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HISTAMINE QUANTIFICATION IN FERMENTED LIVER SAUSAGES OF THE MARCHE REGION AND CHARACTERISATION OF PRO-TECHNOLOGICAL GRAM-POSITIVE BACTERIA

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ABBREVIATIONS

%	Percentage
°C	Degree Celsius
a_W	Water activity
BA	Biogenic Amine
g	Gram
mL	Milliliter
Kg	Kilogram
µL	Microliter
CFU	Colony forming unit
CNC	Coagulase-negative cocci
LAB	Lactic acid bacteria
BHI	Brain Heart infusion
MRS	De Man, Rogosa and Sharpe
MSA	Mannitol Salt Agar
RH	Relative Humidity

Chapter 1
INTRODUCTION

The production of salami through fermentation of meat represents one of the most ancient and effective methods to preserve such a perishable foodstuff. Thousands of years ago, it was discovered that if meat was combined with salt and fragrant herbs followed by a drying phase, the resulting product was edible for a long time. Nowadays, the main principles of meat preservation have not been altered, and a variety of fermented meat products are still manufactured all over the world. Among them, the most well-known goods that have resulted from these processes are dry-fermented sausages and dry-cured hams (Zdolec, 2017).

Fermented meat products are among the most representative traditional foods that have been prepared and enjoyed throughout history by an extended range of civilizations worldwide. These meat products, which exist in a broad range of tastes and textures, play an essential role in local economies, cultures, and gastronomic heritages. To date, there is a significant tendency to enrich our sensory experiences, and many customers and meat companies worldwide are becoming increasingly interested in fermented products (Toldrà, 2022). Such evidences are highlighted also from statistics, which state that about 3% to 5% of all consumed meat is fermented (Hutkins, 2019).

1.1 History of fermented sausages

Since from the civilization of the first societies, the need to preserve food for storage and transport increased. Fresh meat is a nutrient-dense but perishable food, and its storage represented a real challenge for such early civilizations; as a result, meat preservation techniques, including extensive salting, drying and smoking processes under the proper environmental conditions emerged. Like other fermented foods, evidence of fermented meats dates back thousands of years due to their advantageous characteristics: they are ready to eat, easy to be transported and stored, they present a high nutritional value and they are extremely stable in terms of flavor. Indeed, fermentation technique has facilitated meat preservation in periods of food scarcity and have circulated over large geographical distances. Fermented and other salt-dried meat products were also employed as trading commodities or food for merchants travelling throughout Europe (Leroy et al., 2013).

According to history, the production of fermented sausages probably started during the Roman empire in the Mediterranean Sea area, even if some studies report evidence of salami-like food remains in the tomb of Ramses

III (1166 b. C.), suggesting their presence in ancient Egypt. Given the fact that Mediterranean climate is particularly favorable to the ripening process, it is likely that the discovery of fermented sausages in Mediterranean countries could be related to a spontaneous fermentation occurring in meat products (Aquilanti et al., 2016).

The Romans established the basis for the modern Italian ability to produce fermented meats. Other countries of Europe, such as Germany, are likely to have adopted the meat fermentation process considerably later, as it is considered that it was adopted barely 150 years ago (Leroy et al., 2013).

The majority of fermented sausages from Northern and Eastern Europe are smoked, whereas mold-ripened sausages are rare. Traditionally, the sausages were prepared during winter, following curing temperatures up to 15 °C for long time periods. Such process allowed their storage at uncontrolled temperatures for up to a year (Zdolec, 2017).

Production of fermented meats seems to have spread from the Mediterranean to Northern Europe, and from the Northern Europe to the rest of the world through increasing migration events, including United States, South America, Australia, and elsewhere (Tamang et al., 2010).

Because of the development of the cold chain, particularly in Western countries, the preservation role of meat fermentation has become mostly obsolete. Nonetheless, fermented meat products are still quite popular and manufactured in great quantities, particularly in Europe, where many of their variants are produced. Furthermore, meat fermentation has become a technique utilized for the modification and the improvement of the sensory characteristics of meat rather than its preservation (Leroy et al., 2013).

In the early 1900s, it was assumed that beneficial bacteria were responsible for the obtainment of a quality end-products, and the use of microbial "starter cultures" was developed in the 1930s and 1940s, paving the way to the industrial production of fermented preparations. Such approach relies on the transfer of a small portion of fermented meat sausage from the previous production batch to the meat batter of the new production batch, leading to safer results. The described method, named *back-slopping*, allows to use bacteria already well-adapted for the meat environment and generally results in a higher quality product (Hutkins, 2019).

From that moment on, the spontaneous fermentation approach at industrial level was nearly abandoned, also due to the poor level of reliability. Although meat fermentation is perceived as an ancient and established technology, production processes have adjusted over centuries mostly due to enhanced technological standards, the overall process efficiency, and the establishment of safety concerns. Around 50 years ago, numerous technologists have stated that successful meat fermentation process should be rapid and homogenous, since it is known that shorter process times greatly increase profit margins and the competitiveness of the final products; a significant acceleration has been achieved through the inoculation of the sausage batter with commercial starter cultures, such as lactobacilli, pediococci, staphylococci, yeasts and molds (Leroy et al., 2013).

It is reasonable to assume that the value of the raw material, as well as the choice and speed of processing, affect quality more than the fermentation process scale. However, traditional small-scale manufacturing may even fail

to generate high-quality goods, since a lack of knowledge of specific technical issues is more likely. Nonetheless, traditional fermented meats are frequently considered qualitatively higher than commercial ones due to more varied and appreciable unique sensory attributes of the end-products (Leroy et al., 2013).

1.2 Production of fermented sausages

Since traditions and people's preferences drastically vary among nations and regions, the added ingredients and production procedures of fermented meat sausages are combined in very different ways. Nevertheless, the production process normally consists of the following main phases: 1) selection of ingredients; 2) mixing of ingredients; 3) fermentation; 4) drying and ripening.

1.2.1 Selection of ingredients

The two main components in most fermented meat preparations are meat and fat. Indeed, their properties have a significant impact on the characteristics of these products, including sensory, nutritional, safety, and health aspects. When comparing low-, medium-, and high-fat sausages, fat content is typically strictly associated to the sensory characteristics of the final product. In fact, higher levels of free fatty acids (FFAs) and oxidation have been determined in fermented sausages prepared with higher quantities of added fat, whereas a moderate fat content (20%) reached the greatest overall acceptability. Meat is defined as the skeletal muscles of animals which include primarily muscle, fat, and connective tissues, but also vascular, lymphatic, and nervous structures. Pork is the most common meat source for fermented meat products, but beef, lamb, chicken, and turkey are also often used. Usually, ovine and goat meats are not considered for this type of production, since they are characterized by a strong flavor, usually rejected by western consumers (Toldrá, 2015). Noteworthy, meat from old animals is preferred, due to their higher fat and myoglobin contents.

Apart from such two basic components, other key elements involved in fermented sausage production include salt together with spices (pepper, chili pepper, fennel seeds, and garlic), offal, plant materials, and water.

Salt mainly exerts three functions in fermented sausages: shelf-life extension, extraction of the myofibrillar proteins promoting water holding and hydration, and flavor enhancement (Slobodan et al, 2011).

Species are mostly used as flavorings and coloring agents. However, species include varied components, including sugars, nitrates, and metallic ions. Ground pepper, which is often present in all types of sausages around 0.2-0.3%, has a relatively high manganese concentration. Furthermore, several spices include antioxidant molecules that can improve the shelf life of dry-fermented sausages. Indeed, the oxidation of lipids in foodstuffs causes the formation of undesired flavors, resulting in unacceptable products for human consumption. Aside from antioxidant activity, many spices exert antibacterial activities, and the antiseptic potential of spices is confirmed in the corresponding

essential oils. Garlic is one of the most added spices, containing multiple antibacterial components, including allicin, the main recognized active element. Extensive research has been carried out to determine garlic inhibitory properties, and many food-borne pathogens, both Gram-positive and Gram-negative bacteria, have been shown to be inhibited by garlic; however, unlike some pathogens, lactic acid bacteria are typically quite resistant to the antimicrobial activity of spices. Generally, the amounts of spices employed during fermented sausage production process result inadequate to interfere with the growth of pathogens, and thus ineffective as preservatives (Verluyten et al, 2004).

1.2.2 Mixing of ingredients

Meat, and eventually offal, used to manufacture fermented sausages are selected and weighted; then, manually or mechanically, the meat is transferred to the processing area, where it is minced through a meat grinder. Here, the chopping degree varies from 2-5 to 6-12 mm, depending on the type of sausage (Zdolec, 2017). Subsequently, the previously weighed non-meat ingredient mixture is poured into the minced meat, and the semi-finished product is inserted into the mixing machine, which uniformly blends the product. During this phase, the salt causes the muscle tissues to melt and myofibrillar proteins to partially dissolve (Hutkins, 2019).
After homogenization, a uniform, slightly sticky mince, named meat batter, is formed and then stuffed into natural or synthetic casings by means of a funnel attached to the machine, obtaining the fresh meat sausages. Afterwards, the product is generally bound with a specific food twine, which contributes to preserves anaerobic conditions in the batter, promoting the formation of desirable fermentative microbiota while suppressing spoilage bacteria (Leroy et al., 2013).

1.2.3 Fermentation

Fermentation step leads to the production of organic acids which influence firmness, flavor, safety, color, proteolysis, lipolysis, aromatic compound synthesis, and a variety of other factors. The fermentation process is strictly correlated with the environmental conditions, including temperature and humidity. Such process, mediated by microorganisms, is favored by a temperature comprised between 2 to 20 °C and a relative humidity (RH) comprised between 75 to 80%. Therefore, the meat sugars in the meat batter are turned into lactic acid by lactic acid bacteria (LAB), whereas coagulase negative cocci (CNC) reduce nitrate to nitrite, and water slowly evaporates; such general modifications generate the typical flavor of fermented meat sausage, characterized by low pH and low water activity (a_w), resulting in a stable end-product with an extended shelf-life even without a thermal treatment. Acidification by LAB is usually boosted by the addition sugar, and added salt, nitrate or nitrite, as well as some extent of dehydration, are major aspects for the preservation of fermented sausages (Toldra, 2002). Lactic acid production is accelerated by the increase of fermentation temperature and a_w. In Europe, fermentation

temperatures range from 5 to 26 °C, with lower temperatures utilized in the Mediterranean region, and higher temperatures used in Northern Europe. Semi-dry products are often fermented in the United States at temperatures that gradually rise over 35 °C in order to minimize the fermentation time, which generally lasts 12 hours or less (Toldrà, 2015).

1.2.4 Drying and ripening

Meat fermentation is followed by a drying step to further stabilize and enhance the sensorial characteristics of the product, in which anaerobic species of lactic acid bacteria are involved. The drying process, together with salting, stuffing and fermentation, provides various antimicrobial barriers, resulting in a shelf-life of several months (Leroy et al., 2013).

Drying not only reduces the a_w of the product but also influences the hardness and durability of the protein matrix. Moreover, the establishment of the correct drying temperature is pivotal to ensure the quality of the end-product, since many variables, including time, humidity, flow characteristics, the product moisture, size, shape and structure, must be taken into account (Hutkins, 2019).

Another process taking place during drying is casing hardening, which indicates not only an increase in surface hardness but also a significant drop in drying rate, resulting in a heterogeneous dried product with a highly dried surface and a partially dried core. Because of its barrier function, fat (ranging from 5% to 70%) leads to a general decrease of drying rate, but also protects the food surface from over-drying. For this reason, in some production plants, a fat coating is typically performed on the sausage surface during the final phase of the production process (Toldrá, 2015).

A component affecting the drying rate is the air flow speed: as the air velocity increases, a higher flow is generated on the product's surrounding area, resulting in a directly proportional increase of the effective a_w gradient in the product outer layers. The drying parameters usually applied consist of a temperature of 15 °C, a RH of 78-88%, and an air velocity of 0.1 m/s for about 3 days. Based on the rate of drying, fermented sausages can be classified into different classes, as shown in *Table 1* (Tamang, 2010).

Table 1 - Classification of Fermented sausages

Designation	Examples	Moisture Content (%)	Moisture Loss (%)	Moisture: Protein	a_w
Dry (sometimes smoked)	Pepperoni, Milano salami, Genoa salami, *saucisson sec* (France)	25–40	25–50	<2.3:1	0.85–0.86
Semidry (usually smoked)	Summer sausage, *cervelat*, *Thuringer*, *chorizo* (Spain, South America, Philippines), *meguez* (N. Africa), *soudjouk* (Turkey)	40–50	15–30	2.3–3.7:1	0.92–0.94
Undried (spreadable)	*Teewurst*, *Mettwurst*, *Braunschweiger*, *nham* (Thailand)	50–60	10		0.95–0.96

Afterwards, the product is subjected to the ripening phase for about 4 weeks by lowering the temperature to 13-15 °C and the RH to 75-80% (Zdolec, 2017).

During ripening, numerous chemical reactions occur, involving endogenous enzymatic reactions, lipid oxidation and many others, generating a vast number of different chemical compounds (non-volatile and volatile), including mostly desirable flavors and aroma compounds (Krvavica et al, 2012). In more detail, the lipid fraction is subjected to hydrolytic and oxidative modifications, resulting in the production of free fatty acids (FFA) and the oxidation of unsaturated fatty acids, particularly polyunsaturated acids, with the consequent generation of carbonyl compounds. The activity of CNC hydrolytic enzymes, which release peptides and amino acids, and the activity of molds, which breakdown lipids, proteins, and lactic acid, favoring pH rise, alter the scent and flavor of the final product (Molly et al, 1997).

Furthermore, the ripening process guarantees additional and consistent moisture removal, preventing the formation of undesired molds on the surface as well as the acquisition of unfavorable alterations in the sensory qualities and texture of the end-product (Molly et al, 1997).

In the case of European fermented meats, based on the ripening, the fermentation process, the final pH and a_w, in Europe, fermented sausages are subjected to a differentiation between the North and South of the continent (Leroy et al., 2006). North-European fermented sausages tend to be acid (pH of 5.0 or lower) and are fermented more rapidly and at higher temperatures than Mediterranean-type sausages. The latter are prepared using a slower acidification process and are more extensively dried and ripened (Hierro et al., 2015).

Additionally, South-European fermented sausages are often heavily spiced and sometimes overgrown with desirable molds, whereas a smoking step is commonly applied in Northern Europe to inhibit mold growth (Leroy et al., 2013).

1.3 Fermented Liver Sausage

Fermented liver sausage has been part of many population diets for a long time and its acceptability varies among societies. They are broadly classified in traditionally or industrially produced; however, many regions of Europe are mainly characterized by a large number of traditional fermented sausages handcrafted by artisanal manufacturers (Feiner, 2006), as in the case of the Italian fermented liver sausages produced in the Marche region. The production of fermented liver sausages maximizes the utilization of unpopular raw materials, such as the internal organs of animals, thereby acquiring a greater value (Chyr, 2006). During manufacturing, different offal can be incorporated into the sausage mixture, but liver is used in greater proportion. *Table 2* shows the usual ingredients employed in fermented liver sausage manufacturing.

Table 2 - Main ingredients of Fermented liver sausages

Ingredient	**Quantity % per Kg**
Fat & lean bloody waste and offal (heart, tongue, etc.)	64
Pork liver	20-33
Salt	2.9
Ground pepper	0.2
Chili pepper	0.1
Fresh ground garlic	0.1
Nitrates or nitrites	< 0.0003
Sugars	0.3

Depending on the producer, the ingredients may differ in the amount of sugar added, spice combination, additives, and meat composition, whereas the production process may differ, for instance, in the grinding degree, casing diameter, and ripening time (Zdolec, 2017).

For this reason, the following description of the production process of fermented liver sausages represents a typical example generally applied for their manufacturing. Prior to use, all ingredients used to manufacture fermented liver sausages are stored in cold rooms. The preparation of fermented liver sausages begins with the sectioning and inspections of the main ingredients (meat, fat, and liver), in order to determine their overall acceptability. The casings of animal origin are desalted through a series of washing with drinking water, wine and flavors, whereas non-meat ingredients, such as salt, spices, flavorings, and additives, are weighed in proportion to the amount of raw material processed (Toldra, 2015).

The operator manually sizes the amount of meat, fat, and liver that will be processed; then, the sized meat is placed into food containers and transported to the processing area, where it is ground through a meat grinder using a first 10 mm grid, and finally a 3 mm grid. Subsequently, non-meat ingredients are manually added to the meat mixture; once a uniform texture is achieved, the resulting meat batter is inserted in the casings through a partially mechanized process by means of a bagging machine. The semi-finished sausages are then transferred to the drying room, where fermentation occurs for 4-5 days as average. Liver sausages are usually fermented at modest temperatures around 15 °C, however there is a tendency toward the increase of the temperature to promote the proliferation of starter cultures eventually added during the production process. Once fermentation phase is concluded, the sausages are transferred to seasoning rooms where both temperature and humidity are under control; fermented liver sausages are generally dried at a temperature of 20 °C and a RH of 90% for 8-10 hours, followed by another drying cycle at 20 °C and 50% of RH for 20 hours. For the subsequent 5 days, temperature is lowered by 1/1.5 °C every day until the drying process is over. However, these parameters may varies depending on several factors, such as the size of the sausages and tradition. Finally, the ripening stage is carried out at 14-5 °C with 70% of RH for at least 1 month. Fermented fish sausages can be considered ready-to-eat after about 50 days from the beginning of the production process (Krvavica et al, 2012).

1.4 Roles of Microbes in Fermented Sausages

To date, the majority of manufactured products in Europe are prepared using conventional fermentative and ripening processes dependent on the activity of extremely different ecosystems composed by autochthonous microorganisms occurring in both the raw material and in the production environment. Lactic acid bacteria (LAB), whose primary contribution is the acidification and the production of organic acids via carbohydrate fermentation, and the CNC, which includes both micrococci and coagulase–negative staphylococci (CNS), responsible for color development and stabilization of the end-product, proteolysis, lipolysis, and free amino acid decomposition, represent the major microbial groups involved in meat sausage fermentation. Yeasts and molds also play a marginal but significant role in the establishment of a surface layer that protects the lipid component from excessive dehydration as well as oxidation favored by air and light (Aquilanti et al., 2016).

1.4.1 Lactic acid bacteria

The stability of fermented meat products is mainly determined by a combination of acidification driven by LAB and a_w decrease during curing and drying (Toldra, 2015).

LAB are pivotal agents in meat fermentation since they improve the hygienic and sensory quality of the final product. Several LABs have been investigated for their ability to produce bacteriocins and utilized as a bioprotective culture to preserve fresh and processed meat and fish (Fadda et al., 2010).

Bacteriocins can be active against Gram-positive pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*, and exert antimicrobial effects through the prevention of spore germination and the modification of microorganism enzymatic activity. Furthermore, the carbon dioxide generated by heterofermentative LAB might help to preserve the end-product by penetrating the microbial cells of pathogens, thus blocking bacterial enzymes and damaging cell membranes (Steinkraus, 1992).

By producing lactic acid, acetic acid and hydrogen peroxide, LAB proliferation leads to a decrease in pH, thus inhibiting spoilage and pathogenic microorganisms, and contributing to color stabilization and texture enhancement. Low pH values allow organic acids to be lipo-solubilized into cell membranes and reach the cytoplasm of vulnerable microbes. Also, the texture of the product is affected since the action of lactic acid generated by LAB causes the coagulation of sausage proteins. In fact, at pH values about 4.6–4.9, muscle proteins coagulate and lose their water-holding capacity, resulting in improved sliceability, firmness, and cohesiveness of the final product. The acidic environment also promotes ripening and color development (Toldra, 2015).

LAB also generate hydrogen peroxide, able to inhibit the growth of numerous undesired microorganisms (e.g., Enterobacteriaceae, *S. aureus*, Pseudomonadaceae) and thus promoting the preservation of fermented sausages. The antibacterial activity performed by hydrogen peroxide is correlated to the oxidation of sulfhydryl groups, which causes bacterial enzymes to denature; despite its positive action, hydrogen peroxide can also cause color and taste undesirable modifications by interacting with polyunsaturated fatty acids, favoring rancidity (Wood, 2012).

The release/degradation of free amino acids by LAB has a considerable impact on the composition of non-volatile and volatile compounds, and the oxidation of unsaturated free fatty acids is also inhibited. LAB proteolytic enzymes produce short peptides and amino acids that function as direct taste enhancers or as precursors to other flavor components (Hammes et al., 1990).

1.4.2 Coagulase negative cocci

Fermented sausages production relies on the metabolic activities of LAB and catalase-positive cocci, particularly the group of CNC. The traditional use of CNC as meat starter cultures results in a defined color development due to their nitrate reductase activity, whereas their catalase activity prevents oxidative damage. Furthermore, CNC metabolism contributes to flavor, although the precise mechanisms are still difficult to be explained (Sánchez Mainar et al., 2017).

Color formation and stability represents a key characteristic of fermented meat products, as the oxidation might result in an unattractive greyish color related to metmyoglobin production. To prevent such modification, a characteristic stabilized cured color is obtained through the synthesis of nitroso-myoglobin, which is produced by the interaction of muscle-based myoglobin and the highly reactive nitric oxide (NO) molecule. In general, NO is produced during the curing process by the addition of potassium nitrate that functions as a precursor of nitrite, since it is transformed during fermentation by the nitrate reductase enzyme found in many CNC strains (Sánchez Mainar et al., 2017).

The metabolic activity of CNS is crucial to flavor development, especially in Mediterranean fermented meats, characterized by slightly acid taste and complex sensory profiles. Potential contributions of CNC to flavor development can be grouped into four major action modes: (i) carbohydrate fermentation; (ii) amino acid conversion reactions; (iii) lipid β-oxidation; (iv) esterase activities (Toldra, 2015).

1.5 Biogenic amines in fermented sausages

Today, food safety and quality are some of the main concerns of consumers and health agencies around the world. Unfortunately, our current lifestyle and market globalization have led to an increase in the number of people affected by food poisoning with different origins (bacteria, virus, parasites, mold, contaminants, etc.), and some cases of food poisoning can be traced back to chemical and natural toxins (Ruiz-Capillas et al., 2019).

Historically, fermented sausages were considered to be healthy and safe foods. However, eating fermented sausages has lately been linked to health risks due to high levels of saturated fats and NaCl, the presence of nitrite and degradation products, such as nitrosamines, together with the use of smoking that can lead to hazardous chemical molecules. Moreover, from the microbiological point of view, the nutritional composition of fermented sausages allows the proliferation of food pathogens, whereas the metabolic activity of microorganisms can lead to the production of mycotoxins and biogenic amines (BA) (Spano et al, 2010).

The study of BAs in food is important due to the possible toxicological consequences on consumers. Food containing high levels of BAs has been linked to a number of hazardous effects, including histaminic poisoning, food-induced migraines, and hypertension due to interactions with monoamine-oxidase inhibitor drugs (Bover-Cid et al, 2000). Although the consumption of food containing large amounts of BAs can have toxicological

consequences, there is no specific legislation regarding the presence of BAs in foods, with the exception of fishery products, whose maximum acceptable level of histamine is defined. In fact, histamine is targeted as toxic by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) (Ruiz-Capillas et al., 2019). However, since numerous factors are involved, determining the hazardous threshold for BAs is challenging (Bover-Cid et al, 2000).

Fermented foods and beverages, such as dry fermented sausages, represent examples of food products that can accumulate high BA levels, generated by the decarboxylation of its corresponding precursor by fermentative and/or spoilage microorganisms (Alvares et al., 2014). However, since the capacity of microorganisms to decarboxylate amino acids greatly varies among microbial groups and species, detecting bacteria with amino acid decarboxylase activity is pivotal to estimate the BA risk in foods and prevent their synthesis in foodstuff (Spano et al, 2010).

BA formation is influenced by a variety of factors, which can be classified into three categories: raw materials (composition, pH, ion strength, and so on), microorganisms (decarboxylase activity is primarily attributed to Enterobacteriaceae, Pseudomonadaceae, Micrococcaceae, LAB), and processing and storage conditions (fresh, cured, fermented, refrigerated, modified atmosphere). These elements do not act in isolation, but rather have a synergistic influence on the ultimate concentration of BAs in food. As a result, in order to ensure food quality in terms of BAs, it is critical to select appropriate raw materials that minimize the presence of BAs in the end-product and therefore ensure superior quality (Ruiz-Capillas et al., 2019).

Microorganism capability of producing BAs depend on the presence of amino acids decarboxylation genes (Spano et al, 2010). Free histidine can be decarboxylated to histamine, and such activity is mainly conducted by Gram-negative bacteria. However, some Gram-positive bacteria involved in food production, including lactic acid bacteria (LAB) strains of the species *Lactobacillus buchneri*, *Lactobacillus curvatus*, *Lactobacillus helveticus, Lactobacillus hilgardii, Lactococcus lactis, Pediococcus damnosus,* and *Pediococcus parvulus,* also perform this enzymatic activity, and form histamine in cheeses, fermented sausages and beverages (Rossi et al, 2011).

Gram-positive bacteria encompass microbial groups, including LAB, potentially responsible for the production of BAs. In fact, LAB capable of producing BAs are common in the microbiota of fermented foods. They may potentially be responsible for sensory defects in foods, thus causing the danger. However, the identification of one of the possible causes of BA production may represent the risk solution to the BA problem in fermented foods (Alvares et al., 2014).

In addition to the health aspect, BA are also extremely important as markers of food quality and acceptability, which is especially relevant because BAs are present in various amounts in a wide variety of foods and their production is regulated by determined conditions (Ruiz-Capillas et al., 2019).

The most important BAs detected in foods are histamine, tyramine, putrescine, cadaverine and phenylethylamine, which are produced by the decarboxylation of histidine, tyrosine, ornithine, lysine and phenylalanine, respectively.

1.5.1 Histamine

Histamine, together with tyramine, is the most toxic BA known. Moreover, it is thermostable, implying it is not inactivated by heat treatments generally used in food processing and preparation. Histamine is synthesized through decarboxylation of the amino acid histidine. Such reaction is carried out by the microbial enzyme histidine decarboxylase, characterized a different structure depending on the production by Gram-negative or Gram-Positive bacteria. Histidine decarboxylase of Gram-negative bacteria is characterized by the presence of a cofactor pyridoxal phosphate, whereas histidine decarboxylase of Gram-positive bacteria is characterized by the presence of a pyruvoyl group on the active site, encoded by the gene *hdcA* (Belleggia et al., 2021).

In Gram positive bacteria, the genes responsible for the biosynthesis of histamine are usually grouped in a cluster, the histidine decarboxylase cluster (HDC). The HDC of histamine-producing lactobacilli generally involves three genes typically orientated in the same direction: *hdcP*, *hdcA*, and *hdcB*. The first gene of the cluster, *hdcP*, encodes the histidine/histamine antiporter, followed by *hdcA*, which codes for histidine decarboxylase (Diaz et al., 2015).

Histamine intoxication is induced by the ingestion of large quantities of such BA, causing the normal metabolic systems incapable of detoxification. The symptoms of histamine intoxication are allergy-like and include neurological and gastrointestinal consequences, such as headaches, nausea, vomiting, diarrhea, cutaneous pruritus and urticaria, as well as rhinorrhea and hypotension. The illnesses severity caused by histamine exposure varies, although it is generally considered mild as it requires medical treatments on rare occasions. *Lactobacillus parabuchneri* and *L. buchneri*, isolated from fermented pork products, can produce histamine. Also, a recent study dealt with the isolation of LAB populations from dry fermented sausages produced with different starters and two spice mixes at different stages of fermentation. In the same study, tyrosine-decarboxylase and histidine-decarboxylase DNA sequences were found in 44% and 16% of lactobacilli isolates, respectively. Moreover, several *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus casei*/*paracasei* bacteria were identified as tyramine and histamine producers in the same sausages (Ruiz-Capillas et al., 2019).

Although no cases of histamine poisoning have been correlated with fermented sausages, it is not uncommon for such food category to contain histamine along with other BAs. Histamine production has been observed to occur mainly during the first 2 to 4 weeks of ripening and is mostly influenced by the activity of lactic acid bacteria and ripening conditions. According to scientific literature, high contents of histamine are generally not detected in fermented sausages; however, quantities up to 55 mg histamine/100 g have been found in dry fermented sausages. If ingested at such levels, histamine might potentially trigger hazardous reactions in sensitive individuals. Currently, only preventive and monitoring measures allow the control of BA accumulation in foods during the manufacturing process and throughout the food chain, even if in some production processes, including fermentative ones, it results quite impossible to carry out monitoring measures. (Stratton et al, 1991).

Based on the assumption that amino oxidases are responsible for the detoxification of dietary BAs, and enzymes with the similar activity have also been discovered in bacteria, some studies focused on the screening of such

activities in food-isolated microbes. It has been found out that strains of *Staphylococcus* species isolated from Italian artisanal fermented sausages were also shown to be capable of decomposing histamine, thus highlighting their potential use as starter culture (or adjunct cultures) in dry sausages to control and eventually decrease the BAs concentration (Ruiz-Capillas et al., 2019).

Chapter 2
AIM OF THE RESEARCH

In the wide scenario of Italian cured meat, fermented liver sausage represents a peculiar product. First, it has a secular and documented tradition, and such long history slightly changed its processing characteristics, if compared with analogous products. In addition, differently from other cured meats, fermented liver sausage is fermented and ripened for quite long time. The fermented liver sausage (*salsiccia di fegato*) is a traditional specialty of the Marche Region, whose production is spread across the provinces of Ancona, Macerata, Ascoli Piceno, Fermo, and Pesaro Urbino.

Several research on the microbiological and physico-chemical characterization of fermented sausages are already available in the scientific literature; however, even if the presence of BAs in food (and the risks associated with them) has been ascertained for a long time, systematic studies on their presence have only recently been conducted. This increasing research effort not only allowed a better understanding of the genetic and biochemical mechanisms promoting BAs production by foodborne microorganisms, but it also provided key information on how to reduce their accumulation in food and the risks associated with their presence.

To reach such aim, the main strategies adopted in food production process are mostly oriented on the characterization of the microbiota associated with fermented foods, the detection of strains capable of producing histamine, and the use of methods able to prevent BAs accumulation, in particular histamine.

The aim of the study is to enumerate and select the main microbial groups (LAB and CNC) naturally occurring in fermented liver sausages produced in the Marche Region to detect the presence of the gene of Gram-positive bacteria involved in histamine production. Moreover, the direct quantification of histamine was performed to better clarify and evaluate the BA risk in the analysed fermented liver sausages.

Chapter 3
MATERIALS AND METHODS

3.1 Sampling

For the present study, ready-to-eat fermented liver sausages were collected from 20 artisan production plants in the Marche region (Central Italy). For each producer, 3 samples of the same batch were collected for a total of 60 fermented liver sausages. Each fermented liver sausage sample consisted of at least 150 g of whole end-product, collected aseptically and stored under refrigeration (+4 °C) until use. Although all sausages were prepared based on the use of swine meat and liver, the list of other ingredients slightly differed depending on the producer. Therefore, the different formulations of the fermented liver sausages under study are reported in *Table 3*.
For each producer, 50 g of each sausage sample were mixed in aseptic conditions. The resulting pools were labelled as follows: A, B, C, D, E, F, G, H, I, L, M, N, O, P, Q, R, S, T, U, V. All the analyses described in the present study were conducted before sausages expiration date.

Table 3 - List of ingredients for each producer of fermented liver sausages

Ingredients	Producers																			
	A	B	C	D	E	F	G	H	I	L	M	N	O	P	Q	R	S	T	U	V
Pork meat	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Pork fat	•	•	•			•														
Pork belly																				•
Pork liver 15%																•				
Pork liver 20%					•		•										•	•		•
Pork liver 25%	•	•	•			•		•	•	•	•	•	•	•	•					
Pork liver 33%				•															•	
Salt	•	•	•	•	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•
Pepper				•	•		•			•	•	•	•			•	•	•		
Garlic					•						•	•		•			•	•	•	
Chili pepper					•									•				•		
Orange peel					•							•						•		
Wine									•											
Liqueur *(Mistrà)*												•								
Milk powder							•													•
Fructose						•														
Dextrose	•	•		•		•	•	•	•	•			•		•	•			•	•
Lactose																				•
Sucrose	•	•		•				•	•	•				•	•	•			•	•
E300	•	•	•	•			•	•	•	•	•			•	•	•			•	•
E301	•					•	•			•										•
E250							•			•				•						•
E252	•	•	•	•	•	•	•	•	•	•	•	•	•	•		•			•	•

3.2 Microbial analysis

To perform microbiological viable counts and isolation of Gram-positive microorganisms, 10 grams of each pool were added with 90 mL of sterile peptone water in a sterile Stomacher bag. Each sample was homogenized by using a Stomacher 400 Circulator (VWR International PBI, Milan, Italy) at 260 rpm for 2 minutes.
Subsequently, the serial ten-fold dilutions were set up to determine the presence and concentration of the following microorganism groups: presumptive LAB on De Man, Rogosa and Sharpe (MRS) agar (VWR Prolabo Chemicals, Leuven, Belgium), supplemented with 250 mg/L of cycloheximide and incubated at 37 °C for 48 h; CNC on Mannitol Salt Agar (MSA) (VWR Prolabo Chemicals), incubated at 37 °C for 24-48 h. Colonies were counted following the manufacturers' instructions and plates containing between 30 and 300 colonies were considered for viable counting. The results of viable counts, expressed as the log of colony forming units (cfu) per gram of sample, were reported as mean values of two biological and two technical replicates ± standard deviation.

3.2.1 Composition of Selective Solid Media

The specific compositions of the selective solid media used to quantify and isolate LAB and CNC are shown in the following tables:

Table 4 - Composition of MRS culture medium

Component	**Concentration (g/L)**
Enzymatic digest of casein	10g/L
Meat extract	10g/L
Yeast extract	4g/L
Glucose	20g/L
Dipotassium phosphate	2g/L
Sodium acetate	5g/L
Triamonium citrate	2g/L
Magnesium sulphate	0,2g/L
Manganese sulphate	0,05g/L
Tween 80	1.08g/L
Agar	15g/L

Table 5 - Composition of MSA culture medium

Component	Concentration (g / L)
Peptic hydrolyzate of animal tissue	7g/L
Pancreatic casein hydrolysate	5g/L
Meat extract	1g/L
Sodium chloride	75g/L
D-Mannitol	10g/L
Phenol red	0,025g/L
Agar	15g/L

3.3 LAB isolation

For each producer, 10 colonies of presumptive lactobacilli from microbiological viable counts on MRS agar were randomly selected starting from the highest dilution factor, in order to obtain biomass from distinct colonies. For each colony, the cell morphology was examined by using an optical immersion microscope to ensure the proper selection of the culture. The resulting cultures were subsequently sub-cultured twice on MRS agar (VWR Prolabo Chemicals) in the same conditions until purity was reached. A total of 100 isolates of presumptive lactobacilli were obtained from the pool samples.

3.4 CNC isolation

For each producer, whenever possible, 10 colonies of presumptive CNC from microbiological viable counts on MSA and BHI medium were randomly selected starting from the highest dilution factor, in order to obtain biomass from distinct colonies. For each colony, the cell morphology was examined by using an optical immersion microscope to ensure the proper selection of the culture. The resulting cultures were subsequently sub-cultured twice on BHI agar (VWR Prolabo Chemicals) in the same conditions until purity was reached. A total of 83 isolates of CNC were obtained from the pool samples.

Table 6 - Composition of BHI culture medium

Component	Concentration (g / L)
Brain heart infusion solids	17,5g/L
Peptones	10,0g/L
Glucose	2,0g/L
Sodium chloride	5,0g/L
Disodium hydrogen phosphate	2,5g/L

3.5 Conservation of microbial isolates

In order to ensure the proper conservation of the purified isolates, a solution containing glycerol as cryoprotective was utilized for each strain. In more detail, a sterile solution containing glycerol and water (VWR Prolabo Chemicals) was mixed (1:1) with MRS broth (VWR Prolabo Chemicals) and BHI broth (VWR Prolabo Chemicals) for presumptive lactobacilli and CNC, respectively.

The resulting solutions were poured in the corresponding pure sub-culture plates and a L-shaped sterile spreader was utilized to suspend the viable biomass for each isolate. Finally, the resulting suspensions were aliquoted in sterile Eppendorf tubes, and then stored at -80°C until use.

3.6 DNA extraction of microbial isolates

A sterile loop was utilized to aliquot viable biomass from the pure sub-culture plates to perform the DNA extraction. For each strain, the biomass was suspended in an Eppendorf tube containing 300 µL of TE buffer in order to solubilize DNA and protect it from degradation.

The resulting Eppendorf tubes were then treated in a Thermoblock FALC 1352 (Treviglio, Bergamo, Italy) at 100 °C for 10 minutes to ensure microbial cell breakage. A subsequent centrifugation of the samples at 13,000 rpm for 5 minutes allowed to separate the aqueous phase from the biomass, leading to the formation of a precipitate at the bottom, and the supernatant containing the strain DNA, which was transferred into a new Eppendorf tube.

The tubes containing the extracted DNA were then stored at -20°C until use.

3.7 DNA amplification via PCR

To evaluate the effective extraction of microbial isolate DNAs, a PCR with universal prokaryotic primers 27f and 1492r was performed.

Amplification via PCR was carried out using the following primers: 27f [5′-AGAGTTTGATCMTGGCTCAG] and 1492r [5′-GGTTACCTTGTTACGACTT3′-AAGTCGTAACAAGGTAACC].

Table 7 - Reaction mix for DNA amplification

Component	Initial concentration	Final concentration	Volume for each reaction tube
MyFi mix	1 X	1 X	12.5 µL
27f	10 µM	1 µM	0.5 µL
1492r	10 µM	1 µM	0.5 µL
H_2O		Up to final volume (25 µL)	
DNA			2 µL

The PCR was performed using a MyCycler Thermal Cycler (BioRad Laboratories) in a final volume of 25 µL for each reaction tube, according to the reaction conditions depicted in *Figure 1*.

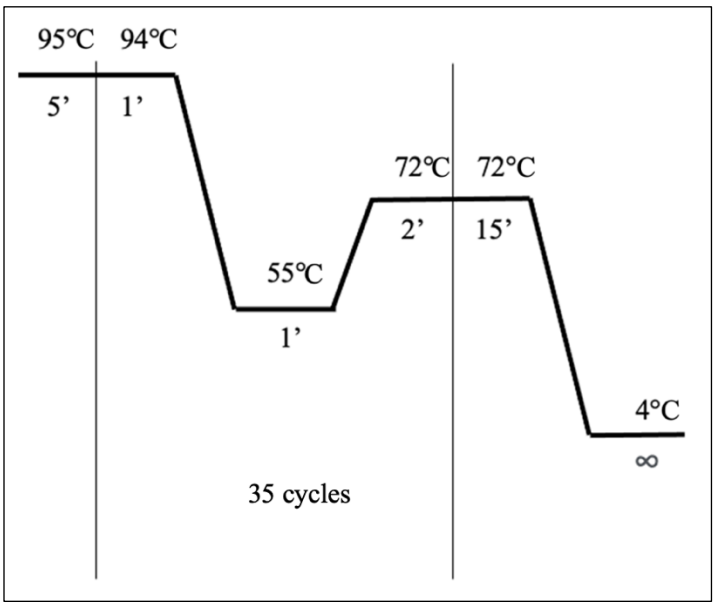

Figure 1 - PCR amplification cycle

3.7.1 Electrophoresis

The amplification was verified by electrophoresis on 1.5% (w/v) agarose gel in 0.5X Tris/Borate/EDTA (TBE) buffer containing 0.5 μg/mL GelRed® Nucleic Acid Gel Stain, 10,000X in water (Biotium, San Francisco Bay Area, USA). 5 μL of each amplicon were mixed with 2 μL of Loading dye and loaded into the dedicated wells of the electrophoresis gel. The electrophoretic run included the HyperLadder™ 1 kb (Meridian Bioscience, Cincinnati, Ohio, USA) as molecular weight standard and was carried out at 75 V for 3.5 h and visualized under UV light (λ= 260 nm). The resulting images were visualized by means of a Canon Powershot G9 camera (Canon Italia S.p.A., Milano, Italy). Correct amplification was verified by comparison with the reference ladder.

3.8 Detection of *hdcA* gene of Gram-positive bacteria on microbial isolates

The reaction mix used in this PCR essay is shown in *Table 8*, in a final volume of 25 μL. The qPCR reactions were carried out using the primer pair Hdc1 (50-TTGACCGTATCTCAGTGAGTCCAT-30) and Hdc2 (50-ACGGTCATACGAAACAATACCATC-30) designed by Fernandez et al. (2006) to amplify a fragment of 174 bp of the *hdcA* gene.

Table 8 - Reaction mix for gene hdcA detection

Component	Initial concentration	Final concentration	Volume for each reaction tube
MyFi mix	1 X	1 X	12,5 μL
Hdc1	30 μM	900 nM	1 μL
Hdc2	30 μM	900 nM	1 μL
Water		Up to final volume (25 μL)	
DNA			2 μL

The PCR was conducted through a MyCycler Thermal Cycler (BioRad Laboratories) in a final volume of 25 μL for each reaction tube, according to the reaction conditions depicted in *Figure 2*.

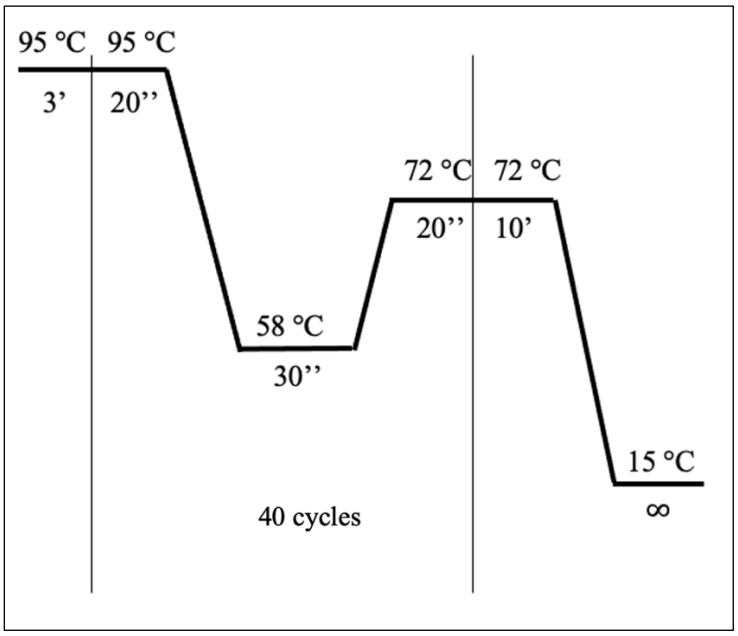

Figure 2 - PCR amplification cycle

3.8.1 Electrophoresis

The PCR amplification was verified by electrophoresis as described in paragraph 2.7.1. The electrophoretic run included the HyperLadder™ 100 pb (Meridian Bioscience, Cincinnati, Ohio, USA) as molecular weight standard.

3.9 Histamine detection

In the present study, the detection of the *hdcA* gene of Gram-positive bacteria on microbial isolates was combined with the detection of histamine in fermented liver sausage samples.

Histamine concentration measurement was carried out using the Histamine Assay Kit (Megazyme, Bray, Ireland). In more detail, 7.5 g of sample were weighted from each producer pool and mashed using a sharp knife. Subsequently, 2 g were transferred into a heat-resistant tube together with 15 mL of previously prepared sample extraction buffer (37.2 g of EDTA plus 750 mL of deionized water, adjusted to pH 8.0 and added with deionized water to reach the final volume of 1 L). The resulting mixture was homogenized; then, the tightly closed tubes were boiled for 20 minutes and left to cool at room temperature.

Again, sample extraction buffer was added in order to reach the precise final volume of 25 mL into a dedicated volumetric flask; the samples were then subjected to filtration by paper filter to remove solid residues and the resulting solutions were used in the assay.

The histamine quantification assay is based on the addition of histamine dehydrogenase to the solution, and an incubation period of 5 minutes at 37 °C, resulting in oxidative deamination of histamine (*Figure 2*).
The reduction of histamine dehydrogenase allows the formation of a colored formazan product that absorbs at a wavelength of 492 nm.

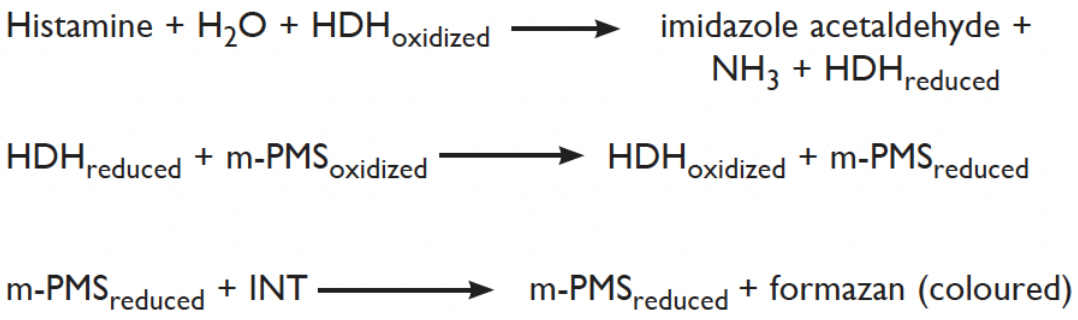

Figure 3 - Reaction mediated by Histamine dehydrogenase

The cuvettes employed in the assay were filled in the following way:

- The sample cuvettes were filled with 200 µL of distilled water, 1 mL of sample, 150 µL of buffer and 10 µL of the electron carrier 1-methoxy-5-methylphenazinium methylsulfate (mPMS) and the tetrazolium salt iodonitrotetrazolium chloride (INT) provided by the Histamine Assay.
- The blank cuvette was filled with 200 µL of distilled water, 150 µL of buffer, 1 mL of sample extraction buffer and 10 µL of INT/mPMS.
- The positive cuvette was filled with 200 µL of distilled water, 150 µL of buffer, 1 mL of previously prepared dilution made of 0.1 mL of histamine (300 mg/mL) and 4.9 mL of Sample Extraction Buffer, and 10 µL of INT/mPMS.

After the first absorbance measurement at 492 nm, performed through a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), another enzyme was employed in the assay: in detail, 1-methoxy-5-methylphenazinium methylsulphate was added to the solution and left to work for 20 minutes at 37 °C. Then, after the resting period, another absorbance measurement at 492 nm was carried out.
The histamine content was calculated using the Megazyme Mega-CalcTM available online (www.megazyme.com). The analyses were performed in three technical replicates, and the results were expressed as the mean of the mg of histamine per Kg of sample ± standard deviation.

Chapter 4
RESULTS AND DISCUSSION

4.1 Microbiological analyses

The results of the microbial characterization of the fermented liver sausages are reported in *Table 9.*

4.1.1 Lactic acid bacteria viable counting

In general, the LAB loads were quite homogeneous, ranging from 5.72 log cfu/g (producer O) to 8.50 log cfu/g (producer E), with an overall mean of 7.61 ± 0,87 log cfu/g.

When compared with the available data from the scientific literature, LAB counts obtained from fermented liver sausages of the Marche Region were higher than those reported for *Cacholeira*, a typical blood sausage containing liver from Portugal, which attested at 6.30 log cfu/g as overall mean (Belleggia et al., 2020). As for *Cacholeira*, also a German-style fermented Turkey sausage analyzed by Alter et al. (2006) was characterized by lower LAB counts, attesting at 5.4 log cfu/g as overall mean.

Such differences can be attributed to the different processing parameters, raw materials, production procedures and environmental conditions, such as temperature, pH or relative humidity employed in the production plant, which can affect the microbial populations growth. Another factor potentially explaining such discrepancy between LAB values is the integrity of the casing and its specific composition. The thickness and/or some physical characteristics of the casing itself could affect the batter inside and, therefore, its microbial composition. For example, the degree of casing permeability may influence the exchange level between the batter and the external environment and may have significant effects on the drying rate, thus on the moisture and a_w of fermented liver sausages. For this reason, greater thickness casing leads to a lower water vapor transmission rate and higher a_w. In fact, the degree of casing permeability to water, gas, and light affects water loss, fat hydrolysis, fat oxidation, as well as pH and a_w, hence on the microbial populations (Serio et al, 2020).

By contrast, LAB loads in fermented liver sausages were almost overlapping with those detected in *Salama da sugo*, a typical cooked fermented sausage produced in Emilia Romagna Region (Italy) and also with those of a fermented sausage produced in Turin, Italy, attesting respectively at 7.84 log cfu/g and 7.03 log cfu/g as overall means (Giardini et al., 2013; Greppi et al, 2015).

The cause of such similarity may be attributed to the production place, as fermented liver sausages of the present study, *Salama da sugo* and Turin fermented sausages were processed in Italy, suggesting the use of similar ingredients and/or production techniques based both on cultural and environmental reasons.

4.1.2 Coagulase-negative Cocci viable counting

CNC loads were heterogenous, ranging from 2.04 log cfu/g (producer P) to 6.80 log cfu/g (producer R) with an overall mean of 3.31 ± 2,45 log cgu/g. However, the values for such microbial group resulted quite low, as no colonies were detected for producer O, and the number of CNC was particularly modest in producer P (2.04 log cfu/g).

By comparing CNC values reported in *Table 9* with data from scientific literature, they did not particularly differ from those of *Cacholeira*, the Portoguese blood sausage containing liver. In fact, the overall mean of CNC in *Cacholeira* attested at 3.75 log cfu/g, with counts comprised between 3.1 log cfu/g and 5.2 log cfu/g (Giardini et al., 2013). Similarly to the Portuguese blood sausage, the typical cooked fermented sausage produced in Emilia Romagna Region (Italy) *Salama da sugo* shows similar CNC values to those reported in the present study. In fact, CNC counts in *Salama da sugo* attested 4.52 log cfu/g as overall mean versus the 3.31 log cfu/g of fermented liver sausages. Another similarity was found with *Ciauscolo*, a traditional Italian salami, which attested at 4.58 log cfu/g as overall mean (Silvestri et al, 2007). Again, the similarity can be attributed to the similar process and environmental conditions, which can favor the growth of certain strains rather than others.

However, when comparing the CNC overall mean of the present study with that of *Soppressata del Vallo di Diano* (6.21 log cfu/g), a traditional dry fermented sausage of Southern Italy, a big discrepancy was noticed (Villani et al., 2007).

The reason behind the lack of CNC vigorous growth in fermented liver sausages could be attributed to the low pH levels that did not increase during the drying phase. In fact, studies conducted by Stavropoulou et al. (2018) about the pH effect on fermented sausages confirm that a pH drop below 5.3 resulted in a poor growth of the CNC communities. In more detail, at the end of the ripening stage of fermented sausages of the same study, the duo of *Staphylococcus xylosus* and *S. saprophyticus* was still detected in the moderately acidified batches, even if in minor proportions, but no CNC were detected from the batches with pH levels below 5.0. Hence, the acidification degree not only influences the composition of the staphylococcal communities during meat fermentations, but also their levels (Stavropoulou et al, 2018).

In addition, differences in production technology of (traditional) fermented sausages, raw materials, additives and climate may significantly influence the affirmation of specific species and strains of CNC (Milicevic at al, 2014).

Concluding, the statistical analysis of LAB and CNC viable counts highlighted a great variability between the 20 fermentations, thus suggesting that the composition of the microbial population of fermented liver sausages from the Marche Region is strongly related to the manufacturing techniques used, as well as to the environmental diversity that characterizes different geographical areas. Notwithstanding this heterogeneity, in all the fermented sausages, the microbiological analyses highlighted the dominance of LAB over CNC and yeasts, thus confirming the pivotal role of LAB in the ripening process of fermented sausages (Coppola et al, 1998).

Table 9 - Viable counts of the fermented liver sausages analyzed samples n.d.: not detected

Producer	Presumptive lactobacilli (MRS)	Coagulase-negative cocci (MSA)
A	8.09 ± 0.03 ^{cd}	6.75 ± 0.07 ^{a}
B	7.86 ± 0.21 ^{de}	5.96± 0.04 ^{bcd}
C	6.89 ± 0.27 ^{gh}	5.67 ± 0.08 ^{cd}
D	8.50 ± 0.04 ^{abc}	4.75 ± 0.01 ^{e}
E	8.50 ± 0.01^{abc}	6.10 ± 0.05 ^{b}
F	8.61 ± 0.09 ^{ab}	6.01 ± 0.07 ^{bc}
G	7.45 ± 0.12 ^{ef}	3.79 ± 0.04 ^{g}
H	8.22± 0.09 ^{bcd}	5.93± 0.06 ^{bcd}
I	8.69 ± 0.14 ^{a}	5.98± 0.20 ^{bcd}
L	8.47 ± 0.00 ^{abc}	4.24 ± 0.12 ^{f}
M	7.82 ± 0.10 ^{de}	4.26 ± 0.08 ^{f}
N	7.05 ± 0.00 ^{fg}	5.67 ± 0.06 ^{cd}
O	5.72 ± 0.02 ^{i}	n.d.
P	7.82 ± 0.13 ^{de}	2.04 ± 0.06 ^{h}
Q	6.12 ± 0.10 ^{i}	5.88± 0.01 ^{bcd}
R	8.17 ± 0.02 ^{cd}	6.80 ± 0.28 ^{a}
S	6.57 ± 0.11 ^{h}	5.63 ± 0.03 ^{cd}
T	7.07 ± 0.05 ^{fg}	4.79 ± 0.04 ^{e}
U	7.93 ± 0.01 ^{d}	4.52 ± 0.06 ^{ef}
V	6.68 ± 0.04 ^{gh}	5.59 ± 0.00 ^{d}
Overall mean	7.61 ± 0,87	3.31 ± 2,45

4.2 Histamine detection

Table 10 reports the concentrations of histamine detected in fermented liver sausages. The histamine content obtained is clearly highly heterogenous, comprised between 7.5 ± 3.1 (producer I) to 313.4 ± 11.7 mg/Kg (producer H).

The high concentration of histamine in some producers, including producer B, G, H, Q, and S, could be attributed to an intense decarboxylase activity during the production process. In fact, it has been demonstrated that histidine decarboxylases can maintain their activity outside the microbial cell and their activity can increase remarkably at temperatures about 40 °C. During the production process of fermented sausages performed at industrial level, in fact, the products remain for several minutes in the optimal range of temperatures for decarboxylase activity, thus favoring the activity of such enzymes. (Giardini et al., 2013).

Frequently, the accumulation of histamine is caused by decarboxylases produced by LAB, even if many microbial groups belonging both to Gram-positive and Gram-negative can contribute to its production. The amount of histamine detected in fermented liver sausages was comparable with that found in *Salama da sugo*, the typical cooked fermented sausage produced in Emilia Romagna Region (Italy), comprised between 22.8 and 191.2 mg/Kg. By contrast, data shown in *Table 10* were lower if compared to those of typical fermented European products reported by Giardini et al. (2013), attesting at about 160 mg/Kg at the end of the ripening stage. In this case, the accumulation of BAs was caused by the decarboxylases produced by LAB, and particularly by *Enterococcus spp*. and *Lactobacillus spp*., even if strains belonging to other genera could contribute to its production (Giardini et al., 2013).

As mentioned above, the results showed in *Table 10* underlined how the amounts of histamine particularly varied among producers and sometimes also within samples of the same producer. Such variability can be attributed to several factors, such as the hygienic quality of raw materials, the storage conditions, and the technological operations used during the production process.

Moreover, to explain the different histamine contents between producers of fermented liver sausages, the shape of the products should be considered, since the product diameters could affect the final result. Such hypothesis is correlated to the degree of fermentation, since LAB are usually closely related to BA production during the fermentation of dry sausages. The ripening process of meat products leads, as a result of the drying process, to a decrease of a_w as well as an increase of salt concentration. Both these phenomena, which have a preservative effect, are less marked in the central part of the sausages with a bigger diameter. Therefore, in thinner sausages, the reduction of microbial growth would be lower than fermented products with a big diameter, where the consequent amine production by the microbial flora may result more intense. Moreover, under anaerobic conditions, mostly in the central section of the sausage, histamine production is favored (Giardini et al., 2013).

Bover-Cid et al. (1999) reported that BAs production by microorganisms in dry fermented sausages could exert a protective role of the microorganism against acidic environmental conditions. Since samples with the lowest pH generally showed the highest amine contents, the acidic environment of fermented liver sausages could explain the differences on the histamine levels observed in the samples of the present study.

A qualitative risk assessment of BAs in fermented foods was undertaken using data from the scientific literature as well as surveys, reports, and consumption statistics from the European Union (EFSA, Scientific Opinion on risk-based control of biogenic amine formation in fermented foods, 2011). Histamine and tyramine resulted the most hazardous BAs, and fermented foods were associated with high BA risks because of the intense microbial activity. Concerning BA risk mitigation options, good hygiene practices to minimize the presence of BA-producing bacteria in raw materials, further microbiological controls, and the use of selected starting cultures are particularly essential. According to the limited available data, no adverse health consequences were reported following the exposure to 50 mg histamine for healthy individuals (EFSA, Scientific Opinion on risk-based control of biogenic amine formation in fermented foods, 2011). Hence, the results obtained in the present study can be considered generally acceptable, except for producer H with values exceeding 300 mg of histamine per kilogram of sample. However, severe health consequences were reported after the exposure to minimal histamine levels for subject affected by histamine intolerance, thus confirming the need of preventing and/or control strategies to histamine formation in foodstuffs (EFSA, Scientific Opinion on risk-based control of biogenic amine formation in fermented foods, 2011).

Table 10 - Histamine content of the analyzed fermented fish sausage samples

Producer	Histamine content (mg/Kg)
A	12.7 ± 1.0 [cd]
B	34.6 ± 18.5 [bc]
C	9.3 ± 4.6 [cd]
D	8.5 ± 4.1 [d]
E	7.7 ± 3.7 [d]
F	12.5 ± 1.4 [cd]
G	24.2 ± 4.6 [cd]
H	313.4 ± 11.7 [a]
I	< 4.2
L	8.3 ± 4.3 [d]
M	16.7 ± 4.4 [cd]
N	12.2 ± 1.7 [cd]
O	13.7 ± 1.7 [cd]
P	11.4 ± 4.3 [cd]
Q	21.7 ± 7.0 [cd]
R	7.5 ± 3.1 [d]
S	57.6 ± 8.1 [b]
T	15.0 ± 3.5 [cd]
U	14.8 ± 0.4 [cd]
V	17.3 ± 3.1 [cd]

4.3 Detection of the *hdcA* gene

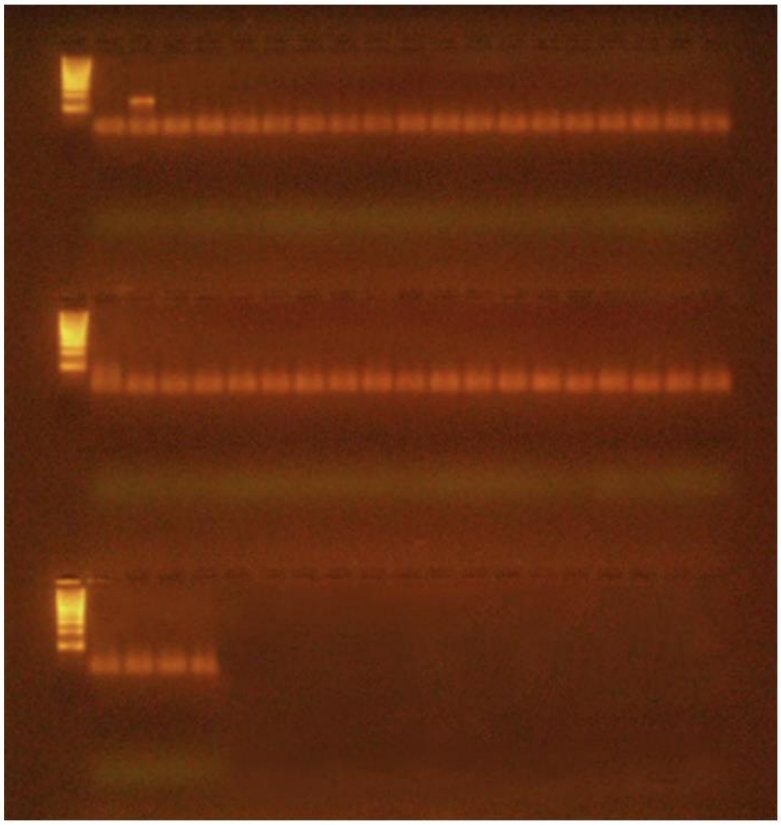

Figure 4 - Agarose Gel Electrophoresis result

To evaluate the histamine-producing potential of the bacterial isolates, a PCR assay was performed in order to verify the presence of the *hdcA* gene of Gram-positive bacteria. The results showed that none of isolates carried the *hdcA* gene, suggesting their inability to produce histamine from histidine. As shown in *Figure 4*, the electrophoresis did not show positive bands among the samples, since no amplification of the *hdcA* gene has occurred during the reaction. As already stated, the ability to produce BAs is widespread among Gram-positive bacteria, e.g. the decarboxylase activity on histidine was detected in strains belonging to the genera *Staphylococcus* and *Bacillus*. On the other hand, histamine-producing bacteria were detected also among Gram-negative bacteria, in particular belonging to Enterobacteriaceae and Pseudomonadaceae isolated from fermented sausages, meats and cheeses. Moreover, yeast strains belonging to the species *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Candida stellata*, *Kloeckera apiculata*, *Metschnikowia pulcherrima* and *Brettanomyces bruxellensis* were capable of BA production (Giardini et al, 2016).

Since the major microbial populations, LAB and CNC, of fermented liver sausages of the present study were likely not able to produce such BA, the decarboxylase activity was attributable to other minor populations of Gram-positive bacteria not isolated through the methods utilized, or to Gram-negative bacteria and yeasts. Another

fundamental aspect must be considered in BAs production: the environmental conditions influencing microbial activities, in particular temperature, salt concentration and pH. These factors can influence BA formation in two ways, since they are responsible for the overall metabolism of BA-producing microorganisms and for the enzymatic reaction itself. Literature data indicate a great variability of microbial decarboxylases activity depending on the environmental factors. Moreover, other aspects influencing the BAs production are the species metabolic pathways, the eventual experimental conditions, the type of matrix (food), but also the great heterogeneity that characterizes the activity of decarboxylase, even within strains of the same species (Giardini et al, 2013).
Below, some of the major environmental factor able to favor the production of histamine are discussed.

4.3.1 Temperature

Temperatures close to optimal growth values, favoring cell proliferation and their metabolism, generally favor the production of BAs, which is often directly proportional to the bacterial load of the system. However, the presence of a large number of decarboxylating cells is not a sufficient condition to explain the final content of BAs.
Studies conducted by Giardini et al. (2016) showed that temperature increase from 16 °C to 44 °C coincided with a faster microbial growth and a more rapid and intense accumulation of BAs. Such studies also investigated the effect of temperature on the activity of a pure commercial decarboxylase enzyme, and the maximum decarboxylation efficiency was registered at a temperature between 30 °C and 37 °C. However, the activity of the same enzyme rapidly decreased while staying at 50 °C for one hour. Regarding fermented foods, the temperature during fermentation and ripening allows the growth and proliferation of the desired microbiota, and the temperature range, usually comprised between 15 and 25 °C, is defined by the chosen protocols. It has been studied that the temperature applied during the first 3 days of fermentation of dry sausages influenced the BA accumulation during all the ripening period (1 month). Moreover, the more the temperature values increased the more the BAs production occurred. Also, the higher fermentation temperatures (and higher relative humidity) favored BAs accumulation in Spanish fermented sausages *Fuet* and *Llonganissa* inoculated with *Lactobacillus curvatus*. Moreover, fermented sausages stored at room temperature after production were characterized by a higher BA content than refrigerated products (Giardini et al, 2016).

4.3.2 Salt concentration

A high salt concentration contributes to the reduction of BA accumulation in foods, because of the resulting reduction of metabolic activities of decarboxylating microorganisms. In particular, Gram-negative bacteria are more inhibited by increasing salt concentrations than Gram-positive bacteria. However, the healthy trend to reduce NaCl concentration contrasts with the utilization of such tool to reduce BA accumulation in foods.

It has been demonstrated by Giardini et al, (2016) that the Gram-positive bacterium *Mycobacterium psychrotolerans* produced more histamine in the presence of a higher salt content (4%) compared to lower salt concentration, even if such salt concentration slowed the growth of the same strain. Hence, stressed cells seem to activate the decarboxylating pathway in the framework of more complex response systems, resulting in more efficient BA production by each single cell. In fermented sausages, BAs are generally accumulated during ripening. However, the accumulation rate decreases with the a_w decrease. To confirm this, products packaged under modified atmosphere – the weight losses are inhibited in such products – continued to accumulate BAs when the packaging was carried out at high a_w (higher than 0.92) (Giardini et al, 2016).
In fermented liver sausages analyzed in the present study the salt content was about 3%. Such salt concentration cannot be considered high enough to guarantee a relevant BA production by Gram-positive bacteria. However, the hypothesis of BA production carried out by Gram-negative bacteria during the storage and/or the initial phases of the production process can be considered, since unsuitable maintenance and/or treatment of raw materials can lead to histamine production by such microbial group.

4.3.3 pH

It is known that decarboxylation is a cell defense mechanism against acidic stress (Giardini et al, 2016). Therefore, various research has been conducted to investigate the correlations between pH and BAs production. In this context, however, the influence of pH varies depending on whether the focus is on the activity of the pure enzyme or the decarboxylase activity of live cells. In any case, it has been thoroughly demonstrated that the transcription of genes of many decarboxylase clusters are induced by low pH (Giardini et al, 2016).
As previously described, histamine values detected in the present study were not considered as high, except for producer H. The reason of such promising results may be attributed to the slower acidification process and the more extensive drying and ripening stages of slightly acid Southern European Fermented sausages respect to Northern ones (Hierro et al. 2015). As a consequence, a lower histamine production is expected.

4.3.4 Additives

In fermented sausage production processes, sugars (mainly glucose, sucrose, and lactose) are added in order to enhance the LAB fermentation process.
Ruiz-Capillas et al, (2007) investigated the influence of different sugar composition and concentration on BA accumulation in *chorizo*, a typical Spanish dry fermented sausage, utilizing three selected pro-technological starter cultures. The highest concentrations of BAs were found at the end of the ripening process in the control sausage with no starter culture irrespective of the use of different sugar concentrations. However, when starter cultures and sugar concentrations equal to 0.5% or 1% were added, the presence of BAs in the sausages decreased considerably

in comparison with lower sugar concentrations. The production of high amounts of BAs was observed when the concentration of sugar in the sausage was only 0.1%, even in sausages added with starter cultures. Moreover, the BA contents significantly increased in sausages with no sugar in their formulation. Therefore, sugar omission is not recommended in fermented sausage formulation since it might increase BAs accumulation during the manufacturing and storage of fermented sausages. Moreover, the amount of sugar added represents a key factor in determining the dynamics among the microbial communities during the fermentation step of sausages, influencing the accumulation of different BAs (Giardini et al, 2016).

However, considering the presence or absence of sugars in each sample of the present study, the BA production is not highly correlated with the presence or absence of sugars. In fact, fermented liver sausages added with sugars usually showed high levels of BAs. Instead, the lowest histamine values detected do not correspond to the samples added with sugars, with some exceptions: producer I, whose sausage formulation includes both sucrose and dextrose, showed a histamine value below 4,2 mg/Kg, together with producer D, L and R, characterized by a histamine concentration around 8 mg/Kg.

4.3.5 Packaging

There is evidence that oxygen can affect the BAs production. For example, it has been demonstrated that the accumulation of some BAs is favored by anaerobic conditions (Giardini et al, 2016). Moreover, the main technologies for food preservation based on atmosphere modification are focused on oxygen exclusion. Nevertheless, in such strategy, the principal aim, in relation to BAs presence, is not the inactivation of decarboxylase activity but the inhibition of microbial population with decarboxylating properties. In this perspective, the atmosphere utilized for packaging can affect the qualitative and quantitative formation of BAs. On this regard, modified atmosphere packaging and vacuum packaging play an important role in the inhibition of spoilage microorganisms and, particularly, decarboxylating bacteria (Giardini et al, 2016).

In the specific case of fermented liver sausages analyzed in the present study, MAP was not employed during packaging, as well as any other oxygen-excluding technique. This could explain the activity of the involved microorganisms and the consequent decarboxylating capability, which lead to histamine production.

4.3.6 Antimicrobial substances

The use of antimicrobial substances can alter the BA profile of foods, interfering with microbial population equilibrium rather than directly affecting decarboxylase enzymatic activity. Nitrate and nitrite salts are extensively employed in fermented sausages for a variety of applications. In fact, they have an impact on the color, taste, and oxidation of cured meat. Furthermore, they are utilized to inhibit dangerous microorganisms, such as clostridia. Nonetheless, their activity can interfere with BA buildup; for example, the addition of nitrate and nitrite to a

Spanish cured meat dramatically enhanced the BA levels (Giardini et al, 2016). The addition of nitrite favored the selection of a superficial microbiota represented by LAB with appropriate decarboxylative activity, which explained this phenomenon. However, as described above, LAB represent the key element of fermented liver sausages. In the present study, LAB starter cultures were not included in any producer formulation, but their application could be recommended to avoid the potential decarboxylases activity of the spontaneous microflora. Moreover, starter cultures can improve the safety of fermented meat products by quick acidification of the meat matrix or by producing antibacterial compounds like bacteriocins. Furthermore, starters may help in the standardization of product qualities and the reduction of ripening periods. Regarding the safety of fermented meat products, LABs are capable of causing fast pH decrease, inhibiting the development of microorganisms with amino acid decarboxylative ability, thus preventing the accumulation of BA in fermented meat products. Besides, starters can compete with the autochthonous, non-starter microbiota throughout ripening and storage, thus likely reducing BA production. Some strains of *Lactobacillus sakei* and *Lactobacillus plantarum* have been shown to reduce the formation/accumulation of BA. Furthermore, *Staphylococcus xylosus* and *Debaryomyces hansenii* strains have been reported to degrade BA in food (Laranjo et al, 2019).

Chapter 5
CONCLUSIONS

The present study offers an analysis on the content of histamine and the microbial groups involved in its production in fermented liver sausages, a traditional preparation handcrafted in the Marche region.

The microbiological viable counts have confirmed the primary role of LAB and CNC in this kind of foods: LAB, through the production of lactic acid, plays a crucial role in the safety, shelf-life and organoleptic quality of fermented liver sausage; CNC mainly contribute to the sensory properties of the final product by means of their enzymatic activities, responsible for the color development and the decomposition of free amino acids and peroxides.

Considering the quite low histamine concentrations detected in the fermented liver sausage samples, it is expected that the consumption of such fermented products does not represent a risk for the health of consumers.

Furthermore, based on the absence of the *hdcA* gene of Gram-positive bacteria in LAB and CNC isolates, it is assumed that the core microbiota of fermented liver sausage samples, which is dominated by these bacterial groups, may not be involved in histamine synthesis. Although the involvement of Gram-positive bacteria cannot be excluded at all, the results also suggests that the decarboxylase activity can be associated to the presence of Gram-negative bacteria. An in-depth investigation on the presence of histamine-producing Gram-negative bacteria in fermented liver sausages is suggested to better understand the source of the detected BA.

While studies on the genetic bases of microorganism decarboxylating activity have led to the development of significant advances and new intuitions on this topic, the role of environmental and technological factors on the overall activity of BA-producing microorganisms requires further research in order to improve the potential intervention on food processes. However, from a strictly microbiological perspective, further study is needed to evaluate and optimize the utilization of microbial cultures capable of degrading BAs via the activity of amino oxidases in fermented foods.

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