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The effect of the selected strain *Lactobacillus sakei subsp sakei* BK18 used as starter culture on physicochemical and microbial characteristics of dry- fermented sausages

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The effect of the selected strain *Lactobacillus sakei subsp sakei* **BK18 used as starter culture on physicochemical and microbial characteristics of dry-fermented sausages.**

Abstract

In Algeria, the sausages are rarely consumed fermented or smoked mostly cured and cooked. Moreover, Lactic acid bacteria (LAB) strain *Lb. sakei subsp sakei* BK18 were selected by phenotypical-genotypical studies of LAB from Algerian *Kaddid* for their safety criteria, appropriate technological characteristics and probiotic activity. This study *in situ* revolves around to formulate a recipes of dry-fermented sausages like Italian *Salami* by the using of selected strain *Lb. sakei subsp sakei* BK18 as starter culture and the available row materials basically low-cost red-meat cuts and fat of Lamb mixed with Turkish meat and salt and spices. After the manufacturing of dry-fermented under controlled conditions, the monitoring of physicochemical and microbial of changes during the repining was investigated comparing to a negative control without starter.

The results of the physicochemical analyses showed that inoculated sausages had lower pH values compared to control sausages. That's where the pH was on the 14th day 5,71 in control batch and 5,64 in started batch. The loss of moisture water activity and water content during the ripening of sausages was not affected by the use of starters.

While the results of the microbiological analysis showed that the addition of starter cultures had a significant effect on microbiological characteristics. the *Coagulase negative cocci* (CNC) counts were the most dominant bacteria, with higher counts in control batches than started batches, except on 3rd ripening day. However, there is no difference in the number of yeasts and molds between the two batches. Otherwise, the starter culture had a bearing on reduction of the number of Coliforms, suggesting the effect of acidic activity of the starter BK18 on the hygienic quality of fermented sausages by reducing counts of *Enterobacteriaceae* and *Clostridia*.

Key words: dry-fermented sausages, *Lactobacillus sakei subsp sakei* BK18, physicochemical, microbial.

Effet de la souche *Lactobacillus sakei subsp sakie* **BK18 utilisée comme culture starter sur les caractéristiques physico-chimiques et microbiennes des saucisses fermentées sèches**

Résumé

En Algérie, les saucisses sont rarement consommées fermentées ou fumées la plupart du temps séchées et cuites. De plus, la souche bactérienne de l'acide lactique (LAB) *Lb. sakei subsp sakei* BK18 a été sélectionnée par des études phénotypiques génotypiques de LAB de *Kaddid* algérien pour leurs critères de sécurité, leurs caractéristiques technologiques appropriées et leur activité probiotique. Cette étude *in situ* tourne autour de la formulation de recettes de saucisses fermentées à sec comme le *Salami* italien par l'utilisation de la souche sélectionnée *Lb. sakei subsp sakei* BK18 comme culture de starter et les matériaux de ligne disponibles essentiellement à faible coût rouge-viandes et gras d'agneau mélangés avec de la viande de dinde et du sel et des épices. Après la fabrication de fermentés secs dans des conditions contrôlées, la surveillance des changements physicochimiques et microbiens pendant la fermentation a été étudiée en comparant avec un témoin négatif sans culture starter**.**

Les résultats des analyses physicochimiques ont montré que le pH des saucisses inoculées était inférieur à celui des saucisses témoins. C'est là que le pH était le 14ème jour 5,71 dans le lot témoin et 5,64 dans le lot inoculé. La perte de l'activité hydrique et de la teneur en eau pendant la maturation des saucisses n'a pas été affectée par l'utilisation des culture starter.

Les résultats de l'analyse microbiologique ont montré que l'ajout de cultures starter avait un effet important sur les caractéristiques microbiologiques. Les *Cocci à coagulase négative* (CCN) étaient les bactéries les plus dominantes, avec des nombres plus élevés dans les lots témoins que dans les lots inoculés, sauf le troisième jour de maturation. Cependant, il n'y a pas de différence dans le nombre de levures et de moules entre les deux lots. Autrement, la culture starter a eu une incidence sur la réduction du nombre de coliformes, suggérant l'effet de l'activité acide du starter BK18 sur la qualité hygiénique des saucisses fermentées en réduisant le nombre d*'Enterobacteriaceae* et de *Clostridia*.

Mots clés : saucisses sèches fermentées, *Lactobacillus sakei subsp sakei* BK18, physique-chimique, microbien

تأثير سلالة BK18 *Lactobacillus sakei subsp sekei* المستخدمة كبادئة

على العناصر الفيزيائية الكيميائية والبيولوجية على النقانق المخمرة الجافة

الملخص

في الجزائر، نادرًا ما يتم استهلاك النقانق المخمرة أو المدخنة في الغالب يتم طهيها. علاوة على ذلك، فان بكتيريا حمض اللاكتيك (LAB) *Lb. sakei subsp sakei* BK18 سلالة تم اختيارها من خلال الدراسات النمطية الظاهرية الوراثية لـ LAB من *القديد* الجزائري لمعايير السلامة والخصائص التكنولوجية المناسبة ونشاط المعززات الحيوية. تدور هذه الدراسة *in situ* حول صياغة وصفات من النقانق المخمرة الجافة مثل *Salami* الإيطالي باستخدام سلالة مختارة من *Lb. sakei subsp sakei* BK18 كبادئة و باستخدام المواد المتاحة بشكل أساسي قطع اللحوم الحمراء منخفضة التكلفة ودهون لحم الضأن الممزوجة بلحم الديك الرومي والملح والتوابل. بعد تصنيع النقانق المخمرة الجافة في ظل ظروف خاضعة للرقابة، تم التحقيق و مراقبة التغيرات الفيزيائية الكيميائية والميكروبية أثناء نضج النقانق بالمقارنة مع التحكم السلبي دون بادئة.

أظهرت نتائج التحليلات الفيزيائية الكيميائية أن النقانق الملقحة لها قيم أقل للأس الهيدروجيني pH مقارنة بنقانق التحكم. حيث كان الاس الهيدروجيني pH في اليوم الرابع عشر 5,71 في دفعة التحكم 5,64 في دفعة البادئة. لم يتأثر فقدان الرطوبة و نشاط الماء ومحتوى الماء أثناء نضج النقانق باستخدام البادئة.

بينما أظهرت نتائج التحليل الميكروبيولوجي أن إضافة البادئة كان له تأثير كبير على الخصائص الميكروبيولوجي. كانت أعداد البكتيريا *Coagulase negative cocci* (CNC) هي الأكثر انتشارًا، مع عدد أكبر في دفعات التحكم مقارنة بالدفعات البادئة، باستثناء يوم النضج الثالث. ومع ذلك، لا يوجد فرق في عدد الخمائر والقوالب بين الدفعتين. خلاف ذلك، كان لثقافة البداية تأثير على تقليل عدد *Coliforms*، مما يشير إلى تأثير النشاط الحمضي للبادئة BK18 على الجودة الصحية للنقانق المخمرة عن طريق تقليل عدد *Enterobacteriaceae*. و*Clostridia* .

الكلمات المفتاحية: النقانق المخمرة الجافة، *Lactobacillus sakei subsp sakei* BK18، الفيزيائية الكيميائية، الميكروبية

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List of abbreviations

- **Unit of measure**

N : Normality

U : Unity

UI : International Unity

rpm : Rotation per minute

- **Other abbreviation**

ANOVA: Analysis of variance

AOAC: Association of Official Analytical chemists

aw: Water activity

BA: Biogenic amines

BPW: Buffered peptone water

CFU: Colony Forming Unit

CNC: *Coagulase negative cocci*

CPC: *Coagulase positive cocci*

DM: Dry matter

DNA: Deoxyribonucleic acid

Eh: Redox potential

EHEC: *Enterohemorrhagic Escherichia coli*

FBUNT: Faculty of Biochemistry-University

GABA: γ-aminobutyric acid

GCC: Gram positive and catalase positive cocci

GRAS: Generally recognized as safe

H: Humidity

ISO: International Organization for Standardization

Kat A: "Kataluein" hemo-dependent catalyst

L: *Listeria*

LAB: Lactic acid bacteria

LABs: Lactic acid bacteria strain

Lb: *Lactobacillus*

M: Moisture

MC: Microbiological Criteria

MRS: de Man Rogosa and Sharpe

N: Number of colonies

OD: Optical Density

OGA: Oxytetracycline glucose agar

P: *Penicillium nalgiovense*

QPS: Qualified Presumption of Safety

RAPD: Randomly Amplified Polymorphic DNA

RIPPS: Ribosomally Synthesized and post translationally modified peptides

RH: Relative Humidity

RTE: Ready to eat

RV: Rappaport Vassiliadis

S : *Salmonella*

SEs: *Staphylococcal enterotoxins*

Subsp: Subspecies

SS: *Salmonella-Shigella* agar

Spp: Species plures

Sp: Species not specified

TC: Total coliforms

TTA: Total Titratable Acidity

TTC: Thermo Tolerant Coliforms

TSW: Tryptone-Salt-Water

WC: Water Content

Introduction

Dry fermented sausages are among one of the main meat-based traditional fermented foods largely produced and consumed not only in Europe, especially Mediterranean regions, but also worldwide. Among them, a few well-known examples include *Saucisson sec* or *Rosette* in France, *Salami* in Italy, or *Fuet* in Spain. In France, nearly 113,000 tons of dry-cured sausages were produced in 2019, which represents about 10% of the global processed meat production. When it comes to product export, scientific data as well as methods to determine overall product quality and safety are required **(Coton et *al*., 2021).**

Fermented sausages are the result of microbiological, biochemical, physical and sensory changes occurring in the meat mixture during ripening under defined conditions of temperature and relative humidity. **(Casaburi et *al*., 2007).** The quality of the final product is closely related to the ripening that takes place during drying. This process, which confers to the product its slice ability, firmness, color and flavor, is characterized by a complex interaction of chemical and physical reactions associated with the microbiological development of the batter flora. **(Ordonez et *al*., 1999).** Today, the need for safe products with standard and desirable technological properties has resulted in the use of starter cultures to produce the dry fermented sausages to control the fermentation and ripening process, inhibiting the growth of other undesirable microorganisms, such as heterofermentative bacteria, biogenic amine and pathogens producer. **(Zaho et *al*., 2011; Tabanelli et *al*., 2012).**

Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation processes because they affect both the technological properties and the microbial stability of the final product. In fact, the use of starter LABs is essential for carbohydrate fermentation and lactic acid generation with subsequent pH drop which contribute to the flavor of the final product through the formation of noticeable acidic and vinegary (acetic acid) tastes. At isoelectric pH, muscle proteins coagulate, lose their water-holding capacity and result in the slice ability, firmness and cohesiveness of the final product. Besides, at this acidic environment ripening is favored as well as color development **(Drosinos et *al*., 2007)**.

Moreover, LAB inhibits growth of spoilage and pathogenic microorganisms through the production of lactic acid and antimicrobial compounds such as bacteriocins, acetic acid, ethanol, acetoin, carbon dioxide and pyruvic acid **(Albano et *al*., 2009)**. The species most used as commercial meat LAB starter cultures are *Lactobacillus plantarum, Lactobacillus sakei, Lacobacillus curvatus, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus pentoseaceus and Pediococcus acidilactici* **(El adab et *al*., 2014).**

The aim of this work was to study the effect of *Lactobacillus sakei subsp sakei* BK18 previously isolated from a traditional salted meat (*Kaddid*) and selected for their technological properties, on the microbiological and physico-chemical characteristics during the ripening of a dry fermented sausage.

Bibliographic Synthesis

Chapter Ⅰ.

Dry fermented sausage generality

Ⅰ.1. Introduction to sausages production:

Sausages are one of the oldest processed foods known to man. Several hundreds of varieties of sausages are produced worldwide with outstanding social and economic relevance. Each local variety within each sausage type (raw, scalded, cooked, or raw and fermented/ripened) reflects the availability of raw materials, the climate conditions of each geographical environment, the cultural and religious conditionings, and the ancestral manufacture knowledge transmitted through generations **(Carballo, 2021).**

Sausage is typically defined as a mixture of ground meat, combined with spices and seasonings. Sausage is often stuffed into some type of casing and linked, but loose sausage products are also available. Originally, sausage was made as a means to salvage value from trimmings and lower-value cuts of meat. As well as adding value to meat, over time, cooked sausage products have become convenient meat products, as they are 'ready-to-eat' (RTE) and are easy to eat in the form of a sandwich **(Knipe, 2014).**

Ⅰ.1.2. Sausage classification:

According to Rust sausage production began with a simple process of salting and drying the meats which helped improve the shelf life of the sausages. Subsequently, aromas and spices were incorporated to improve the organoleptic quality of the product. The product was then stuffed in a container, namely the gastrointestinal tract of animals, to make the product more convenient to eat.

The manufacturing process may vary because seasoned meat is minced, stuffed in casings, and can be smoked, dried, fermented and heated. There is a large variety of sausages and the different variants are linked to the modification of the type of meat, the processing temperature, types of casings and the particle size of the casings **(Hugo CJ and Hugo A, 2015).** Table1 shows the different types of sausages:

Table 1: Sausage classification

Classification	Characteristics	Examples
Raw sausages		
Fresh	Made from fresh comminuted, uncured, non-smoked meats. Must be refrigerated prior to heating by the consumer.	Breakfast sausage (USA), *boerewors* (South Africa), *bratwurst* (Germany), *Merguez* (North Africa), *siskonmakkara* (Finland)
Fermented	Made from comminuted, cured or uncured, fermented and often smoked meats. Not heat-processed.	
Semidry (Quickly fermented)	Stuffed in medium- and large-diameter artificial casings. "Tangy" flavor produced by fermentation. Length of smoking and fermentation depends on type but rarely exceeds a few days. Improved stability of stored refrigerated.	Variety of summer sausages, ex: *cervelats*, *mettwursts* (Lebanon, bologna, (USA)).
Dry (Slowly fermented)		Different types of salamis, ex: *droë wors* (South Africa, not fermented)
Heat-processed sausages		
Smoked precooked	Mostly comminuted, cured, non-fermented. Final cooking before consumption	Chinese pork sausages, *kielbasa*
Emulsion-type	Made from comminuted well-homogenized cured meats, Fat, water and seasoning. Usually smoked, slightly cooked. Ready-to-eat product.	*Frankfurters*, *wieners*, *bologna*, *Mortadella*
Cooked	Made from previously comminuted cooked fresh or cured raw materials. Final cooking afterstuffing. With or without smoking. Ready-to-eat product.	Liver sausage, *Braunschweiger*

I.2. Dry fermented sausage:

Fermentation and drying can be considered to be the oldest way to preserve raw materials. Although the historical origin of fermented meat products remains unknown, fragmentary bibliographical research has traced it back more than 2500 years in China **(Vignolo et *al*., 2010).** Dry fermented sausage can be defined as meat product which is manufactured by chopping and mincing meat and fat with spices, certain additives and starter cultures, then filling into casings and ripening/drying **(Çolak et *al*., 2007).**



Picture 1. dry fermented sausage

I.2.1. Ingredients:

- **Meat:** The selection of meat ingredients for sausages and comminuted meats is an important decision made by meat processors. The first requirement is that the meat be of high quality and as fresh as possible. The second requirement is information on composition and color of each ingredient. Computerized least-cost formulation programs are used to determine the proportions of each ingredient needed to achieve the desired product characteristics at the lowest possible cost. This helps to improve product consistency and uniformity.
- **Non meat ingredients**: are essential to product characteristics, such as salt, and can be restricted by regulations. It is important to check the regulations appropriate to where the product is being manufactured and/or sold to determine the limits of nonmeat ingredient use. Salt is also critical to microbial control, product texture, and binding of

fat and water. Research has been conducted to reduce salt levels in processed meats. Salt is necessary for typical texture and binding qualities of meat, and is used in restructured meats and nuggets **(Heinz and Hautzinger, 2007).**

Table 2: non meat ingredients **(Heinz and Hautzinger, 2007).**

	Function	Level of use and applications
Salt (sodium chloride)	Flavoring, microbial inhibition, protein solubilization (which controls fat and water retention in cooking), product texture, and product adhesion	0–5% in all sausages and many comminuted meat. Dry sausage may be as high as 5%, other sausages 1.5–2.5%, restructured comminuted meats 0.5–1.0% and ground beef 0%. There are no regulatory limits
Spices (Ground pepper, paprika, garlic, mace, pimento and cardamom, red pepper, mustard, rosemary, sage)	stimulate the rate of lactic acid formation, thus extent the shelf life of dry sausages, it enhances flavor and taste.	Ground pepper is usually present in all types of fermented sausages. Some spices are more heavily spiced than smoked sausages
Sweeteners (sucrose, dextrose, lactose, corn syrups, sorbitol)	Provide sweetness, improve water retention, substrate for fermentation	Usually used at 0.5–1.0%. Sucrose and dextrose is unlimited but corn syrups and sorbitol are restricted to 2.0% by regulation
Starter cultures	Produce lactic acid from sugars and lower product pH	Limited to 0.5% (with water) for fermented Sausage

Ⅰ.2.2. The manufacturing of dry and fermented sausages:

The manufacture of dry-fermented sausages is usually considered to entail three main steps: formulation, fermentation, and ripening /drying **(Juan et *al*., 1999):**

A. Formulation:

- **Meat and fat selection:** Removal of bones and fat and sinews trimming. Soft intermuscular fatty tissue must be detached.
- **Mixing:** The chopped meat and fat mass is thoroughly mixed with spices, curing additives, Starter cultures and salt in the cutter (8-12ºC in absence of air).
- **Stuffing:** The meat batter is firmly packed in the stuffer to exclude air pockets. Stuffing by filling natural or synthetic casings is carried out under vacuum.

B. Fermentation: Conditions vary in terms of temperature and duration. Dry sausages: 12-24ºC, for 1-7 days.

C. Drying and/or smoking: Dry sausages slow drying rate (10-15ºC for 4-12 weeks); RH: 65-90%; surface molds are favored at the beginning of drying. If smoking (12-22ºC during 10-45 h depending sausage diameter).

- **Packaging for retail distribution:** Although fermented sausages are shelf-stable they may be packaged under vacuum or modified atmospheres preventing quality deterioration during handling, transportation and storage **(Vignolo, 2010).**

Ⅰ.2.3. Ecological aspects of sausage fermentation:

Most European fermented sausages still follow the traditional procedures in which fermentation and ripening depend on the activities of heterogeneous microbial communities **(Gardini et *al*., 2001; Cocolin et *al*., 2006).** Two wide groups of bacteria largely predominate, lactic acid bacteria (LAB) and the group known as either Coagulase–negative cocci (CNC) or (gram-positive)-catalase-positive cocci (GCC+/CPC), which includes both micrococci and coagulase–negative staphylococci (CNS) **(Aquilanti et *al*., 2016).** Yeasts and filamentous fungi also play a relevant role, through the formation of a superficial film which exerts a protective action against both excessive dehydration and the oxidation of the lipid fraction due to oxygen and light **(Gardini et *al*., 2001; Cocolin et *al*., 2006).** The acidification process that is the result of the fermentation of the sugars into lactic acid by LAB, plays a fundamental role to prevent

spoilage and pathogen outgrowth. *Coagulase-negative cocci* are involved in the proteolytic and lipolytic processes thus playing a central role in the formation of the final organoleptic characteristics **(Hammes and Hertel, 1998),** and contributing to nitrate reduction and color formation, as well as to prevention of rancidity. In addition, the characteristic flavors and surface aspect are due to yeasts and molds **(Cocolin et *al*., 2006).**

LAB are more numerous than CNC during fermentation and ripening, remaining more stable in the ripened products. Within LAB, facultatively heterofermentative lactobacilli generally prevail and, among them, the two psychrotrophic species *Lactobacillus sakei* and *Lactobacillus curvatus* are dominant. Within CNC, *Staphylococcus xylosus* neatly dominates **(Aquilanti et *al*., 2016).**

The selection of specific populations adapted to a specific environment depends on ingredient composition, fermentation and maturation factors **(Rantsiou et *al*., 2005).** The production environment may have an impact on the microbial ecology of the product. For example, **(Greppi et *al*., 2015)** showed that strains isolated from environmental samples were also detected either in the raw materials or in the product. This finding highlights that microorganism may enter in the production plant with the raw materials. Furthermore, it underlines that the production environment is a source of continuous "inoculation", during fermentation and ripening, with strains that may have important technological characteristics and influence the characteristics of the final product.

I.2.4. Safety of Dry Fermented Sausages:

Dry fermented sausages are considered ready - to - eat products, referring to those products that do not undergo thermal treatments before consumption. These products become stable and safe through a sequence of hurdles, some of which are specifically included (NaCl, $NaNO_2$ /$NaNO_3$, ascorbate), while others are indirectly created in the stuffed mix (low Eh, antagonistic substances, low a_w). By means of these hurdles, spoilage and food - poisoning bacteria are inhibited, whereas the desirable organisms, especially LAB, are hardly affected. Apart from LAB, GCC, molds, and yeasts involved in sausage fermentation, beef and pork meat as the major components of cured sausages regularly contain pathogenic bacteria and are often implicated in the spread of food – borne diseases. Chilling inhibits the growth of various pathogens, including mesophilic and psychrotrophic organisms. Good hygienic conditions may not pose sanitary risks, but fermented dry sausage can still harbor pathogenic bacteria **(Mataragas et *al*., 2008)**. Food poisoning from *Staphylococcus aureus*, *Salmonellae*, and

Clostridium has been linked to fermented sausage, while emergent pathogens like *Campylobacter, Yersinia, Listeria monocytogenes,* and EHEC have been involved in outbreaks. Fermented sausage conditions, curing additives, and LAB starter cultures may help control these pathogens **(Vignolo and Fadda 2007; Castellano et *al*., 2008).**

I.3. Chemical hazard in fermented meat product:

I.3.1. Biogenic amines (BA):

Biogenic amines (BA) are nitrogenous compounds derived from amino acids**.** The content and profile of BA present in fermented meat products has been extensively studied Histamine, tyramine, and phenylethylamine are the foremost dietary BA associated with health problems, namely vasoactive and psychoactive reactions: histaminic intoxication, enteric histaminosis causing food intolerance, food-induced migraines, and interactions between tyramine and monoamine oxidase inhibitors **.**The production of fermented meat products involves a hugely diverse microbiota that includes technologically important microorganisms as well as undesired food spoilers and pathogens**.** High levels of BA have traditionally been used as an index of undesired microbial activity in food, which may derive from poor hygiene manufacturing or storage practices**.** Nevertheless, both technological microbiota as well as microbial contaminants may be responsible for producing BA**.** Therefore, it is necessary to effectively control the levels of BA that accumulate in fermented meat products, due to the health risks associated with these compounds **(Laranjo et *al*., 2019).**

I.4. Physicochemical character:

In general, these products have a final pH ranging between 5.2 and 5.8, which is consistent with the lower lactic acid content (0.5% – 1.0%), a moisture lower than 30% **(Vignolo et *al*., 2010).**

Due to a_W which ranges from 0.85 to 0.91, dry fermented sausages exhibit high shelf stability and can be kept without refrigeration. The typical lower a_W values of these products are achieved by air - drying in Mediterranean countries and by smoking in northern countries **(Vignolo et *al*., 2010).**

NaCl is normally added at levels ranging from 2% to 4%, depending on technology and market demands. Salt performs many functions, including microbial growth suppression, a_W reduction, salt - soluble protein release, and prooxidant effects **(Vignolo et *al*., 2010).**

I.5. Microbiological quality:

I.5.1. Microbiological Criteria (MC):

Microbiological Criteria (MC) have been used in food production and the food regulatory context for many years and Codex defines MC as follows **(CAC, 2013**): A microbiological criterion is a risk management metric which indicates the acceptability of a food, or the performance of either a process or a food safety control system following the outcome of sampling and testing for microorganisms, their toxins/metabolites or markers associated with pathogenicity or other traits at a specified point of the food chain **(WHO, 2016).**

I.5.2. Bacteria Searched:

I.5.2.1. Staphylococcus aureus:

Staphylococcus aureus is a gram-positive bacterium, commonly found in the nostrils, on the skin and on the hair of warm-blooded animals, including humans. It can produce a wide variety of virulence factors, including *staphylococcal enterotoxins* (SEs). In literature, 24 different SEs and many variants have been identified; among these, only five (the so-called classic enterotoxins) have been well-defined. Due to their emetic activity, SEs are frequently responsible for staphylococcal food poisoning, when consumers ingest contaminated food **(Grispoldi et *al*., 2021).**

I.5.2.2. The coliform bacteria:

Coliform bacteria are defined as rod-shaped Gram-negative non spore forming and motile or nonmotile bacteria that can ferment lactose with the production of acid and gas when incubated at 35-37 °C. They are a commonly used indicator of sanitary quality of food and water. *Coliforms* can be found in the aquatic environment, in soil and on vegetation; while coliforms themselves are not normally causes of serious disease, they are easy to cultivate, and their presence is used to indicate that other pathogenic organisms of fecal origin may be present. Such pathogens include disease-causing bacteria, viruses, or protozoa and many multicellular parasites. *Coliform* procedures are performed in aerobic respiration aerobics or anaerobic conditions **(Daoliang and Shuangyin, 2019).**

I.5.2.3. *Clostridium* ***sulfite recital:***

Clostridium are rod-shaped, Gram-positive bacteria capable of producing spores. *Clostridia* is one of the largest bacterial classes including more than 150 validly described species. Among these, there are several with enormous biotechnological potential and also a few well-known pathogens. *Clostridia* are obligate anaerobes and not osmotolerant. They are typically involved in the spoilage of foods such as canned or vacuum-packaged foods. *Clostridia* in the public

opinion is associated with production of a bad smell. In most cases, this is caused by butyric acid, one of the major fermentation products **(Lorenzo et *al*., 2018).**

I.5.2.4. *Salmonella* sp:

Salmonellae are a group of facultative anaerobic, Gram-negative, rod-shaped bacteria in only two species: *Salmonella enterica* and *S. bongori* with more than 2600 serovars. these bacteria are present in intestine of mammals, birds (particularly poultry), and man worldwide **(Ellis, 1969; Hoelzer et *al*., 2011),** the presence of *Salmonella spp*. has also been reported in water, sediments, aquatic flora **(Jyoti et *al*., 2011),** and reptiles **(Schro¨ter et *al.*, 2004).** And it one of foodborne and cross-contamination.

I.5.2.5. Yeasts and Molds:

Yeasts are fungi that grow as solitary cells that reproduce by budding and can be classified either to *Ascomycetes* (e.g., *Saccharomyces, Candida*) or *Basidiomycetes* (e.g., *Filobasidiella, Rhodotorula*). The term mold is commonly applied to certain multicellular, filamentous fungi whose growth on foods is usually readily recognized by its fuzzy or cottony appearance Generally, molds are concerned in the spoilage of foods. Additionally, certain species have contaminated and poisoned the food **(Lorenzo et *al*., 2018).**

Chapter Ⅱ.

The effect of lactobacillus sakei in dry fermented sausage

Ⅱ.1. *Lactobacillus sakei:*

Ⅱ.1.1. Originally:

The origin of the name of this species finds its roots in 1934, when it was isolated in as a contaminant from the fermentation of Japanese rice alcohol, saké **(Kagermeier-Callaway and Lauer, 1995).**

The phenotypical heterogeneity of the species and proximity with another resident species Meat, *Lb. curvatus*, has in the past made it difficult to correctly identify strains. by *Lb. sakei* Melibiotic fermentation and hydrolysis of arginine Desiminase are two phenotypic criteria used to distinguish the two species: *Lb. sakei* is positive on both tests while *Lb. curvatus* is negative there **(Montel, 1985, Klein et *al*., 1996)**. Additionally, DNA-DNA hybridization experiments have shown that *Lb. curvatus* and *Lb. sakei* are clearly separated at the genomic level with less than 50% identity **(Champomier et *al*., 1987)**. While the heterogeneity of the species was known, Recent studies have also revealed the genetic diversity of the species at 25%. Variation in the size of the genome **(Chaillou et *al*., 2009).**

Ⅱ.1.2. Definition:

Lb. sakei is a bacterium species of the genus Lactobacillus. It is a facultative heterofermentative able to produce either alcohol or lactic acid from sugars. *Lb. sakei* is used in Europe for the production of traditional dry sausage as starter and can be used for the conservation of fresh meat **(Bredholt et *al*., 2001).** It was therefore of interest for its application in cooked ham and sausages during processing in the factory. Regarding spoilage effect, they have negligible effect on the sensory properties of the products; however, it can lead to the formation of slime textures on cured and processed meat products **(Marshall and Bal'a, 2001).**

In Western Europe, *Lb. sakei* is generally considered as beneficial. Nevertheless, some exopolysaccharide-producing strains have been reported in meat products **(Mallere et *al*., 1998)** and fish products **(Marceau et *al*., 2001)**. They are considered to be deleterious for these products, especially in the Finnish meat and fish industries **(Bjorkroth and Korkeala, 1997)** Strains harboring plasmids associated with hydrogen peroxide production

have also been reported **(Stentz and Zagorec, 1999)**. Due to this important role in meat technology, its, taxonomic status, physiology and metabolism have been studied in relation to its particular habitat.

Ⅱ.1.3. Habitats of the species:

Meat is the natural habitat of *Lb. sakei* even if its natural reservoir remains unknown. he species *Lb. sakei* shows a large diversity of fermented profiles, the difficult yield to Classify on this phenotypic aspect. becomes the dominant flora when the meat is stored under vacuum. Several studies have shown that *Lb. sakei* was able to conduct natural fermentation. in various products such as Greek *Salami* **(Samelis et *al*., 1994)** or dry sausage Italian **(Conter et *al*., 2005)** but not found in Turkish dried sausage **(Adiguzel and Atasever, 2009).** *Lb. sakei* is one of the most common bacteria. used as a starter culture for the fermentation of meat **(Ammor et *al*., 2005, Cocconcelli, 2007).**

Ⅱ.1.4. Adaptation to Habitat:

Lb. sakei is presented in the form of sticks (Figure 2) with rounded ends (0.6 to 0.8 µm per 2 to 3 µm long), often curved or irregular especially in phase stable growth and grouped by pairs or short chains. GC% of chromosome It is 42-44 percent mol. No growth is possible at 45°C even though most strains It can grow from 4°C to 42°C. *Lb. sakei* belongs to the optional heterogenic group **(Klein et *al*., 1996, Stiles and Holzapfel, 1997).**



Picture 2. Morphology of *Lb. sakei* observed by scanning electron microscope **(Plateforme MIMA2, Massy, France).**

Lb. sakei a milk bacterium well adapted to its ecological niche where it is naturally present. In particular, the analysis of the genome highlights the presence of specific equipment that enables *Lb. sakei* to manage oxidative stress, the sources of carbon and essential elements, as well as to proliferate and colonize the surface of the and meat.

Ⅱ.1.5. Diversity of Species:

Studies based on biochemical and physiological characteristics have described There is a large phenotypic heterogeneity of the strains of *Lb. sakei*. Fingerprints of Protein RAPD (Randomly

Amplified Polymorphic DNA) This led to the conclusion that the species *Lb. sakei* could be divided into two subgroups **(Klein et *al*., 1996, Berthier and Ehrlich, 1999**). These subgroups are described as the two subspecies of *Lb. sakei subsp*. **(McLeod et *al*., 2008).**

the study examined the biodiversity of 10 strains of *Lb. sakei* isolated from food in the country, using phenotypic tests and proteomic fingerprint techniques to identify two genetic groups. In 2009 identified 27 islets, and Des Subtractive hybridization experiments revealed new genes absent from the Sequence the Straw. A collection of 73 strains was crushed on the presence or absence of sixty different genes, and a dozen clusters of Seeds were described. The average genome size of *Lb. sakei subsp sakei* is 2100 kb versus 2000 kb for the Carnosus species, making this character ineffective for distinguishing subspecies. **(Chaillou et *al*., 2009).**

Ⅱ.1.6. Taxonomy and phylogeny of *Lb. sakei* group:

Lb. sakei, first described as *Lactobacillus sake*, a spoilage microorganism in the fermented beverage, *sake*, in 1934, was renamed following recommendations of Trüpper and DeClari **(Trupper and DeClari, 1997)**. It is a lactic acid bacterium (LAB). This group of bacteria contains many other species involved in food fermentation and preservation. It is a Gram-positive bacillus which groups by pair or short chain. The GC content is 42–44%. Phenotypic differentiation between strains and other species is usually based on the type of lactic acid isomer produced, sugar fermentation pattern and ammonia production from arginine.

Wide phenotypic heterogeneity within strains of *Lb. sakei* isolated from meat has been reported in many studies based on biochemical and physiological features, especially in sugar fermentation. Difficulties in correctly identifying these strains are also due to the close relatedness between *Lb. sakei* and *Lb. curvatus,* also found in meat products. The need for correct identification, especially of starter strains, has led over the last ten years to several taxonomic studies dealing with the taxonomic status of these species using molecular and genomic methods, thus enabling a clearer distinction between *Lb. sakei* and *Lb. curvatus* **(Champomier-Vergès, 2001).**

Ⅱ.2. Starter Culture in Fermented Sausages:

Ⅱ.2.1. Definition:

A starter culture is a microbiological culture of defined organisms made up of cultivation medium or nutrient liquids colonized by living microorganisms for fermentation **(Pilevar and Hosseini, 2017).** The incorporation of starter culture into the meat during manufacturing of dry-

fermented sausage significantly reduces the ripening period. The use of lactic acid bacteria (LAB) as starter culture produces meat products with desirable characteristics and health benefits, as well as contributing to food safety and quality in terms of physiochemical and sensory characteristics **(Parvez et *al*., 2006; Justo et *al*., 2013).** As far as LAB starter cultivation is concerned, *Lb. sakei, Lb. acidilactici, Lb. curvatus, Lb. pentosus, Lb. casei*, and *Pedicoccus pentosaceus* are the main species used for the fermentation process **(Hugas and Monfort, 1997).**

Ⅱ.2.2. Starter culture selection parameters:

So far, the selection of a starter culture has been based on the screening of a great number of isolates in small-scale food fermentations. A satisfactory performance of the selected starter culture in the process, and an acceptable organoleptic evaluation of the food product are the fundamental characteristics to be found. The behavior of the starter culture in relation to the environmental factors and ripening conditions encountered during a specific production needs to be carefully investigated and standardized in the selection process. It is necessary to understand the properties required and the specific techand recipe for which a strain will be used in order to develop the ideal starter culture **(Hansen, 2002).**

According to **Holzapfel (1997)**, in order to improve product quality, the introduction of starter cultures in traditional small-scale fermentations should incorporate considerations as:

- ✓ rapid metabolic activities (acid production);
- ✓ improved and predictable fermentation processes;
- ✓ desirable sensory attributes;
- ✓ improved safety and reduced hygienic and toxicological risks.
- ✓ Another important factor is the interaction in mixed cultures of selected starter strains, with con-sideration for the behavior of these strains under defined conditions, and within the food matrix.

Other aspects, which should be considered, include:

- ✓ competitive behavior,
- ✓ viability and survival;
- ✓ antagonism against pathogens and spoilage microbes;

- ✓ rate of acid production;
- ✓ organoleptic changes;
- ✓ primary metabolites of fermentation;
- ✓ degradation of antinutritive factors;
- ✓ detoxification;
- ✓ probiotic features

(Holzapfel, 1997). Modern approaches for selection of the best (s) for a process integrate also technical safety and health-promoting features **(Holzapfel, 2002).**

It is essential to know the autochthonous microbiota of fermented food that is to be analyzed because commercial starter cultures usually originate either from substrates or from the processes in which they are applied. Factors that can contribute to the selection of microbial populations typical of a fermentation process are environmental conditions, back-slopping, adaptation and the repeated use of specific tools **(Holzapfel, 1997).**

II.2.3. *Lactobacillus sakei* **as starter culture:**

The species name *Lb. sakei* is derived from its isolation and characterization from *'sake'*, a fermented rice beverage **(Katagiri et *al*., 1934).** This species has a lot of genetic variability, which can be seen when looking at different strains, and on which its numerous features in meat products: starter, spoiler, and protective culture, are most likely based. Because of its resilience to a variety of culture conditions and antibacterial properties, *Lb. sakei* was employed in fermented sausages **(Gelinski et *al*., 2019).** The ability to cope with high NaCl concentrations, acidic pH, or high temperatures vary between *Lb. sakei strains*. The aptitude of *Lb. sakei* strains to fast acidification is an important characteristic as it has an impact on taste and safety, aroma, and bacteriostatic and bactericidal effects. **(Leroy et *al*., 2006).** *Lb. sakei* is also one of the few LAB that has a heme-dependent catalase **(Chaillou et *al*., 2005).**

The role of *Lb. sakei* in the fermentation of different types of meat products of various country origins was reported by several authors **(Nguyen et *al*., 2010; Wanangkarn et *al*., 2014; Fontana et *al*., 2016; Tamang et *al*., 2016).** However, the effect of different strains of *Lb. sakei* species on quality characteristics of dry fermented sausages during fermentation and ripening was not conducted to date. We believe that evaluating the functional features of individual

strains can help in the selection and production of high-quality fermented meats on an industrial scale, whether they are employed as a single strain or a mix of strains.

Ⅱ.3. Improving food safety of fermented sausage:

The growth of harmful microorganisms, such as foodborne pathogens, in fermented sausages can be inhibited by applying starter cultures to them, lowering their pH **(Laranjo et *al*., 2019).** The starter culture should be able to inhibit toxin production by other microorganisms. *P. nalgiovense*, widely used for starter culture, can inhibit the production of toxin-producing fungi, ensuring the safety of fermented sausages **(Bernáldez et *al*., 2013).**

Ⅱ.3.1. Safety of starter culture:

The starter culture should grow well under the physical and chemical conditions of the sausages and be harmless to humans. To ensure the safety of starter cultures, hemolysis, toxin production, and biogenic amine production by the microorganisms in the starter culture must be evaluated **(Jeong and Lee, 2015; Sirini et *al*., 2022).** Some starter cultures produce biogenic amines in fermented sausages, and thus, starter cultures producing no biogenic amines need to be developed, and processing methods such as packaging, additives, hydrostatic pressure, and smoking can also reduce biogenic amines **(Doeun et *al*., 2017; Özogul and Hamed, 2018).**

Ⅱ.3.2. Lactic Acid Bacteria and Their Antimicrobial Activities:

Ⅱ.3.2.1. Biogenic amin:

Recent studies have shown that autochthonous starter cultures may control the accumulation of BA in fermented meat products **(Lorenzo et *al*., 2017).** Combined starter cultures of *Lb. sakei* with either *S. carnosus* or *S. xylosus amine-negative* strains inoculated in fermented sausages resulted in a severe reduction in the contents of tyramine, cadaverine, and putrescine, when compared with spontaneously fermented sausages **(Bover-Cid et *al*., 2000).**

In in vitro studies, most of the *Lb. sakei* strains were amine negative, these observations suggest that starters of *Lb. sakei* would be more suitable than at her spics for the reduction of biogenic amine accumulation during ripening **(Bover-Cid et *al*., 2000).**

Ⅱ.3.2.2. Organic Acid Production

Lactic acid bacteria produce organic acids by fermentation of carbohydrates. Homo-fermentative lactic acid bacteria produce mainly lactic acid, whereas the heterofermentative species produce acetic as well as lactic acid and, to various extents, other acids such as formic, citric, and maleic. The antimicrobial activities of organic acids are primarily associated with

the undissociated forms of the acids. For this reason, the inhibitory activities of organic acids are affected by the pH values of foods. their antimicrobial effects increase with decreasing pH. The inhibitory activities are mainly due to the following:

- ✓ Reduction of pH with an increase of the concentration of H+ in a food. The pH can be reduced to values near or below the minima for growth of pathogens and potent spoilage organisms.
- ✓ Reduction of the intracellular pH of bacteria. The undissociated acid is relatively lipophilic and is, therefore, able to penetrate the bacterial membrane to release H+ in the cytoplasm. Then, in order to survive, the bacteria have to use energy to transport H+ out of cells. This causes energy depletion and cell stress.

Because the antimicrobial activities of organic acids increase as the pH decreases, their activities are greater in fermented sausages of pH 4.5 than in cooked meat products of pH 6.3. It is, however, possible to achieve some inhibitory effects by adding organic acids to products while maintaining a high pH **(Yost ;2014).**

Ⅱ.3.2.3. Bacteriocins:

Bacteriocins are defined as ribosomally-synthesized antimicrobial peptides (AMPs) or proteins from bacteria that can inhibit or kill closely related bacterial strains **(Ibrahim, 2019).** Bacteriocins produced by LAB are useful and generally regarded as safe (GRAS). They are colorless, odorless, tasteless, tolerant to low pH and high temperatures, and degraded by proteolytic enzymes in the human digestive tract due to their proteinaceous nature **(Perez et *al*., 2014).**

Bacteriocins are widely used as food biopreservatives **(Yang et *al*., 2014),** and added in various foods as pure bacteriocin preparations, bacteriocin containing fermentates or producing cultures **(O'Connor et *al*., 2020).**

Bacteriocins are classified based on different criteria including their primary structures, molecular weights, intrinsic function, posttranslational modifications, the presence or absence of unusual amino acids as well as antimicrobial mode of action and immunity mechanisms. Recently, the latest updated bacteriocin classification scheme has been proposed by **(Alvarez-Sieiro et *al*., 2016)** and **(Choi et *al*., 2022).** According to this new classification, bacteriocins can be divided into three major classes including class I, II and III.

- ✓ Class I: Modified bacteriocins or ribosomally synthesized and post translationally modified peptides (RiPPs);

- ✓ Class II: Unmodified bacteriocins;
- ✓ Class III bacteriocins.

Table 3. Biochemical characteristics and inhibitory spectrum of bacteriocins produced by *Lb. sake*i **(Champomier-Vergès,2001).**

Bacteriocin	Producers	Biochemical characteristics	Inhibitory spectrum
sakacin A	*Lb. sakei L706*	class IIa bacteriocin 4309 Da 41 amino acids hydrophobic identical to curvacin A produced by *Lb. curvatus* LTH1174	*Carnobacterium piscicola* *Enterococcus spp. Lb. sakei,* *Lb. curvatus, Lb. brevis* *Leuconostoc* *(Lc.) paramesenteroides* *Listeria (L.) monocytogenes* *S. aureus* *Lactococcus (Lc.) cremoris*
sakacin P (also called sakacin 674)	*Lb. sakei* LTH673 *Lb. sakei* L674	class IIa bacteriocin 4436 Da 43 amino acids hydrophobic	*Lb. curvatus, Lb. delbrueckii,* *Lb. sakei Lb. pentosus, Lb.* *plantarum, Lb. reuteri Lb.* *fructivorans, L. ivanovii,* *En. faecalis,* *Carnobacterium spp.* *L. monocytogenes*
lactocin S (also called sakacin M)	*Lb. sakei* L45 *Lb. sakei* 148	Antibiotic 3769 Da 33 amino acids 50% nonpolar amino acids	*Lactobacillus spp.* *Leuconostoc spp.* *Pediococcus spp*

Ⅱ.4. *Lactobacillus sakei* other features:

Lb. sakei is an interesting candidate for bio-conservation due to its ability to colonize meat products, producing hydrogen peroxide and antibacterials against contaminants in food. **(Bredholt et *al*., 1999).**

Kat A (Homo-dependent Catalyst) reduces hydrogen peroxide and is active in the hem-rich meat ecosystem, minimizing color defects and rancid flavor. (*Lb. sakie 23K* does not produce biogenic amins harmful to human health). **(Chaillou et *al*., 2005).**

Lb. sakei is known for producing bacteriocins, which have inhibitory effects against Enterococcus, Carnobacterium, and *Listeria monocytogenes*. It has also been shown to extend the shelf life of prepared meat dishes, inhibit the growth of *L. monocytogenes* in smoked salmon, and improve health safety during the preparation of French sausages. **(Sobrino et *al*., 1992, Tichaczek et *al*., 1994, Hugas et *al*., 1995, Champomier Vergès et *al*., 2002, Simon et *al*., 2002).**

Lb. sakei has the ability to produce antibacterial compounds, but it may be deprived of essential elements such as iron by endogenous flora. Iron is essential for the growth of pathogenic and altering bacteria, and is virtually inaccessible for pathogenic bacteria, **(Vaughan et *al*., 2004).**

Experimental study

Materiel and Methods

Ⅱ Materiel and Methods

Ⅱ1 Objective of the study:

This study revolves around to formulate a recipes of dry-fermented sausages like Italian *Salami* by the using of selected strain *Lb. sakei subsp sakei* BK18 as starter culture and by using the available row materials basically low cost red-meat cuts and fat of Lamb mixed with Turkish meat and salt and spices .after the manufacturing of dry-fermented under controlled conditions, the monitoring of physicochemical and microbial of changes during the repining will was investigated comparing to a negative control without starter, To meet the objectives of this study, we adopted the experimental approach which includes the following steps:

- Preparation of dry-fermented sausages under laboratory conditions.
- Evaluation of some complementary physicochemical parameters during drying of dry-fermented sausages
- Evaluation of the microbiological quality of dry-fermented sausages.

The work was carried out during the period May 2023 to join 2023 at the pedagogical laboratories of Biology (PLB), Campus of Ouzera- Medea University.

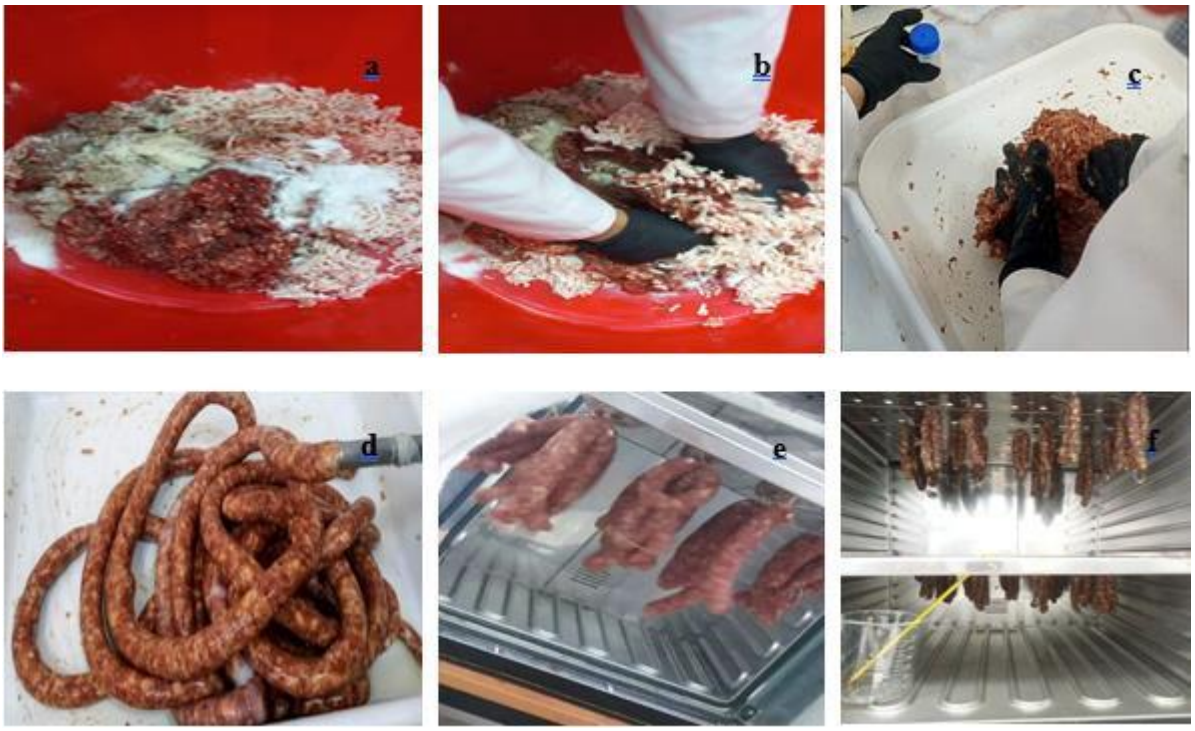
Ⅱ2 Starter culture:

The strain *Lactobacillus sakei subsp sakei* BK18 stored at -25°C in MRS broth with 25 % of glycerol was activated several times on the MRS, pH 6.2 **(de Man, Rogosa, and Sharpe, Conda; Spain)** medium at 30 °C for 18h, then sticked on MRS plate, the purity of the colonies was checked by Gram test. New stocks were made and stored at -25°C for future use.

The strain was previously activated several times until the day of preparation of the sausages by using increased volumes of MRS broth, from 5, 10, 20, until reaching 200 mL, by inoculating the medias with 0.1% (v/v) and incubated under static conditions at 30 °C for 18h. After incubation, the optic density was measured at OD_{600}, and the concentration of cells were calculated by using growth curves if the strains previously studied (Data not published), after that, the bacterial cells are recovered by centrifugation at 6000 rpm for 5 min at 4 °C, washed twice with physiological water pH 7 and resuspended in sterile falcon at the volume of 40 ml and stored at +4 °C until use. The count of viable cells was estimated by serial dilutions and results were expressed with UFC/mL. **(Essid and Hassouna, 2013)**.

Ⅱ□3□ Sausages manufacturing:

Dry fermented sausages used in this work were prepared by using the equipment's of local butcher in the center of Medea. The recipes inspired from **Noelia and *al.* (2015)** dictate the composition of these meat products per kilogram that consists of 2 kg lamp meat, 786 g turkey meat 1333.2 g lamp fat, 151g sodium chloride, 66.6 g sugar, 44.4 g powder milk, 22.2 g chopped garlic, 8.8 g ground red pepper, and 13.2 g ground black pepper. All the ingredients were thoroughly mixed, and meat dough was divided into two batches: one batch was added with the starter *Lb. sakei subsp sakei* BK18 with concentration previously estimated at □4.85×10^5 colony forming units per gram (CFU/g) concentrated in 40 ml of physiological water the other was kept as a control system was inoculated also with 40 mL of sterile physiological water. Afterwards, once the meat dough had been finished, being completely homogenized before casings were stuffed. meat dough was packed and shaped into natural casings (sheep gut) and transported tothe laboratory for drying in ventilled oven **(Memmert; Germany),** the unfermented products were placed in a ripening chamber for 15 days. During the first seven days—fermentationperiod—temperature was kept at 20–24 °C and relative humidity (RH) oscillated to 90–95%. Thenext 8 days— ripening period—temperature was kept at 23 °C and RH range was 80–85%. From each batch, samples were withdrawn toper form physicochemical and microbiological analyses atinitial day0 (d_0) time, d_3, d_7, d_{10}, and d_{14}.



Picture 3. The steps of the manufacturing sausages.

a) weighting of the ingredients; **b)** Mixing of the ingredients; **c)** weighting of the mix and batching (started batch and control batch); d**)** stuffing; **e)** fermentation 7 days (20–24 °C and RH 90–95%; **f)** Drying and repining 8 days at 23 °C and RH 80–85%.

Ⅱ□4□ Physicochemical analysis:

Ⅱ□4□1□ pH determination:

The measurement of pH is performed according to the technique described by **Wang (2000)** and the protocol **AOAC (2007).** Ten grams of the sample is homogenized with 90 ml of distilled water boiled and cooled (1:10 w/v) using a Stomacher mill **(400 circulator; England)** at a speed of 250 rpm for 3 min. The suspension is left to equilibrate for 5 min. The pH of the meat is measured with the pH meter **(Consort C5020; Belgium)** at laboratory temperature by immersing the electrode of the latter in this suspension followed by the reading of the number displayed on the screen. The device is calibrated with buffer solutions pH4 and pH7 and the result is expressedby calculating the average of three readings.



Picture 4. pH measuring by using of pH-meter.

Ⅱ□4□2□ Total titratable acidity (TTA) determination:

The acidity can be accurately titrated with soda, a precise 10 ml sample the total titratable acidity value was determined according to the protocol described by **Sadler and Murphy (2010)**. 10 ml of the meat suspension is placed in a beaker and two drops of the phenolphthalein indicator are added and then titrated with the 0.1 N sodium hydroxide solution while gently stirring the contents of the beaker until a pale pink color change is obtained which lasts a few seconds. Pale pink color change that persists for a few seconds and corresponds to the pH 8.2 turning point of phenolphthalein indicator. The total titratable acidity (TTA) of the sample was expressed as percentage of lactic acid in the dry matter basis.

Ⅱ□4□3□ Moisture determination:

The humidity content is determined according to the official **AOAC method (1998)**. 5 g of the well ground sample is weighed into a tared crucible and dried, then placed in an oven for 18 h at a temperature of 102°C. The weight of the sample is determined after complete drying. The humidity content is calculated by the difference between the initial weight (W_i) of the sample and the final weight (W_f) after drying, the average of two measurements is calculated and thehumidity is expressed as percentage (%) (Weight/weight).

$$\mathbf{H\ (\%) = ((W_i - W_t) - (W_f - W_t))/ (W_i - W_t)\ x100.}$$

The water content (WC) of the sample is expressed in percent (%) of dry matter (DM) according to the following relationship:

$$\mathbf{WC\ (\%MS) = ((W_i - W_f)/W_f)\ x100.}$$

The dry matter (DM) of the sample was obtained according to the following formula:

DM (%) =100 -WC (%)

Where:

W_i: weight of the sample and the crucible before drying (g);

W_f: weight of the sample and crucible after drying (g);

W_t: weight of the tare.

Ⅱ□4□4□ Water activity calculation:

The aw of the meat sample is calculated using an equation relating the water content to the water content and water activity; equation given by **Kouete Kongni (2012)**, and it is obtained from the work of **Ruiz-Ramirez et *al*. (2005).**

$$a_W = (8.139 \text{ Ln } (WC) + 51.14)/100$$

Where:

a_W : represents the water activity;

WC: water content.

Ⅱ□5□ Microbiological quality of samples:

The enumeration of the different flora is done with a colony counter, considering that the that the boxes contain between 30 and 300 colonies. The number per gram of dry-fermented sausages is calculated using the following formula **(ISO 7667 June 1984 V 08-001).**

$$N = \frac{\sum C}{(n_1 + 0 \cdot 1 n_1).d}$$

In which:

$\sum C$: The sum of colonies counted on all retained boxes;

n_1: The number of plates retained for the first dilution;

n_2: The number of plates retained for the second dilution;

d: is the dilution rate corresponding to the first dilution.

If the two boxes contain less than 15 colonies, the arithmetic mean *y* of the colonies counted on two boxes was taken, and the results are expressed in microorganisms per colonies counted on two plates, and the results are expressed in microorganisms per gram as follows:

$$N_E = y/d$$

In which: *d* is the dilution rate of the parent suspension.

Ⅱ□5□1□Preparation of the stock solution and dilutions:

A homogeneous quantity of the sample is aseptically ground in a sterilized mortar sterilized mortar. 10g of the crushed material is transferred into a sterile Stomacher bag. This quantity received 90 ml of tryptone-salt-water supplemented with 1% of $tween_{80}$; the whole was homogenized in the Stomacher at a speed of 250 rpm for 3 min **(NF V 05-010 March 1996).** Thesuspension is left at room temperature for 15 minutes to revive the germs. The solution obtainedis considered as the mother solution. From the stock solution stock solution 10^{-1}dilutions are made up to 10^{-5}.

Ⅱ□5□2□ Enumeration of total coliforms (TC) and thermo-tolerant coliforms (TTC):

The enumeration is carried out on Mac Conkey agar **(Tmmedia, India)**, by plating in mass of 1 ml of the 10^{-2} and 10^{-3} dilution in duplicate followed by incubation at 37°C for 24 hours for total coliforms and at 44 °C for fecal coliforms. Red colonies are counted (Lactose+) colonies are counted and sometimes surrounded by a cloudy halo **(NF ISO 4832 July 1991V 08-016, and NF ISO 08-017 June 1980).**

Ⅱ□5□3□ Research and enumeration of Clostridia:

The plating is done by sampling 5 ml in duplicate of 10^{-1} and 10^{-2} dilutions in sterile screw tubes. These tubes are then treated in a water bath at 80°C for 10 minutes and cooled immediately by immediately cooled by tap water for 10 minutes to destroy the vegetative forms, each tube destruction of vegetative forms, each tube is filled with liver agar **(Cmsmed; Algeria)** and containing sodium sulfite and iron alum with the addition of a layer of sterile petroleum jelly to ensure anaerobiosis. After incubation at 37°C, the black colonies that have grown after incubation at 37°C, the black colonies that have grown in depth are counted after 18h, 48h and 72h **(Guiraud, 2003).**

Ⅱ□5□4□ Research and enumeration of Coagulase negative cocci:

The prepared medium Mannitol-salt agar is poured and solidified in petri dishes. 0.1 ml of each10^{-1} dilution each dilution 10^{-1} and 10^{-2} dilution is inoculated in duplicate by spreading. After24 to 48 hours incubation at 37°C **(Guiraud, 1998).**

Ⅱ□5□5□ Search for Salmonella/Shigella:

The search for *Salmonella/Shigella* was done in three steps **(NF ISO 6579 V 08-013 December 1993):**

- **Non-selective pre-enrichment**: the Stomacher bag containing 10g of the samples received 90ml of buffered peptone water (BPW) **(BiochemChemopharma; France)**, after homogenization, the medium was incubated at 37°C for 20 hours.
- **Selective enrichment**: in 10 ml of Rappaport Vassiliadis (RV) **(Tmmedia; India)**
- broth contained in each sterile screw tube we put 0.1 ml of subculture from pre-enrichments medium then incubated in an oven at 42°C for 18 to 24 h the selectivity of the broth and the relatively high incubation temperature led to the elimination of a large part of the accompanying flora and favor the growth of *Salmonella/Shigella*.
- **Isolation**: from each tube and tube having shown signs of positivity, an isolation is made by isolation is done by plating in spaced streaks (about 0.5 cm) on two solid *Salmonella-Shigella (S-S)* **(Tmmedia; India)** and Hecktoen **(Tmmedia; India)** solid selective media, then the plates are incubated at 37°C for 24 hours.

➢ **Reading**: typical colonies on each medium were selected:

✓ **On S-S**: colorless colonies (lactose-) with black center (H_2S production), the presence of black center presence of black center eliminates the possibility of *Shigella*.

✓ **On Hecktoen:**

- Green or bluish colonies (absence of fermentation of sugars) indicates the possibility of *Shigella* or *Salmonella* not producing of H_2S;
- Green colonies with black center indicate the possibility of *Salmonella;*
- Salmon or yellow colonies eliminate the possibility of *Salmonella* or *Shigella* (Except *Shigella sonnei*).

Ⅱ□5□6□ Enumeration of yeasts and molds:

The OGA **(Conda; Spain)** base medium with Oxytetracycline and Gentamicin is poured and cooled. Inoculation is then carried out in bulk and in duplicate of 1 ml of dilutions 10^{-1} and 10^{-2} the plates are incubated lid up for 5 days at a temperature of 22 °C. A control was made with the **TSE (NF ISO 13 681 V 04-507 April 1996).**

Ⅱ.6. Statistical analysis:

Agar assays were performed by duplicate and the growth curves of *Lb sakei subsp sakei* BK18 by triplicate. The standard mean (SM) and standard deviation (SD) were calculated for microorganisms' enumeration data, results (means OD ± SD) being evaluated by the application of ANOVA to define differences and statistical significances were determined by the Tukey test.

Ⅱ.7. Financial support

Row ingredients of sausages and liver-agar were bought by the students, the plastic materiel such as petri dishes and tips were bought by the director of the Thesis, the other media were purchased by PLB.

Results and Discussion

III. Results and Discussion

Meat fermentation is, in fact, a complex biological phenomenon accelerated by the desirable action of certain microbes in the presence of a variety of synergistically acting or competing species. A great variability in the quality of the products is due to traditional practices and variation in the microorganisms involved in the process. De **Vuyst (2000)** underlines that it is of primordial importance to investigate and analyze the influence of the environment on the performance of a starter culture before using it in a selected product. In order to protect the traditional aspects of these products and to select autochthonous starter cultures to be used, it is essential to understand the microbial dynamics during fermentation **(Rantsiou and Cocolin, 2006).** Therefore, a current quest is to develop indigenous starters that guarantee hygienic quality and improve the sensorial aspects of the product **(Talon et *al*., 2007).** It should, however, be considered that the law allows only the use of qualified presumption of safety (QPS) in the EU, or generally recognized as safe (GRAS) in the US, microorganisms in food production. In Italy, only *Lactobacillus, Pediococcus, Micrococcus, Debaryomyces*, and *Staphylococcus xylosus*, *Staphylococcus simulans* and *Staphylococcus carnosus* are authorized as starters cultures for sausage production **(Gazzetta Ufficiale, 1995).**

The microorganisms that are primarily involved in sausage fermentation include species of lactic acid bacteria, gram-positive and catalase-positive cocci (GCC), molds, and yeasts, mainly including *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus paracasei, Staphylococcus xylosus, Staphylococcus carnosus, Staphylococcus saprophyticus, Kocuria varians, Penicillium nalgiovense, Penicillium chrysogenum*, and *Debaryomyces hansenii* **(Leroy et *al*., 2006).**

The choice of the *Lb. sakei subsp sakie* BK18 strain was based on the selection criteria for starter cultures for meat products described by **Ammor and Mayo (2007)** and **Cocconcelli and Fontana (2008),** particularly the lack of soilage and pathogenesis character, antimicrobial activity, Anti-*Listeria monocytogenes* FBUNT, bacteriocins production, anti-*Listeria monocytogenes* FBUNT biofilm formation, probiotic features, quick growth and acidification in meat-media broth, resistance to 10 % of salt, slight Lamb-myofibrillar protein hydrolysis and γ-aminobutyric acid (GABA) production (Data not published).

Ⅲ1. Physicochemical evaluation of dry-fermented sausages during maturation:

Ⅲ1.1. pH:

The results of pH determination were reported in Fig.1 The pH decreased strongly from the initial value 6.59 in control sausages and 6.60 in started sausages to reached after 7 days of ripening 5.69 and 5.57 in control and started sausages respectively. The pH fall was presumably caused by an accumulation of organic acids, mainly lactic acid, present in this type of sausages as a result of carbohydrate breakdown during fermentation **(Zaho et *al*., 2011; Essid and Hassouna, 2013).** Results showed that inoculated sausages gave lower pH values 5.57 than control sausages 5.69, Many studies reported that sausages inoculated with selected *Lactobacillus* starters gave lower pH values than control sausages **(Aro Aro et *al*., 2010; Casaburi et *al*., 2007, 2008; Cenci-Goca et *al*., 2008; Ruiz-Moyano et *al*., 2011; Zaho et *al*., 2011; Essid and Hassouna, 2013).** Higher pH values in the control samples could be due to the occurrence of autochthonous *Lactobacilli*/LABs with low acidifying activity **(Casaburi et *al*., 2007; Zaho et *al*., 2011; Essid and Hassouna, 2013)** or due to the aconsequent loss of acidifying and deaminase activities **(Essid and Hassouna, 2013; Aro Aro et *al*., 2010**; **Dellaglio et *al.,* 1996).** The rapid growth of LAB at the initial stage of fermentation was beneficial in decreasing the pH of the fermented sausages, which was responsible for reducing undesirable bacteria, such as *Enterobacteriaceae*. After 7 days of ripening, the pH progressively increased until the end of ripening. The increase of pH values at the end of ripening of dry fermented sausages was also observed by **Casaburi et *al*., 2008; Spaziani et *al*., 2009; Ruiz-Moyano et *al*., 2011; Lorenzo and Franco, 2012 and Tabanelli et *al*., 2012; Essid and Hassouna, 2013**. This increase of pH may be due to proteolytic activity generated by microorganisms. Bacterial proteases induce proteolytic degradation, generating peptides, amino acids and amines which have a buffering effect on the organic acids produced by LAB during fermentation **(Benito et *al*., 2007; Ruiz-Moyano et *al*., 2011).**

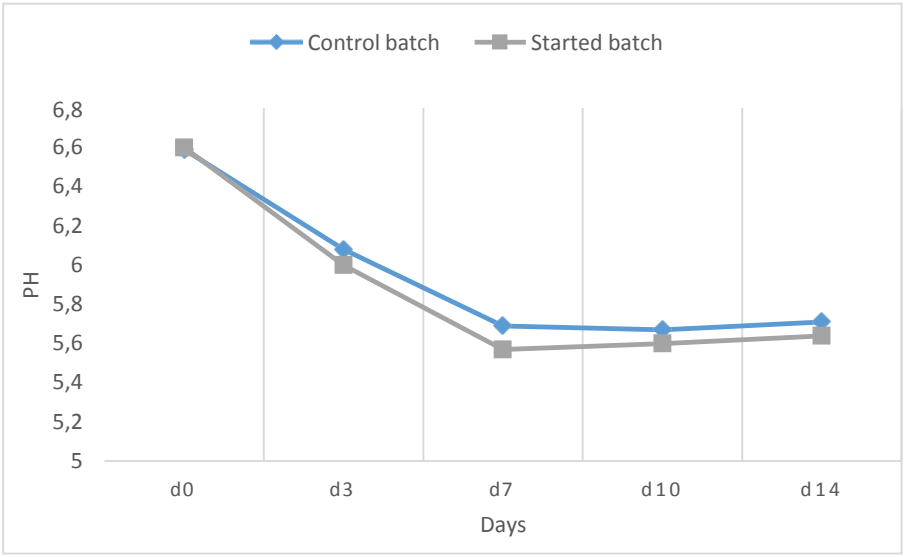


Figure 1. Evolution of pH values during the ripening of dry-fermented sausages**.**

Ⅲ1.2. Total titratable acidity (TTA):

The results of Total titratable acidity (TTA) determination were reported in Fig 2**.** The evolution and change in TTA between the two batches during drying are remarkably different. On d_0 the TTA value was 2.5 % and 2 % in control and started sausages respectively. For inoculated sausages the acidity on d_3 was 3 % and this value is fixed until the end of the drying period. on the other hand, in control sausages, the acidity percentage increased after the 3rd d of drying until it reached 4 %, and decreased to 3 % on the 14th d.

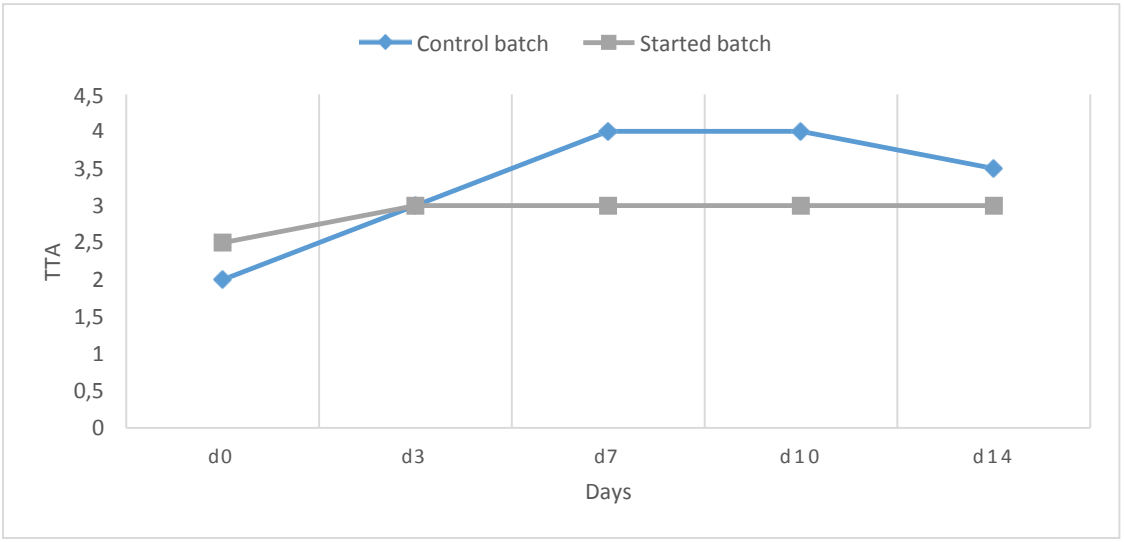


Figure 2. Evolution of (TTA) values during the ripening of dry-fermented sausages**.**

After 7 d of drying, the increase in total titratable acidity and the lowering of pH in the sausages inoculated with the starter culture may be linked to the production of organic acids by the latter. In the control sausages, acidity increased after three days, probably linked to the production of organic acids by autochthonous LABs, particularly heterofermentative lactic acid bacteria, as observed by **Essid and Hassouna (2013).** This value decreased after 7 days of drying,according to **Fonseca et *al*., (2013**), the final decrease in pH during maturation of control sausages may be linked to the development of yeasts and molds capable of consuming lactic acid, as the absence of starter cultures may facilitate their growth**.**

Ⅲ1.3. The moisture (M), water content (WC), water activity (a_w), and dry matter (DM): the moisture content decreased significantly in the inoculated and the control sausages during all the ripening., from initial values of about 51.8 %--18.93 % at the end of ripening (Fig 3). However, no significant difference ($p > 0.05$) was observed between the inoculated and the control sausages during all the ripening. Our results are in line with the study of **Essid and Hassouna, 2013** who found that the addition of *S. xylosus* and *Lactobacillus plantarum* starter culture did not affect the loss of sausage's moisture; **Casaburi et *al*., (2007)** before him also found the same result. On the other hand, other studies reported that moisture content through ripening of sausages could be affected by both processing method and processing time **(Dalmis and Soyer, 2008).**

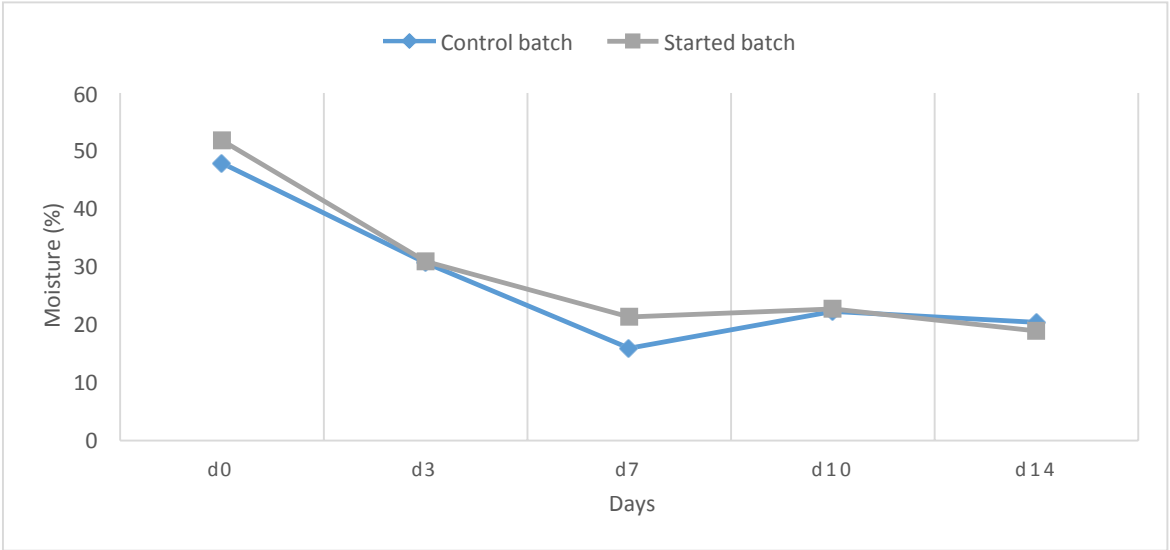


Figure 3. Evolution of Moisture values during the ripening of dry-fermented sausages.

The results of the WC determination were reported in Fig 4**.** the WC decreased significantly in the inoculated and the control sausages during all the ripening. The average initial water

contents of the dry-fermented sausages were similar among batches similar results were obtained by **Lorenzo and franco, (2012)** and in agreement with those reported by other authors in other sausage varieties **(Franco et *al*., 2002; Lizaso et *al*., 1999).**

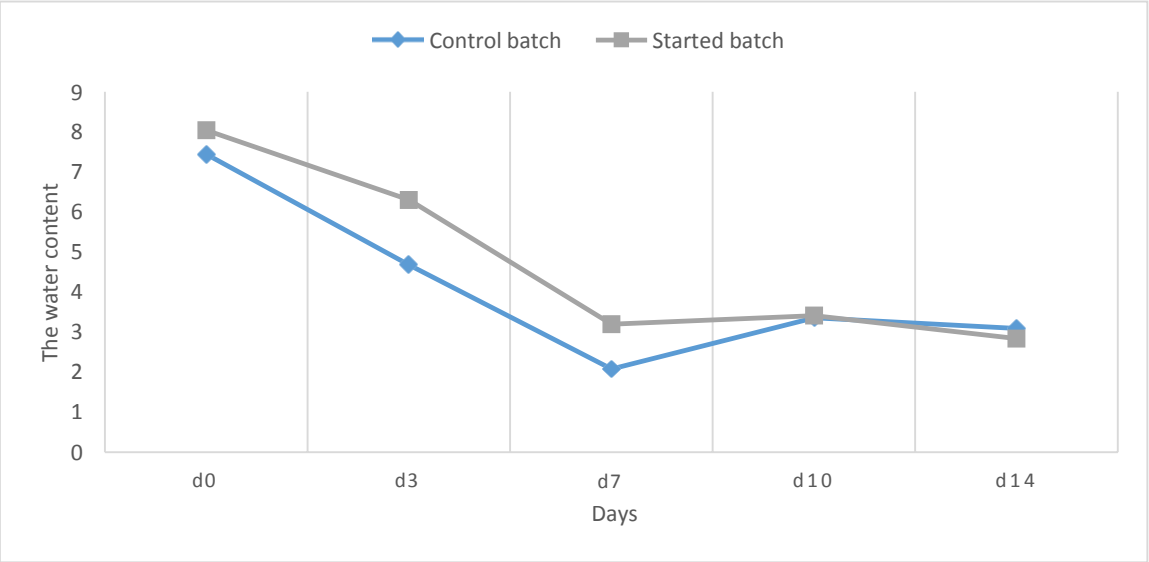


Figure 4. Evolution of the water content values during the ripening of dry-fermented sausages.

The results of the a_W determination was reported in Fig.5. During the ripening process of the dry-fermented sausages, a_W gradually declined over the curing period from an initial value of 0.68 to 0.60 due drying but differences were not significant ($P>0.05$) among batches, Similar results were obtained by **lorenzo and franco, (2012)** as also described by **Olivares et *al*., (2010)** This decrease in the a_W was largely due to the water loss, in fact values showed a positive correlation with moisture content.

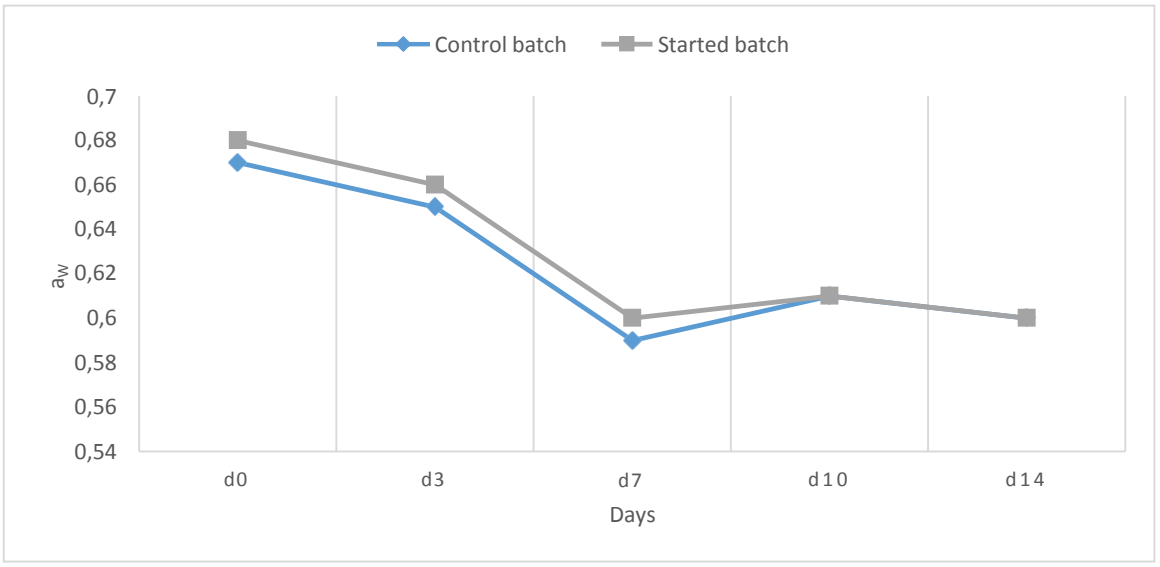


Figure 5. Evolution of the a_W values during the ripening of dry-fermented sausages.

The results of the dry matter determination were reported in Fig 6. the dry matter increased significantly in the inoculated and the control sausages during all the ripening.

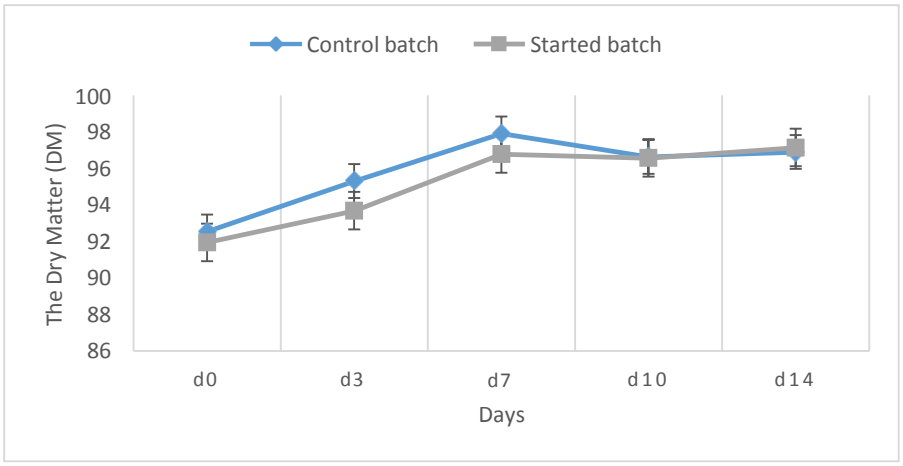


Figure 6. Evolution of the dry matter values during the ripening of dry-Fermented sausages.

Ⅲ2. Microbiological evaluation of dry-fermented sausages:

The evaluation of the microbiological quality of the dry-fermented sausages during maturation by counting and researching the total and thermo-tolerant coliforms, *Coagulase negative cocci*, *Salmonella* and *Clostridia* and the effect of the selected strain *Lactobacillus sakei subsp sakei* BK18 used as starter culture on microbial characteristics of dry-fermented sausages comparing to a control.

Ⅲ.2.1. Evolution of the coagulase negative cocci (CNC):

The results were reported in Fig 7. Initial counts of *Coagulase negative cocci* were 1.2×10^7 CFU/g in the inoculated sausages against 2.2×10^7 CFU/g in the control ones. On the 3rdday of ripening, counts of CNC increased to reach 8.6×10^8 and 2.3×10^9 CFU/g respectively for control and inoculated sausages. At the end of ripening, this number decreased to reach about 5.8×10^7 CFU/g for started sausages and 6.8×10^7 CFU/g for control sausages. Decrease in CNC counts during the last days of ripening for the inoculated and the control sausages suggests the poor competitiveness of CNC due to the intensive growth of LAB associated with a decrease of pH as reported by other works **(Casaburi et *al*., 2008; Samelis et *al*., 1998; Zaho et *al*., 2011; Zdolec et *al*., 2008).**

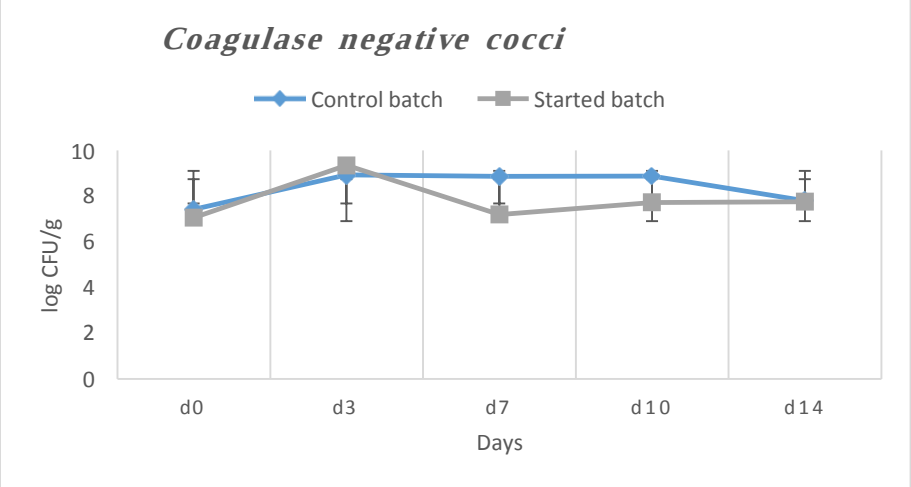


Figure 7. Evolution of CNC values during the ripening of dry-fermented sausages.

Lactic acid bacteria (LAB) and *Coagulase negative cocci* are the most active indigenous microorganisms; first in the acidification process, second in the denitrification, the lipolysis and the proteolysis **(Hammes and Hertel, 1998).**

Ⅲ.2.2. Yeasts and molds:

The results of the total number of yeasts and molds are shown in Figure 8. A considerable decrease in the fungal charge, in particular in the inoculated sausages, observed after 7 days of drying. From the 7th day of drying, an increase in this load is observed in two batches, but it is greater in the control sausages batch. Then it decreases until the end of the ripening they did not show significant differences between inoculated sausages and their control sausages.

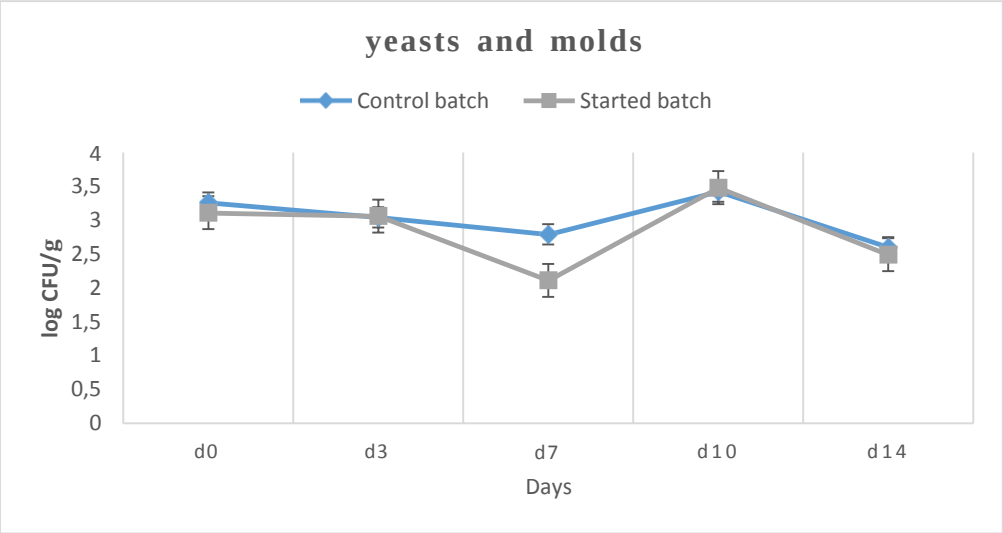


Figure 8. Evolution of yeasts and molds during the ripening of dry-fermented sausages.

Yeast is anaerobic microorganisms that can consume the oxygen remaining in the meat, and also produced proteases and lipases which contribute to the improvement of product's flavor **(Cheng, and Li 2019). Essid and Hassouna (2013),** did not observe any significant effect of the *Lb. plantarum* culture on the number of yeasts and molds during the ripening of Tunisian sausages. Furthermore, **Casaburi et *al*., (2008)** who found that the number of yeasts in control and inoculated sausages increased during the first days of ripening and then underwent a reduction to reach at the end of maturation values between 10^2 and 10^3 CFU/g. and **El Adab et *al*., (2014)** found the same results. Indeed, **Wang et *al*., (2013)** didn't observe the effect of *Lb. sakei* used as a starter culture on the load of yeast and mold during the maturing of sausagesand that yeasts and molds were present in almost similar numbers, while **Tabanelli et *al*., (2012)** observed that the number of molds is slightly higher than that of yeasts in two types of Italian sausages *Felino* and *Milano*.

Ⅲ2.3. Total coliforms and thermo-tolerant coliforms (TTC):

The results of counting total coliforms are illustrated in Fig 9**.** The results show a reduction in the load of coliforms in inoculated sausages. This flora is almost completely disappeared after 14 days of drying in contrast thermo-tolerant coliforms (TTC) are completely absent in inoculated sausages and control sausages. **Cenci-Goga et *al*., (2012)** have been observed a significant reduction in the quantity of coliforms in non-acidified Italian *salame di daino* type sausages started by using a formulation of *Lc. lactis ssp*. lactis 340; *Lc. lactis ssp.* lactis 16;

Lb. casei ssp. casei 208 from cheese. The absence of coliforms in inoculated sausages is explained in part by a decrease in a_W (<0.7 after 7 days) and the proliferation of lactic acid bacteria, which inhibit other viable cells by releasing antimicrobial compounds. According to the findings of various writers, the addition of starting cultures in sausages appears to dramatically reduce the burden of *Enterobacteria* and Gram- bacteria during the maturation phase. **(Essid and Hassouna, 2013 ; Ciuciu Simion et *al*., 2014 ; Lorenzo et *al*., 2014).**

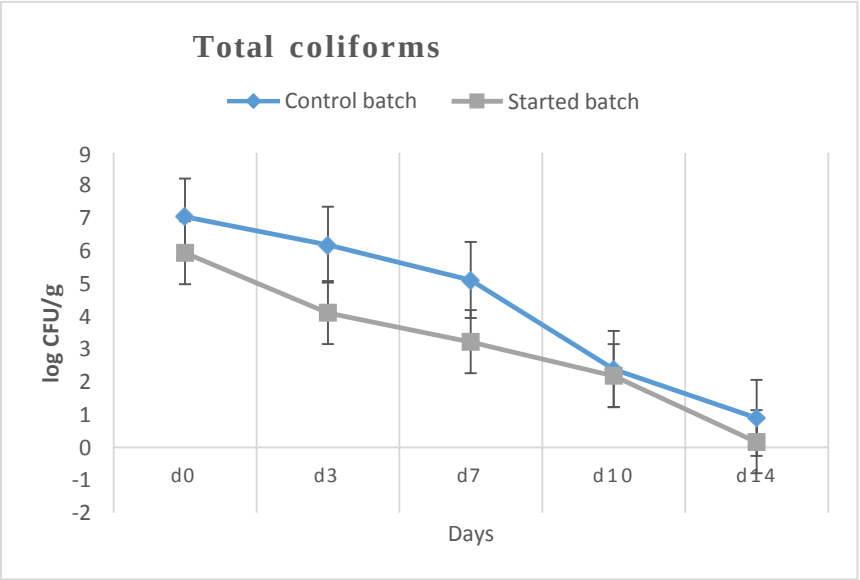


Figure 9. Evolution of total coliforms during the ripening of dry-fermented sausages.

Ⅲ2.4. *Clostridia* and *Salmonella/Shigella:*

The results of searching and counting of *Clostridium* are shown in Figure 10 and the enumeration of *Clostridia* show a decrease from its initial value until 85 CFU/g in control sausages and 8 CFU/g in started sausages and they disappear completely absent on the 3rd day of repining in both batches. Although, no detection of *Salmonella/Shigella* in row sausages of the two batches.

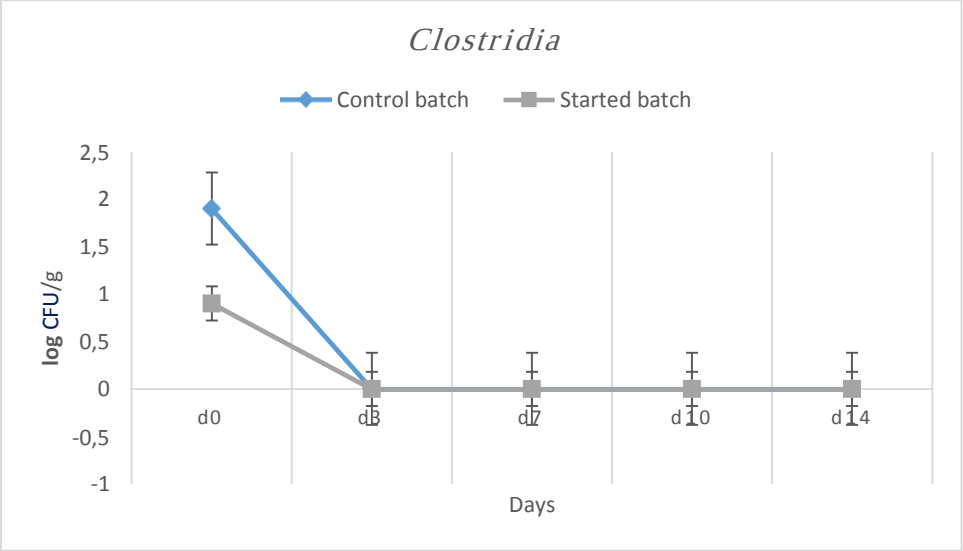


Figure 10. Evolution of sulfite-reducing *Clostridium* during the ripening of dry-fermented sausages.

The detection of putative *Salmonella*, *Staphylococcus* and *Clostridium* in fresh meat is related to the raw material used and the handling conditions during processing **(Casquete et *al*., 2011).** Inhibition of *Staphylococcus* sp, *Salmonella sp* and *Clostridia* is likely due to the intense growth of *Lb. sakei subsp sakei* BK18 which may be accompanied by antimicrobial substances production as by other studies **(Baka et *al*., 2011; Zhao et *al*., 2011, Essid and Hassouna, 2013; Lorenzo et *al*., 2014).** The combined effect of the salting/drying process may also play a role in the reduction of pathogens such as *Salmonella* sp and *sulfite-reducing Clostridium* **(Bennani et *al*., 2000).**

Conclusion and Outlook

Conclusion and Outlook

The use of starter cultures in food fermentations, including sausages, has allowed for a greater level of safety and quality. Undoubtedly, the addition of starter strains in the meat batter to produce sausages makes the fermentation process less prone to modifications, which are responsible for products with deviated organoleptic properties and potentially hazardous due to the presence of foodborne pathogens not inhibited during the process. However, due to the traditional and artisanal aspects that fermented sausages often possess, spontaneous fermentations are still used and represent a common practice especially in the Mediterranean countries. Isolation and selection of new strains of well-known LAB and CNC species from those traditional products represents a possible alternative to the use of commercial starter cultures.

A study *in situ* was carried out comparison to a negative control, at the beginning dry fermented sausages was prepared by using of mixture containing Lamb meat and fat, Turkish meat, spices and salt, and then the sausages were hanged for fermentation and drying for 15 days in the ventilated oven under controlled conditions of temperature and relative humidity.

According to the results a significant effect on the physicochemical changes on started sausages by giving a lower pH value than control sausages which related to the quick growth and acidification of the inoculated strain help to reduce lipid oxidation and excessive proteolytic activity. This latter had an effect on the inhibition of the growth of *Enterobacteriaceae* and *Clostridia* microorganisms responsible of producing biogenic amines and toxins associated with hygienic quality of dry-fermented foods. On the other hand, the starter strain didn't affect the changes of the values of moisture water activity and water content decreasing during the ripening of sausages between the two batches.

Beside the high presence of the starter the results show that the *Coagulase negative cocci* were in high number in two batches, except for 3rd day of ripening counts of CNC were higher in started batch than control. In addition, there is no difference in the count of yeasts and molds between the two batches.

The results of our study open new perspectives. To complete these studies, we propose:

- ✓ Supplement the study with the determination of organic acids and biogenic amines by HPLC;

- ✓ Study the effect of using a mix of starters culture such as *Lb. sakei. subsp sakei* BK18 with other selected LABs and Coagulase Negative-Staphylococci;
- ✓ Determine the shelf-life of dry fermented sausage.

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Annex

Annex I

Composition of culture media, reagents and buffers

- **Liquid media**

TSE (Tryptone-salt-water)

Tryptone	1 g
Sodium chloride	8.5 g
Tween 80	1g/l
Distilled water sqf	1000 ml

pH7.4. Autoclave 120°C for 15min.

Buffered Peptone Water (BPW)

Peptone	20 g
Sodium chloride	5 g
Disodium phosphate	9 g
Monopotassic phosphate	1.5 g
Distilled water sqf	1000 ml

PH 7.2. Autoclave 120°C for 15min.

Bouillon Rappaport Vassiliadis (RV)

Peptone	4.5
Sodium chloride	7g
Monopotassic phosphate	1.4g
Magnesium chloride	16.2g
Malachite green	33mg
Distilled water sqf	1000ml

pH 5.5. Autoclave 120°C for 15min

Physiological water

Sodium chloride	8.5g
Distilled water sqf	1000ml

pH 7. Autoclave 120°C for 15min.

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- **Agar media**

Mac Conkey Agar

Trypsic peptone of gelatin	17g
Peptone of meat and casein	3g
Lactose	10g
Bile salts	5g
Sodium chloride	5g
Neutral red	40mg
Agar	13g
Distilled water sqf	1000ml

pH7.4. Autoclave 120° C for 15min.

Liver meat agar (VF-iron alum)

Liver meat extract	10g
Peptone	20g
Yeast extract	10g
Glucose	5g
Agar	15g
Distilled water sqf	1000ml

pH 7.6. Autoclave 120°C for 15min.

Salmonella-Shigella (S-S)

Peptone	10 g
Meat extract	5 g
Lactose	10 g
Bile salts	6 g
Iron ammoniacal citrate	1 g
Sodium citrate	8.5 g
Sodium thiosulphate	8.5 g
Neutral red	25 mg
Bright green	0.33 mg
Agar	13 g
Distilled water sqf	1000ml

pH 7. Sterilize by boiling for 2 minutes (do not autoclave).

OGA (with oxytetracycline gentamycin)

Yeast extract	5g
Glucose	20g
Agar	16g
Distilled water sqf	1000ml

pH 7. Autoclave 120°C for 15min.

Mannitol-salt agar

peptone	10 g
beef extract	1 g
Sodium chloride	75 g
D-mannitol	10 g
phenol red	25 mg
Agar	15 g
Distilled water sqf	1000ml

pH 7.4. Sterilize at 121°C for 15.

Hécktoen

Proteosis -peptone	12 g
Yeast extract	3 g
Sodium chloride	5 g
Sodium thiosulphate	5 g
Bile salts	9 g
Ammoniacal iron citrate	1.5 g
Salicin	2 g
Lactose	12 g
Sucrose	12 g
Fuschine acid	0.1 g
Bromothymol blue	65 mg
Agar	13 mg
Distilled water sqf	1000ml

PH 7.6. Autoclave 120°C for 15min.

- **Reagents and buffers**

Violet crystal

Solution A

Violet crystal	2g
Alcohol at 95°	20ml

Solution B

NH4 oxalate	0.8g
Distilled water sqf	20ml

Mix solutions A and B.

Ziehl Fuschine

Basic fuschine	1g
Ethyl alcohol 90°	10ml
Phenol	5g
Distilled water sqf	100ml

The solution is diluted to 1/15.

Lugol

Iodine	1g
Potassium iodide	2g
Distilled water sqf	300ml.

Catalase (reagent for highlighting)

Hydrogen peroxide 10 volumes (10%).

Annex Ⅱ

Gram staining

1- Place a drop of sterile physiological water on a clean blade

2- Take a colony sample and mix with the drop of water, strip and dry by fast passage on the flame of a benzene burner

3- Cover the smear with purple crystal for 60 seconds

4- Wash with distilled water for 5 seconds

5- Cover with lugol for 30 seconds

6- Wash with distilled water for 5 seconds

7- Immediately rinse the smear with the alcohol-acetone mixture by tilting the blade and drop by drop until complete disappearance of violet coloring

8-Wash with distilled water for 5 seconds

9- Cover with Fuschine for 15 seconds

10- Wash with distilled water for 10 seconds

11- Place a drop of immersion oil on the smear and observe under a microscope high magnification. Gram+ cells absorb the color of violet crystal and remain purple blue in appearance, unlike Gram-cells which appear distinctly pinkish.