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Prevalence and molecular characterisation of *Salmonella* spp. isolated from *alheira*, a traditional Portuguese meat product

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List of Abbreviations

EFSA= European Food Safety Authority

FBO= Foodborne outbreaks

EU= European Union

BS= Bismuth Sulfite

HE= Hektoen Enteric

XLD= Xylose Lysine Deoxycholate

BET= Ethidium Bromide

VP= Voges Proskauer

ONPG= Ortho-Nitrophenyl-β-Galactoside

TDA= Tryptophan deaminase

DGTP= deoxyadenosine triphosphate

DATP= deoxyguanosine triphosphate

DCTP= deoxycytidine triphosphate

DTTP= dexythymidine triphosphate

A= Adenosine

G= Guanine

C= Cytosine

T= Thymine

PCR= Polymerase chain reaction

TAQ POL= *Thermus aquaticus* polymerase

pH= Potential of hydrogen

aw= Water activity

Abstract

Previous meta-analytical work estimated that in Portuguese meat products intended to be eaten cooked, the overall occurrence of *Salmonella* spp. was 9.7% (95% CI: 7.0–13.4%). One of these meat products is *alheira*, which is a fermented sausage made of poultry/pork meat, bread and seasonings. The main objective of this study was to investigate prevalence, numbers and serovars of *Salmonella* spp. in alheira sausages artisanally produced in Bragança, Portugal. This work was undertaken in three stages: (i) sampling of 52 alheiras from market fairs and local shops/supermarkets; (ii) detection of *Salmonella* using culture methods and enumeration by MPN; and (iii) molecular confirmation of isolates (invA and randomly-cloned chromosomal fragment), and typing of S. Enteritidis (SefA), Typhimurium (fliC) or Pullorum (glgC) by PCR. A comparison of the intrinsic properties and microbial quality/stability between unpacked unbranded alheiras and vacuum-packed alheiras was also conducted.

Analysis of 52 sausage samples revealed the presence of *Salmonella* spp. in 10 samples (incidence of 0.192; 95% CI: 0.108–0.319), although all of these positive samples were unpacked sausages from traditional fairs (n=21), indicating therefore the higher *Salmonella* prevalence in alheiras sold in these establishments (incidence 0.476; 95% CI: 0.283–0.676). *Salmonella* mean concentration in positive enriched samples was 1.792 log MPN/g (s.d 0.916 log MPN/g). All of the 33 biochemically- and serologically-confirmed isolates coded for the invA gene. Multiplex-PCR revealed that only 3 of the positive isolates had the presence of SefA genes; which indicated that 9.1% of the isolates belonged to Enteritidis; while 20 isolates (60.60%) belonged to Typhimurium since they coded for fliC gene. The other 10 isolates (30.3%) were of serovars different from Enteritidis/Typhimurium/Pullorum. From the positive alheiras, Enteritidis or Typhimurium serovar was recovered from two samples, while the other samples harboured at least two serovars.

Salmonella continues to be a frequent contaminant of alheiras produced in Bragança, and, in particular, of those sold in market fairs (47.61%). Not unexpectedly, Typhimurium and Enteritidis represented the prevailing serovars (~70%) since they are linked to pork and poultry meat, the main raw materials of alheira.

In addition, it could be inferred that unbranded, unpacked alheiras traditionally sold in market fairs are very likely to be produced under poor hygienic conditions, because of the significantly higher levels of total coliforms that were recovered from unpacked alheiras (7.958 log CFU/g; s.d. 0.948 log CFU/g) in comparison to branded vacuum-packed alheiras (5.423 log CFU/g; s.d. 1.264 log CFU/g). Unpacked alheiras also presented higher counts of *Pseudomonas* spp. (5.687 log CFU/g; s.d. 0.100 log CFU/g) and psychrotrophic bacteria (7.977 log CFU/g; s.d. 0.726 log CFU/g) than vacuum-packed alheiras (4.527 log CFU/g; s.d. 0.336 log CFU/g; and 6.969 log CFU/g; s.d. 0.295 log CFU/g, respectively), which suggested that the unpacked alheiras have generally a shorter shelf-life, and, more importantly, that both hurdles, vacuum-packaging and cold storage, are key in delaying microbial degradation in this product.

This work has evidenced that microbial stability and safety of the alheiras elaborated by the artisanal producers are not ensured. Thus, artisanal producers must be urgently informed on the implementation of preventive and corrective actions in their current manufacturing processes and hygiene practices; whereas consumers must be advised to fully cook alheira sausages before consumption.

I. Introduction

Food can be defined as any substance that is consumed in its natural state or after cooking. The role of food is to provide the organism with essential elements for growth, restoration and energy needs and reserves. However, sometimes these foods become contaminated during production, processing, transportation and/or manipulation by pathogenic microorganisms such as viruses, parasites and bacteria. These pathogens are responsible for gastrointestinal disease in humans. *Salmonella* spp. is the causative agent of one of the main causes of food poisoning: salmonellosis. *Salmonella* cells can enter the body through the digestive tract. While in a person in good health, a significant amount of cells must be ingested to trigger the infection (salmonellosis), there are susceptible population groups such as infants, young children, the elderly and immune compromised people who can became infected by low doses of *Salmonella* cells (Ralph, 1996).

In Europe, *Salmonella* spp. is the second most commonly reported zoonotic infection in humans, and remains the most common cause of foodborne outbreaks. The joint annual report of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) confirm that the 4 786 outbreaks of food poisoning reported in 2016 decrease in 2017 to achieve 5 079 foodborne outbreaks (EFSA, 2018).

According to EFSA (2018), *Salmonella* was the most frequently reported causative agent in the EU (1 241 FBOs and no waterborne outbreaks; 24.4% of total outbreaks in 25 member states). Outbreaks of salmonellosis had the highest impact on human cases (9 600; 22.1% of all outbreak cases), hospitalisations (2 227; 49.0% of all hospitalisations) and deaths (11; 33.3% of all deaths). S. Enteritidis was by far the most frequently reported *Salmonella* serovar and amounted to 61.1% (n = 758, 23 MS) of *Salmonella* FBO, corresponding to 14.9% – about one seventh – of all reported FBO at the EU level.

A semi-quantitative risk ranking of pathogens in Portuguese-produced pork pointed *Salmonella* spp. as critical (with a mean occurrence of 12.6%; 95% CI: 8.0 – 19.3%) (Xavier *et al*., 2005). These authors estimated by meta-analysis that, in Portuguese meat products, the non-

compliance to EU microbiological criterion for *Salmonella* spp. (9.7%; 95% CI: 7.0 – 13.4%) at sample units level, in the categories 'intended to be eaten cooked' and 'to be eaten raw', were considerably higher than EU levels for ready-to-eat products in comparable categories.

The objective of this study was to further investigate the contamination by *Salmonella* spp. in *alheira*, a meat product intended to be eaten cooked that is artisanally produced in Bragança. The study has been accomplished in six stages:

A. A meta-analysis of primary studies reporting incidences of *Salmonella* spp. in alheira;
B. Periodical sampling of unpacked alheiras sold in market fairs, and vacuum-packed alheiras sold in supermarkets/local shops;
C. Physicochemical and microbiological characterisation of unpacked and vacuum-packed alheiras;
D. Determination of *Salmonella* prevalence and counts using the most-probable-number technique;
E. Biochemical and serological confirmation of *Salmonella* spp.; and
F. Molecular confirmation and typing of *Salmonella* isolates using the PCR technique.

II. Literature Review

2.1 The alheira sausage

In Portugal, there are a wide variety of fermented meat products and their manufacture represents an important income in specific regions, predominantly in the North and the Southern regions. A traditional and naturally fermented meat sausage, typical from Trás-os-Montes region of Northern Portugal is *alheira* (garlic sausage). Traditionally, alheira is produced from pork and poultry meat, pork fat, lard, regional wheat bread, olive oil, mixed with salt, red pepper, garlic and spices. The meats, lard, olive oil and spices are boiled together with water and then, when the finely sliced bread is added, the mass becomes a batter, which is stuffed into natural pig casings and submitted to a drying process for approximately one week (Silva et al., 2019). The product is always cooked, grilled or pan-fried. Despite its wide consumption in Portugal, some research studies have indicated that alheira is one of those meat products that could be of questionable microbiological quality, harbouring the presence of pathogenic micro-organisms (Esteves et al., 2006a,b; 2008).

2.2 Intrinsic factors for bacterial growth

2.2.1 Water activity

It is common to distinguish the water content of a food in two forms: free water and bound water (Mathlouthi, 2001). To express the degree of free water in food, the concept of water activity (aw) is used. The concept of aw has been very useful in food preservation and on that basis many processes could be successfully adapted and new products designed. Water has been called the universal solvent as it is a requirement for growth, metabolism, and support of many chemical reactions occurring in food products.

In fact, it is well known that each microorganism has a critical aw below which growth cannot occur. For instance, pathogenic microorganisms cannot grow at aw <0.86; yeasts and moulds

are more tolerant and usually no growth occurs at aw<0.62. The so-called intermediate moisture foods (IMF) have aw values in the range of 0.65-0.90 (Barbosa-Cánovas, 2003).

2.2.2 Acidity

The acidity of food is often measured by a parameter called pH, whose scale ranges from 0 until 14. When a food is said to be more acidic, the lower is its pH. As with water activity, pH also plays an important role in the stability of food products (Vijayakamur et al., 2017). Inhibition of microorganisms can be achieved by increasing acidity (reducing pH) by the addition of weak acids or by the production of lactic acid by lactic acid bacteria (fermentation). Given the importance of aw and pH in food preservation and stability, a product is considered stable at a pH below 4.5 because, in an acid product, bacteria generally stop developing (Buffet, 2002).

2.2.3 Moisture

Food preservation methods such as drying, freezing and adding salt/sugar work out by lowering the available moisture in foods. Moisture in foods occurs in two forms: (1) water bound to ingredients in the food (proteins, salt, sugars); and (2) free or unbound water that is available for microbial growth (Mathlouthi, 2001). Nonetheless, aw and moisture are not interchangeable concepts and a complex relationship exists between them. Generally speaking, foods with higher moisture content might be expected to have higher aw than dry foods, although the expectation is not necessarily correct. Products with the same water content may have very different water activities. For example, salami and cooked beef have similar total moisture (approximately 60%); however, salami has lower aw than cooked beef (Clemson, 2019).

2.3 Microbiological indicators of food spoilage

2.3.1 Mesophilic bacteria or total viable counts

Bacteria relevant to meat, meat products and other food are divided into three groups according to the temperature range within which they can grow: mesophiles 10–45°C, psychrophiles 0–28°C and psychrotrophs 10– 45°C. Aerobic bacteria have an absolute requirement for oxygen which limits their growth to the meat surface. A high total viable counts (TVC) resulting from severe contamination during slaughter or processing will shorten the shelf-life even in ideal

conditions. Meat will spoil with TVC at 10^6/cm^2 because of off-odours. Slime and discoloration appear at 10^8/cm^2. The main factors determining the time taken for the TVC to reach these levels are the initial count due to contamination during slaughtering and processing, further contamination during storage, temperature, pH and relative humidity. Nonetheless, high TVC also indicates poor hygiene so that contamination with food-poisoning bacteria is likely (FAO, 1991).

2.3.2 Psychrotrophic bacteria

Psychrotrophic microorganisms have the ability to grow at cold temperatures, and are widespread in natural environments and in foods (Gounot, 1986). Mesophiles will not grow below 10°C but psychrotrophs, of which *Pseudomonas* are the more important, will grow down to 0°C. The nearer to 0°C the storage temperature, the slower the growth of the spoilage bacteria and the longer the shelf-life.In chilled meat, psychrotrophic bacteria become the dominant species, mainly *Pseudomonas*, *Achromobacter*, *Micrococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Flavobacterium* and *Proteus* spp.

2.3.3 Lactic acid bacteria

Lactic acid bacteria (LAB) constitute a group of gram-positive bacteria, non-sporing, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. LAB are widespread in nature and commonly occur on all kind of plant materials, on mucous membranes, in saliva and, in feces. Consequently and unavoidably, they are part of the contamination flora of fresh meats after slaughter. Under certain conditions, e.g. in packaged refrigerated meats or raw sausage meats, they are able to compete efficiently with accompanying microorganisms for nutrients, and may reach substantial viable counts. Their metabolic activities may ultimately result in either a desired preservative effect due to the repression of pathogenic and spoilage microorganisms, a desired tasty meat product, such as raw fermented sausage, or in meat spoilage through undesired transformations of raw and cooked meats. Heterofermentative LAB of the *Carnobacterium*, *Leuconostoc* and *Weissella* genera are usually more involved in meat spoilage than the homofermentative *Lactobacillus* and *Pediococcus* genera (Krochel, 2013)

2.3.4 *Pseudomonas*

The pseudomonads represent a large and poorly defined group of microorganisms. The family *Pseudomonadaceae* consists of Gram-negative rods, motile with polar flagella, oxidase-positive, catalase-positive, and they are obligate respiratory bacteria. There are many marine species among the pseudomonads. The spoilage compounds associated with the growth of psychrotrophic *Pseudomonas* spp. are diverse and in many cases species-specific. *Pseudomonas*-mediated spoilage is characterised by ‘fruity’, ‘oniony’ and ‘faecal’ odours from the production of biogenic amines, ketones, aldehydes, esters and non-H_2S sulphur-containing compounds, such as methyl sulphide. *Pseudomonas fragi* and *putida* are predominantly isolated from meat (Rawat, 2015).

Pseudomonas spp. are the most important psychrotrophic bacteria since they give rise to slime and off-odor formation at levels of 10^7–10^8 log CFU/g. The time for this to occur depends on several factors (especially temperature, relative humidity, and initial load) but meat kept in retail packs at a relative humidity of 99.3% usually spoils after 5–10 days at 1°C. If meat is stored under elevated levels of carbon dioxide, the growth of *Pseudomonas* spp. is inhibited and *Lactobacillus* spp. becomes dominant. Such meat can be kept for several weeks at chill temperatures with little evidence of spoilage.

2.4 Microbiological indicators of food hygiene

2.4.1 Total coliforms

Coliforms are defined as rod-shaped Gram-negative non-spore forming and motile or non-motile bacteria which can ferment lactose with the production of acid and gas when incubated at 36°C. Typical genera include *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Escherichia*, which are bacteria that live in the gut of warm-blooded animals, and in the general environment (soils, vegetation and water). Total coliforms count is a hygienic indicator, and high level of coliform counts generally indicates unsanitary condition or poor hygiene practices during or after food production (Snel *et al*., 2005).

2.4.2 *Escherichia coli*

Escherichia coli are a Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination. It is a contaminant of water, and often used as an indicator of the hygienic quality of a food product. Meat products, such as pork and poultry, may contain this microorganism. This can be transmitted by contact with fecal matter during food processing due to failures in manufacturing, hygiene and handling (Percival *et al*., 2014).

2.5 *Salmonella species*

2.5.1 Definition

Salmonella spp.

- Are Gram-negative non-spore-forming bacilli, predominantly motile, moving in all directions by flagella, with cell diameters between 0.7 and 1.5 µm;
- Are optional anaerobes or facultative aerobes (Ralph, 1996);
- Are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources; and
- Have a genome in the form of a chromosome of nearly 5 million base pairs, which encodes around 4,500 proteins (Marianne *et al*., 2003)

Salmonella spp. are mesophilic bacteria with optimal growth temperature close to the body temperature of warm-blooded animals (35 – 43°C). It is usually admitted that most serotypes only grow from 7°C. They support a pH range from 4.5 to 9.0 with an optimum of 6.5 to 7.5 and they are very resistant to desiccation and grow well in aw from 0.945 to 0.999.

2.5.2 Taxonomy

The *Salmonella* genus belongs to the family *Enterobacteriaceae*, of the order *Enterobacteriales*, class of *Gammaproteobacteria* and phylum *Proteobacteria* (Tindall *et al*., 2005). The genus *Salmonella* is divided into two species:

- *S. enterica*, which include the majority species, and
- *S. bongori*, comprising rare species (Porwollik *et al*., 2004).

The species *enterica*, the most frequently isolated (99.4%), is divided into 6 subspecies that include over 2 600 serotypes. Serologically, the characterisation of antigens somatic (O) and flagellar (H), allows the classification of subspecies (Ralph, 1996). In serotypes, those belonging to the subspecies enteric have been assigned a name frequently corresponding to a geographical place; the others have been designated by their antigenic formula.

Salmonella serotypes can be divided into two main groups—typhoidal and nontyphoidal. Nontyphoidal serotypes are more common, and usually cause self-limiting gastrointestinal disease (Ralph, 1996). They can infect a range of animals, and are zoonotic, meaning they can be transferred between humans and other animals. Typhoidal serotypes include *S.* Typhi and *S.* Paratyphi A, which are adapted to humans and do not occur in other animals. In the food sector, *S*. Enteritidis and *S*. Typhimurium predominate, but their relative importance varies over time, regions and food vehicles. However, all serotypes should be considered as potentially pathogenic (ANSES, 2011).

2.5.3 Habitat

S. enterica subspecies are found worldwide in all warm-blooded animals and in the environment; whereas *S. bongori* are restricted to cold-blooded animals, particularly reptiles. *Salmonella* spp. can be isolated from the intestines of many animal species (pigs, cattle) and birds (domestic poultry). In other words, animals are a reservoir of *Salmonella* and their release into the environment is mainly through fecal contamination (Ralph, 1996). *Salmonella* in the fecal matter of animals can contaminate pastures, soils and water and can even survive for several months (Gray, 2001) *Salmonella* can also attach to vectors such as boots, brushes, shovels, wheelbarrow wheels, clothing, hands, equipment, and etc. (CFSPH, 2013).

2.5.4 Culture characteristics

Salmonella spp. has a thick cell wall between 8 and 12 nm. These are Gram-negative stems from 0.3 to 1 µm in width, and length from 1 to 6 µm. They are mobile because of the capilliformes protein filaments: the flagellum. The optimum temperature for grow this 35°C-37°C (Avril *et al*., 2000).

Effective methods for the isolation of *Salmonella* spp. from various foods are important to ensure food quality and safety. The choice of a suitable sampling procedure combined with a sensitive culture method is important for the successful detection of *Salmonella* (Carrique-Mas, 2008). The use of selective and differential plating media is a simple method for the isolation of *Salmonella* spp. A wide variety of selective and differential media has been developed for this purpose, including xylose lysine desoxycholate agar (XLD), Hektoen enteric (HE) agar, and bismuth sulfite (BS) agar, among others (Cooke *et al*., 1999). XLD and HE agar are the most popular media for isolating *Salmonella* spp., and their differentiation abilities rely on biochemical characteristics of *Salmonella*, such as hydrogen sulfide production and the non-fermentation of lactose (Rambach, 1990).

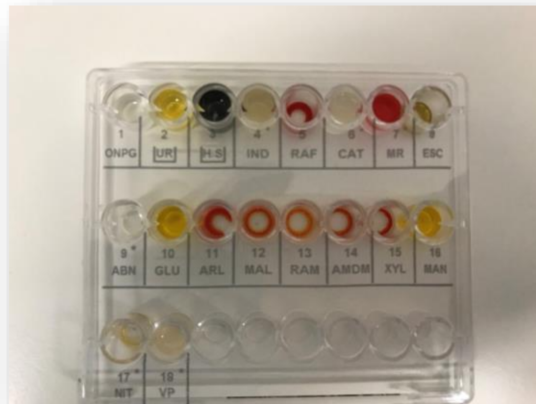
However, these characteristics are shared with other microorganisms, such as *Proteus* and *Citrobacter* (Eigner*et al*., 2001). Thus, numerous false-positive results are observed on these media which require further confirmation testing, a time-consuming and labor-intensive activity (Gaillot *et al*., 1999). BS agar is the medium of choice for the isolation of *Salmonella* enteric serovar Typhi, and it is used for the isolation of atypical salmonellae, such as those which ferment lactose (Coxt, 1993). However, BS agar has several disadvantages, such as low sensitivity and long incubation time for development of the characteristic colony morphology (Park et al., 2012).

2.5.5 Biochemical characteristics

Many differential selective media for the isolation of *Salmonella* spp. are available. In most of these, a limited number of biochemical traits are used to detect salmonellae not only from human and animal fecal specimens but also from food and other materials. Most of these media are prone to deliver high rates of false-positive results, mainly due to *Proteus* and *Citrobacter* strains from the normal flora mimicking the appearance of *Salmonella* strains. Some of the media yield high rates of indeterminate isolates, all of which must be differentiated further by biochemical or serological tests before a final result is obtained (Eigner et al., 2001). The biochemical characteristics of *Salmonella*, which are also used for confirmatory tests, are shown in Table 1. Figure 1 illustrates a biochemical multiple test kit used for confirmation.

Table 1: Biochemical characteristics of *Salmonella* (Bourgeois et al., 1996)

Tests	Reaction	Tests	Reaction
Motility	+	Glucose fermentation with gas production	+
Nitrate reduction	+	Mannitol fermentation	+
Oxidase	-	Maltose fermentation	+
Catalase	+	Lactose fermentation	-
Urease	-	Sucrose fermentation	-
Indole	-	Salicin fermentation	-
H_2S	+	Adonitol fermentation	-
Use of citrate	+	Dulcitol fermentation	+
Sodium malonate	-	Lysine decarboxylase	+
Methyl red	+	Arginine dihydrolase	+
Voges Proskauer (VP)	-	Ornithine decarboxylase	+
Gelatinase	-	Tryptophan deaminase (TDA)	-
Ortho-Nitrophenyl-β-Galactoside (ONPG)	-		



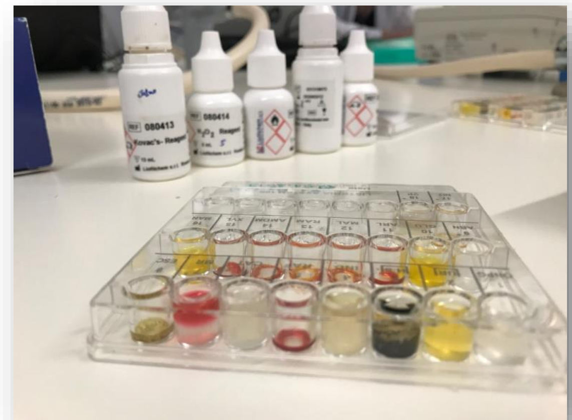


Figure 1: An example of a biochemical multiple tests kit for confirmation

2.6 Contamination of food products of animal origin

2.6.1 Poultry and eggs

Poultry meats are important sources of protein and other nutrients, and, as such, they allow *Salmonella* proliferation. The most common serotypes in poultry are *Salmonella* Enteritidis and *Salmonella* Typhimurium (Afshari *et al*., 2018). Although less specific to poultry, serotype Typhimurium is also widely encountered in chickens, turkeys and ducks. Eggs are important sources of *Salmonella* contamination, being the shell more frequently contaminated than the yolk or egg white (WHO, 2007)

2.6.2 Meat and meat products

Among meat and meat products, *Salmonella* have been often recovered from minced meats, charcuterie (sausages and cured meats) and pre-prepared beef roasts. Food may become contaminated by *Salmonella* during the slaughter of an animal and the processing of its meat (carcasses, jointed meat, minced meat), or by cross-contamination (EFSA, 2017). According to EFSA (2018), the numbers of contaminated sample units reported for the general food category 'meat and meat products' for 2017 (366 362) were lower compared with the years 2013 (410 529) but higher compared with the previous two years (278 254 and 203 683, respectively).

In 2017, with basis on regulation (EC) No. 2073/2005 on microbiological criteria, the highest proportions of *Salmonella*-positive single samples from official control investigations were reported from foods of meat origin intended to be cooked before consumption. A rate of 3.3% of 'minced meat and meat preparations from different species' was positive for *Salmonella* from samples tested in the EU (EFSA, 2018).

2.6.3 Sausages

There are two types of sausages, ready to eat and to be cooked. A sausage must contain at least 50% meat and may also contain rusk (filler), water, salt, sugar, starch, non-meat protein, stabilisers, preservatives, antioxidants, colourants and flavourings. The term meat, however, can include fat, skin, gristle and sinew and can often include meat that has been mechanically recovered from carcasses. However, meat can harbour pathogens such as *Salmonella*. For instance; some "economy" sausages have been found to contain high numbers of *Salmonella* which may not be killed if they are not fully cooked. *Salmonella* has been found in 7.5% of frozen sausages and 9.1% of chilled sausages (Mattick *et al*., 2002).

Generally, the heat treatment minimises the presence and the development of certain bacteria. If sausages are cooked adequately prior to consumption, any *Salmonella* cells present would be killed and there would be no risk of food poisoning. Therefore, the delivery of adequate heat during the cooking of comminuted meat products is important to ensure food safety. To inactivate bacterial pathogens, it is recommended that a temperature of 70°C is achieved in all parts of the sausage for a minimum of 2 min or the equivalent (Ellison *et al*., 1994).

Alheira, a non-ready-to-eat Portuguese sausage has been the subject of research in two studies. One of them inoculated *L. monocytogenes* in alheira batter to determine and characterise its behaviour (Pereira et al., 2015); while the other used a PCR-denaturation gradient gel electrophoresis protocol to characterise the natural microflora of alheira sausage (Albano et al., 2008). Currently, there is limited research on the presence and molecular traits of *Salmonella* in non-ready-to-eat regional meat products from Portugal, such as alheira sausage.

2.6.4 Inactivation treatments in industrial environment

Generally, Gram negative bacteria such as *Salmonella* are susceptible to many disinfectants including 1% sodium hypochlorite, 70% ethanol, 70% propanol, 2% glutaraldehyde, and 4% formaldehyde, as well as phenol, peracetic acid, hydrogen peroxide, quaternary ammonium compounds, and iodophors. *Salmonella* spp. can be resistant to nitrite (CFSPH, 2013). Table 2 lists process criteria of a few interventions that can inactivate *Salmonella*.

Table 2: Inactivation treatments in industrial environment of *Salmonella* spp. (ANSES, 2011)

Disinfectants	Effects of temperature
Susceptible to all disinfectants authorised in food industry subject to follow the recommended terms of use	D* value: D at 60°C = 2-6 min; D70 at °C ≤ 1 min. The heat resistance depends on the serotype and the food matrix A particular strain of S. Senftenberg 775 W has a 30-fold greater heat resistance
Ionization	High pressure
D10 = 0.5 – 0.8 kGy**	-600 MPa for 2 min at 35°C ➞ 5 decimal Reductions of the initial load. -350 MPa for 5 min at 25°C➞ 3 decimal reductions of S. Enteritidis in the mixture of yolk and egg white. -450 MPa for 5 min at 25°C➞ 5 decimal reductions of S. Enteritidis in the mixture of yolk and egg white. -345 MPa for 5 min at 25°C➞ 4 decimal reductions of S. Enteritidis in buffered Peptone water. -Same treatment but at pH 5.5 ➞ 8 decimal reductions of S. Enteritidis.

*D is the time required to divide by 10 the population of the microbiological hazard initially present.

**D10 is the dose (in kGy) required to reduce a population to 10% of its initial workforce

2.6.5 Survival and growth characteristics of Salmonella

The optimum temperature, pH and Aw for growth of *Salmonella* spp. are given in Table 3, as well as the minimum and maximum values (range) for growth. Some examples have also been given for extreme survival characteristic found in the literature (ANSES, 2011).

Table 3: Survival and growth characteristics of *Salmonella* spp.

Settings	Survival (Extreme values)	Growth	
		Optimum	Range
Temperature (°C)	– 23 (butter)	35 - 37	5.0 - 50
pH	/	7 – 7.5	3.8 – 9.5
Aw	0,3 - 0,5 (chocolate)	0.99	0.940 - > 0.999

2.7 Diseases caused by Salmonella

Salmonella is one of the most pathogenic microorganisms that cause foodborne illnesses; it can affect humans as well as a wide variety of animals, indeed, poultry and pigs are the most susceptible to these hazards, and so they constitute important sources of *Salmonella*-contaminated food products, which are also responsible for many outbreaks and infections around the world as gastroenteritis and other special cases (Aubry *et al*., 2018).

2.7.1 Typhoidal Salmonella

Typhoid fever is caused by *Salmonella* serotypes which are strictly adapted to humans or higher primates—these include *Salmonella* typhi, Paratyphi A, Paratyphi B, and Paratyphi C. In the systemic form of the disease, salmonellae pass through the lymphatic system of the intestine into the blood of the patients (typhoid form) and are carried to various organs (liver, spleen, kidneys) to form secondary foci (septic form). Endotoxins first act on the vascular and nervous apparatus, resulting in increased permeability and decreased tone of the vessels, upset of thermal regulation, and vomiting and diarrhoea. In severe forms of the disease, enough liquid and electrolytes are lost to upset the water-salt metabolism, decrease the circulating blood volume and arterial pressure, and cause hypovolemic shock. Septic shock may also develop.

Shock of mixed character (with signs of both hypovolemic and septic shock) is more common in severe salmonellosis. Oliguria and azotemia may develop in severe cases as a result of renal involvement due to hypoxia and toxemia (Townsend *et al*., 2001).

2.7.2 Gastroenteritis due to nontyphoidal Salmonella

Infection with nontyphoidal serotypes of *Salmonella* generally results in food poisoning. Infection usually occurs when a person ingests food that contains a high concentration of the bacteria. Infants and young children are much more susceptible to infection, easily achieved by ingesting a small number of bacteria. In infants, infection through inhalation of bacteria-laden dust is possible.

The organisms enter through the digestive tract and must be ingested in large numbers to cause disease in healthy adults. An infection can only begin after living salmonellae (not merely *Salmonella*-produced toxins) reach the gastrointestinal tract. Some of the microorganisms are killed in the stomach, while the surviving ones enter the small intestine and multiply in tissues. Gastric acidity is responsible for the destruction of the majority of ingested bacteria, but *Salmonella* has evolved a degree of tolerance to acidic environments that allows a subset of ingested bacteria to survive. Bacterial colonies may also become trapped in mucus produced in the esophagus. By the end of the incubation period, the nearby host cells are poisoned by endotoxins released from the dead salmonellae. The local response to the endotoxins is enteritis and gastrointestinal disorder. Clinical symptoms include diarrheal disease and fever (Poppe *et al*., 1998).

According to EFSA (2018), in total, 93 583 human salmonellosis cases were reported in 2017, with 91 662 confirmed cases resulting in an EU notification rate of 19.7 cases per 100 000 population (Table 4). This was a slight decrease by 2.9% compared with 2016 (20.4 cases per 100 000 population). As in the previous year, the highest notification rates in 2017 were reported by the Czech Republic (108.5 cases per 100 000 population) and Slovakia (106.5 cases per 100 000 population), while the lowest rates were reported by Greece and Portugal (< 7.0 cases per 100 000 population). Table 4 summarises human cases and rates of salmonellosis in different countries from 2014 until 2017 (EFSA, 2018).

Table 4: Reported human cases of salmonellosis and notification rates per 100000 population in the EU by country and by year 2014-2017 (EFSA, 2018)

Country	**2017**		**2016**		**2015**		**2014**	
	Confirmed Cases	Confirmed rate	Confirmed cases	Confirmed rate	Confirmed cases	Confirmed rate	Confirmed cases	Confirmed rate
Austria	1.667	19	1.415	16.3	1.544	18	1.654	19.4
Czech Rep	11.473	108.5	11.610	110.0	12.408	117.7	13.255	126.1
Greece	672	6.2	735	6.8	466	4.3	349	3.2
France	7.9993	24.9	8.876	27.7	10.305	32.3	8.880	28.1
Ireland	379	7.9	299	6.3	270	5.8	259	5.6
Italy	3.347	5.5	4.134	6.8	3.825	6.3	4.467	7.3
Poland	8.924	23.5	9.718	25.6	8.245	21.7	8.042	21.2
Portugal	462	4.5	376	3.6	325	3.1	244	2.3
Slovakia	5.789	106.5	5.299	97.7	4.841	89.3	4.078	75.3
EU Total	91.662	19.7	94.425	20.5	94.477	21	92.012	20.7

2.8 The polymerase chain reaction (PCR)

2.8.1 Definition

The gene amplification technique, more commonly known as PCR technique, was discovered in 1988 by the K. Mullis team and earned him the 1993 Nobel Prize in Chemistry. It is a technique for performing several cycles of DNA replication *in vitro* by the action of the ATQ polymerase (thermostable) produced from the *Thermus aquaticus* microorganism, nucleotides, and complementary primers specific targeted as edge amplification sequence (Saiki *et al*., 1988).

PCR is a method for direct detection of the genome of infectious agents by enzymatic amplification. This molecular biology tool is very specific and allows the detection of very small amounts of pathogens in a variety of samples in a fast and reproducible manner (Garibyan *et al*., 2013).

2.8.2 Principle of the PCR

The PCR is a sequence of cycles, repeated in a loop, where each cycle has three levels of temperature. In addition, each of these bearings is characterised by a distinct chemical reaction. On average, a PCR has between 20 and 40 cycles.

The PCR is based on two principles:

1. "DNA polymerases, heat-stable dependent DNA" have properties of enzymatic synthesis and initiation, "specific double-stranded DNA"
2. Hybridisation and elongation of complementary DNA strands is a function of temperature. By controlling the temperature, it is possible to control the enzymatic activity of the DNA polymerases (Mathys *et al*., 2007).

2.8.3 The intervenient of the reaction

2.8.3.1 DNA

DNA (deoxyribonucleic acid, genetic information support) is a chain of "double strand" bases. Each base consists of a sugar (deoxyribose) that carries a nucleotide of choice: adenosine (A), thymine (T), guanine (G) or cytosine (C) (Figure 2). The sugars form together the chain of DNA, linked to each other by their phosphate which binds to the carbon of the next deoxyribose. This is defined as a 5 '-3 ' strand and the 3 '-5 ' strand which reads in the other direction. The 3 '-5 ' strand is a "further copy" of the other strand where C is replaced by G, G by C, A by T and T by A (Asensio Gil, 2007).

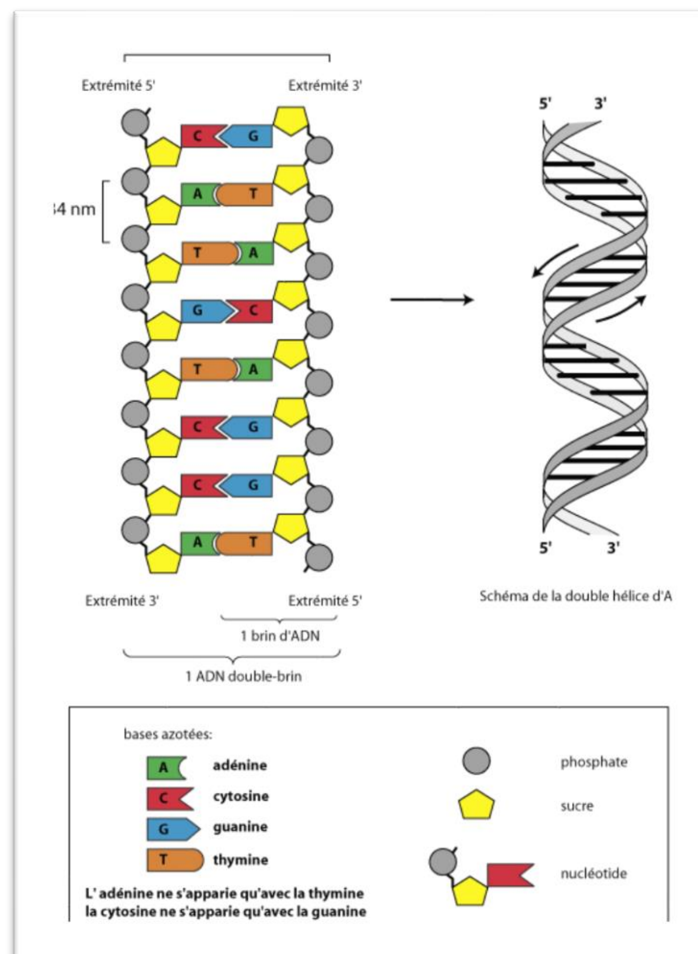


Figure 2: Structure of the DNA molecule

2.8.3.2 Primers

The primer is a short single-stranded DNA sequence of 20 bases generally. A couple of primers are selected so as to frame the area of DNA to be amplified by the primers. A primer is selected on the 5 '-3 ' strand (forward) and the other on the 3 '-5 ' (reverse) complementary strand. The size in number of base pairs of the fragment of DNA framed by the primers, which corresponds to the fragment that will be amplified, is there foreknown as the outset and depends on the choice of the position of the primers. The primers are chosen to frame the sequence of DNA to be amplified. (Asensio Gil, 2007; Poitras *et al.*, 2002)

2.8.3.3 An enzyme

The ATQ polymerase (ATQ Pol) is a heat-resistant DNA reaction extracted from the *Thermusaquaticus* bacterium. Its optimum action temperature is 72ºC and it is able to withstand successive passages at 95ºC, which made it possible to automate the procedure (Asensio Gil, 2007).

2.8.3.4 Nucleotides

The DNTPs (Deoxynucleotides-Tri-phosphates); DGTP, DATP, DTTP, DCTP, are the basic elements used by the ATQ Pol to synthesise complementary DNA strands (Asensio Gil, 2007).

2.8.4 The PCR reaction

The polymerase chain reaction is performed in a reaction mixture that includes the DNA extract (Matrix DNA), the ATQ polymerase, the primers, and the four excess triphosphate deoxyribo nucleosides (DNTP) in a buffer solution. The tubes containing the reaction mixture are subjected to repeated temperature cycles several dozen times in the heating block of a heat-cycler – a device that contains a chamber where the sample tubes are placed and in which the temperature can vary, very quickly and accurately, from 0 to 100ºC per Peltier effect. The device allows programming of the duration and the succession of the cycles of temperature bearings. Each cycle consists of three periods of a few tens of seconds (Poitras *et al.*, 2002).

2.8.4.1 Amplification cycle

a. Denature

The first period takes place at a temperature of 94ºC, known as the denaturing temperature. At this temperature, matrix DNA, which serves as a matrix during replication, is denatured: Hydrogen bonds cannot be maintained at temperatures above 80°C, and double-stranded DNA is separated into single-strand DNA (stranded DNA) (Poitras *et al.*, 2002).

b. Annealing with primer

The second period takes place at a temperature generally between 40 and 70ºC, known as the primer hybridisation temperature. The decrease in temperature allows the hydrogen bonds to be reformed so to the complementary strands to hybridise. The primers "recognise" their complementary sequence on the target DNA strands. They hybridise each on their respective strands. This step lasts one minute to give the primers time to hybridise properly. The higher the hybridisation temperature, the more selective and specific the hybridisation (Poitras *et al.,* 2002).

c. Extension

The temperature of the tube is then increased to 72ºC, allowing the ATQ Pol to add nucleotides to the hybridised primers in the direction 5 ' to 3 '. Nucleotides are not randomly incorporated but based on the target sequence (additional nucleotide). This step lasts one minute. A new strand of DNA, the sequence of which is complementary to that of the target strand, has just been synthesised (Poitras *et al.*, 2002) (Figure 3).

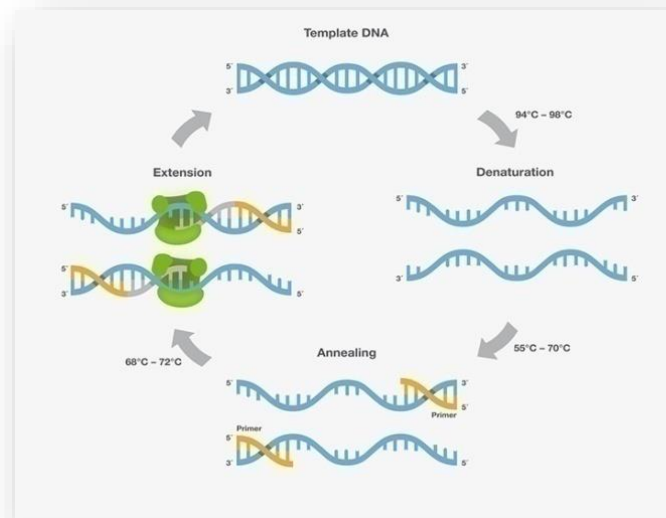


Figure 3: Amplification cycle

2.8.4.2 PCR cycle

PCR products obtained at the end of each cycle serve as a matrix for the next cycle (each cycle sees theoretically doubling the amount of DNA present in the previous cycle), this will recur 35 times (depending on the PCR protocol) and amplification is so called exponential (Mathys *et al.*, 2007) (Figure 4).

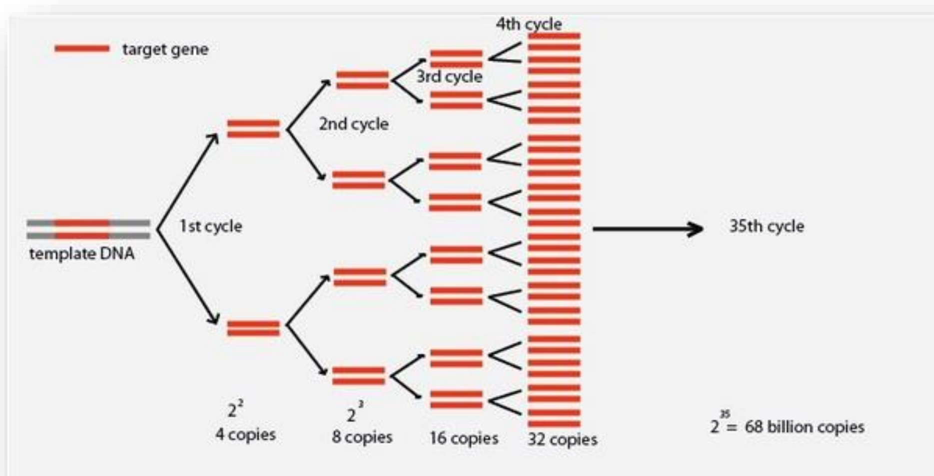


Figure 4: Exponential amplification

2.8.4.3 Visualisation of amplification products

The amplification products are subjected to agarose gel electrophoresis which allows the migration of nucleic acids through the gel added to BET (ethidium bromide which interconnects between the bonds and emits fluorescence). The rate of migration is dependent on the molecular mass, thus the number of bases of the DNA, the presence and the size of the amplicons can be verified (Asensio Gil, 2007).

III. Materials and Methods

3.1 *Meta-analysis of prevalence of Salmonella spp. in alheira sausage*

The objective of this section was to summarise the prevalence of *Salmonella* spp. in *alheira* sausages by meta-analysing the findings from published primary studies. Literature search to identify suitable scientific articles was conducted using PubMed and Web of Science databases for papers indexed since 1990 as well as Google searches using both English and Portuguese terms for combinations of the pathogen (e.g., *Salmonella*) and the meat product under study (e.g., Portuguese sausage, alheira). For inclusion in the meta-analyses, the papers had to meet two requirements: to be an original article and to use an approved microbiological method.

The population was specified as alheira produced in Portugal while the measured outcome was the occurrence defined as the ratio between the number of positive samples (s) and the total number of samples (n). The sought information was found in only five published primary studies, which were considered appropriate for inclusion in the meta-analysis model. Apart from "s" and "n", other information annotated from the primary studies was: year of the survey, sample weight for analysis in grams and origin of the samples (i.e., factories, retail, etc.).

A random-effects meta-analysis model was adjusted to the logit transformation of the incidence or proportion p_j, which was calculated using s_j and n_j taken from each of the five primary studies recovered (j={1, 2, 3, 4, 5}) as,

$$\theta_j = logit\ p_j = \log\left(\frac{p_j}{1-p_j}\right) = \log\left(\frac{s_j}{n_j - s_j}\right) \qquad (1)$$

In order to estimate the meta-analytical or pooled prevalence, weights w^*_j were assigned to the observations as the reciprocal of their variances, as follows,

$$w_j^* = \frac{1}{\sigma^2(\theta_j) + \tau^2} \qquad (2)$$

where the variances $\sigma^2(\theta)$ of the observations were estimated as,

$$\sigma^2(\theta_j) = \frac{1}{n_j p_j (1 - p_j)} \tag{3}$$

andτ^2, the between-study variability, was computed from the Q-statistic

$$\hat{\tau}^2 = \frac{Q - (J - 1)}{\sum_j w_j - \left(\sum_j w_j^2 \Big/ \sum_j w_j \right)} \tag{4}$$

with

$$Q = \sum_j \frac{(\theta_j - \hat{\Theta})^2}{\sigma^2(\theta_j)} \tag{5}$$

and

$$\hat{\Theta} = \frac{\sum_j w_j^* \theta_j}{\sum_j w_j^*} \tag{6}$$

The random-effects meta-analysis model was adjusted in R version 3.6.0 (R Development Core Team) using the 'metafor' package (Viechtbauer, 2010). For the appreciation of the heterogeneity in *Salmonella* prevalence in surveyed alheiras among primary studies, a forest and a Galbraith plot were constructed, while for the assessment of publication bias, a funnel plot was produced. Further information on meta-analysis of prevalence of pathogens and publication bias assessment can be found in Xavier et al. (2014).

3.2 Physicochemical and microbiological characterisation of alheira sausage

The Portuguese sausage, subject of analysis, was alheira, which is a non-ready-to-eat traditional fermented product usually elaborated from poultry, pork, rabbit and/or game meat, generally without the use of protective or starter cultures. For the physicochemical and microbiological characterisation of alheira, a total of 18 alheiras were used. Alheiras in two presentations, bulk (unpacked) and vacuum-packed, were purchased between May and June 2019, taken to the laboratory in a cooler, and analysed immediately. Unpacked alheiras were purchased from market fairs, while branded vacuum-packed alheiras were purchased from local shops and supermarkets, and were kept under refrigeration.

3.2.1 Physicochemical characterisation of alheira sausage

Three physicochemical analyses for alheira sausages were conducted: pH, aw and moisture. The pH was measured in triplicate by introducing a puncture pH-meter (HANNA, Portugal) into the sausage. Values were read off after stabilisation of the measurement. Moisture was determined in triplicate by drying out 5 g of sausage until constant weight in an oven. The water activity measurement was done using an AquaLab equipment (4TE, AquaLab, USA). The sample was carefully placed in a disposable cup making sure it was homogeneously spread. This was repeated three times. For each alheira sausage, the three replicates of pH, moisture and aw were averaged.

3.2.2 Microbiological characterisation of alheira sausage

The microbiological analyses of alheira sausage included six bacterial groups, namely, mesophiles, psychotrophic bacteria, lactic acid bacteria, coliforms, *E. coli* and *Pseudomonas*. Twenty-five grams were sampled within a sausage and placed in a stomacher bag with 225 ml buffered peptone water and homogenised for 2 minutes in a stomacher (Interscience Bag Mixer 400, France). After decimal dilutions to 10^{-5}, one-ml aliquots were inoculated into petrifilms (3M) Aerobic Count Plate, for quantification of mesophiles, and Lactic Acid Bacteria Count, for lactic acid bacteria. Aliquots of one-ml were seeded by incorporation in Plate Count Agar (Liofilchem, Italy) for psychrotrophic counts, while, for the quantification of *Pseudomonas*,

aliquots of 0.5 ml were spread in a selective medium Pseudomonas Agar Base (Oxoid, UK), added with 1% v/v glycerol and supplemented with cetrimide-fucidine-cephalosporin (SR0103, Liofilchem, Italy). The mesophilic and lactic acid bacteria plates were incubated at 35±0.5ºC for 48h, the psychrotrophic ones at 7±0.5ºC for 10 days, and the *Pseudomonas* at 25±0.5ºC for 24 h.

3.3 Qualitative and quantitative analysis of Salmonella spp. in alheira sausages

Alheiras were purchased from three types of establishments: supermarkets, local shops and market fairs. They were analysed in a total of 9 batches, as shown in Table 5.

Table 5: Codes assigned and origin of the alheira sausage samples

Month of purchase	Samples	Code	Origin
03 December 2018	1	2112/S1	Market fairs
	2	2112/S2	
	3	2112/S3	
	4	2112/S4	
18 December 2018	1	2212/S1	Local shop
	2	2212/S2	
	3	2212/S3	
	4	2212/S4	
	5	2212/S5	
	6	2212/S6	
14 January 2019	1	1701/S1	Supermarket
	2	1701/S2	
	3	1701/S3	
	4	1701/S4	
	5	1701/S5	

29 January 2019	1	0102/S1	Local shop
	2	0102/S2	
	3	0102/S3	
	4	0102/S4	
	5	0102/S5	
	6	0102/S6	
06 February 2019	1	0902/S1	Supermarket
	2	0902/S2	
	3	0902/S3	
	4	0902/S4	
	5	0902/S5	
20 March 2019	1	2303/S1	Market fairs
	2	2303/S2	
27 March 2018	1	3003/S1	Market fairs
	2	3003/S2	
	3	3003/S3	
	4	3003/S4	
	5	3003/S5	
	6	3003/S6	
18 June 2019	1	P1	Supermarket
	2	P2	
	3	P3	
	4	V1	
	5	V2	
	6	V3	
	7	W1	
	8	W2	
	9	W3	
24 June 2019	1	A1	Market fairs
	2	A2	
	3	A3	
	4	B1	
	5	B2	

	6	B3	
	7	C1	
	8	C2	
	9	C3	

Once collected, alheiras were transported hermetically in a cooler to the laboratory. Most of the times, they were analysed on the same day of purchase. Else, they were kept at 4ºC and analysed within the next 24 hours. For microbiological analysis, the mass from one alheira was sampled aseptically from all parts of the sausage to make 25 g.

3.3.1 Qualitative analysis

Qualitative analysis of *Salmonella* traditionally requires three steps: pre-enrichment, enrichment and isolation (Figure 5). For the pre-enrichment, twenty-five g sampled from the alheira sausage was placed in a stomacher bag containing 225 ml of buffered peptone water (CM0509, Oxoid, UK), and homogenised for 2 minutes using a stomacher (Interscience Bag Mixer 400, France).

After incubation for 24 hours at 37°C, aliquots of 0.1 ml and 1.0 ml of the pre-enriched culture was inoculated into two selective enrichment broths: Rappaport-Vassiliadis (RV) broth (610175, Liofilchem, Italy) and Tetrathionate Broth (TTB) Base (610183, Liofilchem, Italy), and incubated at 42 and 37°C, respectively. These two selective broths contain active inhibitory agents on microorganisms that compete with *Salmonella* spp. after 24 h incubation, one loop from each of the enriched broths was streaked onto plates of Hektoen enteric (HE) agar (01-216-500, Scharlau, Spain), Bismuth sulfite (BS) agar (610301, Liofilchem, Italy) and Xylose Lysine Deoxycholate (XLD) agar (DSHB3011, Liofilchem, France), and incubated at 37°C for 24 h. The plates were examined for the presence of typical *Salmonella* colonies, transparent colonies with black centers on HE and BS agar, and red colonies with black centers on XLD agar. Suspected colonies were confirmed by conventional biochemical methods and also with serological method as described in the next sections.

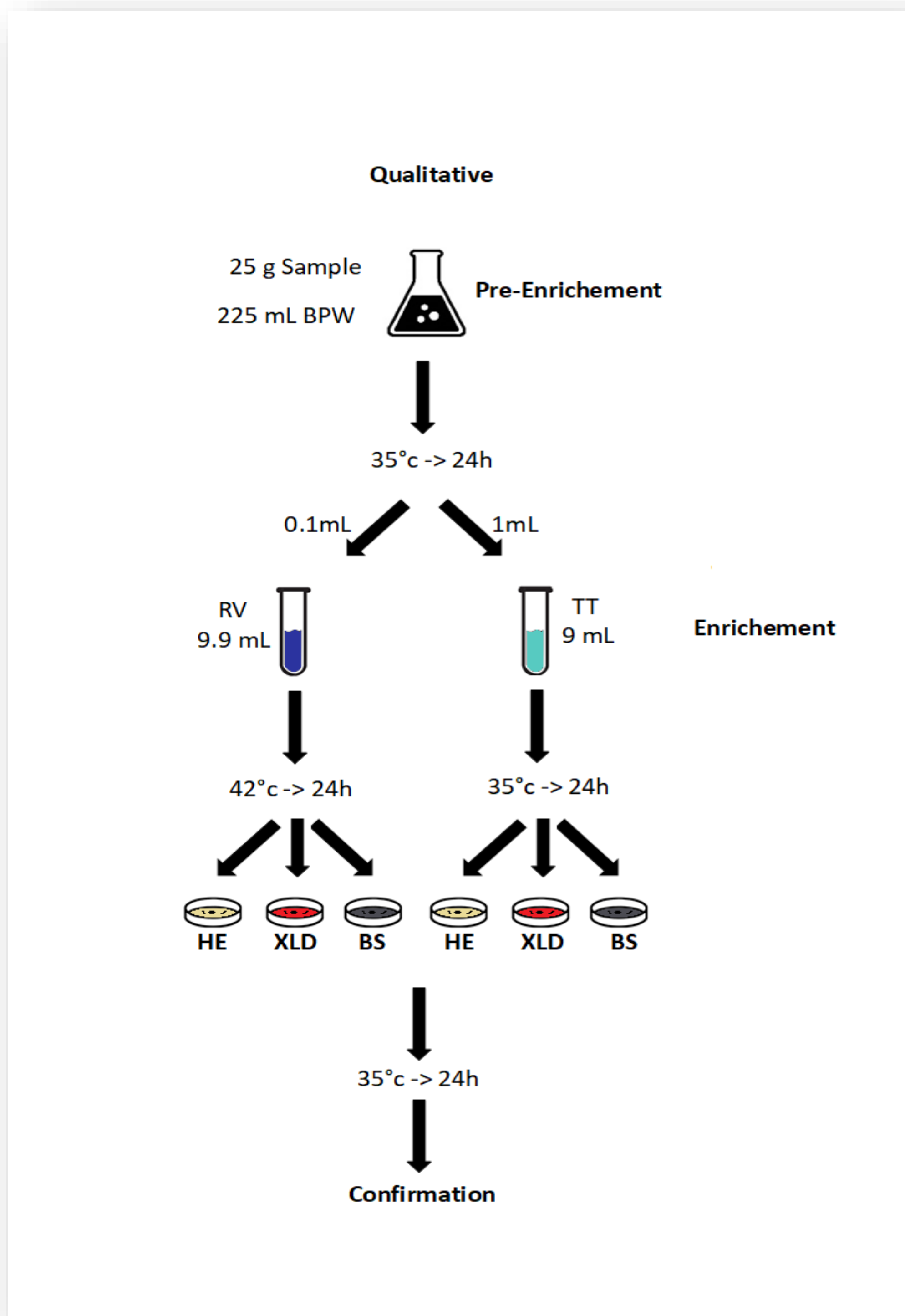


Figure 5: Qualitative analysis of *Salmonella*

3.3.2 Quantitative analysis

The quantitative analysis of *Salmonella* was carried out in parallel with the qualitative analysis, and using the most-probable-number (MPN) technique (Figure 6). After preparation and incubation of the homogenate, 30 ml of the homogenate was then transferred and divided into 3 10-ml tubes; each tube was then diluted to 10^{-2} and 10^{-3} using 9 ml of BPW. All tubes were incubated for 24h at 37°C.

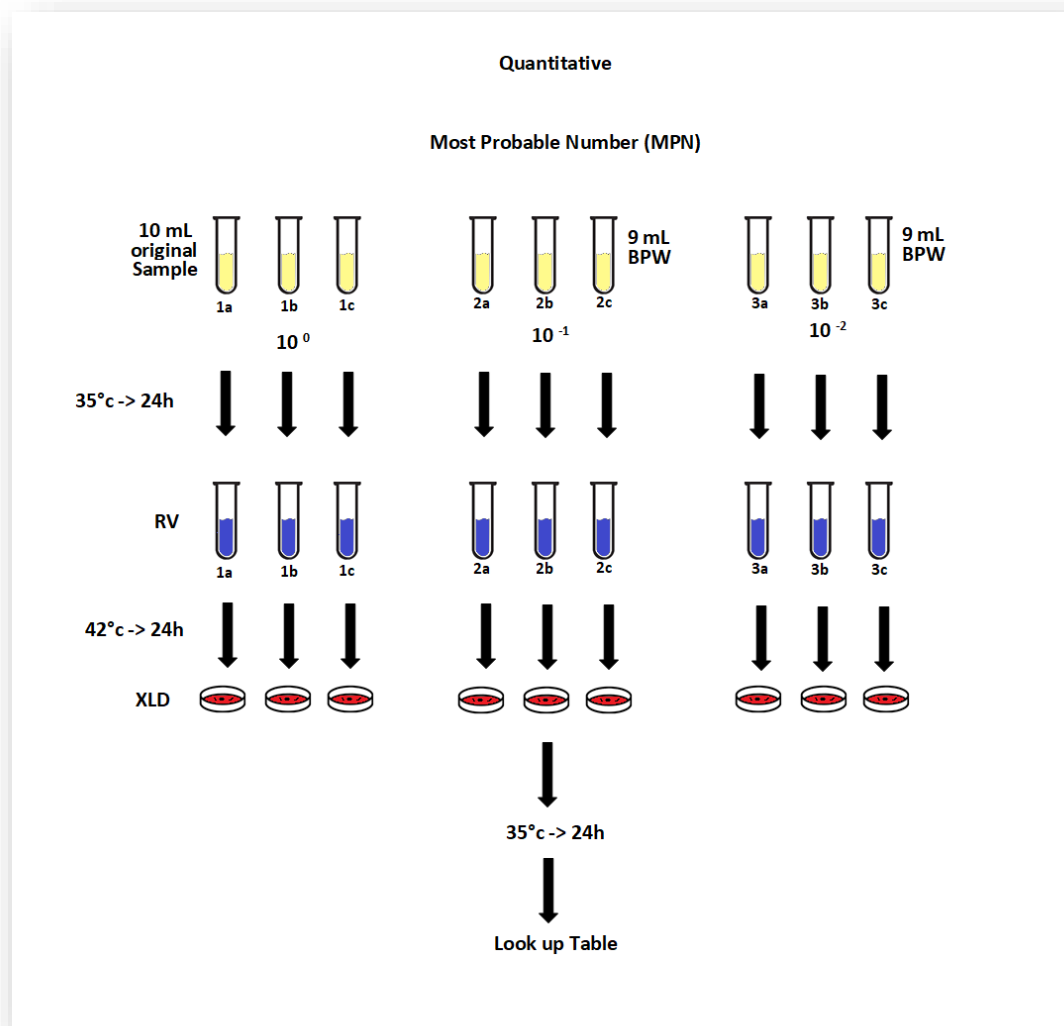


Figure 6: Quantitative analysis of *Salmonella* using the most probable number technique

Afterwards, from each tube, an aliquot of 0.1 ml was transferred into 10 ml of RV broth, and incubated at 42ºC for 24 hours. After this, a loopful from each tube of RV was streaked onto XLD agar. Selective plates were incubated at 37ºC for 24 h. For a plate, to be considered as having a positive results, suspicious colonies of *Salmonella* spp. were confirmed both biochemically and serologically.

After determining if a plate was positive for *Salmonella*, the number of positive plates in each of the three dilutions was counted and expressed as a triplet. The concentration of *Salmonella* was obtained using the MPN statistical table for nine tubes (Table 6). Nonetheless, the concentration of *Salmonella* from a sample was only meaningful if the qualitative analysis gave a positive result for the same sample.

Table 6: An excerpt of a nine-tube most-probable-number (MPN) statistical table*

Number of positive tubes	MPN	95% Confidence Limits
000	<0.30	-
100	0.36	0.02 to 1.7
200	0.92	0.15 to 3.5
210	1.5	0.4 to 3.8
300	2.3	0.5 to 9.4
310	4.3	0.9 to 18.1
311	7.5	1.7 to 19.9
320	9.3	1.8 to 36
321	15	3.0 to 38
330	24	4.0 to 99
331	46	9.0 to 198
332	110	20.0 to 400
333	>110	-

* Based on 3 x 1 g(ml) + 3 x 0.1 g(ml) + 3 x 0.01 g(ml) samples (expressed as organisms per one g)

3.4 Confirmation of Salmonella

From one sample, at least three *Salmonella*-like colonies underwent the confirmation process, which is described in this Section (Figure 7).The choice of the distinct colonies was based on color, size and shape.

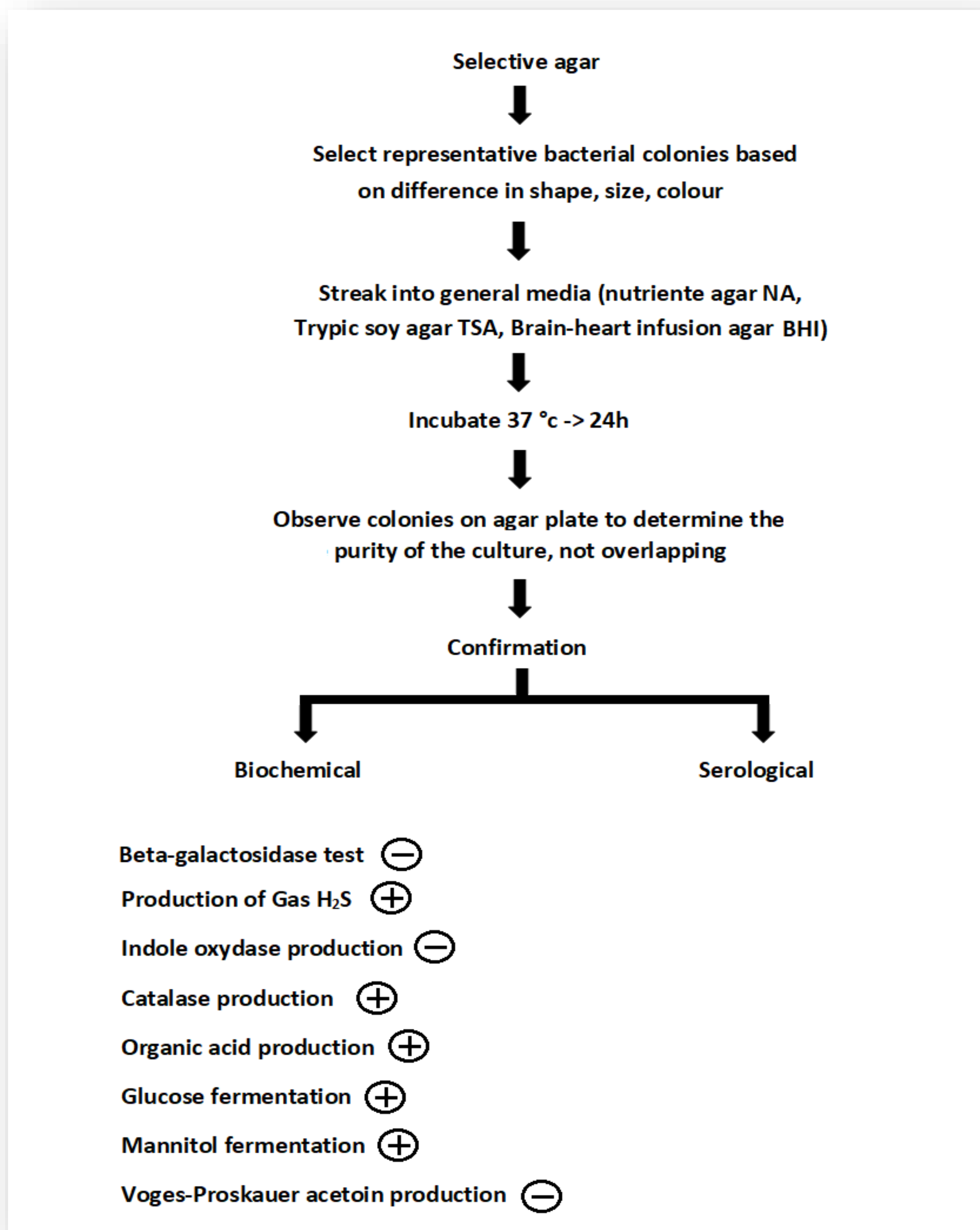


Figure 7: Isolation and purification of *Salmonella* cells

3.4.1 Isolation and purification of colonies

From the plates selected, and after identifying the suspicious bacterial colonies, the centre of the chosen colony was picked and streaked onto Nutrient agar (NA) (610036, Liofilchem, Italy). After incubation at 37°C for 24 hours, the state of the colonies was checked to make sure that the culture was pure. These colonies of *Salmonella* were confirmed biochemically and serologically. In most cases, for confirmation, the purification step was not needed, when suspect colonies in the selective medium were sufficient in number. However, for the molecular characterisation part, this is, for the preparation of frozen stocks, colonies were always purified.

3.4.2 Biochemical confirmation

An isolated or purified suspicious colony was biochemically confirmed. Biochemical confirmation was carried out using the API 20E test kit (Figure 8), which consists of micro tubes containing dehydrated substrates which allow the realization of enzymatic reactions or fermentations of sugars. The reactions produced during the incubation period result in spontaneous color turns or are revealed by the addition of reagents. After inoculation, incubation for 18-24 hours, the confirmation is achieved.

Figure 8: API 20E test system for *Salmonella* confirmation

For *Salmonella* confirmation, the following tests were validated:

- H_2S (+)
- Voges Proskauer (VP) (-)
- Indole (-)
- Catalase (+)
- Urease (-)
- Methyl red (+)

- Mannitol and Glucose fermentation (+)
- Ortho-Nitrophenyl-β-Galactoside(ONPG) (-)

3.4.3 Serological confirmation

The serological confirmation was carried out using the *Salmonella* latex agglutination kit (96151, Liofilchem, Italy). The result is achieved by the presence or absence of coagulation following the addition of a drop of *Salmonella* latex reagent on the mixture of the physiological water and the colony to be identified. After mixing well, the presence of coagulation or precipitation in the drop confirms that the bacterium belongs to *Salmonella* spp.

3.5 Preparation of bacterial frozen stocks

Bacterial glycerol stocks are important for long-term storage. The step of frozen stocks preparations starts with the growth of bacteria in BPW. An amount of 500 µL of the overnight broth culture was added to 500 µL of 30% glycerol contained in a 2-mLcryotube. Figure 8 illustrates the steps that were carried out to prepare frozen stocks of *Salmonella* cells to be used later in molecular typing.

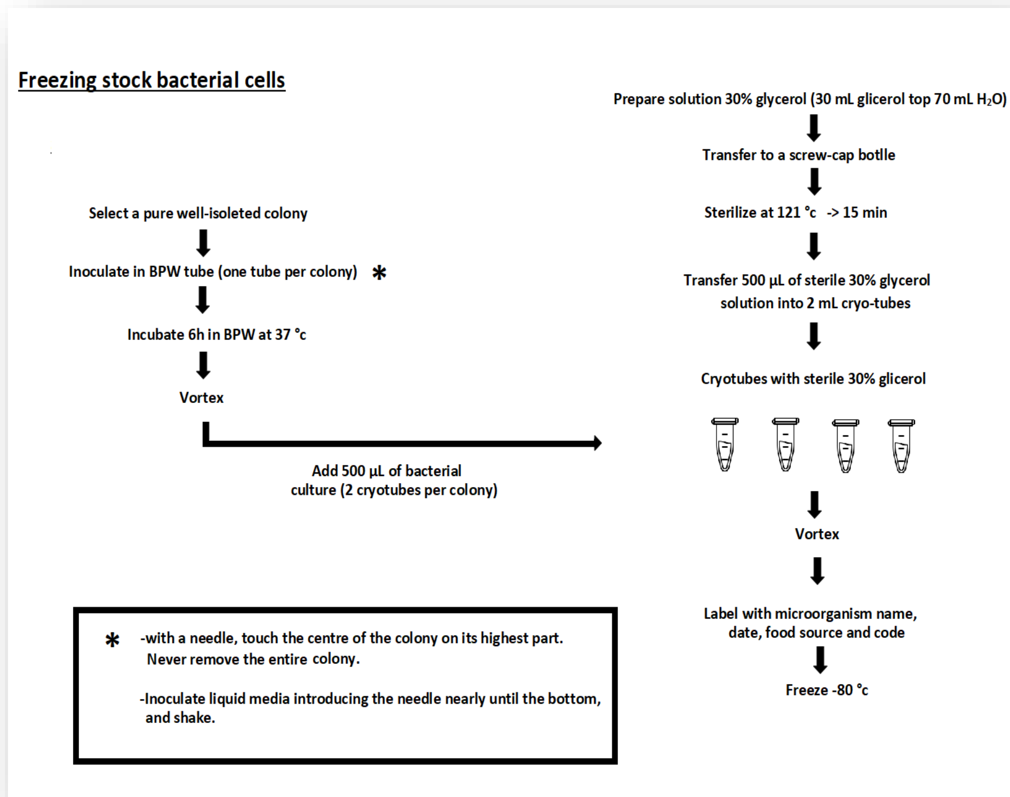


Figure 8: Preparation of frozen stocks of *Salmonella* cells

3.6 Molecular typing of *Salmonella*

3.6.1 Extraction of DNA

For the extraction the DNA, the GF-1 Bacterial DNA extraction kit (Vivantis, GF-BA-100) was used, which is a kit designed for rapid and efficient purification of high weight genomic DNA from Gram-negative or Gram-positive bacteria (Figure 9). This kit contains a treated glass filter membrane fixed to columns to efficiently bind DNA in the presence of high salt, by efficiently applying the principle of a mini-column spin technology and the use of optimised buffers to ensure that only DNA is isolated while cellular proteins, metabolites, salts and other low molecular weight impurities are removed during the washing steps.

Figure 9: GF-1 Bacterial DNA extraction kit

The extraction of DNA was carried out following these steps:

1. Centrifugation: Two ml of bacteria culture grown overnight or culture grown to log-phase was pelleted by centrifugation at 6,000*g for 2 min at room temperature. The supernatant was decanted completely.

2. Re-suspension of pellet: One-hundred µl of Buffer R1 was added to the pellet and the cells re-suspended completely by pipetting up and down.

3. Centrifugation: Digested cells were pelleted by centrifugation at 10,000*g for 3 min. and then decant the supernatant completely.

4. Protein denaturation: The pellet was re-suspended in 180 µl of Buffer R2 and 20 ul of Proteinase K was added, and mixed thoroughly. It was then incubated at 65°C for 20 min in a shaking water bath.

5. Homogenisation: 440 µl of Buffer BG was added and mixed thoroughly by inverting tube several times until a homogeneous solution was obtained. The tube was then incubated for 10 min at 65°C.

6. Addition of ethanol: 200 µl of absolute ethanol was added to each tube and mixed immediately and thoroughly.

7. Loading the column: The sample was transferred into a column assembled in a clean collection tube, centrifuged at 10,000 *g for 1 min then flow through discarded. This step was repeated until centrifuging all the volume of the sample present in the tube was finished.

8. Column washing: The column was washed with 650 µl of wash buffer and centrifuged at 10,000*g for 1 min. Flow through was discarded.

9. Column drying: The column was centrifuged at 10,000*g for 1 min to remove residual ethanol.

10. DNA elution: The column was placed into a clean micro-centrifuge tube, 50 µl of elution buffer was added directly onto the column membrane and let it stand for 2 min. To elute the DNA, it was centrifuge at 10,000*g for1 min.

The extract was put in vial tubes and stored at -20 ° C until used as a PCR template. Table 7 indicates the extracted DNAs, codes and date of extractions.

Table 7: Throughput data of *Salmonella* positive samples for DNA extraction

Samples	Date of sampling	Code of the tubes chosen	Date of DNA extraction	Code chosen for PCR
3	03/12/2018	2112 (S1)*	10/05/2019	2112 (S1)
3	03/12/2018	2112 (S4)*	10/05/2019	2112 (S4)
3	03/12/2018	2112 (S7)*	10/05/2019	2112 (S7)
4	03/12/2018	2112 (S16)	10/05/2019	2112 (S16)
4	03/12/2018	2112 (S17)*	10/05/2019	2112 (S17)
4	03/12/2018	2112 (S20) *	10/05/2019	2112 (S20)
1	27/03/2018	3003 (S1 HE)*	10/05/2019	3003 (S1 HE)
1	27/03/2018	3003 (S 1 2a)*	10/05/2019	3003 (S 1 2a)
2	27/03/2018	3003 (S2 1a) *	10/05/2019	3003 (S2 1a)
2	27/03/2018	3003 (S2 3b)*	10/05/2019	3003 (S2 3b)
4	27/03/2018	3003 (S4 3c)*	10/05/2019	3003 (S4 3c)
6	27/03/2018	3003 (S6 3a)	10/05/2019	3003 (S6 3a)
2	27/03/2018	3003 (S2 2a)	31/05/2019	3003 (S2 2a)
2	27/03/2018	3003 (S1 1b)*	31/05/2019	3003 (S2 1b)
2	27/03/2018	3003 (S2 1c)*	23/05/2019	3003 (S2 1c)
2	27/03/2018	3003 (S2 2c)*	23/05/2019	3003 (S2 2c)
3	03/12/2018	2112 (S3)	23/05/2019	2112 (S3)
3	03/12/2018	2112 (S9)	23/05/2019	2112 (S9)
3	03/12/2018	2112 (S10)	23/05/2019	2112 (S10)
3	03/12/2018	2112 (S14)	28/05/2019	2112 (S14)
3	03/12/2018	2112 (S5)	28/05/2019	2112 (S5)
4	03/12/2018	2112 (S22)*	23/05/2019	2112 (S22)
4	03/12/2018	2112 (S26)*	23/05/2019	2112 (S26)
3	03/12/2018	2112 (S15)	07/06/2019	2112 (S15)
3	03/12/2018	2112 (S8)	07/06/2019	2112 (S8)
3	03/12/2018	2112 (S2)	07/06/2019	2112(S2)
3	03/12/2018	2112 (S12)*	23/05/2019	2112 (S12)
3	03/12/2018	2112 (S19)	23/05/2019	2112 (S19)
3	03/12/2018	2112 (S29) *	23/05/2019	2112 (S29)
3	03/12/2018	2112 (S25)*	28/05/2019	2112 (S25)
A1	28/06/2018	A2 3c*	02/07/2018	A2 3C
B2	28/06/2018	B2 1c	02/07/2018	B2 1c

B2	28/06/2018	B2 2a*	02/07/2018	B2 2a
B2	28/06/2018	B2 XLD*	02/07/2018	B2 XLD
B2	28/06/2018	B2 3c*	02/07/2018	B2 3c
A2	28/06/2018	A2 1c*	02/07/2018	A2 1c
A2	28/06/2018	A2 2b*	02/07/2018	A2 2B
B2	28/06/2018	B2 3a	02/07/2018	B2 3a
B2	28/06/2018	B2 HE*	02/07/2018	B2 HE
A2	28/06/2018	A2 2c*	02/07/2018	A2 2C
B2	28/06/2018	B2 2c*	02/07/2018	B2 2C
A2	28/06/2018	A2 2a*	02/07/2018	A2 2a
B2	28/06/2018	B2 2b*	02/07/2018	B2 2b
A2	28/06/2018	A2 1b*	02/07/2018	A2 1b
B2	28/06/2018	B2 1a*	02/07/2018	B2 1a
A2	28/06/2018	A2 3b*	02/07/2018	A2 3b
A2	28/06/2018	A2 XLD*	02/07/2018	A2 XLD
B2	28/06/2018	B2 1C	02/07/2018	B2 1C

* Positive extracted DNA

3.6.2 Agarose gel

Agarose gel electrophoresisis a method used in biochemistry and molecular biology to separate DNA, RNA or proteins based on their molecular weight. The technique of agarose gel electrophoresis is based on the separation of negatively charged nucleic acids under the effect of an electric field. This separation takes place through the agarose gel matrix: the smaller molecules move faster and migrate further than the larger molecules.

The use of agarose gel allows us to estimate the molecular weight of the DNA fragment after digestion by restriction enzymes and DNA analysis after amplification by PCR (Lee *et al.*, 2012). All amplified products were analysed by electrophoresis using 1.5% agarose gel and visualised by ultraviolet trans-illuminator (Bio-Rad, Universal Hood II, USA) after gel staining with ethidium bromide stain. To prepare an agarose gel, the following procedure was followed:

- One-hundred ml of the TAE solution was transferred to a conical flask.
- 1.5 grams of agarose was weighed and added to the flask.
- The flask was microwaved for 5 min.
- The solution was left to cool, and then 5.4 g of ethidium bromide was added.

3.6.3 PCR amplification

PCR is an automated technique that takes place in a thermocycler. The device contains a heating block where tubes containing the mixture are inserted for the PCR reaction and where the temperatures can vary very quickly and very precisely from 0 to 100°C (Poitras *et al*., 2002).

Amplification was performed as follows:

-15 µl of PCR ready-Mixt FROM Red Extract–N-Amplification plant PCR kit

-1 µl of upstream primer, 1 µl of downstream primer

-2 µl of template DNA

And nuclease-free water up to 30 µl.

The amplification conditions of 284 bp of InvA gene for the thermal cycler were adjusted to:1 cycle at 95°C for 1 min, then 35 cycles at 95°C for 1 min, 64°C for 30 s, 72°C for 30 s, followed by 1 cycle at 94°C for 4 min. For multiplex PCR, the amplification conditions were adjusted to 1 cycle at 94°C for 1 min, 35 cycles at 94°C for 30 s, 56°C for 1 min 30 s, 72°C for 30 s, followed by 1 cycle at 72°C for 10 min. The Duplex PCR was performed, with a wide range of annealing temperatures, where PCR conditions were 1 cycle at 95°C for 5 min, 35 cycle of 95°C for 30 s, 55-65°C for 30 s, and 72°C for 30 s followed by a final extension step at 72°C for 10 min (Ibrahim *et al*., 2016). The primers used are summarised in Table 8.

For molecular confirmation of *Salmonella* spp., a primer set was used for amplification of 284 bp of InvA gene. Another primer set (S139, S141, ST11, ST15, Fli 15, Tym, Self 167, Sef 478 and SG-L) was used for general identification of *Salmonella* spp. as well as typing of *Salmonella* Typhimurium, Enteritidis or Pullorum in a multiplex PCR reaction (Ibrahim *et al*., 2016).

Table 8: Primer sets for *Salmonella* strains (Ibrahim *et al.,* 2016)

Primer	**Salmonella strain**	**Target gene**	**Primer sequence 5'----3'**	**Length**	**Amplicon fragment (bp)**
S 139 **S141**	*Salmonella* spp	invA gene	**-GTGAAATTATCGCCACGTTCG GGCAA** **-TCATCGCACCGTCAAAGGAACC**	26 22	284
ST11	*Salmonella* spp	Randomly cloned chromosomal fragment	**AGCCAACCATTGCTAAATTGGCGCA**	25	429
ST15 **Fli15**	*Salmonella* Typhimurium	fliC	**-GGTAGAAATTCCCAGCGGGTACTG** **-CGGTGTTGCCCAGGTTGGTAAT**	24 22	559
Tym **Sef167**	*Salmonella* Enteritidis	Sef A gene	**-ACT CTT GCT GGC GGT GCG ACTT** **-AGGTTCAGGCAG CGGTTACT**	22 20	312
Sef478 **20 SG-L**	*Salmonella* Pullorum	glgC	**-GGGACATTTAGCGTTTCTTG** **-GATCTGCTGCCAGCTCAA**	20 18	252

3.6.4 PCR mixture composition

The DNA of the *Salmonella* isolates were prepared with a total volume of 30 µl, as shown in Table 9; for each mixture a different DNA sample was chosen. After assembling all reaction components on ice, the reactions were transferred to a thermocycler preheated to the denaturation temperature (95°C).The amplified products were subjected to electrophoresis using 1.5% agarose gel and visualised by ultraviolet transilluminator after gel staining with ethidium bromide stain.

Table 9: PCR reaction mixture composition

Reaction mixture constituents	**Volume of each component**
DNA	2 µl
Primers (n=9)	1 µl (9 µl)
Mix	15 µl
Distilled water	4 µl

IV. Results and Discussion

4.1 Meta-analysis of prevalence of Salmonella in alheira

The systematic review on contamination of alheira sausages with *Salmonella* spp. produced only five published articles (Almeida *et al*., 1998; Esteves *et al*., 2006a; 2008; Elias *et al*., 2015; and Silva *et al*., 2019), which revealed the scarcity of this type of data, and hence justified our study. The incidences of *Salmonella* spp. quantified in the five studies presented some degree of heterogeneity, as implied by the intra-class correlation I^2 of 53.3%. This value indicates that the between-study variability is responsible for 53.3% of the total variability in *Salmonella* prevalence, which is a much higher value than the cut-off rule-of-thumb of I^2=25% that is normally used in meta-analysis. The highest prevalence of *Salmonella* spp. in alheira was reported in the oldest study (33.3% by Almeida et al., 1998), while lower incidences (1.8% and 5.6%) were recovered in the recent surveys of Elias et al. (2015) and Silva et al. (2019), respectively (Figure 10). Nonetheless, despite the heterogeneity in incidence values, the Galbraith or radial plot (Figure 11) shows that this small meta-analytical data set did not present any outlier. Notice that, for the assessment of outliers, the Galbraith graph plots the logit-transformed prevalence values [-∞, +∞], and not the back-transformed ones (to the prevalence scale [0, 1]) as in the forest plot.

The meta-analytical or pooled incidence of *Salmonella* in alheira sausages was estimated at 10.5% (95% CI: 4.4 – 23.0%; Figure 10 and 11), which can be regarded as high even if it is a product intended to be eaten cooked. According to EFSA (2018), this level of non-compliance to the *Salmonella* microbiological criterion (in single sample units) is higher than the EU levels in 2017 for the following related categories: foods for poultry meat preparations intended to be eaten cooked (6.4%) and meat preparations from other species than poultry intended to be eaten cooked (3.3%).

According to EFSA (2018), in 2017 the highest proportions of *Salmonella*-positive single samples from official control investigations by the competent authorities were reported for foods of meat origin intended to be cooked before consumption: 4.8% for fresh chicken and 1.58% for fresh pig meat. Given the high incidence of *Salmonella* in the Portuguese

alheira, as found in this meta-analysis, along with the severity of this pathogen, actions should be enabled to instruct consumers of the importance of fully cooking this raw traditional product and avoiding cross contamination by means of adequate labelling.

As part of this meta-analysis study, publications bias was also investigated by the construction of a funnel plot, which relates the mean incidence value from each primary study with its respective standard error as a measure of the level of confidence in the results of such primary study. Only one publication reported a very high incidence value (highest value in the x- axis of the funnel plot) from small sample sizes (low inverse standard error in the y-axis in the funnel plot). This can be corroborated by the blank right bottom area in the funnel plot (Figure 12). Nevertheless, some caution should be taken in identifying this phenomenon directly as a proof of publication bias, since it is quite common that a small sample size will fail to detect any pathogen. For instance, it is likely that a sample size consisting of five sample units will not have the statistical power to detect *Salmonella* spp. if the true prevalence of this pathogen in alheira sausage were very low. For this reason, in microbiological surveys of absence/presence of pathogens in foods, in order to accurately estimate the pathogen's prevalence, a large sample size is commonly required when its concentration in food is known to below.

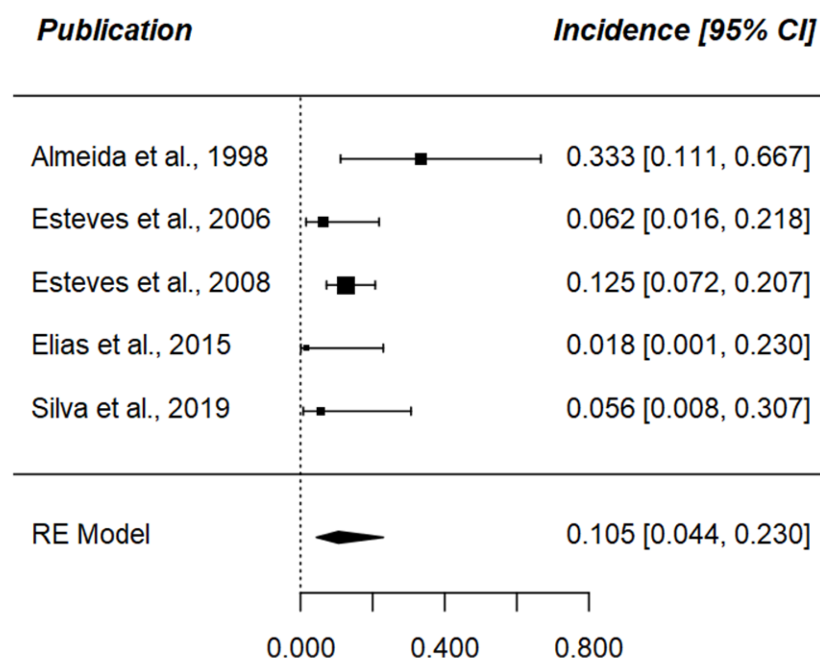


Figure 10 : Forest plot of the random-effects model of incidence of *Salmonella* spp. in alheira sausage

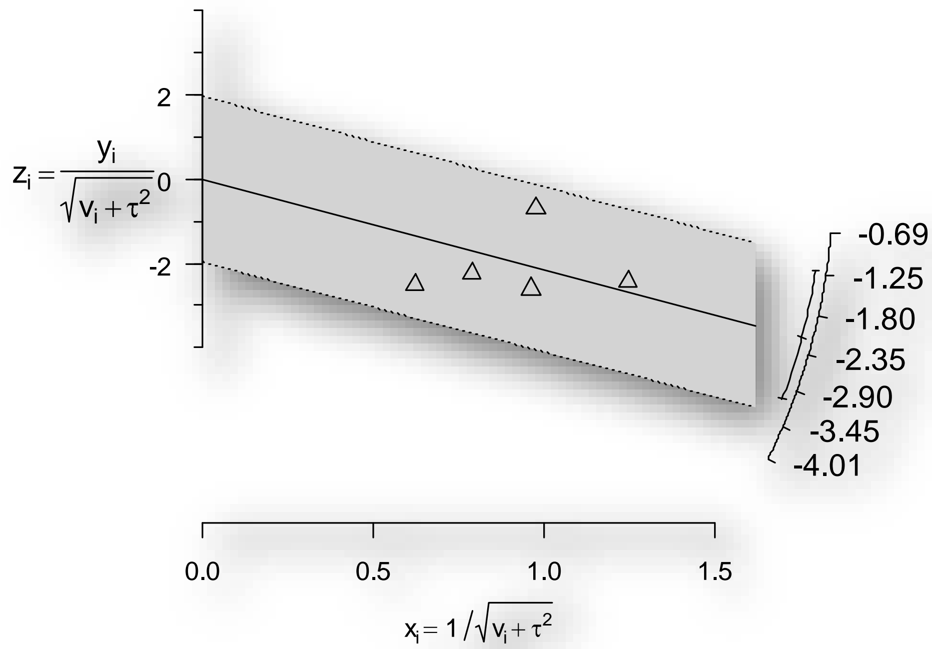


Figure 11: Galbraith plot of the random-effects model of the logit transformation of the incidence of *Salmonella* spp. in alheira sausage

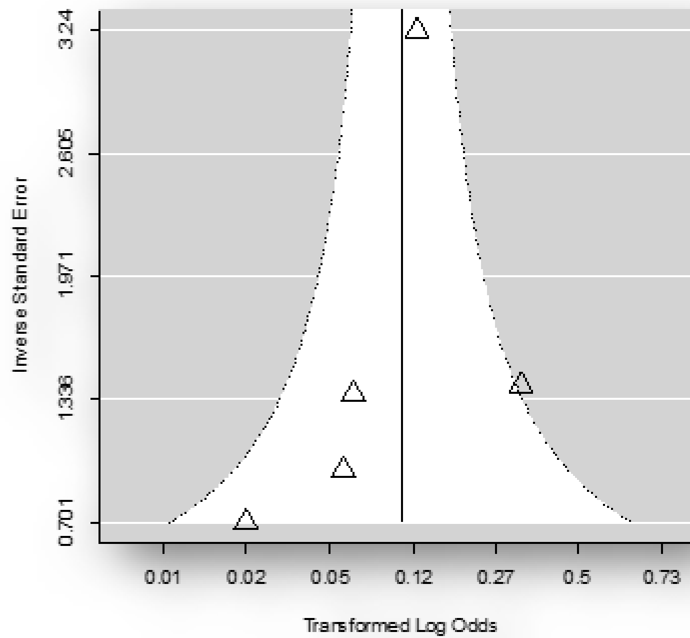


Figure 12: Funnel plot of the random-effects model of incidence of *Salmonella* spp. in alheira sausage

4.2 *Physicochemical and microbiological characteristics of alheira*

The physicochemical and microbiological characteristics of unpacked and vacuum-packed alheiras sausages are summarised in Table 10.

Table 10: Physicochemical and microbiological characterisation of alheiras. Microbial counts are given in log CFU/g

Property	**Unpacked alheira**			**Vacuum-packed alheira**		
	Mean	St. dev	CV	Mean	St. dev	CV*
Aw	0.987[a]	0.004	0.004	0.979[b]	0.008	0.008
pH	4.827[a]	0.563	0.116	4.036[b]	0.090	0.022
Moisture	0.495[a]	0.068	0.137	0.470[a]	0.033	0.071
Mesophiles	7.481[a]	0.715	0.096	6.394[b]	0.436	0.068
Coliforms	7.958[a]	0.948	0.119	5.423[b]	1.264	0.233
Pseudomonas	5.687[a]	0.100	0.017	4.527[b]	0.336	0.074
Psychrotrophic	7.977[a]	0.726	0.091	6.969[b]	0.295	0.042
LAB	9.557[a]	0.197	0.021	9.496[a]	0.242	0.025

*CV: Coefficient of variance
Different superscript letters denote significant differences between unpacked and vacuum-packed alheira at α=0.05

Overall, unpacked alheira had a significantly higher pH (4.82) than the vacuum-packed ones (4.03), although in both cases they fall under the category of an acidic product. Acidity is a consequence of the lactic acid fermentation that takes place during processing. Interestingly, alheira sausage is more acidic than pork or beef sausage (pH=5.50-5.60), according to Puolanne *et al*. (2011).

Unpacked or not, the water activity of bulk and packed alheira (0.987 and 0.978, respectively) indicate that it is a product of low stability, since it contains an important quantity of unbound

water which is favorable for bacterial growth. Alheiras sold unpacked and in a bulk manner are expected to have greater microbial instability than the vacuum-packed ones due to their higher pH and water activity. In terms of moisture content, nevertheless, there was no significant difference between packed and unpacked alheiras.

The greater instability of unpacked alheiras might partly explain the higher counts of mesophiles, coliforms, psychrotrophic and *Pseudomonas* recovered from these sausages in comparison to the packed alheiras. Nonetheless, since mesophiles and coliforms are hygiene indicator microorganisms, results point out that the manufacturing and hygienic conditions of the artisanal establishments where bulk sausages are elaborated might not be as strictly controlled as those in the factories where vacuum-packed alheiras are produced. The level of coliforms in bulk alheiras (7.958 log CFU/g) was found to be much higher than that of vacuum-packed alheiras (5.423 log CFU/g). These results are not surprising since bulk alheiras are commonly produced in artisanal conditions with no brand name associated to them and sold in market fairs at ambient temperature, while vacuum-packed alheiras are branded and sold in supermarkets under refrigeration.

The higher counts of *Pseudomonas* and psychrotrophic bacteria recovered from the unpacked alheira sausages also indicate that these artisanally-produced alheiras will have a shorter shelf-life than the vacuum-packed ones, and, in addition, that by the end of alheira processing, more microbiological deterioration might have occurred in the unpacked sausages than in the vacuum-packed ones. Furthermore, vacuum-packaging inhibits the growth of *Pseudomonas*, since they are aerobic microorganisms. In relation to lactic acid bacteria, no significant difference was found between packed (9.496 log CFU/g) and unpacked alheiras (9.557 log CFU/g).

The presence as well as the behavior of microorganisms in foods is governed by a variety of environmental and ecological factors (Cummings *et al.*, 2012). In the case of alheiras, these include water activity, pH, quality of the fermentation process, ripening and storage temperature, packaging, physical manipulation, among others. The comparison between unpacked and packed alheiras highlighted that, as in other fermented meat products, both vacuum-packaging and cold storage should be applied as hurdles to protect alheira against excessive growth of deteriorating microflora. In addition, packaging prevents direct cross-

contamination of the product with dirty hands, which probably occurs in the market fairs where these products are sold unprotected and at ambient temperature.

4.3 Qualitative and quantitative analysis of Salmonella in alheira

The analysis of the 52 sausage samples showed the presence of *Salmonella* spp. in 10 samples (global incidence 0.192; 95% CI: 0.108–0.319), although all of these positive samples originated from unpacked sausages sourced from market fairs (n=21), indicating therefore the higher prevalence of *Salmonella* in alheira sold in these establishments (incidence 0.476; 95% CI: 0.283–0.676). Table 11 compiles the results according to the date of sampling, displaying both the qualitative and quantitative analyses. In the case of the positive enriched samples, counts in MPN/g have been calculated from the tube triplets. In the present study, and as shown in Table 11, the quantitative analysis showed that *Salmonella* was found in concentrations of <3, 3.0, 16, 35, 36, 120, 150, 210, 1100 and >1100 MPN/g, with an approximate mean concentration of 1.792 log MPN/g (s.d 0.916 log MPN/g) when present.

The prevalence of *Salmonella* in alheira (~19%) was found to be higher than the incidence reported in the literature for other kinds of sausage. For instance, Cabelo *et al*. (2008) reported a frequency of isolation of 11.1% in dry and semidry sausages. In addition, the prevalence of *Salmonella* in alheira in this microbiological survey study was much higher than that estimated by meta-analysis (10.5%; 95% CI: 4.4 – 23.0%). The level of *Salmonella* contamination in alheira is comparable to that of a fresh pork sausage, surveyed by Mürmann *et al*. (2009), where they detected 82 positive samples from a total of 336 (incidence 0.244; 95% CI: 0.2011 – 0.2926), with a MPN count ranging from 0.03 MPN/g to 460 MPN/g.

Unpacked sausages from market fairs were found to have an unacceptably high prevalence of *Salmonella* spp. This pathogen can enter the product through meats contaminated with *Salmonella*, by cross-contamination, and through a large number of interacting factors that can be linked to the inadequacy of hygiene controls at different stages of production as well as in the distribution chain to the consumer. In addition, the absence of packaging induces the product's exposure to different sources of contamination and the rapid microbial growth.

Table 11: Results of qualitative and quantitative analysis of *Salmonella* in alheiras sausage discriminated by sampling batch

Origin	**Starting day**	**Counting day**	**Samples**	**Qualitative test**			**Quantitative test**			**MPN**
				XLD	HE	BS	-1	-2	-3	
Traditional market	03/12/2018	06/12/2018	1	neg	neg	pos	0	0	0	<3.0
Traditional market	03/12/2018	06/12/2018	2	neg	neg	neg	0	0	0	0
Traditional market	03/12/2018	06/12/2018	3	pos	pos	pos	3	3	2	1100
Traditional market	03/12/2018	06/12/2018	4	neg	pos	neg	0	0	1	3.0

Origin	**Starting day**	**Counting day**	**Sample**	**Qualitative test**			**Quantitative test**			**MPN**
				XLD	HE	BS	-1	-2	-3	
Local shop	18/12/2018	22/12/2018	1	neg	neg	neg	0	0	0	0
Local shop	18/12/2018	22/12/2018	2	neg	neg	neg	0	0	0	0
Local shop	18/12/2018	22/12/2018	3	neg	neg	neg	0	0	0	0
Local shop	18/12/2018	22/12/2018	4	neg	neg	neg	0	0	0	0
Local shop	18/12/2018	22/12/2018	5	neg	neg	neg	0	0	0	0
Local shop	18/12/2018	22/12/2018	6	neg	neg	neg	0	0	0	0

Origin	Starting day	Counting day	Sample	Qualitative test			Quantitative test			MPN
				XLD	HE	BS	-1	-2	-3	
Supermarket	14/01/2019	17/01/2019	1	neg	neg	neg	0	0	0	0
Supermarket	14/01/2019	17/01/2019	2	neg	neg	neg	0	0	0	0
Supermarket	14/01/2019	17/01/2019	3	neg	neg	neg	0	0	0	0
Supermarket	14/01/2019	17/01/2019	4	neg	neg	neg	0	0	0	0
Supermarket	14/01/2019	17/01/2019	5	neg	neg	neg	0	0	0	0

Origin	Starting day	Counting day	Sample	Qualitative test			Quantitative test			MPN
				XLD	HE	BS	-1	-2	-3	
Local shop	29/01/2019	01/02/2019	1	neg	neg	neg	0	0	0	0
Local shop	29/01/2019	01/02/2019	2	neg	neg	neg	0	0	0	0
Local shop	29/01/2019	01/02/2019	3	neg	neg	neg	0	0	0	0
Local shop	29/01/2019	01/02/2019	4	neg	neg	neg	0	0	0	0
Local shop	29/01/2019	01/02/2019	5	neg	neg	neg	0	0	0	0
Local shop	29/01/2019	01/02/2019	6	neg	neg	neg	0	0	0	0

Origin	Starting day	Counting day	Sample	Qualitative test			Quantitative test			MPN
				XLD	HE	BS	-1	-2	-3	
Supermarket	06/02/2019	09/02/2019	1	neg	neg	neg	0	0	0	0
Supermarket	06/02/2019	09/02/2019	2	neg	neg	neg	0	0	0	0
Supermarket	06/02/2019	09/02/2019	3	neg	neg	neg	0	0	0	0
Supermarket	06/02/2019	09/02/2019	4	neg	neg	neg	0	0	0	0
Supermarket	06/02/2019	09/02/2019	5	neg	neg	neg	0	0	0	0

Origin	Starting day	Counting day	Sample	Qualitative test			Quantitative test			MPN
				XLD	HE	BS	-1	-2	-3	
Market fair	20/03/2019	23/03/2019	1	neg	neg	neg	0	0	0	0
Market fair	20/03/2019	23/03/2019	2	neg	neg	neg	0	0	0	0

Origin	Starting day	Counting day	Samples	Qualitative test			Quantitative test			MPN
				XLD	HE	BS	-1	-2	-3	
Traditional market	27/03/2019	30/03/2019	1	pos	pos	neg	3	2	2	210
Traditional market	27/03/2019	30/03/2019	2	pos	pos	neg	3	3	3	>1100
Traditional market	27/03/2019	30/03/2019	3	neg	neg	neg	0	0	0	0

Traditional market	27/03/2019	30/03/2019	4	neg	pos	neg	2	2	2	35
Traditional market	27/03/2019	30/03/2019	5	neg	pos	neg	1	3	0	16
Traditional market	27/03/2019	30/03/2019	6	neg	neg	neg	3	1	2	120

Origin	**Starting day**	**Counting day**	**Sample**	**Qualitative test**			**Quantitative test**			**MPN**
				XLD	HE	BS	-1	-2	-3	
Supermarket	18/06/2019	21/06/2019	1	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	2	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	3	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	4	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	5	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	6	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	7	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	8	neg	neg	neg	0	0	0	0
Supermarket	18/06/2019	21/06/2019	9	neg	neg	neg	0	0	0	0

Origin	**Starting day**	**Counting day**	**Sample**	**Qualitative test**			**Quantitative test**			**MPN**
				XLD	HE	BS	-1	-2	-3	
Market fair	24/06/2019	27/06/2019	1	neg	neg	neg	0	0	0	0
Market fair	24/06/2019	27/06/2019	2	pos	neg	neg	2	3	1	36
Market fair	24/06/2019	27/06/2019	3	neg	neg	neg	0	0	0	0

Market fair	24/06/2019	27/06/2019	4	neg	neg	neg	0	0	0	0
Market fair	24/06/2019	27/06/2019	5	pos	pos	neg	3	2	1	150
Market fair	24/06/2019	27/06/2019	6	neg	neg	neg	0	0	0	0
Market fair	24/06/2019	27/06/2019	7	neg	neg	neg	0	0	0	0
Market fair	24/06/2019	27/06/2019	8	neg	neg	neg	0	0	0	0
Market fair	24/06/2019	27/06/2019	9	neg	neg	neg	0	0	0	0

4.4 Extraction of DNA and molecular typing of Salmonella

The bacterial colonies that were confirmed as *Salmonella* spp. by biochemical tests were cultured overnight on nutrient agar for molecular confirmation. After DNA extraction, an agarose gel electrophoresis was performed to ensure presence of DNA in the isolates (Figure 13). The differences found in this agarose gel implied that each isolate had a different concentration of DNA: the higher the concentration of DNA, the intense and bigger the band appears in the gel.

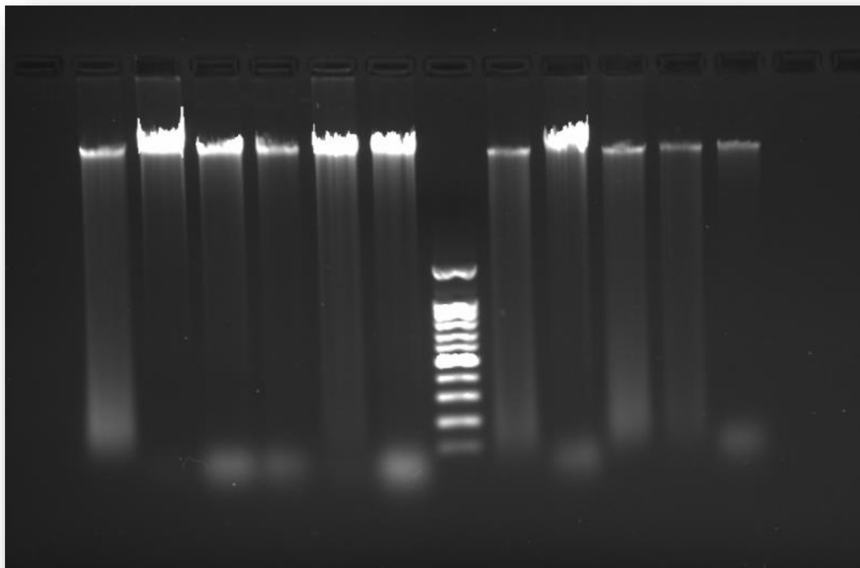


Figure 13: Agarose gel electrophoresis of DNA extracted

Salmonella spp. was detected in 10 samples (19.60 %) out of 52 analysed samples. A total of 48 phenotypically-confirmed *Salmonella* isolates from the 10 samples were investigated in this study by a PCR assay, but only 33 isolates (68.8%) were confirmed to be related to *Salmonella* spp. by the appearance of the InvA specific bands. All of these isolates showed positive bands at 284 bp (Figure 14).

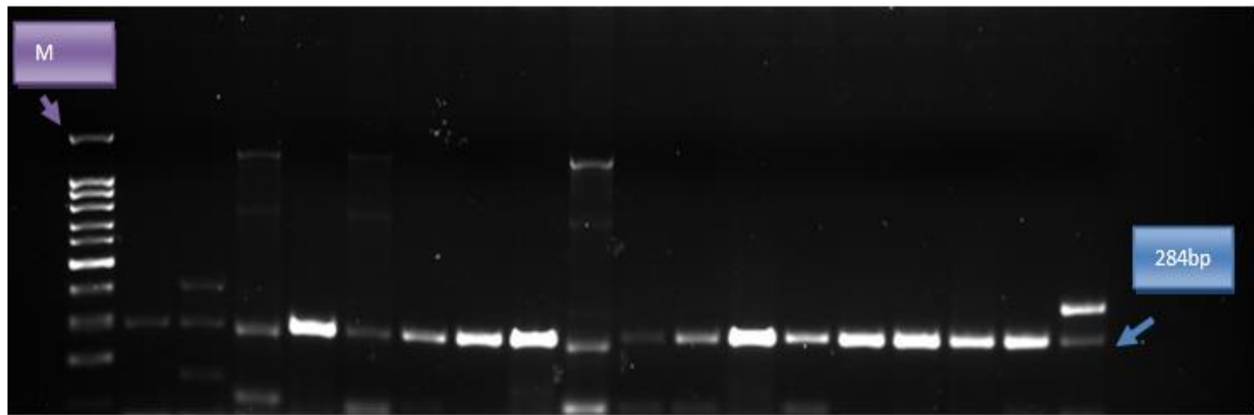


Figure 14:Agarose gel electrophoresis showing amplification of 284 bp of InvAgene of *Salmonella* spp. Lane M: 100 bp DNA ladder; Lane 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19: *Salmonella* spp

The genotype identification was done using multiplex PCR assay with simultaneous characterisation of *Salmonella* spp. using three different primers referring each one to a target gene; FliC, SefA and glgC, which code respectively for *S*. Typhimirium, *S*. Enteritidis and *S*. Pullorum. The results obtained in the agarose gel electrophoresis for the 33 *Salmonella* isolates are shown in Figures 15 and 16. Only three isolates revealed a specific band at 312 bp (SefA) for *Salmonella* Enteritidis. Twenty isolates showed a band at 559 bp related to the target gene fliC, specific to *Salmonella* Typhimirium, while the other 10 isolates had no match with any of the genes investigated. Thus, these 10 isolates must belong to serovars other than Typhimurium, Enteritidis or Pullorum. None of the isolates belonged to *Salmonella* Pullorum. The distributions of the different target genes and the combination of genes in the *Salmonella* isolates are shown, respectively, in Tables 12 and 13.

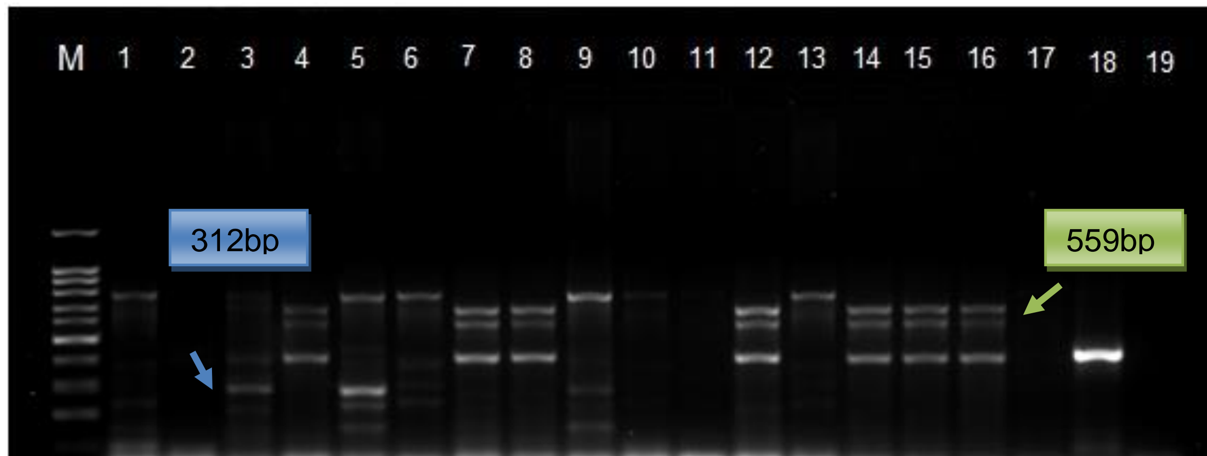


Figure 15: Multiplex-PCR assay using three sets of primers run for 19 *Salmonella* isolates. The fliC gene amplified at 559 bp specific for S. Typhimurium, the glgC gene amplified at 252 bp specific for S. Pullorum, and SefA amplified at 312 bp for S. Enteritidis. Lane M: 100 bp DNA ladder; Lanes 3,5,9: positive control for S. Enteritidis; Lanes 4,7,8,12,14,15,16 positive bands for S. Typhimirium; Lane 1,2,6,9,10,11,13,17,18,19 : other serovars of *Salmonella*.

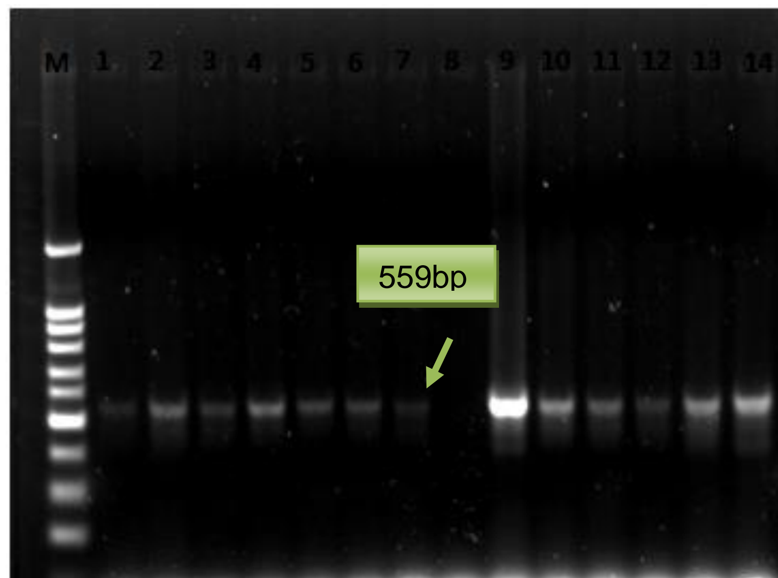


Figure 16: Multiplex-PCR assay using three sets of primers run for 14 *Salmonella* isolates. The fliC gene amplified at 559 bp specific for S. Typhimurium,, the glgC gene amplified at 252 bp specific for S. Pullorum and SefA amplified at 312-bp for S. Enteritidis. Lane M: 100 bp DNA ladder; Lanes 1,2,3,4,5,6,7,9,10,11,12,13,14: S. Typhimirium; Lane 8: another serovar of *Salmonella*

Table 8: Distribution of target genes in 33 extracted DNA samples of *Salmonella*

Number of isolates phenotypicaly confirmed	**Number of positive isolates**	**Virulence genes (n° and % of positive samples to target gene)**		
48	33	**InvA**	**SefA**	**FliC**
		33 **(100%)**	3 **(9.09%)**	20 **(60.60%)**

Table 9: Distribution of the combination of genes in 33 samples of *Salmonella*

Genes combination	**Amplification fragment**	**Number of samples**
InvA, SefA	284 bp, 312 bp	3
InvA, FliC	284 bp, 559 bp	20
InvA only	284 bp	10
Randomly cloned chromosomal	429 bp	0

According to Table 13, the most common serotype of *Salmonella* found in alheira sausage was *Salmonella* Typhimurium (n=20; 60.60%) followed by *Salmonella* Enteritidis (n=3; 9.09%). Indeed, *Salmonella* serovars Enteritidis and Typhimurium have been ranked as the most common ones involved in foodborne outbreaks (Hendriksen et al., 2011). *Salmonella* Enteritidis is commonly associated with poultry and products (eggs and poultry meat) (Hugas et al., 2014), and it considered the main vehicle of *Salmonella* infection and clearly associated with the worldwide epidemic of S. Enteritidis (Antunes et al., 2016). However, salmonellosis have been also linked to the consumption of raw or undercooked pork and pork products; and among the main serovars of porcine origin detected in confirmed human cases is S. Typhimurium (Bonardi, 2017). Thus, the results of *Salmonella* typing suggest that that pork and chicken meat, being the main raw materials of alheira, are very likely to be the sources of contamination. Contaminated pork intestine used as casings cannot be disregarded as a source of *Salmonella* in alheira.

According to a research based in Brazil (Mürmann*et al*., 2009), the most frequently isolated *Salmonella* serovars from a fresh pork sausage were Brandenburg, Panama, Derby and Typhimurium. Additionally, the presence of different serovars with quite fluctuating percentages may be related to different causes which are probably directly or indirectly related to the preparation methods for which the producers are responsible as well as to the composition of the product itself.

Nevertheless, since the abundance of those two serovars, Typhimurium and Enteritidis, is not merely in sausages but in foods in general, the contamination source is not necessarily linked to the meats, but could be also due to poor hygienic standards. Artisanal producers are responsible for enforcing hygiene practices and rules, that include not only personal hygiene but also a correct maintenance of protective equipment (clothing, hairnets, etc.) to avoid the exposure of the product to any kind of contamination (Schiffers, 2011).

V. Conclusion remarks and future perspective

Meat and meat products are the main vehicles of foodborne diseases in humans caused by pathogens such as *Salmonella* spp. This study was undertaken to confirm or otherwise disprove the fact that this microorganism continues to be a frequent contaminant of alheiras produced in Bragança. According to microbiological surveys of alheira sold in traditional fairs, supermarkets and local shops, the prevalence of *Salmonella* was high and was only found in alheiras sold unpacked and kept at ambient temperature in market fairs with a prevalence of 47.6% (95% CI: 28.3–67.6) and with an approximate mean concentration of 1.792 log MPN/g (s.d 0.916 log MPN/g). This finding implies low hygiene standards in their artisanal production process as well as their manipulation.

Furthermore, it could be inferred that alheiras traditionally prepared and sold in market fairs were produced under poor hygienic conditions because of the high levels of total coliforms comparing with the vacuum-packed alheiras sold in supermarkets. Unpacked alheiras also presented higher counts of *Pseudomonas* and psychrotrophic bacteria than the vacuum-packed alheiras, which suggest that the former will eventually have a shorter shelf-life, and, more importantly, that both hurdles, vacuum-packaging and cold storage, are key in delaying microbial degradation in this product. The serovars Typhimurium and Enteritidis represented ~70% of the *Salmonella* isolates from alheiras, which suggest that *Salmonella* may be entering the production process through pork meat, poultry meat, and pig intestine casings.

As a consequence, and considering the local or artisanal producers as responsible for this food quality leak, many safety rules must be urgently enforced, such as the implementation of good manufacturing practices, and good hygiene standards comprising not only hand hygiene but also the use of personal protective clothing and equipment/surfaces cleaning and disinfection plans. In addition to the food safety programmes and controls, artisanal producers must be advised to vacuum-pack their products and to implement an adequate management of the cold chain until the product reaches the consumer. This also entails that fair vendors must commercialise these products under refrigeration. Thus, regional producers must be informed and educated in order to introduce preventive and corrective actions in their current production processes; whereas consumers must be also advised to thoroughly cook alheira before consumption.

VI. References

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