Evaluation of the Resistance of *Salmonella enterica* subsp. *enterica* Isolated from Pigs to Biocides Used in the Agri-food Industry

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Declaration

I declare that this document is an original work of my authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Department of Sanitary Inspection of the Faculty of Veterinary Medicine, University of Lisbon (Lisbon, Portugal), during the period October 2021 – July 2022, under the supervision of Professor João de Bettencourt Barcelos Cota, and within the frame of the Centre for Interdisciplinary Research in Animal Health (CIISA). The thesis was co-supervised at Instituto Superior Técnico by Professor Ana Cristina Anjinho Madeira Viegas.

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Abstract

Combating the persistence of *Salmonella* in the abattoir's environment has become crucial through the implementation of cleaning and disinfection programmes, especially as their susceptibility to biocides can tend to decrease.

The present study aimed to evaluate the resistance of *Salmonella enterica* isolates from pigs slaughtered in abattoirs to biocides used in the agri-food industry.

Forty-four *Salmonella* isolates from slaughtered pigs were used to detect the presence of both efflux pump and quaternary ammonium compound (QAC) biocide resistance genes by PCR. Susceptibility to the biocides Suma Bac D10® (QAC-based formulation) and Mida FOAM 193® (chlorine-based formulation) at three levels of organic matter (absent, low and high) was assessed in twelve selected isolates.

At all levels of organic matter, the susceptibility of the isolates to Suma Bac D10® remained 10x (0.1%) lower than the concentration indicated by the manufacturer (1-4%), in terms of minimum bactericidal and inhibitory concentrations. The presence of resistance genes to QACs did not seem to induce any changes in the susceptibility of the isolates to biocide at the studied formulation concentrations. As for Mida FOAM 193®, decreased susceptibility only occurred with the presence of organic matter at high levels and for biocide concentrations <2.5%, still 4x below the concentration recommended by the manufacturer (10%). Efflux pump genes were detected in all isolates, so the decrease in susceptibility observed for this biocide in the studied conditions could be linked to the barrier effect of organic matter.

In conclusion, the findings obtained revealed the importance of using biocides at concentrations that effectively eliminate *Salmonella* spp. from contaminated surfaces, including the abattoir environment, since their misuse could potentially lead to the persistence of this bacterium.

Keywords

Salmonella enterica subsp. *enterica*; Chlorine-based biocide; QAC-based biocide; Resistance genes; Minimum Inhibitory Concentration; Minimum Bactericidal Concentration.

Resumo

O combate à persistência de *Salmonella* em matadouro é fundamental para combater a disseminação deste agente de toxinfeção, recorrendo, em parte, a programas de lavagem e desinfeção, tendo em conta que a sua suscetibilidade a biocidas poderá ter tendência para diminuir.

O objetivo deste estudo foi a avaliação da resistência em isolados de *Salmonella enterica* de porcos abatidos em matadouro a biocidas utilizados na indústria agro-alimentar.

Utilizou-se quarenta e quatro isolados de *Salmonella* de porcos abatidos em matadouro para detetar por PCR genes associados a resistência a biocidas, tanto codificando bombas de efluxo como relacionados com a resistência a compostos de amónio quaternário (CAQ), tendo sido ainda avaliada a suscetibilidade aos biocidas Suma Bac D10® (formulação baseada em CAQ) e Mida FOAM 193® (formulação à base de cloro) na presença de três níveis de matéria orgânica (ausente, baixa e elevada) em doze isolados selecionados.

Em todos os níveis de matéria orgânica, a suscetibilidade dos isolados ao Suma Bac D10® manteve-se 10x mais baixa (0.1%) que a concentração indicada pelo fabricante (1-4%), em termos de concentrações mínimas bactericida e inibitória, sendo que presença dos genes associados a resistências a CAQs não pareceu induzir alteração da suscetibilidade face ao biocida nas concentrações estudadas.

Quanto ao Mida FOAM 193®, ocorreu diminuição da suscetibilidade na presença de matéria orgânica em nível elevado para concentrações <2.5%, ainda 4x abaixo da concentração recomendada pelo fabricante (10%). Os genes de bombas de efluxo foram detetados em todos os isolados, pelo que a diminuição da suscetibilidade nas condições do presente estudo poderá advir da combinação do efeito barreira da matéria orgânica.

Assim, os resultados obtidos revelam a importância da utilização de biocidas em concentrações que efetivamente eliminam *Salmonella* spp. de superfícies contaminadas, incluindo do ambiente de matadouro, pois a sua utilização incorreta poderá levar à persistência desta bactéria.

Palavras-Chave

Salmonella enterica subsp. *enterica*; Biocida derivado de cloro; Biocida composto por QAC; Genes de resistência; Concentração mínima inibitória; Concentração mínima bactericida.

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List of Abbreviations

µL	Microlitre
ABC	ATP-Binding Cassette
ADBAC	Alkyl dimethyl benzyl ammonium chloride
a_w	Water Activity
CAQ	Composto de Amónio Quaternário
CBB	Chlorine-based biocide
CFU	Colony Forming Units
CO_2	Carbon dioxide
COVID-19	Coronavirus Disease
DDAC	Didecyl dimethyl ammonium chloride
DMT	Drug//Metabolite Transporter
DNA	Deoxyribonucleic Acid
EC	European Commission
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organisation
GHP	Good Hygiene Practice
HACCP	Hazard Analysis and Critical Control Point
HIS	High-level interfering substance
LIS	Low-level interfering substance
LPS	Lipopolysaccharides
MATE	Multidrug and Toxic Compound Extrusion
MBC	Minimum Bactericidal Concentration
MDR	Multidrug resistance
MFS	Major Facilitator Superfamily
MGE	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
min	Minutes
NaCl	Sodium chloride
ng	Nanograms
NIS	No interfering substance
NTS	Non-typhoidal *Salmonella*
OECD	Organisation for Economic Co-Operation and Development
PCR	Polymerase Chain Reaction
PHC	Process Hygiene Criterion

PT03	Product used in veterinary hygiene
PT04	Product used on surfaces in contact with foodstuffs and feeding stuffs
QAC	Quaternary Ammonium Compound
RND	Resistance-Nodulation-Division
rpm	Rotations per minute
s	Seconds
SCV	*Salmonella*-Containing Vacuole
Ser.	Serotype or Serovar
SMR	Small Multidrug Resistance
SPI	*Salmonella* Pathogenicity Island
T3SS	Type III Secretion System
TSA	Tryptone Soy Agar
TSB	Tryptic Soy Broth

List of Software

Primer3Plus software	Primer3Plus picks primers from a DNA sequence (https://www.primer3plus.com/)

Chapter 1: Introduction

1.1. Introduction

Salmonella spp. is a gram-negative rod-shaped bacterium belonging to the family *Enterobacteriaceae* (Olubisose *et al*., 2021), containing only two species, *Salmonella enterica* and *Salmonella bongori* (Lamas *et al*., 2018), the most prominent being *Salmonella enterica* with over 2600 different serotypes identified (Silva *et al*., 2014).

S. enterica was divided into six different subspecies, which were associated with a Roman numeral, namely *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). The Roman numeral V is associated with *S. bongori* (Lamas *et al*., 2018) and is not relevant to the present study.

Most *Salmonella* serotypes are able to infect a wide range of hosts, including a variety of wild, farm and even pet animals, and can therefore be transmitted through a wide variety of foods that may have been in contact with faeces, the most common of which would be eggs, pork, beef, poultry meat, and vegetables (Silva *et al*., 2014).

Salmonella enterica is responsible for several infections, the most relevant being salmonellosis derived from non-typhoidal *Salmonella* (NTS), so it became a global public health problem. Human salmonellosis caused by NTS is characterised as self-limiting gastroenteritis with the main symptoms being diarrhoea, fever, abdominal pain, and vomiting (Campos *et al*., 2019). In some risk groups, such as children, young people, the elderly and the immunocompromised, it may be observed complications of the clinical condition that could be potentially fatal, through possible endocarditis and bacteraemia (Acheson & Hohmann, 2001; Campos *et al*., 2019).

Salmonellosis was one of the most frequent gastrointestinal diseases, with an incidence of 20 cases per 100 000 people in the European Union (EU) in 2019, being one of the major foodborne illnesses in the EU. In the European context, one of the main food vehicles associated with salmonellosis food outbreaks is "pork and products thereof", accounting for 12 % of all *Salmonella* serotypes isolated in the various member states (EFSA, 2021).

In the Portuguese context, the monophasic variant of *S.* Typhimurium (4,[5],12:i:-) became predominant (47.7%), followed by *S.* Rissen (40.9%) and *S.* Derby (11.4%) (Cota *et al*., 2019). These results were in line with previous studies by Caleja *et al*. (2011) and Gomes-Neves *et al*. (2012), in which a higher incidence of the monophasic variant of S. Typhimurium, namely 4,[5],12:i:-, but also of *S.* Rissen and *S*. Derby, were found both in samples collected from pigs and in samples obtained from the environment of several slaughterhouses.

Pigs can be asymptomatic carriers of *Salmonella* spp., however, when exposed to various stresses, as happens when the animals are transported and remain in the lairage, they can become active shedders (Mannion *et al*., 2012). Therefore, as soon as there are pigs infected with *Salmonella* spp. in the slaughterhouse, all of the pig carcasses can be at risk of becoming contaminated by this pathogen

(Berends *et al*., 1997; Mannion *et al*., 2012). Thus, there seems to be a correlation between the number of pigs carrying *Salmonella* spp. in the abattoir, namely in their faeces, and the number of contaminated carcasses at the end of the slaughter line (Berends *et al*., 1997).

In the abattoir, this is problematic because as more animals are together, the more difficult it is to prevent infection by *Salmonella* spp. (Berends *et al*., 1996). This type of situation could be avoided if the interventions carried out to prevent the contamination, persistence and spread of *Salmonella* spp. in the slaughterhouse started to be implemented previously in the farms where the pigs come from, through the use of non-contaminated feed, quarantine of purchased animals and veterinary monitoring (Campos *et al*., 2019). In addition, slaughterhouses should be able to guarantee good hygiene practices (GHPs) and the existence and application of hazard analysis and critical control point (HACCP) programmes (EFSA, 2021).

Additionally, the fact that slaughterhouses used biocides could lead to the development of bacterial resistance and their persistence in the environment. Furthermore, the inappropriate use of biocides could be challenging as the perpetuation of QACs and chlorine-based biocides (CBB) (Poole, 2002) and their slow degradation in the environment could lead to an advantage in the adaptive response of pathogens, namely the genus *Salmonella* (Kampf, 2018).

Accordingly, resistance to biocides could occur due to increased efflux pump activity in the plasma membrane and structural changes in cell wall permeability (Poole, 2002). Regarding *Salmonella* species and in other *Enterobacteriaceae*, one of the most important and ultimately best-studied systems is the AcrAB-TolC complex, since the expression of *acrA*, *acrB* and *tolC* genes ultimately leaded to biocide exit and contributed to multidrug resistance (MDR) (Weston *et al*., 2018). As for resistance to QACs, several resistance genes have been identified in gram-negative bacteria, like *qacE*, *qacEΔ1*, *qacF*, *qacG* and *sugE* genes (Zou *et al*., 2014).

In the present study, the goal was to investigate the resistance of *Salmonella* isolates from pigs slaughtered in a Portuguese abattoir to commercial biocides.

This research involved assessing the susceptibility of *Salmonella* isolates, previously gathered, and studied in terms of pheno- and genotypic characteristics, to two biocides commonly used in the agri-food industry, such as Mida FOAM 193®, which is a CBB, and Suma Bac D10®, which is derived from QAC, to assess the presence of biocide associated resistance genes, namely *acrA*, *acrB*, *tolC*, *qacE*, *qacEΔ1*, *qacF*, *qacH* and *qacI,* by molecular biology methods and to evaluate the resistance and/or susceptibility to the aforementioned biocides determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). Finally, the data obtained was compared with the results obtained in Cota *et al*. (2019) research, in terms of phenotypic and genotypic correlations, deepening the knowledge of how *Salmonella* was found in pigs and pork in a Portuguese slaughterhouse.

Chapter 2: Literature Review

2.1. *Salmonella* spp.

Salmonella spp. is a species of motile, gram-negative, spore-forming, capsule-less, oxygen-consuming, rod-shaping bacillus to facultative anaerobic microscopic organisms, belonging to the family *Enterobacteriaceae* (Doyle *et al*., 2020; Monte & Sellera, 2020; Neish, 2004; Olubisose *et al*., 2021; Popoff & Le Minor, 2015).

In 1880, Karl Joseph Eberth was quick to notice *Salmonella* from samples of patients with typhoid fever, which was once called *Eberthella typhosa* in his tribute. Georg Gaffky effectively isolated this bacillus from patients with typhoid fever, affirming Eberth's discoveries, in 1884. A while later, Salmon, a veterinary pathologist, and his bacteriologist associate Theobald Smith propagated *Salmonella* serovar (ser.) Choleraesuis from pigs, erroneously expecting that this microorganism was the causative agent of hog cholera. Later, Joseph Lignières, a French bacteriologist, proposed the class name *Salmonella* in acknowledgement of Salmon's endeavours (Monte & Sellera, 2020).

Even before there was a consensus on the name for this microorganism, a clinician called William Bud, in 1856, developed a system to prevent typhoid fever from spreading, which involved boiling contaminated linen in water, disinfecting the spills of patients, and making his assistants wash their hands regularly (Canadian Medical Association, 1978).

Salmonella and one of its most commonly associated diseases, typhoid fever, became better known with the case of Mary Mallon. She worked for the Warren family on Long Island (New York, USA) where, three weeks into her employment as a cook, four family members and seven employees began experiencing fever symptoms associated with typhoid, although Mary showed no signs of the disease, thus becoming the first known carrier of *Salmonella* Typhi, being referred to as an asymptomatic carrier. After being detained in the William Parker Hospital, she was allowed to leave as long as she did not return to work as a cook. However, she refused the request and was confined there, spending the rest of her life in the hospital. Mallon died in 1938, after 26 years of strict isolation and having transmitted the disease to 51 people, in which three of whom died. Mary Mallon would always be known as “Typhoid Mary” (Brooks, 1996).

Since then, considerable progress has been made in terms of knowledge of the genus *Salmonella*, namely its main characteristics, species and subspecies discovered and serotypes identified, where it is found and how the pathogenicity and resistance mechanisms are identified.

2.1.1. General Characteristics

As far as *Salmonella* species are concerned, these are gram-negative and bacillus-shaped, with dimensions that can range from 0.7-1.50 µm wide by 2.0-5.0 µm long, and colonies that can be 2-4 mm in diameter (Figure 1). These are normally motile since they have a peritrichous flagellum (Popoff & Le Minor, 2015). This genus has a mesophilic growth temperature of 37 ºC, producing hydrogen sulphide

(H_2S), but cannot hydrolyse urea (Doyle *et al*., 2020). Chemically, some common features are that *Salmonella* spp. can reduce nitrate to nitrite, produce gas from D-glucose, use citrate as a carbon source, and not produce lipase or deoxyribonuclease. *Salmonella* spp. are also indole and urease negative, positive for lysine and ornithine decarboxylase and cannot ferment sucrose, salicin, inositol and amygdalin (Popoff & Le Minor, 2015). *Salmonella* has a genome size of 4.8 Mb, which is ultimately affected by a panoply of extrinsic factors, namely plasmids, lysogenic phage genomes and some mobile genetic elements (MGE) (Neish, 2004). Finally, they have a Deoxyribonucleic Acid (DNA) mol% G+C content that may vary between 50 and 53 (Popoff & Le Minor, 2015).

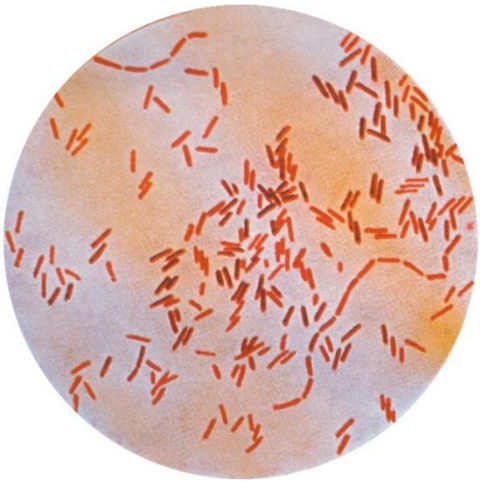

Figure 1. Picture of *Salmonella* Typhi using the Gram stain technique (source: CDC, https://phil.cdc.gov/Details.aspx?pid=2114).

The currently accepted type species for the genus *Salmonella* is *Salmonella enterica* Le Minor and Popoff 1987 (Tindall *et al*., 2005). Since the genus *Salmonella* is closely related to the genera *Yersinia* and *Shigella* and the species *Escherichia coli* (*E. coli*), it was established that it would belong to the family *Enterobacteriaceae* (Neish, 2004).

Despite these more general characteristics that concern most salmonellae, there could be some exceptions in a genus with so many serovars, such as *Salmonella* ser. Gallinarum and *Salmonella* ser. Pullorum which does not have motility or *Salmonella* ser. Typhi not being able to produce gas, even though most of them are aerobic (Popoff & Le Minor, 2015). Whereas this same *Salmonella* ser. Typhi also presents as an exception since it has a capsule unlike the other *Salmonella*e (Neish, 2004).

There are many more differences between specimens of this genus in terms of biochemical characteristics. For example, some strains belonging to the serovar Paratyphi A do not produce hydrogen sulphide. It does not use citrate as a carbon source and are negative for the lysine decarboxylase reaction; *Salmonella* ser. Typhi also does not use citrate as a carbon source and is negative for the ornithine decarboxylase reaction (Popoff & Le Minor, 2015).

Several factors can cause *Salmonella* spp. to adapt to survive, namely adapting to various temperatures, pH, water activity (aw), and extrinsic growth factors, namely salt concentration (Doyle *et al*., 2020).

Concerning pH, *Salmonella* species proliferate in a very wide range of values, varying from 3.9 to 9.5, but it is considered that their optimum growth occurs in pHs varying between 6.5-7.5. As for the salt concentration, *Salmonella* species are inhibited when in the presence of 3 to 4% NaCl; still, as would be expected from such a large group, salt tolerance has already been shown to be influenced by increasing temperatures, being capable to grow in the presence of 6% NaCl at 30 °C (Doyle *et al*., 2020).

As for water activity (a_w), which refers to the ratio between the equilibrium vapour pressure of a foodstuff to the saturated water vapour pressure under the same conditions (FDA, 2018; Sandulachi & Tatarov, 2012), it directly affects the ability of this bacterial genus to developed a capacity to resist dehydration, although the mechanism from which it does so was not well explained. Normally, when the a_w is low, there is an inhibition of the growth of *Salmonella*e; however, it has been found that it can survive on surfaces that can varied from 11 % to 97 % in terms of relative humidity, increasing its ability to survive desiccation. In addition, it can survive a variety of stresses associated with low a_w, as well as a wide range of temperatures and pH, as mentioned above, making the elimination of *Salmonella* from the environment, especially those associated with food processing, a challenge (Doyle *et al*., 2020).

As for their distribution, apart from being able to survive on surfaces (Doyle *et al*., 2020), *Salmonella* spp. can be ubiquitous and survive in several environments (e.g., *S. enterica* ser. Typhimurium) under different conditions, but can also be exclusive to one region (e.g., *S. enterica* ser. Sendai), being isolated only in the same place (Popoff & Le Minor, 2015). It should also be considered that the distribution of specimens belonging to the genus *Salmonella* does not only take into consideration its geographical distribution, but also which hosts it could populate, so it is frequently isolated from mammals, but also other animals, such as amphibians, fish, birds and cold-blooded animals, besides the fact that it can still survive in plants, water and soil (Popoff & Le Minor, 2015; Silva *et al*., 2014).

2.1.2. Taxonomy of the genus *Salmonella*

Salmonella spp. is a rod-shaped gram-negative bacterium of the *Enterobacteriaceae* family (Olubisose *et al*., 2021). *Salmonella enterica* and *Salmonella bongori* are members of this genus, with *S. enterica* being the most important, with over 2500 distinct serotypes documented (Lamas *et al*., 2018).

Salmonella enterica was divided into six subspecies, each designated with a Roman numeral, namely I for *enterica*, II for *salamae*, IIIa for *arizonae*, IIIb for *diarizonae*, IV for *houtenae*, and VI for *indica*. The V is connected with *S. bongori* (Lamas *et al*., 2018) but it is unrelated to the current investigation.

Earlier, it was made a reference to serotypes or serovars. These were ultimately defined as groups belonging to species of microorganisms with different surface structures (CDC, 2020).

As far as the genus *Salmonella* is concerned, it is composed of more than 2500 different serotypes (Lamas *et al*., 2018), most of which belong to *S. enterica* subsp. *enterica* with more than 1500 serotypes, while *S*. bongori has only 22 serotypes representing its species (Lamas *et al*., 2018). More specifically, *S. enterica* subsp. *enterica* (subspecies I) is associated with warm-blooded animals, mainly mammals,

and about 99 % of infections occurred in humans and other mammals; as for *S. bongori* (subspecies V), it is rarer to occur in humans and other mammals, and its incidence is higher in cold-blooded animals and was widespread in the environment (Jajere, 2019). An approximate breakdown of the number of serotypes and usual habitat was taken from Lamas *et al*. (2018) and Brenner *et al*. (2000), which is shown in Table 1.

Table 1. The number of serotypes concerning the species and subspecies of *Salmonella* (adapted from Lamas *et al*. (2018) and Brenner *et al*. (2000)).

Species/subspecies	Number	Serotypes	Usual habitat
S. enterica subsp*. enterica*	I	1531	Warm-blooded animals
S. enterica subsp*. salamae*	II	505	Cold-blooded animals and the environment
S. enterica subsp*. arizonae*	IIIa	99	Cold-blooded animals and the environment
S. enterica subsp*. diarizonae*	IIIb	336	Cold-blooded animals and the environment
S. enterica subsp*. houtenae*	IV	73	Cold-blooded animals and the environment
S. bongori	V	22	Cold-blooded animals and the environment
S. enterica subsp*. indica*	VI	13	Cold-blooded animals and the environment

The numbering of *Salmonella* serotypes is based on the identification of three antigenic determinants that it included, the O, H, and K antigens, with the O and H antigens being the most frequent among *Salmonella* spp. (Jajere, 2019).

The system has undergone some changes to reach a consensus until arriving at the current scheme to characterise *Salmonella* serovars. There have been various scientists who, over time, have evolved the characterisation scheme, from 1926 with Philip Bruce White, in 1934 with Fritz Kauffman until 2007 with Patrick Grimont and François-Xavier Weill, with the contribution of Le Minor and Popoff (Grimont & Weill, 2007; Hardy, 2004).

Initially, the Kauffmann-White scheme was able to list 44 *Salmonella*e serovars, and by the time Kauffmann (1934-1965) retired the list had 958 serotypes. By the time, Le Minor (1965-1989) began contributing to the Pasteur Institute until his retirement, 2267 serotypes had been identified. There were already 2555 *Salmonella* serotypes when Popoff (1989-2003) was no longer with the Pasteur Institute (Grimont & Weill, 2007).

The serotypes followed the Kauffmann-White scheme, in which the somatic, flagellar and capsular antigens of *Salmonella* spp. are named according to their letters representing each one and numbers (Popoff & Le Minor, 2015). Currently, the Kauffmann-White scheme is called White-Kauffmann-Le Minor (Grimont & Weill, 2007) and the Pasteur Institute holds an up-to-date list of *Salmonella* serotypes (Brenner *et al*., 2000).

The O antigen, which is one of the most common, is called somatic and is found in the outermost part of the outer membrane of the cell wall of these bacteria, i.e., on the outside of the lipopolysaccharides (LPS), forming the oligosaccharide components and conferring stability to warmth (CDC, 2020; Jajere, 2019). At first, specimens belonging to this antigen group were designated by letters, however, as there

were not enough letters, the letters were associated with numbers from 51 to 67. Nowadays, the letter O is kept together with associated numbers, and if it is necessary to add more letters to characterize the specimen, this is done with the letter in parenthesis (Grimont & Weill, 2007).

The second most common concerns the H or flagellar antigen which is found on the end part of the flagella and is involved in triggering the host immune response (CDC, 2020; Jajere, 2019). Bacteria, such as *Salmonella*e, containing this antigen have two phases. In phase I, the H antigens lead to an immune identity that can be expressed by the serotypes that possess them; in phase II, they eventually lose their specificity, so the antigens can be found in other serovars (Jajere, 2019).

Finally, there is also the K or capsular antigen. These are the rarest in *Salmonella* serovars as they only exist in serotypes containing capsules since it only exists there. Furthermore, this type of antigen has a subtype characteristic in *Salmonella* serovars. The Vi or virulence antigen is only found in serotypes such as Dublin, Paratyphi C and Typhi (Jajere, 2019).

As for the names given to the serotypes, these have evolved, mainly according to the situation in which they were founded, so they can be named according to the syndrome it causes (e.g., *S.* Typhi), its relationship (e.g., *S.* Paratyphi A, B and C), its hosts specificity (e.g., *S.* Abortusovis) or the geographical location of its discovery (e.g., *S.* London), and these criteria only apply to serovars of *S. enterica* subsp. *enterica*. In the case of serotypes from other subspecies of *S. enterica* or the species *S. bongori*, the rules of the White-Kauffmann-Le Minor scheme apply (Grimont & Weill, 2007).

2.1.3. Pathogenesis of *Salmonella* spp.

Most *Salmonella* serovars can infect a wide variety of hosts, some of which are wild, farm or pet animals, and are transmissible to humans through a wide variety of food products that may have been in contact, directly or indirectly, with faeces, such as eggs, pork, beef, poultry and vegetables (EFSA, 2021; Silva *et al*., 2014). Consequently, *Salmonella* spp. can be referred to as generalist, restricted or host-adapted, depending on the hosts they infect (Silva *et al*., 2014).

Generalist serovars can infect different organisms, for example, *S.* Enteritidis and *S.* Typhimurium (Bonardi, 2017; Silva *et al*., 2014). Generalist serotypes tend to have greater genetic variability, culminating in achieving a greater diversity of results and possibility in clinical terms for the hosts (Silva *et al*., 2014).

As for host-adapted *Salmonella*e, these usually have a specific host but can cause disease in others, as seen in serotypes such as *S.* Choleraesius and *S.* Dublin (Bonardi, 2017; Silva *et al*., 2014).

Finally, host-restricted *Salmonella* serovars are those that are only associated with only one organism in particular, such as the serovars *S.* Typhi and *S.* Paratyphi. This type of specificity leads to a compromise in their distribution, since, at the expense of a greater capacity to grow their population,

specimens of this type have a smaller population, but eventually survive in an environment that shields them from threats (Silva *et al*., 2014).

Despite the above description of the types of interactions that can occur between the serovars of *Salmonella* and associated hosts, it is also relevant to understand their pathogenicity.

This ability is mainly associated with islands of pathogenicity in *Salmonella* (SPIs). SPIs are large fragments of DNA that include pathogenicity determinants (Ritter *et al*., 1995). Also, SPIs are unstable chromosome segments and are only found in pathogenic organisms (Ochman & Groisman, 1996), which have some genes that encode pathogenicity factors (Ritter *et al*., 1995). These are related to microorganisms like *Salmonella* spp. that can colonise their hosts and cause bacterial infections (Lamas *et al*., 2018). Besides that, these reveal a strain-specific phenotype of *Salmonella*, which only becomes apparent when the infection is already occurring (Marcus *et al*., 2000). These SPIs allow *Salmonella* spp. to express the ability to invade cells, notably host epithelial cells, but also to cause widespread infections and to escape the assault of the immune system (Ochman & Groisman, 1996), being critical for bacterial survival within the host organism (Kombade & Kaur, 2021).

The pathogenicity islands enable to arrange species to their phylogenetic relationship, namely *Escherichia coli* and *Salmonella* spp. because, despite having the same common ancestor, these diverge from each other as can be perceived from the presence of the pathogenicity island; *Salmonella* species have the SPI-1 but *E. coli* does not (Bäumler *et al*., 1998; Lamas *et al*., 2018; Li *et al*., 1995). Furthermore, SPIs also allowed the phylogenetic relationship between the *Salmonella* species to be established. It was possible to verify that *S. bongori* is between *E. coli* and *S. enterica* in evolutionary terms (Lamas *et al*., 2018), as this last one in addition to having SPI-1, also contains the SPI-2, whereas *S. bongori* only has the SPI-1 (Lamas *et al*., 2018; Ochman & Groisman, 1996).

To the present day, 17 SPIs are known and described (Kombade & Kaur, 2021), and for the current work, only two of them will be focused on, SPI-1 and SPI-2. These two islands of pathogenicity are important because, in the case of SPI-1, it allows *Salmonella* strains to enter the epithelial cells of hosts and, in the case of SPI-2 it allows its survival within macrophages (Bäumler *et al*., 1998; Lamas *et al*., 2018; Ochman & Groisman, 1996).

As for SPI-1, it has a G+C content of 52%, which is lower than the *Salmonella* genome (Hensel, 2004), and it encodes a secretion system necessary for the translocation of effector proteins into the eukaryotic cells of the host, which was called the type III secretion system (T3SS) and mediates the invasion of non-phagocytic cells by this pathogen, but also acts at the level of inflammation in the intestinal epithelium, and as a result causes diarrhoea to the hosts (Hensel, 2004; Johnson et al., 2018; Kombade & Kaur, 2021).

As for SPI-2, it is composed of two different elements, where the larger element (25 kb) is only found in *Salmonella enterica*, with a G+C content of 43 % and is ultimately responsible for systemic pathogenesis, while the other smaller element (15 kb), with a G+C content of 54 %, is found in both

Salmonella species and it is only involved in anaerobic respiration (Hensel, 2004). The other T3SS is found in this SPI and it is essential for the survival of this pathogen within the hosts macrophages (Hensel, 2004; Johnson et al., 2018; Kombade & Kaur, 2021), through the *Salmonella*-containing vacuole (SCV) that enables its survival and replication within the cell (Kombade & Kaur, 2021).

Both SPIs are implicated in the occurrence of diseases in humans, such as salmonellosis (Lamas *et al*., 2018), so it is important to briefly describe how this is accomplished, as can be seen in Figure 2.

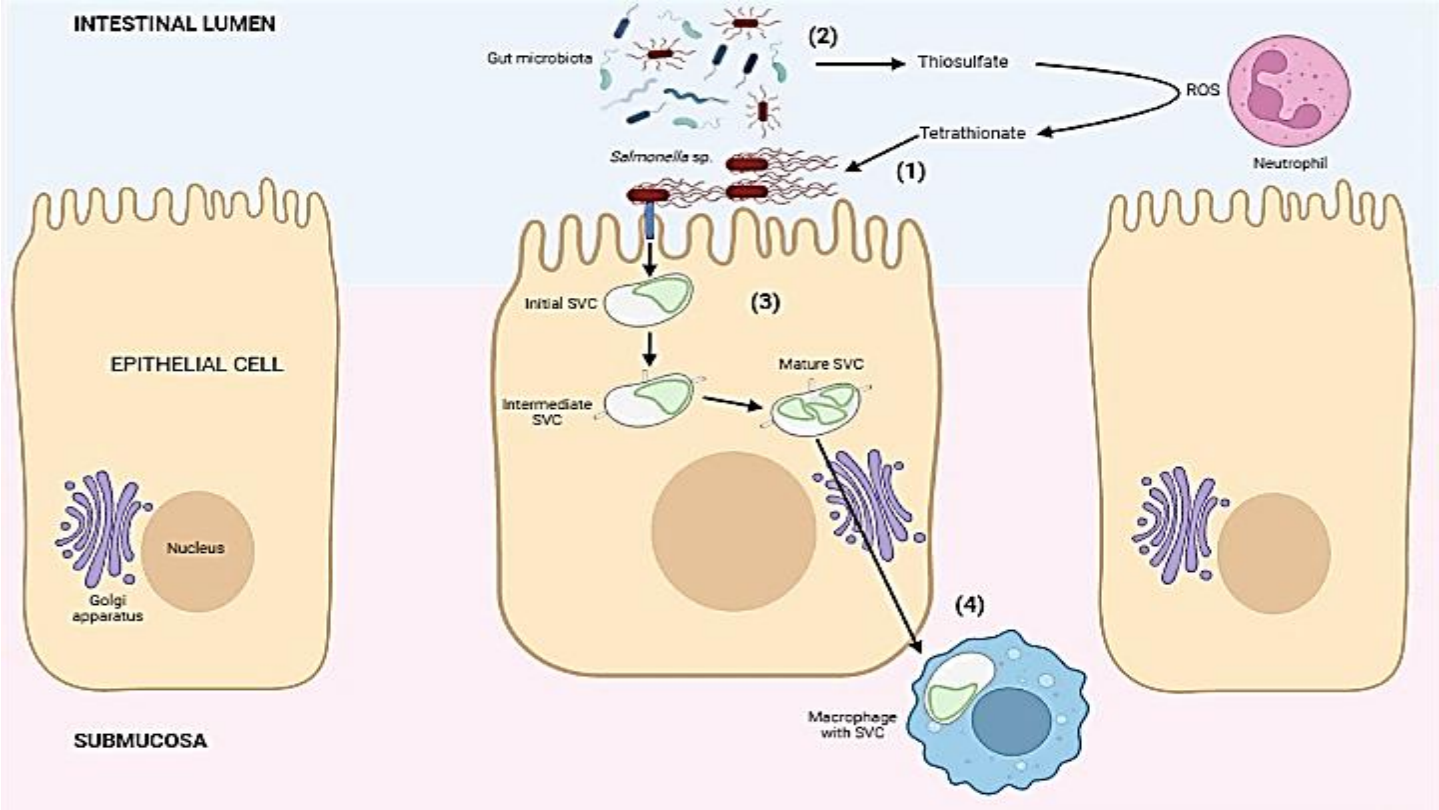

Figure 2. Human pathogenicity model of a *Salmonella* sp. (adapted from Lamas *et al*. (2018) and created with BioRender.com).
Legend: (1) Attachment and invasion (SPI-1 T3SS); (2) *Salmonella* competition with gut microbiota; (3) Intracellular invasion (SPI-2 T3SS); (4) Systemic dissemination of *Salmonella*.

The *Salmonella* microorganism, which tolerates the acidic pH environment of the stomach, reaches the intestine, passes through the mucous layer of the intestine, and attaches to the intestinal epithelium by adhesins. Upon attachment, *Salmonella* expresses the multiprotein complex T3SS at SPI-1 and the pathogenic agent is uptaken by epithelial cells. As a protective reaction, the appendix mucosa converts H_2S produced by the intestinal microbiota into thiosulfates. During the invasion of *Salmonella*, neutrophils are released into the intestinal lumen, converting thiosulfate to tetrathionate. In the cytoplasm, *Salmonella* is present in the SCV and expresses the second T3SS in SPI-2, which is essential for survival and replication in the host cells. Mature SCVs move near to the Golgi apparatus and *Salmonella* replicate. When *Salmonella* passes through the epithelium, it is phagocyted by macrophages, survives in a reaction similar to epithelial cells, and replicates in SCV, in which phagocyte

migration promotes its diffusion within the host through blood circulation (Lamas *et al*., 2018; Marcus *et al*., 2000).

2.1.4. Salmonellosis

Salmonella enterica is responsible for a variety of illnesses, with one of the most common being salmonellosis caused by non-typhoidal *Salmonella* (NTS), which has become a global public health issue (Campos *et al*., 2019).

Given the impact of *Salmonella* spp. and non-typhoidal salmonellosis, it was important to have an approach that coordinated its resolution and combat in various areas, so the One Health paradigm is the most suitable (Silva *et al*., 2014). The One Health approach aims at the integration and union of human and veterinary health, as well as the environment, in order to anticipate and address global health challenges (World Health Organization, 2022).

The understanding and knowledge about a disease, such as non-typhoidal salmonellosis, as well as its pathogenic agent and how it would be found in various environments, such as one as specific as a slaughterhouse, ultimately contributes to the impact of this public health approach.

Human salmonellosis caused by NTS is characterised as self-limiting gastroenteritis with the main symptoms being diarrhoea, fever, abdominal pain, and vomiting (Campos *et al*., 2019), whereby all these symptoms eventually emerge within 8-72 hours after contact has occurred with *Salmonella*e (Doyle *et al*., 2020). Since these symptoms most commonly disappear on their own after 5 days, NTS is considered to be a self-limiting and self-resolving disease, so only fluids and electrolytes are recommended as treatment, instead of antibiotics, as would be indicated for typhoid fever, then in this situation it would only prolong the continuity of the *Salmonella* in the host (Doyle *et al*., 2020).

In some risk groups, such as children, the elderly and the immunocompromised, complications of the clinical condition may be observed which could potentially be lethal, due to endocarditis and bacteraemia (Acheson & Hohmann, 2001; Campos *et al*., 2019; Marus *et al*., 2019).

As shown in Table 1, NTS belonging to *S. enterica* subsp. *enterica* have as their main hosts warm-blooded animals and inhabit their gastrointestinal tracts, especially meat-producing animals (Brenner *et al*., 2000; Campos *et al*., 2019; Lamas *et al*., 2018). The faeces of these animals could contaminate food products directly or indirectly, or through irrigation systems, where its ingestion can lead to the contamination of the host with NTS (Campos *et al*., 2019; Lamas *et al*., 2018).

Although it could seem simple how food becomes contaminated by *Salmonella enterica* and how this pathogen reached the human host, the reality is that this is a complex network of interactions and varied sources, as can be seen in Figure 3. Faeces from farm animals such as pigs and chickens, as well as faeces from wild animals such as foxes among others, can contaminate field irrigation water and horticultural produce as one alternative route of *Salmonella*. Then, these horticultural products or food

from animal origin such as pork, beef and lamb, and their products thereof can reach the market and be consumed by humans, who become contaminated with *S. enterica* serotypes (Lamas *et al*., 2018).

Salmonellosis is one of the most frequent infectious gastrointestinal diseases (EFSA, 2021), with an incidence of 93.8 million worldwide cases of the disease in humans caused by NTS (Campos *et al*., 2019) and with an incidence of 20 cases per 100 000 people in the European Union (EU) in 2019, making *Salmonella* the most reported hazard associate with reported foodborne outbreaks in the EU (EFSA, 2021). In Europe, 83 923 cases of salmonellosis in humans were confirmed in 2019, being the second most common infectious gastrointestinal (Campos *et al*., 2019; EFSA, 2021).

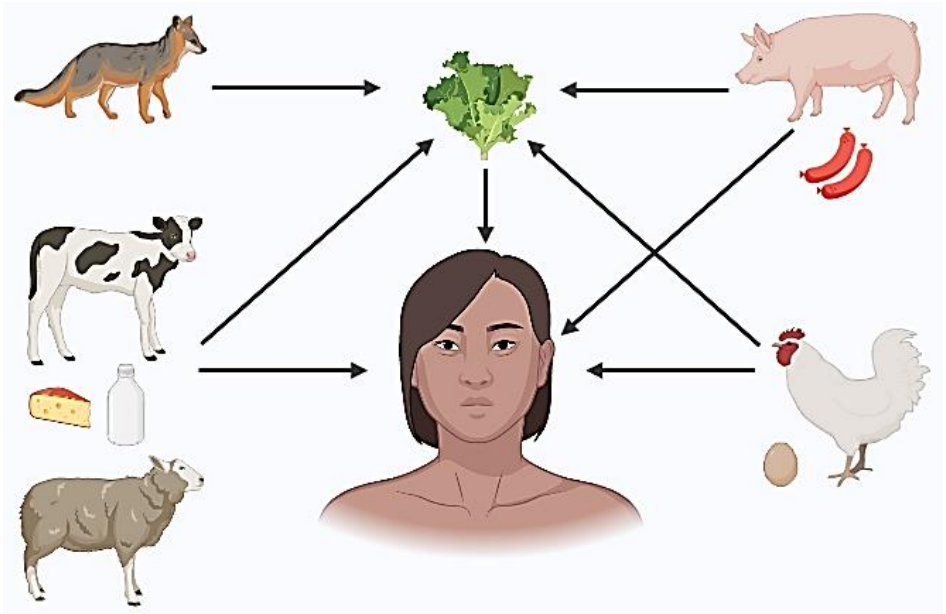

Figure 3. Interaction network between sources of *Salmonella* spp. contamination in humans (adapted from Lamas *et al*. (2018) and created with BioRender.com).

Salmonella spp. causes multiple outbreaks in various places, making it easy to determine how they are disseminated over the world (Majowicz *et al*., 2010). In the European context, one of the main sources of salmonellosis foodborne outbreaks is "pork and products thereof", accounting for 12 % of all *Salmonella* serotypes implicated in the various member states, with *S.* Derby and the monophasic variant of *S.* Typhimurium as two of the most common serovars. Pork and associated products come in second overall, with the majority of foodborne outbreaks being associated with poultry, 70% with broilers, 7% with laying hens, 7% with turkeys, 1% with cattle and the remaining 3% with a variety of sources. As a way of helping to obtain these numbers, the EU implemented mandatory reporting of salmonellosis cases in humans starting with Directive 2003/99/EC (EFSA, 2021).

Also in the EU context, although most salmonellosis cases in the member states were associated with five serotypes, the reality is that only three stand out in terms of being detected in pigs, these being *Salmonella* Typhimurium with 42 %, *S*. Typhimurium monophasic variant with 72.1 % and finally, *S*. Derby with 72 % (Campos *et al*., 2019; EFSA, 2021), although there has also been an increase in the incidence of *S.* Rissen (Campos *et al*., 2019).

Concerning Portugal, there have been attempts to study and verify the possible routes of *Salmonella* contamination, namely at the level of pigs and their carcasses in the slaughterhouse, as in the studies of Cota *et al*. (2019) and Vieira-Pinto *et al*. (2005, 2006). Considering the data declared by EFSA for 2020, Portugal was one of the EU member states with the lowest reported incidence of salmonellosis cases with less than 4.4 cases per 100,000 inhabitants (EFSA & ECDC, 2021).

Although large-scale studies are scarce in abattoirs in Portugal and in pork and pork-associated products, through studies by Cota *et al*. (2019) and Vieira-Pinto *et al*. (2006), it was found that some of the most common serotypes are Rissen, Derby and the monophasic variant of *S.* Typhimurium (such as serotype 4,[5],12:i:-).

Although the prevalence of salmonellosis is still considered high, hygiene programmes in combination with Commission Regulation (EC) No 1441/2007 on microbiological criteria for foodstuffs and associated good hygiene practices by operators could lead to a decrease in the incidence of salmonellosis. Furthermore, in the event of a salmonellosis outbreak, the established measure is the notification of the competent authorities in each country (Campos *et al*., 2019; EFSA, 2021).

2.2. The Importance of Pork Consumption

Pork has been one of the most requested meat products globally since consumption patterns have changed with improving economies around the globe (FAO, 2016; Szűcs & Vida, 2017). In the case of OECD countries, between the decade corresponding to 2003-2013, there was a flattening of meat consumption, but with an increase in the production of certain meats, namely pig meat (González *et al*., 2020; OECD & FAO, 2013), in the projection for the decade 2021-2030, a period initially affected by coronavirus disease caused by SARS-CoV-2 (hereinafter "COVID-19"), there was a drop in meat prices in general, but also the replacement of some types of meat, such as pork, by more affordable meat products, such as various poultry meat (OECD & FAO, 2021).

Given these factors, there was an increasing tendency to look for pigs that have fast-growing characteristics to meet this demand, which ended up being different depending on the region of the globe, with the Asian continent being at the forefront of this increase in request for pork, notably as the People's Republic of China (FAO, 2014; OECD & FAO, 2013; Szűcs & Vida, 2017), which was one of the top 10 producers of this type meat (Figure 4). With the increase in demand for this meat, there was a need to increase its production, particularly on a large scale. The animals started being genetically similar and had the same food and breeding base (FAO, 2014).

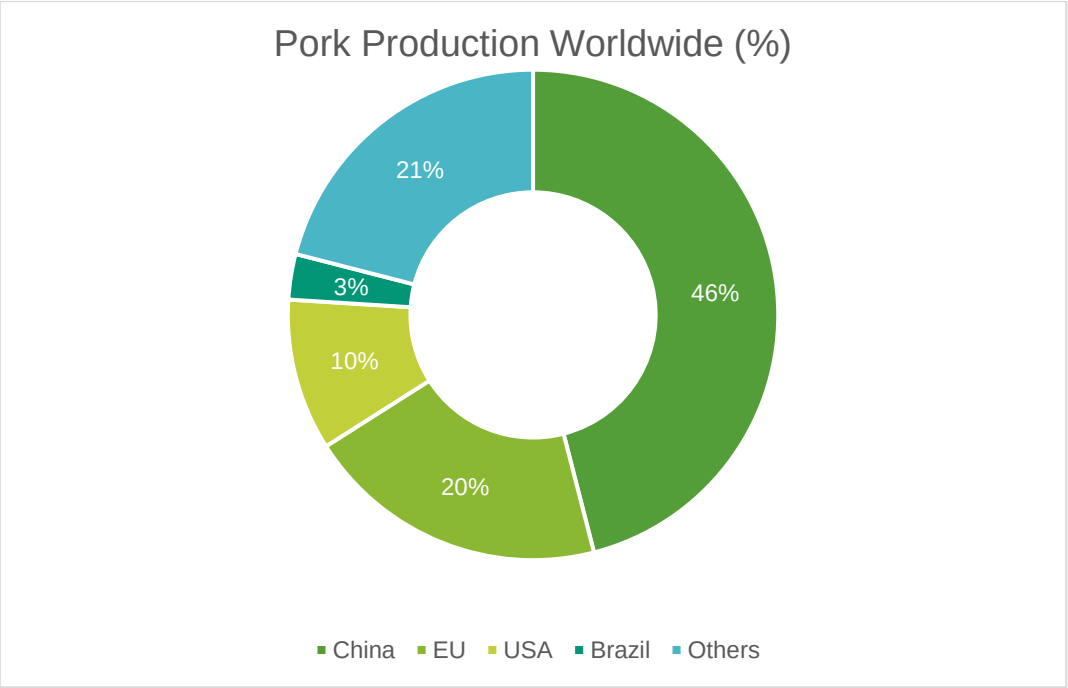

Figure 4. Countries that are the largest producers of pork meat in the world (adapted from Szűcs & Vida (2017)).

However, despite the dynamism of these intensive systems, the reality is that pig production still existed on a traditional basis and on small farms, where little investment was made and where there was a dependence on animals that were closely adapted to the region in which these were raised in (FAO, 2016). This contributed to the fact that meat production, relying also on pork, in developing countries was increasing as investments were not as high as in more developed countries, as was indicated above (FAO, 2016; OECD & FAO, 2013).

According to the Agricultural Outlook 2021-2030 by the Organisation for Economic Co-operation and Development (OECD) and Food and Agriculture Organisation (FAO) (OECD & FAO, 2021), by the year 2030, it is projected that one of the most consumed proteins would be pig meat with an availability of 13.1 %, being overtaken by poultry and sheep meat due to changes in consumption patterns, although it is foreseen an increase up to 127 megatons (Mt) of pig meat consumption, being equivalent to 33 % of the total meat consumption, regarding the same year.

Concerning pork consumption in the EU, its member states will continue to be one of the largest consumers of pork in world terms, reaching a consumption of about 32 Kg/capita/year, by the year 2025 (González *et al*., 2020), however, this trend may decrease until 2030, reaching values around 27 Kg/capita/year (OECD & FAO, 2021).

Although the Agricultural Outlook 2021-2030 predicts that until 2030 there will be an increase in the consumption of pork meat, there were other predictions that indicated that until then there may be a decrease in the consumption of this type of meat (OECD & FAO, 2021). This difference may be related to changes in the dietary patterns of Europeans, which could involve a reduction in the consumption of red meat to around 50 g/day, to avoid comorbidities such as prostate, colorectal and breast cancers

(González *et al*., 2020). In addition, white meats, such as poultry, which are a healthier option (González *et al*., 2020) and more accessible (OECD & FAO, 2021), can contribute to the decrease in the consumption of pork in EU member states.

As for Portugal, in 2020, total meat production reached 902 thousand tons, with 380 thousand tons corresponding to pork (INE, 2021b). However, when considering the year 2021, the period from January to September, meat production increased by 2.4% compared to 2020 and corresponded to the slaughter of almost 4 million pigs (INE, 2021a).

This situation was because, during the pandemic associated with COVID-19, less pork was consumed in Portugal (González *et al*., 2020; INE, 2021b; OECD & FAO, 2013), mainly piglets and fattening pigs, which were marketed more by the catering sector, leading to a decrease in their price by 14.9% (INE, 2021b).

Therefore, it appears that COVID-19 did have negative impacts on meat production and consumption, and this impact was also prominent in pork, particularly in terms of the decrease in its consumption. However, as consumption patterns and consumer demand can change, vigilance is advised in order to check whether the projections regarding the consumption of meat and pork meat (OECD & FAO, 2021) are observed.

2.2.1. Slaughter Technology for Pigs

The "Farm to Fork" strategy was developed by the European Commission (EC) to enable the European continent to be the leader in achieving carbon neutrality by the year 2050. In this way, this strategy presented a change not only in the concern for social and health benefits but also in environmental terms, aiming at the profit of farmers, fishermen and agri-food industry operators in a sustainable way (European Commission, 2020).

Based on this assumption, it became necessary to bring to European consumers the safest and most nutritious food, through knowledge of the entire food processing chain, also relying on institutions and agencies, such as EFSA, to protect consumers from potential hazards that may be present on each point in the chain (EFSA, 2014).

Regarding the slaughtering of pigs, the whole process must be carried out considering the animal's welfare (EFSA *et al*., 2020) and the safety and hygiene of the carcass. The most important ones are stunning, bleeding, scalding, dehairing, singeing, scraping, polishing, evisceration, washing, and cutting of the carcass (De Busser *et al*., 2013; Greig *et al*., 2010; Swart *et al*., 2016). The pig slaughtering process can be seen in Figure 5.

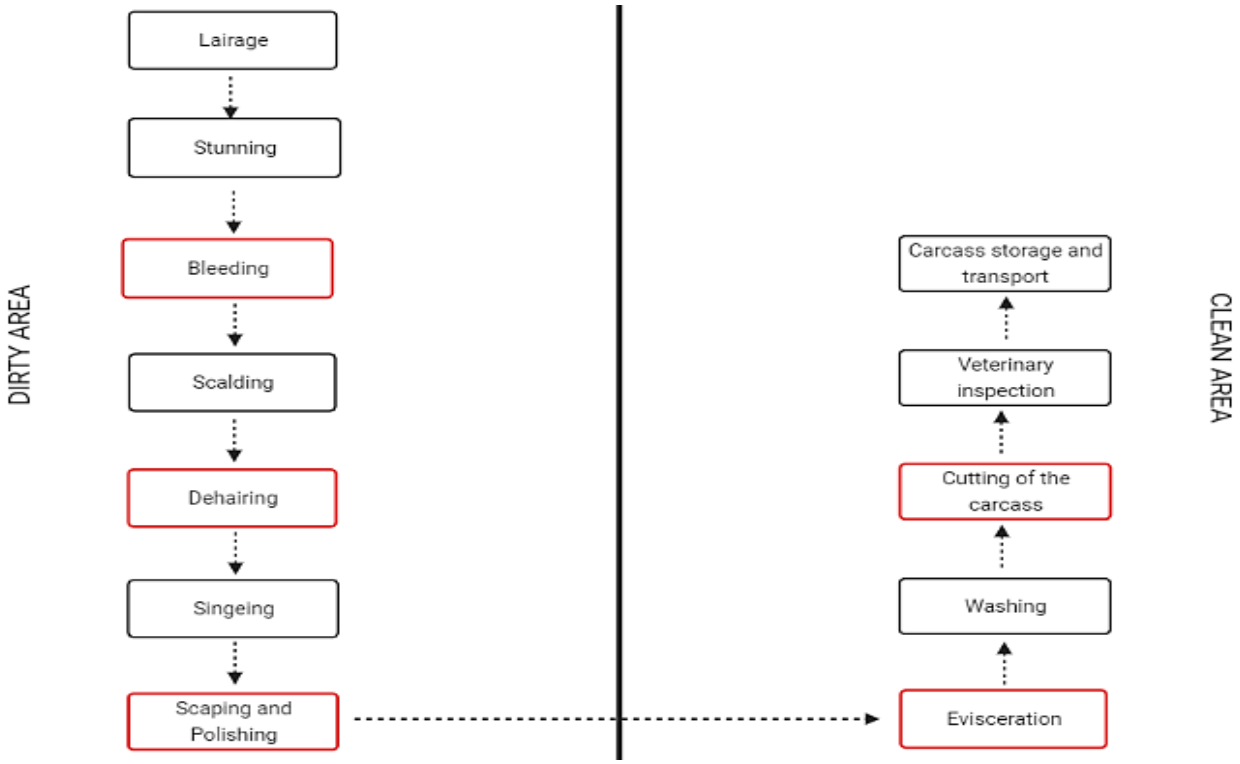

Figure 5. Pig slaughtering process (adapted from De Busser *et al*. (2013); Greig *et al*. (2010) and Swart *et al*. (2016) and created with BioRender.com).
Legend: Red blocks – points where there is greater microbial contamination, namely by *Salmonella* spp.

2.2.1.1. Lairage

The lairage is the place where the pigs are kept at the slaughterhouse to rest from the stress of being transported there, for 2 to 3 hours (De Busser *et al*., 2013) or as long as considered necessary by the official veterinarian for the animals to rest. The biggest threat at this stage are pigs who had been asymptomatic up to this point, and because of the stress of travel, could begin to develop symptoms of *Salmonella*e infection and increase its shedding (Bonardi, 2017).

2.2.1.2. Stunning

After lairage, the pigs are sent for stunning, where diverse methods are employed to render the animals unconscious before bleeding (FAO, 1991) so that the pigs do not feel pain or stress (Wallgren *et al*., 2021). Three different stunning methods are approved, electrical, mechanical and gas stunning, and these must be carried out precisely (Wallgren *et al*., 2021).

Although there is still no data associated with the contamination of *Salmonella* spp. during stunning, the pigs falling and being in contact with the slaughterhouse equipment could potentially be contaminated with that microorganism (Swart *et al*., 2016), nevertheless, the animals should not be stunned on the ground or fall to the ground after stunning.

2.2.1.3. Bleeding

The death of animals is caused by bleeding, after severing the major blood vessels close to the heart with a sticking knife (FAO, 1991; Swart *et al*., 2016). This method has as its main objectives to cause the least possible damage to the carcass, to promote the exsanguination of the animal, to avoid the propagation of bacteria, and the cut should be as small as possible (FAO, 1991).

Normally, there are two types of bleeding that can be used in the abattoir. One is the vertical bleeding, where the animal is hung by one foot from a conveyor belt so that it is upside down to allow to drain all the blood until there was little flow. In horizontal bleeding, on the other hand, the pig is lying on a metal surface (FAO, 1991).

As shown in Figure 5, this step in the slaughter line is among the ones that may render the highest microbial contamination. The contamination by microorganisms could come from the knife that performed the cut during the bleeding, and it was advisable to sanitize it regularly (Swart *et al*., 2016).

2.2.1.4. Scalding

The main purpose of scalding is to enable the removal of pig hair off the skin surface, however, it is also a stage that can contribute to reduce the number of microorganisms present on the pig's body (Greig *et al*., 2010; Swart *et al*., 2016). Two types of scalding can be applied: horizontal and vertical (FAO, 1991; Greig *et al*., 2010; Swart *et al*., 2016).

The scalding tank must have water at an average temperature of 60 °C during horizontal scalding, and the carcasses must be inside for 5-6 minutes (min) (FAO, 1991; Greig *et al*., 2010). Although this is a stage in which high temperatures can contribute to a reduction of pathogens (Swart *et al*., 2016), if the temperature of the tank drops below the temperature specified or the amount of organic matter in the tank raises, there can be an increase in pathogens in the tank, notably *Salmonella*e (De Busser *et al*., 2013; Greig *et al*., 2010).

In vertical scalding, the pig’s body passes through a steam tunnel, and it is considered even better than horizontal scalding in terms of reducing the microbial load since there is no cross-contamination between pigs or reuse of water (Collins & Huey, 2014).

Although the current study focuses on *Salmonella* strains, the fact was that this step of the slaughtering process reduced the number of specimens of this genus and other *Enterobacteriaceae*, mesophilic bacteria, and bacilli, such as *Campylobacter* spp., *Yersinia* spp., and *Listeria* spp. (Greig *et al*., 2010).

2.2.1.5. Dehairing

Dehairing is one of the most contaminating steps of the slaughtering process, involving the removal of the majority of the pigs' hair with a machine that rotates brushes or flaps (FAO, 1991; Swart *et al*., 2016). There is a chance of faecal waste coming out of the pig's anus in this circumstance, but the machine

itself can be a source of dispersal of pathogens throughout the pig's body (Greig *et al*., 2010; Swart *et al*., 2016).

Usually, contamination of the pig carcass with *Salmonella* is linked with this step. However, this form of contamination may be prevented if the machinery is cleaned more often, primarily with water at 60 to 62 °C, the same temperature used in the epilator, to remove any skin remnants that may be adhered to and prevented microbial development (Greig *et al*., 2010).

2.2.1.6. Singeing

Singeing allows the complete removal of the hair that had remained on the pig's skin after the previous stage (Swart *et al*., 2016). This process is based on the use of flames reaching temperatures between 800-1000 ºC for 9-12 seconds (s) (Collins & Huey, 2014; Swart *et al*., 2016).

This stage is one of the most important and most efficient for reducing the microbial loads present on the pig skin (FAO, 1991; Greig *et al*., 2010; Swart *et al*., 2016), with a reduction in aerobic mesophiles and *Enterobacteriaceae* (Greig *et al*., 2010), such as *Salmonella* strains.

2.2.1.7. Shaving and Polishing

After singeing, the carcasses are shaved, which can be either manually or mechanically, and polished to remove the remaining hairs and the scaling of the epidermis that was shed after the treatments of the previous stages (Greig *et al*., 2010; Swart *et al*., 2016).

This procedure can significantly increase the microbial burden on the skin's surface (Swart *et al*., 2016), with polishing alone accounting for 5-15% of the potential contamination (De Busser *et al*., 2013). It may be due to improper cleaning of the material utilized at this stage, which can contribute to a rise in surface pathogens such as mesophiles and *Enterobacteriaceae* such as *Salmonella* spp. (Greig *et al*., 2010).

2.2.1.8. Evisceration

Evisceration is the stage of the slaughtering process where the abdomen is opened and the organs are removed (Greig *et al*., 2010).

It is one of the stages with the highest risk of pig carcass contamination, with 55-90 % of contaminations emerging here (De Busser *et al*., 2013), and is generally caused by the risk of colon or stomach rupture or perforation (Swart *et al*., 2016). Pathogens such as *Salmonella*, *Campylobacter*, and *E. coli* are commonly linked with this step (Greig *et al*., 2010), so the cleansing of the equipment between each carcass after evisceration is recommended to reduce the chance of contamination (Swart *et al*., 2016).

Carcass contamination by *Salmonella* spp. is reported to occur more frequently in previously contaminated pigs rather than in those that had not been contaminated by this bacteria (Berends *et al*.,

1997) with the former case accounting for 70% of carcass contamination and cross-contamination accounting for 30% (Greig *et al*., 2010).

Another major risk comes from the pluck removal, which is associated with the removal of organs such as the tongue, pharynx, oesophagus, trachea, liver, lungs and heart, some of which are consumed, so care must be taken with the possible microbial load they contain (Swart *et al*., 2016), as this is where the liver is found and it is quite likely to find this organ contaminated with *Salmonella* as well as the spleen and lungs (Greig *et al*., 2010).

Ultimately, another way to prevent contamination is though the sanitation and decontamination of the operators and the instruments used to execute evisceration, like GHPs, such as handwashing and disinfection of the material in water at 82 °C, which could reduce carcass contamination by 50 % (De Busser *et al*., 2013; Greig *et al*., 2010).

After evisceration, the carcass is divided in two-halves and the risk of contamination is associated with the equipment used and the operators carrying out this stage (De Busser *et al*., 2013; Swart *et al*., 2016).

2.2.1.9. Veterinary inspection

Regulations (EC) No 852/2004 and No 853/2004 outline how operators of food of animal origin should handle food throughout the primary sector, as well as other guidelines. Both regulations require GHPs to be considered for operators in the primary sector who use HACCP principles. Moreover, Regulation (EC) No 853/2004 summarizes the actions that must be followed in a pig slaughter line to ensure that the carcass is not likely contaminated.

After all the previous steps of the pig slaughter line, a competent meat inspector, usually an official veterinarian or an official auxiliary, is required to inspect the pig carcasses and the offal to check that there is no risk to the consumer (Commission Implementing Regulation (EU) No 2019/627; Swart *et al*., 2016).

Commission Implementing Regulation (EU) No 2019/627 has fixed which are the main criteria to be observed in a carcass with the ultimate goal of being of human consumption; as such, the veterinarian must observe the pig carcass thoroughly without excessive touching but resorting to palpation and incision of the parts considered necessary. By this regulation, during the inspection of the carcass, it must be possible to confirm the absence of any zoonosis, or evidence of microbiological or any other types of contamination, which could lead to the rejection of the carcass, as it is considered to be unfit for human consumption.

Yet, this same method may have certain implications, such as cross-contamination between carcasses by the inspector, since it may be required to use palpation or sharp instruments to perform the incision when necessary, thus the inspector must proceed with extra caution (Swart *et al*., 2016).

For microbiological criteria in food products, Commission Regulation (EC) No 1441/2007, established that, according to the process hygiene criteria, *Salmonella* spp. should be absent in the area tested.

Following inspection approval, the carcasses are washed to remove any residual blood (FAO, 1991), and it is advised that the water is at 80 °C for 14-16 s (De Busser *et al*., 2013). This procedure must take into consideration that it could lead to an increased load of microorganisms on the carcass due to water dissemination (Swart *et al*., 2016). Finally, the carcasses are chilled at 0 °C for a period of 12 to 16 hours to let muscles reach 7 °C or lower and then could be delivered to commercial surfaces (Regulation (EC) No 853/2004, 2004; FAO, 1991).

As a result, the treatment of animals along with the distinct phases of the pig slaughter line, as well as GHPs, could lead to a reduction of *Salmonella* spp. in primary production (De Busser *et al*., 2013) and ensure that the food is safe for the consumer (Cota *et al*., 2019).

2.3. Biocides

Biocides or 'biocidal products' refers to all substances or mixtures that contain or generate active substances capable of destroying, preventing, hindering the action of, or rendering harmless any pathogenic microorganism by means other than physical or mechanical action, under Regulation (EU) No 528/2012.

Biocides are significant agents because they reduce microbial load on many types of surfaces but also inhibit pathogens with MDR from growing (Geraldes *et al*., 2021), which is especially relevant on slaughterhouse surfaces. Thus, biocides are used to disinfect surfaces, but their effectiveness in combating *Salmonella* spp. increases if there is cleaning before disinfection (Møretrø *et al*., 2012). Biocides must meet certain requirements, particularly when used in food processing environments: (i) must have a broad spectrum of antimicrobial activity at low concentrations while also being effective against biofilms; (ii) must be safe and must not be toxic, cause allergies or be flammable; (iii) must not be corrosive, reactive or stain the surfaces they come in contact with; (iv) must be stable in terms of temperature and pH allowing storage for some time; (v) must be able to withstand environmental fluctuations, ground level, dilution and hard water; (vi) must be environmentally friendly and low cost (Møretrø *et al*., 2012; Quinn *et al*., 2011).

Based on all these characteristics associated with biocides, especially those used in the food industry, it is convenient to separate it according to its applicability. Sheldon (2005) divided them into four different classes according to its intrinsic characteristics, namely antiseptics, disinfectants, preservatives and sterilants.

Antiseptics are chemical biocides that can be applied to the skin, killing or inhibiting the growth of vegetative microorganisms (Hernández-Navarrete *et al*., 2014; Sheldon, 2005). Unlike antiseptics,

disinfectants are chemical biocides that are applied to surfaces and kill or inactivate bacterial vegetative cells (Hernández-Navarrete *et al*., 2014; Sheldon, 2005). Preservatives are chemical biocides and are used to prevent the growth of organisms, resulting in the deterioration by microorganisms (Sheldon, 2005). Finally, sterilants are chemical biocides and their main purpose was to kill vegetative and spore-forming bacteria (Sheldon, 2005).

The biocides that are relevant for the present work, according to the European Chemicals Agency (ECHA), belong to the main group of disinfectants, namely to categories PT03 and PT04. Category PT03 concerns veterinary hygiene disinfectants, which are associated with the disinfection of materials and surfaces of animal housing or transport structures. On the other hand, those belonging to category PT04 are those that concern the food area, both for humans and animals, and are used to disinfect equipment and surfaces that come into direct contact with food (European Chemicals Agency, 2018).

A summary of the main types of disinfectants, with their main components, modes of action, types of activity and possible use in the agro-food industry is presented in Table 2. The disinfectants that can have a more complete action and that could interfere with more bacterial cell structures, as well as being at the same time suitable for use in the agri-food industry (categories PT03 and PT04), are the halogens (chlorine compounds), which belong to the oxidizing agents, and the quaternary ammonium compounds (QACs).

For the present study, two biocides were used, taking into account the previous concepts presented, namely Mida FOAM 193® a chlorine-based biocide formulation and Suma Bac D10® a QAC-based biocide formulation, which belonged to the PT03 and PT04 categories. Although there were six different main categories of disinfectants, namely alcohols, alkalis, aldehydes, oxidizing agents, phenolic compounds and quaternary ammonium compounds (Quinn *et al*., 2011; Stull *et al*., 2018), the focus will remain on the biocides Mida FOAM 193® and Suma Bac D10® and their respective categories.

Table 2. Types of disinfectants, main components, modes of action and activity, as well as possible use in the agri-food industry.

Types of disinfectants	Main components	Mode of action	Activity	Used in agri-food industry?	References
Alcohols	Ethanol Isopropanol 2-propanol Propan-2-ol	Protein denaturation	Bactericidal (vegetative cells) Fungicidal Virucidal	Yes (PT03, PT04)	Al-Adham *et al*., 2012; Boyce, 2018; European Chemicals Agency, 2022; Karsa, 2007; Quinn *et al*., 2011; Rutala, 2008; Şahiner *et al*., 2019; Sattar *et al*., 1989; Williams & Worley, 1999
Alkalis	Sodium hydroxide Potassium hydroxide	Hydrolysis of lipids and proteins Damage of cell wall and membranes Leakage of cytoplasm content	Bactericidal (endospores) Virucidal	No (PT02)	European Chemicals Agency, 2022; Fernandes *et al*., 2013; Greenwood *et al*., 2016; Quinn *et al*., 2011
Aldehydes	Glutaraldehyde	Alkylation of sulfhydryl, carboxyl and amino groups Alters the DNA, RNA and protein synthesis	Microbiocidal Bactericidal Sporicidal Virucidal	Yes (PT03, PT04)	Al-Adham *et al*., 2012; European Chemicals Agency, 2022; J.-Y. Maillard, 2013; Quinn *et al*., 2011; Rutala, 2008; Sattar *et al*., 1989
	Formaldehyde	Alkylation of sulfhydryl, carboxyl and amino groups Alters the DNA, RNA and protein synthesis	Microbiocidal Bactericidal Sporicidal Virucidal	Yes (PT03)	Al-Adham *et al*., 2012; European Chemicals Agency, 2022; Karsa, 2007; Quinn *et al*., 2011

Types of disinfectants	Main components	Mode of action	Activity	Used in agri-food industry?	References
Oxidizing agents	Halogens (Chlorine compounds)	Inhibit metabolic processes Protein denaturation Leakage of the cytoplasmic contents Inhibition of enzymatic reactions	Bactericidal Sporicidal Virucidal Fungicidal	Yes (PT03, PT04)	European Chemicals Agency, 2022; Fernandes *et al*., 2013; Karsa, 2007; Stull *et al*., 2018; Williams & Worley, 1999
	Peroxygen compounds (Hydrogen peroxide, peracetic acid, potassium peroxymonosulfate)	Formation of hydroxyl free radicals	Bactericidal Sporicidal Virucidal Fungicidal	Yes (PT03, PT04)	European Chemicals Agency, 2022; Omidbakhsh & Sattar, 2006; Quinn *et al*., 2011; Rutala, 2008
Phenolic compounds	Ortho-phenylphenol Ortho-benzyl-para-chlorophenol	Lysis of intercellular components Interaction with metabolic enzymes	Bactericidal Fungicidal Virucidal	Yes (PT03, PT04)	European Chemicals Agency, 2022; Karsa, 2007; Quinn *et al*., 2011; Russell, 1990; Rutala, 2008
Quaternary ammonium compounds (QACs)	Alkyl dimethyl benzyl ammonium chloride (ADBAC) Didecyl dimethyl ammonium chloride (DDAC)	Denaturation of proteins Interaction with metabolic enzymes Leakage of cytoplasmic contents Coagulation of the cytoplasm	Bactericidal Fungicidal Sporicidal Virucidal	Yes (PT03, PT04)	European Chemicals Agency, 2022; Luz *et al*., 2020; Quinn *et al*., 2011; Stull *et al*., 2018; Walia *et al*., 2017

Legend: QAC – quaternary ammonium compound; ADBAC - Alkyl dimethyl benzyl ammonium chloride; DDAC - Didecyl dimethyl ammonium chloride; PT03 – Product used in veterinary hygiene; PT04 – Product used on surfaces in contact with foodstuffs and feeding stuffs.

2.3.1. Mida FOAM 193®

According to the manufacturer's description, Mida FOAM 193® biocide is a chlorinated alkaline disinfectant detergent in liquid form, foaming with high sanitizing power, and is suitable for the food industry (Christeyns, 2014). The fact that it is a chlorinated alkaline biocide means that it has

characteristics of two categories of disinfectants, alkalis, and oxidative agents, namely the subcategory of chlorine compounds, which makes the Mida FOAM 193® a chlorine-based biocide (CBB).

On the one hand, being a sodium hydroxide makes Mida FOAM 193® an alkaline biocide, a strong base (Quinn *et al*., 2011) and able to reduce the microbial load by inhibiting metabolic processes (Fernandes *et al*., 2013). Biocides of this category tend to alter the pH, via hydroxyl ions, and by saponification of fat (Stull *et al*., 2018), so they ultimately compromise the microbial cell wall and membranes, leading to their rupture and to the leakage of cytoplasmic components (Fernandes *et al*., 2013).

The alkaline biocides are characterised by a slow mode of action, these biocides must be used at high temperatures and their efficiency is affected by pH (Stull *et al*., 2018). In addition, it eventually can become an environmental hazard since it is corrosive on metal surfaces, and can also be hazardous for workers, as it can cause severe burns and irritation of the mucous membranes (caustic effect) (Fernandes *et al*., 2013; Quinn *et al*., 2011; Stull *et al*., 2018). With all these alkaline characteristics, it is found that CBBs, like Mida FOAM 193®, have bactericidal, virucidal, fungicidal and sporicidal capabilities (Stull *et al*., 2018), so it can kill gram-negative bacteria (Quinn *et al*., 2011), such as *Salmonella* strains.

On the other hand, since Mida FOAM 193® is also a sodium hypochlorite-based biocide formulation, it also belongs to the chlorine compounds group of oxidizing agents. Chlorine compounds have as mechanisms of action the denaturation of proteins (Stull *et al*., 2018) and the inhibition of enzyme reactions, and thus these biocides have good antimicrobial capabilities (Fernandes *et al*., 2013).

These chlorine compounds are extremely reactive in water (Fernandes *et al*., 2013), and their mode of action is related to the fact that when this element is added to water and there is the hydrolysis of chloramines and hypochlorite, leading to the formation of hypochlorous acid, which ultimately results in the release of nascent oxygen (Quinn *et al*., 2011). Thus, the main characteristics of sodium hypochlorite are that it is fast-acting and could be affected by pH, it is most often applied in the form of bleach, but it can also be corrosive on metal surfaces and cause irritation to mucous membranes (Stull *et al*., 2018).

Like alkaline disinfectants, these also have bactericidal, fungicidal, virucidal and sporicidal characteristics (Stull *et al*., 2018), particularly against gram-negative bacteria (Quinn *et al*., 2011), such as *Salmonella* strains.

In both categories of this compound, it was found that the pH at which it had the best antimicrobial capacity was pH 5 (Quinn *et al*., 2011), and this was in line with manufacturer usage guidelines, which indicate that Mida FOAM 193® would be most effective when used at a pH between 3-6 (Christeyns, 2014).

2.3.2. Suma Bac D10®

According to the manufacturers, Suma Bac D10® disinfectant is based on quaternary ammonium compounds (QAC), a sequestrant and a buffer, making it an effective biocide for a broad spectrum of microorganisms (Diversey Inc., 2019) and widely used in food processing units (Wu *et al*., 2015).

Its mode of action is associated with the denaturation of proteins by binding to phospholipids in the cell membrane of microorganisms (Stull *et al*., 2018), so QACs interact with enzymes essential to metabolism (Quinn *et al*., 2011). If acting in low concentrations, it could lead to the exit of cytoplasmic contents since it causes the disruption of the interactions between lipids and proteins existing in the plasma membrane; if acting in high concentrations, there is the coagulation of the cytoplasm (Quinn *et al*., 2011; Walia *et al*., 2017).

Biocides such as Suma Bac D10® have as main features that it is most efficient when used at neutral or alkaline pH (Quinn *et al*., 2011; Stull *et al*., 2018), but also at higher temperatures (Stull *et al*., 2018) and it is effective in waters of any type of hardness (Diversey Inc., 2019). These biocides can corrode metal surfaces (Stull *et al*., 2018) and in high concentrations can irritate the respiratory tract and the skin (Quinn *et al*., 2011; Stull *et al*., 2018). In addition, and as indicated above, Suma Bac D10® is a powerful bactericide, but also a fungicide and virucide (Diversey Inc., 2019).

QAC-based formulations, like Suma Bac D10®, are an effective biocide against gram-negative bacteria (Stull *et al*., 2018), such as against *Salmonella* strains, and are, therefore, suitable for the present study.

2.3.3. Mechanisms of Resistance to Biocides by Microorganisms

With the increasing use of disinfectant biocides such as those described above, chlorine-based and QAC-based formulations, in both home and food processing environments, it was found that there was a possibility that bacterial pathogens may come in contact with such formulations at concentrations below the effective level and become more tolerant to it, eventually developing resistance to the biocides (Møretrø *et al*., 2012).

Based on this concern, it is necessary to understand what it means to be in the presence of biocide-resistant bacteria. Biocide-resistant bacteria are all those that do not die when in contact with a concentration of biocide that would cause the death of other bacteria (J.-Y. Maillard, 2018), but also bacteria that can develop and grow at higher concentrations of biocides than other strains (Møretrø *et al*., 2012). If the biocide that has caused the bacteria to acquire resistance is used repeatedly, it can be found a predominance in terms of numbers of these resistant bacteria, which eventually become dominant and end up passing on the genetic material that allows it to be resistant to the more susceptible ones, if it has this capacity (Quinn *et al*., 2011).

Often associated with the concept of bacterial resistance is the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The MIC refers to the minimum concentration

required of an antimicrobial, or in this case a biocide, to inhibit visible bacterial growth during one night of incubation (Levison, 2004). The MBC, on the other hand, is the minimum concentration of a bactericidal agent, which can be the same biocide, that would kill a bacterium (Wiegand *et al*., 2008). Although these concepts were often associated with bacterial resistance, they should be associated with lower susceptibility (J.-Y. Maillard, 2018), as MIC and MBC make it possible to determine how effective a given bactericide is and which is the recommended concentration to be used (J.-Y. Maillard, 2018; Møretrø *et al*., 2012).

Innate resistance, also known as intrinsic or unsusceptible resistance, implies processes that are in bacteria to fight against a drug and are implicit and associated with their functioning (Fraise *et al*., 2012; Poole, 2002; Quinn *et al*., 2011). On the other hand, acquired or extrinsic resistance is all the new mechanisms to combat biocides, usually acquired by mutation or transfer of mobile genetic elements, such as bacteriophages, plasmids or transposons (Fraise *et al*., 2012; Poole, 2002; Quinn *et al*., 2011). Although these two concepts are valid, the reality is that resistance can be more complex and not fit into those two types, so Maillard (2018) defined transient resistance as the expression of resistance mechanisms as a response to bacteria that have been under direct selective pressure.

Thus, once all the concepts are defined, it becomes necessary to explain the three most important biocide resistance mechanisms, these being target alteration and impermeability, efflux pumps (Poole, 2002), and biofilm formation (Fraise *et al*., 2012) paying attention to gram-negative bacteria, such as the genus *Salmonella*.

2.3.3.1. Impermeability and Target alteration

The Gram staining method allows the differentiation between gram-negative and gram-positive bacteria (Denyer & Maillard, 2002). In the present work, the focus is on gram-negative bacteria since the interest lays on *Salmonella* strains' possible mode of action against biocides.

Gram-negative bacteria have a conserved structure in which it contains an outer membrane, a peptidoglycan layer and a periplasmic space (Denyer & Maillard, 2002; Møretrø *et al*., 2012). The outer membrane consists of a bilayer composed of lipopolysaccharides (LPS), phospholipids and membrane-integrated proteins (Denyer & Maillard, 2002). This constitution makes gram-negative bacteria inherently more resistant to biocides than gram-positive bacteria (Denyer & Maillard, 2002; Poole, 2002; Russell, 2003).

Thus, when cell wall impermeability is considered, there are several mechanisms that bacteria used, particularly gram-negative bacteria, such as its hydrophobic outer membrane, the structure of the membrane itself, as mentioned above, as well as the changes that can occur in terms of fatty acid composition (Denyer & Maillard, 2002; Poole, 2002), the latter also being linked to target alteration.

Target alteration is a rare bacterial resistance mechanism, with mutations or interactions with biocide targets being implicated (Poole, 2002). Mutations can occur at various sites, at the level of the cell wall

and outer membrane, cytoplasmic membrane and cytoplasmic constituents (Maillard, 2002). As for the level of interactions, these can be between biocides and specific groups, namely thiol groups (Maillard, 2002).

When dealing with mutations at the membrane level, these are associated with triclosan, since its effect was related to the biosynthesis of fatty acids (Poole, 2002). This mutation has implications in how the enoyl acyl reductase protein was modified, as triclosan affects the FabI enzyme, which is responsible for the previous change (Maillard *et al*., 2013; Poole, 2002; Russell, 2003).

From this, it can be seen that this type of action can have a bactericidal activity, as the biocide can act directly on the cytoplasmic constituents of the affected bacteria, altering the targets on which the biocides would act, however, the exact sites within cells where biocides act are difficult to identify (Maillard, 2002).

Despite the efforts to address this sort of resistance, triclosan was widely used in food processing, and when analysing how *Salmonella* reacts to this substance, resistance can be developed even at subinhibitory levels (Maillard, 2018; Møretrø *et al*., 2012).

2.3.3.2. Efflux Pumps

Efflux pumps exist in various types of bacteria, notably in gram-negative bacteria, and are responsible for the decrease in the concentration of biocides inside bacteria (Maillard, 2018). Efflux pumps can either be encoded on bacterial chromosomes or plasmids (Fraise *et al*., 2012).

Efflux pumps can be divided into five different categories, such as the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxic compound extrusion (MATE) family and the small multidrug resistance (SMR) family (Maillard, 2018; Poole, 2001). The category associated with the SMR family also belongs to a superfamily, the drug/metabolite transporter (DMT) (Poole, 2001).

For gram-negative bacteria, this type of resistance mechanism is associated with resistance to QACs (Maillard, 2018; Møretrø *et al*., 2012; Poole, 2005). The genes that are associated with QAC resistance are *qacE*, *qacEΔ1*, *qacF* and *qacG*, and these are usually associated with mobile genetic elements, specifically, integrons (Poole, 2005; Zou *et al*., 2014), and the first three genes are relevant to the present study.

In addition to this efflux pump system against QACs, other systems are important, namely, the AcrAB-TolC complex (Chowdhury *et al*., 2019; Fraise *et al*., 2012; Møretrø *et al*., 2012; Weston *et al*., 2018), being a mechanism associated with reduced susceptibility to biocides (Fraise *et al*., 2012; Møretrø *et al*., 2012). This AcrAB-TolC system is composed of three parts: (i) the TolC which is an outer membrane protein; (ii) the AcrA which is a periplasmic adaptor protein; and (iii) the AcrB which is an internal membrane transporter (Chowdhury *et al*., 2019).

A mutation was associated with resistance against biocides through this mechanism, namely at the level of regulatory genes (Møretrø *et al*., 2012; Weston *et al*., 2018), specifically when the *ramA* gene was implicated (Weston *et al*., 2018). When the *ramA* gene was over-expressed, then the *acrAB* gene was also over-expressed and bacteria become resistant; when the *ramA* gene was inactivated, then there was a reduction in *acrAB* expression (Bailey *et al*., 2008; Weston *et al*., 2018).

Thus, to combat biocide resistance in gram-negative bacteria such as *Salmonella* spp. using efflux pumps, this system could be inhibited, through its under-expression or by modifying the biocides themselves in such a way as to make the bacteria susceptible to them (Chowdhury *et al*., 2019).

2.3.3.3. Biofilm Formation

A biofilm formation refers to a population of bacteria that are enclosed in a matrix of polymers that have been produced and expelled by them to adhere to a surface. There are distinct regions in the biofilm, such as an aerobic and an anaerobic area, since the biocide cannot reach the anaerobic area of the biofilm, it becomes a physical mechanism of biocide resistance (Greig *et al*., 2010). So, when bacteria are found in the biofilm form, they are more resistant to biocides than those found in their isolated form (planktonic bacteria) (Fraise *et al*., 2012; J.-Y. Maillard *et al*., 2013; Møretrø *et al*., 2012).

This divergence in susceptibility to biocides when in planktonic form or when integrated into a biofilm arises from several mechanisms, such as (i) the difficulty of the biocide in accessing the cells of the biofilm, which is the quenching; (ii) the interactions that can occur between the biocide and the biofilm; (iii) the changes that occur in that environment, in which the limitation of nutrients and oxygen leads to different speeds of bacterial growth; (iv) the increased production of degrading enzymes; (v) the genetic exchange between cells, leading to an increase in the number of mutations or gene transfer; (vi) the presence of quorum sensing; (vii) the existence of persisters or dormant bacteria; and, finally, (viii) biocide efflux (Fraise *et al*., 2012; J.-Y. Maillard *et al*., 2013; Russell, 2003).

As gram-negative bacteria, namely strains of *Salmonella*, are more resistant to biocides in the form of biofilm, which became a problem in the food industry, it is urgent to find solutions to fight this natural formation, particularly the development of anti-biofilm biocides (Møretrø *et al*., 2012).

2.4. Master's Thesis Objective

The present research involves the assessment of the susceptibility of *Salmonella* isolates, previously gathered, and studied in terms of pheno- and genotypic characteristics (Cota *et al*., 2019) to biocides commonly used in the agri-food industry, namely Mida FOAM 193®, which was derived from hypochlorite and sodium hydroxide, and Suma Bac D10®, which was a type of QAC disinfectant.

In addition, biocide resistance genes, namely *acrA*, *acrB* and *tolC*, which are efflux pump encoding genes, and *qacE*, *qacEΔ1* and *qacF/H/I*, which are QACs resistance genes, were detected by polymerase chain reaction (PCR). Also, the phenotypes of resistance and/or susceptibility to biocides

in *Salmonella* isolates were also determined by the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

Finally, all the data obtained was compared with the results obtained in Cota *et al*. (2019) research, in terms of phenotypic and genotypic correlations, through how the application of chlorine-based and QAC-based formulations and organic matter could influence the permanence of *Salmonella* spp. in a Portuguese slaughterhouse.

Chapter 3: Materials and Methods

3.1. Bacterial isolates

In the present study, a collection of 44 *Salmonella* spp. isolates that came from pigs slaughtered in a Portuguese abattoir was analysed and these were obtained by Vanessa Silva in 2014 for her master's thesis.

All *Salmonella enterica* isolates from slaughterhouse pigs used in this study are shown in the following table (Table 3), together with the serovars to which it belongs and the cluster determined for it, taking into account the previous study conducted by Cota *et al*. (2019).

Table 3. List of isolates of *Salmonella enterica* subsp. *enterica* obtained from pigs slaughtered in a slaughterhouse, with the corresponding cluster and serotype (adapted from Cota *et al*. (2019)).

Sample code	Cluster	Serotype	Sample code	Cluster	Serotype
p1	IA	Rissen	ci117	IIB	4,[5],12:i:-
p3	IA		p114	IIB	
p5	IA		ci109	IIB	
p116	IA		p112	IIB	
p58	IA		ci110	IIB	
p4	IA		ci115	IIB	
ci57	IA		p96	IIB	
ci55	IB	Rissen	p104	IIB	
p61	IB		p106	IIB	
p62	IB		p107	IIB	
ce44	IB		p109	IIB	
p25	IB		p115	IIB	
p31	IB		p110	IIB	
ce21	IB		ci104	IIB	
ce37	IB		ci105	IIB	
ci21	IB		ci108	IIB	
p64	IB		p66	III	Derby
p55	IIA	4,[5],12:i:-	p67	III	
p56	IIA		p68	III	
ci111	IIB	4,[5],12:i:-	ce70	III	
p118	IIB		ci68	III	
ci116	IIB		ci38	ind	Rissen

Legend: “p” – isolates that came from the skin before scalding; “ci” – isolates that came from the internal part of the carcass; “ce” – isolates that came from the external part of the carcass; ind – independent cluster; Rissen – *Salmonella enterica* subsp. *enterica* ser. Rissen; Derby – *Salmonella enterica* subsp. *enterica* ser. Derby; 4,[5],12:i:- – monophasic variant of *Salmonella enterica* subsp. *enterica* ser. Typhimurium.

Above it was indicated that the isolates were from the genus *Salmonella*, and in that work, the survey for this microorganism was conducted according to the International Organization for Standardization

(ISO) standard 6579:2002. Thus, the *Salmonella* isolation was carried out, in the first stage, on specific media for *Salmonella*e, namely Xylose Lysine Deoxycholate (XLD) (Liofilchem®, Via Scozia, Italy) and Hektoen Enteric Agar (HEA) (Biolab®, Budapest, Hungary); and, in a second stage, biochemical tests were performed on Triple Sugar Iron (TSI) (Liofilchem®, Via Scozia, Italy) and the urease test (HiMedia®, Einhausen, Germany).

Taking into consideration the previous work of isolating the *Salmonella* specimens, the isolates were coded according to where they were found in the pig carcass, so there were three codes: "p", "ci" and "ce", where "p" indicated that the isolates came from the skin, before scalding, "ci" that the isolates came from the internal part of the carcass and "ce" that it came from the external part of the carcass.

In addition to the *Salmonella* spp. isolates, five control strains were also used in this research, specifically *Salmonella enterica* subsp. *enterica* ser. Typhimurium CECT 443, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538 and *Enterococcus hirae* ATCC 10541. These isolates were kindly provided by Professor Manuela Oliveira (FMV-ULisboa).

All bacterial isolates were inoculated in Brain Heart Infusion (BHI) (VWR® International, Leuven, Belgium) broth for 24 hours (h) at 37 °C. From there 1 mL of the suspension was taken to an Eppendorf, to which 0.5 mL of glycerol was added, leaving a 20 % glycerol suspension, so that it could be stored at -20 °C.

3.2. DNA extraction and quantification of bacterial isolates

All the studied isolates were subjected to DNA extraction followed by its quantification.

The protocol indicated by Moore *et al*. (2004) was followed for the DNA extraction, in this specific case, the protocol indicated as "*Protocol II - Protocol for the extraction of genomic DNA from individual bacterial colonies*" was performed.

Before proceeding with the DNA extraction, all isolates were sown in a BHI broth (VWR International®, Leuven, Belgium) suspension. For that purpose, a loop was collected from each cryotube of each isolate to be sown on plates with BHI agar to grow at 37 ºC for 24 h to obtain isolated colonies. Tubes with 5 millilitres (mL) of BHI broth were prepared and with a loop, a colony was removed from the previously mentioned plates to grow at 37 ºC for 24 h and continue with the extraction protocol.

One mL of the bacterial suspension was transferred from each tube to a clean 1.5 mL Eppendorf and centrifuged (10 000 × g, 10 min) to pellet the bacterial cells, after which the supernatant was discarded. Then, 100 µL of purified and filtered sterile water (0.2 µm filter) (Nalgene®, New York, USA) was added to the cell pellet and placed in a dry bath at 97 °C for 10 min. Finally, the Eppendorf was removed from the dry bath. The cell lysate was centrifuged (15 000 ×g, 10 min). Subsequently, the supernatant containing DNA was transferred to a clean 1.5 mL Eppendorf and placed on ice until frozen (-20 °C).

After DNA extraction, DNA quantification was performed using the NanoDrop™ 2000 (Thermo Fisher Scientific, Massachusetts, United States of America (USA)), where the same purified and filtered sterile water (1 µL) was used as a calibrator suspension (the "blank" suspension). From there, 1 µL of each suspension of DNA extracted from each isolate was used to obtain its quantification in nanograms (ng) per microliter (ng/µL).

3.3. Polymerase Chain Reaction (PCR) amplification

For the detection of biocide resistance genes, the PCR amplification technique was used. PCR amplification was conducted to detect genes encoding efflux pumps belonging to the AcrAB-TolC complex, namely *acrA*, *acrB* and *tolC*, as well as for QACs resistance genes, such as *qacEΔ1*, *qacE* and *qacF/H/I* genes.

The primers for the amplification of *acrA*, *acrB* and *tolC* were designed from their sequences provided by GenBank and using the Primer3Plus software (https://primer3plus.com/). Previously reported primers were used for the amplification of *qacEΔ1*, *qacE* and *qacF/H/I* genes (Zou *et al*., 2014). The sequences of these primers are shown in Table 4.

Each PCR reaction had a total volume of 25 µL and consisted of 12.5 µL of NZYTaq II 2x Green Master Mix (Nzytech®, Lisbon, Portugal), 9.5 µL of nuclease-free water filtered with a 0.2 µm filter (Nalgene®, New York, USA), 1 µL of forward primer (STAB VIDA, Lda., Caparica, Portugal) at a final concentration of 0.4 µM, 1 µL of reverse primer (STAB VIDA, Lda., Caparica, Portugal) at a final concentration of 0.4 µM and 1 µL of DNA template from each isolate. Each PCR protocol varied according to the gene that was being amplified, however, for all of them the initial denaturation of the DNA occurred at 95 °C for 5 min and the final extension occurred at 72 °C for 5 min. As for the denaturation, annealing and elongation steps, these occurred in 30 cycles, and the specificity of each one is shown in Table 5.

After each PCR run, electrophoresis was performed in a 2% agarose gel stained with GreenSafe Premium® (Nzytech®, Lisbon, Portugal), at 90 volts (V). After electrophoresis, the agarose gel was visualized using ChemiDoc XRS+ (BIO-RAD Laboratories, Inc., Algés, Portugal).

Table 4. List of primers sequences used to target genes encoding efflux pumps and QACs.

Primer	Sequence (5' → 3)	Product size (bp)	Accession number
***acrA*-FW**	TGGCAAATGGTTCGCTGAAA	220	MH933961.1
***acrA*-RV**	GGTTTTGTCCCTTCCTGCAG		
***acrB*-FW**	ACGTAATCAGTTGTTCGGCG	242	NC_003197.2
***acrB*-RV**	ATTTCGCTTCGGACATCACG		
***tolC*-FW**	CCGTACTGGCGAATGAAGTG	165	NC_003197.2
***tolC*-RV**	TTTCCGCTTCCTTCAACAGC		
***qacEΔ1*-FW**	AATCCATCCCTGTCGGTGTT	175	JN596280
***qacEΔ1*-RV**	CGCAGCGACTTCCACGATGGGGAT		JN566044
***qacE*-FW**	AAGTAATCGCAACATCCG	258	X68232
qacE-RV	CTACTACACCACTAACTATGAG		
***qacF/H/I*-FW**	GTCGTCGCAACTTCCGCACTG	229	HQ875011
***qacF/H/I*-RV**	TGCCAACGAACGCCCACA		FJ160769 JN596279

Legend: Bp – base pair.

Table 5. Temperatures and times for denaturation, annealing and elongation steps across all 30 cycles for genes *acrA*, *acrB*, *tolC*, *qacEΔ1*, *qacE* and *qacF/H/I*.

Gene	Denaturation		Annealing		Elongation	
	T (°C)	Time (s)	T (°C)	Time (s)	T (°C)	Time (s)
acrA	95	30	53	30	72	30
acrB	95	30	54	30	72	30
tolC	95	30	54	30	72	30
qacEΔ1	95	30	56	25	72	30
qacE	95	30	50	25	72	30
qacF/H/I	95	30	60	30	72	30

Legend: ºC – the temperature in Celsius degrees; s – seconds.

3.4. Determination of Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)

To determine the MICs and MBCs of the studied biocides, twelve out of the forty-four isolates of *Salmonella enterica* subsp. *enterica* were selected. The selection process was carried out with the objective of including isolates harbouring different biocide resistance genes combinations, different Salmonella serotypes and from different clusters. Additionally, and according to EN 1656:2009 standard (EN 1656, 2009), *E. hirae* ATCC 10541, *S. aureus* ATCC 6538 *E. coli* ATCC 10536 and *P. aeruginosa* ATCC 15442 were used as control strains.

3.4.1. Preparation of the Suma Bac D10® and Mida FOAM 193® solutions

Before beginning any experiment, sterile water was used to create solutions for each biocide to be employed. In the case of Suma Bac D10®, eight solutions were created with a starting concentration that allowed for final solutions of 5%, 4%, 3%, 2%, 1%, 0.5 %, 0.25 %, and 0.1 %. The recommended in-use concentrations vary between 1 and 4 %.

In terms of the biocide Mida FOAM 193®, eight solutions were created with an initial concentration that allowed for final solutions of 15%, 12.5 %, 10%, 7.5 %, 5%, 2.5 %, 1%, and 0.5 %, as the recommended concentration is 10%.

3.4.2. Preparation and testing of the controls of the remaining solutions

Initially, a sodium chloride tryptone (NaCl tryptone) diluent solution was prepared, according to EN 1656:2009 (EN 1656, 2009). This NaCl tryptone was prepared by diluting 1 gram (g) of tryptone (Becton, Dickinson & Co., New Jersey, USA) with 8.5 g of sodium chloride (Merck & Co., Inc., New Jersey, USA) in 1 litre (L) of water and then sterilised in an autoclave (121 °C, 20 min).

Since biocides have a contact time established by the manufacturer, which for both Suma Bac D10® and Mida FOAM 193® was 5 min, followed by a washing step which removed the biocides from the treated surfaces, to mimic this effect in the laboratory, the standard EN 1656:2009 (EN 1656, 2009) indicated which neutralisers were most suitable to use according to the group of biocide tested.

In the case of Suma Bac D10®, as it was a biocide based on QACs, the recommended neutraliser was composed of 30 g/L polysorbate 80 (Merck & Co., Inc., New Jersey, USA), 30 g/L saponin (Sigma-Aldrich, St. Louis, Missouri, USA) and 3 g/L lecithin, which in this case was used ovolecithin (The British Drug Houses Ltd., London). For Mida FOAM 193®, as it was based on sodium hydroxide and sodium hypochlorite biocide, its neutralizer was a mixture of 3 – 20 g/L of sodium thiosulfate (Merck & Co., Inc., New Jersey, USA), 30 g/L of polysorbate 80 (Merck & Co., Inc., New Jersey, USA) and 3 g/L of lecithin, which it was used ovolecithin (The British Drug Houses Ltd., London) just like in the neutralizer of Suma Bac D10®.

Also, for the validation of the neutralizer of chlorine-based formulations (like Mida FOAM 193®), since sodium thiosulfate can be used at concentrations ranging from 3 to 20 g/L, the neutralizer with different sodium thiosulfate amounts, such as 3, 5, 8, 10, 15, and 20 g/L, was evaluated with one of the control strains, *E. coli* ATCC 10536. The neutralizer could be made with sodium thiosulfate at a concentration of 3 g/L, according to this preparation experiment.

To verify that the neutralizer did not affect the bacteria under study in any way, a "neutralizer control" was performed, in which 8 mL of neutralizer (which is different according to the biocide, as previously indicated), 1 mL of sterile water and 1 mL of bacterial suspension, previously set at 10^3 CFU/mL, were used. These suspensions were incubated for the time indicated by the producer (5 min for both Suma Bac D10® and Mida FOAM 193®) at room temperature (~ 20 °C). Next, 100 µL of the previous suspension was transferred to be inoculated by scattering into a plaque 10 mL Tryptone Soya Agar (TSA) (Oxoid, Ltd., Hampshire, England) and incubated for 24 h at 37 °C to check if the neutralizers would influence colony growth.

Since organic matter can be present on all surfaces in the abattoir, this scenario must be replicated by employing low and high doses of interfering substances (LIS and HIS, respectively) to simulate the disinfection method by both biocides, since both were tested according to the European Standard (EN 1656, 2009).

The solution of low-level interfering substance (LIS) was prepared by dissolving 3 g of bovine albumin fraction V (NZYTech, Lisbon, Portugal) in 100 mL of water, which was sterilized by membrane filtration with a sterile syringe filter of 0.2 µm (Nalgene®, New York, USA).

The high-level interfering substance (HIS) solution was prepared by mixing and dissolving 50 g of yeast extract (Oxoid, Ltd., Hampshire, England) in 250 mL of water, which was sterilized by autoclave (120 °C, 20 min) and cooled until reached 20 °C ± 1 °C. In another container, 5 g of albumin was dissolved in 25 mL of water and sterilized by a sterile syringe filter of 0.2 µm (Nalgene®, New York, USA). To this last solution, 25 mL of the previous suspension of yeast extract was added.

To assure that both interfering substances did not have any effect on the bacterial viability, it was necessary to perform an “interfering substance control” before each test. A solution was prepared by adding 1 mL of interfering substance (low or high, depending on the experimentation) with 1 mL of bacterial suspension with 10^3 CFU/mL into a test tube, and then incubating for 2 min ± 10 s at room temperature. After incubation, 8 mL of sterile water was added to the solution and followed by a second incubation at room temperature for 10 min ± 10 s. Thereafter, 1 mL of the previous solution was sown by incorporation in TSA (Oxoid, Ltd., Hampshire, England) and incubated for 24 h at 37 °C. After incubation time, the plates were evaluated to detect if there were any adverse effects caused by both interfering substances.

Finally, a "neutralization method validation" was undertaken to validate this procedure. To do so, 24 hours before the procedure, the selected isolates were sown and prepared with the greatest concentration of the biocide, which was 5% for Suma Bac D10® and 15% for Mida FOAM 193®. It was prepared a test tube with 1 mL of interfering substance (high or low concentration, when testing without interfering substance, 1 mL of sterile water was added), 8 mL of tryptone NaCl, and 1 mL of the highest concentration previously of the biocide in test, and then incubated for 5 min ± 10 s at room temperature. Following that, 1 mL of the preceding suspension was added to a test tube with 8 mL of neutralizer (which, as previously said, was specific for each biocide) and 1 mL of sterile water, followed by a 5 min

± 10 s at room temperature. Following that, 1 mL of bacterial suspension containing 10^3 CFU/mL was added to the preceding solution, followed by another 10 min ± 10 s incubation time at room temperature. Finally, 100 µL of the final solution was inoculated by scattering in TSA (Oxoid, Ltd., Hampshire, England) for 24 h at 37 °C.

The purpose of this "neutralization method validation" was to verify that the neutralizers corresponding to each of the biocides tested (Suma Bac D10® and Mida FOAM 193®), even in the presence of low and high interference substances, annulled the action of the biocides and did not prevent bacterial multiplication (Figure 6).

Figure 6. Example of a “neutralization method validation” plaque with *E. coli* ATCC 10536 for Suma Bac D10® after 24 hours of incubation at 37 °C.

3.4.3. MIC determination protocol

Since the protocol for assessing the MICs for Suma Bac D10® and Mida FOAM 193® was based on EN 1656:2009 (EN 1656, 2009), only the particular elements, such as the biocide itself and the neutralising agent, differed. The first stage was to prepare all of the 96-wells plates (VWR International®, Leuven, Belgium) necessary.

Only the wells of columns 5 to 12 in the first plate (designated as "Plate 1”) were filled with 160 µL of biocide, either Suma Bac D10® or Mida FOAM 193®, at the eight different tested concentrations previously mentioned. Columns 1 to 4 were, on the other hand, left blank. Figure 7 illustrated all of these indications.

The wells in columns 1 to 4 on the second plate (designated as "Plate 2") were left empty, while the wells in columns 5 to 12 were filled with 160 µL of neutralizer, one particular for Suma Bac D10® and another one for Mida FOAM 193® (as previously indicated), and 20 µL of pure sterile water.

Finally, in the case of the third plate (defined as "Plate 3"), column 1 was filled with negative control to demonstrate that no contamination occurred in the Tryptone Soya Broth (TSB) (Oxoid, Ltd., Hampshire,

England) medium employed, thus it was only filled with 200 µL of liquid TSB. Column 3's wells were filled with 180 µL of liquid TSB as a positive control to check that bacteria were present in the original suspension. Finally, columns 5 to 12 were successfully employed for MIC testing, therefore they were likewise filled with 180 µL of liquid TSB. Columns 2 and 4 had been left empty.

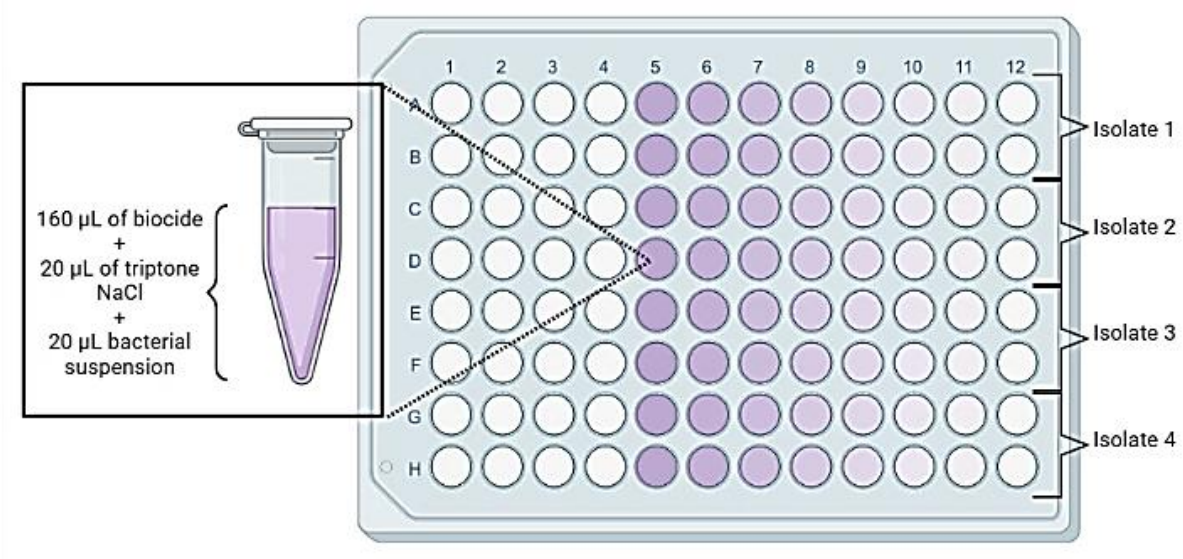

Figure 7. Model of a 96-well plate (defined as "Plate 1") with biocide, a representative for both Suma Bac D10® and Mida FOAM 193® (created with BioRender.com).
Legend: For Suma Bac D10®: column 5 – 5 %; column 6 – 4 %; column 7 – 3 %; column 8 – 2 %; column 9 – 1 %; column 10 – 0.5 %; column 11 – 0.25 %; column 12 – 0.1 %. For Mida FOAM 193®: column 5 – 15 %; column 6 – 12.5 %; column 7 – 10 %; column 8 – 7.5 %; column 9 – 5 %; column 10 – 2.5 %; column 11 – 1 %; column 12 – 0.5 %.

With all plates prepared, all bacterial suspensions of the isolates were prepared in a diluent solution at a concentration corresponding to 0.5 on the McFarland scale (~ 1.5×10^8 CFU/mL). These suspensions were prepared from 24 h bacterial cultures grown on BHI (VWR International®, Leuven, Belgium) agar medium. All MIC tests were performed at room temperature (~20 °C). Positive controls in Plate 3 were first filled with 20 µL of this bacterial suspension.

Since there were three types of conditions to be tested, it was needed to adapt the volumes of the wells in Plate 1. When evaluating MICs without interfering substances, 20 µL of sterile water were added and when testing with low or high interfering substances 20 µL of LIS or 20 µL of HIS were added to the wells.

In columns 5 to 12, the assays were performed in duplicate (A and B correspond to isolate 1, C and D to isolate 2, E and F to isolate 3, and G and H to isolate 4), so 20 µL of bacterial suspension were added to each of the wells in Plate 1, which previously contained the mixture of biocide with the sterile water or with LIS or HIS, depending on which condition it was tested. After the wells in Plate 1 were filled with the described mixture, it was incubated for the contact time instructed by the manufacturer (5 min ± 10 s for both Suma Bac D10® and Mida FOAM 193®) at room temperature, during which time it was stirred at 700 rotations per minute (rpm).

From the suspension of each well in Plate 1, 20 µL were transferred to Plate 2, which already contained the mixture of neutralizing solution and water. For the following, it was necessary to incubate at room temperature for the time indicated in the European Standard (for both neutralizers are 5 min ± 10 s), with a stirring of 700 rpm.

After Plate 2 finished its stirring time, 20 µL of suspension were removed from each well and transferred to Plate 3, resulting in a mixture of 180 µL of liquid TSB and 20 µL of the suspension from Plate 2 (a mixture of biocide, neutralising substance, purified sterile water or with LIS or HIS, and bacterial suspension), and Plate 3 was incubated for 24 h at 37 °C. Plate 3 scheme can be visualized in Figure 8.

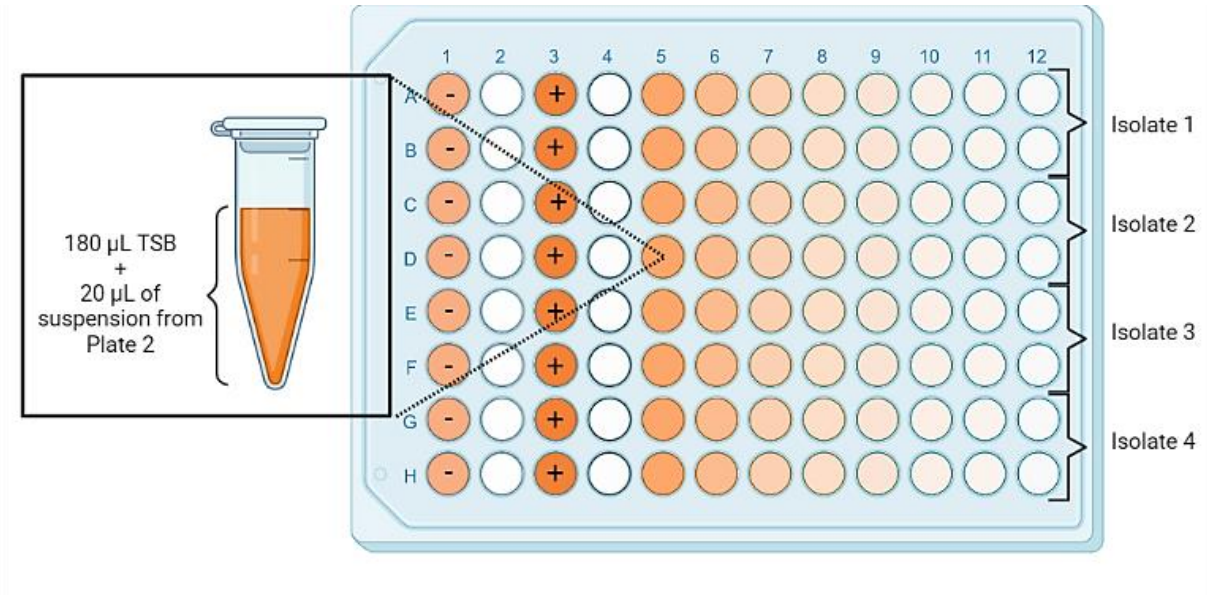

Figure 8. Representation of Plate 3 for both Suma Bac D10® and Mida FOAM 193® (created with BioRender.com). Legend: "-" - negative controls; "+" - positive controls. For Suma Bac D10®: column 5 – 5 %; column 6 – 4 %; column 7 – 3 %; column 8 – 2 %; column 9 – 1 %; column 10 – 0.5 %; column 11 – 0.25 %; column 12 – 0.1 %. For Mida FOAM 193®: column 5 – 15 %; column 6 – 12.5 %; column 7 – 10 %; column 8 – 7.5 %; column 9 – 5 %; column 10 – 2.5 %; column 11 – 1 %; column 12 – 0.5 %.

After 24 hours of incubation, it was possible to evaluate in which wells cell multiplication may have occurred. The MIC is the minimum concentration of Suma Bac D10® or of Mida FOAM 193® at which it was possible to visually verify that bacterial multiplication was inhibited. This meant that on Plate 3 there could be wells that either (i) showed opacity, which meant that bacterial multiplication had occurred and the concentration of the biocide under testing was not effective in inhibiting bacteria; or (ii) there could be translucent wells, which meant that the concentration of the biocide tested had been effective in inhibiting bacterial multiplication.

To evaluate the reproducibility, an approach based on the Clinical & Laboratory Standards Institute (CLSI) recommendations was used, in which MIC determination findings could deviate by one dilution (CLSI, 2018; Humayoun *et al*., 2018; Riesenberg *et al*., 2016). In this study, the reproducibility test was performed on 10 % of the 12 isolates tested.

For this, the random selection function of Microsoft Excel (Microsoft Corporation, Washington, USA) was used to select 2 isolates and perform the MICs in the three organic matter assays (NIS, LIS and HIS). This assay aimed to verify if the results obtained in the MICs with the selected isolates were maintained, obeying the principle that it could differentiate at ±1 concentration level of biocide tested (CLSI, 2018; Humayoun *et al*., 2018; Riesenberg *et al*., 2016) (Annex Tables 4 and 5, Annex 3).

3.4.4. MBC determination protocol

Once the MICs had been determined, the values associated with the MBC were also determined. To did so, 5 µL were taken from the wells where no bacterial multiplication had been observed and transferred onto TSA plates for 24 hours at 37 °C to assess for colony formation.

The objective of the MBC was to determine what was the minimum bactericidal concentration for each of the biocides, Mida FOAM 193® and Suma Bac D10®, at the three levels of organic matter (NIS, LIS and HIS). The spawned concentration at which it did not show bacterial multiplication would be considered the minimum bactericidal concentration or MBC of the biocide tested in that organic matter assay. This procedure can be seen in the following Figure 9.

The same 10% reproducibility assay was carried out for the MBCs, using the same 2 isolates randomly selected for the previous MIC procedure. This test was used to verify if the MBCs values previously observed for the three organic matter assays (NIS, LIS and HIS) were maintained, and could diverge in ±1 level of biocide concentration (CLSI, 2018; Humayoun *et al*., 2018; Riesenberg *et al*., 2016) (Annex Tables 4 and 5, Annex 3).

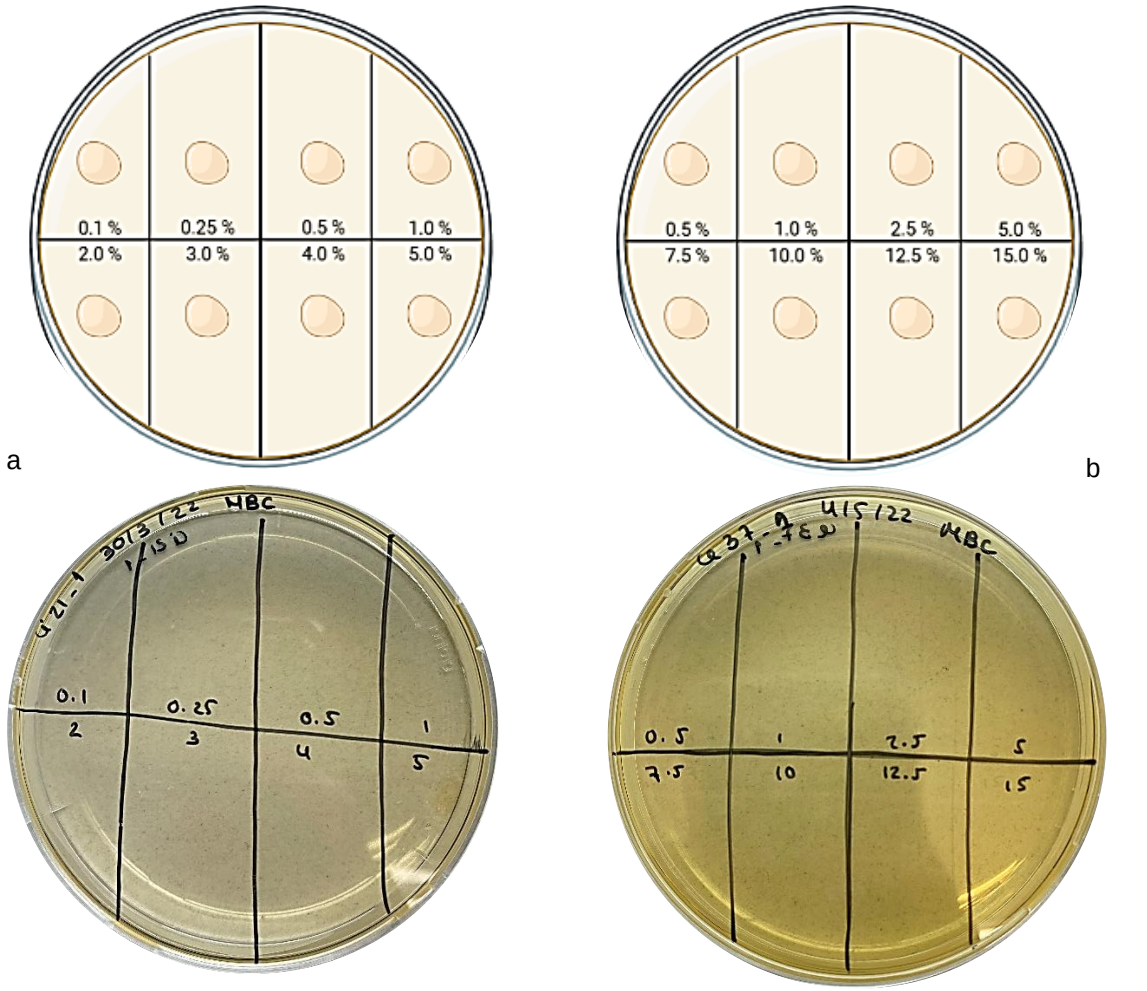

Figure 9. Example of a division of an MBC plate for Suma Bac D10® (a and c) and Mida FOAM 193® (b and d) (created with BioRender.com). In c we can see the isolate ci21 of *Salmonella* spp. into an MBC plate of Suma Bac D10®. In d we can observe the isolate ce37 of *Salmonella* spp. into an MBC plate of Mida FOAM 193®.
Legend: Each yellow drop on both plates corresponds to 5 uL of inoculation spot at concentrations where no growth occurred on the plates where MICs were performed, for the biocides Suma Bac D10® and Mida FOAM 193®.

3.5. Data analysis

All the data analysis and the graphs shown in this study were performed using Microsoft Excel® (Microsoft Corporation®, Washington, USA).

Chapter 4: Results

4.1. Biocide susceptibility genotypes of bacterial isolates

PCR amplification aimed to identify the presence of the *acrA*, *acrB* and *tolC* genes, which encoded efflux pumps, and the *qacEΔ1*, *qacE* and *qacF/H/I* genes, which are genes associated with resistance to QACs.

It was possible to determine the genotype of the 44 *Salmonella* isolates studied, together with *S. enterica* subsp. *enterica* ser. Typhimurium CECT 443 which was used as a PCR reaction control (n = 45) since its genome is known. The results obtained are shown in Table 6.

Table 6. Detection of the resistance genes *acrA*, *acrB*, *tolC*, *qacEΔ1*, *qacE* and *qacF/H/I* by PCR amplification in *Salmonella* spp. isolates and correspondent genotype (adapted from Cota *et al*. (2019)).

Sample code	Genes						Genotype	Cluster	Serotype
	acrA	*acrB*	*tolC*	*qacEΔ1*	*qacE*	*qacF/H/I*			
CECT 443	+	+	+	-	-	-	*acrA/acrB/tolC*	—	—
ci57	+	+	+	-	-	-	*acrA/acrB/tolC*	IA	Rissen
p1	+	+	+	-	-	-	*acrA/acrB/tolC*		
p3	+	+	+	-	-	-	*acrA/acrB/tolC*		
p4	+	+	+	-	-	-	*acrA/acrB/tolC*		
p5	+	+	+	-	-	-	*acrA/acrB/tolC*		
p58	+	+	+	-	-	-	*acrA/acrB/tolC*		
p116	+	+	+	-	-	-	*acrA/acrB/tolC*		
ce21	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*	IB	Rissen
ce37	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*		
ce44	+	+	+	-	-	+	*acrA/acrB/tolC/qacF/H/I*		
ci21	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*		
ci55	+	+	+	-	-	-	*acrA/acrB/tolC*		
p25	+	+	+	-	-	+	*acrA/acrB/tolC/qacF/H/I*		
p31	+	+	+	-	-	+	*acrA/acrB/tolC/qacF/H/I*		
p61	+	+	+	-	-	+	*acrA/acrB/tolC/qacF/H/I*		
p62	+	+	+	-	-	+	*acrA/acrB/tolC/qacF/H/I*		
p64	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*		
p55	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*	IIA	4,[5],12:i:-
p56	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci104	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*	IIB	4,[5],12:i:-
ci105	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci108	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci109	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci110	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci111	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci115	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci116	+	+	+	-	-	-	*acrA/acrB/tolC*		
ci117	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*		

Sample code	Genes						Genotype	Cluster	Serotype
	acrA	*acrB*	*tolC*	*qacEΔ1*	*qacE*	*qacF/H/I*			
p96	+	+	+	-	-	-	*acrA/acrB/tolC*	IIB	4,[5],12:i:-
p104	+	+	+	-	-	-	*acrA/acrB/tolC*		
p106	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*		
p107	+	+	+	-	-	-	*acrA/acrB/tolC*		
p109	+	+	+	+	-	+	*acrA/acrB/tolC/qacEΔ1/qacF/H/I*		
p110	+	+	+	-	-	-	*acrA/acrB/tolC*		
p112	+	+	+	-	-	-	*acrA/acrB/tolC*		
p114	+	+	+	-	-	-	*acrA/acrB/tolC*		
p115	+	+	+	-	-	-	*acrA/acrB/tolC*		
p118	+	+	+	-	-	-	*acrA/acrB/tolC*		
ce70	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*	III	Derby
ci68	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*		
p66	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*		
p67	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*		
p68	+	+	+	+	-	-	*acrA/acrB/tolC/qacEΔ1*		
ci38	+	+	+	-	-	+	*acrA/acrB/tolC/qacF/H/I*	ind	Rissen

Legend: "+" – positive; "-" – negative; “p” – isolates that came from the skin before scalding; “ci” – isolates that came from the internal part of the carcass; “ce” – isolates that came from the external part of the carcass.

Figure 10 shows an electrophoresis gel used for the identification of the *acrA* gene amplicons in 13 *S. enterica* subsp. *enterica* isolates, to exemplify the results obtained in Annex Table 1. The same procedure was applied to the other genes studied.

From Table 6 it could be seen that all 45 isolates evaluated were found to contain the genes that encoded efflux pumps from the complex AcrAB-TolC, *acrA*, *acrB* and *tolC* (100%).

From the same Table 6, it was possible to verify that the distribution of the resistance genes to QACs, *qacE*, *qacEΔ1* and the *qacF/H/I* complex, in the population of isolates tested (n=45) had a more varied distribution, with the *qacE* gene not being detected in any isolate (0 %).

Regarding isolates belonging to cluster IA (n = 7), all of which were *S*. Rissen, it was found that only efflux pumps encoding genes were present, but not the QACs resistance genes, and their genotype was *acrA*/*acrB*/*tolC*.

As for the isolates belonging to cluster IB (n = 10), which also belong to *S*. Rissen, this group turned out to be more heterogeneous regarding the genes for resistance to biocides, revealing three different genotypes. The most predominant genotype was *acrA*/*acrB*/*tolC*/*qacF/H/I*, present in 5 isolates (p25, p62, p61, ce44 and p 31) from this cluster. Next, the *acrA*/*acrB*/tolC/*qacEΔ1*/*qacF/H/I* genotype was present in 4 isolates of this cluster (ce37, ce21, ci21 and p64). Finally, isolate ci55 had the *acrA*/*acrB*/*tolC* genotype, being found in only one of this cluster.

As for cluster IIA (n = 2), in the two isolates that it contained and that belonged to the monophasic variant (4,[5],12:i:-) of *S. enterica* subsp. *enterica*, the genotypes diverged from each other. For isolate p55, its genotype was *acrA*/*acrB*/*tolC*/*qacEΔ1*, whereas for isolate p56 its associated genotype was *acrA*/*acrB*/*tolC*.

In cluster IIB (n = 19), where all belonged to serotype 4,[5],12:i:- of *S*. Typhimurium, there were three genotypes identified here. Most isolates in this cluster (n = 15) contained the *acrA*/*acrB*/*tolC* genotype, while 3 isolates (p106, p109 and ci104) contained the *acrA*/*acrB*/*tolC*/*qacEΔ1*/*qacF/H/I* genotype and one of them (ci117) had the *acrA*/*acrB*/*tolC*/*qacEΔ1* genotype.

As for cluster III (n = 5), in which all isolates belonged to *S*. Derby, the studied genotype was *acrA*/*acrB*/*tolC*/*qacEΔ1*.

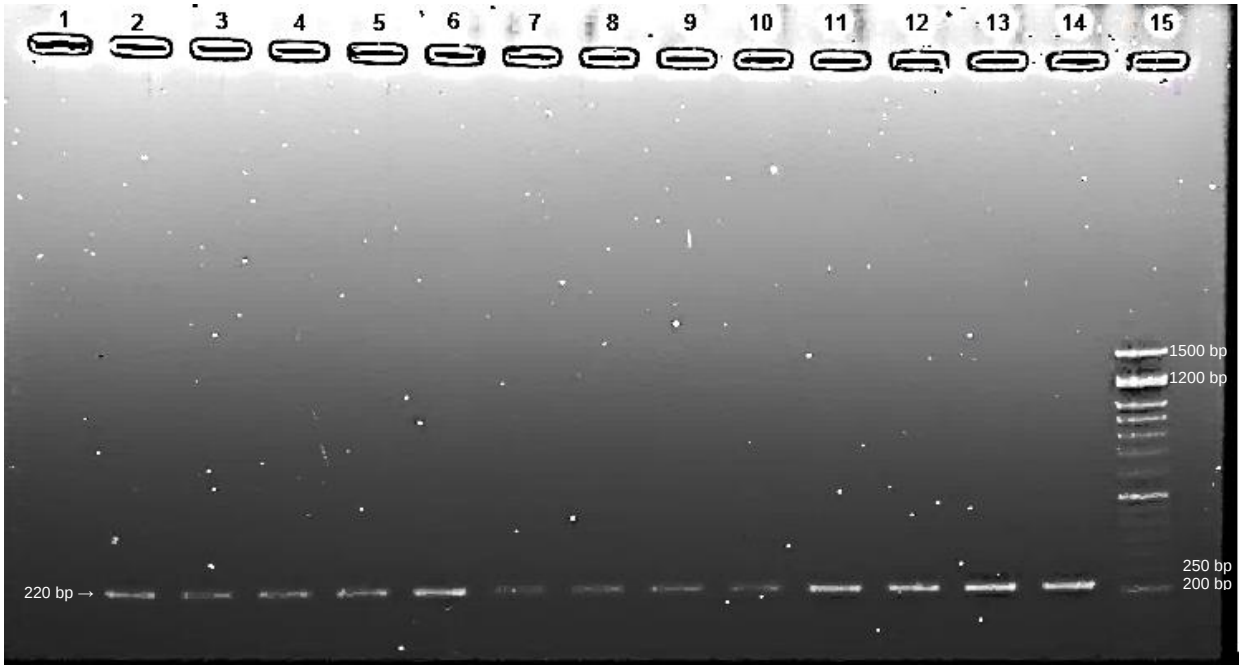

Figure 10. Detection of gene *acrA* (product size of 220 base pairs) by electrophoresis in 13 isolates of *S. enterica* subsp. *enterica*.
Legend: 1) Negative control; 2) ce37; 3) ce70; 4) p1; 5) p25; 6) p56; 7) p62; 8) p66; 9) ci38; 10) ci57; 11) ci105; 12) ci111; 13) ci115; 14) *S*. Typhimurium CECT 443; 15) Ladder VI (Nzytech®, Lisbon, Portugal).

Finally, isolate ci38, which did not fit in any cluster and which belongs to *S*. Rissen, presented the genotype *acrA*/*acrB*/*tolC*/*qacF/H/I*.

How these genotypes had higher or lower incidences concerning the set of these isolates was visible in Figure 11. It was evident that the majority of isolates in the study only harboured genes that code for efflux pumps, corresponding to 55.5% (n = 25) of the total. As for the genotypes *acrA*/*acrB*/tolC/*qacEΔ1* (n = 7) and *acrA*/*acrB*/*tolC*/*qacEΔ1*/*qacF/H/I* (n = 7), each represented 15.6% and, finally, the combination *acrA*/*acrB*/*tolC*/*qacF/H/I* (n = 6) represented 13.3% of the total isolates.

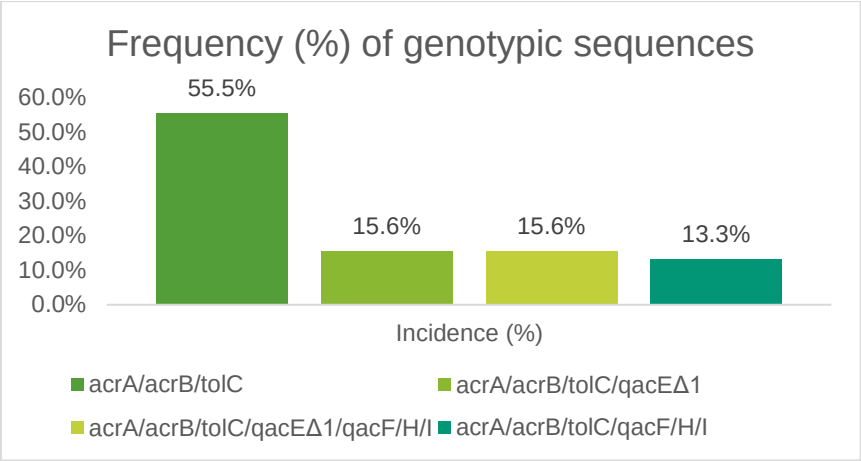

Figure 11. Frequency (percentage, %) of the genotypic sequences present in the 44 *Salmonella* isolates under study.

4.2. Determination of MICs and MBCs of bacterial isolates

Tables 7 and 8 shows the mean values obtained for MICs and MBCs for the 12 isolates of *S. enterica* subsp. *enterica* for the biocides Suma Bac D10® and Mida FOAM 193®, respectively. The results regarding these reference strains can be found in Annex 2 (Annex Tables 2 and 3).

Regarding the Suma Bac D10® biocide, either in the assays without interfering substance (NIS), with low interfering substance (LIS) or with high interfering substance (HIS), it was verified that for all *S. enterica* isolates, the MIC value obtained was the lowest of the concentrations studied (0.100 %). For the same three conditions (NIS, LIS and HIS), as the lowest concentration tested (0.100 %) had a bactericidal effect (MBC) (Table 7).

As for the MIC values for the biocide Mida FOAM 193® (Table 8), in the NIS assay, the results obtained were the lowest concentration used for this biocide (0.500 %) in all isolates tested. In the case of the value obtained for MBC, in the same condition (NIS), there were no changes compared to the MIC values (0.500 %).

In the LIS assay, the result for isolate ce37 changed in comparison with the other isolates, presenting a MIC value 1.5 times higher (0.750%) than the others, which remained at concentrations of 0.500%. As for the MBCs under these conditions, the same results were observed, with 0.750 % for the isolate ce37 and 0.500 % for the others.

Table 7. Suma Bac D10® medium values of MICs and MBCs for all *S. enterica* subsp. *enterica* isolates tested, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	Cluster	MIC (%)			MBC (%)		
		NIS	LIS	HIS	NIS	LIS	HIS
ce37	IB	<0.100	<0.100	<0.100	0.100	0.100	0.100
ci21	IB	<0.100	<0.100	<0.100	0.100	0.100	0.100
p64	IB	<0.100	<0.100	<0.100	0.100	0.100	0.100
ce21	IB	<0.100	<0.100	<0.100	0.100	0.100	0.100
p1	IA	<0.100	<0.100	<0.100	0.100	0.100	0.100
ci38	ind	<0.100	<0.100	<0.100	0.100	0.100	0.100
p55	IIA	<0.100	<0.100	<0.100	0.100	0.100	0.100
p56	IIA	<0.100	<0.100	<0.100	0.100	0.100	0.100
ci104	IIB	<0.100	<0.100	<0.100	0.100	0.100	0.100
ci117	IIB	<0.100	<0.100	<0.100	0.100	0.100	0.100
p109	IIB	<0.100	<0.100	<0.100	0.100	0.100	0.100
ce70	III	<0.100	<0.100	<0.100	0.100	0.100	0.100
ci117	IIB	<0.100	<0.100	<0.100	0.100	0.100	0.100
p109	IIB	<0.100	<0.100	<0.100	0.100	0.100	0.100
ce70	III	<0.100	<0.100	<0.100	0.100	0.100	0.100
$\bar{x}$		0.100	0.100	0.100	0.100	0.100	0.100
σ		0.000	0.000	0.000	0.000	0.000	0.000

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; $\bar{x}$ – medium MIC and MBC value for each group; σ – corresponding standard deviation of each population; "p" – isolates that came from the skin before scalding; "ci" – isolates that came from the internal part of the carcass; "ce" – isolates that came from the external part of the carcass.

Finally, for the HIS assays, the MIC values were variable. The lowest observable MIC value corresponded to *S. enterica* isolates ci38 and ci117 with a value of 0.500 %, while isolates p1, p55, p56, p109 and ce70 had a value of 1.000 % and, finally, ce37, ci21, p64, ce21 and ci104 presented a value of 2.500 %. Concerning the MBC values for this assay, the results also varied depending on the isolates. The isolates ci38 and ci117 had an MBC value greater than the MIC value, increasing 1.5 times (0.750 %) and 2 times (1.000 %), respectively, as did the isolate ce70 which increased the MBC value 1.75 times compared to the MIC value (1.750 %). The remaining isolates, ce37, ci21, p64, ce21, p1, p55, p56, ci104 and p109, maintained the same MIC values in MBCs (Table 8).

Table 8. Mida FOAM 193® medium values of MICs and MBCs for all *S. enterica* subsp. *enterica* isolates tested, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	Cluster	MIC (%)			MBC (%)		
		NIS	LIS	HIS	NIS	LIS	HIS
ce37	IB	<0.500	0.750	<2.500	0.500	0.750	2.500
ci21	IB	<0.500	<0.500	<2.500	0.500	0.500	2.500
p64	IB	<0.500	<0.500	<2.500	0.500	0.500	2.500
ce21	IB	<0.500	<0.500	<2.500	0.500	0.500	2.500
p1	IA	<0.500	<0.500	<1.000	0.500	0.500	1.000
ci38	ind	<0.500	<0.500	0.500	0.500	0.500	0.750
p55	IIA	<0.500	<0.500	<1.000	0.500	0.500	1.000
p56	IIA	<0.500	<0.500	<1.000	0.500	0.500	1.000
ci104	IIB	<0.500	<0.500	<2.500	0.500	0.500	2.500
ci117	IIB	<0.500	<0.500	0.500	0.500	0.500	1.000
p109	IIB	<0.500	<0.500	<1.000	0.500	0.500	1.000
ce70	III	<0.500	<0.500	1.000	0.500	0.500	1.750
$\bar{x}$		0.500	0.521	1.542	0.500	0.521	1.667
σ		0.000	0.069	0.828	0.000	0.069	0.738

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; $\bar{x}$ – medium MIC and MBC value for each group; σ – corresponding standard deviation of each population; “p” – isolates that came from the skin before scalding; “ci” – isolates that came from the internal part of the carcass; “ce” – isolates that came from the external part of the carcass.

An evaluation of the averages regarding MICs and MBCs of the biocides Suma Bac D10® and Mida FOAM 193® in relation to the three levels of organic matter used in the trials (NIS, LIS and HIS) was carried out and are represented in Figures 12 and 13, respectively.

Regarding the biocide Mida FOAM 193®, the results of the MICs and MBCs averages had variations throughout the various levels of interfering substances. In the case of the NIS test, the mean MIC and MBC values were equal to each other (0.500 %). For the LIS assay, it was found that the mean MIC values increased by 1.042 times from the previous assay (0.521 %). Finally, for the HIS assay, the mean MIC and MBC values were higher than in the previous assay, with the MIC value increasing 2.959 times (1.542 %) and the mean MBC value increasing 3.199 times (1.667 %) when compared with the mean values of the LIS assay.

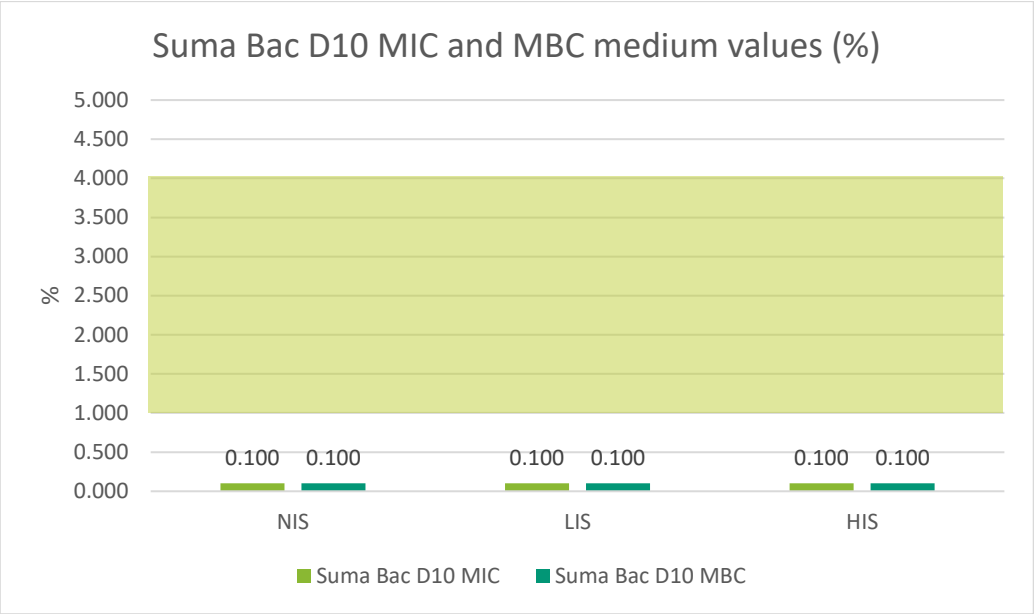

Figure 12. Average minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) values obtained for Suma Bac D10® for the low interference substance (NIS), low interference substance (LIS) and high interference substance (HIS) assays (percentage, %).

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimal Inhibitory Concentration; MBC – Minimal Bactericidal Concentration; Green block – concentration indicated by the fabricant (1.000 – 4.000 %).

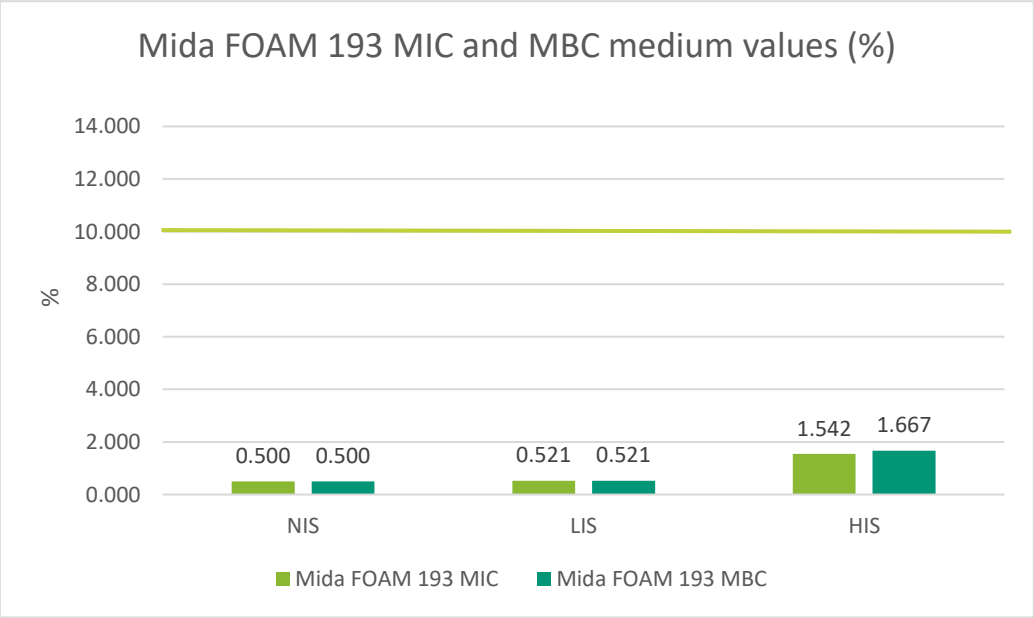

Figure 13. Average minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) values obtained for Mida FOAM 193® for the low interference substance (NIS), low interference substance (LIS) and high interference substance (HIS) assays (percentage, %).

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimal Inhibitory Concentration; MBC – Minimal Bactericidal Concentration; Green line – concentration indicated by the fabricant (10.000 %).

To check the reproducibility of the results, both for MICs and MBCs, using the same conditions for the biocides Mida FOAM 193® and Suma Bac D10®, 10% of the total isolates tested were chosen (CLSI, 2018; Humayoun *et al*., 2018; Riesenberg *et al*., 2016). The results obtained in these reproducibility tests confirmed the results obtained in the MICs and MBCs performed for the two biocides in the three different organic matter conditions (NIS, LIS and HIS), since it obeys the CLSI rule (CLSI, 2018). The results obtained for these replicates can be found in Annex 3 (Annex Tables 4 and 5).

In general, for Suma Bac D10®, there was no variability in the results obtained in the three conditions for MICs and MBCs, maintaining the value of the minimum concentration tested for both cases (0.100 %).

In the case of the biocide Mida FOAM 193®, the results determined for the three tests were in agreement with the results previously tested.

4.3. Relationship between biocide susceptibility genotypes and phenotypes determined by MBCs

Tables 9 and 10 gather the genotypic information previously determined by PCR for the 12 isolates of *S. enterica* subsp. *enterica* selected together with the mean values determined for the MBCs of the biocides Suma Bac D10® and Mida FOAM 193®, respectively. In addition, it also contains the serotypes that each isolate belongs to and the clustering that was previously determined in the study conducted by Cota *et al*., 2019.

Overall, for the 44 isolates of *S. enterica* subsp. *enterica* all these isolates were found to contain the *acrA*, *acrB* and *tolC* genes, however, the detection of the *qacEΔ1*, *qacE* and the *qacF/H/I* gene complex was more variable (Table 6).

For the 12 isolates screened for susceptibility to Suma Bac D10® biocide and whose susceptibility can be related to the *qac* genes, there were different genotypes present. Six of these 12 isolates contained the *qacEΔ1/qacF/H/I* genotype (ce37, ci21, p64, ce21, ci104 and p109), and three contained only the genotype associated with the *qacEΔ1* gene (p55, ci117 and ce70) and one contained only the genotype associated with the *qacF/H/I* gene complex (ci38). QAC resistance genes were not detected in isolates p1 and p56. Independently of the genotype determined for these isolates, it could be verified that the mean values determined for the MBCs, in the three conditions (NIS, LIS and HIS), did not change, maintaining the lowest value of biocide concentration tested (0.100 %). All these results could be seen in Table 9.

The same 12 isolates tested for susceptibility to the biocide Mida FOAM 193®, which decreased susceptibility could be associated with the genes belonging to the AcrAB-TolC complex, all contained the genotype *acrA/acrB/tolC* (Table 10). However, the mean MBC values determined for the three conditions (NIS, LIS and HIS) varied. In the NIS and LIS conditions, the MBC values remained at 0.500

%, which was the lowest concentration of biocide tested, except for isolate ce37 in which the MBC value was 0.750 % in the LIS conditions. In the higher organic matter setting (HIS), the mean values of associated MBCs ranged between 0.750 % and 2.500 %, being higher than in the other previous conditions.

Table 9. Association between the genotypic sequences determined by PCR for 12 isolates of *S. enterica* subsp. *enterica* and the medium values determined for the MBCs of the biocide Suma Bac D10®, under the conditions of no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	Serotype	Cluster	Genotype sequence	MBC (%)		
				NIS	LIS	HIS
ce37	Rissen	IB	*qacEΔ1/qacF/H/I*	0.100	0.100	0.100
ci21	Rissen	IB	*qacEΔ1/qacF/H/I*	0.100	0.100	0.100
p64	Rissen	IB	*qacEΔ1/qacF/H/I*	0.100	0.100	0.100
ce21	Rissen	IB	*qacEΔ1/qacF/H/I*	0.100	0.100	0.100
p1	Rissen	IA	–	0.100	0.100	0.100
ci38	Rissen	ind	*qacF/H/I*	0.100	0.100	0.100
p55	4,[5],12:i:-	IIA	*qacEΔ1*	0.100	0.100	0.100
p56	4,[5],12:i:-	IIA	–	0.100	0.100	0.100
ci104	4,[5],12:i:-	IIB	*qacEΔ1/qacF/H/I*	0.100	0.100	0.100
ci117	4,[5],12:i:-	IIB	*qacEΔ1*	0.100	0.100	0.100
p109	4,[5],12:i:-	IIB	*qacEΔ1/qacF/H/I*	0.100	0.100	0.100
ce70	Derby	III	*qacEΔ1*	0.100	0.100	0.100

Legend: MBC – Minimum Bactericidal Concentration; NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; “p” – isolates that came from the skin before scalding; “ci” – isolates that came from the internal part of the carcass; “ce” – isolates that came from the external part of the carcass.

Table 10. Association between the genotypic sequences determined by PCR for 12 isolates of *S. enterica* subsp. *enterica* and the medium values determined for the MBCs of the biocide Mida FOAM 193®, under the conditions of no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	Serotype	Cluster	Genotype sequence	MBC (%)		
				NIS	LIS	HIS
ce37	Rissen	IB	*acrA/acrB/tolC*	0.500	0.750	2.500
ci21	Rissen	IB	*acrA/acrB/tolC*	0.500	0.500	2.500
p64	Rissen	IB	*acrA/acrB/tolC*	0.500	0.500	2.500
ce21	Rissen	IB	*acrA/acrB/tolC*	0.500	0.500	2.500
p1	Rissen	IA	*acrA/acrB/tolC*	0.500	0.500	1.000

Sample code	Serotype	Cluster	Genotype sequence	MBC (%)		
				NIS	LIS	HIS
ci38	Rissen	ind	*acrA/acrB/tolC*	0.500	0.500	0.750
p55	4,[5],12:i:-	IIA	*acrA/acrB/tolC*	0.500	0.500	1.000
p56	4,[5],12:i:-	IIA	*acrA/acrB/tolC*	0.500	0.500	1.000
ci104	4,[5],12:i:-	IIB	*acrA/acrB/tolC*	0.500	0.500	2.500
ci117	4,[5],12:i:-	IIB	*acrA/acrB/tolC*	0.500	0.500	1.000
p109	4,[5],12:i:-	IIB	*acrA/acrB/tolC*	0.500	0.500	1.000
ce70	Derby	III	*acrA/acrB/tolC*	0.500	0.500	1.750

Legend: MBC – Minimum Bactericidal Concentration; NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; “p” – isolates that came from the skin before scalding; “ci” – isolates that came from the internal part of the carcass; “ce” – isolates that came from the external part of the carcass.

Chapter 5: Discussion and Conclusions

5.1. Discussion

5.1.1. Biocide susceptibility genotypes of bacterial isolates

The control of pathogens, such as *Salmonella* spp., within abattoirs, is of supreme importance, and this is only possible through good hygiene practices (Hill *et al*., 2016). To this end, the use of biocides becomes an essential part of this process, as these are important for the reduction or elimination of pathogens (Geraldes *et al*., 2021), particularly in food processing environments such as slaughterhouses.

Some genes are associated with the resistance of *Enterobacteriaceae*, such as *Salmonella* strains, to biocides. This resistance may be dependent on genes encoding efflux pumps, such as *acrA*, *acrB* and *tolC* (Chowdhury *et al*., 2019), as well as the genes responsible for resistance to QACs, such as *qacEΔ1*, *qacE* and *qacF/H/I* genes (Zhang *et al*., 2016; Zou *et al*., 2014), so in the present study, presence or absence of these genes in isolates of *S. enterica* was evaluated. The results corresponding to these genes can be seen in Table 6.

The presence of the genes *acrA*, *acrB* and *tolC* in all of the isolates examined (100%) was consistent with the fact that these genes are present on their chromosomes and were constitutively expressed since the proteins its express are components of the cell membrane structure itself (Chowdhury *et al*., 2019).

Furthermore, as all the genotypes determined, which were *acrA/acrB/tolC/qacEΔ1*, *acrA/acrB/tolC/qacEΔ1/qacF/H/I* and *acrA/acrB/tolC/qacF/H/I*, which together accounted for 44.5 % (15.6 %, 15.6 % and 13.3 % respectively), contain these three genes, which could participate in a mechanism of action of the bacterial cell against biocides. Indeed, in previous studies, such as those by Mangalappalli-Illathu *et al*. (2008) and Weston *et al*. (2018) it had been shown that in order to occur susceptibility to biocides, there must be upstream repressors of the AcrAB-TolC complex on *Salmonella* cells when exposed to biocides.

As for QACs resistance genes, the *qacE* gene was not present in any of the studied isolates (Table 6), since genes of this type were found in mobile genetic elements, such as plasmids, which is following previous studies that also did not detect the presence of the same gene (Chuanchuen *et al*., 2007; Kücken *et al*., 2000; Paulsen *et al*., 1993; Zou *et al*., 2014).

Furthermore, according to the study by Paulsen and his colleagues (Paulsen *et al*., 1993) and later confirmed by the study of Kazama *et al*. (1999), it was possible to verify that the *qacEΔ1* gene was a gene that is derived from *qacE*, being a mutant version of this last one. Just as happened in this work, in which it was not detect the presence of *qacE* in any of the studied isolates, in the research conducted by Chuanchuen *et al*. (2007), Kücken *et al*. (2000), Zhang *et al*. (2016) and Zou *et al*. (2014) the

identification of this gene did not occur or occurred in such particular cases that did not correspond to a significant sample of the population tested.

This was because the *qacE* gene belongs to class 1 integrons, which were mobile genetic elements but had two conserved regions at 3' and 5' and a variable region compared to a cassette integrated therein (Kücken *et al*., 2000). As the *qacEΔ1* gene was a mutated, derived version of *qacE*, the latter was not commonly detected (Chuanchuen *et al*., 2007).

Regarding the work of Zou *et al*. (2014), these genes are commonly found in *Enterobacteriaceae*, including *Salmonella* spp., which could impair the use of QACs biocides as an effective measure for decontaminating surfaces. On the other hand, in the studies by Chuanchuen *et al*. (2007), Kücken *et al*. (2000) and Zhang *et al*. (2016), it was shown that *qac* genes were not widespread, and in the present study, only 20 of 45 isolates contained these genes (Table 6 and Figure 11), which could lead to changes in the susceptibility to biocides containing QACs, so it should be interesting to verify how these isolates perform when exposed to other biocides similar to Suma Bac D10®.

The same thing happens for the *qacF/H/I* gene complex, it is found in mobile genetic elements (Wu *et al*., 2015; Zou *et al*., 2014) and had a fairly high degree of similarity with the *qacE* gene (Kazama *et al*., 1998; Ploy *et al*., 1998). Similar to the *qacF* gene, the *qacH* and *qacI* genes belonged to class 1 integrons and were therefore mobile genetic elements, their incidence being consequently very variable in gram-negative bacteria, namely *Enterobacteriaceae* (Hegstad *et al*., 2010; Jiang *et al*., 2017; Kampf, 2018).

The primers used to detect the presence of *qacF*, *qacH* and *qacI*, did not allow to perceive their isolated occurrence, and so, it will be necessary, in the future, to resort to an identification of which gene was present in the isolates that had been tested positive for this complex, which in this case concerns 28.9 % of the isolates with the genotypes *acrA/acrB/tolC/qacEΔ1/qacF/H/I* and *acrA/acrB/tolC/qacF/H/I* (Figure 11). Besides, as shown by Hegstad *et al*. (2010), the *qacH* gene is usually associated with clinical strains of *Staphylococcus aureus*, which is a gram-positive bacterium, so it should been checked if in the *qacF/H/I* complex that was detected the *qacH* gene would indeed be present since this isolates were from *S. enterica*, which was a gram-negative bacterium, and collected from apparently healthy animals sent for slaughter.

The distinction by sequencing between *qacF*, *qacH* and *qacI* genes, although interesting to be detected in the future, was not part of the methods stipulated for the present work.

Although the entire population of *S. enterica* (n= 44) was tested to verify if the genes that were present could act against the biocides studied, the susceptibility study was only performed on a selection of those (n= 12) to verify which phenotype they would present.

5.1.2. Determination of MICs and MBCs of bacterial isolates and their relationship between biocide susceptibility genotypes and phenotypes determined by MBCs

One of the ways in which possible contamination of pig carcasses by *S. enterica* can be prevented in a slaughterhouse is through good hygiene practices and disinfection programs, and it was necessary to make this assessment in the case of the Portuguese abattoir by determining the MICs and MBCs of the different biocide formulations that could be used in these structures.

The establishment of the optimum conditions for the application of biocides is important because it allows the prediction of the possible interactions that existed between their concentrations and the bacterial activity, through the establishment of concentration threshold values of biocides and the way it can affect the susceptibility of bacteria (Geraldes *et al*., 2021). Although there are no breakpoints for biocides such as there are for antimicrobials, namely as gathered by CLSI (CLSI, 2018), this did not imply a total absence of them, since biocides need to have an optimum use concentration to be approved and placed on the market, and this value is used as a reference.

The first considerations for the discussion according to the results obtained were for the Suma Bac D10® biocide and then for the Mida FOAM 193® biocide.

For the three tested conditions of organic matter, absent, low and high (NIS, LIS and HIS), concerning the biocide Suma Bac D10®, the *S. enterica* isolates tested showed high susceptibility to that biocide. This was indicated by the MIC and MBC medium values remaining unchanged throughout the three assays at the lowest tested concentration of Suma Bac D10® (0.100 %), as could be seen in Table 7 and Figure 12. Thus, it did not appear that as organic matter increases the susceptibility of the isolates to the biocide decreases since the results remained constant throughout the experiments.

One of the possible reasons for obtaining this type of results in the Suma Bac D10® biocide assays is related to the fact that the *S. enterica* isolates studied, being gram-negative bacteria, contained a layer of lipopolysaccharides (LPS) on the outside of its plasma membrane (Denyer & Maillard, 2002; J.-Y. Maillard, 2018; Poole, 2002; Russell, 2003). This can lead to a higher susceptibility to Suma Bac D10®, because it contributes for the disintegration of the plasma membrane through its interaction with it, resulting in the escape of the entire cytoplasmic contents of *S. enterica* to the exterior (McBain *et al*., 2004).

Nevertheless, in the studies conducted by Møretrø *et al*. (2012) and by Kampf (2018), it was found that *Salmonella* spp. isolates, when routinely exposed to QAC-based disinfectants, such as Suma Bac D10®, could result in the acquisition of some type of resistance due to the characteristics of the biocide, but also by prolonged exposure to subinhibitory concentrations.

The presence of QAC resistance genes, such as the *qacEΔ1* gene and the *qacF/H/I* gene complex, on the isolated strains tested is variable (Table 6) through the mean values of the minimum bactericidal

concentration in 12 isolates tested (Table 9) did to follow the same pattern. The presence of some of these genes in 10 of 12 *S. enterica* isolates, either alone or in combination, did not seem to influence the susceptibility of the isolates to the tested biocide formulation. However, contrary to the results obtained in the present study, the studies by Zhang *et al*. (2016) and Zou *et al*. (2014) indicate that resistance genes to QACs, such as the *qacEΔ1* gene and the *qacF/H/I* gene complex, seemed to suggest that their presence could lead to altered susceptibility or even resistance to QAC-based formulations, such as the biocide Suma Bac D10®.

Although the genes *qacEΔ1* and the *qacF/H/I* complex had been tested, studies show that there was a relationship between tolerance to QAC-based formulations, such as Suma Bac D10®, and the expression of the efflux system AcrAB-TolI, which is dependent on the expression of the genes *acrA*, *acrB* and *tolC*, in *S. enterica* isolates (Guo *et al*., 2014; Karatzas *et al*., 2007; Møretrø *et al*., 2012). As expected, our results confirm the presence of the genes *acrA*, *acrB* and *tolC* (Tables 6 and 10), but the impact of the AcrAB-TolC complex expression pathway on the susceptibility to biocides in *S. enterica* needed to be further studied.

Next, the discussion of the results obtained with MIC and MBC concerning Mida FOAM 193® biocide is presented. Similarly, to the previous biocide, the evaluation of Mida FOAM 193® was made for three different contact conditions with organic matter, absent, low and high (NIS, LIS and HIS), to verify if there was low or high susceptibility to this biocide, as shown in Table 8 and Figure 13.

In the case of the absence of organic matter tested (NIS) and very similar to what happened with the other biocide tested, there were no changes in the MIC and MBC values. In the test with LIS, it was found that, overall, both MIC and MBC did not change, except for the isolate of *S. enterica* ce37 (*S.* Rissen), which seemed to be less susceptible, with the lowest concentration of the biocide not being effective, but slightly above it (0.750 %), both for MIC and MBC. Finally, in the HIS assay, it was found that organic matter may influence the results regarding the MICs and MBCs values obtained, in agreement with previous studies (Aryal & Muriana, 2019; Veasey & Muriana, 2016), since there was lower susceptibility to the biocide tested at concentration values lower than 2.500 % (4 times lower than the 10 % concentration indicated by the manufacturer), and on average the MIC value was relatively lower than the MBC value, with 1.542 % and 1.667 % respectively (Table 8 and Figure 13).

The isolate ce37 (*S.* Rissen), in the LIS assay, showed an average value of 0.750 % (Table 8), for MIC and MBC. The fact that it showed a value between two tested concentrations (0.500 % and 1.000 %) allows the difference between a bacteriostatic and a bactericidal formulation to be verified, and normally a bacteriostatic effect is a result of the use of a biocidal formulation in low concentrations (Maillard, 2002). As the MBC had the same value as the MIC, it was found that this concentration of chlorine-based biocide formulation (Mida FOAM 193®) had bactericidal activity on the isolate ce37.

Contrary to the biocide tested previously, it was found that here the increase in the organic matter could have implications on the efficacy of the biocide Mida FOAM 193® on the *S. enterica* isolates tested since it went from a total susceptibility to the biocide in the absence of organic matter (NIS) at very low

concentrations of the biocide formulation to a partial tolerance at the same biocide concentration but with high organic matter levels (HIS).

According to the same studies carried out by Cavalli *et al*. (2018) and Maillard (2005, 2013), several factors are indicated that could contribute to the strong influence of organic matter, such as the formation of a protective barrier around the bacteria, the formation of bacteria aggregates and the neutralization of the biocide, reducing its availability in the environment. Furthermore, as it was possible to verify through the results obtained in contact with HIS, in which only with concentrations lower than 2.500 % of Mida FOAM 193®, there was the lower susceptibility of the *S. enterica* isolates tested, it was found to be in line with the study conducted by Marriott *et al*. (2018) in which the higher the concentration of chlorine-based biocide the more likely it was to act as an antimicrobial, because it affected the membranes of the bacteria cells, caused damage at the DNA level, inhibited synthesizing proteins, oxidized respiratory components of cells or even acted several of these factors at the same time.

Another aspect to consider based on our results is related to the presence of possible biocide resistance genes, in this case, linked to efflux pumps, such as those of the AcrAB-TolC complex. In Table 10, it can be seen that all 12 isolates of *S. enterica* tested contained the genotype *acrA/acrB/tolC*, thus indicating that it contained the efflux pumps of the AcrAB-TolC complex. The fact that all these isolates contained this genotype was indicative, as above, that these genes were constitutively expressed and its synthesised proteins were part of the plasma membrane itself (Chowdhury *et al*., 2019). This efflux pump mechanism has as its main objective to pump harmful components out of the cells, even if these components are not specific (Fraise *et al*., 2012), as is the case of the biocide Mida FOAM 193®. As indicated in the studies by Bailey *et al*. (2008), Lawler *et al*. (2013) and Ricci & Piddock, 2009), later reviewed by Weston *et al*. (2018), when *Salmonella* is exposed to a biocide formulation an increase in *ramA* expression occurs. As the RamA protein is responsible for activating *acrAB* and *tolC*, then when its production increases there is an increase in the expression of the efflux pumps of the AcrAB-TolC complex, leading to *Salmonella* being MDR.

When the tested isolates of *S. enterica* were in contact with Mida FOAM 193® with none or little organic matter (NIS and LIS assays), it was found that there was a high susceptibility to the biocide (Tables 8 and 10 and Figure 13). As the *acrA/acrB/tolC* genes were present (Table 10), it would be expected that expulsion of the biocide Mida FOAM 193® out of the cell through the efflux pumps of the AcrAB-TolC complex would occur (Fraise *et al*., 2012), however, this did not happen under the conditions studied.

In contrast, concerning the HIS assay, it was verified that at concentrations of 2.500 %, this combination of mechanisms could be effective since lower susceptibility from the isolates was observed (Tables 8 and 10 and Figure 13). However, at higher concentrations it was still found that the isolates had susceptibility to the biocide, so even this high amount of organic matter could not be sufficient to act as protection at high concentrations of Mida FOAM 193® (> 2.500 %) nor could the efflux pump mechanism belonging to the AcrAB-TolC complex be able to effectively expel the higher concentrations of biocide.

Lastly, it was relevant to indicate that both Suma Bac D10® and Mida FOAM 193®, according to the European Chemicals Agency (ECHA) categories, are disinfectants for food and feed areas (PT04), so these have a mode of action for a large spectrum of microorganisms and bactericidal activity (Christeyns, 2014; Diversey Inc., 2019; European Chemicals Agency, 2018; Stull *et al*., 2018), namely bacteria such as *S. enterica*. Because Suma Bac D10® and Mida FOAM 193® could be used on slaughterhouse surfaces and equipment, it is important that it not only meets European safety and hygiene regulations (Regulation (EC) No 853/2004, 2004; Regulation (EC) No 854/2004, 2004; Commission Regulation (EU) 2021/382, 2021) but also retains its stability without losing efficacy (European Chemicals Agency, 2018), given that the products have been pre-tested and approved for market entry and had its indications for use.

According to the results of the biocide Suma Bac D10® (Table 7 and Figure 12), it could be seen that there was always susceptibility to the biocide by the tested *S. enterica* isolates, since the concentration remained at the lowest (0.100 %) in MBCs and lower in the MIC (< 0.100 %), regardless of the amount of organic matter (NIS, LIS and HIS). However, the same was not true for the results of the biocide Mida FOAM 193® (Table 8 and Figure 13), since, when in the presence of HIS, there was lower susceptibility to the biocide at lower concentrations (< 2.500 %), which could eventually resulted in the misapplication of Mida FOAM 193® (European Chemicals Agency, 2018).

Furthermore, the study initiated by Cota *et al*. (2019) contributed to the knowledge that the implementation of GHPs in the abattoir is important to prevent potential *Salmonella* contamination in the abattoir, as well as that the distribution and persistence of this pathogen in the abattoir is differentiated according to serovars. The present study, a follow-up of the study indicated above, contributed to verify that, regardless of the location of collection of isolates in the abattoir and the serovars identified, the biocides tested are effective in eliminating *S. enterica* isolates collected from pigs slaughtered from the abattoir. Moreover, both biocides demonstrated their efficacy in inhibitory and bactericidal action to the tested *S. enterica* isolates at various levels of organic matter.

These findings pointed out the importance of using biocides at concentrations that effectively eliminate *S. enterica* from the contaminated surfaces, using adequate biocide formulations, namely QAC-based compounds (such as Suma Bac D10®) or chlorine-based formulations (such as Mida FOAM 193®), as the susceptibility was observed even when exposed to concentrations markedly below the recommended by the manufacturers.

5.2. Conclusions

Both Suma Bac D10® (QAC-based formulation) and Mida FOAM 193® (chlorine-based formulation) appeared to be efficient in the elimination of the *S. enterica* isolates tested that were previously collected from pig carcasses in a Portuguese abattoir, and no phenotypic resistances were detected.

From the genotypes determined and associated with a susceptibility study to the biocide Suma Bac D10®, it was found that the presence of the *qacEΔ1* genes and the *qacF/H/I* gene complex did not

appear to produce phenotypic effects on the *S. enterica* isolates tested when exposed to the previously indicated formulation under the three conditions studied in the assays (NIS, LIS and HIS), since there were no changes in the results throughout the assays concerning this biocide.

The three levels of organic matter tested (NIS, LIS and HIS) did not influence the susceptibility of *S. enterica* isolates to the biocide Suma Bac D10®, maintaining the same bacterial concentration throughout the experiments, remaining below the concentration range of 1 – 4 % recommended by the manufacturer. In the case of the biocide Mida FOAM 193®, there was a lower susceptibility to the biocide on the isolates tested in the assay with high organic matter, verified by the increase of the MBC values in this assay, even though it remained below the concentration of 10% recommended by the manufacturer.

5.3. Future perspectives

Further studies should be carried out to verify whether the susceptibility profile of the biocide's chlorine-based and QAC-based formulation is maintained when *Salmonella* isolates are exposed to subinhibitory concentrations, especially when high levels of organic matter were present. It would also be relevant to determine the number of generations and the conditions required to reverse the resistance phenotype if indeed it existed.

It would also be important to perform the testing of these biocide formulations against *Salmonella* spp. in a biofilm form, and also for other pathogens prevalent in the slaughterhouse environment, as well as the determination of efficiency factors for these already studied formulations. Another relevant aspect was to establish the antimicrobial resistance profile and to determine a possible multidrug resistance profile for several genera of pathogens prominent in abattoirs, to establish a relationship with the biocide resistance profile.

Finally, since there was a lack of documentation on the optimal conditions of application of biocides according to the different stages of bacterial cells, as well as the fact that there were no breakpoint values for the application of these biocides (except those indicated by manufacturers for their application), as there were for antimicrobials, it would be relevant to have a study that could aggregate this information, at least concerning slaughterhouses in Portugal.

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Chapter 6: Annexes

Annexe 1

DNA quantification of bacterial isolates

DNA quantification was performed on a total of 49 bacterial isolates, which are divided into 44 isolates of *S. enterica* subsp. *enterica* from the slaughterhouse and 5 isolates of control strains, such as *S. enterica* subsp. *enterica* CECT 443, *P. aeruginosa* ATCC 15442, *E. coli* ATCC 10536, *E. hirae* ATCC 10541 and *S. aureus* ATCC 6538, where the latter 4 isolates are controls stipulated by EN 1656 (EN 1656, 2009). The isolates of *S. enterica* subsp. *enterica* from slaughterhouses in Portugal are indicated by the code's "p", "ci" and "ce" as explained above. The data concerning the quantification of these isolates are stipulated in Annex Table 1.

Annex Table 1. DNA quantification (ng/µL) of all isolates under study.

Sample code	DNA quantification (ng/µL)	Sample code	DNA quantification (ng/µL)
p1	759.9	**p96**	749.6
p3	999.7	**p104**	754.9
p4	809.7	**ci104**	948.8
p5	733.3	**ci105**	755.8
ce21	765.6	**p106**	613.1
ci21	611.0	**p107**	860.9
p25	677.2	**ci108**	798.4
p31	1198.2	**p109**	1080.2
ce37	743.2	**ci109**	763.4
ci38	704.0	**p110**	1096.6
ce44	580.2	**ci110**	755.3
p55	1063.0	**ci111**	798.8
ci55	627.8	**p112**	963.1
p56	619.5	**p114**	740.3
ci57	759.2	**p115**	978.2
p58	744.3	**ci115**	717.6
p61	702.4	**p116**	838.5
p62	508.6	**ci116**	960.7
p64	839.3	**ci117**	878.0
p66	693.4	**p118**	973.1
p67	1086.6	***S. enterica* CECT 443**	893.9
p68	753.6	***P. aeruginosa* ATCC 15442**	1407.2
ci68	804.0	***E. coli* ATCC 10536**	769.1
ce70	683.1	***E. hirae* ATCC 10541**	920.0
		***S. aureus* ATCC 6538**	430.6

Legend: "p" - means skin in Portuguese, being these isolates taken from the pig carcass skin; "ci" - means internal part of the carcass in Portuguese, which is the place from where the samples were taken; "ce" - means external part of the carcass in Portuguese, so the samples were taken from that area of the carcass.

Overall, the DNA extractions from each of the isolates (n = 49) highlighted here went quite well, so the extraction method worked. This is demonstrated by the fact that the DNA quantification for the isolates had a minimum value of 430.6 ng/µL (*S. aureus* ATCC 6538) and a maximum value of 1407.2 ng/µL (*P. aeruginosa* ATCC 15442).

From Annex Table 1, it is possible to divide the results of DNA quantification into five categories: 0 – 400 ng/µL, 401 – 700 ng/µl, 701 – 900 ng/µL, 901 – 1100 ng/µL and 1101 – 1500 ng/µL. Thus, we found that none of the isolates had a DNA extraction that fell into the first category, but this has already happened in the categories with higher DNA concentrations.

For the 401 – 700 ng/µL category, we verified that 20.4 % of the isolates (n = 10) are present in this, with values between 430.6 ng/µL, which had previously been indicated as belonging to *S. aureus* ATCC 6538, and 693.4 ng/µL, which belongs to the *Salmonella* p66 isolate.

The subsequent category, which is in the range 701 – 900 ng/µL, has the most isolates, corresponding to 53.1 % of all isolates (n = 26). The lowest value in this category is 702.4 ng/µL and is associated with the *Salmonella* p61 isolate, while the highest value corresponds to 893.9 ng/µL and belongs to the *S. enterica* CECT 443 isolate.

As for the category of 901 – 1100 ng/µL, this represents the second category where more isolates fall, corresponding to 22.4 % (n = 11) of the total of isolates, whereby the minimum associated value is found in 920.0 ng/µL of *E. hirae* ATCC 10541 and the maximum associated value concerns 1096.6 ng/µL of the *Salmonella* p110 isolate.

Finally, the last category in the range 1101 – 1500 ng/µL, is the one with the lowest number of isolates, corresponding to 4.1 % of the total (n = 2), containing the minimum value of 1198.2 ng/µL associated with the *Salmonella* p31 isolate and the maximum value of 1407.2 ng/µL, which, as indicated above, concerns *P. aeruginosa* ATCC 15442.

Annexe 2

Determination of MICs and MBCs of reference strains

The reference strains *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 10536, *Enterococcus hirae* ATCC 10541 and *Staphylococcus aureus* ATCC 6538 were used as controls in the MIC and MBC assays for the biocides Suma Bac D10® and Mida FOAM 193®, at three different organic matter concentrations, absent (NIS), low (LIS) and high (HIS). The results obtained in the tests can be seen in Annex Tables 2 and 3.

Annex Table 2. Suma Bac D10® medium values of MICs and MBCs for reference strains tested, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	MIC (%)			MBC (%)		
	NIS	LIS	HIS	NIS	LIS	HIS
EC ATCC 10536	0.100	0.100	0.100	0.100	0.100	0.100
EH ATCC 10541	0.100	0.100	0.100	0.100	0.100	0.100
PA ATCC 15442	0.100	0.100	0.100	0.100	0.100	0.100
SA ATCC 6538	0.100	0.100	0.100	0.100	0.100	0.100

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; EC – *Escherichia coli*; EH – *Enterococcus hirae*; PA – *Pseudomonas aeruginosa*; SA – *Staphylococcus aureus*.

Annex Table 3. Mida FOAM 193® medium values of MICs and MBCs for reference strains tested, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	MIC (%)			MBC (%)		
	NIS	LIS	HIS	NIS	LIS	HIS
EC ATCC 10536	0.500	1.000	1.750	0.500	1.000	1.750
EH ATCC 10541	0.500	0.500	0.500	0.500	1.000	2.500
PA ATCC 15442	0.500	0.500	0.500	0.500	1.000	2.500
SA ATCC 6538	0.500	0.500	0.500	0.500	0.500	1.000

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; EC – *Escherichia coli*; EH – *Enterococcus hirae*; PA – *Pseudomonas aeruginosa*; SA – *Staphylococcus aureus*.

Annexe 3

Determination of the replicas MICs and MBCs of bacterial isolates

To verify if the MICs and MBCs tests, in all three conditions, were properly carried out, it was necessary to use replicas of them. Reproducibility tests are performed with 10 % of the total isolates, which in this study concerns 2 isolates of *S. enterica*, tested under the same conditions for the biocides Suma Bac D10® and Mida FOAM 193®. The isolates were selected with the random function of Microsoft Excel (Microsoft Corporation, USA), p1 and p64. To assess reproducibility, we employ a technique based on Clinical & Laboratory Standards Institute (CLSI) recommendations, in which MIC determination findings may deviate by ± one dilution (CLSI, 2018; Humayoun *et al*., 2018; Riesenberg *et al*., 2016). The results obtained by the replicates can be visualized in Annex Tables 4 and 5.

Annex Table 4. Suma Bac D10® medium values of MICs and MBCs for the replicas selected (p1 and p64) to test, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	MIC (%)			MBC (%)		
	NIS	LIS	HIS	NIS	LIS	HIS
p1	0.100	0.100	0.100	0.100	0.100	0.100
p64	0.100	0.100	0.100	0.100	0.100	0.100

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; "p" - means skin in Portuguese, being these isolates taken from the pig carcass skin.

Annex Table 5. Mida FOAM 193® medium values of MICs and MBCs for the replicas selected (p1 and p64) to test, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (percentage, %).

Sample code	MIC (%)			MBC (%)		
	NIS	LIS	HIS	NIS	LIS	HIS
p1	0.500	0.500	1.000	0.500	0.500	1.000
p64	0.500	1.000	1.000	0.500	1.000	1.000

Legend: NIS – no interfering substance; LIS – low interfering substance; HIS – high interfering substance; MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; "p" - means skin in Portuguese, being these isolates taken from the pig carcass skin.