where it lives as a saprophyte (Vazquez-Boland et al., 2001).

Animals may become contaminated by drinking contaminated water, eating contaminated pasture, or being ensiled. Nevertheless, the presence of *Listeria monocytogenes*

in faeces does not always indicate that an animal has been infected with the pathogen (Esteban et al., 2009). Due to its ongoing oral and faecal cycles, the ruminant family appears to be crucial in the prevalence of this microbe in rural areas. In its asymptomatic form, the bacteria can be found in a variety of animals, including other mammals, birds, fish, and invertebrates (Fleming et al., 1985).

Listeria monocytogenes can survive in food in a variety of physiological states, including viable, non-viable, and damaged cells. Most frequently, therapeutic procedures that include applying high temperatures, drying, irradiation, or exposure to chemical substances result in cells that are harmed. The damaged state of *Listeria monocytogenes* cells in food is reversible under ideal circumstances, and they may once more turn pathogenic (Donnelly, 2002). Additionally, these bacteria are still capable of creating biofilms, which are resistant structures linked to certain surfaces using polysaccharide matrices (Mah and O'Toole, 2001). According to Lado and Yousef (2007), these structures are created through the adherence of cells to the surface, cell deposition, colonization, biofilm formation, and development, as well as the generation of a matrix of extracellular polymeric substances (polysaccharides), proteins, and nucleic acids. Long-term adhesion of these structures to materials used in the food business raises the possibility of cross-contamination. The cleaning process of a food sector with relation to equipment is not always effective since biofilms can stick to a variety of surfaces where efficient penetration of detergents and disinfectants does not occur (Lado and Yousef, 2007).

2.2. Transmission to animals and Clinical signs of *Listeria monocytogenes*

Listeria monocytogenes can infect people and several animal species with invasive diseases, particularly tiny ruminants. Bovine, sheep and goats can become infected through their teat canals or through contaminated feed. Both infection paths expose consumers either directly, through the consumption of raw milk, or indirectly. Many dairy producers create fresh cheese made from unpasteurized sheep and goat milk, which is a high-risk commodity. Surprisingly, very little scientific research has been done on the hygienic and food safety aspects of dairy products that are sold directly to consumers (Schoder et al., 2023).

There are several transmission routes of *Listeria monocytogenes* on small ruminant on-farm dairies. One of the possible routes is the introduction of the bacterium into the dairy environment through contaminated feed or water. Another is the presence of *Listeria monocytogenes* in the faeces of infected animals, which can contaminate the milking equipment and subsequently the milk. Additionally, poor hygiene practices during milking and milk handling can also contribute to the transmission of *Listeria monocytogenes* (Schoder et al., 2023). Small ruminant on-farm dairies can be a potential source of *Listeria monocytogenes* transmission to humans through contaminated dairy products. Preventing transmission requires a

comprehensive approach that includes good hygiene practices, regular testing, and proper cleaning and disinfection (Schoder et al., 2023). Preventing *Listeria monocytogenes* transmission on small ruminant on-farm dairies requires a multi-faceted approach. This includes implementing good hygiene practices during milking and milk handling, regularly testing milk for *Listeria monocytogenes* contamination, and ensuring that feed and water sources are not contaminated with the bacterium. Proper cleaning and disinfection of milking equipment and facilities is also crucial in preventing transmission (Schoder et al., 2011).

Listeria monocytogenes, as an intracellular pathogen, possesses various virulence factors, including cell adhesion factors, hemolysins (listeriolysin O and two phospholipases), and proteins facilitating cell-to-cell movement (Murray et al., 2002). This bacterium can lead to two distinct forms of listeriosis: non-invasive and invasive. Non-invasive listeriosis typically occurs in individuals immunocompromised. Symptoms in humans, such as fever, nausea, vomiting, and diarrhea, manifest within 20 hours of consuming contaminated food. This form of the disease is usually self-limiting, lasting between 27 and 42 hours, and most patients recover without the need for antibiotic therapy (Douglas and Bronze, 2008). In contrast, invasive listeriosis affects individuals in high-risk groups (pregnant women, elderly and immunocompromised people), and emerges 20 to 30 days after consuming food contaminated with *Listeria monocytogenes*. However, invasive listeriosis is more severe and requires medical attention (Douglas and Bronze, 2008). Listeriosis typically presents with symptoms such as fever and muscle discomfort, which may occasionally be preceded by diarrhea or other gastrointestinal issues. When individuals experience an invasive infection, it means the bacteria migrate from the intestinal tract to either the bloodstream, resulting in a bloodstream infection, or the central nervous system, leading to meningitis. Although there have been instances where listeriosis symptoms appeared up to two months after consuming contaminated food, they are typically apparent within several days. The treatment for listeriosis involves the administration of antibiotics (CDC, 2015).

Poorly acidified silage has long been regarded for being the principal source of bacterial contamination in ruminants, where *Listeria monocytogenes* can actually reproduce in large numbers. It has been observed that ruminants fed large amounts of silage expel bacteria in their stools more frequently and in greater numbers, and they also manifest clinical sickness more frequently (Bagatella et al., 2022). The seasonality of clinical listeriosis cases in ruminants in the northern hemisphere, which rise during winter and peak in spring, has also been connected to silage feeding during hibernal indoor dwellings (Bagatella et al., 2022). Different epidemiological investigations, however, have been unable to establish a connection between eating silage and listeriosis outbreaks, casting doubt on the widely held belief that silage is the only source of infection. Furthermore, studies in the southern hemisphere commonly document

listeriosis instances in ruminants that are unrelated to silage eating, occur during the warmest months of the year, or at the changeover between the rainy and dry seasons. It looks likely that additional factors contribute to infection given that listeriosis infections can occur all year long in both hemispheres and with varied diets (Bagatella et al., 2022). Although local bacterial implantation at body surfaces (keratoconjunctivitis, dermatitis) or ascending genital tract infection are rare causes of listeriosis, mouth infection is the most common source. After an oral infection, *Listeria monocytogenes* may colonize the gastrointestinal tract and either cause self-limiting enteritis or be shed by asymptomatic carriers, however *Listeria monocytogenes* frequently breaches the gastro intestinal barrier and results in invasive illness (Bagatella et al., 2022).

Much of the surveillance information regarding *Listeria monocytogenes* in animals and their feed is the result of non-standardized monitoring programs across different member states. There are no compulsory reporting obligations in place for this matter. Listeriosis in animals, which can occur through various transmission routes, including the ingestion of contaminated feed like subpar silage, is one of the routes. Information concerning the presence of *Listeria monocytogenes* in animal feed is only gathered as part of clinical investigations conducted on farm animals. As a result, data on *Listeria monocytogenes* in animal feed is rarely accessible (EFSA, 2021).

2.3. Transmission to humans and Clinical signs of *Listeria monocytogenes*

Listeria monocytogenes possesses several virulence factors that enable it to cause disease in humans. These include the ability to invade and replicate within host cells, as well as the production of toxins that damage host tissues. The bacterium also has the ability to form biofilms on surfaces, which can make it difficult to eradicate from food processing environments (Kawacka et al., 2020).

After consuming contaminated food, individuals with *Listeria monocytogenes* infection may experience symptoms as soon as a few hours or as late as two to three days later. It could take up to three months for more severe cases of listeriosis to manifest (FDA, 2022). Healthy people seldom get sick from *listeria* infections, but infants, pregnant women, and adults with weakened immune systems can die from the illness. With prompt antibiotic treatment, *listeria* infection symptoms can be reduced and treated (WHO, 2018). According to Gray and Killinger (1966) and Vazquez-Boland et al. (2001), human listeriosis typically manifests as one of three clinical syndromes, including febrile gastroenteritis, maternal-fetal/neonatal listeriosis, or bacteremia with or without cerebral infections like meningitis, meningoencephalitis, rhombencephalitis, or brain abscess. Endocarditis, peritonitis, septic arthritis, and endophthalmitis are less frequent focused infections caused by hematogenous dissemination

(Doganay, 2003). Focal infections have also been reported to include cholecystitis, infections of prosthetic joints, and infections of vascular grafts. Additionally, cutaneous listeriosis can be problematic for people who have eczematous skin and work with infected animals (Douglas and Bronze, 2008). There have been numerous reports of listerial gastroenteritis epidemics, which mainly affect healthy people. Most patients appear with diarrhea, fever, stomach discomfort, chills, headache, and myalgias after an incubation period of 6-49 h (median 20-25 h). This is a self-limited illness with median durations of fever and diarrhea of 27 and 42 hours, respectively. Most patients recover without the need for antibiotics (Douglas and Bronze, 2008).

Listeria invasive illness during pregnancy, most women come with a bacteremic sickness that includes fever, chills, headache, and leukocytosis 6-7 days before being diagnosed. Cultures of the placenta, amniotic fluid, and cervix may yield the organism. Preterm delivery and neonatal infection are other potential complications. Complications may also include spontaneous abortion or stillbirth, particularly if infection occurs early in the pregnancy. Due to transplacental transfer of maternal bacteremia or exposure during passage via a colonized vaginal canal, up to two-thirds of surviving neonates born to listeriosis-positive mothers acquire overt neonatal illness. Neonatal listeriosis can either be an early infection (occurring within the first 5-7 days of delivery) or a late (Douglas and Bronze, 2008). Early illness is frequently visible at delivery and connected to maternal infection. It might manifest as meningitis, bacteremia, or pneumonia. Typical symptoms include meconium stains, respiratory difficulty, fever, lethargic behaviour, jaundice, and rash. While late-onset infection typically manifests as meningitis and is associated with contamination via the mother's vaginal tract during childbirth (Chan et al., 2018).

Listeria non-perinatal invasive infection with *Listeria monocytogenes* often manifests as bacteremia, either with or without a clear centre of infection, or as a central nervous system (CNS) infection, which can include meningitis, meningoencephalitis, brainstem encephalitis (rhombencephalitis), and brain abscess. Fever, altered sensorium, and headache are the most common symptoms of *Listeria* meningitis/meningoencephalitis in patients between the ages of 4 and 50 (Brouwer et al., 2006). Brain abscess and brain stem encephalitis are two less frequently seen CNS illnesses. Rhombencephalitis occurs in 10% of *Listeria* CNS infections and is characterized by a prodrome of headache, nausea, vomiting, and fever, followed by progressive brainstem and cerebellar dysfunction (Douglas and Bronze, 2008).

The medications usually administered are ampicillin and penicillin, while trimethoprim/sulfamethoxazole is a suitable second-line treatment for patients who are allergic to penicillin. Antibiotic resistance is rare among human isolates of *Listeria* spp.. owever, isolates are frequently partially or completely resistant to cephalothin, cefotaxime, cefepime,

monobactams, D-ofloxacin, and nalidixic acid. Therefore, it is not advised to use cephalosporins to treat Listeria infections. Tetracycline resistance. The importance of antibiotic tolerance is another therapy factor to consider. The high case fatality rate of listeriosis reflects both the patients' underlying condition and the aggressiveness of the infection. Infants and patients with underlying diseases or who were on immunosuppressant drugs had a mortality rate of over 30%, while healthy individuals showed no signs of death (Douglas and Bronze, 2008).

The surge in antimicrobial resistance within *Listeria monocytogenes* is associated with several factors. The development of antimicrobial resistance in *L. monocytogenes* can be linked to the uptake of antibiotic resistance genes from other harmful microorganisms. Additionally, the main factors contributing to resistance in *L. monocytogenes* include the acquisition of conjugative transposons, the presence of active efflux-related genes, and mutations in its ribosomal and chromosomal components (Elsayed et al., 2022). Multiple drug resistance has been observed in *Listeria monocytogenes* strains found in both food and environmental samples worldwide. In Northern Ireland, a study by Walsh et al. (2001) detected 0.6% of *Listeria monocytogenes* in retail foods with resistance. Moreover, in the United States, *Listeria monocytogenes* isolates from food and animal sources (n = 167) exhibited resistance rates of 1.8% to ciprofloxacin, 9% to tetracycline, 73% to sulfonamide, and 100% to nalidixic acid (Matle et al., 2020).

2.4. Epidemiology of *Listeria monocytogenes* in Bovine and Human

The occurrence of human listeriosis and notable outbreaks resulting in numerous fatalities has notably risen in various countries. This increase is primarily attributed to changing dietary habits, with many people consuming ready-to-eat (RTE) foods, like processed beef meat that have been identified as an important source of transmission of *Listeria monocytogenes*. Additionally, factors like the globalization of food trade, demographic shifts including an ageing population, and the presence of immune-compromising disease have elevated the risk of listeriosis. The adoption of sequencing techniques for detecting and classifying listeriosis outbreaks has also led to a higher number of reported cases. Epidemiological surveillance studies have revealed that human listeriosis is more commonly reported in high-income and industrialized nations due to robust foodborne disease monitoring systems (Matle et al., 2020).

One of the recent significant occurrences in past years was the South African outbreak that occurred between 2017 and 2018. This outbreak had more than 1,000 laboratory-confirmed cases and more than 200 fatalities, it was the greatest *Listeria* outbreak. Whole-genome sequencing (WGS) permitted to pinpoint the source of the outbreak as a ready-to-eat processed meat item. The outbreak strain was also found in the manufacturing facility where the product was being processed. Following that, recalls were announced in South Africa and 15 other

African nations where the product had been sold. The European Union (EU)/European Economic Area (EEA) had 2555 confirmed cases of listeriosis as of 2016, with the highest rates found in infants under 1 year old (1.3 per 100 000 population) and the elderly over 64 years of age (1.6 per 100 000) (Desai et al., 2019). Interestingly, since the European Union (EU) started collecting human surveillance data, most listeriosis cases have been reported in people over 64 years of age. The number and proportion of cases reported for this age group has increased steadily from 2008 and continued to increase in 2017 and 2018. Human cases almost doubled in the age group greater than 84 years in the same period (Farber et al., 2020). Considering the definitions of Law No. 81/2009, Order No. 15385-A/2016, Portugal includes listeriosis in the group of infectious diseases that when diagnosed have to be reported. However, there is a lack of information available about the prevalence of listeriosis in Portugal. Some information can be found in the studies conducted by Almeida et al. (2006) that demonstrated that the incidence rate of listeriosis had increased over the years and that it was associated with a high mortality rate. Further, investigations should be performed in order to better understand the current epidemiology of listeriosis in Portugal.

During the same period, there were several reported cases of *Listeria* infection linked to the consumption of contaminated meat, such as deli-sliced meats in Brooklyn, New York (CDC, 2023). Almost all the outbreaks lead to at least one death and several hospitalizations.

2.5. Preventive measures of *Listeria monocytogenes* in the meat industry

The meat industry is particularly susceptible to Listeria contamination due to the nature of the product and the processing methods used. Therefore, it is essential for meat processors to implement preventive measures to control *Listeria monocytogenes.* According to Giaccone and Catellani (2016), strict management of *Listeria monocytogenes* in agricultural and food-processing contexts as well as prevention and control of listeriosis in animals used for food production are crucial steps in ensuring both human and animal health. Tompkin (2002) provided a six-step listeria control program for environments that deal with food. The first is the prevention of Listeria *species'* development and establishment in devices or other areas where they could contaminate RTE foods, and the second is the installation of a sampling program to evaluate how well the control program is working. Third, a rapid and effective response when the sampling program gains positive results for Listeria species; Fourth, follow-up sampling to confirm that the source of contamination has been found and eliminated; The fifth, short-term assessment of the previous 4–8 samplings to facilitate early detection of problems and trends; and the sixth, long-term assessment at suitable intervals (quarterly, annually, etc.) to identify widely dispersed contamination events and to gauge the severity of those events.

Another factor that contributes to the growth of *Listeria monocytogenes* in meat products is pH. *Listeria monocytogenes* can grow at a pH range of 4.4-9.6, with an optimum pH range of 6.0-7.5. Meat products typically have a pH range of 5.5-6.2, which is within the optimal range for *Listeria monocytogenes* growth. Therefore, controlling pH levels in meat products is crucial to prevent the growth of this pathogen (Schlech, 2000).

Water activity (aw) is another critical factor that affects the growth of *Listeria monocytogenes* in meat products. Water activity refers to the amount of free water available for microbial growth, and it is measured on a scale from 0 to 1. *Listeria monocytogenes* can grow at aw levels as low as 0.92, which is higher than many other foodborne pathogens. Meat products typically have a high aw, which provides an ideal environment for the growth of *Listeria monocytogenes*. Therefore, controlling aw levels in meat products is essential to prevent the growth of this pathogen (Farber and Peterkin, 1991).

In addition to these factors, recent studies have also identified other factors that contribute to the growth of *Listeria monocytogenes* in meat products. These include the presence of other microorganisms, such as lactic acid bacteria and *Enterobacteriaceae*, which can provide nutrients and create an environment conducive to *Listeria monocytogenes* growth (Ribeiro, et al, 2018). The use of certain processing techniques, such as vacuum packaging and modified atmosphere packaging, can also create an environment that promotes the growth of *Listeria monocytogenes* (Ferreira et al., 2011).

One of the preventive measures against *Listeria monocytogenes* is the use of antimicrobial agents. Several studies have shown that natural antimicrobial compounds such as essential oils, organic acids, and bacteriocins can inhibit the growth of *Listeria monocytogenes* in meat products. For example, a study conducted by Wang et al. (2018) found that a combination of thyme essential oil and organic acid reduced *Listeria monocytogenes* counts in pork loin by 2.5 log CFU/g after 14 days of storage at 4°C (Kurpas et al., 2018).

Another approach to prevent *Listeria monocytogenes* growth in meat products is the use of bacteriophages. Bacteriophages are viruses that infect and kill bacteria, including *Listeria monocytogenes*. Several studies have demonstrated the effectiveness of bacteriophages in reducing *Listeria monocytogenes* counts in meat products. For instance, a study showed that the application of a cocktail of bacteriophages reduced *Listeria monocytogenes* counts in dry-cured ham by 1.5 log CFU/g after 60 days of storage at 15°C (Saraiva et al., 2018).

In addition to antimicrobial agents and bacteriophages, other preventive measures can be implemented to control *Listeria monocytogenes* growth in meat products. These include good manufacturing practices (GMPs), such as proper sanitation, hygiene, and temperature control, as well as the use of hurdle technology, which involves combining multiple preventive measures to create barriers against microbial growth (Farber et al., 2020). One of the most effective

preventive measures is sanitation. Meat processing facilities should be cleaned and sanitized regularly to prevent the growth and spread of *Listeria monocytogenes*. This includes cleaning and sanitizing all equipment, surfaces, and utensils that come into contact withencounter meat products. One of the most effective preventive measures is sanitation. Meat processing facilities should be cleaned and sanitized regularly to prevent the growth and spread of *Listeria monocytogenes*. This includes cleaning and sanitizing all equipment, surfaces, and utensils that come into contact with encounter meat products. Additionally, employees should practice good personal hygiene, such as washing their hands frequently and wearing clean clothing (European Guide for Good Hygiene Practices, 2016).

Meat processors should also implement a Hazard Analysis and Critical Control Points (HACCP) program. HACCP is a systematic approach to identifying potential hazards in food production and implementing controls to prevent them. By implementing a HACCP program, meat processors can identify potential sources of Listeria contamination and take steps to prevent it (Kurpas et al., 2018).

Overall, preventing cross-contamination of *Listeria monocytogenes* in slaughterhouses requires a combination of good hygiene practices, separation of raw and cooked meats, and monitoring of the environment for the presence of the bacterium. By following these guidelines, slaughterhouses can help ensure the safety of their meat products and protect consumers from illness (Valderrama et al., 2016).

The use of natural antimicrobial agents, such as essential oils and plant extracts, can also be an efficient preventive approach against *Listeria monocytogenes* in meat products. According to the study of Bahrami et al. (2022), *Listeria monocytogenes* in meat products can also be prevented by using bacteriophages. Since the approval of the first phage-based product in 2006 as "generally recognized as safe" for controlling *Listeria monocytogenes* in meat and poultry products, there has been a growing effort to develop new phage-based technologies for pathogen control in postharvest foods. The primary organizations responsible for approving bacteriophage cocktails for use in the agri-food sector are the EFSA and the US Food and Drug Administration (FDA). The production of commercially available bacteriophage cocktails must adhere to good manufacturing practices (Połaska and Sokołowska, 2019). The application of bacteriophages PhageGuard Listex™ (formerly Listex™ P100) to assorted foods (for example; cooked ham) has been shown to be effective at reducing contamination with *L. monocytogenes* (Połaska and Sokołowska, 2019).

In conclusion, preventing *Listeria monocytogenes* growth in the meat industry is crucial to ensure food safety and protect public health. The use of preventive measures such as GMPs and hurdle technology can effectively control *Listeria monocytogenes* contamination in meat products.

3. Objectives

The aim of this work was to assess the frequency of *L. monocytogenes* in bovine carcasses at a slaughterhouse, comparing results from slaughterhouse online sampling with those obtained in carcasses after cooling. The isolates of *L. monocytogenes* obtained were characterized regarding their antibiotic resistance.

4. Materials and methods

4.1. Evaluation of the presence of *Listeria monocytogenes* in bovine carcasses at a slaughterhouse

The presence of *Listeria monocytogenes* in bovine carcasses at a slaughterhouse was assessed with the goal of ensuring food safety and protecting public health. The primary objective was to detect whether *Listeria monocytogenes* had been found on the carcasses, as its presence had the potential to result in the contamination of meat products and subsequent foodborne illnesses.

4.1.1. Sampling beef carcasses at a slaughterhouse

Between Jan 2023 and May 2023, Bovine carcasses were sampled (*n*=72) according to the recommendations of ISO 17604:2003, which detail sampling techniques for identifying and counting microorganisms on the surface of recently butchered (red) meat animals, on different days of slaughtering. The sampling was done online in the final slaughter line after Veterinary inspection and in the cold storage for temperature equalization after the cooling tunnel (Table 1).

Table 1. Sample information.

N	Date	Location	NUMBER OF SAMPLES
1	12th Jan	meat cold storage	1-13
2	3rd Fev	slaughterhouse line	14-33
3	25th March	slaughterhouse line	34-52
4	8th May	meat cold storage	53-72

When it comes to evaluating and searching for *Listeria monocytogenes* on beef cattle half carcasses, various sampling sites can be considered. The sampling was done on the cow carcasses in a hanging position, from both individual hind legs (left and right), the brisket, the forelegs and the neck of each half carcass (Figure 1). Sampling from these areas allows for a comprehensive assessment of the presence of Listeria monocytogenes on the carcasses. Each carcass was swabbed with a sterile gauze (previously prepared) utilizing an area delimitator of 100 cm^2 (Figure 2), to obtain a total area sampled of 1,000 cm^2. To ensure maximum contact with the surface area the gauze was gently pressed on the surface and then moved with back-and-forth motions. Between each carcass sampling, the metal delimitator was disinfected with alcohol (70%) and the gloves were changed. The sterile gauze was prepared in the laboratory of before sampling. Before the sampling, the gauze was moistened with a sterile isotonic saline solution (Braun, Portugal) and then sterilized. After collection, all samples were transported back to the Laboratory of Food Technology of the Faculty of Veterinary Medicine (University of Lisbon) in cooled conditions (3 ± 2ºC) and analyzed on the same day.

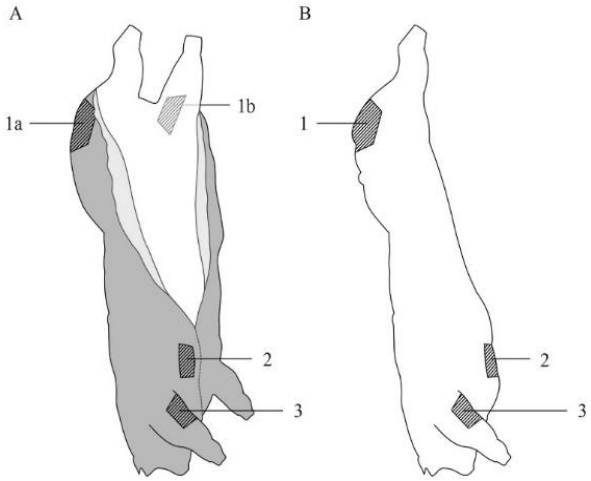

Figure 1. Cow carcass sampling sites (1a-eft hind leg; 1b- right hind leg; 2- of one carcass half brisket; 3- inside foreleg) (Demaître et al., 2021).

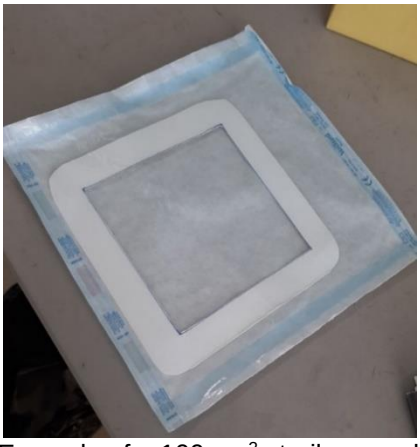

Figure 2. Example of a 100 cm^2 sterile area delimitator.

When it comes to evaluating and searching for *Listeria monocytogenes* on beef cattle half carcasses, various sampling sites can be considered. These sites include the both half neck, both half foreleg, both half brisket, and individual left and right hind legs (especially hip area). Sampling from these areas allows for a comprehensive assessment of the presence of *Listeria monocytogenes* on the carcasses.

4.1.2. Detection of *Listeria monocytogenes*

The detection of *Listeria monocytogenes* was performed according to ISO 11290-1:2017. To each gauze it was added 225 mL of half-Fraser broth (Scharlau, Spain) followed by 30s homogenization on the Stomacher (blender bag, VWR STERILE, USA). All samples were then incubated at 30°C for 24h. After incubation, 0,1 mL of culture was inoculated in 10 mL of Fraser broth (Scharlau, Spain) and then incubated for 24h at 37ºC. Additionally, a loop was also inoculated in a plate of Agar *Listeria* according to Ottaviani and Agosti (ALOA, BioMérieux, France) and incubated at 37°C for 24h. From the 10 mL inoculated Fraser broth, after incubation, a loop was taken and inoculated in a plate of ALOA and incubated for 24 at 37°C. In both plates of ALOA, characteristic *Listeria* blue-coloured colonies were selected (Figure 3). Each characteristic colony selected was inoculated in Tryptic Soy Agar (TSA, Scharlau, Spain) and incubated for 24h at 37°C. Following this, the plates were observed using a magnifying glass to identify characteristics indicative of *Listeria spp.*, such as colony shape, size, and colour. From this plate, several characteristic colonies were then selected and further purified in a new plate of TSA and again incubated for 24h at 37°C.

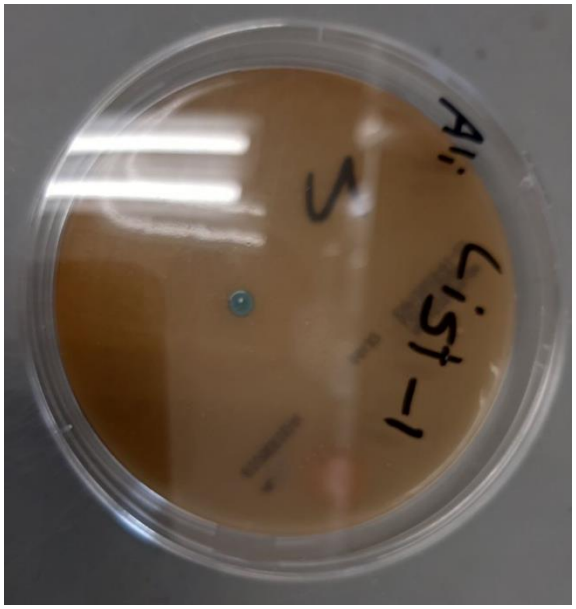

Figure 3. *Listeria monocytogenes* colony in the ALOA medium

4.1.3. Preservation of *Listeria monocytogenes* isolates

After the colonies of *Listeria* spp. had been purified, the next step involved preserving isolate samples in cryotubes (ClearLine, Italy), by the addition of a loop of fresh culture to the vial. Cryotubes were composed 1.5 mL of Brain Heart Infusion Broth (BHI, Scharlau, Spain) with 15% glycerol (Merck, Germany). The cryotubes were then stored at -80°C.

4.1.4. DNA Extraction

For DNA extraction the Chelex 100 method adapted from Talon et al. (2007) was utilized. A loop of fresh culture was suspended in 1 mL of 1X Tris.EDTA Buffer Solution (TE, Nzytech, Portugal). The culture-containing eppendorf was centrifuged for 5 minutes at 12,000 rpm and then placed on ice. The supernatant was then removed, and the resulting pellet was resuspended in 300 µl of 6% Chelex (Merck, Germany) solution and subsequently vortexed for 10-15 seconds. The sample was then subjected to an 8-minute incubation at 100°C. After incubation, the samples were vortexed for 10 seconds and immediately chilled on ice for approximately two minutes. The tube was once again centrifuged for 5 minutes at 12,000 rpm, and 80 µL of the supernatant was then transferred to a new 1.5 mL Eppendorf. The extracted DNA samples are stored at -20°C.

4.1.5. DNA Concentration Measurement using NanoDrop

The NanoDrop One (ThermoFisher, USA) was utilized to measure the concentration and purity of the DNA extracted. The purity ratios utilized were A260/A280 and A260/A230. The concentration was considered adequate when it was higher than 100 ng/μL.

4.1.6. Identification of *Listeria monocytogenes* isolates by PCR

Identification of *Listeria monocytogenes* was performed by PCR according to Simon, Gray, and Cook (1996). The PCR amplification was performed from 5μL of sample DNA, with Mastermix being done according to the volumes presented in Table 2.

Table 2. Master mix composition according to the PCR protocol of *listeria monocytogenes* (Simon, Gray, and Cook, 1996).

REAGENTS FOR PCR MIXING	VOLUME	INITIAL CONCENTRATION	FINAL CONCENTRATION
H2O	28.9 µL	-	-
PE-Buffer KCL Tris-HCL pH 8,3	5 µL	10 x 500 mM 100 mM	1x 50 mM 10 mM
MgCl2	2.5 µL	50 mM	2.5 mM
dNTPs mix	0.3 µL	10 mg	600 µM (150 µM)
Primers (x2)	0.3 µL	50 µM	0.3 µM
BSA	5 µL	10 mg/mL	1µg/µL
Taq Polimerase	0.2 µL	5 U/ µL	1U
Total	42.5 µL	-	-

For this PCR two primers were utilized Lip1 and Lip2 (Table 3). The thermocycler conditions utilized are described in Table 4. For each PCR there was a negative control, where the DNA was substituted by water and a positive control, the reference strain *Listeria Monocytogenes* CECT 934.

Table 3. Primers (Simon, Gray, and Cook, 1996).

PRIMERS	SEQUENCE
Lip 1	5'GAT ACA GAA ACA TCG CTT GGC 3'
Lip 2	5'GTG TAA CTT GAT GCC ATC AGG 3'

The PCR conditions for Listeria monocytogenes, adapted from the study by Simon, Gray, and Cook (1996), which are: 94 ºC (2 min)/ 94 ºC (30 sec), 55 ºC (30 sec), 74 ºC (1 min) * 40 cycles / 74 ºC (5 min), 4 ºC.

Table 4. Master mix composision according to the PCR protocol of Listeria genus (adapted from Ryu et al., 2013).

REAGENTS FOR PCR MIXING	VOLUME	INITIAL CONCENTRATION	FINAL CONCENTRATION
H2O	16.3 µL	-	--
PE-Buffer KCL Tris-HCL pH 8.3	2.5 µL	10x	1X
MgCl2	3 µL	25 mM	3 LmM
dNTP	0.5 µL	10 mM	800 µM (200 µM)
prs-F	0.25 µL	20 mM	0.3 µM
prs-R	0.25 µL	20 mM	0.3 µM
Taq	0.2 µL	5 U / µL	1U
Total	25 µL		-

All samples that were found to not be *Listeria monocytogenes* in the previous PCR, undertake another PCR to confirm if they belonged to the Listeria genus. This PCR protocol was adapted from Ryu et al. (2013) (Table 5).

Table 5. Primers (Ryu et al., 2013).

ID	PRIMERS	SEQUENCE	SIZE	VOLUME	INITIAL CONCENTRATION	FINAL CONCENTRATION
Genus	Prs-F	GCTGAAGAGATTGCGAAAGAAG	370	0.25	20 pmol/µL	0.2 pmol/µL
	Prs-R	CAAAGAAACCTTGGATTTGCGG				

4.1.7. Electrophoresis

To create a mixture for sample loading, 2 µl of Gel red is mixed with 2 µl of Bromophenol blue. Subsequently, 5 µl of the DNA sample is added to the Gel red and Bromophenol blue mixture, and 5 µl of the final mixture is pipetted into the designated well of the electrophoresis gel for each sample. A 2% w/v Agarose gel in 1x TBE buffer was utilized and the gel electrophoresis was run for 1h at 90V. The ladder (5 µL) used was the 100 lanes NZYDNA ladder VI (NZYTech, Portugal). In conjunction with the ChemiDoc system, the Image Lab Software was employed to capture and analyze the generated images of electrophoresis gel.

4.1.8. Calculation of Prevalence

After obtaining the PCR and electrophoresis results, the prevalence was calculated through the following formula:

Prevalence = (Number of Positive Carcasses) / (Total Number of Sampled Carcasses) * 100

For the cold storage area: Prevalence = (14 / 39) * 100 ≈ 35.90%

For the slaughter line: Prevalence = (1 / 33) * 100 ≈ 3.03%

4.1.9. Antibiograms

4.1.9.1. Antibiogram protocol for *Listeria monocytogenes*

EUCAST's guidelines and breakpoints for antimicrobial susceptibility testing were used to choose the following antibiotics tested: Meropenem (MERO), Penicillin (PENI), Ampicillin (AMPI), Ciprofloxacin (CIPRO), LINE (Linezolid), Erythromycin (ERY), Trimethoprim/Sulfamethoxazole (SXT), Gentamicin (GENTA), and Tetracycline (TETRA). The antibiotic discs (MERO, PENI, AMPI, CIPRO, LINE, ERY, SXT, GENTA, TETRA; Liofilchem, Italy) were stored according to the manufacturer's guidelines. For quality control the strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were utilized. The bacteria were prepared by streaking a well-isolated colony of *Listeria monocytogenes* onto a TSA plate, which is then incubated overnight at 37 ºC.

In the Antibiotic Susceptibility Testing (Kirby-Bauer Disc Diffusion Method), a bacterial suspension was created from a pure *Listeria monocytogenes* culture in TSA (Triptone soya Agar, Sharlau, Spain), by taking a loop of culture and making a bacterial suspension in sterile sodium chloride (NaCl) at 0.5 McFarland using a densimat (BioMerieux, France). This suspension was then spread evenly over the entire surface of a Mueller-Hinton Fastidious Agar (BioMerieux, France) plate using a sterile cotton swab. The antibiotic discs were placed on the inoculated agar plate. The plates were incubated at the appropriate *Listeria monocytogenes*

growth temperature 37°C for 24 hours. After incubation, the diameter of the zones of inhibition around each antibiotic disc was measured using a ruler or caliper. The zone diameters to a zone interpretation chart (EUCAST's guidelines and breakpoints for antimicrobial susceptibility specific to *Listeria monocytogenes* were compared.

5. Results and Discussion

5.1 Identification of *Listeria monocytogenes* and *Listeria* spp.

A comprehensive record of all PCR reactions was conducted (Table 6). The data include a series of PCR events carried out on different dates, ranging from January to July of 2023. Additionally, a record of all gel electrophoresis images was also performed, Figure 4 and Figure 5 are examples of gel electrophoresis obtained during this work.

Table 6. PCR information based on date, protocol, number of samples.

Date	Type of PCR protocol	Number of Samples	Description
25Jan	*Listeria monocytogenes*	1-11	-
30 jan	*Listeria monocytogenes*	12-22	-
2 fev	Repeat, *Listeria monocytogenes*	15,16,17,22	Because the previous answer was not clear
13March	*Listeria monocytogenes*	23-43	-
20 March	Repeat, *Listeria monocytogenes*	25,36, 42	-
4 April	*Listeria genus*	37,40, 41	-
5 abril	*Listeria genus*	23-42	-
6 April	*Listeria monocytogenes*	23-42	-
14 April	*Listeria monocytogenes*	43-65	-
19 April	Repeat, *Listeria monocytogenes*	44,45,46,58,62,64,65	Repeated without dilution
20Jun	*Listeria monocytogenes*	66-105	-
21 Jun	*Listeria monocytogenes*	106-143	Because the previous answer was not clear
29 Jun	*Listeria monocytogenes*	92-117	-
5 July	*Listeria genus*	92-117	-

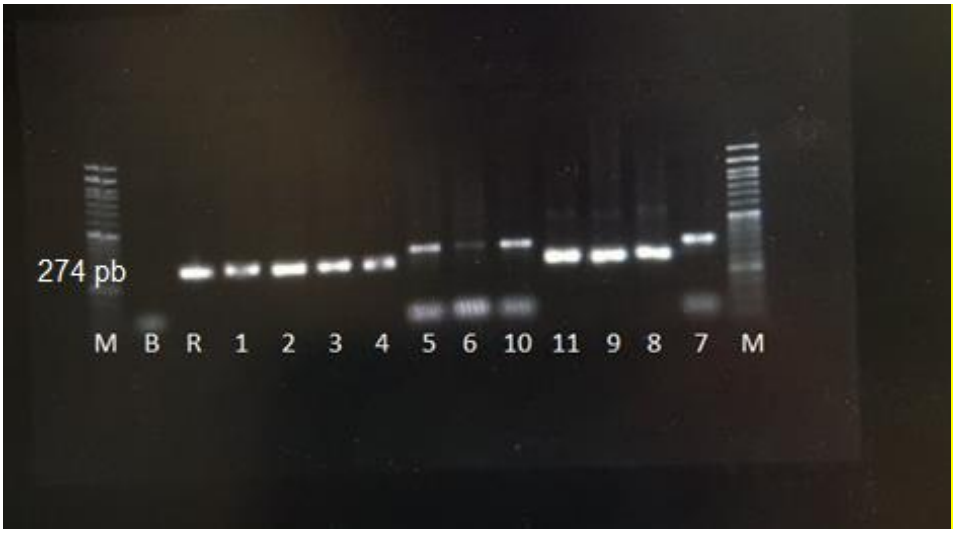

Figure 4. Gel Electrophoresis run on 31 January of 2023 as an example of a result of the PCR for identification of *Listeria monocytogenes*. From left to right it can be seen that in the first and last lanes have the ladder VI, lane 2 the negative control, lane 3 the positive control, lane 4 to 14 the samples tested.

Figure 5. Gel Electrophoresis was run on 6 July of 2023 as an example of a result of the PCR for identification of Listeria genus. From left to right it can be seen that the first and last lanes have the ladder VI, lane 2 the negative control, lane 3 the positive control, and lane 4 to 15 the samples tested. The reference is the strain *Listeria monocytogenes* CECT 934

5.2 Prevalence of *Listeria monocytogenes*

In the context of the provided data, a total of 72 carcasses (33 carcasses from cold storage and 39 carcasses from the slaughter line) were sampled, with the aim of assessing the presence of *Listeria monocytogenes* and *Listeria* spp. contamination. Of the 72 carcasses, 22 were found to be positive for *Listeria* spp., with *Listeria monocytogenes* being identified in 15

out of the 22 (Table 7). Out of the 15 carcasses positive for the pathogen in the study, one was sampled at the slaughter line while the rest were all sampled when the carcass was in the cold storage stage (Table 8). This could indicate potential contamination of the carcass during the slaughter which could mean that *Listeria monocytogenes* is present in the processing facility which could be a concern in general. This is while, generally, out of 72 sampled animals, 22 animals showed the presence of *Listeria* spp. The higher number of carcasses in the cold storage indicates once again the presence of *Listeria monocytogenes* on the processing facility at the same time also shows how these bacteria can survive very low temperatures in contrast with other microorganisms (Abeysundara et al., 2019).

With the data obtained it was possible to determine the prevalence of *Listeria monocytogenes* in slaughter line and in the cold storage. In the cold storage area, the prevalence was of approximately 35.90%, while in the slaughter line, it was approximately 3.03%. These results highlight a substantial difference in the prevalence of *Listeria monocytogenes* between the two areas, with the cold storage area showing a significantly higher prevalence compared to the slaughter line. This underscores the importance of targeted control measures and hygiene practices, particularly in the cold storage area, to mitigate the risk of contamination and ensure food safety.

Table 7. Number of sampled animals, colonies, and results.

Total numbers of animals sampled	Total positive results for Listeria	Total positive results for *Listeria monocytogenes*, from cold storage	Total positive results for *Listeria monocytogenes*, from slaughter line
72	22	14	1

Table 8. Sampled carcasses that tested positive for Listeria or *Listeria monocytogenes*.

N	Date of sampling	Animal's Number	Result	The place of sampling
1	12-01-2023	75	Listeria	meat cold storage
2	12-01-2023	78	*Listeria monocytogenes*	meat cold storage
3	12-01-2023	79	*Listeria monocytogenes*	meat cold storage
4	12-01-2023	81	*Listeria monocytogenes*	meat cold storage
5	12-01-2023	83	*Listeria monocytogenes*	meat cold storage
6	12-01-2023	84	*Listeria monocytogenes*	meat cold storage
7	12-01-2023	73	*Listeria monocytogenes*	meat cold storage
8	12-01-2023	72	*Listeria monocytogenes*	meat cold storage
9	03-02-2023	79	*Listeria monocytogenes*	Slaughterhouses line
10	08-05-2023	151	Listeria	meat cold storage
11	08-05-2023	147	Listeria	meat cold storage
12	08-05-2023	144	Listeria	meat cold storage
13	08-05-2023	157	*Listeria monocytogenes*	meat cold storage
14	08-05-2023	158	*Listeria monocytogenes*	meat cold storage
15	08-05-2023	160	Listeria	meat cold storage
16	08-05-2023	163	*Listeria monocytogenes*	meat cold storage
17	08-05-2023	164	Listeria	meat cold storage
18	08-05-2023	166	*Listeria monocytogenes*	meat cold storage
19	08-05-2023	168	Listeria	meat cold storage
20	08-05-2023	169	*Listeria monocytogenes*	meat cold storage
21	08-05-2023	173	*Listeria monocytogenes*	meat cold storage
22	08-05-2023	172	*Listeria monocytogenes*	meat cold storage

In total 143 suspicious colonies were collected during the study, 7 were identified as *Listeria* spp., while 51 were specifically identified as *Listeria monocytogenes*. The majority of *Listeria monocytogenes* isolates identified (93%) were isolated from carcasses in cold storage while only 7% originated from the slaughterhouse line (Figure 6). This demonstrates once again the slaughter line in the processing facility is contaminated with *Listeria monocytogenes* which is not uncommon (1 item found). This contamination can also justify the higher presence in the cold storage, since these bacteria can grow in much lower temperatures than other bacteria which can multiply more rapidly in the cold storage (Abeysundara et al., 2019) .This considerable percentage highlights the significance of maintaining strict hygiene and proper storage conditions in cold storage facilities to mitigate the risk of pathogen contamination.

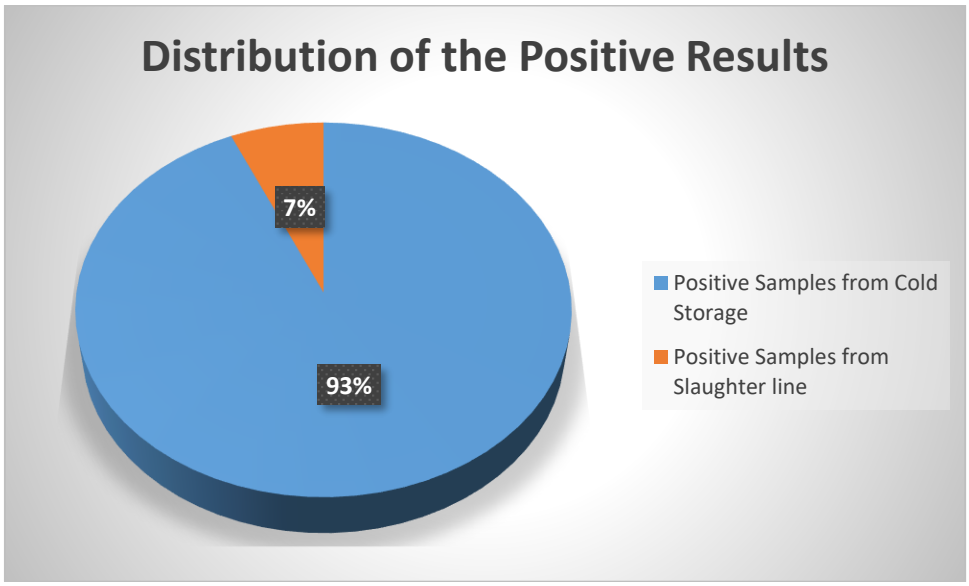

Figure 6. Distribution of positive results at cold storage and slaughterhouse line.

5.3 Antibiotic resistance profiles

After identification of *Listeria* spp. and *Listeria monocytogenes* six isolates were selected to study the antibiotic resistance profile (Figures 7 and 8).

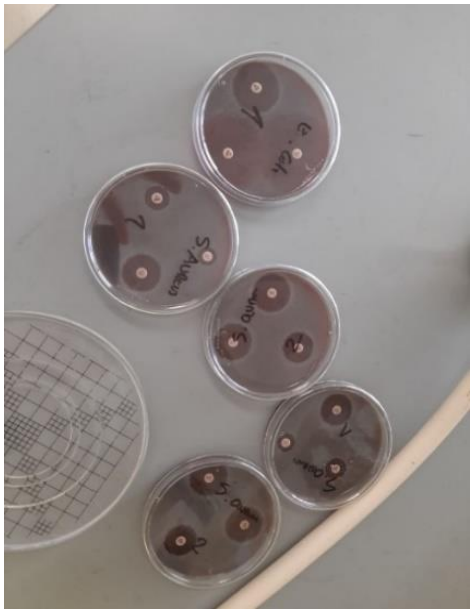

Figure 7. Antibiogram using antibiotic discs.

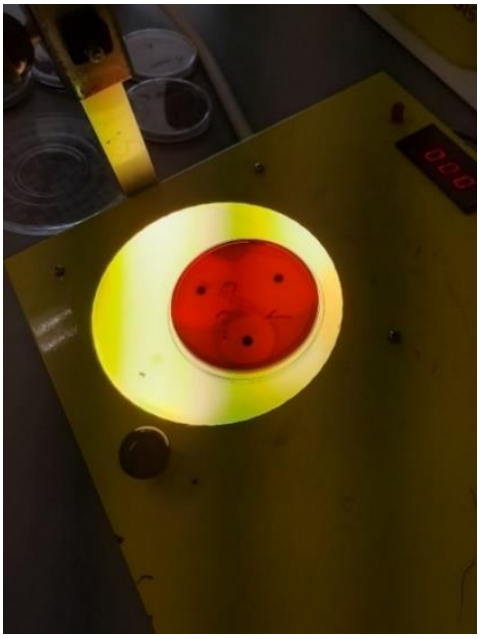

Figure 8. Measurement of zone diameter values.

Table 9 shows the zone diameter values (in millimeters) for each antibiotic disk, indicating the inhibition zones around the discs on the agar plate. These values are used to determine whether the bacteria are sensitive or resistant to the antibiotics. All six isolates were susceptible to ampicillin, meropenem, linezolid, gentamicin, and tetracycline. Resistance to ciprofloxacin was found in all isolates except one, which originated from bovine carcass under refrigeration. Interestingly this same isolate was the only one found to be resistant to sulfamethoxazole/trimethoprim and erythromycin. Also, there is a study on the resistance in beef isolates, which shows that most of the strains isolated from raw meat, food, or food-processing environments were susceptible to antimicrobials except oxacillin; some isolates were also resistant to ampicillin, clindamycin, gentamicin, tetracyclines, and penicillin. Several isolates were resistant to oxacillin (72.2%) or clindamycin (37.0%) (Wieczorek et al., 2012). Another study showed more than 90 % of the *Listeria monocytogenes* isolates from beef cattle resisted ampicillin, penicillin and erythromycin and more than 75 % resisted vancomycin (Obaidat, 2020). These findings indicate that *Listeria monocytogenes* of beef origin can be considered a public health concern.

Table 9. Recorded results of antibiogram for samples.

Date		8th May		12th Jan		3rd Fev	
Sampling Location		Cold Storage				Slaughter Line	
Antibiotic	Halo diameter Range	Number of the Carcasses					
		163	172	73	84	79	79
Peni	S⩾13mm	20	12	21	19	17	19
	R<13mm	20	12	21	19	18	19
Ampi	S⩾16mm	21	19	24	23	21	22
	R<16mm	21	19	24	23	21	22
Cipro	S⩾50	20	45	21	21	19	19
	R<24	20	45	21	21	19	20
Mero	S⩾26	28	27	31	32	31	28
	R<26	28	27	31	31	31	28
Line	S⩾22	25	25	24	25	25	24
	R<22	25	25	25	25	25	25
Ery	S⩾25	27	18	28	27	25	27
	R<25	27	18	28	27	25	27
SXT	S⩾29	33	16	35	35	35	34
	R<29	33	16	35	35	35	34
Genta	S⩾18	23	22	24	24	22	22
	R<18	23	22	24	24	23	22
Tetra	S⩾25	30	25	31	27	26	26
	R<25	30	25	31	28	26	26

6. Conclusions

The investigation showed several critical findings. Among the 72 animals sampled, approximately 30.56% were identified as carriers of Listeria, which 20.83% of that belonged to *Listeria monocytogenes*, highlighting the concerning prevalence rate. The presence of 143 suspicious colonies in samples emphasizes the need for continuous attention in monitoring and suggests potential lapses in control measures. Moreover, the detection of *Listeria monocytogenes* in 14 positive samples from meat cold storage and one positive result along the slaughter line underscores the susceptibility of the processing chain to contamination at various stages.

The antibiogram analysis conducted on six *Listeria monocytogenes* exhibited sensitivity to Ampicillin, Meropenem, Linezolid, Gentamicin, Tetracycline, and Sulfamethoxazole/Trimethoprim (SXT) in *Listeria monocytogenes* was found in all carcasses while Resistance to Erythromycin, Penicillin, and SXT in *Listeria monocytogenes* was found in one carcass. In addition, resistance to Ciprofloxacin in *Listeria monocytogenes* was found in all

carcasses. These findings highlight the importance of accurate antibiotic selection when treating infections, caused by *Listeria monocytogenes*, with several effective options available for treatment.

Considering these findings, it is crucial that slaughterhouses adopt comprehensive monitoring, sanitation, and control measures to minimize the threat of *Listeria monocytogenes* contamination and ensure the safety of food products originating from bovine carcasses. continuous attention and proactive interventions are important to safeguard public health and maintain the integrity of the food supply chain. This emphasizes the need for targeted control measures, particularly in the cold storage area, to reduce the risk of contamination and ensure food safety. The relatively high occurrence of *Listeria monocytogenes* carriers among the sampled animals raises significant concerns for food safety, underscoring the vital role of stringent hygiene and sanitation practices.

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