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STUDY OF THE MICROBIOTA OF FERMENTED LIVER SAUSAGES PRODUCED IN THE MARCHE REGION (CENTRAL ITALY)

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ABBREVIATIONS

%	Percentage
°C	Degree Celsius
a_W	Water activity
g	Gram
mL	Milliliter
CFU	Colony forming unit
CNC	Coagulase-negative cocci
DFD	Dark, firm and dry
LAB	Lactic acid bacteria
MRS	De Man, Rogosa and Sharpe
MSA	Mannitol Salt Agar
PAB	Pseudomonas Agar Base
pH	Potential of hydrogen
RB	Rose Bengal Chloramphenicol Agar
PSE	Pale, soft and exudative
RH	Relative Humidity
SBA	Slanetz Bartley Agar
Spp.	Species
VRBGA	Violet Red Bile Glucose Agar

1 INTRODUCTION

There is a growing trend towards the consumption of fermented foods as consumers are increasingly interested in natural products with unique sensorial characteristics. Fermented sausages are widely consumed globally, especially in Europe. Just in Germany, Spain, France, and Italy, more than 600×10^6 kg of fermented sausages are consumed every year, which is about 3% to 5% of the total meat consumed in the countries *per annum* (Hutkins, 2019). Therefore, in Europe, fermented sausages play a fundamental role both economically and in the consumers' diet. Moreover, fermented sausages are also recognized as authentic and traditional foods. In fact, most fermented sausages are produced using traditional procedures, that mainly involve a fermentation process without the use of starter cultures. Such fermentation process depends on the development of naturally occurring microbiota, generally dominated by lactic acid bacteria (LAB) and coagulase negative cocci (CNC) (Santa *et al*., 2012). Specifically, the metabolic activity of LAB is mainly characterized by the production of lactic acid, whose accumulation in the sausage batter causes a decrease in pH and thus the inhibition or suppression of the growth of spoilage and pathogenic bacteria. Furthermore, lactic acid supports the establishment of a favorable environment for desired chemical, physical and microbiological modifications during the transformation process. The resulting products are characterized by distinctive sensory properties and a remarkable microbiological stability (Kröckel, 2013). The latter aspect is essential in ensuring food safety, as the raw materials are particularly susceptible to rapid deterioration. Moreover, since raw meat contains abundant macronutrients and micronutrients composition, it is ideally suitable for microorganism growth, and its preservation represented a real challenge to early civilizations. Consequently, different meat preservation techniques emerged, such as salting, drying and fermentation.

1.1 History of Fermented Sausages

Fermentation, combined with salting and drying, is one of the earliest methods of meat preservation that dates back to 4000 BC. Even though the exact origin is still unknown, the earliest sources documenting sausage production are linked to the Sumerians. Furthermore, iconographic materials and remains of salami in the tomb of Ramses III (1166 BC) indicate their existence in ancient Egypt. Sausages are also described in the Odyssey, written by the famous Greek author and poet Homer around 800 and 700 BC. In fact, the name *salami*, used to define fermented sausage, seems to have originated in the ancient Greek town of Salamis (Zeuthen, 2007). Similarly, fermented sausages were also prepared during the Roman Empire using different ingredients, such as pork, beef, blood, fat, and meat wastes (Toldra *et al*., 2015). Indeed, it is said that sausage manufacture has been perfected by Roman butchers, who were tasked to prepare some for Caesar's legions during the conquest of Gaul, as fermented sausages were praised for aiding the retention

of vigor and health (Toldra, 2007). In Asia, a Chinese type of sausage made from goat and lamb meat, named *Lup Cheong*, was also reported around 589-420 BC from the North and Southern Dynasty.

The practice of meat fermentation was used not only to preserve highly perishable raw material, but also to use and enhance meat leftovers and entrails (Toldra, 2007). Therefore, meat leftovers and entrails were cut into small pieces with further addition of salt and various additives, followed by stuffing into casings, air drying and/or smoking. Such production sequence led to an accumulation of several antimicrobial hurdles. Stuffing into casings creates anaerobic conditions that suppress the activity of many spoilage microorganisms (Leroy *et al*., 2013) while salting lowers water activity values and thus protects the meat against several spoilage and pathogenic microorganisms.

To date, there is still a doubt about the first sausage inoculations with microorganisms that promote the fermentation process. However, the fermentation technique remained widely unclear until the findings of Louis Pasteur in the late 19th century, who first unraveled the mechanisms of microbial processes (Benninga, 1990; Alba-Lois & Segal-Kischinevzky, 2010). Thereafter, it was ascertained that microorganisms were responsible for several modifications in sausages, such as lactic acid production and nitrate reduction.

In the last decades, the combined effect of fermentation with salting, drying and smoking is still utilized to obtain products with a longer shelf life and unique sensory qualities. The manufacturing of fermented sausages continued to spread to various regions of the Mediterranean area, as shown by the salami known as Genoa, Milano, and Lombardi. In general, the Mediterranean countries produced highly seasoned, non-smoked products, identified as Southern European products. On the other side, Northern European countries developed fermented sausages that were slightly spiced, heavily smoked, moist, and higher in salt content (Talon *et al*., 2007).

1.2 Production of Fermented Sausages

The production of fermented sausages is characterized by the use of different recipes and processing procedures, carried out both at small-scale level and industrialized level. However, the production process generally involves the following basic phases: i) selection of ingredients, ii) formulation, iii) fermentation, iv) drying and ripening (Figure 1).

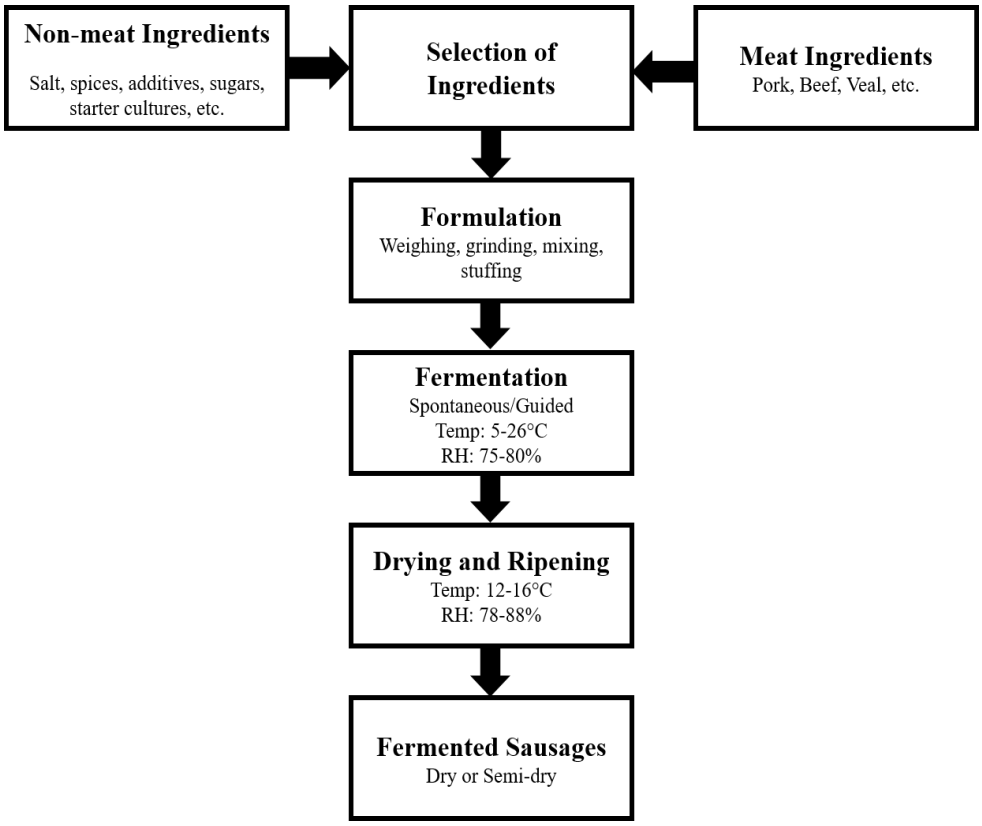

Figure 1: Basic process flow diagram for fermented sausages

1.2.1 Selection of ingredients

The ingredients to produce fermented sausages are mainly composed of meat and other components of animal origin. Generally, lean muscle tissue, fat and sometimes entrails of pork origin are selected, although beef, veal, and other kinds of meat are used in some formulations. Meat from adult animals, characterized by firm fat with high melting point and low content of polyunsaturated fatty acids (PUFA), is usually preferred as it has a lower water content, a higher fat content, which supports the drying process, and a higher myoglobin content, important for the color of the end-product (Zdolec, 2017). The non-meat ingredients generally include salt and spices, whereas the use of additives and sugars is usually optional.

1.2.2 Formulation

Initially, chilled or slightly frozen meat and frozen fat are chopped in order to obtain a clear cut of the meat, and to prevent the fat “smearing” respectively. The chopping degree depends on the type of sausage and varies from 2-5 mm to 6-12 mm for moderately and coarsely chopped meat, respectively (Zdolec, 2017). Salt addition, whose amounts vary depending on the recipe, usually follows. Concomitantly, spices, sugars and nitrates or nitrates are added. Pepper, garlic, fennel seeds and chili pepper are the mostly used spices, and the exact variety and quantity used depend on the recipe. Regarding curing agents, sodium and

potassium nitrates and nitrites are commonly used in accordance with EU Reg. EC 1333/2008 on food additives and subsequent modifications (Aquilanti *et al.*, 2016).

Once non-meat ingredients have been added and blended into the meat batter, the latter is placed in a kneader and then in a stuffing machine. Fresh sausages are obtained by filling batter into natural or synthetic casings.

Subsequently, the product is usually bound with a food grade twine. Such technique maintains anaerobic conditions of the meat batter, in order to favor the growth of desired fermentative microbiota and suppress the spoilage microorganisms usually contaminating fresh meat, such as Pseudomonadaceae (Leroy *et al.*, 2013).

1.2.3 Fermentation

The fermentation process can proceed spontaneously or can be guided by means of back-slopping (which is rarely used) or by starter cultures (Hutkins, 2019). Spontaneous and back-slopping fermentations represent traditional methods: the former relies on the selective development of the natural microbiota of starting raw materials, while the latter is based on the addition of a portion of a finished product from a successful fermentation to the starting raw materials. While spontaneous fermentation may present a higher degree of unpredictability of the entire process, back-slopping method offers safer results, as it selects for microbiota well suited for the growth in the sausage environment. Moreover, the repeated transfers of microbial populations from the end-products will likely give a more consistent and characteristic product (Hutkins, 2019). However, both methods are more subjected to slow or failed fermentations, contamination and inconsistent quality. On the other hand, the use of starter cultures consists in the addition of selected microorganisms that are directly inoculated into the meat batter, in order to achieve faster and more standardized fermentations and thus a more consistent quality (Hutkins, 2019).

During the fermentation process, the microorganisms require both intrinsic and extrinsic factors for their growth. Consequently, in the case of standardized manufacturing, processing room conditions are often adjusted to certain levels of temperature and humidity. On the other hand, traditional manufacturing often relies on the natural environmental conditions, and the temperatures usually range from 5 to 26 °C and in some cases reaching above 30 °C, depending on the area of production with relative humidity (RH) of around 75 to 80% (Toldra *et al.*, 2015; Zdolec, 2017). These differences greatly influence the rate and extent of fermentation, resulting in either slow or fast fermentations. Therefore, sausages undergo different periods of fermentation that may range from 2 to 4 days and even up to 7 days or more.

Regardless of different temperature and humidity conditions, the fermentation process causes physico-chemical and microbial modifications on fresh sausages, generally including a reduction of pH and a radical change in the microbial composition, favoring aciduric microbiota. In detail, lactic acid bacteria (LAB) ferment available sugars forming lactic acid, whereas coagulase negative cocci (CNC) reduce nitrate to

nitrite. The acid environment favors the reaction of nitrite with myoglobin, forming nitric oxide myoglobin, a key molecule for the characteristic color of the final product and the suppression of deleterious microorganisms (e.g. Clostridia) (Ordóñez *et al*., 1999).

1.2.4 Drying and Ripening

During this phase, in the case of standardized manufacturing, the product is exposed to different temperatures, humidity values and air velocities in processing rooms, in order to determine the rate of drying. Temperatures of 14-16 °C with relative humidity of 78-88% and air velocity of about 0.1 m/s, are often recorded during this process that usually lasts between 2 to 4 days (Hui 2012; Toldra *et al*., 2015; Zdolec, 2017). By the end of drying, different levels of a_w can be registered, while the lactic acid fermentation ends and the growth of molds on the surface of the sausages may take place (Hui & Evranuz, 2012).

Finally, the product undergoes a ripening period generally characterized by lower temperatures and humidity values between 12-15 °C and 75-80%, respectively (Zdolec, 2017). The duration of this phase varies depending on the specific productive process, in fact some products undergo a short ripening period of less than 4 weeks, whereas others undergo a long ripening period of more than 4 weeks (Aquilanti *et al*., 2016). The ripening step ensures a steady removal of moisture from the fermented sausage and thus prevents the growth of undesirable molds on the surface and the acquisition of undesirable changes in the sensory characteristics and texture of the end-product (Hui & Evranuz, 2012). Furthermore, several chemical changes occur due to the activity of endogenous enzymes and microbial enzymes. In fact, the aroma and taste of the final product are modified by the activity of CNC hydrolytic enzymes, that release peptides and amino acids, and the activity of molds, that breakdown fats, proteins and lactic acid, thus favoring pH increase (Ordóñez *et al*., 1999; Bruna *et al*., 2003).

In the last decades, the several differences in the recipes and/or in the production processes of fermented sausages made their classification necessary. The criteria used in the classification of such heterogeneous products include: i) the animal species used to obtain the raw material, ii) the fat content, iii) the type of tissue, iv) the degree of comminution of the meat ingredients, v) the spices and other non-meat ingredients added, vi) the casing diameter, vii) the degree of fermentation, viii) the ripening duration and ix) the physico-chemical properties of the end-product (Wood, 2012; Aquilanti *et al*., 2016). In this regard, two main categories of fermented sausages have been identified, which are Northern European products and Southern European products, based on the rate of fermentation, the ripening process and final pH and a_w. Northern European products, such as German and Hungarian salami, are generally fast fermented, smoked and their pH reach values below 5. Whereas, Southern European products, such as salami produced in the main countries of the Mediterranean area (Italy, France, Greece, Spain), are mostly slow-fermented, not smoked and their pH reach values close to 5 or comprised between 5.3 and 6.2. Moreover, Southern

European products are distinguished in dry sausages, when the ripening period lasts for more than 4 weeks reaching final a_w values less than 0.90, and semi-dried sausages, when the ripening period lasts less than 4 weeks reaching final a_w values between 0.90 and 0.95. Moreover, Southern Europe products usually contain microorganisms colonizing the sausage surface, including molds, yeasts and Gram-positive cocci (Aquilanti *et al.*, 2016).

1.3 Fermented Liver Sausage

The fermented liver sausage has been in existence for many years and its acceptability varies among societies (Feiner, 2006). The production of this delicacy maximizes the utilization of unpopular raw materials, such as the internal organs of animals, thereby acquiring a greater value (Chyr, 1978). During manufacturing, different offal can be incorporated into the sausage mixture, but liver is used in greater proportion, hence it is called fermented liver sausage.

Fermented liver sausage is manufactured according to procedures passed down from generation to generation which rely on the use of pork meat from pigs raised in local farms. The main ingredients are shown in Table 1-1 and include not only pork liver but other offal, such as tongue and heart. The meat and fat of pork are also used together with their bloody lean and fat waste products. Other ingredients are salt, spices (ground pepper, chili pepper, crushed garlic), sugars (lactose, fructose, dextrose, saccharose) and curing agents (nitrates and nitrates). In some formulations, ingredients such as wine, port and brandy are used as flavoring materials (Feiner, 2006).

Table 1-1: General Formulation for Fermented Liver Sausage

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
Crushed garlic	0.1
Nitrates or nitrites	< 0.0003
Sugars	0.3

The table shows basic ingredients and portions may differ from each producer.

1.3.1 Manufacturing Procedure

Selection of ingredients and their roles: A careful selection of ingredients represents the key of the entire production of fermented liver sausage. Such a step acts as a filtration phase, preventing materials of unacceptable quality from being used during the process (Essien, 2003). The selected meat ingredients should present a total microbial count below 10^5 colony forming units (cfu)/g, while the microbial pathogens *Listeria monocytogenes* and *Salmonella* should be absent (Hui, 2012). Moreover, lean meat pH should range between 5.5 and 6.0, while fresh liver pH should result slightly higher, at around 6.4 (Feiner, 2006). The high degree of free water in meat (75%) and water activity (0.99-0.98), especially in liver which also contains abundant blood, makes the raw material more susceptible to spoilage. Therefore, meat should be kept in cold rooms at less than 4°C until use to reduce the proliferation of undesired microorganisms (Feiner, 2006).

The amount of pork fat used is usually comprised between 25 and 35%. Practically, any pork fat type from different cuts can be used in the product formulation, including shoulder, legs, loin, neck and belly. However, frozen fat is preferred to avoid its "smearing" during the grinding step. An optimal fat composition could improve the flavor, the juiciness and the texture of the final product. Generally, the ratio of pork meat to fat of 2:1 appears the best choice in order to obtain a desirable final texture.

Regarding offal, pork liver usually amounts to 20 and 33% of the final composition. Liver naturally contains a high amount of glucose, that is quickly metabolized by certain microbial groups during the fermentation process (Hutkins, 2019). Moreover, liver is a natural emulsifier of fat and water that allows the formation of a three-dimensional matrix, inhibiting the interaction of fat particles thereby stabilizing the meat batter (Feiner, 2006). Furthermore, pork liver has a major impact on the overall taste and color of the final product.

Salt represents a key ingredient in fermented liver sausage, and its amount ranges between 2 and 3%. Its main functions are to enhance the flavor of the end-product and limit the microbial growth by reducing the water activity of the product (Feiner, 2006; Wijnker *et al.*, 2006).

Spices are usually required in small amounts and constitute about 0.4 g/kg of the sausage. They contain carbohydrates, fats, proteins, ash and a complex variety of chemical compounds, including volatile and non-volatile oils that deeply characterize the aromatic profile of the final product (Sebranek, 2009). In fact, during the production process, the volatile oils are broken down into aroma compounds, such as aldehydes, esters and alcohols, contributing to the sausage flavor (Sebranek, 2009). Carbohydrates from spices constitute an additional source of fermentable sugars, stimulating the growth of lactic acid bacteria (LAB) and thus lactic acid formation. Meanwhile, specific spices play different roles in the product: i) chili pepper contains nitrates and nitrites, known for its antimicrobial activity especially against several pathogens, including *Clostridium botulinum* (Verluyten *et al*., 2004; Colavita *et al*., 2014); ii) black pepper contains

manganese ions which act as stimulatory components on LAB, affecting the acidification rate during the fermentation process (Toldra *et al*., 2015); iii) garlic contains antioxidants which are important inhibitors of lipid oxidation, contributing to the longer shelf-life of the end-product.

Nitrates (NO_3) and nitrates (NO_2) are important for the preservation of the product and can be added at very low levels to meat preparations in the form of sodium and potassium nitrates and nitrites, in accordance to EU Reg. EC 1333/2008 on food additives (Aquilanti *et al*., 2016). Firstly, nitrate is transformed into nitrite by the activity of bacteria. Consequently, nitrite is reduced to nitric oxide (NO), a process favored by a decrease in pH during fermentation. Then, nitric oxide (NO) turns the large amount of hemoglobin contained in pork liver into nitroso-hemoglobin, which contributes to the color of the final product (Feiner, 2006). Nitrates (NO_3) and nitrates (NO_2) also i) prevent the growth and the production of toxins of pathogenic microorganisms, such as *Clostridium botulinum* and *Staphylococcus aureus*, and ii) act as antioxidants, stabilizing the product against oxidative rancidity (Chyr, 1978; Toldra, *et al*., 2015).

Fermentable sugars (lactose, fructose, dextrose and saccharose) represent a nutrition source for fermenting bacteria, including LAB. Moreover, such compounds improve sensorial and rheologic properties of the end-product (Campbell-Platt & Cook, 1995).

Casings are used in order to maintain the shape of the meat sausage and to control certain parameters, including moisture content, oxygen levels and UV light, thereby increasing the product shelf-life (Essien, 2003). Moreover, casings minimize product weight loss during the drying and the ripening steps. Two types of casings can be used during the production process: natural or artificial casings. Natural casings are obtained from the small and large intestine, urinary bladder, stomach and rectum of pigs, sheep, goats, cattle and other animals (Dikeman & Devine, 2014). Artificial casings are produced with collagen from cattle skin, cellulose (edible) or plastic (not edible). However, most fermented liver sausages are prepared using natural casings, produced from the pig caecum or large intestine, in order to support the traditional taste of the product (Çaglar *et al*., 2018).

Ingredients receiving: During receiving, the aforementioned ingredients are verified based on the approved supplier list, and preliminary checks are carried out to assess the quality of the goods. Such checks are based on both hygienic-sanitary parameters, including the expiration date and the temperature of perishable products, and commercial parameters, including the order conformity (quantities, types, etc. of the goods). If the product results compliant, it is accepted and transferred to the appropriate storage room. Otherwise, the non-conformity of a product is recorded on a specific non-compliance form.

Storage: Meat and other perishable products are stored in cold rooms or in special refrigerators, where the temperature must be maintained and monitored below 4 °C (Feiner, 2006), in order to retard the growth and the proliferation of microorganisms, and allow an easier grinding step (Hui, 2012). Non-perishable products are stored in the food warehouse or in the lockers and shelves of the processing rooms.

Processing: During processing phase, meat and fat are subjected to grinding using a 10 mm grid, followed by a second passage at 3 mm. Subsequently, the mixture of non-meat ingredients (salt, spices, curing salts, sugars, antioxidants and other preservatives) is manually added to the meat batter. Afterwards, the semi-finished product is inserted into a mixing machine for uniform homogenization. The sausage batter is placed into a bagging machine, where it slides into a natural casing through a funnel connected to the machinery (the operations are partly mechanized and partly manual). The stuffing and filling steps are carried out in a way to minimize air inclusions into casings (Lücke, 2000) and, once completed, the obtained fresh sausage is manually or automatically tied by specific machines associated with the filling equipment (Hui, 2012). The fresh sausages are hung on suitable trolleys and stored in the drying room at controlled temperature and relative humidity (RH), usually above 20 °C and 80% respectively, for about 4 days.

The fermentation process is generally guided by the natural microbiota present in the raw materials and processing environment (Toldra *et al*., 2015; Aquilanti *et al*., 2016). At the end of fermentation, the temperature and RH are maintained above 20 °C and 90% respectively for 8-10 hours, followed by an adjustment of RH to around 50% for about 20 hours. Subsequently, cycles of 24 hours are carried out for 5 days or more, lowering the temperature by 1-1.5 °C every day. However, the physiochemical parameter of temperature and RH vary, depending on the environmental conditions. At the end of the drying phase, the sausages are transferred to the seasoning room for ripening process, carried out at a temperature of about 15 °C and RH of 70% for at least 30 days. However, these values are purely indicative and greatly vary both for technological reasons and for the size of fermented sausages. After the production, a period comprised between 30 and 50 days should pass, before eating. The final product usually has a dark brown color and a semi-soft consistency, depending on the recipe and the manufacturing process (Belleggia *et al*., 2020).

Figure 2: Fermented liver sausage

During fermentation, drying and ripening phases, fermented liver sausages undergo complex microbiological and physico-chemical modifications that induce significant changes in the characteristics of the end-product. Some of the major modifications include the microbial evolution and a marked reduction in pH and water activity.

1.4 Roles of Pro-technological Microbiota in Fermented Sausages

1.4.1 Lactic acid bacteria

LAB play a key role in fermented sausages by contributing to safety and the development of desirable sensorial properties (Lücke, 2000; Cizeikiene *et al.,* 2013). Regarding the safety role, LAB have anti-microbial effect related to the production of organic acids, hydrogen peroxide, ethanol, carbon dioxide and antimicrobial substances, such as bacteriocins (Dalié *et al.,* 2010). In more detail, lactic and acetic acid reduce the pH and thus inhibit the growth of spoilage and pathogenic bacteria, including Pseudomonadaceae, Enterobacteriaceae, *S. aureus* and *C. botulinum* (Ockerman & Basu, 2010). In fact, low pH levels allow the lipo-solubilization of organic acids into the cell membranes and therefore the arrival at the cytoplasm of susceptible microorganisms (Parada *et al.,* 2007; Ockerman & Basu, 2010; Reis *et al.,* 2012). Also, hydrogen peroxide, produced by LAB, can prevent the growth of several microorganisms (Pseudomonadaceae, Enterobacteriaceae, *S. aureus*), contributing to fermented sausage preservation (Dalié *et al.,* 2010). The antimicrobial effect of hydrogen peroxide results from the oxidation of sulfhydryl groups that causes the denaturation of bacterial enzymes (Šušković *et al*., 2010; Angula, 2017). However, peroxides can lead to color and flavor defects through the interaction with polyunsaturated fatty acids, thus promoting their rancidity (Wood, 2012).

Moreover, LAB produce a wide variety of bacteriocins, which are active against Gram-positive pathogens, such as *L. monocytogenes*, *S. aureus*, *C. perfringens* and *B. cereus* (de L Agüero *et al.,* 2020). Such bacteriocins exert antimicrobial effects through different mechanisms, including the inhibition of spore germination and the alteration of the enzymatic activity of microorganisms (Parada *et al.,* 2007). Finally, carbon dioxide produced by heterofermentative LAB can contribute to the preservation of the final product. In fact, carbon dioxide can penetrate into the microbial cells, inhibiting bacterial enzymes and disrupting the cell membranes (Steinkraus, 1992).

LAB also contribute to the flavor, texture and color of the product through different mechanisms (Maksimović *et al.,* 2015). For flavor improvement, LAB proteolytic enzymes generate short peptides and amino acids that act as direct flavor enhancers or precursors of other flavor compounds (Sanz *et al.,* 2002). The texture of the product is modified by the action of lactic acid produced by LAB that induces the coagulation of sausage proteins. Finally, the decrease of pH values due to the presence of lactic acid contributes to the development and stabilization of the end-product color (Hammes *et al.,* 1990).

Enterococcus faecalis and *Enterococcus faecium* are the enterococci species most frequently isolated from different types of fermented sausages (Milićević *et al.,* 2014). Such a LAB group play an important role in flavor development, bio-protection and lactate production (Zdolec, 2017). However, some enterococci species may cause the spoilage of meat products, the transmission of mobile antibiotic-resistance genes, opportunistic infections in humans and the production of toxic substances, such as biogenic amines.

1.4.2 Coagulase negative cocci

The enzymes produced by CNC influence the organoleptic properties of fermented sausages, including the color and flavor. As regards flavor, the lipolytic and proteolytic activity of CNC promote the release of short chain fatty acids and peptides which are flavor precursors. Moreover, such short chain fatty acids are further broken down to carbonyls, distinct flavors of fermented sausages (Ockerman & Basu, 2010; Heo *et al*., 2020). As regards color, nitrate reductase produced by CNC reduces nitrate to nitrite, that allow the production of nitroso-myoglobin, a key component for the characteristic color of fermented sausages (Rebecchi *et al.,* 2020). CNC also prevent lipid oxidation through the synthesis of catalase and superoxide dismutase (Kamiloğlu *et al.,* 2016).

1.4.3 Eumycetes

Yeasts have oxygen-scavenging properties thereby contributing to the color of the end-product, meanwhile their lipolytic and proteolytic activity produces free fatty acids, peptides and free amino acids which contribute to the flavor (Flores *et al.,* 2015). Yeasts also ferment different sugars to produce volatile compounds, such ethanol, acetaldehyde and ethyl acetate (Mendonça *et al.,* 2013). They also metabolize lactic acid, increasing the pH of fermented sausages (Flores *et al*., 2015).

Molds uniformly colonize the external part of the sausage, especially the surface of the casing, where they exert different roles: i) they allow a homogenous dehydration and thus improve the texture of the product; ii) they prevent fat oxidation by degrading peroxides; iii) they reduce oxygen levels on the product surface, avoid oxidative processes and improve meat color; iv) they contribute to the flavor of the final product by breaking up fats, proteins and lactic acid, thus favoring pH increase, especially when natural casings are used (Bruna *et al.*, 2003; Toldra *et al.,* 2015). However, some mold strains can lead to quality and appearance defects and can produce mycotoxins, toxic substances for humans (Zdolec, 2017).

1.5 Microbial Evolution During Processing of Sausages

The entire production process of fermented sausages is characterized by a dynamic activity of several groups of microorganisms. The microorganisms play a fundamental role in the quality, safety and sensory properties of the end product, based on their ability to transform food in a beneficial (pro-technological microorganisms) or in an undesired way (spoilage and pathogenic microorganisms) (Bezirtzoglou *et al*.,

2000; Zhengchao *et al*., 2020). The microorganisms derive from the raw materials, including casings and spices, the processing equipment and also the product handlers (de Souza Sant'Ana, 2016). Since raw meat is a nutrient-rich substrate with suitable pH and a_w for microbial growth, it carries a wide variety of microorganisms that derive from slaughtering environments, the skin, hooves and intestines of the animal (Nychas *et al*., 2008). The microorganisms mainly consist of bacteria, but even yeasts and molds are present (Table 1-2). The main bacterial groups and species are usually Staphylococcaceae, Micrococcaceae, Enterobacteriaceae, LAB, Moraxellaceae, Pseudomonadaceae, *Flavobacterium* and *Brochothrix thermosphacta*.

The populations of such microorganisms vary depending on the meat cut, the year of processing and mainly the working environment (de Souza Sant'Ana, 2016). In fact, an investigation carried out on fresh pork working environments revealed that bacteria populations on the cut surfaces usually range from 3 to 5 log cfu/cm^2, while Enterobacteriaceae and Pseudomonadaceae mean counts range from 0.6 to 2.2 log cfu/cm^2 and from 1.1 to 4.4 log cfu/cm^2, respectively (Caldara *et al*., 2014).

Table 1-2: Microorganisms found in Fresh Pork

Bacteria		**Eumycetes**	
Gram-positive	**Gram-negative**	**Yeasts**	**Moulds**
Lactic acid bacteria	Pseudomonadaceae	*Saccharomyces*	*Penicillium*
Coagulase negative cocci	Enterobacteriaceae	*Candida*	*Aspergillus*
Bacillus	*Moraxella*	*Hansenula*	*Cladosporium*
B. themosphacta	*Aeromonas*	*Debaryomyces*	*Thamnidium*
Clostridium	*Flavobacterium*	*Trichosporon*	*Mucor*
	Campylobacter	*Rhodotorula*	*Monilia*
	Vibrio	*Torulopsis*	

The Table 1-2 shows the main microbial groups isolated from fresh pork meat. However, such microbial profile is altered during chill storage, especially under aerobic conditions, as Gram-negative bacteria, including *Acinetobacter,* Pseudomonadaceae and Enterobacteriaceae, predominate over Gram-positive bacteria, such as LAB and CNC (de Souza Sant'Ana, 2016).

On the other hand, spices contain a heterogenous microbiota, including Enterobacteriaceae and fungi (Garbowska *et al.,* 2015), while natural sausage casings contain species belonging to *Bacillus*, Pseudomonadaceae, *Clostridium*, *Micrococcus*, *Proteus*, *Lactobacillus* and other species of halophilic bacteria, which represent a part of the initial microbiota during the sausage production.

During the first operations of sausage preparation, chilled raw meat is exposed to higher temperatures above 4 °C, favorable for microbial growth. Subsequently, meat grinding provides a greater surface area that promotes the further growth and proliferation of microorganisms, in particular aerobic, facultative

anaerobic or aerotolerant ones (Sofos, 1994). Research studies have shown that, by the end of sausage batter preparation, typical microbial populations include Pseudomonadaceae, Enterobacteriaceae, LAB, CNC, yeasts and molds. In particular, viable counts ranged between 3 to 5 log cfu/g for Pseudomonadaceae and Enterobacteriaceae, between 2 to 5 log cfu/g for LAB and CNC, and between 2 to 4 log cfu/g for yeasts and molds (Zdolec, 2017). However, the addition of salt, spices and nitrates and/or nitrites into the ground meat modify the microbial equilibrium. After the mixing phase, the salt lowers the a_w and thus inhibits a large part of Gram-negative bacteria, including several alterative and pathogenic microorganisms. Nitrates and/or nitrites act by inhibiting specific pathogenic microorganisms, such as *S. aureus* and *C. botulinum*, and the germination of *C. botulinum* spores (Hui & Evranuz, 2012).

During fermentation and ripening phases, the sausage microbiota will be generally dominated by Gram-positive bacteria, which mainly consist, based on investigations that started in the 1960's, of LAB and CNC, while yeasts and molds are found in smaller numbers (Hui, 2012; Toldra *et al*., 2015; Zdolec, 2017). Extrinsic factors, such as temperature and humidity, and intrinsic factors, such as pH, a_w and redox potential (Eh), on the sausage batter could promote the proliferation of pro-technological microorganism and the suppression of undesirable Gram-negative bacteria (Zdolec, 2017).

In more detail, under anaerobic conditions, LAB metabolize sugars into lactic acid, whose accumulation in the sausage batter leads to a distinct reduction in pH from initial values of 5.8 to 6 to final values of 4.9 to 5.3 (Toldra *et al.*, 2015). The low pH inhibits non-aciduric microorganisms, while LAB rapidly grow and generally attest from 7 to 9 log cfu/g in the first 3 days of fermentation (Hui, 2012; Toldra *et al*., 2015). Due to their competitiveness in the sausage environment, LAB become the most dominant bacterial group and their high viable counts remain constant till the end of the ripening phase (Hui & Evranuz, 2012). Moreover, LAB generally include facultatively heterofermentative species belonging to *Lactobacillus, Pediococcus* and *Leuconostoc*.

CNC, especially *Staphylococcus* spp. and *Kocuria* spp., have been reported to constitute the second most dominant microbial group during fermentation and ripening phases (Hui, 2012). CNC release the enzyme nitrate reductase, which reduces nitrate to nitrite and thus allows the production of nitroso-myoglobin, important for the characteristic color of the product (Aquilanti *et al.,* 2016). Since CNC are poor competitors in the presence of active LAB, their levels are usually lower. However, CNC show a rapid increase in the first days of fermentation, starting from low levels of approximately 3 log cfu/g and reaching counts of 5 to 6 cfu/g or more after the first days of fermentation. Their counts have also been shown to increase during ripening, due to the slight increase in pH that occurs after the end of lactic acid fermentation (Hui, 2012; Toldra, 2007). Furthermore, the proteolytic activity of CNC also contributes to the pH increase during the end of the ripening phase which usually ranges from 5.0 to 5.3, and sometimes around 6.2 (Aquilanti e*t al.*, 2016)

Enterococci represent one of the autochthonous LAB group that can be found in high numbers during the sausage fermentation, as they contribute to flavor development due to glycolytic, proteolytic and lipolytic activities. They have been shown to increase from levels of 2-4 log cfu/g to 4-6 log cfu/g during early fermentation and remain constant until the end of ripening. However, other studies show a decrease in their counts at the end of maturation, depending on the season of manufacture (Toldra *et al.*, 2015; Zdolec, 2017)

Although LAB and CNC are the main actors during sausage fermentation, the activity of eumycetes has been reported in some fermented sausages, especially those produced in France, Italy, and Spain (Hui & Evranuz, 2012). Their growth is reported to increase in the last stages of fermentation till the end of the ripening time, with levels above 5 log cfu/g. Yeasts metabolize the lactic acid present in the sausage, thereby contributing to the pH increase during such maturation phase, while molds create an external protective layer to keep the product moist and avoid its excessive drying (Flores *et al.*, 2015).

The end of ripening is characterized by a gradual and constant reduction of microbial groups less resistant to low pH and a_w, including members of Enterobacteriaceae, Pseudomonadaceae and *Bacillus*. On the other hand, LAB, CNC, enterococci and eumycetes, usually defined as pro-technological microorganisms, survive for a longer period (Ockerman & Basu, 2010; Hui, 2012). Pathogenic microorganisms, such as *L. monocytogenes* and shiga toxin-producing *Escherichia coli* (STEC), can also survive for a long time at low temperatures and low pH conditions (Hui & Evranuz, 2012).

1.6 The Microbiota of Traditional Italian Fermented Sausages

Scientific studies on pro-technological microorganisms of fermented sausages began in 1960 and are still evolving (Hui & Evranuz, 2012). In Italy, many investigations on local traditional fermented sausages (raw materials, processing environment, end-product, etc.) have already been carried out based on both conventional methods and modern molecular biology approaches.

Many Italian traditional fermented sausages are prepared according to a wide variety of recipes. In detail, the recipes are generally composed of different types and quantities of meat cuts (which derive mostly from pork) and spices, while they sometimes include additives (curing salts and fermentable sugars). These recipe variations greatly contribute to the unique sensorial properties of the end-product. For instance, Southern Italian fermented meats commonly have a stronger flavor than those produced in Northern and Central Italy, due to the type and quantity of spices used (Aquilanti *et al.*, 2016). The processing conditions, especially during fermentation, drying and ripening steps, are also of great importance to obtain a specific end-product. The modification of such processing conditions, including the temperature and humidity, rely on the activities of heterogenous microbial communities. The different processing conditions and the use of various recipes influence the composition and dynamics of the microbiota of the product and its final features (Aquilanti *et al.*, 2016). Indeed, it is known that the quality

of fermented sausages is a result of several characteristics, especially the type of microbiota, one of the most important variables involved. Therefore, in order to deeply characterize the microbiota of fermented sausages, numerous investigations have been carried out on such traditional preparations produced in Northern, Central and Southern Italy. The products under study have received protected denominations at EU level with PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) status or certifications as traditional products (TP), whereas others are named based on the location where the production takes place, although such denominations are not protected at EU level (Aquilanti *et al.,* 2016).

Several products have been investigated from Northern Italy, including *salame Milano* (TP), *soprèssa* (from Veneto region), *traditional piedmontese sausage, salame mantovano* (TP), *salame cremonese, salame bergamasco, salame piacentino* (PDO) and traditional fermented sausages of Friuli Venezia Giulia, using a combination of culture-dependent and -independent approaches. The main microbial groups of Northern Italy products were LAB and CNC with an average microbial load of about 8.0 log cfu/g and 6.0 log cfu/g, respectively. In detail, the main LAB species identified were *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus piscium, Lactobacillus paracasei, Lactobacillus fermentum, Leuconostoc mesenteroides* and *Pediococcus* spp., while the predominant CNC species was *Staphylococcus xylosus*.

In Central Italy, the main studies focused on the microbial ecology of Ciauscolo (PGI), which revealed a clear dominance of LAB over CNC, with counts mostly higher than 7.5 log cfu/g for LAB and lower than 5.0 log cfu/g for CNC. Moreover, the most frequent LAB species were *Lb. sakei* and *Lb. curvatus*, while *S. xylosus* was found with the highest frequency among CNC.

Finally, in Southern Italy there are many different types of fermented sausages, known with the generic names of *salame*, *soppressata*, or *salsiccia*. The products under study include *salame Napoli*, *soppressata molisana* (TP), *soppressata del Vallo di Diano* (TP), *Salame di senise, Salsiccia sarda* (TP) and *Suino nero dei Nebrodi*. The microbiological analysis on such preparations revealed a microbiota dominated by LAB, with an average microbial load of about 9.7 log cfu/g, followed by CNC, with an average microbial load of about 6.0 log cfu/g. In detail, it was revealed a clear predominance of the LAB species *Lb. sakei* and *Lb. curvatus*, while *S. xylosus* was the most frequent species in CNC.

Overall, the main bacterial communities involved in the production of Italian fermented sausages include LAB, with an average microbial load of about 8.0 log cfu/g, and CNC, with an average microbial load of about 5.0-6.0 log cfu/g. The main species of LAB comprise *L. sakei* and *L. curvatus*, while the main species of CNC comprise *S. xylosus* (Aquilanti *et al*., 2016).

Table 1-3: Enumeration results compiled for lactic acid bacteria (LAB) and coagulase negative cocci (CNC) of fermented dry sausages manufactured in Italy

Salami	LAB	CNC	Eumycetes	Enterococci	pH	Aw	Ref
		Counts	log cfu/g				
Salame Milano	8.0	6.9	-	5.0	5.00	0.91	Rebecchi *et al*., 1998
Salame mantovano, Salame di Cremona, Salame bergamasco	8.0	4.0-6.0	<5	5.3	5.00	0.91	Pisacane *et al*., 2015 Cocolin *et al*., 2009
Salame Piacentino	8.2	4.0-7.5	8.0	6.2	6.60	0.89	Di Cagno *et al.*, 2008
Traditonal fermented sausages of Fruili Venezia Giulia	4.0-9.0	5.5- 8.8	<3	6.0	5.65	0.92	Cocolin *et al*., 2001 Cocolin *et al*., 2001 Comi *et al*., 200
Traditional Piedmontese sausage	8.0	7.0	4.0	-	6.80	-	Greppi *et al.*, 2015
Ciasculo	7.5	5.0	4.7-5.0	-	5.59	0.88-0.95	Silvestri *et al.*, 2007 Belleggia *et al.*, 2020
Salame Napoli	8.0 - 8.8	6.2 -7.0	4.0	-	5.22	-	Coppola *et al.*, 1995 Coppola *et al.*, 2000
Soppressata molisana	8.0	4.0- 5.0	-	-	6.00	-	Coppola *et al.*, 1997 Coppola *et al.*, 1998
Soppressata del Vallo di Diano	6.7-9.3	4.2- 7.3	2.0-4.0	3.0-6.0	6.30	-	Villani *et al*., 2007
Salame di senise	7.0	6.0	-	-	4.60	0.82	Baruzzi *et al.*, 2006
Salsiccia and soppressata of Basilicata	8.0	6.0	-	-	-	-	Bonomo *et al.*, 2000 Bonomo *et al.*, 2009
Salsiccia sarda	8.2-10	5.4- 9.1	3.0-6.0	-	5.73	-	Mangia *et al.*, 2008
Suino nero dei Nebrodi	7.0	6.9	5.0	5.0	6.00	-	Francesca *et al.*, 2013

2 AIM OF THE RESEARCH

Fermented sausages can also be broadly distinguished into traditionally and industrially manufactured sausages. However, many parts of Europe are still characterized by a high number of traditional fermented sausages prepared by artisanal producers. This is the case of the Marche Region in Central Italy, renowned for its traditional fermented pork sausages. In particular, the fermented liver sausage *(salsiccia di fegato)* is a typical product from the Region. Its production is spread across the provinces of the Marche region, including Ancona, Macerata, Ascoli Piceno, Fermo, and Pesaro and Urbino.

The scientific literature includes several studies about the microbiological and physico-chemical characterization of fermented sausages. However, to the authors' knowledge, there is no information about the main characteristics and the dynamics of the microbiota of liver-based fermented sausages. Therefore, new data would be needed to clarify if the use of such ingredient can modify and how, the main features of the end-product.

The aim of the study is to characterize for the very first time, the microbial biodiversity of fermented liver sausages (*salsiccia di fegato*). To this end, 10 samples of ready-to-eat fermented liver sausages were collected from 5 different producers (2 samples from the same batch for each producer) of the Marche Region.

The analyses conducted include i) microbiological analysis, such as the enumeration of several microbial groups (LAB, CNC, enterococci, eumycetes, Pseudomonadaceae and Enterobacteriaceae) and ii) physico-chemical analyses, such as the measurement of pH, water activity, total titratable acidity and concentration of organic acids (acetic and lactic acid).

3 MATERIALS AND METHODS

3.1 Sampling

Ten samples of ready-to-eat fermented liver sausage (*salsiccia di fegato)* were randomly collected from five artisan production plants located in different geographical areas of the Marche region (Central Italy). For each producer, two samples of the same batch were collected after 30 days of production. In more detail, the producers were labelled as PA, PB, PC, PD and PE. Each sample consisted of 200 g of fermented liver sausage produced with different recipes (Table 3-1) and without the use of starter cultures. The samples were stored under refrigeration at 4°C until analysis.

Table 3-1: Ingredients from different producers of the fermented liver sausage

Producer	**PA**	**PB**	**PC**	**PD**	**PE**
Pork meat	x	x	x	x	x
Pork fat	x				x
Pork liver % 20			x		x
25				x	
33		x			
Salt	x	x	x	x	
Pepper		x	x		x
Garlic					
Milk powder			x		
Fructose				x	
Dextrose		x		x	x
Sucrose		x			
Chili pepper			x		
Orange peel			x		
E300 (antioxidant)	x	x			x
E301 (antioxidant)				x	x
E250 (preservative)					x
E252 (preservative)	x	x	x	x	x

3.2 Microbial Analyses

For each sample, the sausage casing was removed aseptically using a sterile scalpel. Ten g-aliquots of each sample were added to 90 mL of sterile peptone water in a sterile Stomacher bag. Each sample was homogenized using a Stomacher 400 Circulator (VWR International PBI, Milan, Italy) at 260 rpm for 3 minutes. Ten-fold serial dilutions were prepared with the same diluent and total viable counts (TVC) of the following microorganisms were carried out onto the opportune selective solid media: i) presumptive lactic acid bacteria (LAB) on De Man, Rogosa and Sharpe (MRS) agar (VWR Prolabo Chemicals, Leuven, Belgium) added with cycloheximide (250 mg/L) and incubated at 37 °C for 48-72 hours; ii) coagulase negative cocci (CNC) on Mannitol Salt Agar (MSA) (VWR Prolabo Chemicals) incubated at 37 °C for 48 hours; iii) Enterobacteriaceae on Violet Red Bile Glucose Agar (VRBGA) (VWR Prolabo Chemicals) incubated at 37 °C for 24 hours; iv) enterococci on Slanetz Bartley Agar (SBA) (VWR Prolabo Chemicals) incubated at 37 °C for 24-48 hours; v*)* Pseudomonadaceae on Pseudomonas Agar Base (PAB) added with cetrimide-fucidin-cephalosporin (CFC) selective supplement (VWR International, Milan, Italy) and incubated at 30 °C for 24–48 h; and vi) eumycetes on Rose Bengal Chloramphenicol agar (RB) (VWR Prolabo Chemicals) incubated at 25 °C for 72-96 hours. Colonies were counted following the manufacturers' instructions and plates containing between 30 and 300 colonies were considered. 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

On the basis of the microbial groups to be enumerated, different culture media were used, whose composition is shown in the following tables:

Table 3-2: 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

De Man, Rogosa and Sharpe (MRS) (WVR Chemicals) (De Man *et al.*, 1960).

Table 3-3: 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

Mannitol Salt Agar (MSA) (WVR Chemicals) (Chapman, 1945).

Table 3-4: Composition of VRBGA culture medium

Component	Concentration (g / L)
Enzymatic digest of animal tissues	7g/L
Yeast extract	3g/L
Glucose	10g/L
Sodium chloride	5g/L
Bile salts	1,5g/L
Neutral red	0,03g/L
Crystal violet	0,002g/L
Agar	15g/L

Violet Red Bile Glucose Agar (VRBGA) (WVR Chemicals) (Garofalo *et al*., 2017).

Table 3-5: Composition of RB culture medium + Chloramphenicol

Component	Concentration (g / L)
Mycological peptone	5.00g/L
Dibasic potassium phosphate	1.00g/L
Magnesium sulfate	0.50g/L
Glucose	10.00g/L
Rose bengal	0.05g/L
Agar	15.00g/L
Chloramphenicol	0.10g/L

Rose Bengal Chloramphenicol Agar (RB) (WVR Chemicals) (Belleggia *et al*., 2020).

Table 3-6: Composition of PBA medium

Component	Concentration (g / L)
Gelatinized peptone	16g/L
Hydrolyzed caseins	10g/L
Potassium sulfate	10g/L
Magnesium chloride	1,6g/L
Agar	11,5g/L

Pseudomonas Agar Base (PAB) (WVR Chemicals) (Mead & Adams, 1977).

Table 3-7: Composition of SBA medium

Component	Concentration (g / L)
Triptose	20g/L
Yeast Extact	5g/L
Glucose	2g/L
Dibasic potassium phosphate	4g/L
Sodium azide	4g/L
Triphenyl tetrazolium chloride (TTC)	1g/L
Agar	10g/L

Slanetz Bartley Agar (SBA) (WVR Chemicals) (Slanetz & Bartley, 1957).

3.3 Physico-chemical Analyses

Water activity (a_w) was determined in accordance with the ISO 21807:2004 standard method using the Aqualab 4TE apparatus (Meter Group, Pullman, USA).

The pH of fermented liver sausage was determined at the core of the products using a pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy).

Total titratable acidity (TTA) was measured on 10 g-aliquots of fermented liver sausage samples, previously homogenized in 90 mL of distilled water using a Stomacher 400 Circulator (VWR International PBI, Milan, Italy) at 260 rpm for 3 min. The results were expressed as the total volume (mL) of a 0.1 N NaOH solution used to achieve a fixed endpoint pH of 8.3.

3.3.1 Determination of Lactic Acid Content

To determine the presence and concentration of lactic acid a D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit provided by Megazyme (Bray, Ireland) was used with the following procedure:

- sample dilution: considering that the assay is linear over the range of 0.5 to 30 μg of D- /L-lactic acid per assay, the sample solution was diluted sufficiently to reach a concentration of lactic acid between 0.005 and 0.30 g/L. If the lactic acid concentration of the sample is below the determination limit, more sample is weighed out or, alternatively, the sample volume can be increased in accordance with the manufacturers' instruction;
- sample clarification, through the use of the following solutions: i) Carrez I solution, obtained by dissolving 3.60 g of potassium hexacyanoferrate in 100ml of distilled water, kept at room temperature; ii) Carrez II solution, obtained by dissolving 7.20g of zinc sulphate in 100ml of distilled water, kept at room temperature; iii) NaOH 100 mM. To clarify the samples, 5g of each

were placed in a 100 mL volumetric flask and 60 mL of distilled water were added. Subsequently, 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH 100 mM were added to the flask, mixing after each addition. Finally, distilled water was added to fill the flask to the mark and the entire content was filtered with filter papers;

- determination of lactic acid content: after the addition of the chemical reagents supplied by the kit, the filtered solution to be quantified was pipetted into cuvettes. Then read on the spectrophotometer at a wavelength of 340 nm. The results were expressed as the grams of lactic acid per 100 g of sample and were reported as mean values of two biological and two technical replicates ± standard deviation.

3.3.2 Determination of Acetic Acid Content

To determine the presence and concentration of acetic acid an Acetic Acid (Acetate Kinase Manual Format) test kit provided by Megazyme (Bray, Ireland) was used with the following procedure:

- sample dilution: considering that the assay is linear over the range of 0.3 to 25 μg of acetic acid per assay, the sample solution was diluted sufficiently to reach a concentration of acetic acid between 0.003 and 0.25 g/L. If the acetic acid concentration of the sample is below the determination limit, more sample is weighed out or, alternatively, the sample volume can be increased in accordance with the manufacturers' instruction;
- sample clarification (the method follows the same procedures of the sample clarification for the determination of lactic acid content);
- determination of acetic acid content (the method follows the same procedures of the determination of lactic acid content). The results were expressed as the grams of acetic acid per 100 g of sample and were reported as mean values of two biological and two technical replicates ± standard deviation.

3.4 Statistical analysis

The Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used to assess differences within the fermented liver sausage samples by one-way analysis of variance (ANOVA). The software JMP Version 11.0.0 (SAS Institute Inc., Cary, NC) was used to perform all tests.

4 RESULTS AND DISCUSSION

The quality aspects of traditional fermented liver sausage (*salsiccia di fegato*) produced in the Marche Region of Central Italy are greatly attributed to the fermentation and ripening processes, which rely on the activities of microorganisms naturally present in the raw materials and the processing environments. However, the knowledge about the microbiological and physico-chemical features of fermented liver sausage is still limited. Therefore, the aim of this study is to generally describe the microbiota of such a product by determining the presence of several microbial groups and its physico-chemical properties.

4.1 Microbiological Analyses

The samples were subjected to microbiological culture-dependent assays to characterize the main microbial populations, along with spoilage and pathogen indicator microorganisms. The results of viable counts on fermented liver sausage samples are shown in Table 4-1.

Table 4-1: Results of total viable counts of bacteria and eumycetes in fermented liver sausages

Producer	Presumptive mesophilic lactobacilli	Coagulase-negative staphylococci	Enterococci	Enterobacteriaceae	Pseudomonadaceae	Eumycetes
PA	6.96±0.08 [b]	6.75±0.22 [a]	4.60±0.9 [ab]	<1.00 [a]	<1.00 [b]	4.53±0.04 [bc]
PB	8.07±0.76 [a]	4.33±0.03 [b]	5.44±0.57 [a]	1.86±1.62 [a]	1.89±1.04 [a]	5.51±0.64 [ab]
PC	8.35±0.21 [a]	4.99±1.31 [ab]	4.04±0.07 [b]	0.90±0.62 [a]	0.58±0.68 [b]	5.80±0.23 [a]
PD	8.01±0.70 [ab]	4.66±1.18 [b]	5.69±0.07 [a]	0.25±0.50 [a]	<1.00 [b]	5.16±0.16 [abc]
PE	8.23±0.36 [a]	4.47±1.03 [b]	3.78±1.01 [b]	0.87±0.62 [a]	<1.00 [b]	4.19±0.74 [c]
Overall mean	7.93±0.68	5.04±1.22	4.71±0.90	0.78±1.01	0.49±0.90	5.04±0.74

For each producer means followed by different letters are significantly different ($P < 0.05$).
Values are expressed as mean ± standard deviation.

The results revealed an active microbial community mainly composed of LAB, CNC, enterococci and eumycetes. Mean counts for presumptive mesophilic lactobacilli ranged between 6.96±0.08 (producer PA) and 8.35±0.21 log cfu/g (producer PC), with an overall mean of 7.93±0.68 log cfu/g. The overall mean value can be compared with those published in other similar studies on Italian traditional fermented sausages by Coppola *et al.* (1998), Rebecchi *et al*. (1998), Cocolin *et al.* (2001), Silvestri et al. (2007), Villani *et al*. (2007), Di Cagno *et al.* (2008) and Belleggia *et al.* (2020) in which the viable counts of

presumptive mesophilic lactobacilli ranged from 7.5 to 8.2 log cfu/g. The significantly different mean value for producer PA (6.96±0.08 log cfu/g), in comparison with the other producers with means above 8 log cfu/g, can be correlated to the use of different ingredients (sugars) and processing conditions which influence the growth of LAB. Moreover, the specific ingredients and processing conditions used by the producer PA may have established more favorable conditions for the competition with other microbial groups, such as CNC. As reported by Lucke (1994), when acid production is slow or delayed, as evidenced by low counts of LAB and low lactic acid content values of producer PA, the non-pathogenic CNC may become more competitive, in fact the viable counts of CNC of the same producer registered the higher mean value of 6.75±0.22 log cfu/g. For all producers, presumptive mesophilic lactobacilli counts were higher compared to the remaining microbial populations analyzed. This finding is in agreement with previous studies on Italian traditional fermented sausages, which showed the dominance of the same microbial group (Aquilanti *et al.*, 2016). Therefore, the competitiveness of LAB in the sausage environment, where they play an acidifying role thereby suppressing non-aciduric microbial populations, including spoilage and pathogenic microorganisms, is confirmed.

The viable counts of CNC ranged from 4.33±0.03 cfu/g (PB) to 6.75±0.22 cfu/g (PA), with an overall mean of 5.04±1.22 log cfu/g. Such values are comparable with the CNC mean counts of 4.0 to 7.5 log cfu/g from fermented sausages, reported by Silvestri *et al.* (2007), Villani *et al*. (2007), Di Cagno *et al.* (2008) and Cocolin *et al.* (2009). It is noteworthy that producer PA showed the highest average mean of CNC (6.75±0.22 cfu/g), which is statistically different compared to the other producers. Moreover, since the mean counts of CNC generally registered lower values, CNC activity may be limited in fermented liver sausages, reflecting their poor competitiveness in the presence of active aciduric bacteria (Mauriello *et al.*, 2004). Also, several scientific studies showed differences in the CNC growing during the ripening phase of fermented sausages, reaching a final value from 3 to 9 log cfu/g. Such extremely varied growth is based on the sausage type, environment conditions, competitiveness with other microbial groups, pH, salt and other factors (Milićević *et al.,* 2014).

Enterococci showed viable counts ranging from 3.78±1.01 to 5.69±0.07 log cfu/g, with an overall mean value of 4.71±0.90 cfu/g. The values resulted lower than those reported in other studies about the microbial characterization of fermented sausages, in which the enterococci counts ranged from 5 to 6 log cfu/g (Rebecchi *et al*., 1998; Comi *et al*., 2005; Di Cagno *et al*., 2008 and Milićević *et al.,* 2014). Nonetheless, their presence confirms the contribution of enterococci even during the transformation processes of fermented liver sausages. As described above, the main role of enterococci is to produce lactate, which causes a drop in pH and improves flavor of the end-product. However, enterococci can induce the transmission of mobile antibiotic resistance genes to other microorganisms and release biogenic amines, including cadaverine and putrescine, whose accumulation is undesired due to their potential toxicity for humans (Mižáková *et al.,* 2002; del Rio *et al.,* 2019).

The overall mean counts of Enterobacteriaceae and Pseudomonadaceae were less than 1 log cfu/g, at 0.78±1.01 and 0.49±0.90 log cfu/g respectively. Both count values were lower than those reported by Francesca *et al*. (2013), in which, both Enterobacteriaceae and Pseudomonadaceae showed similar levels of approximately 5 log cfu/g. However, regarding Enterobacteriaceae, the results obtained for this study were similar to those reported by Belleggia *et al*. (2020) for *Ciauscolo* salami showing an average count of 1.82 cfu/g. Enterobacteriaceae are a large family of Gram-negative bacteria that includes many pathogenic bacteria. For this reason, the presence of such microbial family group is considered as an indicator of the hygiene and safety of fermented sausages. On the other hand, the Pseudomonadaceae family include the spoilage species *Pseudomonas fragi, Pseudomonas fluorescens, Pseudomonas lundensis* and *Pseudomonas putida*. Such microbial group generally acts by secreting enzymes responsible for the catabolism of sulphur-containing amino acids which cause defects in texture and flavor of fermented meats. However, the low levels of both groups in fermented liver sausages are evidence of good manufacturing practices and further indicate the effectiveness of the preparation methods used, including the addition of salt, nitrates and nitrites.

Regarding eumycetes, mean counts ranged between 4.19±0.74 (PE) and 5.80±0.23 log cfu/g (PC), with an overall mean of 5.04±0.74 log cfu/g. Similar yeast counts on *Ciauscolo* salami, a typical fermented sausage of the Marche Region, were reported Silvestri *et al*. (2007) and Belleggia *et al*. (2020), ranging between 4.7 and 5.0 log cfu/g. As described before, yeasts have been reported to play a role in the metabolism of lactic acid present in the sausage, thereby contributing to the increase in pH during the ripening phase. Regarding molds, they create a protective layer to avoid excessive drying and maintain the product moist (Flores *et al*., 2015). However, the activity of eumycetes is mostly reported in typical fermented sausages from the Mediterranean countries, thus explaining their presence even in fermented liver sausages (Hui & Evranuz, 2012). Indeed, the Mediterranean climatic conditions do not allow for extensive drying and therefore promotes the growth of eumycetes (Leroy *et al*., 2010).

4.2 Physico-chemical Measurements

The results of the physico-chemical analysis, including a_w, pH, TTA, lactic and acetic acid concentrations, of fermented liver sausages are shown in Table 4-2.

The water activity values were comprised between 0,807±0,002 (PA) and 0,905±0,001 (PC), with an overall mean of 0,847±0,042. The values were slightly lower than those reported for *Ciauscolo* by Aquilanti *et al.* (2007), Silvestri *et al.* (2007) and Belleggia *et al.* (2020). Such variation in water activity levels can be attributed to the difference in the processing conditions that include the duration of drying and ripening phases, and the fat content and the varying amounts of solutes, such as salt or sugar, of fermented liver sausages (Vignolo *et al.*, 2010; Belleggia *et al.*, 2020).

Table 4-2: Physico-chemical parameters of fermented liver sausage

Producer	Water Activity (aw)	pH	Total titratable acidity (mL NaOH 0.1M)	Lactic acid	Acetic acid
PA	0.807±0.002 [a]	5.60±0.03 [a]	25.25±1.44 [b]	0.164±0.112 [c]	0.032±0.003 [a]
PB	0.869±0.060 [a]	4.89±0.21 [d]	26.78±0.59 [ab]	1.056±0.031 [ab]	0.026±0.003 [a]
PC	0.905±0.001 [a]	5.26±0.02 [b]	22.10±2.17 [c]	0.799±0.015 [abc]	0.020±0.008 [a]
PD	0.809±0.002 [a]	5.16±0.03 [bc]	28.15±0.85 [a]	0.570±0.060 [bc]	0.030±0.005 [a]
PE	0.849±0.005 [a]	4.99±0.02 [cd]	21.13±0.85 [c]	1.330±0.367 [a]	0.020±0.005 [a]
Overall mean	**0.847±0.042**	**5.18±0.27**	**24.68±2.99**	**0.784±0.448**	**0.025±0.006**

For each producer means followed by different letters are significantly different ($P < 0.05$)
TTA, total titratable acidity
Values expressed as mean ± standard deviation

The combined effect of the aforementioned aspects resulted in a_w levels below 0.90, which are suitable for the growth of Gram-positive bacteria, especially pro-technological microorganisms such as LAB and CNC, and eumycetes, which proliferate at even lower a_w levels. On the other hand, a_w levels below 0.90 are unsuitable for most Gram-negative bacteria, which normally favor levels above 0.97. It is indeed well known that the a_w level is a fundamental physico-chemical parameter for determining the shelf-life of fermented meat products and food in general (Porto-Fett *et al*., 2008).

The TTA of fermented liver sausage samples ranged between 21.13±0.85 (PE) and 28.15±0.85 mL of 0.1 N NaOH (PD), with an overall mean of 24.68±2.99 mL of 0.1 M NaOH. Moreover, the levels of organic acids showed a higher concentration of lactic acid compared to acetic acid. In fact, lactic acid content of fermented liver sausage samples was comprised between 0.164±0.112 (PA) and 1.330±0.367 g/100g (PE), with an overall mean of 0.784±0.448; meanwhile, acetic acid content was comprised between 0.026±0.003 (PB) and 0.032±0.003 g/100g (PA), with an overall mean of 0.025±0.006. The results obtained by Belleggia *et al.* (2020) for *Ciauscolo* salami at the point of fermentation were 0.614 ± 0.047 and 0.696 ± 0.090 g/100g for lactic acid, and 0.055 ± 0.010 and 0.059 ± 0.00 g/100g for acetic acid. The different levels of organic acids produced depend on the amount of glycogen present in the raw materials (i.e., meat muscle and, in the case of fermented liver sausages, pork liver), the addition of fermentable sugars, the production conditions and the diversity of LAB microbiota (Milićević *et al.*, 2018). In this regard, higher amounts of lactic acid compared to acetic acid suggest the prevalent contribution of homo-fermentative LAB species

respect to hetero-fermentative LAB species during the acidification process (Belleggia *et al*., 2020). It is noteworthy that the results showed a correlation between LAB loads and the amounts of lactic acid produced. In fact, producer PA showed the lowest LAB mean count (6.96±0.08) with a correspondingly low lactic acid content (0.164±0.112). The quality of acidification plays an important role in the palatability and the overall quality of the final product. Generally, in fermented sausage products, lactic acid is preferred compared to acetic acid, which instead is associated with off flavors.

The pH of fermented liver sausage samples ranged between 4.89±0.21 and 5.60±0.03, with an overall mean of 5.18±0.27. In addition, scientific studies on *Ciauscolo* salami showed similar pH values, ranging between 4.98±0.01 and 5.69±0.01 (Aquilanti *et al.,* 2007; Belleggia *et al.,* 2020). Interestingly, fermented liver sausage samples with the highest pH values (5.60±0.03 from producer PA) also showed the highest CNC counts (6.75±0.22 from producer PA). Likewise, the other samples showed a similar pattern, evidencing a correlation between pH values and CNC counts. These results confirm the lower competitiveness of CNC with low pH levels, while LAB remain the most competitive microbial group in the same conditions. Besides, the pH affects the growth and mortality of undesirable microorganisms because of its potential effects on enzymatic activities and denaturation of proteins. For this reason, pH plays an important role in the safety, shelf-life and sensorial quality of fermented sausages (Andrés-Bello *et al*., 2013.

5 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The present study offers a first look on microbiological and physico-chemical characteristics of fermented liver sausage. In accordance with other scientific studies on fermented sausages, the microbiota was dominated by LAB. The product acidification carried out by LAB through the production of lactic acid plays a crucial role in the safety, shelf-life and organoleptic quality of fermented liver sausage. Another fundamental microbial group identified in fermented liver sausages is represented by CNC, which mainly contributes to the organoleptic properties of the final product by means of its enzymatic activities, responsible for the color development and the decomposition of free amino acids and peroxides. Regarding enterococci and eumycetes, they play a secondary but significant role in fermented liver sausage fermentation, as their viable counts result quite similar to those of CNC. Generally, eumycetes contribute to define the final pH and sensorial characteristics of the product through their proteolytic and lipolytic activities. The low counts of hygiene and sanitary indicator microorganisms belonging to the Enterobacteriaceae and Pseudomonadaceae families indicate good manufacturing practices during the production of the analyzed fermented liver sausages.

5.2 Recommendations

Further studies on the isolation, identification and characterization of the major microbial species and strains occurring in the production of fermented liver sausages are needed. Such information will provide a better understanding of the evolution dynamics of the microbiota, highlighting the role of the microbial species present. Furthermore, the pro-technological properties of beneficial microorganisms of fermented liver sausages must be evaluated for their potential use as starter cultures in food fermentation.

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