UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ISOLATION AND IDENTIFICATION OF FOODBORNE PATHOGENS OF SPECIAL INTEREST IN FOOD SAFETY

Doctoral Thesis

PhD in Biotechnology

Author:

Aya Boukharouba

Director:

Dra. Salut Botella Grau

Valencia, December of 2021

AGRADECIMIENTOS

Me gustaría empezar dando las gracias a mi familia, y en especial, a mis padres que me dieron la vida y dedicaron la suya a mi éxito y mi felicidad. Seguidamente, a mis hermanos por su apoyo y a mi sobrino, el angelito de mi mundo.

También me gustaría hacer mención a mi directora Mª Salud Botella, por su paciencia, su esfuerzo y su profesionalidad durante cada una de las etapas de la Tesis Doctoral.

Me gustaría también agradecer a María Antonia, Ana Jiménez, Jorge García y Manuel Hernández por su ayuda y amabilidad, así como a Ana González, Nancy y demás miembros del departamento, que me han ayudado con cualquier problema que haya podido surgir, y gracias a quienes he podido disponer de todo el material necesario para las pruebas y ensayos.

Agradezco también la ayuda de mis amigos Alejandro Fenollar y Miguel García-Ferrús por su inquebrantable apoyo.

A todas esas personas,

… GRACIAS

RESUMEN

La seguridad alimentaria es una prioridad para la población y en la actualidad cobra mayor importancia por ciertas tendencias alimentarias como el consumo de alimentos crudos y la distribución generalizada de alimentos orgánicos, que pueden ser la causa de enfermedades transmitidas por alimentos.

Para garantizar la seguridad alimentaria, la detección de estos microorganismos debe realizarse de manera rápida y eficiente. Par eso, el método de cultivo microbiológico se considera el oficial para la detección de estos patógenos. Sin embargo, adolece de importantes inconvenientes, ya que no solo requiere mucho tiempo, sino que también es laborioso y consume muchos recursos. Además, puede ser limitado con respecto a la detección de bacterias fisiológicamente alteradas y/o estresadas durante el almacenamiento y la conservación.

En este trabajo se ha desarrollado un protocolo sencillo y rápido para la detección simultánea de *E. coli*, *L. monocytogenes*, *S. aureus* y *S. enterica* en alimentos, mediante la combinación de una etapa de co-cultivo en medio líquido y la detección por PCR múltiple.

Se ha evaluado la eficiencia de varios medios de enriquecimiento y se seleccionó el agua de peptona tamponada como el medio óptimo para el co-cultivo de las cuatro bacterias diana. También se optimizaron las condiciones de PCR múltiple y se aplicaron tanto a co-cultivos como a muestras de alimentos inoculados artificialmente, lechuga orgánica y carne picada.

Después de la optimización, la PCR múltiple desarrollada fue capaz de detectar las cuatro bacterias simultáneamente, hasta con una inoculación inicial de 10^0 UFC/mL. En presencia de ambas matrices alimentarias inoculadas, tras la etapa de co-cultivo, la PCR múltiple pudo detectar simultáneamente las 3 bacterias *E. coli*, *S. enterica* y *L. monocytogenes*, mientras que *S. aureus* se ha detectado por PCR simplex, a partir del mismo extracto de ADN del co-cultivo.

Los resultados obtenidos permiten concluir que el uso de un paso de co-cultivo en Agua peptona tamponada, antes de la detección por PCR simple y múltiple, puede facilitar la detección simultánea de las cuatro bacterias potencialmente presentes en las matrices alimentarias. La presencia o ausencia de la bacteria diana en los alimentos se confirma en

unas 30 horas, lo que reduce el tiempo requerido para la detección en comparación con el tiempo mínimo de 7 días por método cultural. Asimismo, permite reducir el número de medios de cultivo y reactivos, para el aislamiento e identificación de bacterias que no son detectadas por PCR y que no están presentes en las matrices alimentarias, lo que supone un importante ahorro económico.

RESUM

La seguretat alimentària sempre és una prioritat per a la població i en l' actualitat cobra major importància per certes tendències alimentàries, com el consum d' aliments crus i la distribució generalitzada d' aliments orgànics, que poden ser la causa de malalties transmeses per aliments.

Per garantir la seguretat alimentària, la detecció d' aquests microorganismes s' ha de realitzar de manera ràpida i eficient. Per a això, el mètode de cultiu microbiològic es considera l' oficial per a la detecció d' aquests patògens. Però, hi ha importants inconvenients, ja que no només requereix més temps, sinó que també és laboriós i consumeix molts recursos. A més, pot ser limitat pel que fa a la detecció de bacteris fisiològicament alterats i/o estressats durant l'emmagatzematge i la conservació.

En aquest treball s'ha desenvolupat un protocol senzill i ràpid per a la detecció simultània d' *E. coli, L. monocytogenes, S. aureus* i *S. enterica* en aliments, mitjançant la combinació d' una etapa de co-cultiu en medi líquid i la detecció per PCR múltiple.

S'ha avaluat l'eficiència de diversos mitjans d'enriquiment i s'ha seleccionat l'aigua de peptona tamponada com el medi òptim per al co-cultiu dels quatre bacteris diana. També es van optimitzar les condicions de PCR múltiple i es van aplicar tant a co-cultius com a mostres d'aliments inoculats artificialment, enciam orgànic i carn picada.

Després de l'optimització, la PCR múltiple desenvolupada va ser capaç de detectar els quatre bacteris simultàniament, fins a una inoculació inicial de 10^0 UFC/mL. En presència d' ambdues matrius alimentàries inoculades, després l' etapa de co-cultiu, la PCR múltiple va poder detectar simultàniament els 3 bacteris: *E. coli, S. enterica* i *L. monocytogenes,* mentre que *S. aureus* s' ha detectat per PCR simple, a partir del mateix extracte d' ADN del co-cultiu.

Els resultats obtinguts permeten concloure que l' ús d' un pas de co-cultiu en Aigua de peptona tamponada, abans de la detecció per PCR simple i múltiple, pot facilitar la detecció simultània dels quatre bacteris potencialment presents en les matrius alimentàries. La presència o absència del bacteri diana en els aliments es confirma en unes 30 hores, la qual cosa redueix el temps requerit per a la detecció en comparació amb el temps mínim de 7 dies per mètode cultural. Així mateix, permet reduir el nombre de mitjans de cultiu i reactius, per a l' aïllament i identificació de bacteris que no són detectats

per PCR i que no estan presents en les matrius alimentàries, la qual cosa suposa un important estalvi econòmic.

ABSTRACT

Food safety is a priority for the population and is nowadays more important than ever due to certain dietary trends such as the consumption of raw foods and the widespread distribution of organic foods, which may be the cause of foodborne diseases.

To ensure food safety, the detection of these microorganisms must be done quickly and efficiently. Although, the microbiological culture method is considered to be the official method for the detection of these food-borne pathogens, it suffers from significant drawbacks, such as time-consuming, laborious and expensive, in addition it may be limited regarding the detection of physiologically altered and/or stressed bacteria, during storage and preservation.

In this work has been developed a simple and rapid protocol for the simultaneous detection of *E. coli*, *L. monocytogenes*, *S. aureus* and *S. enterica* in food, by combining a liquid co-culture step and detection by multiplex PCR.

The efficiency of several enrichment media was evaluated and buffered peptone water was chosen as the optimal medium for the co-culture of the four target bacteria. Then, optimized multiplex PCR conditions were applied to both the co-cultures and the samples of artificially inoculated foods, organic lettuce and ground meat.

After optimization, the developed multiplex PCR was able to simultaneously detect the four bacteria, up to an initial inoculation of 10^0 CFU/mL. In the presence of the two inoculated food matrices, after a co-culture step, the multiplex PCR could simultaneously detect the 3 bacteria: *E. coli*, *S. enterica* and *L. monocytogenes*, whereas, *S. aureus* has been detected by simplex PCR, from the same co-culture DNA template.

The results obtained allow conclusion that the use of a co-culture step in Buffered Peptone Water, before detection by simplex and multiplex PCR, can facilitate the simultaneous detection of the four bacteria potentially present in the food matrices. The presence or the absence of the target bacteria in food is confirmed in approximately 30 hours, which reduce the time required for the detection compared to the minimum time of 7 days by cultural method. Also, it allows to reduce the number of culture media and reagents, for the isolation and identification of bacteria that are not detected by PCR and which are not initially present in the food matrices, which represents a significant economic savings.

ABBREVIATIONS

×g: G-force (the relative centrifugal force)

°C: degree Celsius
µL: Microliter
µm: Micrometer
µM: Micromolar
ALOA: Chromogenic Listeria Agar Base of Ottaviani and Agosti
API: Analytical Profile Index (miniaturized biochemical tests)
a_w: Water activity
BGBLB: Brilliant Green Bile Lactose Broth
bp: Base pair
BPA: Baird-Parker Agar
BPW: Buffered Peptone Water
CAB: Chromogenic Agar Base CM1007
CDC: Centers for Disease Control and Prevention
CE: European Commission
CECT: Spanish Type Culture Collection (Colección Española de Cultivos Tipo)
CFU: Colony-Forming Unit
CO_2: Carbon dioxide
DAEC: Diffuse-adhering *E. coli*
DEC: Diarrheagenic *E. coli*
DNA: Deoxyribonucleic Acid
dNTPs: Deoxynucleotide triphosphates
EAEC: Enteroaggregative *E. coli*
EC: European Commission
EDTA: Ethylenediaminetetraacetic acid
EDTA-Na_2: Ethylenedinitrilo Tetraacetic acid disodium salt
EEO: Electroendosmosis
EFSA: European Food Safety Authority
EHEC: Enterohemorrhagic *E. coli*
EIEC: Enteroinvasive *E. coli*
ELISA: Enzyme Linked Fluorescent Assay
EN: European standard (Norma Europea)
EPEC: Enteropathogenic *E. coli*

ETEC: Enterotoxigenic *E. coli*

EXPEC: Extra-intestinal pathogenic strains of *E. coli*

EYEPT: Egg Yolk Emulsion Potassium Tellurite

F/R: Forward/ Reverse

FB: FRASER Broth

FBH: FRASER Broth half concentration

g: gram (gramme)

GC: Giolitti-Cantoni

GMP: Good Manufacturing Practices

h: hour

H_2O_2: Hydrogen peroxide

H_2S: Hydrogen sulfide

HIV: Human immunodeficiency viruses

HUS: Hemolytic Uremic Syndrome

IMViC: Biochemical tests (Indole, Methyl-Red, Voges-Proskauer and Citrate)

ISO: International Organization for Standardization

KOH: Potassium hydroxide

LB: Luria-Bertani

log_{10}: Decimal logarithm (common logarithm)

LT: Thermolabile enterotoxins

M: Molar (mol/L)

MAEC: Neonatal meningitis *E. coli*

$MgCl_2$: Magnesium Chloride

Milli-Q water: Ultrapure water (Water purified by Millipore)

min: minute

MKTTn: Müller-Kauffmann Tetrathionate Broth

mL: Milliliter

mM: Millimolar

MR: Methyl-Red

MRSA: Methicillin-resistant *Staphylococcus aureus*

NB: Nutrient Broth

NH_4: Ammonium cation

NO_2: Nitrogen dioxide

NO_3: Nitrate

PAL: PALCAM

PCA: Plate Count Agar

PCR: Polymerase Chain Reaction

pg: Picogramme

pH: Potential Hydrogen

RD: Royal Decree (Real Decreto)

RNA: Ribonucleic Acid

rpm: revolutions per minute

RTE: Ready-To-Eat

RV: Rappaport-Vassiliadis

SSSLE: Multi-pathogen enrichment broth for simultaneous growth of *S. enterica*, *S. aureus*, *S. flexneri*, *L. monocytogenes* and *E. coli* O157:H7

ST: Thermostable enterotoxins

STEC: Shiga Toxigenic *Escherichia coli*

TAE: Tris-Acetate-EDTA

Taq: Thermostable DNA polymerase, named according to Thermus aquaticus.

TBX: Tryptone Bile X-glucuronide

TE: Tris-EDTA

Tm: Melting Temperature

TSI: Triple Sugar Iron

UNE: Acronym for A Spanish Standard (Una Norma Española)

UPEC: Uropathogenic *E. coli*

UV: Ultraviolet

VP: Voges-Proskauer

VRBGA: Violet Red Bile Glucose AGAR

XLD: Xylose Lysine Deoxycholate

TABLE OF CONTENTS

INDEX TABLES

INDEX FIGURES

1. INTRODUCTION

Foodborne diseases are a significant global health concern, as unsanitary food is not only a threat to the health of populations, it can also have bad repercussions on the socio-economic development of countries. Most of the pathogens involved have a zoonotic origin and according to the European Food Safety Authority's report on Zoonoses in Europe, Salmonellosis is ranked second among zoonotic diseases with the highest incidence in the European Union, followed by Shiga toxin-producing *Escherichia coli* (STEC) infection in third position, *Listeria* infections in fourth position and in the category of microbiological contaminants subject to food safety criteria, the Enterotoxins of *Staphylococcus aureus* are mentioned in second position after Histamine (EFSA, 2021; ELIKA, 2021).

The microbiological criteria of foodstuffs in the European Union countries are covered by the 2073/2005 Regulation and its modifications. The microbiological criterion is described as "the criterion which defines the acceptability of a product, a batch of food products or a process, on the basis of the absence, the presence or the number of microorganisms and/or quantity of toxins/metabolites per unit of mass, volume, area or lot".

These criteria are divided into two classes, the food safety criteria, which define the acceptability of a product or a batch of products and whose non-compliance would mean the non-marketing of the product or the batch, or a withdrawal from the market of commercialized products. The second class concerns the criteria relating to process hygiene, which make it possible to assess whether the operation of the production process is acceptable or not. Established according to the type of food, these criteria define the microorganisms to be analyzed and the tolerance limits for each, through sample analysis protocols by applying standardized techniques, most often belonging to the standards of the International Organization for Standardization (ISO). Their development and modifications are carried out according to the prevalence reports established by the European Food Safety Authority (EFSA).

Among the microorganisms and toxins usually looked for in the context of food safety criteria, we can distinguish *Listeria monocytogenes*, *Salmonella* sp., Shiga toxin-producing *Escherichia coli* (STEC), and Staphylococcal Enterotoxins, in addition to the histamine *Cronobacter* spp., and *Enterobacter sakazakii*. Regarding the criteria that

define the hygiene of the process, the enumeration of *Enterobacteriaceae* and Total Aerobic Mesophilic bacteria are mainly taken into account, in addition to *Salmonella* sp., *Escherichia coli*, Coagulase-positive Staphylococci and *Bacillus cereus*.

Therefore, the development of a rapid method for the simultaneous detection of the four bacteria *Escherichia coli*, *Salmonella* sp. *Listeria monocytogenes* and *Staphylococcus aureus* in food, would be a good initiative to save time, facilitate analyzes and minimize the required resources, in comparison to detection by cultural methods.

1.1. *Escherichia coli*

1.1.1. Historical antecedents

In 1884, the German pediatrician and microbiologist Theodor Escherich began a study on the microorganisms present in the guts, by working on the newborn stools, to determine their role in digestion, as well as their probable involvement in some enteric diseases. From this study, he discovered a fast-growing bacterium that he named "*Bacterium coli commune*", which made him the first to have isolated and described this bacterium in 1885. Years later, in 1919, Castellani and Chalmers proposed to rename the microorganism *Escherichia coli*, to honor his work (Shulman et al., 2007; Blount, 2015).

1.1.2. Current taxonomic situation

According to the classification of the List of Prokaryotic names with Standing in Nomenclature (LPSN, 2021), the species *Escherichia coli* belongs to the genus *Escherichia*, which is part of the family *Enterobacteriaceae*, of the order *Enterobacteriales*, Class *Gammaproteobacteria*, Phylum *Proteobacteria*, Subkingdom *Negibacteria*, Kingdom *Bacteria*. The Genus *Escherichia* is composed of 6 other species with a validly published and correct name in addition to *Escherichia coli*, all shown in **Table 1**.

Table 1. Current taxonomic situation of *Escherichia coli*

Kingdom	*Bacteria*	Cavalier-Smith, 2002
Subkingdom	*Negibacteria*	Cavalier-Smith, 2002
Phylum	*Proteobacteria*	Garrity et al., 2005
Class	*Gammaproteobacteria*	Garrity et al., 2005
Order	*Enterobacteriales*	Garrity and Holt, 2001
Family	*Enterobacteriaceae*	Rahn, 1937
Genus	*Escherichia*	Castellani and Chalmers, 1919
Species	*Escherichia coli*	Migula, 1895, Castellani and Ch., 1919
	Escherichia hermannii	Brenner et al., 1982a
	Escherichia vulneris	Brenner et al., 1982b
	Escherichia fergusonii	Farmer et al., 1985

Escherichia albertii	Huys et al., 2003
Escherichia marmotae	Liu et al., 2015
Escherichia ruysiae	Van Der Putten et al., 2021

Source: LPSN (Last revision 11/11/2021)

1.1.3. Characteristics

Escherichia coli is a Gram-Negative bacterium, a rod-shaped microorganism with an average size of 1.1 to 1.5 µm wide by 2 to 6 µm long, although this may vary considerably depending on the growth conditions (Desmarchelier and Fegan, 2011; Leung and Gallant, 2014).

Usually motile in liquid by peritrichous flagella. Many strains produce other appendages with filamentous structures such as Fimbriae; bristle-like short and tiny fibers composed of Fimbrillin protein, arising in very large numbers for both motile and non-motile strains, allowing the bacteria to attach to other surfaces, including other cells and host tissues (Blount, 2015; Sangwan, 2016).

Another important structure, the Pili, a thick tubular structure, composed of Pilin protein, extended from the surface of Gram-negative bacteria, in small numbers (less than 1-10 per cell), used during bacterial conjugation, for the gene transfer and attachment. Usually arranged singly or in pairs, *E. coli* is non-spore forming, but some strains may have capsules or microcapsules (Dodd et al., 2017; Holban and Grumezescu, 2018; Aryal, 2019).

On Nutrient agar, unpigmented colonies, appear in shades of beige, greyish or off-white colors, with a shiny surface. They generally, appear as large opaque or translucent discs, ranging from 2 to 3 mm of diameter, thick and low convex, smooth, moist, with an entire fixed margin and a steady growth pattern. Mucoid forms may appear. In broth, the growth appears as an intense turbidity with a deposit in the bottom of the container, which disperses after shaking (Brenner and Farmer, 2007; Sangwan, 2016; Gillespie, 2018).

E. coli is an aerobic and facultative anaerobic microorganism, which means that it can grow in the presence or the absence of oxygen, possessing both respiratory and fermentative metabolism. Under anaerobic conditions, it can grow from the carbohydrate fermentation process, which results in the production of a mixed acids (lactate, acetate and formate) as well as Carbon Dioxide gas. On the other hand, the bacterium can develop by anaerobic respiration. This alternative respiratory pathway is linked to its nitrate reductase enzyme, which allows the use of NO_3, NO_2 or fumarate as a final electron

acceptor. This explains its adaptation inside and outside the intestine (Batt, 2014; Sangwan, 2016; Todar, 2020).

As a Chemoorganotrophic and Heterotrophic bacteria (Chemoorganoheterotrophic), *E. coli* derives its energy from organic carbon compounds, which are used simultaneously as a carbon source. Versatile, it can grow easily and abundantly on simple culture media as well as synthetic ones, transforming Glucose or other carbohydrates into molecular component that constitute the cell (Steiner et al., 2006; Rivas et al., 2015; SLU, 2019).

Although it is not considered to be extremophile bacteria; non-thermophilic, non-halophilic, non-acidophilic, most *E. coli* strains have the ability to grow over a wide temperature range, around 10 to 50 °C, with a maximum growth rate between 37 and 42 °C (Dworkin et al., 2006; Euzéby, 2010; Blout, 2015).

Regarding pH, *E. coli* can grow in a pH range of about 5.5 to 8.0, with optimum growth at neutrality. Some diarrhea genic *E. coli* have the ability to survive at pH=2. In addition, strains with the capacity to develop in foods with a water activity (a_w) of less than 0.95 may occur (Desmarchelier and Fegan, 2011; WHO, 2018).

Classified among coliforms for its ability to ferment Lactose within 48 h, this characteristic is considered to be the main test for various *E. coli* of *Shigella* spp. and *Salmonella* spp. (Dworkin et al., 2006; Batt, 2014). Moreover, its enzymatic diversity allows the use of many other substrates. The study of enzymatic activities and the sugar fermentation allows the identification and differentiation between strains (Euzéby, 2010; Aryal, 2018).

According to usual biochemical laboratory tests, *E. coli* is positive for catalase, Indole production, Nitrate reductase and Methyl Red tests. However, most strains are negative on the Voges Proskauer and Oxidase tests, do not use Citrate and do not produce H_2S (Dworkin and al., 2006; Sangwan, 2016; Aryal, 2018).

This species is generally found in two environments, their primary habitat is the gastrointestinal tract of warm-blooded organisms, as part of the normal microbiota of the human and animal intestinal tract. However, it is regularly evacuated from the host to the outside world along with the intestinal mucosa, which is continuously excreted with the feces. Consequently, the secondary habitat is the environment, where *E. coli* can be

mainly isolated from plants, soil, water and sediment. It might also be found, albeit less commonly in food (Tenaillon et al., 2010; Gordon, 2013; Blount, 2015).

E. coli strains are divided into serogroups and serotypes, according to the antigenic composition. Developed in 1947, the method of serogrouping carried out by sero-agglutination was subsequently considered as the gold standard, for differentiation between multiple *E. coli* strains (AFSSA, 2003; Dodd et al., 2017; DebRoy et al., 2018).

The serogroup of the strain is defined by the somatic antigen or "O antigen", which is part of the lipopolysaccharide in the outer membrane of *E. coli*, exposed on the cell surface and providing antigenic specificity for each strain. There are at least 188 serogroups O that have been identified and currently recognized (Stenutz et al., 2006; Bos et al., 2007; Dodd et al., 2017).

Identification of the serotype, within the serogroup, is carried by the identification of the complementary antigens, from the structures presented on the surface, such as the flagellar antigen "antigen H" or the capsular antigen "antigen K" and their combination with the somatic antigen "O". The flagellar antigen "antigen H", is present in motile strains with considerable diversity, due to the multiple types of flagellin composing the flagella. There are also many strains which express a third kind of antigens, the capsular antigen "antigen K", a polysaccharide antigen, eventually (not always) present in the envelope. Although important in pathogenesis, it is occasionally used in serotyping. Regarding fimbriated strains, the classification can also be based on the fimbrial antigen "antigen F" (Campos et al., 2004; Desmarchelier and Fegan, 2011; Rivas et al., 2015; Dodd et al., 2017; Aryal, 2020).

Although serotyping remains limited, this technique is a useful epidemiological tool, because it allows to distinguish between different *E. coli* strains including those belonging to particular serotypes, which have been identified as pathogens responsible for various diseases, ranging from simple diarrhea to severe systemic infections even fatal (Kaper et al., 2004; Desmarchelier and Fegan, 2011; DebRoy et al., 2018).

1.1.4. Pathogenicity

Although most *E. coli* strains are harmless, several groups can cause serious food-borne illnesses, due to their adhesion capacities and host cells invasion, as well as virulence factors such as the production of enterotoxins, verotoxins, colicins and siderophores. Transmitted to humans mainly through consumption of contaminated water

and uncooked or undercooked food their mode of action is similar to that of other mucosal pathogens, following multiple stages, including the colonization of a mucosal site and then multiplication, which results in the destruction of host tissues (Steiner et al., 2006; FAO, 2017; WHO, 2018).

Pathogenic *E. coli* strains are grouped according to their mechanisms of pathogenicity, such as their attachment and invasion strategies, their virulence factors, such as toxin production, or even the clinical symptoms. However, their nomenclature is relatively complex, because of the names given and their incoherent uses in the literature, as well as the continued emergence of new pathotypes. This complexity of nomenclature may also be due to the overlaps of existing pathogenesis mechanisms, generated by the similarity of virulence characteristics, between various pathotypes (Kaper et al. 2004; Rivas et al., 2015).

Depending on the clinical symptoms caused, *E. coli* strains include intestinal pathogens, which may be responsible for diarrhea and other intestinal illnesses, while extra-intestinal pathogens may induce disease at non-intestinal sites. These extra-intestinal pathogenic strains of *E. coli* (EXPEC) include strains of Uropathogenic *E. coli* (UPEC) associated with urinary tract infections, as well as neonatal meningitis *E. coli* (MAEC) which can cause meningitis and bacteremia. Regarding the intestinal pathogens group, also known as Diarrheagenic *E. coli* (DEC), it includes 6 pathotypes: Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and Diffuse-adhering *E. coli* (DAEC) (DebRoy et al., 2018).

Enteropathogenic *E. coli* (EPEC)

In order to bind, this strain produces intimin, an adhesion factor that induces a modification of the enterocyte cytoskeleton, resulting in the disappearance of microvilli, mainly responsible for infant diarrhea, the symptoms of which may appear as early as the third day after infection, including abdominal pain and persistent secretory diarrhea, which may progress to hemorrhagic colitis, leading to dehydration or even death, without adequate treatment. Moreover, vomiting and fever might occur (Kaper et al., 2004).

Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli*, also known as Shiga Toxin-producing *E. coli* (STEC), are responsible for multiple disorders, ranging from simple diarrhea to hemorrhagic

diarrhea, which might be associated with severe kidney damage, called Hemolytic Uremic Syndrome (HUS), with a significant risk of chronic kidney disease and even death. EHEC colonize the digestive tract by adhering to enterocytes through the intimin, then release toxins (shigatoxins), inducing vascular, intestinal and even cerebral damages (Tarr et al., 2005).

Enterotoxigenic *E. coli* (ETEC)

The ETECs are responsible for moderate to acute watery diarrhea, without blood or mucus, associated with mild fever with nausea and abdominal cramps, headache and muscle soreness. Main pathogens of "traveler's diarrhea", commonly referred to as "turista". The symptoms appear 2 hours after ingestion of the bacteria.

These strains mainly colonize the mucous membrane of the small intestine, through colonization factors (pilis or fimbriae), in addition to their ability to produce thermostable enterotoxins (ST) as well as thermolabile enterotoxins (LT) (Kaper et al., 2004).

Enteroaggregative *E. coli* (EAEC)

EAECs are commonly involved in watery diarrhea, in tourists traveling to developing countries, in addition to children and adults infected with HIV. Known as strains that do not secrete LT or ST enterotoxins, they adhere to the epithelial cells of the intestine through the fimbriae, forming clusters "aggregates". The characteristic symptoms of this type of infection are watery secretory diarrhea with blood and mucus, associated with moderate fever (Pierard et al., 2012).

Enteroinvasive *E. coli* (EIEC)

EIECs are responsible for dysenteric syndromes. Their mechanisms of pathogenicity are based on the invasion of the intestinal epithelium, after adhesion to the intestinal villi, and the bacteria penetrate the cell by endocytosis, then multiply until the infected cells are destroyed, and join, after that adjacent epithelial cells, causing a strong inflammatory reaction.

The clinical symptoms resulting from this infection, can be very similar to those caused by ETEC, including diarrhea with blood and mucus, associated with abdominal cramps and high fever. Generally, infections caused by these strains are more associated with epidemics than isolated cases (Van Den Beld and Reubsaet, 2012).

Diffuse-adhering *E. coli* (DAEC)

DAEC is responsible for watery diarrhea without blood or leukocytes, especially in children between 4 and 5 years old. These strains are characterized by their mode of adhesion to intestinal epithelial cells through fimbrial adhesins, producing a cytopathic effect on infected cells, by the formation of large cellular extensions which wrap around the bacteria (Steiner et al., 2006).

1.1.5. *Escherichia coli* in food

The usual presence of *E. coli* in the human intestine and thus in the fecal matter, has led to the tracking of thermotolerant coliforms in general and these bacteria specifically, in nature, in food and water as an indicator of fecal pollution. Although most forms of *E. coli* are not dangerous, their levels are checked during examinations, because as this provides indications on the potential presence of harmful digestive origin bacteria (Breyer, 2017; Todar, 2020).

According to estimates by the World Health Organization, around 600 million people worldwide fall ill each year from the consumption of contaminated food and water, leading to nearly 420,000 deaths (WHO, 2020).

From 1999 to 2006, 433 outbreaks related to waterborne transmission of *E. coli* have been reported in Spain, causing 24610 cases, 213 hospitalizations and 2 deaths. In 2014, the O157: H7 serotype caused 5955 confirmed cases in the European Union countries. The 46 cases of Shigatoxigenic *Escherichia coli* (STEC) infections confirmed during 2015 in Spain were related to the consumption of contaminated food and water (BES, 2008; BES, 2015).

As of 2018, Shigatoxigenic *Escherichia coli* is considered the third cause of foodborne zoonotic disease in the European Union, with 8161 reported cases including 126 confirmed cases in Spain, due to the consumption of meat beef, water from tap and wells, as well as juices and other products derived from fruits and vegetables. In 2019, a total of 7775 confirmed cases of Shigatoxigenic Escherichia coli infections in humans were reported in 27 countries of the European Union, including a total of 42 epidemics that caused 50 hospitalizations and 1 death, mainly related to consumption of meat beef and milk, in addition to tap and wells water (Civieta, 2021; EFSA, 2021).

1.1.6. *Escherichia coli* detection in food

In Spain as in the European Union, the microbiological criteria required for food are established by (EC) 2073/2005 Regulation and its amendments. Therefore, the acceptability of a product, a batch or a process, is defined by the absence or the presence as well as the number of microorganisms, in addition to the quantity of toxins or metabolites present by unit (mass, volume, surface). Thus, two classes are established: food safety criteria and process hygiene criteria.

The hygiene criteria include specifications for *E. coli*, applicable at the end of the manufacturing process for products such as shelled crustaceans and molluscs, pre-cut fruits and vegetables, unpasteurized fruit and vegetable juices, minced meat as well as meat preparations. The analytical reference methods for the detection and enumeration of beta-glucoronidase positive *E. coli*, mentioned in the regulation are the protocols ISO 16649-1, ISO 16649-2 and ISO 16649-3. These standards are effective when the bacteria level is low (less than 100/g or 10/mL), or when the target microorganism is physiologically weakened and stressed, such as a recovery from dried or frozen products. Concerning the detection of the *E. coli* O157:H7 serotype, the ISO 16654 standard describes a precise protocol for the detection of *Escherichia coli* belonging to the O157 serogroup.

Although these traditional detection methods have the advantage of detection at very low growth rates and of being applicable on complex matrices, their uses are expensive and long, requiring several days, which could represent a danger for the consumer. Therefore, the application of faster protocols that could replace the isolation steps or even the identification of bacteria, would save time and facilitates the work. Among these methods, immunological techniques such as the Enzyme Linked Fluorescent Assay (ELISA) method which generates a result in 2 hours after a 24-hour enrichment phase, or genetic methods based on the in vitro amplification of DNA or RNA specific sequences such as the polymerase chain reaction (PCR) and its variants, which allow the detection of injured and stressed bacteria, generally difficult to grow on culture media (Bouvier, 2011; Palomino and González, 2014).

1.2. *Salmonella* sp.

1.2.1. Historical antecedents

In 1818, Professor Pierre Bretonneau described Typhoid fever, an infectious disease named by the French pathologist Pierre Louis in 1829, from the ancient Greek tûphos which means "vapor rising to the brain, fever or delusion" (Marineli et al., 2013).

William Budd, confirmed the faecal-oral transmission route of this disease in 1838, after finding what he called "the poison" in the feces of affected patients. The pathogen responsible for this disease was determined in the end of the 19th century, by the German pathologist Karl Joseph Eberth, who was the first to describe a bacillus found in the abdominal lymph nodes and spleen of a patient in 1879. Its discovery was subsequently confirmed by numerous German and English bacteriologists including Robert Koch, after the publication of his research in 1880 and 1881 (Roumagnac et al., 2006; Emmeluth et al., 2009).

Cultivating these bacteria was only possible four years later, in 1884, when the German bacteriologist Georg Theodor August Gaffky, succeeded in growing this microorganism in pure culture (Marineli et al., 2013).

In 1896, the French bacteriologist Fernand Georges Isidore Widal demonstrated the antigenic diversity of *Salmonella* strains by serodiagnosis. The same year, the first effective vaccine developed to prevent Typhoid, by the British immunologist Almroth Edward Wright was tested on the military (Grimont et al., 2000; Bhan et al., 2005).

A year later, the medical researcher Theobald Smith, who worked as a laboratory assistant in the research team of the pathologist Daniel Elmer Salmon, discovered *Salmonella choleraesuis*, which would later be known as *Salmonella enterica* (Hardy, 1999).

The name *Salmonella* was only used in the 1900s, at the suggestion of the French professor Joseph Léon Lignières, to name this pathogen which was discovered by the Salmon team, in his honor (Marques et al., 2006).

1.2.2. Current taxonomic situation

According to the classification of the List of Prokaryotic names with Standing in Nomenclature, the Genus *Salmonella*, belongs to the family of *Enterobacteriaceae*, of the

order *Enterobacteriales*, which is part of the Class *Gammaproteobacteria*, Phylum *Proteobacteria*, Subkingdom *Negibacteria*, Kingdom *Bacteria* (LPSN, 2021).

At present, the Genus *Salmonella* is composed of 9 species with a validly published name, including three species with a correct name; *Salmonella bongori* (Reeves et al., 1989), *Salmonella enterica* (Le Minor and Popoff, 1987) and *Salmonella subterranea* (Shelobolina et al., 2005), all mentioned in **Table 2.**

Table 2. Current taxonomic situation of *Salmonella* sp.

Kingdom	*Bacteria*	Cavalier-Smith, 2002
Subkingdom	*Negibacteria*	Cavalier-Smith, 2002
Phylum	*Proteobacteria*	Garrity et al., 2005
Class	*Gammaproteobacteria*	Garrity et al., 2005
Order	*Enterobacteriales*	Garrity and Holt, 2001
Family	*Enterobacteriaceae*	Rahn, 1937
Genus	*Salmonella*	Lignières, 1900
Species	*Salmonella arizonae*	Kauffmann, 1964
	Salmonella bongori	Reeves et al., 1989
	Salmonella choleraesuis	Weldin, 1927
	Salmonella enterica	Le Minor and Popoff, 1987
	Salmonella enteritidis	Castellani and Chalmers, 1919
	Salmonella paratyphi	Ezaki et al., 2000
	Salmonella subterranea	Shelobolina et al., 2005
	Salmonella typhi	Warren and Scott, 1930
	Salmonella typhimurium	Castellani and Chalmers, 1919

Source: LPSN (Last revision 11/11/2021)

1.2.3. Characteristics

Salmonella species are Gram-Negative bacteria, rod-shaped (bacillus) with an average size of 0.7 to 1.5 µm diameter by 2 to 6 µm in length, depending on growing conditions. Non-spore-forming, the species are predominantly motile, with peritrichous flagella (Fàbrega and Vila, 2013; Andino and Hanning, 2015; Chaves et al., 2016).

Considered as Chemoorganotroph bacteria, *Salmonella* is aerobic and facultative anaerobe microorganism, which means that they can grow in the presence of oxygen by respiration, and survive without oxygen through anaerobic respiration by fermentation of organic compounds. Along with positive catalase and negative oxidase, these bacteria have the ability to ferment glucose and other monosaccharides, resulting in the production of acids and gases, including hydrogen sulfide (H_2S). However, they do not have the capacity to ferment lactose and sucrose, using neither arginine, nor hydrolyze urea. Species of this Genus have a citrate permease, which allows the use of citrate as a carbon source (Cabrera et al., 2013; Wang and Hammack, 2014).

Regarding growth parameters, *Salmonella* strains are not extremophiles and they may be destroyed from 60 °C for 15 min. Although they can survive at a minimum temperature of 5.2 °C and a maximum of 46.2 °C, its optimal growth requires an incubation temperature between 35 and 43 °C. In addition, these bacteria adapt easily to acidic pH and alkaline (basic), which can grow at pH ranging from 3.8 to 9.5, with optimal growth at neutral pH between 7 and 7.5. Moreover, it is very resistant to low water activities up to 0.93, which allows its development even in dry conditions. After 18 to 24 hours of incubation at 37 °C, *Salmonella* colonies are generally low convex, smooth type (S), circular with regular edges, measuring 2 to 3 mm in diameter, and more translucent than coliform colonies (Chaves et al., 2016; ELIKA, 2021).

The antigenic characteristics are represented by the somatic antigens O (lipopolysaccharide) located on the surface of the outer membrane, the flagellar antigens H as well as the capsular antigen Vi, a polysaccharide antigen common to serovars such as *Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin (Ryan et al., 2017).

1.2.4. Pathogenicity

Several species of the Genus *Salmonella* may be responsible of enteric infectious diseases, including *Salmonella enterica* subsp. enterica, which is found in the intestines of warm-blooded animals. Therefore, direct contact with fecal matter is the main route of transmission, in addition to cases of cross-contamination during food processing (Wu et al., 2016).

Diseases caused by this pathogen occur through ingestion of contaminated food or water, which is the most common form of transmission, then through contact with livestock or domestic animals and contaminated surfaces, in addition to the transmission human-to-human by the faecal-oral route. Infections may develop in multiple ways, gastroenteritis, bacteremia, typhoid fever or in the case of asymptomatic carriers, without any symptoms. This is usually related to the infectious dose, strain, age and state of health of the infected person (Forsythe, 2003; Chaves et al., 2016).

Typhoid fever is a potentially severe systemic infection of enteric origin, which could be caused by 4 serotypes of *Salmonella*; *Salmonella* Typhi, Paratyphi A, Paratyphi B and Paratyphi C. It is characterized by general malaise, abdominal pain, diarrhea, skin rash and fevers, with temperature up to 40 °C. These symptoms appear because of the entry of bacteria through the digestive tract, even at low infectious dose, after a relatively

long incubation period ranging from 1 to 3 weeks. These microorganisms pass through the intestinal mucosa, invade the intestinal lymphoid tissue and penetrate in the mesenteric lymph nodes, then into the lymph, to end up in the bloodstream, resulting in bacteremia. An appropriate antibiotic treatment is generally recommended, in order to avoid infection, spread and complications such as intestinal perforation and hemorrhage. Moreover, even after recovery, the patient potentially remains a carrier of the microorganism for months (Wang and Hammack, 2014; LaRock et al., 2015; Key et al., 2020; Cohn et al., 2021).

Another foodborne illness caused by these bacteria, the salmonellosis is considered one of the most important diarrheal infections in the world. It is mainly caused by the consumption of contaminated food and is characterized by abdominal pain, vomiting and diarrhea. These symptoms appear after an incubation period of 12 to 72 hours, from the ingestion of a large infectious dose and they last for 4 to 7 days. Although most cases are mild, it could be potentially fatal for people with weakened immune systems, such as those immunocompromised, persons over 60, and young children under 5, due to the severe dehydration caused by the disease (Wang and Hammack, 2014; Cohn et al., 2021).

1.2.5. *Salmonella* sp. in food

The development of these diseases is mainly caused by the consumption of raw or ready-to-eat products, which have not been subjected to heat treatment, sufficiently effective to inactivate this microorganism, especially in foods such as unpasteurized dairy products, meats, eggs products and their derivatives, as well as raw fruits and vegetables, which could potentially be contaminated by soil or polluted irrigation water (Ricke, 2021).

Salmonellosis is the second most common zoonosis in the European Union (EU), especially in Spain where it comes just after campylobacteriosis. In 2015, 94625 cases were confirmed in the European Union, with an increase of 1.9 % compared to 2014. According to the annual report of the Microbiological Information System a total of 5215 non-typhoidal *Salmonella* isolates were reported in Spain, through 11 different communities. The prevalence of these bacteria mainly concerned meats and meat products requiring cooking before consumption, of which 6.5 % positive samples for chicken meat and 4.6 % for turkey meat. However, the rates were lower for both pork meat (1.7 %) and beef meat (0.2 %) (EFSA and ECDC, 2016).

Despite the large number of reported cases, it would appear that 60-80 % of cases are not recorded, because for healthy people this disease does not require treatment (ELIKA, 2021).

In 2018, 1580 outbreaks of salmonellosis related to the consumption of contaminated water and food were reported, resulting in 94203 human cases reported by the European Surveillance System, in 28 countries of the European Union countries. Of the 91857 confirmed cases, 8872 were in Spain of which 53.1 % were in households and 31.5 % in the catering sector (RENAVE, 2018; EFSA and ECDC, 2019; AESAN, 2021).

In 2019, 87923 cases of salmonellosis were reported in the European Union, including 5103 cases in Spain, which represents a fairly significant decrease, compared to the rates in previous years. According to the Centers for Disease Control and Prevention (CDC), the types of food concerned were mainly meat (beef, chicken, and pork), eggs, vegetables like cabbage and even processed foods like breaded chicken and pies of frozen meat (EFSA and ECDC, 2019; Civieta, 2021).

1.2.6. *Salmonella* sp. detection in food

According to the microbiological criteria established by (EC) 2073/2005 Regulation, the presence of *Salmonella* spp. is not tolerated in food and its detection (presence/absence), as well as its enumeration must be determined by the application of the UNE-EN ISO 6579 protocol. The detection of these bacteria is carried out according to the four basic steps, pre-enrichment of the sample in Buffered Peptone Water, selective enrichment in the two selective broths Muller-Kaufman tetrathionate and Rappaport-Vassiliadis, isolation from the two selective broths incubated, on the solid selective medium Xylose Lysine Deoxycholate agar (XLD), then confirmation by biochemical and serological tests. Although, this method is known as the "gold standard", the fact that it requires several days is a huge drawback, which is why the development of molecular biology has made it possible to develop new, faster detection methods such as detection by PCR.

Among the alternative methods the use of Bioreceptors, a biological recognition molecule which makes it possible to distinguish the target, among the most commonly used; aptamer, nucleic acid probe, bacteriophage and lectin, in addition to antibodies, which have been recognized as a standard recognition factor of commercial rapid detection kits used in food safety, including lateral flow immunochromatographic strips

and enzyme immunoassay. There are also rapid detection methods based on optical detection and electrochemical identification methods, such as Optical Sensing, Colorimetry, Fluorescence Analysis and Photothermal Detection (Wang et al., 2021).

1.3. *Listeria monocytogenes*

1.3.1. Historical antecedents

In 1924, the bacteriologist E. G. D. Murray, after having isolated a gram-positive bacilli in the blood of rabbits, could not associate it with any known bacterial genus. Thus, in 1926, he described and named these bacteria *Bacterium monocytogenes*, according to the induced increase in monocytes. A year later, John Lister renamed it *Listerella hepatolytica* (Murray et al., 1926).

In 1940, Joseph Lister was able to isolate these bacteria from humans, thus the nomenclature of *Listeria monocytogenes*, was proposed by his collaborators, then accepted by the Approved Lists of Bacterial Names, to honor this British scientist, who also discovered that sterilization of surgical instruments, before operations, reduces the risk of infection (Euzèby and Tindall, 2004).

Unfortunately, Lister's observations could not establish sufficient awareness about this pathogen, in the field of infectious diseases and food microbiology, during that period. The pathogenicity of these bacteria was only realized in 1949, during an epidemic of newborns listeriosis in Germany, where the pathogen was classified in the Genus *Corynebacterium* by the bacteriologist J. Potel, according to the detection of granulomas called "granulomatosis infantiseptica" in various organs such as the liver, spleen, brain, lungs and skin (Hof, 2002).

At the same time, the research of H. P. R. Seeliger, who examined these pathogens isolated from similar lesions, demonstrated the motility of these bacteria, a fact ncompatible with *Corynebacteria*, but rather with *Listeria*. Over the next few years, a new era of listeriosis research had begun, led by Seeliger whose goal was to raise awareness of the dangers of *Listeria*, thus becoming the pioneer of listeriosis research (Hof, 2002).

Today, listeriosis is considered as a typical foodborne illness and a threat to public health because of its severity, its long incubation period and its high case fatality rate. The epidemics reported were mainly related to leafy vegetables and certain soft cheeses (WHO, 2018).

1.3.2. Current taxonomic situation

According to the classification of the List of Prokaryotic names with Standing in Nomenclature (LPSN, 2021), the Species *Listeria monocytogenes* are included in the Genus *Listeria*, of the family *Listeriaceae*, which is part of the Class *Bacilli*, of the Phylum *Firmicutes*, Kingdom *Bacteria* (Gibbons and Murray, 1978; Cavalier-Smith, 2002; Ludwig et al., 2010).

At present, the Genus *Listeria* is composed of 27 species with a validly published and correct name, all mentioned in **Table 3.**

Table 3 . Current taxonomic situation of *Listeria monocytogenes*

Kingdom	*Bacteria*	Cavalier-Smith, 2002
Phylum	*Firmicutes*	Gibbons and Murray, 1978
Class	*Bacilli*	Ludwig et al., 2010
Order	*Bacillales*	Prévot, 1953
Family	*Listeriaceae*	Ludwig et al., 2010
Genus	*Listeria*	Pirie, 1940
Species	*Listeria monocytogenes*	Pirie, 1940
	Listeria aquatica	Den Bakker et al., 2014
	Listeria booriae	Weller et al., 2015
	Listeria cornellensis	Den Bakker et al., 2014
	Listeria cossartiae	Carlin et al., 2021
	Listeria costaricensis	Núñez-Montero et al., 2018
	Listeria denitrificans	Prévot, 1961
	Listeria farberi	Carlin et al., 2021
	Listeria fleischmannii	Bertsch et al., 2013
	Listeria floridensis	Den Bakker et al., 2014
	Listeria goaensis	Doijad et al. 2018
	Listeria grandensis	Den Bakker et al., 2014
	Listeria grayi	Errebo Larsen and Seeliger, 1966
	Listeria immobilis	Carlin et al., 2021
	Listeria innocua	Seeliger, 1983
	Listeria ivanovii	Seeliger et al., 1984
	Listeria marthii	Graves et al., 2010
	Listeria monocytogenes	Pirie, 1940
	Listeria murrayi	Welshimer and Meredith, 1971
	Listeria newyorkensis	Weller et al., 2015
	Listeria portnoyi	Carlin et al., 2021
	Listeria riparia	Den Bakker et al., 2014
	Listeria rocourtiae	Leclercq et al., 2010
	Listeria rustica	Carlin et al., 2021
	Listeria seeligeri	Rocourt and Grimont, 1983
	Listeria thailandensis	Leclercq et al., 2019
	Listeria valentina	Quereda et al., 2020
	Listeria weihenstephanensis	Lang Halter et al., 2013

Source: LPSN (Last revision 11/11/2021)

1.3.3. Characteristics

L. monocytogenes is a gram-positive rod-shaped short bacteria (0.5 to 2.0 μm in length and 0.4 to 0.5 μm diameter) with blunt-ended. Nonsporulating, these bacteria

present a characteristic tumbling motility between 20 and 25 °C, due to the polar peritrichous flagella, however it is not mobile at 37 °C. The arrangement of this microorganism may be in single or double cell, but it can also exist as long chains, depending on growth (Arun, 2008; Soares et al., 2013; Allerberger et al., 2015).

Aerobes and facultative anaerobe, with catalase positive and oxidase negative, these intracellular bacteria are considered psychrotrophic microorganisms, as they can grow at temperature of -0.4 °C, with optimal development ranging between 30 and 37 °C and can maintain growth up to 50 °C. Moreover, it is very tolerant of extreme environmental stress conditions, such as its adaptation to a wide pH ranging from 4.1 to 9.6, with optimal growth between pH 6 to 8 and water activity around 0.9 to 0.97. In addition to its ability to grow at high salt concentrations of 10 % and survive between 20 and 30 % (Chaves et al., 2016; Jamshidi and Zeinali, 2019).

These bacteria are easy to grow on ordinary media, where they give smooth convex colonies of 1 to 2 mm, translucent and round with regular edges. On blood agar, colonies are smaller, gray and surrounded by an indistinct zone of beta hemolysis (Sauders et al., 2012).

L. monocytogenes have the ability to produce lecithinase, ferment glucose with production of acids without gas, in addition to hydrolyzing esculin. However, it is devoid of nitrate reductase and does not produce indole. Regarding the antigenic characteristics, they are expressed by the presence of somatic antigens (O) and flagellar (H) (Linke et al., 2014; Jamshidi and Zeinali, 2019).

1.3.4. Pathogenicity

L. monocytogenes is responsible for the development of listeriosis, a sever and potentially fatal foodborne infection, that occurs after ingestion of contaminated food. It can lead to sepsis and infection of the central nervous system in healthy adults and fever in pregnant women, resulting to abortion, premature birth or severe neonatal infections in the newborn (Angelidis et al., 2015; Ledlod et al., 2020).

Once inside the body, this pathogen passes through the intestinal epithelium and spreads through the bloodstream and lymph, causing damage to affected organs, mainly the liver and spleen. Its incubation period extends from a few days to two months, which complicates the diagnosis, it is longer in maternal forms compared to septicemic or

neurological forms, which lasts only a few days (Ramaswamy et al., 2007; Chaves et al., 2016).

There are two types of listeriosis: invasive and non-invasive. The invasive one is characterized by symptoms such as fever, muscle pain, endocarditis, pneumonia or meningitis, it mainly affects immunocompromised hosts and pregnant women. Non-invasive listeriosis has symptoms such as diarrhea, fever, muscle pain and headache, which go away after 3 days; it usually affects healthy people. In addition to ingestion of contaminated food, transmission may occur through cross-contamination or direct contact between people (Pizarro-Cerdá and Cossart, 2019).

1.3.5. *Listeria monocytogenes* in food

Food susceptible to be contaminated are mainly raw or ready-to-eat foods, such as dairy products (milk and cheese), meat, leafy vegetables which are generally eaten under or uncooked. Since refrigeration of food does not protect against the danger of *L. monocytogenes*, due to its ability to grow at low temperatures, it is recommended to vulnerable people and especially pregnant women, to eat only well-cooked foods (Doménech et al., 2015; Ledlod et al., 2020; ELIKA, 2021).

In Spain from 2015 to 2018, among the listeriosis cases reported to the National Epidemiology Centre (RENAVE), 1369 cases were confirmed including 124 deaths. In 2017, two outbreaks in Madrid caused 301 cases and three other outbreaks in Andalusia, Aragon and Castile La Mancha caused 433 cases in 2018, due to the consumption of contaminated meat (RENAVE, 2020).

The following year, an outbreak of listeriosis in Andalucia, also linked to meat consumption, caused 223 confirmed cases, 3 deaths, 2 cases of abortion and 3 stillbirths, which brought the incidence of this disease to a total of 548 cases in 2019 (Centro de Coordinación de Alertas y Emergencias Sanitarias, 2019; Civieta, 2021).

September 2021, an alert report was addressed to the Ministry of Health and Families, about the presence of *Listeria monocytogenes* in a batch of fresh goat and cow cheese, which was distributed in the provinces of Cadiz, Huelva and Seville (Consejería de Salud y Familias de la junta de Andalucía, 2021).

1.3.6. *Listeria monocytogenes* detection in food

According to Regulation (EC) 2073/2005, *L. monocytogenes* is bacteria whose presence in food is not tolerated. To guarantee the microbiological quality of food, the standardized protocols applied are UNE-EN ISO 11290-1 for the detection (presence/absence) of bacteria and UNE-EN ISO 11290-2 for enumeration.

Microbiological controls carried out according to cultural techniques should follow a pattern of four basic steps: pre-enrichment of the sample in Half-concentration Fraser Broth incubated 24 to 26 hours at 30 °C, then an enrichment in Fraser Broth incubated 24 hours at 37 °C, isolation on selective solid media Ottaviani and Agosti (ALOA) and PALCAM media, for 24 to 48 hours incubation at 37 °C, then confirmation by morphological and biochemical analysis.

Although the effectiveness of this method is proven, the fact that it requires so much time (4 to 5 days), as well as the need for several specific selective media complicate the analyses, especially for the detection of several pathogens from a single sample. Therefore, the need for a rapid result has led to the development of a large number of alternative commercial methods, although based on different principles, they all require at least a phase of selective enrichment before the application of a test, such as the immunoenzymatic test, nucleic acid hybridization test or Polymerase Chain Reaction (PCR).

Some rapid methods apply immunocapture step, by using a support coated with antibodies specific for the species *L. monocytogenes*, to sort positive and negative samples within 2 to 3 days. However, confirmation of a positive result by isolation on selective medium and identification remain necessary (AFFSA, 2020; AFNOR, 2021).

1.4. *Staphylococcus aureus*

1.4.1. Historical antecedents

Staphylococci were first described in the late 1800s by Robert Koch and Louis Pasteur, as "Micrococci" from the Greek kokkos (meaning berry) (Bhunia, 2008; Paterson et al., 2014).

In the 1870s, the Scottish Surgeon Alexander Ogston was the first to isolate and observe *Staphylococcus*, after microscopic examination of pus extracted from surgical

abscesses, identifying it as the causative agent of inflammation and suppuration of wounds (Thomer et al., 2016; Khan, 2017; Rasheed and Hussein, 2021).

His studies led him in 1882 to baptize them *Staphylococcus*, according to their microscopic appearance and their arrangement in cluster, derived from the Greek words "staphyle" (bunch of grapes) and "kokkos" (berry), to differentiate them from streptococci, which are arranged in chains and also responsible for post-surgical infections (Gnanamani et al., 2017; Rasheed and Hussein, 2021).

In 1884, the German Surgeon and bacteriologist Anton J. Rosenbach, isolated and cultivated *Staphylococcus* strains of human origin, which he named according to the color of the colonies, *Staphylococcus aureus* from the Latin word "*aureus*" meaning golden, for the colonies with yellow to golden color, and *Staphylococcus albus* from the Latin word "albus" meaning white, which will later be renamed *Staphylococcus epidermidis* (Sejvar, 2013; Rasheed and Hussein, 2021).

In the 1900s, with the emergence of the first official bacterial classifications, both Genus *Staphylococcus* and *Micrococcus* were grouped into the family *Micrococcaceae*. However, the recent use of molecular phylogenetic data, associated with chemical analyzes has led to the creation of the *Staphylococcaceae* family (Wattam et al., 2014).

In 1914, the British scientist Barber discovered that staphylococci had the ability to produce a toxic substance, which was the cause of staphylococcal food poisoning (Barber, 1947; Bhunia, 2008).

After the introduction of methicillin in 1959, for the treatment of infections caused by penicillin-resistant *Staphylococcus aureus*, isolates of *S. aureus* having acquired resistance to methicillin (Methicillin-resistant *Staphylococcus aureus*, MRSA) were observed by Professor Patricia Jevons in October 1960, then reported in 1961 in the British Medical Journal of the United Kingdom, quickly followed by other European countries, Japan, Australia and the United States (Mark et al., 2002).

1.4.2. Current taxonomic situation

According to the classification of the List of Prokaryotic names with Standing in Nomenclature (LPSN, 2021), the Species *Staphylococcus aureus* is part of the Genus *Staphylococcus*, of the Family *Staphylococcaceae*, which is part of the Class *Bacilli*, of the Phylum *Firmicutes*, Kingdom *Bacteria*. At present, the Genus *Staphylococcus* is

composed of 68 species with a validly published name, of which 61 species with a correct name, all mentioned in **Table 4.**

Table 4. Current taxonomic situation of *Staphylococcus aureus*

Kingdom	*Bacteria*	Cavalier-Smith, 2002
Phylum	*Firmicutes*	Gibbons and Murray, 1978
Class	*Bacilli*	Ludwig et al., 2010
Order	*Bacillales*	Prévot, 1953
Family	*Staphylococcaceae*	Schleifer and Bell, 2010
Genus	*Staphylococcus*	Rosenbach, 1884
	Staphylococcus agnetis	Taponen et al., 2012
	Staphylococcus argensis	Hess and Gallert, 2015
	Staphylococcus argenteus	Tong et al., 2015
	Staphylococcus arlettae	Schleifer et al., 1984
	Staphylococcus aureus	Rosenbach, 1884
	Staphylococcus auricularis	Kloos and Schleifer, 1983
	Staphylococcus borealis	Pain et al., 2020
	Staphylococcus caeli	MacFadyen et al., 2019
	Staphylococcus caledonicus	Newstead et al., 2021
	Staphylococcus canis	Newstead et al., 2021
	Staphylococcus capitis	Kloos and Schleifer, 1975
	Staphylococcus caprae	Devriese et al., 1983
	Staphylococcus carnosus	Schleifer and Fischer,1982
	Staphylococcus casei	Madhaiyan et al., 2020
	Staphylococcus caseolyticus	Schleifer et al., 1982
	Staphylococcus chromogenes	Hájek et al.,1987
	Staphylococcus coagulans	Madhaiyan et al., 2020
	Staphylococcus cohnii	Schleifer and Kloos, 1975
	Staphylococcus condimenti	Probst et al., 1998
	Staphylococcus cornubiensis	Murray et al., 2018
	Staphylococcus croceilyticus	Madhaiyan et al., 2020
Species	*Staphylococcus debuckii*	Naushad et al., 2019
	Staphylococcus delphini	Varaldo et al., 1988
	Staphylococcus devriesei	Supré et al., 2010
	Staphylococcus durrellii	Fountain et al., 2019
	Staphylococcus edaphicus	Pantůček et al., 2018
	Staphylococcus epidermidis	Evans, 1916
	Staphylococcus equorum	Schleifer et al., 1984
	Staphylococcus felis	Igimi et al., 1989
	Staphylococcus fleurettii	Vernozy-Rozand et al., 2000
	Staphylococcus gallinarum	Devriese et al., 1983
	Staphylococcus haemolyticus	Schleifer and Kloos, 1975
	Staphylococcus hominis	Kloos and Schleifer, 1975
	Staphylococcus hyicus	Devriese et al., 1978
	Staphylococcus intermedius	Hájek, 1976
	Staphylococcus jettensis	De Bel et al., 2013
	Staphylococcus kloosii	Schleifer et al., 1984
	Staphylococcus lentus	Schleifer et al., 1983
	Staphylococcus lloydii	Fountain et al., 2019
	Staphylococcus lugdunensis	Freney et al., 1988
	Staphylococcus lutrae	Foster et al., 1997
	Staphylococcus massiliensis	Al Masalma et al., 2010
	Staphylococcus microti	Nováková et al., 2010

Staphylococcus muscae	Hájek et al., 1992
Staphylococcus nepalensis	Spergser et al., 2003
Staphylococcus pasteuri	Chesneau et al., 1993
Staphylococcus petrasii	Pantůček et al., 2013
Staphylococcus pettenkoferi	Trülzsch et al., 2007
Staphylococcus piscifermentans	Tanasupawat et al., 1992
Staphylococcus pragensis	Madhaiyan et al., 2020
Staphylococcus pseudintermedius	Devriese et al., 2005
Staphylococcus pseudoxylosus	MacFadyen et al., 2019
Staphylococcus pulvereri	Zakrzewska-Czerwińska et al., 1995
Staphylococcus rostri	Riesen and Perreten, 2010
Staphylococcus roterodami	Schutte et al., 2021
Staphylococcus saccharolyticus	Foubert and Douglas, 1948
Staphylococcus saprophyticus	Shaw et al., 1951
Staphylococcus schleiferi	Freney et al., 1988
Staphylococcus schweitzeri	Tong et al., 2015
Staphylococcus sciuri	Kloos et al., 1976
Staphylococcus simiae	Pantůček et al., 2005
Staphylococcus simulans	Kloos and Schleifer, 1975
Staphylococcus singaporensis	Chew et al., 2021
Staphylococcus stepanovicii	Hauschild et al., 2012
Staphylococcus succinus	Lambert et al., 1998
Staphylococcus ureilyticus	Madhaiyan et al., 2020
Staphylococcus vitulinus	Webster et al., 1994
Staphylococcus warneri	Kloos and Schleifer, 1975
Staphylococcus xylosus	Schleifer and Kloos, 1975

Source: LPSN (Last revision 11/11/2021)

1.4.3. Characteristics

Staphylococcus aureus is a Gram-positive round-shaped (cocci) bacteria, with a diameter ranging between 0.5 and 1 µm. Immobile and non-spore-forming, the cells are usually grouped in clusters as a bunch of grapes, but can also appear isolated, in diplococci or in short chains (Becker et al., 2004; Sievert et al., 2013).

It is aerobic and facultative anaerobic bacteria, with positive catalase and negative oxidase and the ability to ferment glucose without production of gas, in addition to degrading mannitol (Nandy et al., 2013).

Grows easily on basal medium, it forms convex, smooth, creamy, opaque and pigmented colonies, with a typical golden-yellow color, due to its high content of carotenoids, in aerobiosis. As mesophilic bacteria, the optimum temperature for growth is around 37 °C, however it is thermosensitive, because its growth is slowed down by cold and it is effectively destroyed by high temperatures (Liu et al., 2005; Wu and Su, 2014).

It requires a neutral pH around 7 for optimal growth, however it is considered as halophilic microorganism, due to its ability to develop at high concentrations of NaCl. In

addition it is relatively resistant to bacterial inhibitors such as crystal violet and potassium tellurite. *S. aureus* is also characterized by free coagulase (staphylocoagulase), thermonuclease (thermostable DNAse), beta hemolytic activity, in addition to its ability to liquefy gelatin (Eisenstein, 2008; Monteith et al., 2021).

1.4.4. Pathogenicity

Although *S. aureus* is a commensal microorganism that colonizes the skin, anterior nostrils, and mouths of healthy humans, its huge arsenal of virulence factors, enzymes and toxins make it a dangerous opportunistic pathogen, which can act as an intracellular and extracellular pathogen, causing various pyogenic and systemic infections, acute, chronic infections and even fatal diseases. These virulence factors contribute to its adhesion, persistence, protection and evasion of the host's immune defenses, with overlapping roles during disease processes (Gorwitz et al., 2008; Becker, 2018).

Among the virulence factors composing the cell wall, Protein A is an immunogen capable of interfering with the opsonization and phagocytosis processes, thereby inducing a hypersensitivity reaction. Some species have a polysaccharide capsule with antiphagocytic activity, which protects them from the action of polymorphonuclear cells. Regarding the enzymatic proprieties, the Catalase and the free Coagulase of *S. aureus* have a role in its resistance to opsonization and phagocytosis, Phospholipase C induces the destruction of tissues, Lipases facilitate the propagation of this microorganism through the skin and subcutaneous tissues, Staphylokinase which gradually eliminates fibrin and Hyaluronidase which hydrolyzes tissues by acting on the intracellular matrix, both contribute to the spread of this microorganism in the adjacent tissues. In addition, these bacteria have the capacity to produce DNAses, proteases and phosphatases, which interact during the infectious process (Stewart, 2017).

Regarding toxins, *S. aureus* has α-hemolysin, a toxin that damages the membranes of certain cells such as polymorphonuclear in humans and erythrocytes of certain animal species. Exfoliative or epidermolytic toxins with proteolytic activity, which cause the separation of the outermost layers of the epidermis and epithelial desquamation, thus inducing "scalded skin syndrome" or "Ritter's Disease". Toxic Shock Syndrome Toxin 1 (TSST-1), which is common in women who use vaginal tampons during menstruation, causes sudden vascular collapse, shock and erythematous rash, scaly, nausea, vomiting, myalgia, kidney and liver dysfunction and even neurological disorders that can lead to death. These bacteria have also a Leucocidin, an exotoxin with a toxic effect on the

membranes of the polymorphonuclear of humans, as well as Enterotoxins (A, B, C, D and E) which are thermostable and resistant to gastric enzymes, responsible for food poisoning after ingestion, with diarrhea and vomiting (Castro et al., 2018; Grace and Fetsch, 2018).

In addition to its pathogenic capacities, this microorganism is capable of easily acquiring resistance to multiple antibiotics, such as *S. aureus* resistant to methicillin (MRSA) (Becker, 2018).

Generally, two types of illnesses caused by *S. aureus* are distinguished, pyogenic and/or systemic infections which can affect all organs and organ systems, respectively, as well as toxin-mediated diseases, such as staphylococcal food poisoning caused by Staphylococcal Enterotoxins (Becker, 2018).

Staphylococcal food poisoning is one of the most common causes of food poisoning in the world. It is usually self-limiting, manifesting after a short incubation period, 2 to 6 hours after ingestion of food containing Staphylococcal Enterotoxins, symptoms of acute gastroenteritis such as nausea, violent vomiting, abdominal cramps, diarrhea and fever can be observed (Kérouanton et al., 2007; Castro et al., 2018).

1.4.5. *Staphylococcus aureus* in food

The foods most affected by the presence of *S. aureus* are raw milk, soft and semi-soft cheeses made from cow's milk or from unspecified animals, ice cream, pre-cut fruits and vegetables, meat products of chicken or other animal species, processed food products and prepared meals, such as pasta, sauces, pastries and more particularly the ready-to-eat food (Grace and Fetsch, 2018).

From 2008 to 2011, 137 outbreaks of foodborne illness caused by *Staphylococcus* spp. and *Staphylococcus aureus* have been declared in Spain, inducing 1577 cases and 33 hospitalizations (Espinosa et al., 2015).

According to the European Food Safety Authority in 2014, 393 food-borne outbreaks caused by Staphylococcal toxins were reported in 12 member states of the European Union, the majority of the cases were localized in France involving 264 hospitalizations and 2 deaths, related to mixed foods, chicken and pork broiler meats, cheese and dairy products, fish and fish products, in addition to vegetables and fruit juices (EFSA-ECDC, 2015a; Castro et al., 2018). In the same year, 26 outbreaks of

staphylococcal food poisoning were reported in Japan, the main sources of which were packed lunches and rice balls (Jin and Yamada, 2016).

In 2016, according to the results of the monitoring carried out by Spain on milk, cheese, meat, bakery products, vegetables, ready meals and other processed food products, 201 (21 %) of the samples were positive for *Staphylococcus* spp. out of a total of 940 samples analyzed (EFSA, 2017).

In 2019, 16 strong evidence outbreaks and 58 weak evidence outbreaks caused by *S. aureus* enterotoxin poisoning were reported in 13 countries in the European Union, including Spain. Among these outbreaks, the two most important ones were reported in Hungary causing 380 illnesses and in France causing 300 cases including one hospitalization. The most serious outbreaks was reported in Italy which caused 70 cases including 44 (62 %) hospitalizations, nevertheless no deaths were reported. According to the European Food Safety Authority, a decrease in the number of foodborne disease outbreaks caused by *S. aureus* has been recorded, compared to 2018 in France and Spain (EFSA, 2021).

1.4.6. *Staphylococcus aureus* detection in food

The presence of *S. aureus*, this commensal microorganism of human skin and respiratory mucous membranes, in food indicates a deficiency in food and processing environment hygiene, mainly associated with improper food handling, in addition to a favorable growth conditions, such as non-compliance with the cold chain during storage and preparation (Valero et al., 2009; Argudín et al., 2010; Castro et al., 2018).

According to the microbiological criteria established by (EC) 2073/2005 Regulation, Staphylococcal Enterotoxins should not be detected in 25 g of dairy food, such as cheese, milk powder and whey powder.

The amount of Staphylococcal Enterotoxin capable of causing disease is obtained from a number of cells greater than 10^5 CFU/g of food, therefore the enumeration of coagulase positive Staphylococci is necessary (Bhatia et al. Zahoor, 2007).

The presence of coagulase positive *Staphylococcus aureus* is tolerated within the limits established by the (EC) 2073/2005 Regulation, depending on the type of food: less than 10^5 CFU/g for raw milk cheeses, less than 10^2 CFU/g for powdered milk and whey and less than 10^4 CFU/g for milk-based cheeses, subjected to less heat treatment than pasteurization and ripened cheese made from milk or whey pasteurized or having

undergone a more intense heat treatment than pasteurization, as well as shelled and shelled products of cooked crustaceans and molluscs. The enumeration should be determined by the application of the protocols mentioned in the regulation, UNE-EN ISO 6888-1 and UNE-EN ISO 6888-2 (Castro et al., 2018).

The diagnosis of Staphylococcal food poisoning is mainly established by the detection of Staphylococcal Enterotoxins in the food consumed or the presence of *S. aureus* producing Enterotoxins at a cellular concentration exceeding 10^6 CFU/mL, in addition to the presence of Enterotoxins with a large number of microorganisms in vomit, or the presence of the *S. aureus* strain in the stool of affected patients after infection (EFSA, 2009; Castro et al., 2018).

The presence of the microorganism or enterotoxin genes can be quickly and efficiently detected by Polymerase Chain Reaction (PCR), including multiplex PCR detection methods, which are sensitive enough to detect all known determinants of Enterotoxins from contaminated foods, even without the presence of viable bacteria or which cannot be cultured. However, the presence of Enterotoxin genes does not mean that the microorganisms carrying the genes are able to produce them in sufficient quantity to cause disease, under the conditions of food storage (Hait et al., 2014; Stewart, 2017).

Another alternative method to detect Enterotoxins is the Mass Spectrometry analysis, however this type of approach can only be applied to culture supernatants and not as a direct measure of Enterotoxins in food. In addition to the commercially available Reversed Passive Latex Agglutination assays which have a sensitivity of 0.5 ng/mL, or the enzyme-linked immunosorbent assay (ELISA) methods, which can detect 0.1 to 1.0 ng of Enterotoxin per gram of food (Attien et al., 2014; Stewart, 2017).

2. OBJECTIVES

Foodborne illness may represent a threat to community health all over the world and until today, food safety has been ensured by the detection of these microorganisms using conventional microbiological methods, which although effective, require time and a lot of resources, especially for the detection of several bacteria from a single sample, where each microorganism must be detected and isolated according to its own protocols, with several different culture media. Molecular detection tools such as multiplex PCR can be a good alternative for a rapid and efficient simultaneous detection of multiple bacteria in food. However, with detection by multiplex PCR, the more the number of targets and primers used increases, the more the detection sensitivity decreases, which is problematic for low cell concentrations, as in the case of *Salmonella* sp. or *L. monocytogenes*, the limit of which is the absence of 1 CFU/25 g. Consequently, the use of a co-culture step in a common medium, before detection by multiplex PCR would increase the number of cells initially present in the food, thus allowing their detection.

Thereby the **main objective** of this work is the development of a quick and easy method for the simultaneous detection of *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus* and *Listeria monocytogenes* in foods, by combining a co-culture step in liquid medium and detection by multiplex PCR.

To achieve this main objective, the following **specific objectives** have been proposed:

1. Analyze several samples of environmental water and raw food in order to detect the target bacteria, using both methods, the microbiological method by applying UNE-EN ISO protocols and the molecular method by simplex PCR.
2. Assess various liquid culture media, to determine the optimal co-culture medium for the simultaneous growth of the four target bacteria.
3. Develop and optimize a multiplex PCR system, for the detection of the four target bacteria.
4. Reduce detection time and improve sensitivity by combining a co-culture step and multiplex PCR for the detection of the four target bacteria, from food matrices in the presence of background microbiota.

3. MATERIAL AND METHODS

3.1. Analysis of samples by cultural method, for the detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*

3.1.1. Samples studied

The food samples were collected, at random, in small shops (butchers and greengrocers) and supermarkets in Valencian Community (Spain) and their analysis was initiated within 24 h after the collection.

A total of 73 food samples were collected and tested between 2016 and 2018, including raw and Ready-To-Eat (RTE) food products, which can be eaten directly without or with little cooking, comprising 43 samples of animal origin products: 12 samples of fresh minced meat, 10 cold meats (pâtés) and 21 fresh cheeses, as well as 30 samples of vegetable origin products, mainly leafy vegetables: 10 lettuces, 10 spinach, 10 chards. In addition, to 15 environmental surface water samples were collected between February and June 2018, in the Alboraya and Vera regions of the Valencian Community.

3.1.2. Analysis of samples by cultural methods

3.1.2.1. Sample preparation

Tweenty-five g of each food sample were mixed with 225 mL of sterilized Buffered Peptone Water (BPW) (Scharlau-Spain), in a Stomacher bag and homogenized during 5 min, for the pre-enrichment step according to UNE-EN ISO 16649-3:2015, UNE-EN ISO 6579-1:2017 and UNE-EN ISO 6888-1:2000 protocols, relating to the research and detection of *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* respectively, to be finally incubated at 36 °C ± 2 °C for 18 h ± 2 h.

For the detection of *Listeria monocytogenes*, 25 g of each food sample was mixed with 225 mL of sterile Enrichment Broth FRASER Base (Scharlau-Spain) half concentration (FBH), supplemented with Listeria UVMII Selective Supplement 06-111-LY01 (Scharlau-Spain) and Ferric Ammonium Citrate Supplement 06-112-LY01 (Scharlau-Spain), in a Stomacher bag and homogenized for 5 min, as a pre-enrichment step of the UNE-EN ISO 11290-1:2018 protocol for the research and detection of *L. monocytogenes*, then the mixtures were incubated 25 h ± 1 h at 30 °C.

The water samples were analyzed according to the UNE-EN ISO 9308-1:2014 standard, using membrane filtration method for water with low microbiota content, where

225 mL of each water sample were filtered through 0.45 µm membranes (Sartorius Stedim Biotech™- Germany). The membranes were immersed into sterile Stomacher bag containing 225 mL of sterilized BPW (for *E. coli*, *Salmonella* spp. and *S. aureus* detection) and FBH with supplements (for *L. monocytogenes* detection), then homogenized for 5 min to finally be incubated under the same conditions mentioned above.

3.1.2.2. Enumeration of *Enterobacteriaceae*

The enumeration of *Enterobacteriaceae* was carried out from a few analyzed samples, following the horizontal method for the detection and enumeration of *Enterobacteriaceae* without the pre-enrichment, detailed in standard UNE-EN ISO 21528-2:2018.

One mL taken from the sample homogenized in BPW before the incubation was incorporated into a 9 mL sterile distilled water tube, then the serial decimal dilutions were carried out. From each decimal dilution 1 mL was inoculated, in duplicate and in depth with approximately 15 mL of molten medium Violet Red Bile Glucose AGAR (VRBGA) medium (Scharlau-Spain) at 44-47 °C, then after solidification an additional layer of medium (about 5 mL) was poured over the first one, to avoid invasive growth. After solidification, the dishes were incubated for 24 ± 2 h at 37 °C. Then, the enumeration of the characteristic purple/pink colonies was done.

3.1.2.3. Isolation and identification of *Escherichia coli*

For the isolation and identification of *E. coli* from food or water, there is no specific standardized method or a unique reference. Therefore, we had to develop our own protocol based on the multiple UNE-EN ISO standards, related to this microorganism (UNE-EN ISO 9308-3:1999; UNE-EN ISO 16654:2002 and UNE-EN ISO 21150:2016). The protocol mainly provides the isolation by three successive steps; a pre-enrichment in non-selective broth Buffered Peptone Water (BPW) (Scharlau-Spain), enrichment in selective broth Brilliant Green Bile Lactose Broth (BGBLB) (Difco^{TM}-BD-France) and isolation on selective solid media Tryptone Bile Glucuronic Agar (TBX) (Scharlau-Spain) and Endo Agar (ENDO) (BBL^{TM}-France). Then identification of the suspected colonies by biochemical tests.

After sample collection, BPW pre-enrichment and incubation (as mentioned above), the selective enrichment step was carried out by inoculating 1 mL of the pre-

enrichment mixture obtained after incubation, in a BGBLB tube for 24 h, at 37 °C. Then the isolation on solid selective media was done by streak, from the selective enrichment broth (BGBLB) after incubation. The two selective media used allowed a clear distinction between the typical *E. coli* colonies, which are blue to blue-green on the TBX and from the dark pink to rose-red with green metallic sheen on ENDO. Both media were incubated at 37 °C for 24 hours. After incubation, the characteristic suspicious colonies were plated on Plate Count Agar (PCA) (Scharlau-Spain) and incubated for 24 h at 37 °C, to be identified by catalase test, gram stain, biochemical tests IMViC. In the case of conclusive results for all those tests, the final confirmation would be established by API® 20E strip (BioMerieux-France).

The suspected cultures, with a positive catalase, short bacilli gram-negative, indole-positive, RM-positive, VP-negative and citrate-negative (IMViC ++--), were subjected to the last identification test, Api 20E strips (BioMerieux-France). This standardized and rapid system, allowing the identification of *Enterobacteriaceae* and other gram-negative bacteria, is composed of several miniaturized biochemical tests, the results of which need to be compared to a database. Results obtained are compared with the manufacturer's database to identify the bacteria. Finally, the confirmed *E. coli* strains were stored in cryotubes (Microbank™-ProLab Diagnostics-Canada) at -20 °C.

3.1.2.4. Isolation and identification of *Salmonella* spp.

The isolation and identification of *Salmonella* spp. from food and water was carried out according to the UNE-EN ISO 6579-1:2017 protocol, comprising a pre-enrichment step in non-selective Buffered Peptone Water (BPW) broth (Scharlau-Spain), mentioned previously, an enrichment step in two selective liquid media, the Rappaport Vassiliadis-R10 Broth (RV) (DifcoTM-BD-France) and Müller-Kauffmann Tetrathionate Broth Base (MKTTn) (Scharlau-Spain) mixed with an iodine/iodide solution and the Brilliant Green-Novobiocin selective supplement 06-017LYO1 (Scharlau-Spain). The two media were incubated 24 h, at 41.5 °C ± 0.5 °C for RV and 37 °C for MKTTn. After incubation, the isolation step was carried out from the selective cultures obtained, by streaking inoculation on two solid selective media, the Chromogenic Agar Base CM1007 (CAB) (Oxoid-United Kingdom) with the supplement SR0194E (Oxoid-United-Kingdom) and the Xylose-Lysine-Deoxycholate Agar (XLD) (Scharlau-Spain), the plates were incubated 24 h at 37 °C. Then, the plates were examined to identify characteristic colonies of *Salmonella* spp. on the two selective media which are red-pink colonies with or without

black center on XLD and magenta colonies on CAB medium. Some characteristic colonies were isolated on PCA for biochemical testing. Among the tests carried out are Catalase, Gram stain and IMViC, which were mentioned above, but also and more particularly the Triple Sugar Iron test (TSI) (Merck KGaA-Darmstadt-Germany).

The Triple Sugar Iron test demonstrates the ability of a microorganism to ferment glucose, lactose and sucrose, as well as the ability to produce hydrogen sulfide (H_2S), which is performed by stabbing the center of the medium TSI to the bottom of the tube, then finalize the inoculation with the same loop, by streaking the surface of the slanted medium. The color change of the medium from red to yellow is a sign of acidification, which is due to sugars fermentation, the blackening of the medium results from the reduction of Sodium Thiosulfate to H_2S, which produces Iron sulfide by reacting with the ferric ions of the medium, as well as air bubbles at the bottom of the tube, which can even crack the agar, indicating production of gases such as CO_2. Cultures considered to be typical of *Salmonella* spp. should get the slanted part of the medium in red (alkaline), which means the non-use of lactose and sucrose, and the deep part in yellow (acidification), which is a sign of glucose fermentation. Furthermore, in most cases, agar blackening is noted, which is due to the production of H_2S.

The resulting suspected cultures resulting with Catalase-positive, Gram-negative of bacillus-form, Indole-negative, RM-positive, VP-negative, Citrate-positive (IMViC - + - +), as well as a TSI results: Glucose-positive, Lactose/Sucrose-negative and H_2S-positive, were subjected to the last identification test using the Api 20E strips (BioMerieux-France). The strains confirmed as *Salmonella* spp. by Api 20E, were stored in cryotubes (Microbank™-ProLab Diagnostics-Canada) at -20 °C, for further use.

3.1.2.5. Isolation and identification of *Staphylococcus aureus*

To detect and isolate *S. aureus* in food and water samples, we applied steps inspired from the UNE-EN ISO 6888-1:2000 protocol. After a pre-enrichment step in a non-selective medium BPW, mentioned previously, a selective enrichment step was carried out by inoculating 1 mL of pre-enrichment culture, at the bottom of a 19 mL tube of Giolitti-Cantoni Broth (GC) (Scharlau-Spain), freed of air bubbles by preheating at 65 °C for 5-10 min and supplemented with the 06-011-100 Potassium Tellurite Solution 3.5 % (Scharlau-Spain), before inoculation. In order to provide an anaerobic atmosphere, the

surface of the medium was covered with 2 mL of Vaseline oil, Extra pure (Scharlau-Spain) before incubation at 37 °C for 24-48 h, examined daily.

After the appearance of a black precipitate at the bottom of the tube, or in the whole of the medium, which is a sign of potassium tellurite reduction, the isolation step was carried out, from this dark zone, by streak on the selective medium Baird Parker Agar Base (BPA) (Scharlau-Spain) supplemented with the Egg Yolk Emulsion Potassium Tellurite (EYEPT) 06-026-100 (Scharlau-Spain). After 24-48 h of incubation at 37 °C, the characteristic colonies of *S. aureus* should be round, convex, black and shiny, with an opaque halo around the colonies, which is due to the reaction of lecithinase (lypolytic activity).

These characteristic suspect colonies were cultured on PCA to be subjected to a Catalase test, Gram stain and specially to demonstrate the hemolysis activity. To do this, the cultures were inoculated on Blood Agar Base (Columbia) (Scharlau-Spain) medium mixed with 5 % Sheep Blood Defibrinated (Thermo Scientific™-Oxoid ™-United Kingdom) and incubated for 24 h at 37 °C. The characteristic colonies of *S. aureus* on this medium produce total hemolysis with a complete digestion of hemoglobin, resulting in a large area around the colony with distinct edges, where the medium becomes transparent and shows the light-yellow color of the nutrient base.

Finally, the suspect cultures showing a catalase-positive test, gram-positive with cocci form, in cluster arrangement and a beta-hemolysis activity, were subjected to the last identification test by Api Staph strip (BioMerieux-France). The strains confirmed by Api Staph as being *S. aureus* were stored in cryotubes (Microbank™-ProLab Diagnostics-Canada) at -20 °C, for further use.

3.1.2.6. Isolation and identification of *Listeria monocytogenes*

The detection and isolation of *L. monocytogenes* were carried out according to the steps mentioned in the UNE-EN ISO 11290-1:2018 protocol. After the pre-enrichment step in Enrichment Broth Fraser Base Half concentration (FBH) (Scharlau-Spain), supplemented with Listeria UVMII Selective Supplement 06-111-LY01 (Scharlau-Spain) and Ferric Ammonium Citrate Supplement 06-112-LY01 (Scharlau-Spain), previously mentioned, 0.1 mL of the pre-enrichment culture was added to 10 mL tube of Enrichment Broth Fraser Base (FB) medium with supplements, as a selective enrichment step, then incubated at 37 °C for 24 h.

From the selective enrichment culture, the isolation step was carried out by streaking on two selective media Palcam Agar Base (PAL) (Scharlau-Spain), mixed with a Selective Supplement 06-110LY01 (Scharlau-Spain) and Chromogenic Listeria Agar Base of Ottaviani and Agosti (ALOA) (Oxoid-United Kingdom), with the OCLA Selective Supplement SR0226E (Oxoid-United Kingdom) and the BrillianceTM Listeria Differential Supplement SR0228E (Oxoid-United Kingdom), then incubated for 24 to 48 h at 37 °C. The characteristic colonies of *L. monocytogenes*, which are gray-green, round with a concave center, surrounded by dark-brown to black halos on PAL medium and round convex with a shiny blue to blue-green color, surrounded by an opaque halo on ALOA medium, were cultured on PCA in order to be subjected to multiple tests such as catalase, gram stain and demonstration of hemolysis activity.

Presumptive cultures with Catalase-positive, Gram-positive, as well as a small, straight and thin rounded ends bacillus shape and, above all, a clear area of beta-hemolysis on Blood Agar, which indicate a hemolysis activity, were submitted to a final identification step by Api Listeria strip. The strains confirmed by Api Listeria as *L. monocytogenes*, were stored in cryotubes (Microbank™-ProLab Diagnostics-Canada) at -20 °C, for further use.

3.2. Analysis of samples by molecular method, for the detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*

3.2.1. Reference strains

In this work, four reference strains were used as positive controls: *Escherichia coli* CECT 101, *Salmonella enterica* CECT 4266, *Listeria monocytogenes* CECT 936 and *Staphylococcus aureus* CECT 435, procured from the Spanish Type Culture Collection (CECT, Valencia, Spain).

Cultures were prepared from culture stocks, stored in cryotubes (Microbank™-ProLab Diagnostics-Canada) at -20 °C, by streaking on Plate Count Agar (PCA-Scharlau), the different bacterial strains were cultured separately and the plates were incubated for 24 h at 37 °C. After incubation, each strain was inoculated by the spread plate method (lawn) on the agar surface, to obtain abundant growth of each pure culture and sufficient template for DNA extraction, then incubated for 24 h at 37 °C.

3.2.2. DNA extraction

For the analyzed samples, 1 mL aliquots collected from selective enrichment steps (BGBLB, GC, RV, MKTTn, FR) were subjected to DNA extraction by kit.

After 2 min of centrifugation at 16000 ×g (Centrifuge Sigma-Germany), the supernatant was removed and the pelleted cells were used for DNA extraction, following the manufacturer's Gram-positive extraction protocol, of GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), which requires the use of Lysozyme (BioChemica-PanReac AppliChem-ITW Reagents). The eluted DNA was stored at -20 °C, to be used as a PCR template.

As a positive control template, the amount of fresh pure culture, from each reference strains *Escherichia coli* CECT 101, *Salmonella enterica* CECT 4266, *Listeria monocytogenes* CECT 936 and *Staphylococcus aureus* CECT 435, were homogenized separately in 1 mL of Tris-EDTA Buffer (TE buffer-PanReac AppliChem; 0.1 mM EDTA-Na_2, 10 mM Tris, pH 8.0), then subjected to DNA extraction by kit, previously mentioned.

3.2.3. Selection of primers

One of the most critical steps is the selection of target DNA sequences to amplify, in order to design the specific primers pair for each target gene. The design of primers is usually done using open-source online software.

For the detection of the four studied bacteria, the pairs of primers used were selected from other studies and presented in **Table 5**. To detect *E. coli*, GADA 670-F/R primers pair targeting the Glutamate Decarboxylase enzyme-encoding gene (*gadA*) was chosen from the work of McDaniels et al. (1996). For *S. enterica* primers pair SalinvA 284-139/141 was chosen from the Rahn et al. (1992) work, targeting the encoding gene of Invasion protein A (*invA*). In addition to the LM 404-F/R primers pair from Wu et al. (2004) targeting the Listeriolysin-encoding gene (*lisA*) of *L. monocytogenes* and Nuc 484-F/R primers pair, targeting the encoding gene of *S. aureus* Thermostable Nuclease (*nuc*), from the work of Xu et al. (2006). The primers used were synthesized by TIB MOLBIOL (Syntheselabor GmbH-Germany).

Table 5. Primers used

Strains	Primers	Sequences	Size	References
E. coli	GADA/F	ACCTGCGTTGCGTAAATA	670 bp	McDaniels et al., 1996
	GADA/R	GGGCGGGAGAAGTTGATG		
S. aureus	Nuc/F	CTTTAGCCAAGCCTTGACGAAC	484 pb	Xu et al., 2006
	Nuc/R	AAAGGGCAATACGCAAAGAGGT		
L. monocytogenes	LM404/F	ATCATCGACGGCAACCTCGGAGAC	404 bp	Wu et al., 2004
	LM404/R	CACCATTCCCAAGCTAAACCAGTGC		
S. enterica	SalinvA139	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	Rahn et al., 1992
	SalinvA141	TCATCGCACCGTCAAAGGAACC		

3.2.4. Primers validation by simplex PCR and temperature gradient

Each pair of primers was previously validated individually by simplex PCR, in a similar reaction mixture containing its own target DNA (extracted by kit), under the same amplification conditions. These conditions chosen as a starting point, were established and optimized from the PCR kit manufacturer recommendations, based on the conditions used in the original studies of each primers pair from Rahn et al. (1992), McDaniels et al. (1996), Wu et al. (2004) and Xu et al. (2006), in addition to the comparison of several study conditions, working with the same primers pairs, to obtain reagents concentrations and the most suitable temperatures, for an effective running of all primers.

During this evaluation, several annealing temperatures were used and the temperature gradient assay (55, 56, 57, 58, 60 °C) was performed to assess the performance of each primers pair in PCR simplex amplification to determine the optimum common temperature between the four pairs of primers.

For each test, a reaction mixture with distilled Milli-Q water was used, instead of the DNA template, as a negative control and amplifications were carried out using a Thermal Cycler (Eppendorf AG-Germany). Then, 5 µL of each PCR products were mixed with Ready-to-Load (GeneRuler-Thermo Scientific) and subjected to an electrophoresis for 80 min at 80 Volts on 1.5 % agarose gel (Agarose D1 low EEO-Conda/TAE buffer-PanReac AppliChem; 0.05 M EDTA-$Na_2 \cdot 2H_2O$, 1 M Acetic Acid glacial, 2 M Tris). Amplification products were visualized under UV transilluminator (Vilber Lourmat-France).

3.2.5. Evaluation of primers sensitivity in simplex PCR

The sensitivity of the primers was determined separately, by evaluating the detection limits of the four simplex PCRs. To do this, each reference strain was cultured individually in 10 mL of Nutrient Broth medium (NB) (Difco™- BD-France) and incubated under 150 rpm agitation for 24 h at 37 °C, in order to obtain an approximate cell concentration of 10^8 CFU/mL.

After incubation, serial dilutions of the overnight cultures were carried out, in 9 mL tubes of 0.85 % sterile physiological saline solution. Then, cell concentrations were determined by a viable counting, in duplicate on the corresponding selective media (TBX, BPA, PAL and XLD). Plates were incubated for 24-48 h at 37 °C.

1 mL aliquots collected from each suspension (10^8-10^0 CFU/mL) were used for DNA extraction by kit (previously mentioned). Then from each resulting DNA, 4 µL were used to perform a simplex PCR, according to the conditions previously established.

After amplification, 5 µL of each resulting PCR products, were mixed with Ready-to-Load, separated by electrophoresis on 1.5 % agarose gel at 80 Volts for 80 min, then visualized using a UV transilluminator.

3.2.6. Analysis of samples by simplex PCR

The detection of the four bacteria from water and food samples by molecular method was carried out by simplex PCR assays, using DNAs extracted from the aliquots of selective enrichment media and the four specific pairs of primers mentioned in **Table 5**.

For each detection, a positive control and a negative control were prepared. For the positive control, DNA extracted from the reference strains was added to the reaction and sterile distillated Milli-Q water was used instead of DNA, in the negative control reaction.

3.3. Simultaneous co-culture and detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* by multiplex PCR

3.3.1. Selection of a co-culture medium

The effect of several selective and non-selective broths on the growth of the four studied microorganisms was compared, according to the maximum population rates obtained under the same culture conditions (24 h at 37 °C), in order to select the most suitable co-culture broth for the 4 target bacteria.

3.3.1.1. Effect of various culture broths on individual growth

The individual growth of each target bacteria was evaluated in several commercial non-selective broths; Luria-Bertani Broth (LB) (DifcoTM-BD-France), Nutrient Broth (NB) (Difco™-BD-France) and Buffered Peptone Water (BPW) (Scharlau-Spain).

Likewise, selective culture broths recommended in UNE-EN ISO protocols, for the selective enrichment of each studied bacteria were tested; BGBLB, RV, GC and FB with supplements for *E. coli*, *S. enterica*, *S. aureus* and *L. monocytogenes*, respectively.

The selective synthetic broth developed by Chen et al. (2015), SSSLE broth, for the simultaneous growth of *S. enterica*, *S. aureus*, *S. flexneri*, *L. monocytogenes* and *E. coli*, was also tested in our work.

To do this, 1 CFU/mL of pure culture of each reference strains cultivated for 24 h on PCA, were separately inoculated in 10 mL of culture broths tested, then incubated for 24 h at 37 °C for NB, BPW, LB, BGBLB, GC, FB, SSSLE and at 41.5° ± 0.5°C for RV.

The maximum population rates obtained were determined after incubation by counting on the respective selective media described in the UNE-EN ISO protocols (TBX, XLD, BPA and PAL). The Plates were incubated at 37 °C during 24-48 h. The number of CFU/mL was determined according to UNE-EN ISO 7218:2008 standard.

3.3.1.2. Buffered Peptone Water as co-culture broth

3.3.1.2.1. Inoculum preparation

Fresh pure cultures of each reference target strain were cultivated separately, in 10 mL of BPW then incubated for 24 h at 37 °C with 150 rpm shaking, to obtain an approximate cell concentration of 10^8 CFU/mL.

After incubation, overnight cultures were submitted to decimal dilutions in 9 mL BPW tubes, to preserve cell integrity and maintain viability, then cell concentrations were estimated by counting on respective selective media described in the UNE-EN ISO protocols, mentioned above. The number of CFU/mL was determined according to the formula, mentioned in UNE-EN ISO 7218:2008 standard.

3.3.1.2.2. Effect of BPW on individual and co-culture growth

To evaluate the BPW recovery abilities, from low initial inoculum concentrations, in individual pure cultures, three inoculum levels were used 10^3, 10^2, 10^1 CFU/mL, in a

final volume of 10 mL, for each target strain. Then, the BPW recovery abilities in co-culture, from low initial inoculum concentrations were tested, also on three inoculum levels with equal concentration between the four target bacteria, for a final volume of 10 mL, in several experiments:

-**Experiment 1:** 10^3, 10^3, 10^3, 10^3 CFU/mL,

-**Experiment 2:** 10^2, 10^2, 10^2, 10^2 CFU/mL and

-**Experiment 3:** 10^1, 10^1, 10^1, 10^1 CFU/mL, then incubated 24 h at 37 °C.

The initial inoculum concentrations and the recovery rates of each experiment were determined by counting, on respective selective media, previously mentioned. All plates were incubated 24-48 h at 37 °C and the number of CFU/mL was determined according to the formula mentioned in UNE-EN ISO 7218:2008 standard, and 1 mL aliquots from each suspension culture were frozen at -20 °C, to be tested by multiplex PCR.

3.3.1.2.3. Effect of BPW on co-culture growth, from artificially inoculated food matrix

To evaluate recovery abilities, during co-culture from an artificially inoculated food matrix, with the presence of background microbiota, two kinds of ready-to-eat food were tested, eco-organic lettuce and raw minced meat. The samples were purchased from local stores and used fresh, without prior heat treatment, to preserve their background microbiota.

Each sample was handled, according to the same protocol: 10 g of sample were artificially inoculated with the appropriate pure culture dilutions (prepared previously and mentioned in **3.3.1.2.1**), to obtain an initial inoculum concentration of 10^3 CFU/mL for each strain. Then 90 mL of sterile BPW was added to the Stomacher bag containing the inoculated sample. The mixtures were homogenized for 5 minutes, then incubated for 24 h at 37 °C.

Initial inoculum concentrations and recovery rates were determined by counting, on the respective selective media and all plates were incubated during 24-48 h at 37 °C. The number of CFU/mL was determined after incubation and 1 mL aliquots of each resulting co-culture were frozen at -20 °C, to be tested by multiplex PCR.

3.3.2. Development and Optimization of multiplex PCR

To detect simultaneously the four microorganisms from a single sample, with an effective means that is simpler, faster and economical, we have relied on the development of a multiplex PCR, using several sets of primers specific to each template, in a single reaction.

However, the practical development of a multiplex PCR is fastidious, as its complexity increases with the number of amplified targets and the primers pairs used. It usually requires numerous optimization tests to ensure a stable reaction, with the best balance between specificity and detection sensitivity, for all the target genes present in the reaction (Oscorbin et al., 2021).

Many factors may influence the multiplex PCR, among the most important; the quality of the DNA matrix, the targeted sequences, the design of the primers, the quantity of reaction reagents used and the Thermal Cycler program applied (temperatures and number of cycles) (Chuang et al., 2013).

3.3.2.1. *In silico* validation of primers

Prior to multiplexing, the specificity of each primers pair was rigorously analyzed *in silico*, using the Primer-Blast program Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their correspondence with the targeted genes, and ensure the absence of homologous sequences in non-target microorganisms, thus avoiding any non-specific amplification.

Another factor related to primers that might influence detection results, is the formation of secondary structures. The control of these structures was made through primers analysis, by an open source program, the Multiple Primer Analyzer (Thermo Fisher Scientific).

With the same program, the four primers pairs were compared with each other, to confirm the absence of any complementarity, thus avoiding the risk of primers dimerization during multiplexing (Huang et al., 2018).

3.3.2.2. Development of multiplex PCR

After the *in silico* validation of the primers, the multiplexing was carried out by a progressive integration of the primers, in combination of two, three, then all 4 in a single reaction, with their respective DNA targets.

The multiplex PCR was carried out according to the conditions resulting from simplex PCRs temperature gradient.

All components of the reaction mixture: 2.9 µL Reaction Buffer 10×NH_4, 3 mM $MgCl_2$ Solution, 0.20 mM dNTP's (dNTP Mix-BIOLINE) and 2.5 Units Taq DNA polymerase (BIOTAQ™ DNA Polymerase-BIOLINE), were mixed with the 4 primers pairs (0.4 µM GADA670, 0.4 µM Nuc484, 0.4 µM LM404 and 0.2 µM SalinvA284). 1 µL of each target DNA (extracted by kit) was added, resulting in 4 µL of DNA template for a total volume of 29 µL (amplification conditions showed in **Table 14** of RESULTS AND DISCUSSION section). After amplification, 5 µL of PCR product were subjected to electrophoresis, according to the conditions mentioned previously.

To verify the presence of a possible cross-hybridization during the multiplexing step, which could lead to a mispriming between the four primers and the four DNA templates, each target DNA was individually incorporated into a reaction mixture, composed of all primers pairs in addition to the reagents required for the multiplex PCR reaction, previously mentioned.

3.3.2.3. Optimization of multiplex PCR

To obtain the best simultaneous detection of the four microorganisms, optimization tests were carried out to resolve the detection problems resulting from multiplexing. Such as removing artifacts and non-specific products by increasing the annealing temperature (58, 59, 60, 61, 62 °C), by reducing the number of cycles (30, 25, 20) or by increasing the amount of $MgCl_2$ (3.2, 3.5, 4 mM). All of these variations were first done separately and then combined.

To improve detection sensitivity, the modifications were mainly based on increasing the amount of dNTP's (0.22, 0.3, 0.4 mM), Taq DNA polymerase or even the DNA template.

To homogenize the detection intensity between the four specific amplicons, primers quantities were adjusted, by decreasing the quantity for amplicons with high band intensity and increasing it for the weakest.

The integrity and the quantity of DNA recovered after extraction is a very important factor, which might significantly influence the PCR detection result. Therefore, the DNA purification method by commercial kit, with Lysozyme, was compared with the DNA

extraction by thermal lysis (the boiling method) without lysozyme, according to Zhang et al. (2012).

Both methods were applied on aliquots of 1 mL, collected from individual cultures and co-cultures of reference strains, made in BPW.

3.3.2.4. Evaluation of multiplex PCR specificity

The multiplex PCR specificity was evaluated by determining the ability of this protocol to distinguish the target bacteria from the non-target ones.

To do this, several reaction mixtures were prepared, containing the 4 pairs of primers, all the components necessary for amplification and the extracted DNA from pure cultures, including: *Escherichia coli* CECT 425, *Escherichia coli* CECT 418, *Escherichia coli* CECT 4558, *Citrobacter freundii* CECT 401, *Micrococcus luteus* CECT 245, *Staphylococcus epidermidis* CECT 231 and the 3 laboratory isolates *Listeria innocua*, *Listeria grayi*, *Bacillus cereus*.

All the strains used were cultured separately on PCA, for 24 h at 37 °C and the DNA templates were extracted by the DNA purification kit previously mentioned.

3.3.2.5. Evaluation of multiplex PCR sensitivity

To determine the sensitivity of the multiplex PCR, the protocol applied was the same as that mentioned for the evaluation of the simplex PCRs sensitivity. Based on the use of DNA extracted by kit, from decimal dilutions of each pure culture (10^8-10^0 CFU/mL).

The DNA template used for this evaluation is composed of the four target DNAs (1 µL ×4) with an equivalent cell concentration (CFU/mL) for each strain, added separately to the reaction mixture with the rest of necessary reagents.

In addition, the DNA templates DNAs used in each PCR mixture were measured using the Qubit 4 Fluorometer (Thermo Fisher Scientific). Then, the amplification was carried out, according to the optimized conditions.

3.3.3. Detection limits by multiplex PCR from the co-culture

To evaluate the detection limits from co-culture carried out in BPW, with and without food matrices, all the aliquots collected after incubation were subjected to a DNA

extraction by kit. Then the resulting DNAs were used as templates in several multiplex PCRs, according to the optimized conditions.

4. RESULTS AND DISCUSSION

4.1. Analysis of samples by cultural method, for the detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*

Raw and Ready-To-Eat food samples were analyzed by cultural and molecular methods, including 43 animal origin samples (minced meat, cold meat, fresh cheese), 30 vegetable origin samples, mainly leafy vegetables (lettuce, spinach, chard). In addition to 15 environmental surface water samples, from two agricultural canals in the areas of Alboraya and Vera (Valencian Community-Spain), to detect four target bacteria: *Escherichia coli, Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes.*

4.1.1. Enumeration of *Enterobacteriaceae*

According to the Regulation CE N° 2073/2005 issued by the European Commission, concerning the microbiological criteria applicable to foodstuffs, the *Enterobacteriaceae* enumeration might be used as an indicator of microbiological risk, because even if most of its species are not dangerous, this group also includes pathogenic species. Therefore, the presence of this family may induce the establishment of controls, targeting specific pathogens.

Relevant for routine monitoring, it is used to assess the hygienic quality of food and water, because their presence can be related to original raw material contamination, to cross-contamination with the environment (soil or irrigation water), or to inappropriate treatment during handling, such as compromising the cold chain or a poor sanitation of processing environment and equipment. For this reason, EFSA's recommendations regarding the controls of *Enterobacteriaceae*, include both manufacturing environment as well as the finished product.

In order to determine the number of *Enterobacteriaceae* in the analyzed food and water samples, the VRBGA medium was used and for each type of food/water results obtained from the multiple enumerations, were expressed as an average. After analysis of *Enterobacteriaceae* enumeration results, presented in **Table 6,** it was observed that the analyzed animal origin products were more loaded with enterobacteria (3.08×10^3-2.49×10^6) than vegetable origin products (4.30×10^3-1.71×10^5) and environmental water samples (with an average of 4.73×10^4).

Table 6. Enumeration of *Enterobacteriaceae*

Animal origin products (CFU/g)	**Minced meat**	1.17×10^5
	Cold meat	3.08×10^3
	Fresh cheese	2.49×10^6
Vegetable origin products (CFU/g)	**Lettuce**	5.34×10^3
	Spinach	4.30×10^3
	Chard	1.71×10^5
Environmental water (CFU/mL)	**Alboraya area**	2.81×10^4
	Vera area	6.64×10^4
	Water average	4.73×10^4

Among these samples, fresh cheese was the one with the highest number of *Enterobacteriaceae*, a fact that has been reported in several studies, concerning different Mediterranean cheeses (Psoni et al., 2003; Macedo et al., 2004), and correlated with certain organoleptic characteristics such as aroma, taste and texture (Dahl et al., 2000; Morales et al., 2004), which contribute to the sensory characterization of cheese (Chaves et al., 2006; Martín et al., 2018). The rate of *Enterobacteriaceae* resulting from our analysis seems to be the same as the results of Armas et al. (2020) in Cuba, but higher than results mentioned by Ashkezary et al. (2020) in Italy and Espinoza et al. (2020) in Ecuador. According to the regulation CE N° 2073/2005, pasteurized milk and other pasteurized liquid dairy products with an *Enterobacteriaceae* level higher than 10 CFU/mL are considered unsatisfactory, therefore, a control of the heat treatment efficiency, prevention of recontamination and quality control of raw materials should be done.

Followed by minced meat, in second position, which might be due to the fact that these were samples of raw and fresh food. *Enterobacteriaceae* enumerations obtained from raw minced meat are significantly higher than those mentioned in the work of Siriken (2004); Elmali and Yaman (2005) in Turkey, Phillips et al. (2008) in Australia, Abdelrahman et al. (2014) in Egypt, Atlabachew and Mamo (2021) in Ethiopia. The high incidence of *Enterobacteriaceae* in ground beef constitutes a potential danger to public health, such as histamine poisoning, especially when consumed raw (with little or no cooking). This type of contamination can be due to the rupture of the intestine or the use of contaminated water during evisceration and slaughter, but also related to handling

during the preparation process (slicing, mincing, weighing and packaging) in butcher's shops (Wong et al., 2004; Scheutz et al., 2012). According to the regulation CE N° 2073/2005, meat and carcasses before refrigeration with an *Enterobacteriaceae* level higher than 1.5-2.5 $\log_{10}$ CFU/cm^2, are considered unsatisfactory and improvements in hygiene during slaughter, in addition to a review of the process should be carried out.

Among the animal origin samples, the food least loaded with *Enterobacteriaceae* was the cold meat kind, this may be related to the fact that it is not a raw food and that for their preparation preservatives are usually used. Generally, for this category, food samples showing an *Enterobacteriaceae* level higher than 10^4 CFU/g are considered unsatisfactory (Gilbert et al., 2000). In our case, although the maximum rate has not been reached and the cold meat samples are considered satisfactory, efforts still need to be made by the supermarket in order to reduce the rate and improve the sanitary quality of this type of food.

Regarding the analyzed vegetable origin products, the number of *Enterobacteriaceae* recorded although lower than that found in animal origin products, it nevertheless remains high. Among the leafy vegetables tested, we noticed that the rate obtained from chard samples (*Beta vulgaris*) was much higher than those obtained from lettuce (*Lactuca sativa*) and spinach samples (*Spinacia oleracea*). This could be due to the size, the shape and texture of the leaves, as chard leaves are larger fan-shaped in a cluster form (Aycicek et al., 2006; Korire et al., 2016), which increases the contact surface in the event of possible contamination, by polluted irrigation water or during manual picking, transport and storage for example. In addition to its crumpled and blistered texture, which facilitates the adhesion of pathogens, unlike spinach and lettuce, which have smaller and smoother leaves.

The counts of *Enterobacteriaceae* recorded from the analysis of vegetables products seem to coincide with the results obtained by Nguz et al. (2005) in Zimbia, Abadias et al. (2008) and Oliveira et al. (2010) in Spain, Seow et al. (2012) in Singapore, Cardamone et al. (2015) in Italy, Al-Kharousi et al. (2016) in Oman, Kłapeć et al. (2016) in Poland. Slightly higher than the results of Johannessen et al. (2002) in USA and Ryu et al. (2014) in Korea, and lower than Viswanathan and Kaur (2001) in India, Aycicek et al. (2006) in Turkey and Al-Holy et al. (2013) in Saudi Arabia results.

The presence of *Enterobacteriaceae* can be due, in addition to polluted irrigation water, to the contact with soil contaminated by animal excrement, or in the case of organic

farming, which requires the use of organic fertilizers, such as manure and slurry. Since the vegetables analyzed were raw, fresh, unpackaged and purchased from greengrocers, contamination may also occur during destocking and displaying operations, or even during weighing.

However, we have observed that most microbiological quality guide recommend the use of *Enterobacteriaceae* enumeration only for the evaluation of the microbiological quality of Ready-To-Eat food, taken at the sale point and judge the enumeration from raw foods, like vegetables or fruits as irrelevant, since these types of foods generally have a high level of *Enterobacteriaceae*, as part of their normal microbiota (Gilbert et al., 2000; Amador et al., 2010).

The environmental water analyzed samples from the two zones Alboraya and Vera (Valencia-Spain), showed a relatively similar load of *Enterobacteriaceae*, which exceeds the limits, ranging between 10^2 and 10^3 CFU/100 mL, established in Spain and recommended by the European Union for use as irrigation water (RD 1620/2007). It therefore, falls into the category of water that cannot be used directly for agricultural purposes, because their direct contact with food would represent a significant risk for human health, if purification treatments were not implemented beforehand (Alcalde-Sanz and Gawlik, 2017).

4.1.2. Isolation and identification of *Escherichia coli*

According to the regulation CE N° 2073/2005 issued by the European Commission, concerning the microbiological criteria applicable to foodstuffs, then modified by the CE N° 1441/2017 and CE N° 229/2019, the presence of *E. coli* in food is tolerable at defined limit, beyond which the products are considered non-compliant and unsatisfactory. For foods like ground meat intended for raw consumption, the level of *E. coli* must be less than 50 CFU/g, and less than 10^2 CFU/g for fresh cheese. The criteria developed by the Federation of Trade and Distribution (FDC-Fédération du commerce et de la distribution) indicate that the level of *E. coli* present in foods such as cold meat should not exceed 10 CFU/g for product of a satisfactory sanitary quality (FCD, 2020). Regarding raw vegetables and green products, the number of *E. coli* should be between 10 and 10^2 CFU/g (Gilbert et al., 2000; Pascual and Calderón, 2010). According to Alcalde-Sanz and Gawlik (2017) the water used for all food crops intended for raw consumption including roots, which are the edible part in direct contact with water and where all irrigation methods authorized, must be of "A category", with an *E. coli* level not exceeding 10 CFU/100 mL.

Analysis of the environmental water and food samples (results presented in **Table 7**), showed that *E. coli* was present in the water samples with a rate 10/15 (66.67 %), which is slightly higher than the results obtained by Holvoet et al. (2013) in Belgium. Since this bacterium is a part of the humans and warm-blooded animal's intestinal microbiota, its survival time in the main habitat is two days (Groisman and Winfiel, 2003; Reshes et al., 2008). It grows and divides only inside and will not withstand long outside due to external factors (Groisman and Winfiel, 2003). Depending on the environment where it is found, *E. coli* can survive for about 1 day in water and from 1 to 2 days, approximately, in soil (Larrea et al., 2013). Therefore, its continued detection in water could only be due to constant contact or transfer of feces (Groisman and Winfiel, 2003). Hence this microorganism is used as an indicator of environment fecal contamination (Jimenez et al., 1989). Its presence in surface water is generally due to the direct discharge of livestock excreta, which has access to watercourses, runoff from manure piles or drainage from spreading plots and pastures (CAC, 2013). The contamination of environmental waters by pathogens represents a major health risk, which might cause intoxications, infections and even epidemics, but also a threat for the future of the water supply, especially from the point of view antibiotic resistance genes transfer, because according to Martínez (2009) about 90 % of bacteria in water are resistant to more than one antibiotic and 20 % are resistant to at least five. Among the most resistant, *Escherichia coli*, *Staphylococcus aureus*, *Klebisiella pneumoniae*, followed by *Salmonella* spp (WHO, 2018).

Table 7. Results of the isolation and identification of *Escherichia coli*

Origin products	Analyzed samples		Positive samples
Animal	Minced meat	12	05/12 (41.66 %)
	Cold meat	10	00
	Fresh cheese	21	01/21 (4.76 %)
	Total	43	06/43 (13.95 %)
Vegetable	Lettuce	10	04/10 (40 %)
	Spinach	10	02/10 (20 %)
	Chard	10	01/10 (10 %)
	Total	30	07/30 (23.33 %)
Water	Water	15	10/15 (66.67 %)

The presence rate of *E. coli* in leafy vegetables was 7/30 (23.33 %), of which 40 % of lettuce samples (04/10), 20 % of spinach samples (2/10) and 10 % of chard samples (01/10) contaminated. Based on these results, *E. coli* prevalence in vegetables food is relatively close to the rates mentioned in the work of Olivera et al. (2010) in Spain, Al-

Kharousi et al. (2016) in Oman, Kłapec et al. (2016) in Poland. However, it remains higher than those mentioned in the work of Abadias et al. (2008) in Spain, Santos et al. (2012) in Portugal, Cardamone et al. (2015) in Italy.

Regarding the presence of *E. coli* in animal origin food, the rate of contaminated samples was 6/43 (13.95 %) of which 05/12 (41.66 %) samples of minced meat and 1/21 (4.76 %) samples of fresh cheese. The recorded results of *E. coli* prevalence in minced meat during analyzes are relatively close to those mentioned in the work of Gwida et al. (2014) in Egypt, and lower than those of Abdelrahman et al. (2014) Egypt. As for the prevalence of *E. coli* in fresh cheese, our results are quite close to those described in the work of Rosengren et al. (2010) in Sweden and much lower than those of Ombarak et al. (2016) in Egypt and Vásquez et al. (2018) in Peru.

According to Amador et al. (2010) in Portugal, the presence of *E. coli* in the deli meat samples was around 15.4 %, which is very far from the results of our analysis, because no cold meat sample has been tested positive for the presence of *E. coli,* which could be due to the heat treatment carried out during the transformation and the preservatives used in the preparation, but above all, to the absence of recontamination during the handling process, which means that the standards of Good Manufacturing Practices (GMP) have been correctly applied, resulting in satisfactory products.

From the samples analyses carried out, we were able to observe that the efficiency of the TBX medium with 16/23 (69.56 %) positive samples was clearly higher than that obtained using ENDO 13/23 medium (56.52 %). However, the fact that only 6/23 (26.08 %) of the positive samples were obtained by both media at the same time, demonstrates the need to combine the two selective culture media. These results are presented in **Table 8**.

Table 8. Efficacy comparison of the two selective media TBX and ENDO

Samples	ENDO	TBX	ENDO & TBX
Minced meat	4/23	4/23	3/23
Fresh cheese	0/23	1/23	0/23
Lettuce	2/23	2/23	0/23
Spinach	2/23	2/23	2/23
Chard	1/23	1/23	1/23
Water	4/23	6/23	0/23
TOTAL	13/23	16/23	6/23
%	56.52 %	69.56 %	26.08 %

In addition to *E. coli*, several *Enterobacteriaceae* species were isolated from the analyzed samples and identified by Api 20E strip (results shown in **Table 9**). These cultures were tested by Api strip because they were suspected of belonging to *E. coli* species, getting all the characteristics required by cultural methods on selective media, Gram stains and biochemical tests. These bacteria are mainly part of the commensal microbiota of humans or animal digestive tract, which are usually isolated from the environment (soil, water, wastewater, plants), their presence in food is mainly due to direct or cross contamination. These microorganisms can behave as opportunistic pathogens, inducing urinary and respiratory infections, wound superinfection and nosocomial infections in hospital environment and even septicemia in people with weakened immune system. Clinically, certain members of the *Enterobacteriaceae* family are among the most feared agents for their pathogenicity (Nhung et al., 2007), but also for their ability to acquire antibiotic resistance genes (Ruimy et al., 2010), which once integrated, can be transferred to other bacteria (Mathur et al., 2005).

Table 9. Identification of several strains by Api strip 20E, from analyzed samples

Origin	Strains	Samples
Animal	*S. liquefaciens*	5/43 (11.63 %)
	E. cloacae	3/43 (6.78 %)
	P. mirabilis	2/43 (4.65 %)
	S. marcescens	1/43 (2.32 %)
	A. hydrophila	1/43 (2.32 %)
	H. alvei	1/43 (2.32 %)
	R. ornithinolytica	1/43 (2.32 %)
	K. oxytoca	1/43 (2.32 %)
	C. koseri	1/43 (2.32 %)
Vegetable	*P. luteola*	2/30 (6.67 %)
	P. oryzihabitans	2/30 (6.67 %)
	P. mirabilis	2/30 (6.67 %)
	P. rettgeri	1/30 (3.33 %)
	M. morganii	1/30 (3.33 %)
	A. baumanni	1/30 (3.33 %)
	P. aeruginosa	1/30 (3.33 %)
	P. stuartii	1/30 (3.33 %)
	C. freundii	1/30 (3.33 %)
	R. ornithinolytica	1/30 (3.33 %)
	K. pneumoniae	1/30 (3.33 %)

Among the identified bacteria, *Klebsiella oxytoca* which can cause colitis (Hoenigl et al., 2012), *Klebsiella pneumoniae* which is usually found in the upper airways of humans and warm-blooded animals, feared for its ease of acquiring antibiotic resistance genes (Rashid and Ebringer, 2007), also isolated from lettuce samples in the work of Al-Holy et al. (2013) in Saudi Arabia. *Enterobacter cloacae* considered in recent years as a

nosocomial infectious agent (Barnes et al., 2003), was also isolated in samples of deli meat with a prevalence of 5.1 % in the study of Amador et al. (2010) in Portugal. *Citrobacter koseri* which might be transmitted vertically from mother to fetus, thus causing meningitis and septicemia (McPherson et al., 2008), and *Citrobacter freundii* which represents 29 % of the opportunistic infections (Whalen et al., 2007). *Raoultella ornithinolytica* usually isolated from food samples, as mentioned by Gwida et al. (2014) in Egypt, the prevalence of which was 2 % from raw meat, which may be responsible for bronchopulmonary and intra-abdominal infections, in addition of histamine shock due to its ability to produce histamine. In 2009, a link was established between this bacterium and Enteric Fever-Like Syndrome (Morais et al., 2009).

4.1.3. Isolation and identification of *Salmonella* spp.

According to the regulation CE N° 2073/2005 issued by the European Commission, concerning the microbiological criteria applicable to foodstuffs, then modified by the CE N° 1441/2017 and CE N° 229/2019, the absence of *Salmonella* spp. is required in food intended for human consumption, to obtain compliant products. For foods such as ground meat intended for raw consumption or meat preparations such as cold meat, *Salmonella* spp. should be undetectable in 25 g of food. According to Gilbert et al. (2000), for dairy products such as fresh cheese, *Salmonella* spp. must be absent in 25 g of food for satisfactory quality. The same goes for raw vegetables according to Pascual and Calderón (2010).

Based on the analysis of the samples, no *Salmonella* spp. was isolated and confirmed by Api 20E. The absence of *Salmonella* spp. could be due to a low rate, less than 1 CFU/25 g of food, which would mean that the analyzed products comply with the established requirements, or the present cells could be stressed and injured, which would make them unable to grow on culture medium. Our results are in accordance with numerous work such as Bohayhuck et al. (2006) in Canada, Amador et al. (2010) in Portugal, Al-Holy et al. (2013) in Saudi Arabia, Abdelrahman et al. (2014) and Gwida et al. (2014) in Egypt, Perdoma et al. (2015) in Venezuela, Kłapeć et al. (2016) in Poland, Vásquez et al. (2018) in Peru, Belhaj and Elamrani (2019) in Morocco. Unlike the results obtained by Siriken et al. (2004) in Turkey, Philips et al. (2008) in Australia, Espinoza et al. (2020) in Ecuador, Atlabachew and Mamo (2021) in Ethiopia, where the prevalence of *Salmonella* spp. was between 1.1 and 51.43 %.

However, several strains isolated from the selective culture media XLD and CAB, with *Salmonella* spp. characteristics (Characteristic colonies on selective media, gram, cell form, IMViC biochemical tests), were identified by Api 20E strip (results shown in **Table 9**).

The majority were non-pathogenic *Enterobacteriaceae*, which might behave as opportunistic pathogens. *Serratia* was the predominant Genus, present in 6/43 (13.95 %) of animal origin samples, including *Serratia liquefaciens* 5/43 (11.63 %) and *Serratia marcescens* 1/43 (2.32 %). These bacteria, are part of the digestive tract, usually isolated from soil, water, plants and vegetables, in addition to being responsible for mastitis in dairy animals, their ability to form biofilms makes them dangerous in hospital environment, because they can induce urinary tract infections, nosocomial infections and bacteremia in immunocompromised (Engel et al., 2009).

Regarding the analyzed samples of plant origin, more specifically leafy vegetables, the most isolated bacteria were of *Pseudomonas* genus 5/30 (16.67 %), including *Pseudomonas luteola* 2/30 (6.67 %), an opportunistic pathogen that can cause meningitis and septicemia (Chihab et al., 2004), and *Pseudomonas oryzihabitans* 2/30 (6.67 %), which are generally found in soil, stagnant water and even in drinking water supplies, it might cause Peritonitis, Endophthalmitis and bacteremia (Dussart-Baptista et al., 2007). In addition to *Pseudomonas aeruginosa* 1/30 (3.33 %), found in water environments, it infects vegetables such as lettuce, inducing symptoms of soft rot, as well as animals. For humans, it might be a very resistant and dangerous pathogen, whose ability to form biofilms makes it responsible of nosocomial infections, leading to a mortality rate of 50 % of immunocompromised patients (Rahme et al., 1997). *Proteus mirabilis* isolated and identified from animal origin products 2/43 (4.65 %) and from leafy vegetables 2/30 (6.67 %), is part of the normal microbiota of human and animal digestive tract. However it can also behave as an opportunistic pathogen (Kim et al., 2003), as well as *klebsiella pneumoniae* or *Morganella morganii*, which might be responsible for food poisoning due to histamines (Dworkin et al., 2005), in addition to *Providancia stuartii* and *Providencia rettgeri*, which have a major clinical role in urinary tract infections, particularly on urinary catheters, and bacteremia (Chamberland et al., 2013). Also, *Acinetobacter baumannii*, usually found in wet sludge, ponds and even plants, which is not part of the human commensal microbiota and whose transmission results from anthropization or through the hands in clinical environment (Bonnin et al., 2013).

During our analyzes *Aeromonas hydrophila* was isolated from minced meat sample and identified by Api 20E, this bacterium considered as an infectious agent, is generally a sign of food contamination by water, which can multiply even at 4 °C, inducing severe gastroenteritis and poisoning even if the contaminated food is refrigerated, in addition to extra-digestive infections and bacteremia, due to its ability to produce enterotoxins associated with hemolysins and cytotoxins (Janda and Abbott, 2010).

Finally, *Hafnia alvei* strain was isolated from a sample of fresh cheese. This enterobacteria is not pathogenic and is generally found in animal manure and it is also used as a probiotic (Janda and Abbott, 2006).

4.1.4. Isolation and identification of *Staphylococcus aureus*

According to the regulation CE N° 2073/2005 issued by the European Commission, concerning the microbiological criteria applicable to foodstuffs, then modified by the CE N° 1441/2017 and CE N° 229/2019, the presence of *S. aureus* in food is tolerated up to a defined level, beyond which the products are considered non-compliant and unsatisfactory. As for fresh cheese, the level of which must not exceed 10 CFU/g and beyond 10^5 CFU/g, the detection of staphylococcal enterotoxins must be carried out. Regarding meat products such as minced meat prepared by butcher, or cold meat preparations, the rate must be less than 10^2 CFU/g (BOE 211/86; FCD, 2020). According to Gilbert et al. (2000), the rate of *S. aureus* should be less than 10^2 CFU/g in fresh vegetables to be considered as a product of acceptable quality, and less than 20 CFU/g for a satisfactory product.

Even if the presence of *S. aureus* in the environment and more particularly its isolation from different types of available water (for human consumption, animals, agriculture, recreation) have been reported in multiple studies around the world (Charoenca and Fujioka, 1995; Hunter, 1997; Ibarluzea et al., 1998; Cheung et al., 2002; Harakeh et al., 2006; Faria et al., 2009; Adesoj et al., 2019), we could not find clear standards for the presence of *S. aureus* in environmental surface or irrigation waters.

Among all the minced meat samples analyzed, the presence of *S. aureus* was confirmed in 5/12 (41.67 %) samples, this rate is higher than those mentioned in various studies such as Siriken et al. (2004) in Turkey, Cohen et al. (2008) and Oumokhtar et al. (2008) in Morocco, Philips et al. (2008) in Australia, Chaalal et al. (2018) and Titouche et al. (2020) in Algeria as well as Atlabachew and Mamo (2021) in Ethiopia. However,

other works have published much higher rates, such as Belhaj and Elamrani (2019) in Morocco. The contamination of minced meat with *S. aureus*, if not of animal origin, might occur in butcher shops due to improper handling, such as handling paper money, or poor knowledge of hygiene practices, non-compliance with the cold chain, the usual use of equipment that are difficult to clean, but also the handling of carcasses in a narrow space, inducing permanent contact with workers, who could be asymptomatic carriers (Belhaj and Elamrani, 2019).

The presence of *S. aureus* in the fresh cheese samples analyzed, was confirmed in 8/21 (38.10 %) samples, which is higher than the results obtained by Jamali et al. (2015) in Iran, but significantly lower than those obtained from several studies about the hygienic quality of fresh cheese, such as Rosengren et al. (2010) in Sweden, Silva et al. (2010) in Brazile, Perdoma et al. (2015) in Venezuela, Vàsquez et al. (2018) in Peru, Espinoza et al. (2020) as well as Mendoza et al. (2020) in Ecuador. The main source of *S. aureus* contamination is probably dairy animals (in cases of mastitis) or even the milking process and equipment, resulting in contaminated milk. In addition to the water and the environment, which are also important sources, one of the most frequently mentioned sources of contamination, in research on the hygiene of fresh dairy products, is human handling (Bergonier et al., 2003; Jorgensen et al., 2005c; Rosengren et al., 2010).

From the analysis of leafy vegetables, the presence of *S. aureus* was confirmed in 3/30 (10 %) samples, including 1/10 sample for each kind (lettuce, spinach and chard), which is lower than the rate reported by Al-kharousi et al. (2016) in Oman from radishes. Other studies on the hygienic quality of raw vegetables have not isolated *S. aureus*, such as Al-Holy et al. (2013) in Saudi Arabia, Cardamone et al. (2015) in Italy as well as Klapec et al. (2016) in Poland.

Finally, the presence of *S. aureus* was confirmed in 1/15 (6.67 %) water sample, a rate equivalent to the work of Lechevallier and Seidler (1980) in USA, after analysis of rural drinking water samples. Harakech et al. (2006) in Lebanon reported a higher rate, with 45 % of *S. aureus* isolated strains resistant to at least 1 antibiotic, as well as the work of Adesoj et al. (2019) in Nigeria and Santos et al. (2020) in Brazil. These results are presented in **Table 10**.

Table 10. Isolation and identification of *Staphylococcus aureus*

Origin products	Analyzed samples		Positive samples
Animal	Minced meat	12	05/12 (41.66 %)
	Cold meat	10	00
	Fresh cheese	21	08/21 (38.10 %)
	Total	43	13/43 (30.23 %)
Vegetable	Lettuce	10	01/10 (10 %)
	Spinach	10	01/10 (10 %)
	Chard	10	01/10 (10 %)
	Total	30	03/30 (10 %)
Water	Water	15	01/15 (6.67 %)

Studies such as Al-Bahry et al. (2014), Kadariya et al. (2014) and Ho et al. (2015) suggest that the presence of *S. aureus* in raw food is mainly due to nasal carriers or human skin disease, whose main transmission pathway is through the hands. However even if there is no association with the development of infection through drinking water (WHO, 2017a) and it is not a bacterial indicator of fecal contamination, its presence in drinking or environmental water might be a serious threat on public health, especially if these strains carry antibiotics resistance genes, such as the MRSA strains, which could be transferred between bacteria (Pavlov et al., 2004; Percival et al., 2004; Abulreesh and Organji, 2011; Adesoji et al., 2019). According to study of Zieliński et al. (2020), MRSA (methicillin resistant *S. aureus*) strains were isolated from air samples of wastewater treatment plants, and from upper respiratory tract mucous membranes swabs, of plant workers which were allegedly contaminated with bioaerosols.

In addition to *S. aureus*, several strains of the Genus *Staphylococcus* were identified during this analysis by Api Staph strips (results shown in **Table 11**). Most isolates are part of the normal microbiota of human and animal skin, which could behave as opportunistic pathogens with the characteristic of easily acquiring antibiotics resistance genes, especially in hospital environment, such as; *Staphylococcus cohnii* ssp. Urealyticus (1 sample of minced meat), which might be the cause of bacteremia and urinary tract infections or even meningitis (Soldera et al., 2013), *Staphylococcus epidermidis* (1 sample of fresh cheese), a human and animal commensal found on the skin and mucous membranes, which have the ability to produce biofilms. Although not pathogenic it can be responsible for skin or nasal infections (Levinson, 2010) and *Staphylococcus capitis* (1 sample of chard), which is mainly present on the neck, face, scalp, ears and scrotum of humans, with an ability to produce biofilms, it is considered as

an opportunistic pathogen, associated with prosthetic endocarditis (Van Der Zwet et al., 2002).

Table 11. Identification of *Staphylococcus* from samples

Origin	Strains	Samples
Animal	*S. lentus*	3/43 (6.98 %)
	S. xylosus	2/43 (4.65 %)
	S. cohnii	1/43 (2.32 %)
	S. epidermidis	1/43 (2.32 %)
Vegetable	*S. lentus*	4/30 (13.33%)
	S. capitis	1/30 (3.33 %)
Water	*S. haemolyticus*	1/15 (6.67 %)

Among these different species *Staphylococcus lentus* was the most isolated, present in 3/43 (6.98 %) of animal origin analyzed samples, including 2/12 (16.67 %) of minced meat, 1/21 (4.76 %) fresh cheese and 4/30 (13.33 %) of leafy vegetables. This commensal species colonizes the skin of animals and workers, it is generally isolated from raw milk because of its association with mastitis in cattle. Its infectious effect on humans has recently been noted (Stepanović et al., 2005). Also, identified from 1/12 (8.33 %) meat sample and 1/21 (4.76 %) fresh cheese sample, *Staphylococcus xylosus* was isolated from 2/43 (4.65 %) animal origin analyzed samples. This bacterium is part of the traditional cheese microbiota, generally isolated from raw milk, cheese and sausage. It is rarely mentioned in human infections but can, nevertheless, behave as an opportunistic pathogen (Gozalo et al., 2010).

Regarding the water samples, *Staphylococcus haemolyticus* was isolated from 1/15 (6.67 %) of the analyzed samples. Although it is part of the human and domestic animal microbiota, this strain might be responsible for localized or systemic infections, due to its ability to produce biofilms, enterotoxins and/or hemolysins. Therefore it is recognized as an agent of nosocomial infection in hospitals (Fredheim et al., 2009).

According to the results of cultural method analysis, the rate of samples with a single microorganism was 25.58 % from animal origin products, of which 4.65 % contaminated only with *E. coli* and 20.93 % contaminated with *S. aureus*. 26.66 % of leafy vegetables, with 20 % contaminated with *E. coli* and 6.66 % contaminated with *S. aureus* and 60 % of the water samples were contaminated with *E. coli*. This shows a predominance of *E. coli* in the samples of leafy vegetables and water, unlike *S. aureus*, which was mostly isolated from animal origin products.

Regarding the number of samples with both *E. coli* and *S. aureus*, the highest rate was 9.30 % from animal origin products, followed by 6.67 % from water samples, then 3.33 % of leafy vegetable samples. All these results are shown in **Table 12**.

Table 12. Results of target microorganisms present in a single sample

Analyzed samples	Samples with one target microorganism		Samples with *E. coli* and *S. aureus*
	Only by *E. coli*	Only by *S. aureus*	
Animal origin	02/43 (4.65 %)	09/43 (20.93 %)	**04/43 (9.30 %)**
	11/43 (25.58 %)		
Vegetable origin	06/30 (20 %)	02/30 (6.66 %)	**01/30 (3.33 %)**
	8/30 (26.66 %)		
Water	09/15 (60 %)	00	**01/15 (6.67 %)**
	09/15 (60 %)		

4.1.5. Isolation and identification of *Listeria monocytogenes*

L. monocytogenes is a dangerous microorganism, the presence of which is not authorized according to the Regulation CE N° 2073/2005 issued by the European Commission, concerning the microbiological criteria applicable to foodstuffs, subsequently amended by the CE N° 1441/2017 and CE N° 229/2019, *L. monocytogenes* should not be detected in 25 g of foods such as minced meat intended for raw consumption, meat preparations such as cold meats and dairy products such as fresh cheeses. According to Gilbert et al. (2000) the presence of *L. monocytogenes* in vegetables should not exceed 20 CFU/g, for satisfactory products.

No *L. monocytogenes* was isolated and confirmed by Api Listeria from the samples analyzed. The absence of this strain would mean that the food products analyzed comply with the established requirements. These results are identical to those obtained in the work of Al-Holy et al. (2013) in Saudi Arabia as well as Belhaj and Elamrani (2019) in Morocco. On the other hand, the results of *L. monocytogenes* prevalence, obtained by Vitas et al. (2003) in Spain, Wu et al. (2015) in China, Byrnea et al. (2016) in Brezil, were between 2.22 and 34.9 %.

Multiple strains isolated from the selective culture media PALCAM and ALOA, showing characteristics similar to those of *L. monocytogenes*, were identified by Api Listeria strip (results shown in **Table 13**), including *Listeria grayi* in 1/43 (2.32 %) animal origin samples and 3/30 (10 %) vegetable origin samples, as well as *Listeria ivanovii* in 1/43 (2.32 %) animal origin samples and in 1/30 (3.33 %) vegetable origin samples. This

bacterium is known to be responsible of gastrointestinal diseases after ingestion and even for sepsis, it is therefore considered as opportunistic enteric human pathogen (Guillet et al., 2010).

Table 13. Identification of *Listeria* strains by Api Listeria from samples

Origin	Strains	Samples
Animal	*L. ivanovii*	01/43 (2.32 %)
	L. grayi	01/43 (2.32 %)
Vegetable	*L. grayi*	03/30 (10 %)
	L. ivanovii	01/30 (3.33 %)

4.2. Analysis of samples by molecular method, for the detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*

4.2.1. Selection of primers

The primers mentioned in **Table 5** (part 3.2.3), were chosen because they have all the characteristics required for multiplexing such as an adequate sequence length, within the preferable limits of 16-28 nucleotides, a GC percentage between 40-60 %, a melting temperature (T_m) approximately around 50-62 °C and no common "GC" nucleotide termini (Chuang et al., 2013). Moreover, the amplicon sizes resulting from the amplification for *E. coli* (670 bp), *S. aureus* (484 bp), *L. monocytogenes* (404 bp) and *S. enterica* (284 bp) are quite distinct from each other, to be clearly identified on the electrophoresis gel. But also, because their effectiveness has been proven in numerous studies, especially in multiplex PCR (Duplex and Triplex) Kim et al. (2006), Germini et al. (2009), Yuan et al. (2009), Zhang et al. (2012), Rajabzadeh et al. (2018) and Wei et al. (2018).

4.2.2. Primers validation by simplex PCR and temperature gradient

Correct running of each primers pair was confirmed separately by simplex PCR. When the amplification of *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enterica* specific genes, produced amplicons of different sizes corresponding to 670 bp, 484 bp, 404 bp, 284 bp, respectively, appearing as distinct and bright bands on electrophoresis gel, without any non-specific product. This means that each pair of primers tested was sufficiently sensitive and specific, to detect its target strain.

To prepare each simplex PCR, the reaction mixtures were composed of 2.9 µL Reaction Buffer 10×NH_4, 3 mM $MgCl_2$ Solution, 2.5 Units Taq DNA polymerase (BIOTAQ™ DNA Polymerase-BIOLINE), 0.20 mM dNTPs (dNTP Mix-BIOLINE) and

the respective pair of primers, for 4 µL of target DNA in a total volume of 29 µL. The primers concentrations were 0.40 µM GADA670, 0.40 µM Nuc484, 0.40 µM LM404 and 0.20 µM SalinvA284. In order to find an optimal common temperature between the four primers, temperature gradients were carried out under the same conditions (simplex PCR), with the annealing temperature as a single variable. The results are shown in **Figure 1**.

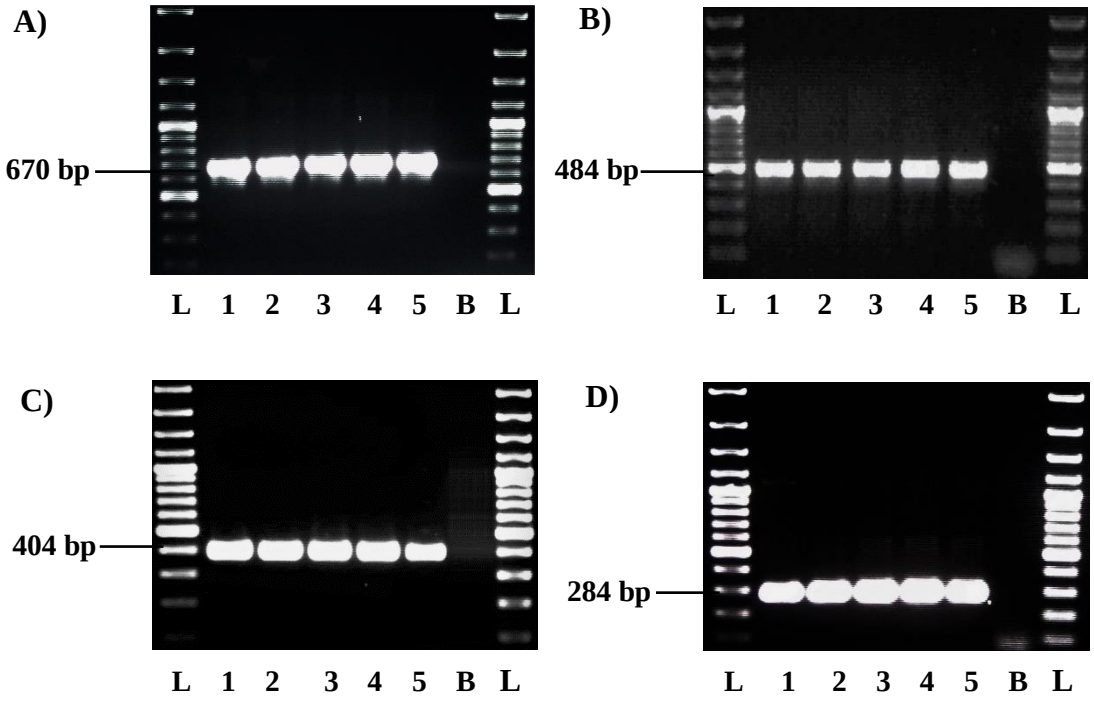


Figure 1. Annealing temperature gradient, for simplex PCR:

A: Temperature gradient of *E. coli* primers: L: 100 bp DNA ladder. Lane 1 to 5 shows the amplicon results of *E. coli*, with annealing temperature changes 55, 56, 57, 58, 60 °C. B: Negative control; **B: Temperature gradient of *S. aureus* primers:** L: 100 bp DNA ladder. Lane 1 to 5 shows the amplicon results of *S. aureus*, with annealing temperature changes 55, 56, 57, 58, 60 °C. B: Negative control; **C: Temperature gradient of *L. monocytogenes* primers:** L: 100 bp DNA ladder. Lane 1 to 5 shows the amplicon results of *L. monocytogenes*, with annealing temperature changes 55, 56, 57, 58, 60 °C. B: Negative control; **D: Temperature gradient of *S. enterica* primers:** L: 100 bp DNA ladder. Lane 1 to 5 shows the amplicon results of *S. enterica*, with annealing temperature changes 55, 56, 57, 58, 60 °C. B: Negative control.

For *E. coli* and *S. aureus*, the intensity was high and almost constant, even with the increase of annealing temperature; however, a slight increase in band intensity was recorded at 58 °C and 60 °C. For *L. monocytogenes*, the intensity was strong and constant from 55 °C to 58 °C, with a slight decrease at 60 °C. Unlike *S. enterica*, where the lowest intensity was observed at 55 °C, the highest and stable from 56 °C to 60 °C. Therefore,

58 °C was the selected annealing temperature and the resulting conditions, which will be used for next steps, are shown in **Table 14**.

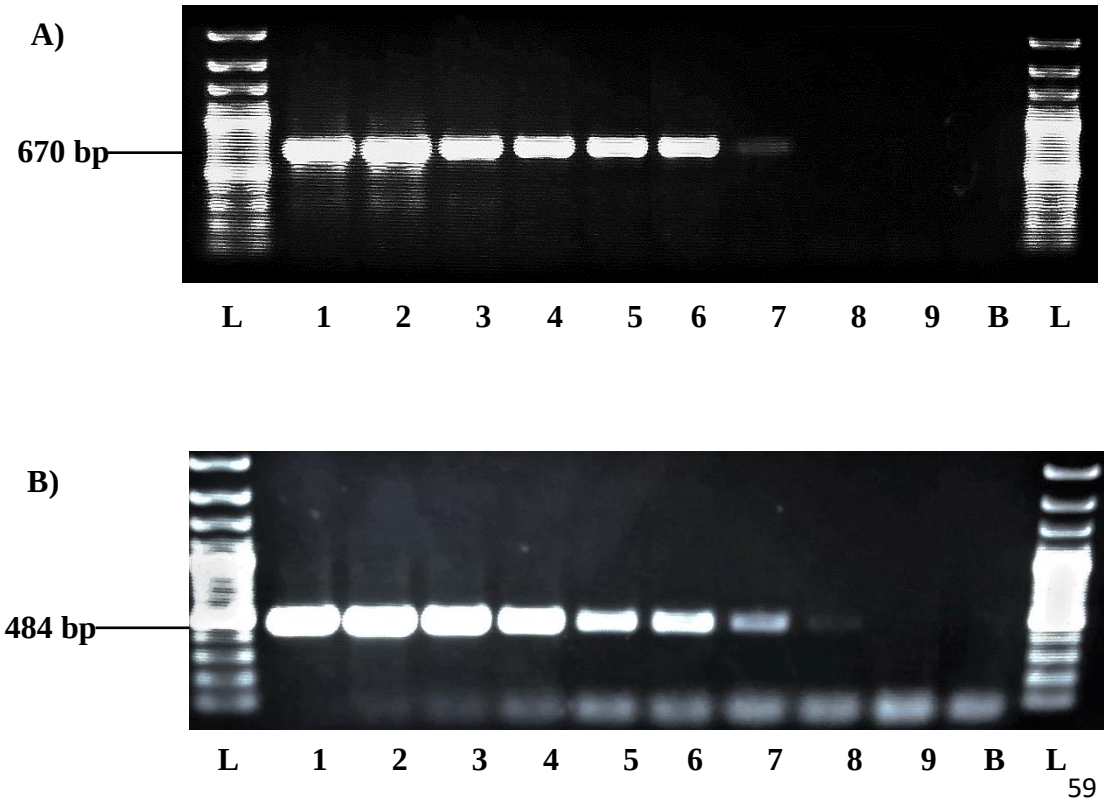
Table 14. Simplex PCRs conditions

PCR conditions		
Initial Denaturation	2 min	94 °C
Cycles	35	
Denaturation	30 s	94 °C
Annealing	30 s	58 °C
Extension	60 s	72 °C
Final Extension	7 min	72 °C

4.2.3. Evaluation of primers sensitivity in simplex PCR

To determine the sensitivity of each pair of primers, under the same conditions, at an annealing temperature of 58 °C, several simplex PCRs were carried out according to a decreasing cell concentration, determined by viable counting on the respective selective media and expressed in CFU/mL.

According to the results presented in **Figure 2**, the detection limits for *S. aureus* were 10^1 CFU/mL and 10^2 CFU/mL for *E. coli*, *L. monocytogenes* and *S. enterica*. This means that, although this temperature is more favorable for the detection of *S. aureus*, it remeans correct for the detection of the other three bacteria studied. Consequently, these conditions and this annealing temperature (58 °C) will be used for the preliminary multiplexing tests.



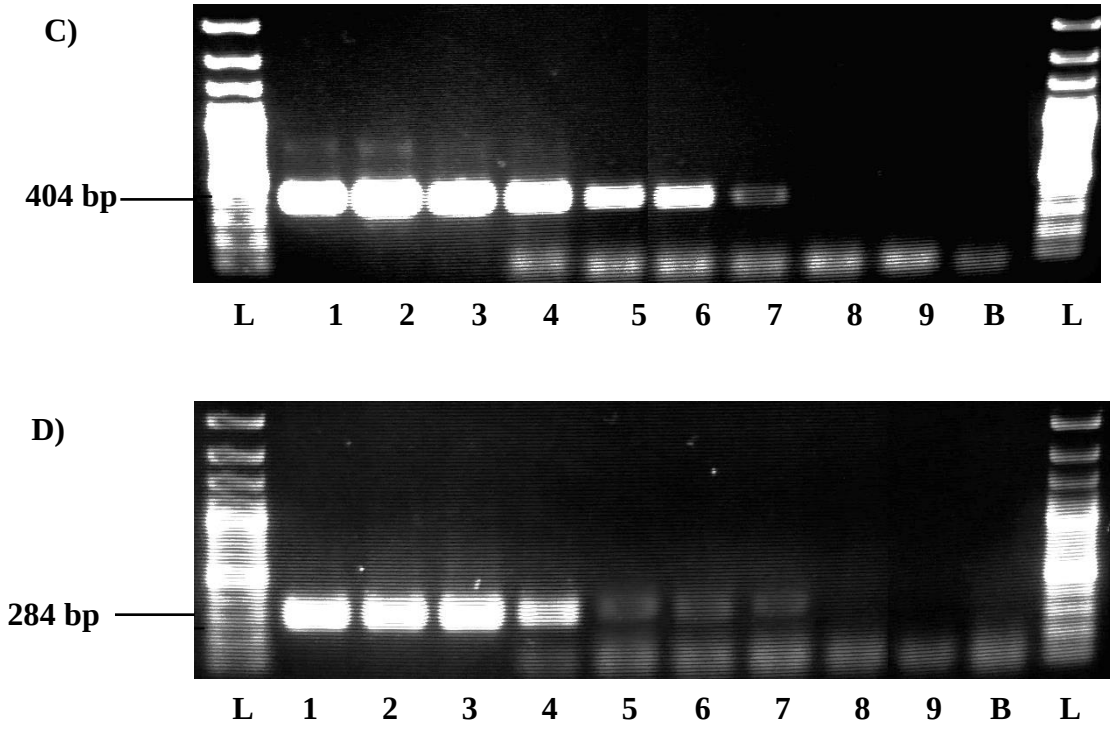


Figure 2. Detection sensitivity of primers, in Simplex PCR:

A: Sensitivity of *E. coli* primers GADA 670 bp: L: 100 bp DNA ladder. Lane 1 to 9 shows the amplicon results of *E. coli*, with change of cell concentration used 10^8–10^0 CFU/mL. B: Negative control; **B: Sensitivity of *S. aureus* primers Nuc 484 bp:** L: 100 bp DNA ladder. Lane 1 to 9 shows the amplicon results of *S. aureus*, with change of cell concentration used 10^8–10^0 CFU/mL. B: Negative control; **C: Sensitivity of *L. monocytogenes* primers LM 404 bp:** L: 100 bp DNA ladder. Lane 1 to 9 shows the amplicon results of *L. monocytogenes*, with change of cell concentration used 10^8–10^0 CFU/mL. B: Negative control; **D: Sensitivity of *S. enterica* primers SalinA 284 bp:** L: 100 bp DNA ladder. Lane 1 to 9 shows the amplicon results of *S. enterica*, with change of cell concentration used 10^8–10^0 CFU/mL. B: Negative control.

4.2.4. Analysis of samples by simplex PCR

For the detection of *E. coli*, 39 samples were tested by simplex PCR, at annealing temperature of 58 °C, using DNA extracted from selective enrichment aliquots. After comparing these results (shown in **Table 15)**, we found that the detection of *E. coli* by cultural method (Api 20E) and by PCR coincides with 74.36 % (29/39) of the samples tested, including 53.85 % (21/39) of positive detections and 20.51 % (8/39) of negative detections. Above all, the use of PCR allowed additional detection of *E. coli* from 25.64 % (10/39) of the samples tested compared to the cultural method.

Table 15. Comparison of detection results by cultural method and by simplex PCR

Strains	Samples tested by PCR	Cultural and PCR (same results)	Cultural and PCR (different results)
E. coli	39	21/39 (Both positive) 08/39 (Both negative)	10/39 (PCR-positive)
		74.36 %	**25.64 %**
S. enterica	23	10/23 (Both negative)	13/23 (PCR-positive)
		43.48 %	**56.52 %**
S. aureus	26	14/26 (Both positive)	12/26 (PCR-positive)
		53.85 %	**46.15 %**
L. monocytogenes	31	12/31 (Both negative)	19/31 (PCR-positive)
		38.71 %	**61.29 %**

Regarding the detection of *S. enterica* 23 samples were tested by simplex PCR, using DNA extracted from selective enrichment aliquots. The results obtained after comparison with the detection by cultural method (Api 20E), which did not lead to any positive results, showed similarities of negative results of 43.48 % (10/23) of the tested samples, but above all the PCR allowed the detection of *S. enterica* from 56.52 % (13/23) of the tested samples.

For the detection of *S. aureus*, 26 samples were tested by simplex PCR, using DNA extracted from selective enrichment aliquots. After a comparison with the detection results obtained by cultural method (Api Staph), we noticed that 53.85 % (14/26) of the tested samples were positive with both detection methods, and additional detections of *S. aureus* were confirmed in 46.15 % (12/26) of the tested samples by PCR, compared to the cultural method.

In order to detect *L. monocytogenes*, 31 samples were tested by simplex PCR, using DNA extracted from aliquots of selective pre-enrichment and enrichment, then the results obtained were compared with the cultural detection method (Api Listeria), which gave no positive results. 38.71 % (12/31) of the tested samples were negative with both detection methods. However *L. monocytogenes* has been detected in 61.29 % (19/31) of the samples tested by PCR.

This difference between the two detection methods does not necessarily mean that the detection by PCR is more efficient. Since the PCR is based on the amplification of the present DNA, it will give a positive detection even if the cell is dead or if there is only DNA in the sample. Other possibilities; the quantity of cells present in the samples was not important enough to be detected, which would mean that the standards were correctly applied, or that the cells were not in good physiological state, they could be too stressed

or damaged by storage temperature, by the pH, or the preservatives products, to grow on culture medium. However, this method is faster, easier and above all more specific.

Therefore, its use in addition to detection by conventional cultural method, would increase the efficiency of food analyzes, especially for fastidious pathogens, or in poor physiological condition, which are difficult to develop on culture media, or even in case of dangerous strains such as *Salmonella* spp. and *L. monocytogenes*, the presence of which is not tolerated.

4.3. Simultaneous co-culture and detection of *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* by multiplex PCR

4.3.1. Selection of a co-culture medium

4.3.1.1. Effect of various culture broths on individual growth

Based on the evaluation of individual cultures performed in several media, the selective co-enrichment medium SSSLE was included in the comparison due to its promising results, to enrich specifically *S. aureus*, *S. enterica*, *S. flexneri*, *L. monocytogenes* and *E. coli*, showed the lowest performance. Although formulated on the basis of BPW composition, with inhibitors for the selectivity against food background microbiota and growth promoters, such as esculin for *L. monocytogenes* and mannitol for *S. aureus*, this broth was the least effective, with the lowest growth rate for *E. coli* and no growth for *S. enterica*, *S. aureus* or *L. monocytogenes.*

Concerning the growth rate resulting after 24 h incubation in non-selective media, it varied between 10^8 and 10^{10} CFU/mL for *S. aureus*, exceeded 10^8 CFU/mL for *E. coli* and for *S. enterica* and *L. monocytogenes* growth was ranging between 10^7 and 10^8 CFU/mL. However, growth rates obtained from individual cultures produced in selective broths, usually used in the selective enrichment step of ISO standards, were much lower for *S. aureus* in GC, for *E. coli* in BGBLB and for *S. enterica* in RV, compared to the rates obtained from individual cultures in non-selective broths. All these results are shown in **Table 16**.

Table 16. Individual culture growth in CFU/mL

Bacteria	Selective enrichment broths (BGBLB, RV, GC, FB)	NB	BPW	LB	SSSLE
E. coli	9.30×10^7	1.48×10^9	$1{,}19\times10^9$	2.73×10^8	5.20×10^5
S. enterica	6.83×10^7	3.29×10^8	$4{,}10\times10^8$	$4{,}26\times10^7$	0
S. aureus	1.74×10^8	7.70×10^{10}	1.07×10^9	$7{,}88\times10^8$	0
L. monocytogenes	8.65×10^9	8.15×10^8	$9{,}97\times10^7$	3.49×10^8	0

Similar results were mentioned for the growth of *S. enterica* serotype Enteritidis, in RV broth by Yu et al. (2010). Unlike FB broth, which obtained the highest growth rate for *L. monocytogenes*. According to Yu et al. (2010) and Chen et al. (2015), who obtained similar results, the growth of *L. monocytogenes* is promoted by the esculetin resulting from the hydrolysis of esculin, which reacts with ferric ions of the medium.

Among the several culture broth evaluated in individual culture, the two media showing the best recovery performance, for the four target bacteria were NB and BPW, with a slightly higher rate for *S. aureus* and *L. monocytogenes* in NB, which may be due to the presence of beef extract in its composition. Abd El-Salam et al. (2010) obtained similar results for *L. monocytogenes* growth in NB.

Although NB broth had better performance during individual growth, BPW seems to be a better choice as a co-culture broth, because the growth rates between the 4 bacteria were relatively close and the difference between them was less important than that obtained from NB, especially between *S. aureus* (7.70×10^{10} CFU/mL) and *S. enterica* (3.29×10^{8} CFU/mL), which may be important during a simultaneous culture, to guarantee a balanced growth and to avoid accentuating the potential effect of competition, that could favor the growth of one bacteria at the expense of the others and thus affecting the detection. Moreover, to identify a possible inhibitory effect on the PCR during meat samples analysis, the chosen co-culture broth should not contain any kind, thereby NB medium which is composed of beef extract was not selected.

Alarcon et al. (2004) obtained very good results with BPW, for the simultaneous detection of *Salmonella* spp., *S. aureus* and *L. monocytogenes* from artificially inoculated meat sample in BPW. Same thing in the work of Wang and Suo (2011), who obtained good detection after 16 h of growth in BPW, using meat artificially contaminated by *Escherichia coli* O157 and *Salmonella* Enteritidis, without any growth inhibitor effect of the background microbiota. In addition to its growth performance and its high ability to elute the bacteria from leafy vegetables such as lettuce, mentioned in the work of Rajabzadeh et al. (2018), BPW was chosen as a co-culture broth because it does not contain any kind of meat extract in its composition, it can ensure a relatively balanced growth between the 4 target bacteria and it is generally used in the pre-enrichment step during food analysis of ISO protocols.

4.3.1.2. Buffered Peptone Water as co-culture broth

4.3.1.2.1. Effect of BPW on individual and co-culture growth

To evaluation the recovery ability of BPW, individual pure cultures and co-cultures were carried out from low initial inoculum concentrations (10^3, 10^2, 10^1 CFU/mL). All the results are presented in **Table 17**.

Table 17. Recovery rates values in CFU/mL of individual and co-culture growths in BPW

Strains	Initial inoculum	Individual culture	Co-culture
E. coli	10^3 CFU/mL	8.55×10^8	7.70×10^8
S. aureus		3.27×10^8	1.70×10^6
L. monocytogenes		2.10×10^7	4.60×10^5
S. enterica		2.20×10^8	1.60×10^7
E. coli	10^2 CFU/mL	7.50×10^8	5.65×10^8
S. aureus		4.60×10^8	2.70×10^5
L. monocytogenes		1.75×10^7	1.38×10^5
S. enterica		1.28×10^8	9.95×10^6
E. coli	10^1 CFU/mL	5.15×10^8	4.20×10^8
S. aureus		1.32×10^8	4.20×10^5
L. monocytogenes		1.50×10^7	6.85×10^4
S. enterica		1.08×10^8	9.75×10^6

After incubation, the results of the individual cultures showed relatively stable and close rates, over 10^8 CFU/mL for *E. coli*, *S. aureus* and *S. enterica*, and over 10^7 CFU/mL for *L. monocytogenes*. The variation in the initial inoculum concentrations induced a slight decrease in the recovery rates, *E. coli* obtained the highest and most stable recovery rates (8.93-8.71 $\log_{10}$ CFU/mL), closely followed by *S. aureus* (8.51-8.12 $\log_{10}$ CFU/mL) and *S. enterica* (8.34-8.03 $\log_{10}$ CFU/mL). *L. monocytogenes* obtained the lowest recovery rates (7.32-7.18 $\log_{10}$ CFU/mL), which nevertheless remains widely detectable by most biomolecular tools. Consequently, the initial inoculum concentration, even very low, did not affect the recovery rate obtained in individual culture.

Regarding the co-cultures, the recorded recovery rates were more affected by the competition effect of the simultaneous growth, than decrease in the inoculum concentration. This competitive effect was demonstrated by the comparison between the growth rates obtained from individual cultures and from co-cultures, for each target bacteria. From the same initial inoculation concentration, the maximum density of all target microorganisms in co-cultures decreased, compared to individual cultures. Gram-negatives seem to be the least affected, with an average decrease between 0.09-1.10 $\log_{10}$

CFU/mL, unlike Gram-positives, which showed a more significant decrease in co-culture, with an average decrease of 2.03-2.39 log_{10} CFU/mL. This competitive effect during simultaneous growth has been mentioned in co-enrichment media works, such as Gehring et al. (2012) or Chen et al. (2015).

The growth rates of *E. coli* remained stable, meaning it was not affected by the presence of other bacteria, while *S. enterica* showed a slight decrease. Regarding *L. monocytogenes* the growth rates recorded during co-culture were lower than in individual culture, which is in accordance with Daley et al. (2014), who showed that the competitive effect exerted by *Enterobacteriaceae* could lead to a decrease ranging from 1 to 4 logs, in *L. monocytogenes* population during 48 h of co-enrichment. Kim and Bhunia (2008) explains that the lower growth rate for *L. monocytogenes*, known to be a slow-growing bacteria and a poor competitor, was likely due to the rapid growth of *E. coli* and *Salmonellae*, which used up most of the nutrients and depleted the culture medium.

Although the maximum population rate of *S. aureus* in co-culture was not the lowest (6.23-5.62 log_{10} CFU/mL), it was nevertheless the most affected by the presence of other bacteria, showing the greatest decrease in growth rate during co-culture, compared to the individual culture. Some studies have reported the antagonistic effect of *E. coli* on the growth of *S. aureus* in nutrient broth at 37 °C, this inhibition decreased with the reduction of *E. coli* inoculum, although *E. coli* does not cause any obvious inhibition of *S. aureus* on spot plates, it could nevertheless strongly suppress the growth of *S. aureus* in liquid medium (Quinto et al., 2020).

Although the recovery of the four studied microorganisms was lower in co-culture, compared to the recovery from individual cultures, the rates remain more than enough for detection by multiplex PCR.

4.3.1.2.2. Effect of BPW on co-culture growth, from artificially inoculated food matrix

To evaluate the BPW recovery capacity, from co-culture in the presence of food matrices, two types of food were artificially inoculated at 10^3 CFU/mL of each target bacteria.

The two samples (lettuce and minced meat) were chosen as RTE food models, because they are usually consumed with little or without any prior heat treatment. Lettuce mainly contaminated by soil or irrigation water and minced meat mainly by handling,

could be vectors of Shiga toxin-producing *E. coli* (STEC), *Salmonella* spp., *L. monocytogenes* and *S. aureus* (Herman et al., 2015; Zilelidou et al., 2016; Latha et al., 2017; Rajabzadeh et al., 2018).

Regarding the co-culture in the presence of lettuce, the recovery rates were around 10^8 CFU/mL for the Gram-negatives, thus dominating the Gram-positives, which showed a recovery of about 10^6 CFU/mL. For the artificially inoculated minced meat sample, the recorded recovery rates were lower than those resulting from the lettuce sample, ranging 10^7 CFU/mL for the Gram-negatives and up to 10^5 CFU/mL for *L. monocytogenes*, unlike *S. aureus* which had a higher recovery rate. These results are shown in **Table 18**.

Table 18. Recovery rates values of co-culture growth in CFU/mL, in BPW with and without food matrices initially inoculated at 10^3 CFU/mL

Strains	Co-culture Without matrix	Co-culture with Lettuce matrix	Co-culture with Minced meat matrix
E. coli	7.70×10^8	2.00×10^8	5.20×10^7
S. aureus	1.70×10^6	8.75×10^6	4.45×10^7
L. monocytogenes	4.60×10^5	1.30×10^6	4.85×10^5
S. enterica	1.60×10^7	1.32×10^8	5.50×10^7

However, compared to the BPW co-culture without food matrix, a slight increase of cell density was recorded in the presence of lettuce, around 0.92 Log_{10} CFU/mL for *S. enterica*, 0.71 Log_{10} CFU/mL for *S. aureus*, as well as 0.45 Log_{10} CFU/mL for *L. monocytogenes*, inducing a decrease of 0.59 Log_{10} CFU/mL for *E. coli*.

The same observation was made, for the co-culture in the presence of minced meat, compared to the BPW co-culture without food matrix, where the cell concentrations recovered were higher for *S. aureus* of 1.42 Log_{10} CFU/mL. For *S. enterica* and for *L. monocytogenes*, the difference was not as significant. Regarding *E. coli*, a decrease of 1.17 Log_{10} CFU/mL was recorded, compared to the co-culture without matrix.

These results attest that, there was no significant inhibitory effect of the food matrix, nor its background microbiota during the co-culture in BPW, so it is not necessary to use selective agents. Moreover, the presence of food matrices seems to have enriched the co-culture, thus boosting the growth of less favored and dominated bacteria, during a co-culture.

4.3.2. Development and Optimization of multiplex PCR

4.3.2.1. *In silico* validation of primers

In silico evaluation of pairs of primers, selected from the literature, using the Primer-Blast program, showed 100 % homology between each sequence and its target gene, on the first hundred alignment results, which means that each pair of primers detects only its own target microorganism and there is no risk of interference with other bacteria, during multiplexing.

Each pair of primers submitted to the Multiple Primer Analyzer program, first analyzed individually, showed no possibility of secondary structures production, which might be due to intramolecular interactions (Hairpins) and intermolecular interactions (Self Dimer or Cross Dimer). These structures affect the annealing with template in the wrong way, by significantly reducing the availability of primers in the reaction, resulting in unnecessary amplifications and poor yield (Oscorbin et al., 2021). Then, from the comparison of the four pairs of primers with each other, no complementarity was detected, which might lead to hybridization between primers, leading to non-specific amplifications.

Therefore, the *in silico* analysis confirms, on the one hand, the specificity of each pair of primers for its target DNA and on the other hand, the compatibility of their simultaneous use during multiplexing, due to the lack of complementarity (data not displayed by its extension).

4.3.2.2. Development of multiplex PCR

The development of multiplex PCR, capable of simultaneously detecting four microorganisms in a single reaction (quadruplex PCR), was carried out gradually, initially, progressively, first, by the combination of primers-template in duplex and triplex PCR. These results are shown in **Figure 3**.

Among the combinations carried out, all the duplex PCRs were able to clearly and correctly detect the two microorganisms, without any non-specific product. Bands intensity of *E. coli*, *S. aureus* and *L. monocytogenes* on electrophoresis gel was strong, but for *S. enterica*, the intensity was the weakest in each duplex combination. Regarding the combinations in triplex PCR (shown in **Figure 3**), the presence of artifacts between 1200 and 1300 bp, at high intensity was recorded for two combinations and a significant decrease in *S. aureus* and *S. enterica* bands intensity, for the other triplex combinations.

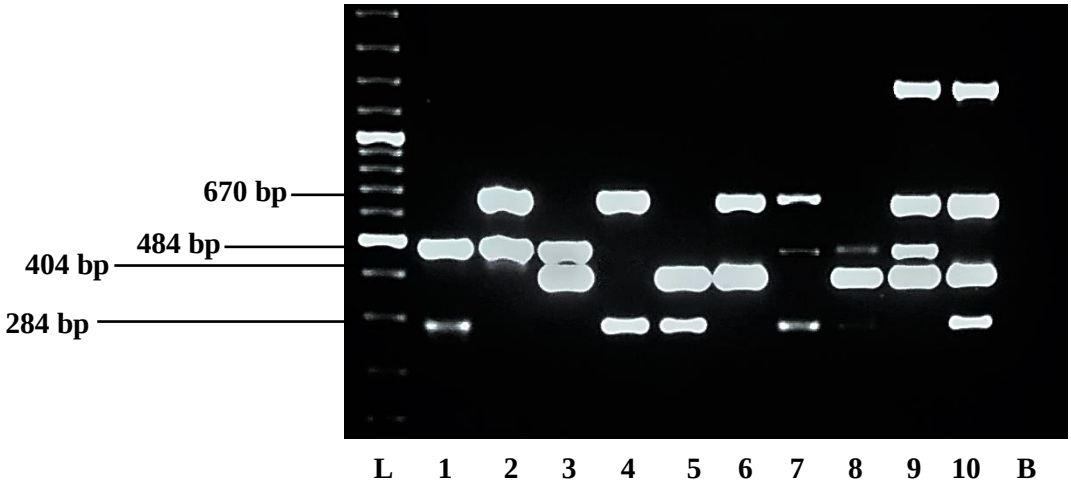


Figure 3. Combination of primers in duplex and Triplex PCRs:

L: 100 bp DNA ladder. Lane 1 to 6 shows amplicon results of duplex PCRs combinations (Mix PCR + 2 primers pairs + 2 targets DNA). Lane 7 to 10 shows amplicon results of triplex PCRs combinations (Mix PCR + 3 primers pairs + 3 targets DNA). The primers GADA 670 bp, Nuc 484 bp, LM 404 bp and SalinA 284 bp for the detection of *E. coli, S. aureus, L. monocytogenes* and *S. enterica* respectively. B: Negative control.

Then, the mix of all primers pairs and their target DNAs, in a single reaction, resulted in the amplification of all targets, whose bands were all clear and distinct (result shown in **Figure 4**; Lane M). Unfortunately, an artifact production was recorded, one high intensity band and a second less intense, between 1200-1300 bp. These non-specific products cannot be linked to the specificity of the primers, which has been proven *in silico* and *in vitro*, nor to contamination because the negative control (B) was negative. Although this experiment was performed in triplicate, the presence of these non-specific bands during the multiplex PCR could not be clearly explained.

Regarding the individual introduction of each target DNA in a PCR reaction containing the four pairs of primers, under the same conditions (results are shown in **Figure 4)**, the amplicons resulting from the amplification of each target gene were very clear and distinct, without any nonspecific product. Thus the absence of cross-hybridization, which might lead to poor priming, confirms the specificity of the primers towards their target genes.

Although the amplification of the target genes was clear, the band intensities were not homogeneous. From the reaction mixture composed of all the primers and their target DNAs, the signal intensity emitted by *E. coli* and *L. monocytogenes* amplicons was much higher. Compared to the amplification results, from the reaction mixture composed of all

the primes with a single DNA target, the order of intensity was the same. However, a significant decrease of *S. aureus* and *S. enterica* band intensity was recorded.

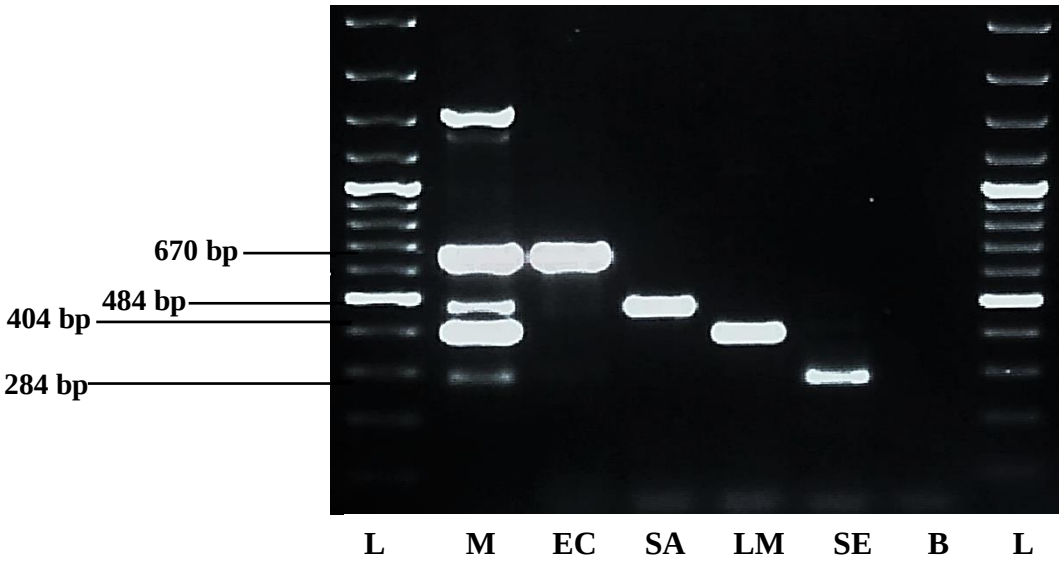


Figure 4. Development of multiplex PCR:

L: 100 bp DNA ladder. M: Multiplex PCR (Mix PCR + DNA *E. coli* 101 CECT, *S. aureus* 435 CECT, *L. monocytogenes* 936 CECT and *S. enterica* 4266 CECT + Their primer pairs GADA 670 bp, Nuc 484 bp, LM 404 bp and SalinA 284 bp, respectively). EC: Mix PCR + DNA *E. coli* 101 CECT + The 4 primer pairs. SA: Mix PCR + DNA *S. aureus* 435 CECT+ The 4 primer pairs. LM: Mix PCR + DNA *L. monocytogenes* 936 CECT + The 4 primer pairs. SE: Mix PCR + DNA *S. enterica* 4266 CECT+ The 4 primer pairs. B: Negative control.

This means that during multiplexing, there was a competition effect between the different amplification ways, where the reactions of *S. aureus* and *S. enterica* were the least favored, compared to *E. coli* and *L. monocytogenes*. Consequently, although multiplex PCR was able to clearly detect the four microorganisms in a single reaction, optimization tests are needed to improve detection efficiency.

4.3.2.3. Optimization of multiplex PCR

Multiplex PCR optimization tests aimed to eliminate artifacts and non-specific products, by reducing the number of cycles during amplification from the initial number of 35 cycles to 30, 25, 23 and 20 cycles, as well as increasing the annealing temperature from the initial temperature chosen 58 °C to 59, 60 and 62 °C. The results summarizing some steps of this long and very complex process are shown in **Figure 5**.

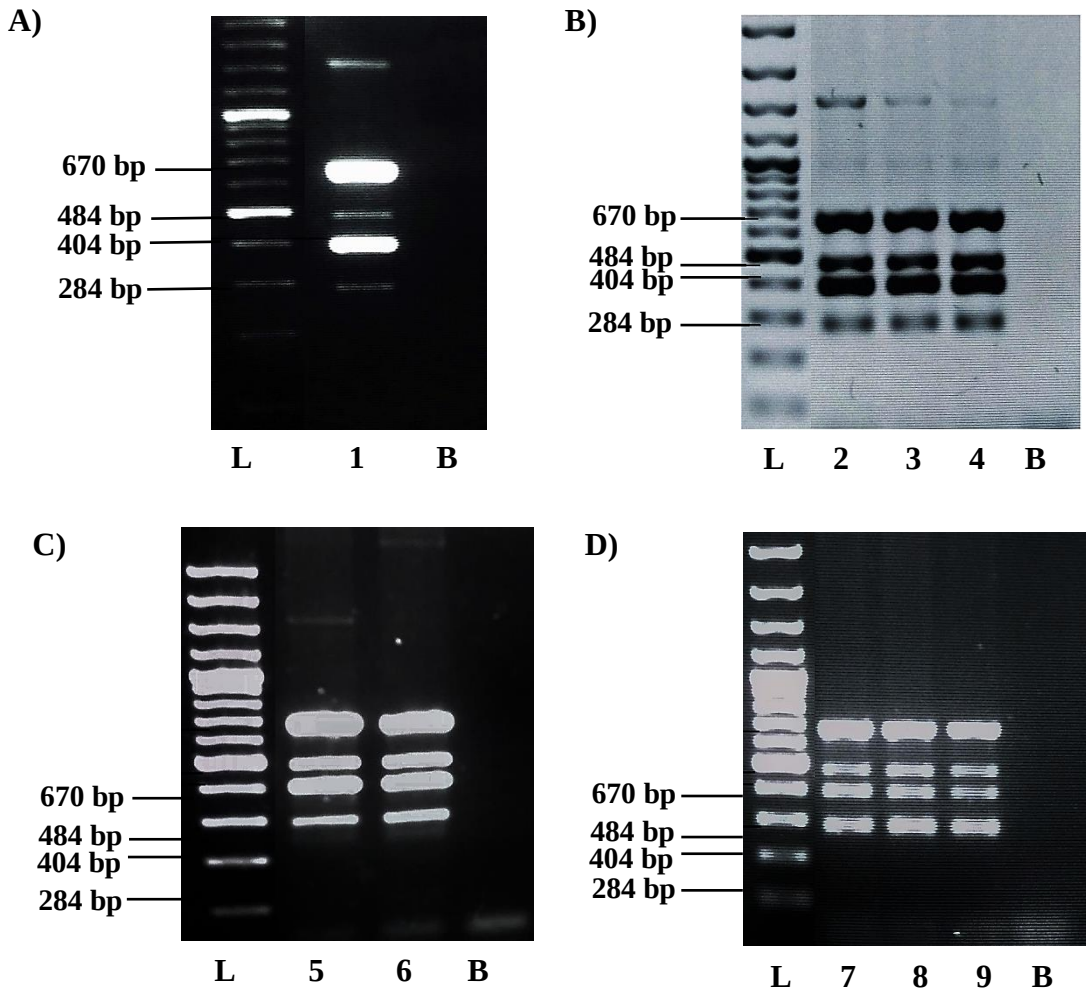


Figure 5. Optimization of multiplex PCR:

A-D: Multiplex PCR reactions (Mix PCR + DNA *E. coli* 101 CECT, *S. aureus* 435 CECT, *L. monocytogenes* 936 CECT and *S. enterica* 4266 CECT + Their primer pairs GADA 670 bp, Nuc 484 bp, LM 404 bp and SalinA 284 bp, respectively). L: 100 bp DNA ladder. B: Negative control. **A:** Optimization by reducing the number of cycles to 30. 1: Multiplex PCR 30 cycles/58 °C; **B:** Optimization by reducing the number of cycles to 25, annealing temperature variable. 2: 58 °C. 3: 59 °C. 4: 60 °C; **C:** Optimization by reducing the number of cycles to 23, annealing temperature variable. 5: 58 °C. 6: 59 °C; **D:** Optimization by reducing the number of cycles to 20, increasing annealing temperature. 7: 59 °C. 8: 60 °C. 9: 62 °C.

The changes in the annealing temperature and the number of cycles, which were first applied separately did not lead to significant results regarding the presence of the non-specific products, hence the annealing temperature and the number of cycles variations were combined. From this combination we noticed that the increase in annealing temperature and the decrease in the number of cycles resulted in the disappearance of the non-specific bands, in addition to the improvement of the homogeneity between the of the four target amplicons intensity.

According to the amplification results the best detection from the point of view of non-production of artifacts and the homogeneity of intensity between the 4 target amplicons were obtained with the conditions of annealing temperatures between 59 °C and 60 °C and a number of cycles of 20. However, with these conditions the multiplex PCR detection limits were seriously affected, which could be due to the low number of amplification cycles. Concequentely, the selected annealing temperature was 59 °C and the number of cycles was maintained at 35.

Although the variations in the annealing temperature improved the homogeneity of intensity between the four amplicons, variations in the quantities of primers had to be made, in order to find the right balance. To do this, the amounts of *E. coli* (0.4 µM) and *L. monocytogenes* (0.4 µM) primers, which present the most intense and invariable bands, were reduced compared to those of *S. aureus* (0.4 µM) and *S. enterica* (0.2 µM), whose primers quantities have been increased. From these variations, the most homogeneous detection of the 4 target amplicons was obtained with the primers quantities of: 0.2 µM GADA670, 0.16 µM LM404, 0.46 µM SalinvA284 and 1 µM Nuc484.

In addition to equilibrating the primers, the amounts of $MgCl_2$ (3 mM) and dNTPs (0.20 mM) were slightly increased to 3.4 mM and 0.22 mM respectively, because of the large number of amplification pathways, in a single reaction.

One of the main factors affecting the PCR sensitivity is the establishment of a reliable DNA extraction procedure (Omiccioli et al., 2009; Ma et al., 2014). On this basis, the impact of the DNA extraction method on the PCR multiplex detection was evaluated. After amplification, the detection from DNA matrix extracted by purification method, using GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich) with Lysozyme was correct and very clear, for each DNA amplified individually or simultaneously, from pure cultures and co-cultures of reference strains (results shown in **Figure 6A**). On the other hand, amplifications performed from DNA extracted by thermal lysis method (results shown in **Figure 6B**), although individually correct, which confirms its effectiveness on Gram-positive and Gram-negative, even without lysozyme, the multiplex PCR could only detect three of the four targets. From the comparison of the two methods, it seems that with the thermal lysis method, the amount and the quality of the DNA recovered are much lower than those resulting from the extraction by kit. This affects the multiplex PCR detection, thus inducing false negative results.

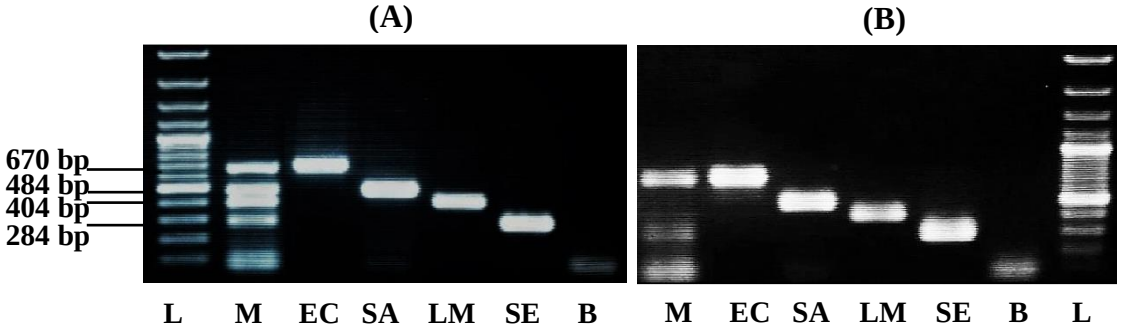


Figure 6. Multiplex PCR after optimization and impact of DNA extraction methods:

A: DNA extracted per kit; **B:** DNA extracted thermal lysis method. L: 100 bp DNA ladder. M: Multiplex PCR (Mix PCR + DNA *E. coli* 101 CECT, *S. aureus* 435 CECT, *L. monocytogenes* 936 CECT and *S. enterica* 4266 CECT + Their primer pairs GADA 670 bp, Nuc 484 bp, LM 404 bp and SalinA 284 bp, respectively). EC: Mix PCR + DNA *E. coli* 101 CECT + The 4 primer pairs. SA: Mix PCR + DNA *S. aureus* 435 CECT+ The 4 primer pairs. LM: Mix PCR + DNA *L. monocytogenes* 936 CECT + The 4 primer pairs. SE: Mix PCR + DNA *S. enterica* 4266 CET+ The 4 primer pairs. B: Negative control.

Which means that, although the boiling method is economical, saves time and labor-less (Zhang et al., 2014; Wei et al., 2018), DNA extraction by kit is much more efficient in terms of the quantity and quality of recovered DNA, which is important during the detection from small amounts of matrix or from co-cultures.

From the optimization results of the four simplex PCRs and all the optimization tests of the multiplex PCR, which were focused on the annealing temperature, the number of cycles, the balance of primers quantities and the adjustment of $MgCl_2$ and dNTPs quantities, the best detection results were obtained with the following conditions (presented in **Figure 6A**): 2.9 µL Reaction Buffer 10×NH_4, 3.4 mM $MgCl_2$ Solution, 2.5 Units Taq DNA polymerase (BIOTAQ ™ DNA Polymerase-BIOLINE), 0.22 mM dNTPs (dNTP Mix-BIOLINE), primers concentrations 0.2 µM GADA670, 1 µM Nuc484, 0.16 µM LM404 and 0.46 µM SalinvA284, in a total volume of 29 µL. The amplification was carried out according to a program with an initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at **59 °C** for 30 s, extension at 72 °C for 60 s and a final extension step at 72 °C for 7 min.

4.3.2.4. Evaluation of multiplex PCR specificity

The specificity of the multiplex PCR was assessed by PCR reactions containing the pairs of primers, separately mixed with various DNAs, extracted from multiple reference strains and laboratory isolates.

After amplification, under optimized conditions, the only positive detection was recorded for the strains *E. coli* 425 CECT, *E. coli* 418 CECT and *E. coli* 4558 CECT, with 670 bp amplicons. On the other hand, for the reference strains *Citrobacter freundii* 401 CECT, *Micrococcus luteus* 245 CECT and *Staphylococcus epidermidis* 231 CECT or the three laboratory isolates, *Bacillus cereus*, *Listeria innocua* and *Listeria grayi*, no detection was recorded. This confirms that the multiplex PCR is sufficiently selective, to differentiate between target and non-target bacteria.

4.3.2.5. Evaluation of multiplex PCR sensitivity

The multiplex PCR sensitivity was evaluated, when the four pairs of primers and their target DNAs were mixed in a single reaction. The results presented in **Figure 7** show a very clear detection of the four microorganisms, with intense bands at 10^8 CFU/mL, of each target bacteria. Unfortunately, by reducing the cell concentration to 10^7 CFU/mL, all the bands remain present, but they lose a lot of their intensity, especially *S. aureus* and *S. enterica*. According to Yuan et al. (2009), this difference in intensity occurs in a traditional multiplex PCR because of the disproportionate amplification between different primers, which cannot be avoided during the whole reaction, because each primers pair has different amplifying efficiency. At 10^6 CFU/mL cell concentration for each microorganism, the system could only detect three microorganisms *E. coli*, *L. monocytogenes* and *S. enterica*. According to estimates carried out using the Qubit 4 fluorometer (Thermo Fisher Scientific), the detection limits of the quadruplex PCR is approximately equivalent to 10 pg/µL of each DNA template, in the same reaction.

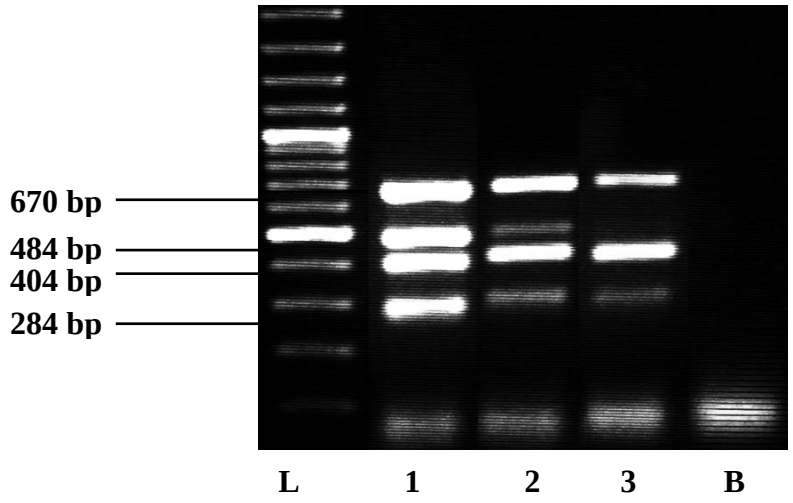


Figure 7. Multiplex PCR sensitivity:

L: 100 pb DNA ladder. 1: Multiplex PCR 10^8 CFU/mL, 2: Multiplex PCR 10^7 CFU/mL, 3: Multiplex PCR 10^6 CFU/mL (Mix PCR + DNA *E. coli* 101 CECT, *S. aureus* 435 CECT, *L. monocytogenes* 936 CECT and *S. enterica* 4266 CECT + Their primer pairs GADA 670 pb, Nuc 484 pb, LM 404 pb and SalinA 284 pb, respectively. B: Negative control.

This means that even after optimization, the efficiency of the multiplex remains low, especially for the detection of bacteria such as *L. monocytogenes* or *Salmonella* spp., whose detection of 1 CFU in 25 g of food is not tolerated. We assume that the low sensitivity is due to the primers, which were designed for a simplex system. On this point, Zhang et al. (2012) and Wei et al. (2018) who used the same primers, for the simultaneous detection of *S. aureus* and *Salmonella* spp. in a triplex system, mentioned that there was fierce competition between the different amplification pathways. To improve the detection sensitivity of the multiplex system, the initial concentration of microorganisms must be amplified, by a preculture in a single medium.

4.3.3. Detection limits by multiplex PCR from the co-culture

4.3.3.1. Detection limits from BPW co-culture recovery

The aliquots collected from each co-culture without food matrix, were tested by multiplex PCR, to evaluate the effect of BPW as a co-culture medium, on the detection limits. The multiplex PCR detection was able to successfully amplify DNA fragments of *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enterica*, up to 10^0 CFU/mL of initial inoculum, confirming the capabilities of BPW as a co-culture medium for multiplex detection based on PCR. The detection results are presented in **Figure 8**.

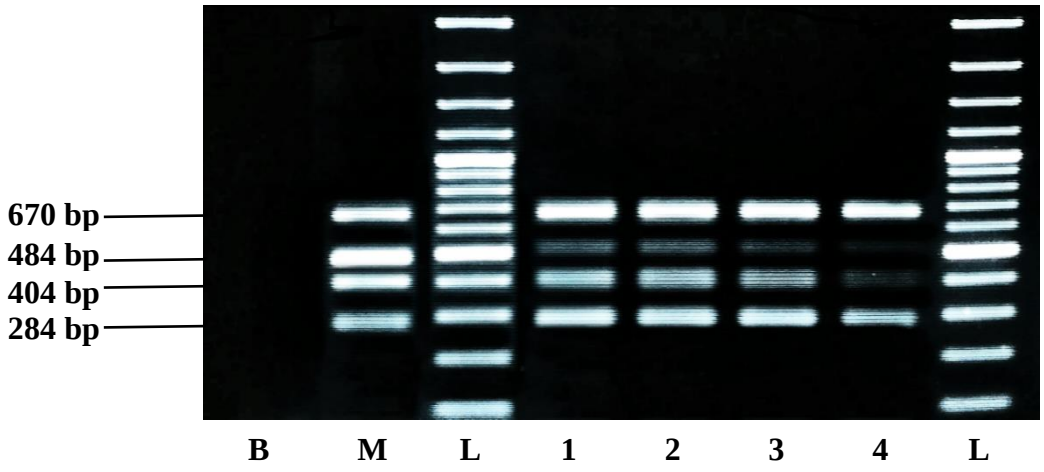


Figure 8. Multiplex PCR detection, from co-cultures recovery made in BPW:

B: Negative control. L: 100 bp DNA ladder. M: Multiplex PCR as a positive control (target bacteria *E. coli* 101 CECT, *S. aureus* 435 CECT, *L. monocytogenes* 936 CECT and *S. enterica* 4266 CECT were tested with their primer pairs GADA670, Nuc484, LM404 and SalinA 284 respectively). Lane 1 shows multiplex PCR results from recovery co-culture, of BPW initially inoculated at 10^3 CFU/mL. Lane 2 shows multiplex PCR results from recovery co-culture, of BPW initially inoculated at 10^2 CFU/mL. Lane 3 shows multiplex PCR results from recovery co-culture, of BPW initially inoculated at 10^1 CFU/mL. Lane 4 shows multiplex PCR results from recovery co-culture, of BPW initially inoculated at 10^0 CFU/mL.

Although the intensity of the *E. coli* and *S. enterica* bands was strong and invariable from 10^3 to 10^0 CFU/mL of initial inoculum, with a predominance of *E. coli*, the intensity of the bands clearly decreased for *S. aureus* and *L. monocytogenes* at 10^1 CFU/mL and 10^0 CFU/mL of initial inoculum.

This difference in band intensity in multiplex PCR detection may be linked to the difference in cell concentrations between the target bacteria, which could agree with Markoulatos et al. (2002), who attests that, the amount of bacterial DNA matrix is a critical factor, which deserves to be optimized. However, according to Yuan et al. (2009), the disproportionate amplification between the different primers cannot be avoided throughout the whole reaction, in a traditional multiplex PCR, because each pair of primers has different amplifying efficiencies. Furthermore, Zhang et al. (2012) and Wei et al. (2018) who used the same primers for the detection of *S. aureus* and *Salmonella* spp., mentioned that there was fierce competition between the different amplification pathways, in a simultaneous detection.

4.3.3.2. Detection limits from BPW co-culture recovery, with artificially inoculated food matrix

The multiplex PCR carried out using aliquots collected from the co-cultures, in the presence of an eco-organic lettuce sample, artificially inoculated with 10^3 CFU/mL of each target strain, made it possible to clearly detect the presence of the three microorganisms *E. coli*, *S. enterica* and *L. monocytogenes*, despite the background microbiota, without any non-specific products. Regarding the co-culture from the artificially inoculated ground meat sample, in the presence of background microbiota, a multiplex PCR carried out using the initial co-culture inoculum (before incubation), could only detect *S. enterica* (Line 2). On the other hand, by using the aliquote of co-culture recovery (after incubation), the multiplex PCR was able to detect the 3 microorganisms *E. coli*, *S. enterica* and *L. monocytogenes* (Line 3), which demonstrates the real impact of BPW on the co-culture and its effect on the detection limites. The detection results are shown in **Figure 9**.

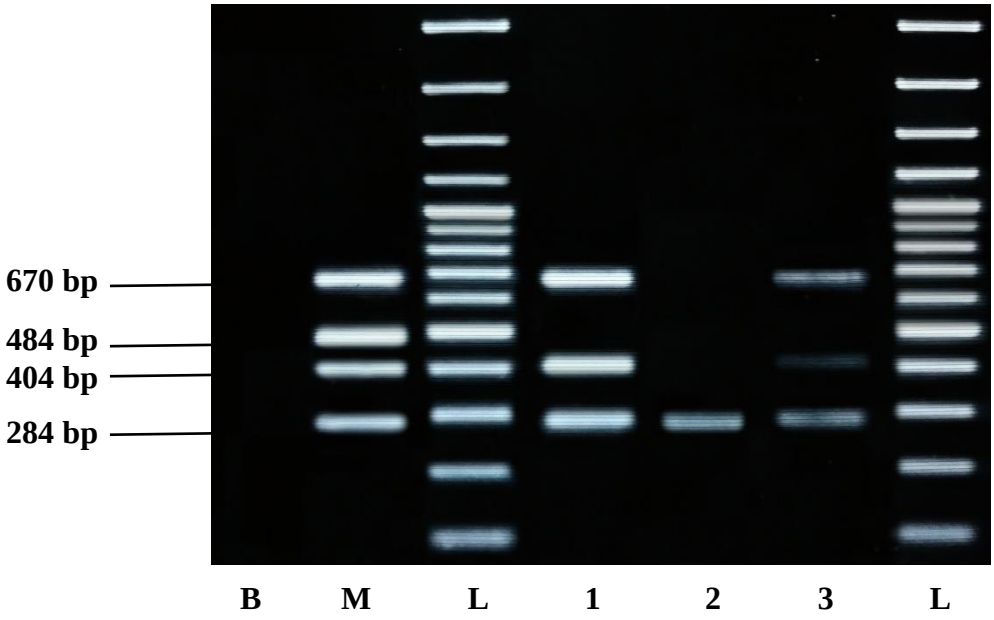


Figure 9. Multiplex PCR detection from co-cultures recovery made in BPW, with the presence of food matrices artificially inoculated, under background microbiota:

B: Negative control. L: 100 bp DNA ladder. M: Multiplex PCR as a positive control (target bacteria *E. coli* 101 CECT, *S. aureus* 435 CECT, *L. monocytogenes* 936 CECT and *S. enterica* 4266 CECT were tested with their primer pairs GADA670, Nuc484, LM404 and SalinA 284 respectively). Lane 1 shows multiplex PCR results from the co-culture recovery made in BPW, of lettuce artificially inoculated (10^3 CFU/mL), in the presence of background microbiota. Lane 2 shows multiplex PCR results from the co-culture made in BPW, of minced meat artificially inoculated (10^3 CFU/mL), in the presence of background microbiota (before incubation). Lane 3 shows multiplex PCR results from the co-culture recovery made in BPW, of minced meat artificially inoculated (10^3 CFU/mL), in the presence of background microbiota (after incubation).

The difference in band intensity on detection between the two food matrices, could be due to the difference in cell concentrations recovered, which were greater from lettuce, or to a possible inhibitory effect of the meat. According to Garrido et al. (2013) and Rajabzadeh et al. (2018), fats and glycogen are considered as inhibitors and could affect a PCR at multiple steps.

S. aureus could not be detected by multiplex PCR from the aliquots tested (Line 1, 2 and 3 of **Figure 9**). However, it was clearly detected by simplex PCR, from co-culture aliquots of the two artificially inoculated samples (results shown in **Figure 10**), with a slight increase of intensity after incubation, compared to the detection from the initial inoculum

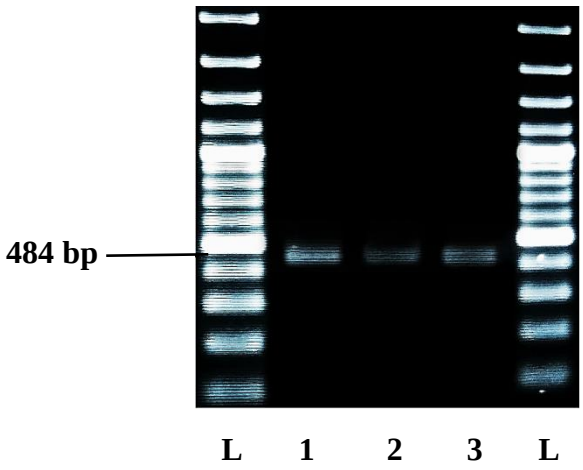


Figure 10. Simplex PCR detection using the primers pair Nuc484 of *S. aureus*, from co-cultures recovery made in BPW, with the presence of food matrices artificially inoculated:

L: 100 bp DNA ladder. Lane 1 shows *S. aureus* detection by simplex PCR, from the co-culture recovery made in BPW, of lettuce artificially inoculated (10^3 CFU/mL), in the presence of background microbiota. Lane 2 shows *S. aureus* detection by Simplex PCR, from the co-culture made in BPW, of minced meat artificially inoculated (10^3 CFU/mL), in the presence of background microbiota (before incubation). Lane 3 shows *S. aureus* detection by Simplex PCR results from the co-culture recovery made in BPW, of minced meat artificially inoculated (10^3 CFU/mL), in the presence of background microbiota (after incubation).

We assume that the problem is related to the sensitivity of the primers used and that they should be replaced by more efficient ones, or completely design a new pair of specific primers, targeting the *S. aureus* thermonuclease gene, by taking account of the conditions such as annealing and melting temperatures, in addition to the compatibility of use in the presence of the three other target bacteria primers, without detection specificity or sensitivity issues upon simultaneous amplification, which could save a lot of time and effort spent in optimization assays.

Wei et al. (2018) mentioned that the detection of *S. aureus* with the Nuc484 primers of Xu et al. (2006) was not characterized by a high sensitivity compared to *Salmonella* spp. primers invA284 of Rahn et al. (1992), in artificially contaminated food products. Moreover, Zhang et al. (2012) asserted that to use these primers pairs, the biomass of *S. aureus* had to be greater than that of other bacteria, for a perfect amplification during multiplex PCR, due to a possible competition, established by a preferential amplification. The preferential amplification of a target in multiplex PCR could be induced by the presence of a low template concentration according to Elizaquível and Aznar (2008). In our case, the template-primers combinations of *E. coli*, *S. enterica* and *L. monocytogenes* seem to be more favored, which means that most of the common resources of PCR mix

go in the direction of their own amplifications (Mukhopadhyay and Mukhopadhyay, 2007), thus affecting the detection of *S. aureus*.

Although multiplex PCR could not detect the four microorganisms from artificially inoculated food, due to a lack of *S. aureus* primers sensitivity, the results showed that BPW broth can effectively support the simultaneous growth of *E. coli*, *S. aureus*, *L. monocytogenes* and *S. enterica*, in the presence of a ready-to-eat food matrix, with a background microbiota and that the use of a co-culture before detection by multiplex PCR, had a positive effect on improving detection sensitivity.

4.4. Methodology proposed to analyze a food sample

The analysis of a single sample by cultural method necessitates a period of at least 7 days and involves the application of a protocol for each microorganism present in the sample until its identification.

In order to reduce the needed time and culture media, we propose in our work a protocol for the simultaneous detection of the four bacteria *E. coli*, *S. enterica*, *L. monocytogenes* and *S. aureus*, which is the the principal objective of this work, after the pre-enrichment of the sample in BPW as a co-culture medium during 24 h at 37 °C, in order to increase the cell concentration of the bacteria initially present in the food matrix.

However, the fact that PCR detects only DNA and not living cells makes it an incomplete analytical method, especially when it comes to apply standards based on bacterial counts. Therefore, combining the classical and molecular method based on the ISO standards, in the same protocol would allow an efficient and rapid analysis, according to the proposed protocol: 25 g of sample are subjected to a pre-enrichment step in BPW (previously mentioned), in addition, the research and detection of *L. monocytogenes* is applied by the pre-enrichment of 25 g of sample in the FBH medium, then incubate for 24 h to 30 °C.

After incubation, aliquots of 1 mL co-culture BPW are collected and subjected to DNA extraction by kit (GenElute™ Bacterial Genomic DNA Kit, Sigma-Aldrich), according to the Gram-positive protocol by using lysozyme (which takes approximately 2 hours). At the same time, the enrichment step, is initiated for each bacteria, in the event of a positive detection by simplex and multiplex PCR.

The DNA matrices obtained from the extraction are subjected to multiplex PCR following the conditions: 2.9 µL Reaction Buffer 10×NH_4, 3.4 mM $MgCl_2$ Solution, 2.5

Units Taq DNA polymerase (BIOTAQ ™ DNA Polymerase-BIOLINE), 0.22 mM dNTPs (dNTP Mix-BIOLINE), primers concentrations 0.2 µM GADA670, 1 µM Nuc484, 0.16 µM LM404 and 0.46 µM SalinvA284, in a total volume of 29 µL, carried out according to a program with an initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 60 s and a final extension step at 72 °C for 7 min (total time about 3 hours).

Since the current conditions of this multiplex system do not allow detection of *S. aureus*, a simplex PCR detection for this bacteria is carried out in parallel, using the same DNA extracted from BPW co-culture, pending the improvement of the multiplex PCR.

After amplifications, the PCR product is mixed with Ready-to-Load and subjected to an electrophoresis for 60 min at 80 Volts on 1.5 % agarose gel, then, the amplification products is visualized under UV transilluminator.

If the PCR results are positif the following steps will be applied for the bacteria that were detected by PCR, as the enrichment steps in selective broths for 24 h of incubation at 37 °C for FB, GC, and BGBLB media and at 42 °C for RV media. After an isolation step on selective solide media PAL, BP, TBX and XLD used respectively for the isolation of *L. monocytogenes*, *S. aureus*, *E. coli* and *Salmonella* sp., the suspect colonies are subjected to identification by biochemical test and Api strips confirmation.

Therefore, resources such as culture media and reagents will be used only for present bacteria, also the selective media can be inoculated directly from BPW, thus saving time and money. Because this method can confirme the presence or the absence of the target bacteria in food in approximately 30 hours, compared to the cultural method. Moreover, it directs the research axis by focusing only on the bacteria potentially present in food, which makes it possible to reduce the number of media used in vain, for bacteria which are not initially present.

CONCLUSIONS

1. For *E. coli*, among the 39 analyzed samples, 21 were positive by simplex PCR from which only 11 samples were obtained with isolates. For the 13 PCR positive samples of *S. enterica* and the 19 PCR positive samples of *L. monocytogenes*, none isolates were obtained. Regarding *S. aureus*, all the 14 PCR positive samples, got isolates. Therefore, combining the PCR detection and the cultural method, would increase the efficiency of food analysis.
2. BPW was chosen as a co-culture medium because of its good capacities to support the growth of the four bacteria in individual culture with stable and close recovery rates (between 10^8-10^9 CFU/mL). After 24 hours, recovery rates obtained in co-cultures with artificially inoculated food matrices at 10^3 CFU/mL, were not affected by the background microbiota, ranging 10^7-10^8 CFU/mL for *E. coli* and *S. enterica*, 10^6-10^7 CFU/mL for *S. aureus* and 10^5-10^6 CFU/mL for *L. monocytogenes*.
3. The detection limits of the multiplex PCR after optimization are equivalent to 10 pg/µL of each individual DNA and up to 10^0 CFU/mL from the co-culture recovery of the four target bacteria. However it detects only *E. coli*, *L. monocytogenes* and *S. enterica* from the co-culture recovery of artificially inoculated matrices at 10^3 CFU/mL. Which means that for *S. aureus*, more sensitive primers must be designed.
4. The protocol proposed for the simultaneous detection of several bacteria in food, which includes a BPW pre-enrichment step and a detection by multiplex PCR, can confirm the absence of bacteria in the sample in approximately 30 hours, to continue until the isolation step in case of positive PCR, while cultural methods requires at least 7 days. Thereby, by focusing the detection steps only on the bacteria potentially present detected by PCR in the sample, avoids the unnecessary use of culture media reagents and the Api strips for the isolation and identification of bacteria that are absent, which represents a significant saving of time and money.

REFERENCES

Abadias, M., Usall, J., Anguera, M., Solsona, C., and Viñas, I. (2008). Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *International Journal of Food Microbiology*, *123*, 121-129.

Abd El-Salam, A. F., Abd El-Ghany, Z. M., and El-Tahan, M. H. (2010). The comparison between different enrichment broth media and selective solid media for growing of *Salmonella typhimurium* and *Listeria monocytogenes*. *Journal of Agricultural Chemistry and Biotechnology*, *7*, 351-363.

Abdelrahman, H., Meawad, A., and Shaheen, H. (2014). Quantitative and Qualitative Studies on *Enterobacteriaceae* in Ground Beef. *Suez Canal Veterinary Medicine Journal*, *19*(2), 77-88. Doi: 10.21608/scvmj.2014.65324.

Abulreesh, H. H., and Organji, S. R. (2011). The prevalence of multidrug-resistant *staphylococci* in food and the environment of Makkah, Saudi Arabia. *Research Journal of Microbiology*, *6*(6), 510-523. Doi: 10.3923/jm.2011.510.523.

Adesoji, A. T., Onuh, J. P., Bagu, J., and Itohan, S. A. (2019). Prevalence and antibiogram study of *Staphylococcus aureus* isolated from clinical and selected drinking water of Dutsin-Ma, Katsina state, Nigeria. *African Health Sciences*, *19*(1), 1385-1392. https://dx.doi.org/10.4314/ahs.v19i1.11.

Agence Française de Sécurité Sanitaire des Aliments, AFSSA. (2003, April). *Bilan des connaissances relatives aux Escherichia coli producteurs de Shiga-toxines (STEC)*.

Agence française de sécurité sanitaire des aliments, AFFSA. (2020, July 28). *Rapport de la Commission d'étude des risques liés à Listeria monocytogenes*.

Agencia Española de Seguridad Alimentaria y Nutrición, AESAN. (2020). *Salmonelosis*. http://www.aesan.gob.es/AECOSAN/web/seguridad_alimentaria/subdetalle/salmonela.htm.

Al Masalma, M., Raoult, D., and Roux, V. (2010). *Staphylococcus massiliensis* sp. nov., isolated from a human brain abscess. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 1066-1072.

Alarcoän, B., Garciäa-Canas, V., Cifuentes, A., Gonzaälez, R., and Aznar, R. (2004). Simultaneous and Sensitive Detection of Three Foodborne Pathogens by Multiplex PCR, Capillary Gel Electrophoresis, and Laser-Induced Fluorescence. *Journal of Agricultural and Food Chemistry*, *52*, 7180-7186.

Al-Bahry, S., Mahmoud, I., Al-Musharafi, S., and Sivakumar, N. (2014). *Staphylococcus aureus* contamination during food preparation, processing and handling. *International Journal of Chemical Engineering and Applications*, *5*, 388.

Alcalde Sanz, L., and Gawlik, B. (2017). *Water Reuse in Europe-Relevant guidelines, needs for and barriers to innovation*. Luxembourg: Publications Office of the European Union.

Al-Holy, M., Osaili, T., El-Sayed, S., AlShammari, E., and Ashankyty, I. (2013). Microbiological Quality of Leafy Green Vegetables Sold in the Local Market of Saudi Arabia. *Italian Journal of Food Science*, *25*(4), 446-452. https://www.researchgate.net/publication/316861273.

Al-Kharousi, Z. S., Guizani, N., Al-Sadi, A. M., Al-Bulushi, I. M., and Shaharoona, B. (2016). Hiding in Fresh Fruits and Vegetables: Opportunistic Pathogens May Cross Geographical Barriers. *International Journal of Microbiology*. http://dx.doi.org/10.1155/2016/4292417.

Allerberger, F., Bago, Z., Huhulescu, S., and Pietzka, A. (2015). Listeriosis: the dark side of refrigeration and ensiling in Zoonoses. In: A. Sing, (ed.), *Infections Affecting Humans and Animals-Focus on Public Health Aspects* (pp.249-286). Heidelberg, German: Springer Verlag.

Amador, P., Fernandes, R., Brito, L., and Prudêncio, C. (2010). Antibiotic resistance in *Enterobacteriaceae* isolated from portuguese Deli Meats. *Journal of Food Safety, 31*, 1-20. https://doi.org/10.1111/j.1745-4565.2010.00258.x.

Andino, A., and Hanning, I. (2015). *Salmonella enterica*: survival, colonization, and virulence differences among serovars. *The Scientific World Journal*. Doi: 10.1155/2015/520179.

Angelidis, A. S., Kalamaki, M. S., and Georgiadou, S. S. (2015). Identification of non-*Listeria* spp. Bacterial isolates yielding a β-d-glucosidase-positive phenotype on Agar *Listeria* according to Ottaviani and Agosti (ALOA). *International Journal of Food Microbiology, 193*, 114-129. https://doi.org/10.1016/j.ijfoodmicro.2014.10.022.

Argudín, M. A., Mendoza, M. C., and Rodicio, M. R. (2010). Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins, 2*, 1751-1773.

Armas, Y. F., Amaya, M. A., Alemán, Y. R., Díaz, D. R., and Vasallo, A. M. (2020). Evaluation of the hygienic-sanitary quality of artisan fresh cheeses in Mayabeque province, Cuba. *Revista de Salud Animal, 42*(2).

Arun, K. B. (2008). *Foodborne Microbial Pathogens: General mechanism of pathogenesis for foodborne pathogens*. New York, United States: Springer.

Aryal, S. (2018, April 28). *Biochemical Test of Escherichia coli*. Microbe Notes Online Microbiology and Biology Notes. https://microbenotes.com/biochemical-test-of-escherichia-coli-e-coli/

Aryal, S. (2019, June 29). *Morphology of E. coli*. Microbe Notes Online Microbiology and Biology Notes. https://microbenotes.com/morphology-of-e-coli/

Ashkezary, M. R., Bonanno, A., Todaro, M., Settanni, L., Gaglio, R., Todaro, A., Alabiso, M., Maniaci, G., Mazza, F., and Di Grigoli, A. (2020). Effects of adding solid and molten chocolate on the physicochemical, antioxidant, microbiological, and sensory properties of ewe's milk cheese. *Journal of Food Science, 85*(3). Doi: 10.1111/1750-3841.15045.

Association française de normalisation, AFNOR. (2021, December 13). *Listeria monocytogenes*. Certify the analytical performances of test kits. https://nf-validation.afnor.org/en/food-industry/listeria-monocytogenes/.

Atlabachew, T., and Mamo, J. (2021). Microbiological Quality of Meat and Swabs from Contact Surface in Butcher Shops in Debre Berhan, Ethiopia. *Journal of Food Quality*. https://doi.org/10.1155/2021/7520882.

Attien, P., Sina, H., Moussaoui, W., Zimmermann-Meisse, G., Dadié, T., Keller, D., Riegel, P., Edoh, V., Kotchoni, S.O., Djè, M., Prévost, G., and Baba-Moussa, L. (2014). Mass spectrometry and multiplex antigen assays to assess microbial quality and toxin

production of *Staphylococcus aureus* strains isolated from clinical and food samples. *BioMed Research International*, 485620.

Aycicek, H., Oguz, U., and Karci, K. (2006). Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. *International Journal of Hygiene and Environmental Health*, *209*(2), 197-201.

Barber, M. (1947). Staphylococcal infection due to penicillin-resistant strains. *British Medical Journal*, *2*, 863-865

Barnes, B. J., Wiederhold, N. P., Micek, S. T., Polish, L. B., and Ritchie, D. J. (2003). Enterobacter cloacae ventriculitis successfully treated with cefepime and gentamicin: case report and review of the literature. *Pharmacotherapy*, *23*(4), 537-542. Doi: 10.1592/phco.23.4.537.32126.

Batt, C. A. (2014). ESCHERICHIA COLI | *Escherichia coli*. In: *Encyclopedia of Food Microbiology* (pp. 688-694). Academic Press. Doi:10.1016/B978-0-12-384730-0.00100-2.

Becker, K. (2018). Pathogenesis of *Staphylococcus aureus*. In: A. Fetsch, (ed.), *Staphylococcus aureus* (pp. 1-26). Amsterdam, Netherlands: Elsevier.

Becker, K., Harmsen, D., Mellmann, A., Meier, C., Schumann, P., Peters, G., and von Eiff, C. (2004). Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. *Journal of Clinical Microbiology*, *42*(11), 4988-4995. Doi: 10.1128/JCM.42.11.4988-4995.2004.

Belhaj, K., Khamri, M., Omari, A., Elamrani, A., and Belbachir, C. (2019). Microbiological quality of minced meat sold in butcher shops of Oujda city, Morocco. *Moroccan Journal of Biology*, *15*, 10-17. https://www.researchgate.net/publication/334387866.

Bergonier, D., de Cremoux, R., Rupp, R., Lagriffoul, G., and Berthelot, X. (2003). Mastitis of dairy small ruminants. *Veterinary* Research, *34*, 689-716.

Bertsch, D., Rau, J., Eugster, M. R., Haug, M. C., Lawson, P. A., Lacroix, C., and Meile, L. (2013). *Listeria fleischmannii* sp. nov., isolated from cheese. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 526-532.

Bhan, M. K., Bahl, R., and Bhatnagar, S. (2005). Typhoid and paratyphoid fever. *Lancet*, *366*, 749-62.

Bhatia, A., and Zahoor, S. (2007). *Staphylococcus aureus* enterotoxins: a review. *Journal of Clinical and Diagnostic Research*, *1*, 188-197.

Bhunia, A. K. (2008). *Staphylococcus aureus*. In: *Foodborne Microbial Pathogens*. New York, USA: Springer.

Bohayhuck, V. M., Gensler, G. E., King, R. K., Manninen, K. I., Sorensen, O., Wu, J. T., Stiles, M. E., and McMullen, L. M. (2006). Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. *Journal of Food Protection, 6*, 2176-2182.

Boletín Epidemiológico Semanal, BES. (2008). Vigilancia epidemiológica de brotes de transmisión hídrica en España 1999-2006. *Sistema de Información Microbiológica*, *16*(3), 25-36.

Boletín Epidemiológico Semanal, BES. (2015). Comentario epidemiológico de las enfermedades de declaración obligatoria y sistema de información microbiológica en 2014. *Sistema de Información Microbiológica*, *23*(5), 60-79.

Boletín Oficial del Estado. (1986). Orden de 14 de enero de 1986 por la que se aprueba la norma de calidad para carnes picadas de vacuno, ovino y porcino destinadas al mercado interior. *Presidencia del Gobierno*, *18,* 2935-2937.

Boletín Oficial del Estado. (2007). Real Decreto 1620/2007, de 7 de diciembre, por el que se establece el régimen jurídico de la reutilización de las aguas depuradas. *Ministerio de la Presidencia*, *294*, 50639-50661.

Bonnin, R. A., Cuzon, G., Poirel, L., and Nordmann, P. (2013). Multidrug-Resistant *Acinetobacter baumannii* Clone, France. *Emerging infectious diseases*, *19*(5), 822-823.

Bos, M. P., Robert, V., and Tommassen, J. (2007). Biogenesis of Gram-negative bacterial outer membrane. *Annual Review of Microbiology*, *61*(6), 191-214

Bouvier, M. (2011). Mise au point de méthodes de détection des souches d'*Escherichia coli* productrices de Shiga-toxines (STEC). file:///D:/methodes%20de%20detection.pdf.

Brenner, D. J., and Farmer, J. J. III. (2007). Family I. *Enterobacteriaceae* Rhn 1937, Nom. Fam. Cons. Opin. 15, Jud. Comm. 1958a, 73, Ewing, Farmer, and Brenner 1980, 674; Judicial Commission 1981, 104. In: G. Garrity, D. J. Brenner, N. R. Krieg, and J. R. Staley (eds.), *Bergey's Manual of Systematic Bacteriology* (Vol. 2, pp. 587-850). New York, United States: Springer.

Brenner, D. J., Davis, B. R., Steigerwalt, A. G., Riddle, C. F., McWhorter, A. C., Allen, S. D., Farmer III, J. J., Saitoh, Y., and Fanning, G. R. (1982). Atypical biogroups of *Escherichia coli* found in clinical specimens and description of *Escherichia hermannii* sp. nov. *Journal of Clinical Microbiology*, *15*(4), 703-713.

Brenner, D. J., McWhorter, A. C., Leete Knutson, J. K., and Steigerwalt, A. G. (1982). *Escherichia vulneris*: a new species of *Enierobacteriaceae* associated with human wounds. *Journal of Clinical Microbiology*, *15*(6), 1133-1140.

Breyer, M. (2017, June 05). *The Brighter Side of E. coli: 9 Ways the Oft-Maligned Bacteria Does a Lot of Good*. https://www.treehugger.com/the-brighter-side-of-e-coli-ways-the-oft-maligned-bacteria-does-4863180.

Byrne, V. D. V., Hofer, E., Vallim, D. C., and Almeida, R. C. D. C. (2016). Occurrence and antimicrobial resistance patterns of *Listeria monocytogenes* isolated from vegetables. *Brazilian Journal of Microbiology*, *47*(2), 438-443. Doi: 10.1016/j.bjm.2015.11.033.

Cabrera, C., León, G., Tejeda, F., Ramírez, B., and Flores, M. (2013). Preliminary study to investigate *Salmonella* sp. and *E. coli* O157:H7 in ground beef sold in supermarkets in Puebla city, Mexico. *CienciaUAT*, *8*(1), 64-69.

Campos, L. C., Franzolin, M. R., and Trabulsi, L. R. (2004). Diarrheagenic *Escherichia coli* Categories among the Traditional Enteropathogenic *E. coli* O Serogroups. *The Memórias do Instituto Oswaldo Cruz*, *99*(6), 545-552.

Cardamone, C., Aleo, A., Mammina, C., Oliveri, G., and Di Noto, A. M. (2015). Assessment of the microbiological quality of fresh produce on sale in Sicily, Italy: preliminary results. *Journal of Biological Research*, *22*(3), 1-6.

Carlin, C. R., Liao, J., Weller, D., Guo, X., Orsi, R., and Wiedmann, M. (2021). *Listeria cossartiae* sp. nov., *Listeria immobilis* sp. nov., *Listeria portnoyi* sp. nov. and *Listeria*

rustica sp. nov., isolated from agricultural water and natural environments. *International Journal of Systematic and Evolutionary Microbiology, 71*, 4795.

Castellani, A., and Chalmers, A. J. (1919). *Manual of Tropical Medicine* (3rd ed.). New York, United States: Williams Wood and Company.

Castro, A., Silva, J., and Teixeira, P. (2018). *Staphylococcus aureus*, a Food Pathogen: Virulence Factors and Antibiotic Resistance. In: A. M. Holban, and A. M. Grumezescu, (eds.), *Foodborne Diseases* (pp. 213-238). Academic Press.

Cavalier-Smith, T. (2002). The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. *International Journal of Systematic and Evolutionary Microbiology, 52*, 7-76. Doi: 10.1099/00207713-52-1-7.

Centro de Coordinación de Alertas y Emergencias Sanitarias. (2019). Informe de fin de seguimiento del brote de listeriosis 27 de septiembre de 2019. Ministerio de Salud y Familia.
https://www.mscbs.gob.es/profesionales/saludPublica/ccayes/alertasActual/listeriosis/docs/Informe_cierre_Listeriosis_20190927.pdf.

Chaalal, W., Chaalal, N., Bourafa, N., Kihal, M., Diene, S. M., and Rolain, J-M. (2018). Characterization of *Staphylococcus aureus* Isolated from Food Products in Western Algeria. *Foodborne Pathogens and Disease, 15*(6). Doi: 10.1089/fpd.2017.2339.

Chamberland, R. R., McElvania, T. E., Burnham, C. A. D., and Kennedy, D. J. (2013). Renal abscess caused by a *Providencia stuartii* isolate biochemically misidentified as 109 *Pasteurella*. *Journal of Clinical Microbiology, 51*, 2775-2777.

Charoenca, N., and Fujioka, R. S. (1995). Association of staphylococcal skin infections and swimming. *Water Science and Technology, 31*, 11-17.

Chaves, L. C., De Angelis, M., Martuscelli, M., Serio, A., Paparella, A., and Suzzi, G. (2006). Characterization of the *Enterobacteriaceae* isolated from an artisanal Italian ewe's cheese (Pecorino Abruzzese). *Journal of Applied Microbiology*, *101*(2), 353-360.

Chaves, R. D., Martinez, R. C. R., Rezende, A. C. B., Rocha, M. D., Oteiza, J. M., and Sant'Ana, A. de S. (2016). *Salmonella* and *Listeria monocytogenes* in ready-to-eat leafy vegetables. In: P. Kotzekidou (ed.), *Food Hygiene and Toxicology in Ready-to-Eat Foods* (pp. 123-149). Academic Press. https://doi.org/10.1016/B978-0-12-801916-0.00008-X

Chen, J., Tang, J., Bhunia, A. K., Tang, C., Wang, C., and Shi, H. (2015). Development of a multi-pathogen enrichment broth for simultaneous growth of five common foodborne pathogens. *Journal of General and Applied Microbiology*, *61*(6), 224-231. Doi: 10.2323/jgam.61.224.

Chesneau, O., Morvan, A., Grimont, F., Labischinski, H., and el Solh, N. (1993). *Staphylococcus pasteuri* sp. nov., isolated from human, animal, and food specimens. *International Journal of Systematic Bacteriology*, *43*, 237-244.

Cheung, A. L., Projan, S. J., and Gresham, H. (2002). The genomic aspect of virulence, sepsis, and resistance to killing mechanisms in *Staphylococcus aureus*. *Current Infectious Disease Reports, 4*(5), 400-410. Doi: 10.1007/s11908-002-0006-2.

Chew, K. L., Octavia, S., Lai, D., Lin, R. T. P., and Teo, J. W. P. (2021). *Staphylococcus singaporensis* sp. nov., a new member of the *Staphylococcus aureus* complex, isolated from human clinical specimens. *International Journal of Systematic Bacteriology*, *71*, 5067.

Chihab, W., Alaoui, A. S., and Amar, M. (2004). Chryseomonas luteola Identified as the Source of Serious Infections in a Moroccan University Hospital. *Journal of Clinical Microbiology*, *42*(4), 1837-1839. Doi: 10.1128/JCM.42.4.1837-1839.2004.

Chuang, L. Y., Cheng, Y. H., and Yang, C. H. (2013). Specific primer design for the polymerase chain reaction. *Biotechnology Letters*, *35*(10), 1541-1549. Doi: 10.1007/s10529-013-1249-8.

Civieta, Ó. F. (2021, may 3). *Las intoxicaciones alimentarias transmitidas de animales a humanos más comunes en España*. https://www.businessinsider.es/principales-intoxicaciones-alimentarias-espana-846781.

Cohen, N., Filliol, I., Karraouan, B., Badri, S., Carle, I., Ennaji, H., Bouchrif, B., Hassar, M., and Karib, H. (2008). Microbial Quality Control of Raw Ground Beef and Fresh Sausage in Casablanca (Morocco). *Environmental Health*, *71*(4), 51-55.

Cohn, A. R., Cheng, R. A., Orsi, R. H., and Wiedmann, M. (2021). Moving Past Species Classifications for Risk-Based Approaches to Food Safety: *Salmonella* as a Case Study. *Frontiers in Sustainable Food Systems*, 5, *652132*.

Conseil des Académies Canadienne. (2013). L'eau et l'agriculture au Canada: vers une gestion durable des ressources en eau. Le comité d'experts sur la gestion durable de l'eau des terres agricoles au Canada, Ottawa. *Conseil des académies canadiennes*, 298.

Consejería de Salud y Familias de la junta de Andalucía. (2021, September 16). Alerta alimentaria por presencia de *Listeria monocytogenes* en queso fresco de cabra y vaca de Andalucía. https://www.juntadeandalucia.es/organismos/saludyfamilias/areas/seguridad-alimentaria/alertas-alimentarias/paginas/listeria-queso-alerta.html.

Dahl, S., Tavaria, F. K., and Malcata, F. X. (2000). Relationships between flavour and microbiological profiles in Serra da Estrela cheese throughout ripening. *International Dairy Journal*, *10*(4), 255-262.

Dailey, R. C., Martin, K. G., and Smiley, R. D. (2014). The effects of competition from non-pathogenic foodborne bacteria during the selective enrichment of *Listeria monocytogenes* using buffered Listeria enrichment broth. *Food Microbiology*, *44*, 173-179. Doi: 10.1016/j.fm.2014.05.004.

Danemark, M., Alcamo, I. E. and Heymann, D. L. (2006). Fièvre typhoïde. *Infobase Publishing*, 8-26.

De Bel, A., Van Hoorde, K., Wybo, I., Vandoorslaer, K., Echahidi, F., De Brandt, E., Schumann, P., Ieven, M., Soetens, O., Pierard, D., and Vandamme, P. (2013). *Staphylococcus jettensis* sp. nov., a coagulase-negative staphylococcal species isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 3250-3256.

DebRoy, C., Fratamico, P. M., and Roberts, E. (2018). Molecular serogrouping of *Escherichia coli*. *Animal Health Research Reviews*, *19*(1), 1-16.

Den Bakker, H. C., Warchocki, S., Wright, E. M., Allred, A. F., Ahlstrom, C, Manuel, C. S., Stasiewicz, M. J., Burrell, A., Roof, S., Strawn, L. K., Fortes, E., Nightingale, K. K., Kephart, D., and Wiedmann, M. (2014). *Listeria floridensis* sp. nov., *Listeria aquatica* sp. nov., *Listeria cornellensis* sp. nov., *Listeria riparia* sp. nov. and *Listeria grandensis* sp. nov., from agricultural and natural environments. *International Journal of Systematic and Evolutionary Microbiology*, *64*, 1882-1889.

Desmarchelier, P., and Fegan, N. (2011). Pathogens in milk *Escherichia coli*. In: *Encyclopedia of Dairy Sciences* (2nd ed., pp. 60-66). London, United Kingdom: Academic Press.

Devriese, L. A., Hajek, V., Oeding, P., Meyer, S. A., and Schleifer, K. H. (1978). *Staphylococcus hyicus* (Sompolinsky, 1953) comb. nov. and *Staphylococcus hyicus* subsp. chromogenes subsp. nov. *International Journal of Systematic Bacteriology*, *28*, 482-490.

Devriese, L. A., Poutrel, B., Kilpper-Bälz, R., and Schleifer, K. H. (1983). *Staphylococcus gallinarum* and *Staphylococcus caprae*, two new species from animals. *International Journal of Systematic Bacteriology*, *33*, 480-486.

Devriese, L. A., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A., and Haesebrouck, F. (2005). *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 1569-1573.

Dodd, C. E. R., Aldsworth, T. G., and Stein, R. A. (2017). *Foodborne Diseases* (3rd ed.). Academic Press. Elsevier.

Doijad, S. P., Poharkar, K. V., Kale, S. B., Kerkar, S., Kalorey, D. R., Kurkure, N. V., Rawool, D. B., Malik, S. V. S., Ahmad, R. Y., Hudel, M., Chaudhari, S. P., Abt, B., Overmann, J., Weigel, M., Hain, T., Barbuddhe, S. B., and Chakraborty, T. (2018). *Listeria goaensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *68*, 3285-3291.

Doménech, E., Jimenez, A., Amoros, J. A., Ferrus, M. A., and Escriche, I. (2015). Prevalence and antimicrobial resistance of *Listeria monocytogenes* and *Salmonella* strains isolated in ready-to-eat foods in Eastern Spain. *Food Control*, *47*, 120-125. https://doi.org/10.1016/j.foodcont.2014.06.043.

Dussart-Baptista, L., Bodilis, J., Barray, S., Frébourg, N., Fournier, M., Dupont, J-P., and Jouenne, T. (2007). Recurrent recovery of *Pseudomonas oryzihabitans* strains in a karstified chalk aquifer. *Water Research*, *41*(1), 96-102. Doi: 10.1016/j.watres.2006.10.007.

Dworkin, M. M., Falkow, S., Rosenberg, E., Schleifer, K-H., and Stackebrandt, E. (2005). *Prokaryotes: Proteobacteria: Gamma and Epsilon Subclass* (3rd ed.). Springer.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K-H., and Stackebrandt, E. (2006). *The Prokaryotes* (3rd ed., Vol. 6, pp. 62-64). New York, United States: Springer.

Eisenstein, B. I. (2008). Treatment challenges in the management of complicated skin and soft-tissue infections. *Clinical Microbiology and Infectious*, *14*, 17-25. Doi: 10.1111/j.1469-0691.2008.01922.x.

Elizaquível, P., and Aznar, R. (2008). A multiplex RTi-PCR reaction for simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimally processed vegetables. *Food Microbiology*, *25*(5), 705-713. http s://doi.org/10.1016/j.fm.2008.03.002.

Elmali, M., and Yaman, H. (2005). Microbiological Quality of Raw Meat Balls: Produced and Sold in the Eastern of Turkey. *Pakistan Journal of Nutrition*, *4*(4), 197-201. Doi: 10.3923/pjn.2005.197.201.

Emmeluth, D., d'Alcamo, I. E., and Heymann, D. L. (2009). Fièvre typhoïde. *Infobase Publishing*, 8-17.

Engel, H. J., Collignon, P. J., Whiting, P. T., and Kennedy, K. J. (2009). Serratia sp. bacteremia in Canberra, Australia: a population-based study over 10 years. *European Journal of Clinical Microbiology & Infectious Diseases*, *28*(7), 821-824.

Enright, M. C. Ashley Robinson, D., Randle, G., Feil, E. J., Grundmann, H., and Spratt, B. G. (2002). The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). *Proceedings of the National Academy of Sciences of the United States of America*, *99*(11), 7687-7692.

Errebo Larsen, H., and Seeliger, H. P. R. (1966). A mannitol fermenting *Listeria*, *Listeria grayi* sp. n. Proceedings of the Third International Symposium on Listeriosis July 13-16, Bilthoven, Utrecht, Kingdom of the Netherlands: Rijks Instituut voor de Volksgezondheit.

Espinosa, L., Varela, C., Martínez, E. V., and Cano, R. (2015). Brotes de enfermedades transmitidas por alimentos. España, 2008-2011. *Boletín epidemiológico semanal*, *22*(11), 130-136.

Espinoza, F. E., Murrieta, A. F., Córdova, M. F., and Nevárez, G. C. (2020). Analysis microbiological of fresh cheeses commercialized in the Babahoyo city. *Journal of science and research*, *5*. Doi:10.5281/zenodo.4432656.

European Centre for Disease Prevention and Control, ECDC (2016, October 27). Multi-country outbreak of *Salmonella* Enteritidis phage type 8 MLVA type 2-9-7-3-2 and 2-9-6-3-2 infections. https://www.ecdc.europa.eu/en/publications-data/joint-rapid-outbreak-assessment-multi-country-outbreak-salmonella-enteritidis.

European Food Safety Authority and European Centre for Disease Prevention and Control, EFSA and ECDC (2015a). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA Journal*, *13*, 4329.

European Food Safety Authority and European Centre for Disease Prevention and Control, EFSA and ECDC. (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal*, *14*(12). https://doi.org/10.2903/j.efsa.2016.4634.

European Food Safety Authority and European Centre for Disease Prevention and Control, EFSA and ECDC. (2019). The European Union One Health 2018 Zoonoses Report. *EFSA Journal*, *17*(12). https://doi.org/10.2903/j.efsa.2019.5926.

European Food Safety Authority, EFSA. (2009). Assessment of the public health significance of methicillin-resistant Staphylococcus aureus (MRSA) in animals and foods. *EFSA Journal*, *993*, 1-73.

European Food Safety Authority, EFSA (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal*, *15*(12), 5077. Doi: 10.2903/j.efsa.2017.5077.

European Food Safety Authority, EFSA. (2021). The European Union One Health 2019 Zoonoses Report. *EFSA Journal*, *19*(2), 6406. Doi: 10.2903/j.efsa.2021.6406.

Euzeby, J. P. (2005). Validation list no. 102. List of new names and new combinations previously effectively, but not validly, published. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 547-549.

Euzeby, J. P. (2010). Notification list. Notification that new names and new combinations have appeared in volume 60, part 3 of the IJSEM. *International Journal of Systematic and Evolutionary Microbiology*, *60*(3), 1253-1255.

Euzéby, J. P. (2010, juin 01). *Dictionnaire de bactériologie vétérinaire*. https://web.archive.org/web/20130127030220/http://www.bacterio.cict.fr/bacdico/nomstaxons.html

Evans, A. C. (1916). The bacteria of milk freshly drawn from normal udders. *Journal of Infectious Diseases, 18*, 437-476.

Ezaki, T., Amano, M., Kawamura, Y., and Yabuuchi, E. (2000). Proposal of *Salmonella paratyphi* sp. nov., nom. rev. and request for an opinion to conserve the specific epithet paratyphi in the binary combination *Salmonella paratyphi* as nomen epitheton conservandum. *International Journal of Systematic and Evolutionary Microbiology, 50*, 941-944.

Fàbrega, A., and Vila, J. (2013). *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clinical Microbiology Reviews*. *26*(2), 308-41. Doi: 10.1128/CMR.00066-12.

Faria, C. I., Vaz-Moreira, E., Serapicos, O. C. N., and Manaia, C. M. (2009). Antibiotic resistance in coagulase negative *Staphylococci* isolated from wastewater and drinking water. *Science of the Total Environment*, *407*(12), 3876-3882. Doi: 10.1016/j.scitotenv.2009.02.034.

Farmer III, J. J., Fanning, G. R., Davis, B. R., O'Hara, C. M., Riddle, C., Hickman-Brenner, F. W., Asbury, M. A., Lowery III, V. A., and Brenner, D. J. (1985). *Escherichia fergusonii* and *Enterobacter taylorae*, two new species of *Enterobacteriaceae* isolated from clinical specimens. *Journal of Clinical Microbiology*, *21*, 77-81.

Fédération du Commerce et de la Distribution. (2019, Novembre 15). Critères microbiologiques applicables à partir de 2020 aux marques de distributeurs, marques premiers prix et matières premières dans leur conditionnement initial industriel. *FCD*, 1-61. http://www.fcd.fr/documentation-fcd/.

Food and Agriculture Organization, FAO. (2017). *Preventing Escherichia coli in food*. https://www.fao.org/food/food-safety-quality/a-z-index/e-coli0/en/.

Forsythe, S. J. (2003). *Alimentos seguros: Microbiología*. Zaragoza, Spain: Acribia.

Foster, G., Ross, H. M., Hutson, R. A., and Collins, M. D. (1997). *Staphylococcus lutrae* sp. nov., a new coagulase-positive species isolated from otters. *International Journal of Systematic Bacteriology*, *47*, 724-726.

Foubert, E. L. J., and Douglas, H. C. (1948). Studies on the anaerobic micrococci. I. Taxonomic considerations. *Journal of Bacteriology, 56*, 25-34.

Fountain, K., Gibbon, M. J., Loeffler, A., and Feil, E. J. (2019). Closed genome sequences of *Staphylococcus lloydii* sp. nov. and *Staphylococcus durrellii* sp. nov. isolated from captive fruit bats (*Pteropus livingstonii*). *International Journal of Systematic Bacteriology, 71*.

Fredheim, E. G. A., Klingenberg, C., Rohde, H., Frankenberger, S., Gaustad, P., Flægstad, T., and Sollid, J. E. (2009). Biofilm Formation by *Staphylococcus haemolyticus*. *Journal of Clinical Microbiology*, *47*(4), 1172-1180. Doi:10.1128/JCM.01891-08.

Freney, J., Brun, Y., Bes, M., Meugnier, H., Grimont, F., Grimont, P. A. D., Nervi, C., and Fleurette, J. (1988). *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., two species from human clinical specimens. *International Journal of Systematic Bacteriology*, *38*, 168-172.

Fundación Vasca para la Seguridad Alimentaria, ELIKA. (2021, July 05). *Salmonella*. https://seguridadalimentaria.elika.eus/fichas-de-peligros/salmonella/.

Garrido, A., Chapela, M. J., Román, B., Fajardo, P., Vieites, J. M., and Cabado, G. (2013). In-house validation of a multiplex real-time PCR method for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *International Journal of Food Microbiology*, *164*(1), 92-98.

Garrity, G. M., and Holt, J. G. (2001). Taxonomic Outline of the *Archaea* and *Bacteria*. In: G. M. Garrity, D. R. Boone, and R. W. Castenholz (eds.), *Bergey's Manual of Systematic Bacteriology*, (2nd ed., pp. 155-166). New York, United States: Springer.

Garrity, G. M., Bell, J. A., and Lilburn, T. (2005). Phylum XIV. Proteobacteria phyl. nov. In: D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (eds.), *Bergey's Manual of Systematic Bacteriology*, (2nd ed., Vol. 2). New York, United States: Springer.

Gehring, A. G., Albin, D. M., Bhunia, A. K., Kim, H., Reed, S. A., and Tu, S. I. (2012). Mixed culture enrichment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, and *Yersinia enterocolitica*. *Food Control*, *26*(2), 269-273. Doi: 10.1016/j.foodcont.2012.01.047.

Germini, A., Masola, A., Carnevali, P., and Marchelli, R. (2009). Simultaneous detection of *Escherichia coli* O175: H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Control*, *20*, 733-738.

Gibbons, N. E., and Murray, R. G. E. (1978). Proposals Concerning the Higher Taxa of Bacteria. *International Journal of Systematic Bacteriology*, *28*, 1-6.

Gilbert, R. J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C. D., Richards, J., Roberts, D., and Bolton, F. J. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. *Communicable disease and public health*, *3*(3), 163-167.

Gillespie, C. (2018, April 13). *Colony Characteristics of E. coli*. Sciencing. https://sciencing.com/colony-characteristics-ecoli-8507841.html.

Gnanamani, A., Hariharan, P., and Paul-Satyaseela, M. (2017). *Staphylococcus aureus*: Overview of Bacteriology, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach. In: *Frontiers in Staphylococcus aureus.* Doi: 10.5772/67338.

Gordon, D. M. (2013). The ecology of *Escherichia coli*. In: M. S. Donnenberg (eds.), *Escherichia coli* (2nd Edition, pp.3-20). Boston, United States: Academic Press.

Gorwitz, R. J., Kruszon-Moran, D., McAllister, S. K., McQuillan, G., McDougal, L. K., Fosheim, G. E., Jensen, B. J., Killgore, G., Tenover, F. C., Kuehnert, M. J. (2008). Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004. *The Journal of Infectious Diseases*, *197*, 1226-1234.

Gozalo, A. S., Hoffmann, V. J., Brinster, L. R., Elkins, W. R., Ding, L., and Holland, S. M. (2010). Spontaneous *Staphylococcus xylosus* infection in mice deficient in NADPH oxidase and comparison with other laboratory mouse strains. *Journal of the American Association for Laboratory Animal Science*, *49*, 480-486.

Grace, D., and Fetsch, A. (2018). *Staphylococcus aureus*-A foodborne pathogen: Epidemiology, detection, characterization, prevention, and control: An overview. In: A. Fetsch, (ed.), *Staphylococcus aureus* (pp. 1-26). Amsterdam, Netherlands: Elsevier.

Graves, L. M., Helsel, L. O., Steigerwalt, A. G., Morey, R. E., Daneshvar, M. I., Roof, S. E., Orsi, R. H., Fortes, E. D., Milillo, S. R., den Bakker, H. C., Wiedmann, M., Swaminathan, B., and Sauders, B. D. (2010). *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 1280-1288.

Gray, M. J., Zadoks, R. N., Fortes, E. D., Dogan, B., Cai, S., Chen, Y., Scott, V. N., Gombas, D. E., Boor, K. J., and Wiedmann, M. (2004). *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Applied and Environmental Microbiology*, *70*(10), 5833-5841.

Gray, M. L., and Killinger, A. H. (1966). *Listeria monocytogenes* and listeric infections. *Bacteriological Reviews*, *30*(2), 309-82.

Grimont, P. A. D., Grimont, F., and Bouvet, P. J. M. (2000). *Salmonella*. In: J. Freney, F. Renaud, W. Hansen, C. Bollet (eds.), *Précis de Bactériologie clinique* (pp.137-115). Paris, France: Editions ESKA.

Guillet, C., Join-Lambert, O., Le Monnier, A., Leclercq, A., Mechai, F., Mamzer-Bruneel, M. F., Bielecka, M. K., Scortti, M., Disson, O., Berche, P., Vazquez-Boland, J., Lortholary, O., and Lecuit, M. (2010). Human listeriosis caused by *Listeria ivanovii*. *Emerging Infectious Diseases*, *16*(1), 136-138. Doi: 10.3201/eid1601.091155.

Gwida, M., Hotzel, H., Geue, L., and Tomaso, H. (2014). Occurrence of *Enterobacteriaceae* in Raw Meat and in Human Samples from Egyptian Retail Sellers. *International Scholarly Research Notices*. http://dx.doi.org/10.1155/2014/565671.

Hait, J. M., Tallent, S. M., and Bennett, R. W. (2014). Screening, detection, and serotyping methods for toxin genes and enterotoxins in *Staphylococcus* strains. *Journal of AOAC International*, *97*, 1078-1083.

Hájek, V. (1976). *Staphylococcus intermedius*, a new species isolated from animals. *International Journal of Systematic Bacteriology*, *26*, 401-408.

Hajek, V., Devriese, L. A., Mordarski, M., Goodfellow, M., Pulverer, G., and Varaldo, P. E. (1986). Elevation of *Staphylococcus hyicus* subsp. chromogenes (Devriese et al., 1978) to species status: *Staphylococcus chromogenes* (Devriese et al., 1978) comb. nov. *Systematic and Applied Microbiology*, *8*, 169-173.

Hajek, V., Ludwig, W., Schleifer, K. H., Springer, N., Zitzelsberger, W., Kroppenstedt, R. M., and Kocur, M. (1992). *Staphylococcus muscae*, a new species isolated from flies. *International Journal of Systematic Bacteriology*, *42*, 97-101.

Harakeh, S. H., Yassin, H., and El-Fadel, M. (2006). Isolates of *Staphylococcus aureus* and *Saprophyticus* resistant to antimicrobials isolated from the Lebanese aquatic environment. *Marine Pollution Bulletin*, *52*(8), 912-919. Doi: 10.1016/j.marpolbul.2005.12.008.

Hardy, A. (1999). Food, hygiene, and the laboratory: A short history of food poisoning in Britain, circa 1850-1950. *Social History of Medicine*, *12*(2), 293-311.

Hauschild, T., Stepanovic, S., and Zakrzewska-Czerwinska, J. (2010). *Staphylococcus stepanovicii* sp. nov., a novel novobiocin-resistant oxidase-positive staphylococcal

species isolated from wild small mammals. *International Journal of Systematic Bacteriology*, *33*, 183-187.

Herman, K. M., Hall, A. J., and Gould, L. H. (2015). Outbreaks attributed to fresh leafy vegetables, United States, 1973-2012. *Epidemiology and Infection*, *143*(14), 3011-3021. Doi: 10.1017/S0950268815000047.

Hess, S., and Gallert, C. (2015). *Staphylococcus argensis* sp. nov., a novel staphylococcal species isolated from an aquatic environment. *International Journal of Systematic and Evolutionary Microbiology*, *65*, 2661-2665.

Ho, J., Boost, M. V., and O'donoghue, M. M. (2015). Tracking sources of *Staphylococcus aureus* hand contamination in food handlers by spa typing. *American Journal of Infection Control*, *43*(7), 759-761. Doi: 10.1016/j.ajic.2015.03.022.

Hoenigl, M., Valentin, T., Zarfel, G., Wuerstl, B., Leitner, E., Salzer, H. J. F., Posch, J., Krause, R., and Grisold, A. J. (2012). Nosocomial Outbreak of *Klebsiella pneumoniae* Carbapenemase Producing Klebsiella oxytoca in Austria. *Antimicrobial Agents and Chemotherapy*, *56*(4), 2158-2161. Doi: 10.1128/AAC.05440-11.

Holban, A. M., and Grumezescu, A. M. (2018). *Foodborne Diseases Handbook of Food Bioengineering* (Vol. 15). Academic Press. Elsevier.

Huang, C. S., Oberbeck, N., Hsiao, Y-C., Liu, P., Johnson, A. R., Dixit, V. M., and Hymowitz, S. G. (2018). Crystal Structure of Ripk4 Reveals Dimerization-Dependent Kinase Activity. *Structure*, *26*(5), 767-777. Doi: 10.1016/j.str.2018.04.002.

Hunter, P. R. (1997). *Waterborne Disease: Epidemiology and Ecology*. United Kingdom: John Wiley and Sons.

Ibarluzea, J., Moreno, B., Zigorraga, C., Castilla, T., Martinez, M., and Santamaria, J. (1998). Determinants of the microbiological water quality of indoor swimming pools in relation to disinfection. *Water Research*, *32*, 865-871.

Igimi, S., Kawamura, S., Takahashi, E., and Mitsuoka, T. (1989). *Staphylococcus felis*, a new species from clinical specimens from cats. *International Journal of Systematic Bacteriology*, *39*, 373-377.

ISO 16649-1 (2018). Microbiology of the food chain−Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli−Part 1: Colony-count technique at 44 degrees C using membranes and 5-bromo-4-chloro-3-indolyl beta-D-glucuronide.

ISO 16649-2 (2001). Microbiology of food and animal feeding stuffs−Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*−Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide.

ISO 16649-3 (2015). Microbiologie de la chaîne alimentaire−Méthode horizontale pour le dénombrement des *Escherichia coli* bêta-glucuronidase positive−Partie 3: Recherche et technique du nombre le plus probable utilisant le bromo-5-chloro-4-indolyl-3 ß-D-glucuronate

ISO 16654. (2001). Microbiologie des aliments−Méthode horizontale pour la recherche des *Escherichia coli* O157.

Jamali, H., Paydar, M., Radmehr, B., Ismail, S., and Dadrasnia, A. (2015). Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control*, *54*, 383-388.

Jamshidi, A., and Zeinali, T. (2019). Significance and Characteristics of *Listeria monocytogenes* in Poultry Products. *International Journal of Food Science*. https://doi.org/10.1155/2019/7835253

Janda, J. M., and Abbott, S. L. (2006). The Genus *Hafnia*: from Soup to Nuts. *Clinical Microbiology Reviews*, *19*(1), 12-28. Doi: 10.1128/cmr.19.1.12-28.2006.

Janda, J. M., and Abbott, S. L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35-73. Doi: 10.1128/CMR.00039-09.

Jin, W., and Yamada, K. (2016). Staphylococcal enterotoxins in processed dairy products. In: P. Kotzekidou, (ed.), *Food Hygiene and Toxicology in Ready-to-Eat Foods* (pp. 1-18). Academic Press: Elsevier.

Johannessen, G. S., Loncarevic, S., and Kruse, H. (2002). Bacteriological analysis of fresh produce in Norway. *International Journal of Food Microbiology*, *77*(3), 199-204.

Jorgensen, H. J., Mork, T., and Rorvik, L. M., (2005c). The occurrence of *Staphylococcus aureus* on a farm with small-scale production of raw milk cheese. *Journal of Dairy Science*, *88*, 3810-3817.

Kadariya, J., Smith, T. C., and Thapaliya, D. (2014). *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International*. Doi: 10.1155/2014/827965.

Kaper, J. B., Nataro, J. P., and Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, *2*(2), 123-140.

Kauffmann, F. (1964). Das Kauffmann-White-Schema. In: E. Van Oye (eds.), *The world problem of salmonellosis* (pp.21-46). The Hague, Netherlands: Junk Publications.

Kérouanton, A., Hennekinne, J. A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A., and De Buyser, M. L. (2007). Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. *International Journal of Food Microbiology*, *115*, 369-375.

Key, F. M., Posth, C., Esquivel-Gomez, L. R., and Hübler, R. (2020). Emergence of human-adapted Salmonella enterica is linked to the Neolithization process. Nature Ecology and Evolution, *4*(3), 324-333.

Khan, M. F. (2017). Brief History of *Staphylococcus aureus*: A Focus to Antibiotic Resistance. *EC Microbiology*, *5*, 36-96.

Kim, B. N., Kim, N. J., Kim, M. N., Kim, Y. S., Woo, J. H., and Ryu, J. (2003). Bacteraemia due to tribe *Proteeae*: a review of 132 cases during a decade (1991-2000). *Scandinavian Journal of Infectious Diseases*, *35*(2), 98-103.

Kim, J., Demeke, T., Clear, R. M., and Patrick, S. K. (2006). Simultaneous detection by PCR of *Escherichia coli*, *Listeria monocytogenes* and *Salmonella typhimurium* in artificially inoculated wheat grain. *International Journal of Food Microbiology*, *111*, 21-25.

Kłapeć, T., Cholewa, A., Cholewa, G., Dutkiewicz, J., and Wójcik-Fatla, A. (2016). Microbiological characterization of vegetables and their rhizosphere soil in Eastern Poland. *Annals of Agricultural and Environmental Medicine*, *23*(4), 559-565. Doi: 10.5604/12321966.1226846.

Kloos, W. E., and Schleifer, K. H. (1975). Isolation and characterization of staphylococci from human skin. II. Description of four new species: *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus hominis*, and *Staphylococcus simulans*. *International Journal of Systematic Bacteriology*, *25*, 62-79.

Kloos, W. E., Schleifer, K. H., and Smith, R. F. (1976). Characterization of *Staphylococcus sciuri* sp. nov. and its subspecies. *International Journal of Systematic Bacteriology*, *26*, 22-37.

Korir, R. C., Parveen, S., Hashem, F., and Bowers, J. (2016). Microbiological quality of fresh produce obtained from retail stores on the Eastern Shore of Maryland, United States of America. *Food Microbiology*, *56*, 29-34.

Lambert, L. H., Cox, T., Mitchell, K., Rossello-Mora, R. A., Del Cueto, C., Dodge, D. E., Orkand, P., and Cano, R. J. (1998). *Staphylococcus succinus* sp. nov., isolated from Dominican amber. *International Journal of Systematic Bacteriology*, *48*, 511-518.

Lang Halter, E., Neuhaus, K., and Scherer, S. (2013). *Listeria weihenstephanensis* sp. nov., isolated from the water plant Lemna trisulca taken from a freshwater pond. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 641-647.

LaRock, D. L., Chaudhary, A., and Miller, S. I. (2015). *Salmonellae* interactions with host processes. *Nature Reviews Microbiology*, *13*(4), 191-205.

Latha, C., Anu, C. J., Ajaykumar, V. J., and Sunil, B. (2017). Prevalence of *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Salmonella enterica* Typhimurium in meat and meat products using multiplex polymerase chain reaction, *Veterinary World*, *10*(8), 927-931.

Le Minor, L., and Popoff, M. Y. (1987). Request for an Opinion. Designation of *Salmonella enterica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*. *International Journal of Systematic Bacteriology*, *37*, 465-468.

Lechevallier, M. W., and Seidler, R. J. (1980). Staphylococcus aureus in Rural Drinking Water. *Applied and Environmental Microbiology*, *30*(4), 739-742.

Leclercq, A., Clermont, D., Bizet, C., Grimont, P. A., Le Fleche-Mateos, A., Roche, S. M., Buchrieser, C., Cadet-Daniel, V., Le Monnier, A., Lecuit, M., and Allerberger, F. (2010). *Listeria rocourtiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 2210-2214.

Leclercq, A., Moura, A., Vales, G., Tessaud-Rita, N., Aguilhon, C., and Lecuit, M. (2019). *Listeria thailandensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *69*, 74-81.

Ledlod, S., Bunroddith, K., Areekit, S., Santiwatanakul, S., and Chansiri, K. (2020). Development of a duplex lateral flow dipstick test for the detection and differentiation of *Listeria* spp. and *Listeria monocytogenes* in meat products based on loop-mediated isothermal amplification. *Journal of Chromatography B*. https://doi.org/10.1016/j.jchromb.2019.121834.

Leung, J. M., and Gallant, C. V. (2014). Infections due to *Escherichia* and *Shigella*. In: *Reference Module in Biomedical Sciences*. Elsevier.

Levinson, W. (2010). *Review of Medical Microbiology and Immunology* (11th ed.). New York: McGraw-Hill.

Lignieres, J. (1900). Maladies du porc. *Bulletin of the Society for Central Medical Veterinarians, 18*, 389-431.

Linke, K., Ruckerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., and Stessl, B. (2014). Reservoirs of *Listeria* species in three environmental ecosystems *Applied and Environmental Microbiolog, 80*, 5583-5592.

List of Prokaryotic names with Standing in Nomenclature, LPSN. (2021, October 11). https://www.bacterio.net/.

Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., Fierer, J., and Nizet, V. (2005). *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *Journal of Experimental Medicine, 202*(2), 209-215. Doi: https://doi.org/10.1084/jem.20050846.

Liu, S., Jin, D., Lan, R., Wang, Y., Meng, Q., Dai, H., Lu, S., Hu, S., and Xu, J. (2015). *Escherichia marmotae* sp. nov., isolated from faeces of *Marmota* himalayana. *International Journal of Systematic and Evolutionary Microbiology, 65*(7), 2130-2134.

Ludwig, W., Schleifer, K-H., and Whitman, W. B. (2009). Class I. *Bacilli* class nov. In: P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K-H. Schleifer, W. B. Whitman (eds.), *Bergey's Manual of Systematic Bacteriology* (2nd ed., Vol. 3, The Firmicutes, pp.19-20), New York, USA: Springer.

Ma, K., Deng, Y., Bai, Y., Xu, D., Chen, E., Wu, H., Li, B., and Gao, L. (2014). Rapid and simultaneous detection of *Salmonella*, *Shigella*, and *Staphylococcus aureus* in fresh pork using a multiplex real-time PCR assay based on immunomagnetic separation. *Food Control, 42*, 87-93

Macedo, A. C., Tavares, T. G., and Malcata, F. X. (2004). Influence of native lactic acid bacteria on the microbiological, biochemical and sensory profiles of Serra da Estrela cheese. *Food Microbiology, 21*(2), 233-240.

MacFadyen, A. C., Drigo, I., Harrison, E. M., Parkhill, J., Holmes, M. A., and Paterson, G. K. (2019). *Staphylococcus caeli* sp. nov., isolated from air sampling in an industrial rabbit holding. *International Journal of Systematic and Evolutionary Microbiology, 69*, 82-86.

Madhaiyan, M., Wirth, J. S., and Saravanan, V. S. (2020). Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family *Staphylococcaceae*. *International Journal of Systematic Bacteriology, 70*, 5926-5936.

Marineli, F., Tsoucalas, G., Karamanou, M., and Androutsos, G. (2013). Mary Mallon (1869-1938) and the history of typhoid fever. *Annals of gastroenterology, 26*(2), 132-134.

Markoulatos, P., Siafakas, N., and Moncany, M. (2002). Multiplex polymerase chain reaction: a practical approach. *Journal of Clinical Laboratory Analysis, 16*, 47-51.

Martin, A., Contreras, M. A., Robles, M. D., Moreno de la Torre, C., Martínez, F., and Robles, M. D. (2018). Aislamiento e identificación de Enterobacterias a partir de Quesos Adobera. *Congreso Internacional Inocuidad de Alimentos*. http://www.egnosis. udg.mx.

Martínez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution, 157*, 2893-2902.

McDaniels, A. E., Rice, E. W., Reyes, A. L., Johnson, C. H., Haugland, R. A., and Stelma Jr., G. N., (1996). Confirmational identification of *Escherichia coli*, a comparison of genotypic and phenotypic assays for glutamate decarboxylase and β Dglucuronidase. *Applied and Environmental Microbiology, 62*, 3350-3354.

McPherson, C., Gal, P., and Ransom, J. L. (2008). Treatment of *Citrobacter koseri* infection with ciprofloxacin and cefotaxime in a preterm infant. *Annals of Pharmacotherapy*, *42*(7), 1134-1138. Doi: 10.1345/aph.1L008.

Mendoza, Y. M. F., Muñoz, J. F. G., Lozano, J. M. A., Andrade, E. F. M., and Mario, L. (2020). Evalución de la presencia de *Staphylococcus aureus* en queso fresco artesanal del mercado municipal del cantón junín de la provincia de manabi. *Alimentos Hoy, 28*(49).

Monteith, A. J., Miller, J. M., Maxwell, C. N., Chazin, W. J., and Skaar, E. P. (2021). Neutrophil extracellular traps enhance macrophage killing of bacterial pathogens. *Science Advances, 7*(37). Doi:10.1126/sciadv.abj2101.

Morais, V. P., Daporta, M. T., Bao, A. F., Campello, M. G., and Andrés, G. Q. (2009). Enteric fever-like syndrome caused by *Raoultella ornithinolytica* (*Klebsiella ornithinolytica*). *Journal of clinical microbiology*, *47*(3), 868-869. https://doi.org/10.1128/JCM.01709-08.

Morales, P., Feliu, I., Fernandez-Garcia, E., and Nunez, M. (2004). Volatile compounds produced in cheese by *Enterobacteriaceae* strains of dairy origin. *Journal of Food Protection*, *67*(3), 567-573. Doi: 10.4315/0362-028x-67.3.567.

Mukhopadhyay, A., and Mukhopadhyay, U. K. (2007). Novel multiplex PCR approaches for the simultaneous detection of human pathogens: *Escherichia coli* 0157:H7 and *Listeria monocytogenes*. *Journal of Microbiological Methods, 68*, 193-200.

Murray, A. K., Lee, J., Bendall, R., Zhang, L., Sunde, M., Slettemeas, J. S., Gaze, W., Page, A. J., and Vos, M. (2018). *Staphylococcus cornubiensis* sp. nov., a member of the *Staphylococcus intermedius* Group (SIG). *Journal of Systematic and Evolutionary Microbiology, 68*, 3404-3408.

Murray, E. G. D., Webb, R. A. and Swann, H. B. R. (1926). A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. *The Journal of Pathology and Bacteriology, 29*, 407-439.

Nandy, P., Roy, S., Thakur, A. R., and Chaudhuri, S. R. (2013). Comparative study on characterization of Available: three staphylococcal isolates from varied origin. https://tspace.library.utoronto.ca/handle/1807/36824.

Naushad, S., Kanevets, U., Nobrega, D., Carson, D., Dufour, S., Roy, J. P., Lewis, P. J., and Barkema, H. W. (2019). *Staphylococcus debuckii* sp. nov., a coagulase-negative species from bovine milk. *International Journal of Systematic and Evolutionary Microbiology, 69*, 2239-2249.

Nguz, K., Shindano, J., Samapundo, S., and Huyghebaert, A. (2005). Microbiological evaluation of fresh-cut organic vegetables produced in Zambia. *Food Control, 15*(5), 623-628.

Nunez-Montero, K., Leclercq, A., Moura, A., Vales, G., Peraza, J., Pizarro-Cerda, J., and Lecuit, M. (2018). *Listeria costaricensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *68*, 844-850.

Ogston, A. (1881). Micro-Organisms in Surgical Diseases. *British Medical Journal*, 369-75.

Oliveira, M., Usall, J., Vinas, I., Anguera, M., Gatius, F., and Abadias, M. (2010). Microbiological quality of fresh lettuce from organic and conventional production. *Food Microbiology*, *27*, 679-684.

Ombarak, R. A., Hinenoya, A., Awasthi, S. P., Iguchi, A., Shima, A., Elbagory, A-R. M., and Yamasaki, S. (2016). Prevalence and pathogenic potential of *Escherichia coli* isolates from raw milk and raw milk cheese in Egypt. *International Journal of Food Microbiology*, *221*, 69-76. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.01.009.

Omiccioli, E., Amagliani, G., Bandi, G., and Magnani, M. (2009). A new platform for real time PCR detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157 in milk. *Food Microbiology*, *26*, 615-622.

Oren, A., and Garrity, G. M. (2014). Notification list. Notification that new names and new combinations. *International Journal of Systematic and Evolutionary Microbiology*, *64*(6), 2930-2931.

Oren, A., and Garrity, G. M. (2021). Notification list. *International Journal of Systematic and Evolutionary Microbiology*, *71*(7), 5006.

Oscorbin, I. P., Shevelev, G. Y., Pronyaeva, K. A., Stepanov, A. A., Shamovskaya, D. V., Mishukova, O. V., Pyshnyi, D. V., and Filipenko, M. L. (2021). Detection of SARS-CoV-2 RNA by a Multiplex Reverse-Transcription Loop-Mediated Isothermal Amplification Coupled with Melting Curves Analysis. *International Journal of Molecular Sciences*, *22*(11), 5743. Doi: 10.3390/ijms22115743.

Oumokhtar, B., Berrada, H., and Huidson, W. (2008). Analyse microbiologique de la viande hachée bovine commercialisée à Fès, Maroc. *Les Technologies de laboratoire*, *12*, 4-10.

Pain, M., Wolden, R., Jaen-Luchoro, D., Salva-Serra, F., Iglesias, B. P., Karlsson, R., Klingenberg, C., and Cavanagh, J. P. (2020). *Staphylococcus borealis* sp. nov., isolated from human skin and blood. *International Journal of Systematic and Evolutionary Microbiology*, *70*, 6067-6078.

Palomino, C., and González, Y. (2014). Técnicas moleculares para la detección e identificación de patógenos en alimentos: ventajas y limitaciones. *Revista Peruana de Medicina Experimental y Salud Publica*, *31*(3), 535-546.

Pantucek, R., Sedlacek, I., Indrakova, A., Vrbovska, V., Maslanova, I., Kovarovic, V., Svec, P., Kralova, S., Kristofova, L., Keklakova, J., Petráš, P., and Doškař, J. (2018). *Staphylococcus edaphicus* sp. nov., Isolated in Antarctica, Harbors the mecC Gene and Genomic Islands with a Suspected Role in Adaptation to Extreme Environments. *Applied and Environmental Microbiology*, *84*.

Pantucek, R., Sedlacek, I., Petras, P., Koukalova, D., Svec, P., Stetina, V., Vancanneyt, M., Chrastinova, L., Vokurkova, J., Ruzickova, V., Doškař, J., Swings, J., and Hájek, V. (2005). *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 1953-1958.

Pantucek, R., Svec, P., Dajcs, J. J., Machova, I., Cernohlavkova, J., Sedo, O., Gelbicova, T., Maslanova, I., Doskar, J., Zdrahal, Z., Růžičková, V., and Sedláček, I. (2013). *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. petrasii subsp. nov. and *S. petrasii* subsp. croceilyticus subsp. nov., isolated from human clinical specimens and human ear infections. *Systematic and Applied Microbiology, 36*, 90-95.

Pascual, A. M. R., and Calderón, P. V. (2010). *Microbiología alimentaria: Metodología analítica para alimentos y bebidas*. (2nd ed.). Madrid: Díaz de Santos.

Paterson, G. K., Harrison, E. M., and Holmes, M. A. (2014). The emergence of mecC methicillin-resistant *Staphylococcus aureus*. *Trends in Microbiology, 22*(1), 42-7.

Pavlov, D., de Wet, C. M. E., Grabow, W. O. K., and Ehlers, M. M. (2004). Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *International Journal of Food Microbiology, 92*, 275-287.

Percival, S. I., Chalmers, R. M., Embrey, M., Hunter, P. R., Sellwood, J., and Wyn-jones, P. (2004). *Microbiology of water borne diseases*. United States of America: Elsevier academic press.

Perdomo, C., Gutiérrez, F., García, O., Acevedo, I., Bastidas, Z., and Kowalski, A. (2015). Caracterización fisicoquímica y bacteriológica de queso blanco artesanal en la parroquia buría, estado lara, Venezuela. *Gaceta de Ciencias Veterinarias, 20*(2), 35-44.

Phillips, D., Jordan, D., Morris, S., Jenson, I., and Sumner, J. (2008). A National Survey of the Microbiological Quality of Retail Raw Meats in Australia. *Journal of Food Protection, 71*(6), 1232-1236.

Pierard, D., De Greve, H., Haesebrouck, F., and Mainil, J. (2012). O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: respective role of cattle and humans. *Veterinary Research, 43*(1), 13.

Pirie, J. H. H. (1940). *Listeria*: change of name for a genus of bacteria. *Nature, 145*, 264.

Pizarro-Cerdá, J., and Cossart, P. (2019). Microbe Profile: *Listeria monocytogenes* a paradigm among intracellular bacterial pathogens. *Microbiology, 165*(7), 719-721. Doi:10.1099/mic.0.000800.

Prévot, A. R. (1961). Traité de Systématique Bactérienne (Vol. 2). Paris, France: Dunod.

Probst, A. J., Hertel, C., Richter, L., Wassill, L., Ludwig, W., and Hammes, W. P. (1998). *Staphylococcus condimenti* sp. nov., from soy sauce mash, and *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. utilis subsp. nov. *International Journal of Systematic Bacteriology, 48*, 651-658.

Psoni, L., Tzanetakis, N., and Litopoulou-Tzanetaki, E. (2003). Microbiological characteristics of Batzos, a traditional Greek cheese from raw goat's milk. *Food Microbiology, 20*(5), 575-582.

Quereda, J. J., Leclercq, A., Moura, A., Vales, G., Gomez-Martin, A., Garcia-Munoz, A., Thouvenot, P., Tessaud-Rita, N., Bracq-Dieye, H., and Lecuit, M. (2020). *Listeria valentina* sp. nov., isolated from a water trough and the faeces of healthy sheep. *International Journal of Systematic and Evolutionary Microbiology, 70*, 5868-5879.

Quinto, E. J., Marín, J. M., Caro, I., Mateo, J., and Schaffner, D. W. (2020). Modelling Growth and Decline in a Two-Species Model System: Pathogenic Escherichia coli O157:H7 and Psychrotrophic Spoilage Bacteria in Milk. *Foods, 9*(3), 331. Doi: 10.3390/foods9030331.

Rahme, L. G., Tan, M-W., Le, L., Wong, S. M., Tompkins, R. G., Calderwood, S. B., and Ausubel, F. M. (1997). Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(24), 13245-13250.

Rahn, K., De Grandis, S. A., Clarke, R. C., McEwen, S. A., Galan, J. E., Ginocchio, C., Curtiss III, R., and Gyles, C. L. (1992). Amplification of an invA gene sequence of *Salmonella enterica* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes*, *6*, 271-279.

Rahn, O. (1937). New principles for the classification of bacteria. In: *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten Und Hygiene*, *96*, 273-286.

Rajabzadeh, S., Bahreini, M., and Sharifmoghadam, M. R. (2018). A rapid method for separating and concentration of food-borne pathogens using elution from ready-to-eat vegetables. *Iranian Journal of Microbiology*, *10*(6), 385-393.

Ramaswamy, V., Cresence, V. M., Rejitha, J. S., Lekshmi, M. U., Dharsana, K. S., Prasad, S. P., and Vijila, H. M. (2007). *Listeria* review of epidemiology and pathogenesis. *Journal of Microbiology, Immunology and Infection*, *40*(1), 4-13.

Rasheed, N. A., and Hussein, N. R. (2021). *Staphylococcus aureus*: An Overview of Discovery, Characteristics, Epidemiology, Virulence Factors and Antimicrobial Sensitivity, *European Journal of Molecular & Clinical Medicine*, *8*(3), 1160-1183.

Rashid, T., and Ebringer, A. (2007). Ankylosing spondylitis is linked to *Klebsiella* the evidence. *Clinical Rheumatology*, *26*(3), 858-864. Doi: 10.1007/s10067-006-0488-7.

Red Nacional de Vigilancia Epidemiológica, RENAVE. (2018). Protocolo de vigilancia de fiebre tifoidea y paratifoidea (*Salmonella typhi* y *Salmonella paratyphi*). https://www.isciii.es/QueHacemos/Servicios/VigilanciaSaludPublicaRENAVE/EnfermedadesTransmisibles/Documents/PROTOCOLOS/Protocolo%20de%20Vigilancia%20de%20Fiebre%20Tifoidea%20y%20Paratifoidea.pdf.

Red Nacional de Vigilancia Epidemiológica, RENAVE. (2020). Resultados de la vigilancia epidemiológica de las enfermedades transmisibles. Informe anual años 2017-2018.
https://www.isciii.es/QueHacemos/Servicios/VigilanciaSaludPublicaRENAVE/EnfermedadesTransmisibles/Documents/INFORMES/INFORMES%20RENAVE/ultimo%20informe.pdf.

Reeves, M. W., Evins, G. M., Heiba, A. A., Plikaytis, B. D., and Farmer, J. J. (1989). Clonal nature of *Salmonella typhi* and its genetic relatedness to other *Salmonellae* as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. *Journal of Clinical Microbiology*, *27*, 313-320.

Reglamento (CE) 2019/229 de la Comisión, de 7 de febrero de 2019, por el que se modifica el Reglamento (CE) Nº 2073/2005, relativo a los criterios microbiológicos aplicables a los productos alimenticios, en lo que se refiere a determinados métodos, al criterio de seguridad alimentaria para Listeria monocytogenes en las semillas germinadas y a los criterios de higiene de los procesos y de seguridad alimentaria relativos a los zumos de frutas y hortalizas no pasteurizados (listos para el consumo). *Diario Oficial de la Unión Europea*, *L37*, 106-110.

Reglamento (CE) Nº 1441/2007 de la Comisión, de 5 de diciembre de 2007, que modifica el REGLAMENTO (CE) Nº 2073/2005, relativo a los criterios microbiológicos aplicables a los productos alimenticios. *Diario Oficial de la Unión Europea*, *L322*, 12-29.

Reglamento (CE) Nº 2073/2005 de la Comisión, de 15 de noviembre de 2005, relativo a los criterios microbiológicos aplicables a los productos alimenticios. *Diario Oficial de la Unión Europea*, *L338*, 1-30.

Ricke, S. C. (2021). Strategies to Improve Poultry Food Safety, a Landscape Review. *Annual Review of Animal Biosciences*, 9(1), 379-400.

Riesen, A., and Perreten, V. (2010). *Staphylococcus rostri* sp. nov., a haemolytic bacterium isolated from the noses of healthy pigs. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 2042-2047.

Rivas, L., Mellor, G. E., Gobius, K., and Fegan, N. (2015). *Detection and Typing Strategies for Pathogenic Escherichia coli*. New York, United States: Springer. Doi: 10.1007/978-1-4939-2346-2345.

Rocourt, J., and Grimont, P. A. D. (1983). *Listeria welshimeri* sp. nov. and *Listeria seeligeri* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *33*, 866-869.

Rosenbach, F. J. (1884). *Microorganismen bei den Wund-Infections-Krankheiten des Menschen.* Wiesbaden, Deutschland: J. F. Bergmann.

Rosengren, A., Fabricius, A., Guss, B., Sylvén, S., and Lindqvist, R. (2010). Occurrence of foodborne pathogens and characterization of Staphylococcus aureus in cheese produced on farm-dairies. *International Journal of Food Microbiology*, *44*, 263-269. Doi: 10.1016/j.ijfoodmicro.2010.10.004.

Roumagnac, P., Weill, F. X., Dolecek, C., Baker, S., Brisse, S., Chinh, N. T., Le, T. A., Acosta, C. J., Farrar, J., Dougan, G., and Achtman, M. (2006). Evolutionary history of *Salmonella typhi*. *Science*, *14*, 1301-1304.

Ryan, M. P., O'Dwyer, J., and Adley, C. C. (2017). Evaluation of the Complex Nomenclature of the Clinically and Veterinary Significant Pathogen *Salmonella*. *BioMed Research International*, 1-6. Doi: 10.1155/2017/3782182.

Ryu, J. H., Kim, M., Kim, E. G., Beuchat, L. R., and Kim, H. (2014). Comparison of the microbiological quality of environmentally friendly and conventionally grown vegetables sold at retail markets in Korea. *Journal of Food Science*, *79*(9), 1739-1744.

Sangwan, A. (2016, May 16). *Escherichia Coli* (*E. coli*): *Meaning, Morphology and Characteristics*. Biology Discussion. https://www.biologydiscussion.com/bacteriology/systematic-bacteriology/escherichia-coli-e-coli-meaning-morphology-and-characteristics/30821.

Santos, G. A. C., Dropa, M., Rocha, S. M., Peternella, F. A. S., and Razzolini, M. T. P. (2020). *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) in drinking water fountains in urban parks. *Journal of Water and Health*, *18*(5), 654-664. Doi: 10.2166/wh.2020.042.

Santos, M. I., Cavaco, A., Gouveia, J., Novais, M. R., Nogueira, P. J., Pedroso, L., and Ferreirad, A. S. S. (2012). Evaluation of minimally processed salads commercialized in Portugal. *Food Control*, *23*(1), 275-281. https://doi.org/10.1016/j.foodcont.2011.06.022.

Sauders, B. D., Overdevest, J., Fortes, E., Windham, K., Schukken, Y., Lembo, A., and Wiedmann, M. (2012). Diversity of *Listeria* species in urban and natural environments. *Applied and Environmental Microbiolog*, *78*, 4420-4433.

Scheutz, F., Teel, L. D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N. A., Melton-Celsa, A. R., Sanchez, M., Persson, S., and O'Brien, A. D. (2012). Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclatura. *Journal of Clinical Microbiology*, *50*, 2951-2963.

Schleifer, K. H., and Kloos, W. E. (1975). Isolation and characterization of staphylococci from human skin. I. Amended descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and descriptions of three new species: *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, and *Staphylococcus xylosus*. *International Journal of Systematic Bacteriology*, *25*, 50-61.

Schleifer, K. H., Geyer, U., Kilpper-Balz, R., and Devriese, L. A. (1983). Elevation of *Staphylococcus sciuri* subsp. *lentus* (Kloos et al.) to Species Status: *Staphylococcus lentus* (Kloos et al.) comb. nov. *Systematic and Applied Microbiology*, *4*, 382-387.

Schleifer, K. H., Kilpper-Balz, R., and Devriese, L. A. (1984). *Staphylococcus arlettae* sp. nov., *S. equorum* sp. nov. and *S. kloosii* sp. nov. : three New coagulase-negative, novobiocin-resistant species from animals. *Systematic and Applied Microbiology*, *5*, 501-509.

Schleifer, K. H., Kilpper-Bälz, R., Fischer, U., Faller, A., and Endl, J. (1982). Identification of "*Micrococcus candidus*" ATCC 14852 as a strain of *Staphylococcus epidermidis* and of "Micrococcus caseolyticus" ATCC 13548 and *Micrococcus varians* ATCC 29750 as members of a new species, *Staphylococcus caseolyticus*. *International Journal of Systematic Bacteriology*, *32*, 15-20.

Schleifer, K-H., and Bell, J. A. (2009). Family VIII. *Staphylococcaceae* fam. nov. In: P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K-H. Schleifer, W. B. Whitman (eds.), *Bergey's Manual of Systematic Bacteriology*, (2nd ed., Vol. 3, The *Firmicutes*, p.392), New York, USA: Springer.

Schutte, A. H. J., Strepis, N., Zandijk, W. H. A., Bexkens, M. L., Bode, L. G. M., and Klaassen, C. H. W. (2021). Characterization of *Staphylococcus roterodami* sp. nov., a new species within the *Staphylococcus aureus* complex isolated from a human foot infection. *International Journal of Systematic and Evolutionary Microbiology*, *71*, 4996.

Seeliger, H. P. R. (1981). Apathogen listerien: *L. innocua* sp. n. (Seeliger and Schoofs, 1977). *Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene*, *249*, 487-493.

Seeliger, H. P. R., Rocourt, J., Schrettenbrunner, A., Grimont, P. A. D., and Jones, D. (1984). *Listeria ivanovii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *34*, 336-337.

Sejvar, J. J. (2013). Neuroinfections (What Do I Do Now?). *Emerging Infectious Diseases*, *19*(9), 1553.

Seow, J., Ágoston, A., Phua, L., and Yuk, H-G. (2012). Microbiological quality of fresh vegetables and fruits sold in Singapore. *Food Control*, *25*(1), 39-44.

Shaw, C., Stitt, J. M., and Cowan, S. T. (1951). Staphylococci and their classification. *Journal of General Microbiology*, *5*, 1010-1023.

Shulman, S. T., Friedmann, H. C., and Sims, R. H. (2007). Theodor Escherich: The First Pediatric Infectious Diseases Physician. *Clinical Infectious Diseases, 45*, 1025-1029. Doi: 10.1086/521946.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., Kallen, A., Limbago, B., and Fridkin, S. (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection Control and Hospital Epidemiology*, *34*, 1-14.

Silva, G. S. E., Reséndiz, M. R., Hernández, Z. J. S., Pérez, A. R. and Pérez, B. J. (2010). El Queso fresco Artesanal de vaca: Calidad Bacteriológica. Memorias, XI Simposio Iberoamericano sobre Conservación y Utilización de Recursos Zoogenéticos.

Siriken, B. (2004). The Microbiological Quality of Ground Beef. *Revue de Médecine Vétérinaire, 155*(12), 632-636. https://www.researchgate.net/publication/236217315.

Soares, D., Lerin, L. A., Cansian, R. L., Oliveira, J. V., and Mazutti, M. A. (2013). Inactivation of *Listeria monocytogenes* using supercritical carbon dioxide in a high-pressure variable-volume reactor. *Food Control*, *31*(2), 514-518.

Soldera, J., Nedel, W. L., Cardoso, P. R., and d'Azevedo, P. A. (2013). Bacteremia due to *Staphylococcus cohnii* ssp. *urealyticus* caused by infected pressure ulcer: case report and review of the literature. *Sao Paulo Medical Journal*, *131*, 59-61.

Spergser, J., Wieser, M., Taubel, M., Rossello-Mora, R. A., Rosengarten, R., and Busse, H. J. (2003). *Staphylococcus nepalensis* sp. nov., isolated from goats of the Himalayan region. *International Journal of Systematic Bacteriology*, *53*, 2007-2011.

Stecher, B., and Hardt, W. D. (2011). Mechanisms controlling pathogen colonization of the gut. *Current Opinion in Microbiology*, *14*, 82-91.

Stecher, B., Berry, D., and Loy, A. (2013). Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. *FEMS Microbiology Reviews*, *37*, 793-829.

Steiner, T. S., Theilman, N. M., and Guerrant, R. L. (2006). Enteric *Escherichia coli* infections. In: R. L. Guerrant, D. H. Walker, and P. F. Weller (eds.), *Tropical infectious diseases: principles, pathogens and practice*, (Vol. 1, pp. 201-219). Churchill Livingstone. Elsevier.

Stenutz, R., Weintraub, A., and Widmalm, G. (2006). The structures of *Escherichia coli* O polysaccharide antigens. *FEMS Microbiology Review*, *30*(3), 382-403.

Stepanović, S., Dakić, I., Martel, A., Vaneechoutte, M., Morrison, D., Shittu, A., Jezek, P., Decostere, A., Devriese, L. A., and Haesebrouck, F. (2005). A comparative evaluation of phenotypic and molecular methods in the identification of members of the *Staphylococcus sciuri* group. *Systematic and Applied Microbiology*, *28*(4), 353-357.

Stewart, G.C. (2017). Staphylococcal Food Poisoning. In: C. Dodd, T. Aldsworth, R. Stein (eds.), *Foodborne Diseases* (3rd ed., pp. 1-14). Academic Press.

Stewart, G. C. (2017). Staphylococcal Food Poisoning. In: C. E. R. Dodd, T. Aldsworth, R. A. Stein, D. O. Cliver, H. P. Riemann, (eds.), *Foodborne Diseases* (pp. 1-14). Academic Press: Elsevier.

Supre, K., De Vliegher, S., Cleenwerck, I., Engelbeen, K., Van Trappen, S., Piepers, S., Sampimon, O. C., Zadoks, R. N., De Vos, P., and Haesebrouck, F. (2010). *Staphylococcus*

devriesei sp. nov., isolated from teat apices and milk of dairy cows. *International Journal of Systematic and Evolutionary Microbiology*, *60*, 2739-2744.

Swedish University of Agricultural Sciences SLU. (2019, October 10). *Glycolysis and Fermentation. Veterinary bacteriology: information about important bacteria*. https://www.vetbact.org/index.php?displayextinfo=109.

Tanasupawat, S., Hashimoto, Y., Ezaki, T., Kozaki, M., and Komagata, K. (1992). *Staphylococcus piscifermentans* sp. nov., from fermented fish in Thailand. *International Journal of Systematic Bacteriology*, *42*, 577-581.

Taponen, S., Supre, K., Piessens, V., Van Coillie, E., De Vliegher, S., and Koort, J. M. (2012). *Staphylococcus agnetis* sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. *International Journal of Systematic and Evolutionary Microbiology*, *62*, 61-65.

Tarr, P. I., Gordon, C. A., and Chandler, W. L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet*, *365*(9464), 1073-1086.

Tenaillon, O., Skurnik, D., Picard, B., and Denamur, E. (2010). The population genetics of commensal *Escherichia coli*. *Nature Reviews Microbiology*, *8*, 207-217.

Thomer, L., Schneewind, O., and Missiakas, D. (2016). Pathogenesis of *Staphylococcus aureus* Bloodstream Infections. *Annual review of pathology*, *11*, 343-64.

Titouche, Y., Houali, K., Ruiz-Ripa, L., Vingadassalon, N., Nia, Y., Fatihi, A., Cauquil, A., Bouchez, P., Bouhier, L., Torres, C., and Hennekinne, J. A. (2020). Enterotoxin genes and antimicrobial resistance in *Staphylococcus aureus* isolated from food products in Algeria. *Journal of Applied Microbiology*, *129*(4), 1043-1052. Doi: 10.1111/jam.14665.

Todar, K. (2020). *Pathogenic E. coli*. Web Review of Todar's Online Textbook of Bacteriology. "The Good, the Bad, and the Deadly". http://textbookofbacteriology.net/e.coli.html.

Tong, S. Y., Schaumburg, F., Ellington, M. J., Corander, J., Pichon, B., Leendertz, F., Bentley, S. D., Parkhill, J., Holt, D. C., Peters, G., and Giffard, P. M. (2015). Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: the non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *65*, 15-22.

Trulzsch, K., Grabein, B., Schumann, P., Mellmann, A., Antonenka, U., Heesemann, J., and Becker, K. (2007). *Staphylococcus pettenkoferi* sp. nov., a novel coagulase-negative staphylococcal species isolated from human clinical specimens. *International Journal of Systematic and Evolutionary Microbiology*, *57*, 1543-1548.

UNE-EN ISO 11290-1 (2018). Microbiology of the food chain−Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp.−Part 1: Detection method (ISO 11290-1:2017).

UNE-EN ISO 16649-3 (2015). Microbiology of the food chain−Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*−Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (ISO 16649-3:2015, Corrected version 2016-12-15)

UNE-EN ISO 16654 (2002). Microbiology of food and animal feeding stuffs−Horizontal method for the detection of *Escherichia coli* O157 (ISO 16654:2001).

UNE-EN ISO 21148 (2017). Cosmetics−Microbiology−General instructions for microbiological examination (ISO 21148:2017).

UNE-EN ISO 21150 (2016). Cosmetics−Microbiology−Detection of *Escherichia coli* (ISO 21150:2015)

UNE-EN ISO 21528-2 (2018). Microbiology of the food chain−Horizontal method for the detection and enumeration of *Enterobacteriaceae*−Part 2: Colony-count technique (ISO 21528-2:2017, Corrected version 2018-06-01).

UNE-EN ISO 6579-1 (2017). Microbiology of the food chain−Horizontal method for the detection, enumeration and serotyping of *Salmonella*−Part 1: Detection of *Salmonella* spp. (ISO 6579-1:2017).

UNE-EN ISO 6888-1 (2000). Microbiology of food and animal feeding stuffs−Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species)-Part 1: Technique using Baird-Parker agar medium (ISO 6888-1:1999)

UNE-EN ISO 7218 (2008). Microbiology of food and animal feeding stuffs−General requirements and guidance for microbiological examinations (ISO 7218:2007).

UNE-EN ISO 9308-1 (2014). Water quality−Enumeration of *Escherichia coli* and coliform bacteria−Part 1: Membrane filtration method for waters with low bacterial background flora (ISO 9308-1:2014).

UNE-EN ISO 9308-3 (1999). Water quality−Detection and enumeration of *Escherichia co*li and coliform bacteria−Part 3: Miniaturized method (Most Probable Number) for the detection and enumeration of *E. coli* in surface and wastewater (ISO 9308-3:1998).

Valero, A., Pèrez- Rodríguez, F., Carrasco, E., Fuentes-Alventosa, J. M., García-Gimeno, R. M., and Zurera, G. (2009). Modelling the growth boundaries of *Staphylococcus aureus*: effect of temperature, pH and water activity. *International Journal of Food Microbiology, 133*, 186-194.

Van Den Beld, M. J., and Reubsaet, F. A. (2012). Differentiation between *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and noninvasive *Escherichia coli*. *European Journal of Clinical Microbiology & Infectious Diseases*, *31*(6), 899-904. Doi: 10.1007/s10096-011-1395-7.

Van Der Putten, B. C. L., Matamoros, S., Mende, D. R., Scholl, E. R., Consortium, C., and Schultsz, C. (2021). *Escherichia ruysiae* sp. nov., a novel Gram-stain-negative bacterium, isolated from a faecal sample of an international traveller. *International Journal of Systematic and Evolutionary Microbiology, 71*(2), 4609. Doi: 10.1099/ijsem.0.004609.

Van Der Zwet, W. C., Debets-Ossenkopp, Y. J., Reinders, E., Kapi, M., Savelkoul, P. H. M., Van Elburg, R. M., Hiramatsu, K., and Vandenbroucke-Grauls, C. M. J. E. (2002). Nosocomial spread of a *Staphylococcus capitis* strain with heteroresistance to vancomycin in a neonatal intensive care unit. *Journal of Clinical Microbiology, 40*(7), 2520-2525. Doi:10.1128/JCM.40.7.2520-2525.2002.

Varaldo, P. E., Kilpper-Bälz, R., Biavasco, F., Satta, G., and Schleifer, K. H. (1988). *Staphylococcus delphini* sp. nov., a coagulase-positive species isolated from dolphins. *International Journal of Systematic Bacteriology, 38*, 436-439.

Vásquez, A. V., Salhuana, G. J. G., Gerardo, G. J., Jiménez, D. L. A., and Abanto, R. L. M. (2018). Evaluation of the bacteriological quality of fresh cheeses from

CAJAMARCA. *Ecología Aplicada*, *17*(1), 45-51. Doi: http://dx.doi.org/10.21704/rea.v17i1.1172.

Vernozy-Rozand, C., Mazuy, C., Meugnier, H., Bes, M., Lasne, Y., Fiedler, F., Etienne, J., and Freney, J. (2000). *Staphylococcus fleurettii* sp. nov., isolated from goat's milk cheeses. *International Journal of Systematic Bacteriology*, *50*, 1521-1527.

Viswanathan, P., and Kaur, R. (2001). Prevalence and growth of pathogens on salad vegetables, fruits and sprouts. *International Journal of Hygiene and Environmental Health*, *203*(3), 205-213.

Vitas, A. I., Aguado, V., and Garcia-Jalon, I. (2003). Occurrence of *Listeria monocytogenes* in fresh and processed foods in Navarra (Spain). *International Journal of Food Microbiology*, *90*, 349-356. Doi: 10.1016/S0168-1605(03)00314-3.

Wang, H., and Hammack, T. S. (2014). *Salmonella*: Detection by Classical Cultural Techniques. In: C. A. Batt, and M. L. Tortorello, (eds.), *Encyclopedia of Food Microbiology*, (2nd ed., pp.332-338). Academic Press. https://doi.org/10.1016/B978-0-12-384730-0.00297-4

Wang, M., Zhang, Y., Tian, F., Liu, X., Du, S., and Ren, G. (2021). Overview of Rapid Detection Methods for Salmonella in Foods: Progress and Challenges. Foods, *10*(10), 2402. https://doi.org/10.3390/foods10102402.

Wang, Y., and Suo, B. (2011). A new 7-plex PCR assay for simultaneous detection of shiga toxin-producing *Escherichia coli* O157 and *Salmonella Enteritidis* in meat products. Journal of Consumer Protection and *Food Safety*, *6*, 441-447. https://doi.org/10.1007/s00003-011-0696-1.

Warren, S. H., and Scott, W. M. (1930). A New Serological Type of *Salmonella*. *The Journal of Hygiene*, *29*, 415-417.

Wattam, A. R., Abraham, D., Dalay, O., Disz, T. L., Driscoll, T., Gabbard, J. L., Gillespie, J. J., Gough, R., Hix, D., Kenyon, R., Machi, D., Mao, C., Nordberg, E. K., Olson, R., Overbeek, R., Pusch, G. D., Shukla, M., Schulman, J., Stevens, R. L., Sullivan, D. E., Vonstein, V., Warren, A., Will, R., Wilson, M. J., Yoo, H. S., Zhang, C., Zhang, Y., and Sobral, B. W. (2014). PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Research*, *42*, 581-91. Doi: 10.1093/nar/gkt1099.

Webster, J. A., Bannerman, T. L., Hubner, R. J., Ballard, D. N., Cole, E. M., Bruce, J. L., Fiedler, F., Schubert, K., and Kloos, W. E. (1994). Identification of the *Staphylococcus sciuri* species group with EcoRI fragments containing rRNA sequences and description of *Staphylococcus vitulus* sp. nov. *International Journal of Systematic Bacteriology*, *44*, 454-460.

Wei, C., Zhong, J., Hu, T., and Xihong, Z. (2018). Simultaneous detection of *Escherichia coli* O157:H7, *Staphylococcus aureus* and *Salmonella* by multiplex PCR in milk. *3 Biotech*, *8*(76). https://link.springer.com/article/10.1007/s13205-018-1086-5.

Weldin, J. C. (1927). The colon-typhoid group of bacteria and related forms. Relationships and classification. *Iowa State Journal of Science*, *1*, 121-197.

Weller, D., Andrus, A., Wiedmann, M., and den Bakker, H. C. (2015). *Listeria booriae* sp. nov. and *Listeria newyorkensis* sp. nov., from food processing environments in the USA. *International Journal of Systematic and Evolutionary Microbiology*, *65*, 286-292.

Whalen, J. G., Mully, T. W., and Enlgish, J. C. 3rd. (2007). Spontaneous *Citrobacter freundii* infection in an immunocompetent patient. *Archives of dermatology*, *143*(1), 124-125.

Wong, T., Whyte, R. J., Cornelius, A. J., and Hudson, J. A. (2004). Enumeration of *Campylobacter* and *Salmonella* on chicken packs. *British Food Journal*, *106*(9), 651-662.

World Health Organization, WHO. (2018, February 20). Listeriosis. https://www.who.int/news-room/fact-sheets/detail/listeriosis.

World Health Organization, WHO. (2018, February 7). *Escherichia coli* (*E. coli*). https://www.who.int/news-room/fact-sheets/detail/e-coli.

World Health Organization, WHO. (2020, April 30). *Food safety*. https://www.who.int/news-room/fact-sheets/detail/food-safety.

World Health Organization, WHO. (2017a). Guidelines for Drinking Water Quality: Incorporating First Addendum. *World Health Organization*, Geneva, Switzerland.

Wu, F., Xu, X., Xie, J., and Yi, S. (2016). Molecular Characterization of Salmonella enterica Serovar Aberdeen Negative for H_2S Production in China. PLOS One, *11*(8).

Wu, S. J., Chan, A., and Kado, C. I. (2004). Detection of PCR amplicons from bacterial pathogens using microsphere agglutination. *Journal of Microbiological Methods*, *56*, 395-400.

Wu, S., Wu, Q., Zhang, J., Chen, M., Yan, Z., and Hu, H. (2015). *Listeria monocytogenes* Prevalence and Characteristics in Retail Raw Foods in China. *PLoS ONE*, *10*(8). Doi: 10.1371/journal.pone.0136682.

Wu, X., and Su, Y-C. (2014). Effects of frozen storage on survival of *Staphylococcus aureus* and enterotoxin production in precooked tuna meat. *Journal of Food Science*, *79*(8), 1554-1559.

Xu, Y. P., Cheng, W., Shao, Y. C., and Chen, F. S. (2006). Detection of *Salmonella* spp., *Escherichia coli* and *Staphylococcus aureus* by multiplex PCR. *Microbiology China*, *33*, 89-94.

Yu, Y. G., Wu, H., Liu, Y. Y., Li, S. L., Yang, X. Q., and Xiao, X. L. (2010). A multipathogen selective enrichment broth for simultaneous growth of *Salmonella enterica* serovar *Enteritidis*, *Staphylococcus aureus*, and *Listeria monocytogenes*. *Canadian Journal of Microbiology*, *56*(7), 585-597.

Yuan, Y., Xu, W., Zhai, Z., Shi, H., Luo, Y., Chen, Z., and Huang, K. (2009). Universal Primer-Multiplex PCR Approach for Simultaneous Detection of *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. in Food Samples. *Journal of Food Science*, *74*(8).

Zakrzewska-Czerwinska, J., Gaszewska-Mastalarz, A., Lis, B., Gamian, A., and Mordarski, M. (1995). *Staphylococcus pulvereri* sp. nov., isolated from human and animal specimens. *International Journal of Systematic Bacteriology*, *45*, 169-172.

Zhang, Q. Y., Zhou, W. W., Zhou, Y., Wang, X. F., and Xu, J. F. (2012). Response surface methodology to design a selective co-enrichment broth of *Escherichia coli*, *Salmonella* spp. and *Staphylococcus aureus* for simultaneous detection by multiplex PCR. *Microbiological Research*, *167*, 405-412.

Zhang, Z., Wang, L., Xu, H., Aguilar, Z. P., Liu, C., Gan, B., Xiong, Y., Lai, W., Xu, F., and Wei, H. (2014). Detection of non-emetic and emetic *Bacillus cereus* by propidium

monoazide multiplex PCR (PMA-mPCR) with internal amplification control. *Food Control, 35*(1), 401-406. https://doi.org/10.1016 /j.food cont .2013 .07.035.

Zieliński, W., Korzeniewska, E., Harnisz, M., Hubeny, J., Buta, M., and Rolbiecki, D. (2020). The prevalence of drug-resistant and virulent *Staphylococcus* spp. in a municipal wastewater treatment plant and their spread in the environment. *Environment International, 143*. https://doi.org/10.1016/j.envint.2020.105914.

Zilelidou, E., Manthou, E., and Skandamis, P. (2016). Growth differences and competition between *Listeria monocytogenes* strains determine their predominance on ham slices and lead to bias during selective enrichment with the ISO protocol. *International Journal of Food Microbiology, 235*, 60-70.