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The decontaminating effects of lactic acid on pig-associated *Salmonella* and *Yersinia enterocolitica*

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I. Table of contents

II. List of figures

III. List of tables

IV. List of abbreviations

Abbreviation	Description
µL	microliter
ail	attachment-invasion locus
API	Analytical Profile Index
ATP	Adenosine TriPhosphate
ASPs	Acid Shock Proteins
ATR	Acid Tolerance Response
a_w	water activity
bp	basepair
BSA	Bovine Serum Albumine
BPW	Buffered Peptone Water
C	Celsius
CCPs	Critical Control Points
cfu	colony forming unit
CIN	Cefsulodin Irgasan Novobiocin agar
cm	centimeter
CRMOX	Congo-Red Magnesium OXalate agar
DMSO	DiMethyl Sulphoxide
EDTA	EthyleneDiamineTetraAcetate
ERIC	Enterobacterial Repetitive Intergenic Consensus sequence
ESBL	Extended-Spectrum Beta-Lactamase
EU	European Union
fur	ferric uptake regulator
g	Gram
GRAS	Generally Recognized As Safe
GMP	Good Manufacturing Process
HACCP	Hazard Analysis Critical Control Point
inv	invasion
ISO	International Organization of Standardization
kDa	Kilo Dalton
KIA	Kligler Iron Agar

L	Liter
LPS	Lipopolysaccharide
M	Molar
MALDI-TOF MS	Matrix-Associated Laser Desorption Ionization–Time Of Flight Mass Spectrometry
mL	milliliter
mm	millimeter
mM	millimolar
MSRV	Modified Semi-solid Rappaport Vassiliadis
PSB	Peptone Sorbitol Bile broth
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
rpm	rounds per minute
SE	Standard Error
spp.	species
TAC	Total Aerobic Count
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TBX	Tryptone Bile X-glucuronide agar
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soya Broth
TSI	Triple Sugar Iron agar
V	Voltage
WIV-ISP	Scientific Institute of Public Health
XLD	Xylose Lysine Deoxycholate
Yops	*Yersinia* outer membrane proteins
YstA	*Yersinia* stable toxin-A
pYV	*Yersinia* Virulence plasmid

V. English abstract

Despite numerous efforts, pig carcasses are often contaminated with human pathogenic bacteria such as *Salmonella* and *Yersinia* after slaughter. Recent research demonstrated that 60% and 53% of the Belgian pig carcasses are contaminated with *Salmonella* and *Yersinia enterocolitica* bioserotype 4/O:3, respectively (Biasino *et al*, 2016; Van Damme *et al*, 2017). To decrease the microbial load on the pig carcass, the sector needs to apply better decontaminating techniques. Lactic acid is proposed as a potential decontaminant that can be applied during slaughter or after evisceration. In Europe, lactic acid is already used to decontaminate bovine carcasses (Official Journal of the European Union, 2013). In contrast to bovine carcasses, it is still not allowed to use lactic acid as a decontaminant for pig carcasses in the European Union (Official Journal of the European Union, 2004). In addition, there is less data about the difference in decontaminating effects of lactic acid on specific serotypes of *Salmonella* and *Y. enterocolitica*.

Therefore, the lactic acid sensitivity of specific strains and serotypes of *Salmonella* and *Y. enterocolitica* isolated from pig carcasses was investigated. In addition, the effect of lactic acid on different Enterobacteriaceae spp., which are currently used as an indicator for *Salmonella* contamination (Ghafir *et al*, 2008), was investigated. Another interesting aspect that was investigated, is the difference in sensitivity towards lactic acid between bacteria that are coming from the carcass and the slaughterhouse environment.

Firstly, different bacterial strains grown at 25°C and 37°C, were exposed in vitro to 5% lactic acid solution and subsequently stored for 48h at 2°C. Next, the effect of the treatment was examined by measuring the difference of surviving cells between the treated and control cells at several timepoints before and after treatment. In a preliminary test, the lactic acid sensitivity was further examined by artificially inoculating the bacteria on pig skin. Subsequently, the skin was treated with lactic acid and stored for 48h in cooling.

There were no differences in lactic acid sensitivity found between serotypes of *Salmonella* and between bioserotypes of *Y. enterocolitica*. The exposure of a 5% lactic acid solution to different *Salmonella* serotypes grown in culture media resulted in a reduction of approximately 1 $\log_{10}$ cfu/mL when the strains were grown at 37°C and 2 $\log_{10}$ cfu/mL at 25°C. *Y. enterocolitica* showed a reduction of 1.5 $\log_{10}$ cfu/mL at both temperatures after exposure to lactic acid. *E. coli* and *K. pneumoniae* showed a lower lactic acid sensitivity than *Salmonella* and *Y. enterocolitica*. In addition, the experiments on pig skin showed only a minor effect of the treatment. The results of this study indicate that lactic acid can reduce the number of pig-associated pathogens in culture media. However, on pig skin, the lactic acid treatment conditions have to be further examined.

VI. Nederlandse samenvatting

Na het slachtproces zijn varkenskarkassen, ondanks vele inspanningen, nog altijd vaak gecontamineerd met humaan pathogene bacteriën zoals *Salmonella* en *Yersinia enterocolitica*. Recent onderzoek toont aan dat respectievelijk 60% en 53% van de Belgische varkens karkassen gecontamineerd zijn met *Salmonella* en *Y. enterocolitica* bioserotype 4/O:3 (Biasino *et al*, 2016; Van Damme *et al*, 2017). Om deze bacteriën te verwijderen van het karkas heeft de varkenssector betere decontaminerende technieken nodig. Het gebruik van melkzuur is voorgesteld als decontaminerende techniek dat kan gebruikt worden na het slachtproces. In de Europese Unie is het al toegestaan om melkzuur te gebruiken op runderkarkassen (Official Journal of the European Union, 2013), maar het is nog steeds niet toegestaan voor varkenskarkassen (Official Journal of the European Union, 2004). Daarenboven zijn er nog niet veel gegevens beschikbaar over het verschil in melkzuurgevoeligheid tussen verschillende *Salmonella* en *Y. enterocolitica* serotypes.

In dit onderzoek zijn stammen van verschillende *Salmonella* en *Y. enterocolitica* serotypes, geïsoleerd van varkenskarkassen, onderzocht naar de gevoeligheid voor melkzuur. Daarnaast wordt het effect van melkzuur onderzocht bij andere Enterobacteriaceae spp. die momenteel worden gebruikt als indicator organismen voor *Salmonella* contaminatie (Ghafir *et al*, 2008). Hiernaast wordt ook het verschil in gevoeligheid onderzocht tussen bacteriën die opgegroeid zijn bij karkas-temperatuur (37°C) en slachthuisomgeving temperatuur (25°C).

De verschillende stammen werden opgegroeid bij 25°C en 37°C in groeimedium, alvorens blootgesteld te worden aan 5% melkzuur. Hierna werden ze voor 48 uur geïncubeerd bij 2°C. Vervolgens werd het effect van de melkzuurbehandeling onderzocht door het verschil tussen behandelde en onbehandelde cellen te bekijken. In een voorbereidend onderzoek werd de melkzuurgevoeligheid verder onderzocht op varkenshuid.

Tussen de verschillende serotypes van *Salmonella* en tussen de bioserotypes van *Y. enterocolitica* werd er geen verschil in melkzuurgevoeligheid gevonden. De melkzuur behandeling in groeimedium resulteerde respectievelijk in een reductie van 1 en 2 $\log_{10}$ kve/ml wanneer de *Salmonella* serotypes waren opgegroeid bij 37°C en 25°C. De *Y. enterocolitica* bioserotypes vertoonden een reductie van 1,5 kve/ml bij beide temperaturen na de melkzuurbehandeling. Twee Enterobacteriaceae spp., *E. coli* en *K. pneumoniae,* vertoonden een lagere gevoeligheid voor de melkzuurbehandeling dan *Salmonella* en *Y. enterocolitica*. De resultaten van het in vitro onderzoek tonen aan dat melkzuur humaan pathogenen afkomstig van varkens kan doden. Bij het onderzoek op varkenshuid, was er een klein effect van de melkzuur behandeling merkbaar, maar dit moet verder onderzocht worden.

VII. Acknowledgement

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1 Introduction

1.1 Pig slaughter in Belgium

Belgium is a world supplier of pork meat. In 2014, almost 12 million pigs were slaughtered in Belgian slaughterhouses, which resulted in 1 million tons of pork (Algemene Directie Statistiek, 2016). In 2014, 814 734 tons of the produced pork was transported to other countries (**Figure 1**). In total, 90% of the exported pork was transported to European countries such as Germany (32%), Poland (24%), the Netherlands (11%) and France (5%). Outside the European Union (EU), the People's Republic of China (2%), the Philippines (1%) and Hong-Kong (1%) are the most important importers of Belgian pork (Belgian Meat Office, 2015).

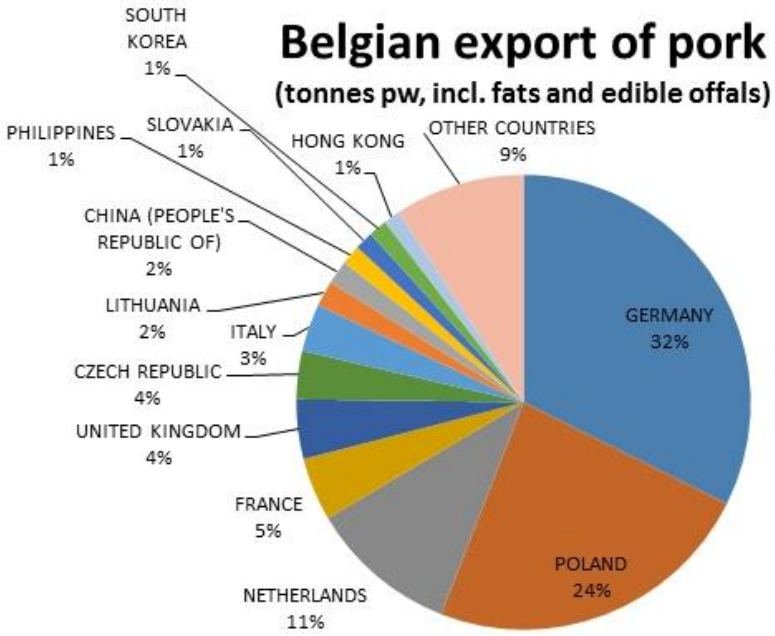


Figure 2: Belgian export of pork. (Belgian Meat Office, 2015)

The production of fresh pork is a complex process that starts with raising and breeding pigs, followed by slaughtering, processing the pig carcasses in meat cutting plants and finally, transportation and distribution of the meat products. Of all the steps in the pork production chain, slaughter is the most important step in contaminating the pig carcasses but also the easiest step where adaptations can be implemented to avoid the contamination (EFSA, 2010; Miller *et al*, 2005).

Before entering the slaughter process, the pigs are kept in lairage, which provides a buffer to supply the slaughter line and allows the pigs to recover from the stress of transport. **Figure 2** describes the pig slaughter process, which begins with the stunning of the pigs and ends with the cooling and chilling step of the carcasses (Swart *et al*, 2016). There are two main zones in a slaughterhouse: the 'dirty' zone and the 'clean' zone, which are physically separated from each other (Arguello *et al*, 2013; Botteldoorn *et al*, 2003). The 'dirty' zone starts with the stunning and killing of the pig. This zone is named 'dirty zone' because the pigs are not yet washed and still covered with feces and dirt. After the polishing step the 'clean' zone starts. At this moment the carcasses are cleaned and the microbial load on the carcass has been drastically reduced (Alban & Stärk, 2005; Berends *et al*, 1997). Further, contamination of the carcass by puncturing the intestines or by handlings of the personnel or the slaughter equipment becomes a risk for (re-)contamination of the carcass (Berends *et al*, 1997; Borch *et al*, 1996; EFSA, 2010).

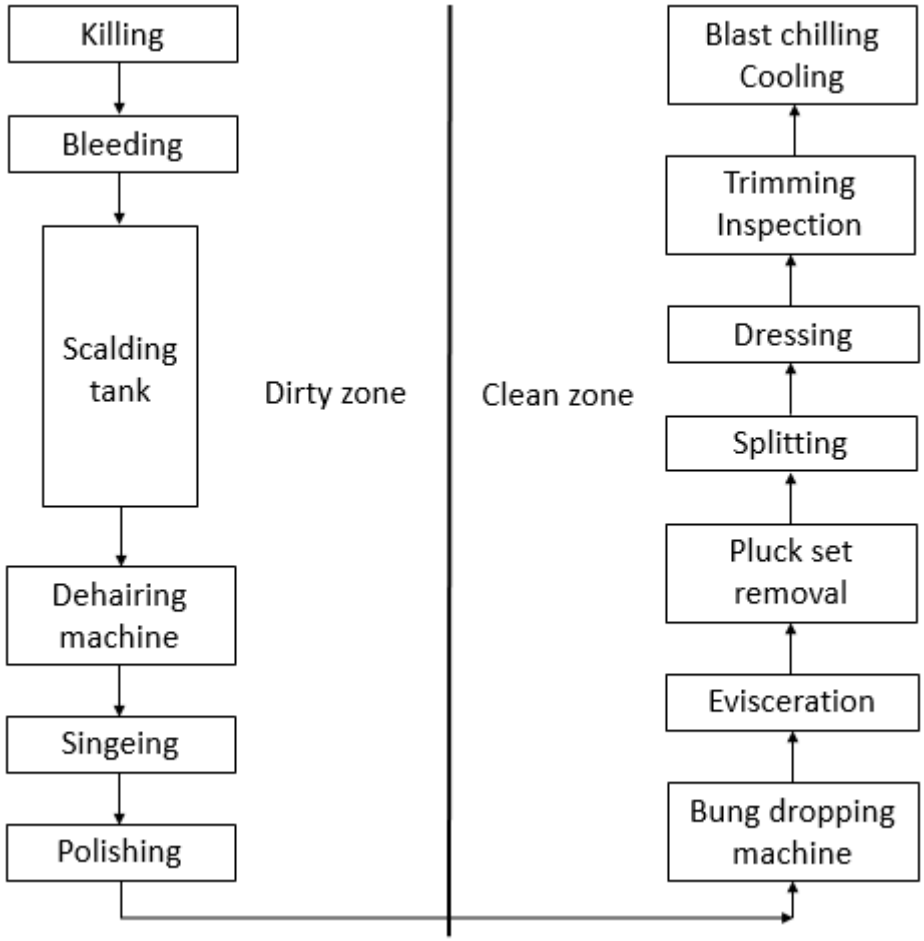


Figure 2: Scheme of the different stages of processing lines in the Belgian pig slaughterhouses.

1.1.1 Stunning, sticking and bleeding

After lairage at the slaughterhouse, the pigs are stunned by an electric shock or in a gas chamber. In the first method, the pigs are stunned by applying an electric field to the head of the pig. In the gas stunning method, pigs are stunned using predominantly CO_2 gas. The gas stunning treatment results in relaxation of the muscles, as opposed to the electrical stunning method. On the other hand, the gas stunning method may lead to increased fecal shedding, which contains a risk for contamination (EFSA, 2010). Subsequently, the stunned pigs are killed by using a long knife to penetrate all the way through the transverse aortic arch. Another method, which is not frequently used, is by incising the main artery in the neck, so called 'sticking'. Finally, the pig bleeds for a while before entering the scalding bath (Swart *et al*, 2016).

1.1.2 Scalding and dehairing

During scalding, the pigs are submerged into a hot water bath of maximum 60°C for 6 to 8 minutes. The purpose is to clean the pig and open the hair follicles. Alternatively, spray scalding system can be used in which hot water is sprayed on the pig (Swart *et al*, 2016). The purpose of the dehairing step is to remove the bulk of hair by using a dehairing machine. The dehairing machine consists of a rotating drum equipped with scraper blocks at the inside. They rotate the carcasses into the drum and remove the hair from the skin. During this 10s during proces, the muscles of the pig carcass relax and fecal matter may be spread and contaminate its own skin and the skin of the next pigs (Borch *et al*, 1996; Pearce *et al*, 2004).

1.1.3 Singeing and polishing

During singeing, the pig carcasses are exposed to very high temperatures (approximately 800 -1000°C) in a singeing machine, consisting of two heated half shells closing on the carcass or a heated tunnel. Singeing for 10s raises the surface temperature of the carcass to approximately 100°C. As a result, all the remaining hairs left after the dehairing step are removed. The remaining dirt and fecal material on the surface of the carcass is burned or dried. When properly performed, this step is considered to be the most effective stage for decreasing the microbial load on the carcass (Alban & Stärk, 2005; Berends *et al*, 1997; Pearce *et al*, 2004). Several areas of the carcass such as the deeper layers of the skin, the hair follicles, the base and orifices of the ear or the deeper skin folds are less easy to decontaminate and bacteria can still survive the singeing step (Berends *et al*, 1997).

During polishing, the carcasses are transported through a tunnel with a car-wash-like series of brushes with flaps. It aims to remove all the dried dirt and hair debris. The polishing contains a risk for spreading the surviving bacteria to other places on the carcass. Furthermore, the polishing machine is difficult to clean and sterilize. In addition, bacteria may become established on the surfaces of the brushes, which leads to a risk for cross contamination (Borch *et al*, 1996; Gill, 2009; Pearce *et al*, 2004). The polishing step is not universally implemented, in some slaughterhouses it is replaced by a manual washing step (Bolton *et al*, 2002).

1.1.4 Evisceration and pluck set removal

During evisceration, the gut is removed from the carcass. First, the rectum is loosened by circumcising the rectum with the bung dropping machine consisting of a probe and a sharp rotating cylinder. Next, the belly of the pig is opened mechanically by using a small hook. After the belly opening, the gut consisting of the colon, cecum, small intestine, stomach and spleen is removed manually and put on a scale for the subsequent post-mortem inspection (Swart *et al*, 2016).

At this stage, there is a high risk for fecal contamination of the carcass. By opening the belly, it is possible that the intestines or the stomach are punctured and as a result contaminating the carcass and slaughter equipment. Similarly, while removing the gut, feces can leak out of the intestines by additional puncturing during manual loosening. The equipment and knives should be sterilized regularly by washing with water at 85°C between successive carcasses. However, when the sterilization of the equipment is not done properly or regularly, it still poses a threat for cross contamination (Berends *et al*, 1997; Botteldoorn *et al*, 2003; Duggan *et al*, 2010).

The pluck set is subsequently removed and consists of the tongue, pharynx including the tonsils, esophagus, trachea, heart, lungs and liver. First, the chest cavity is opened from the front, exposing the interior of the carcass. Then, the pluck set is removed manually and stacked on hook at the corresponding gut containing scale. At this stage, there is a high risk for cross contamination. The oral cavity including the tonsils and tongue can contain high numbers of pathogenic *Yersinia.* When incising these organs with the knife, it can be contaminated with the pathogenic bacteria and subsequently poses a risk for cross contamination of the next carcass (Borch *et al*, 1996; Van Damme *et al*, 2017).

1.1.5 Splitting and dressing

At this stage, the carcass is cut in two from tail to the neck by a mechanical saw. Like the other equipment, the saw is sterilized inside the machine between successive carcasses. However, the inside of the machine poses a small risk for contamination, because it is unreachable and therefore hard to clean. Next, the kidneys and surrounding fat is removed manually during the dressing step (Swart *et al*, 2016).

1.1.6 Trimming and inspection

Trimming is defined as the inspection of the carcass by the slaughterhouse personnel. All visible contamination is removed manually with a knife, that is sterilized between actions. Next, meat inspectors examine the carcasses, intestines, and pluck set for abnormalities. This includes looking for indicators of disease or infection. Inspectors can make incisions and perform palpations. If they find visible contamination, abscesses or swellings, the carcass is put on a separate line and the contamination, abscess or swelling is removed (Swart *et al*, 2016).

1.1.7 Blast chilling and cooling

At the blast cooling stage, the carcasses are cooled very fast by blowing cold air (-20°C). The temperature of the exterior of the carcass is lowered rapidly. The temperature goes below the freezing point and ice crystals, which can kill bacteria, are formed. Moreover, the water activity (a_w) is lowered by the cold air which dries the exterior of the carcass. In addition, the lipid bilayer of the membrane will be destroyed, which cause permanent damage to the cell (Chang *et al*, 2003). During chilling, the carcasses are cooled at 4°C in a storage room for 24h or over the weekend before transporting them to the cutting plant. Chilling alters the level and composition of the bacterial numbers and flora on the carcass and is gradually dominated by psychrotrophic microorganisms during extended storage (Wheatley *et al*, 2014).

1.1.8 Contamination degree of the pig carcass during slaughter

During several stages of the slaughter process such as dehairing and evisceration, the pig carcasses can be contaminated with bacteria, mostly Enterobacteriaceae including pathogenic *Salmonella* and *Yersinia* spp., coming from the pigs or from the contaminated equipment (Berends *et al*, 1997; Borch *et al*, 1996). The total aerobic count (TAC) and the number of Enterobacteriaceae on the pig carcass fluctuate greatly during slaughter. After stunning and bleeding, the total aerobic count reduces drastically to 2-3 $\log_{10}$ colony forming units (cfu)/cm² (Bolton *et al*, 2002; Pearce *et al*, 2004; Spescha *et al*, 2006). A study in an Irish abattoir confirmed these reductions in TAC. A reduction of almost 4 $\log_{10}$ cfu/cm² from stunning to singeing was reported (Wheatley *et al*, 2014). Subsequently, the numbers increase after polishing and evisceration, before declining during inspection and chilling to approximately 3 $\log_{10}$ cfu/cm^2 (Wheatley *et al*, 2014; Spescha *et al*, 2006).

The Enterobacteriaceae contamination follows the same trend as the TAC, as shown in **figure 3**. There is a reduction from 4 to 1 $\log_{10}$ cfu/cm² after scalding and singeing, an increase during polishing and evisceration (Wheatley *et al*, 2014). While TAC stabilizes during inspection and chilling (Wheatley *et al*, 2014; Spescha *et al*, 2006).

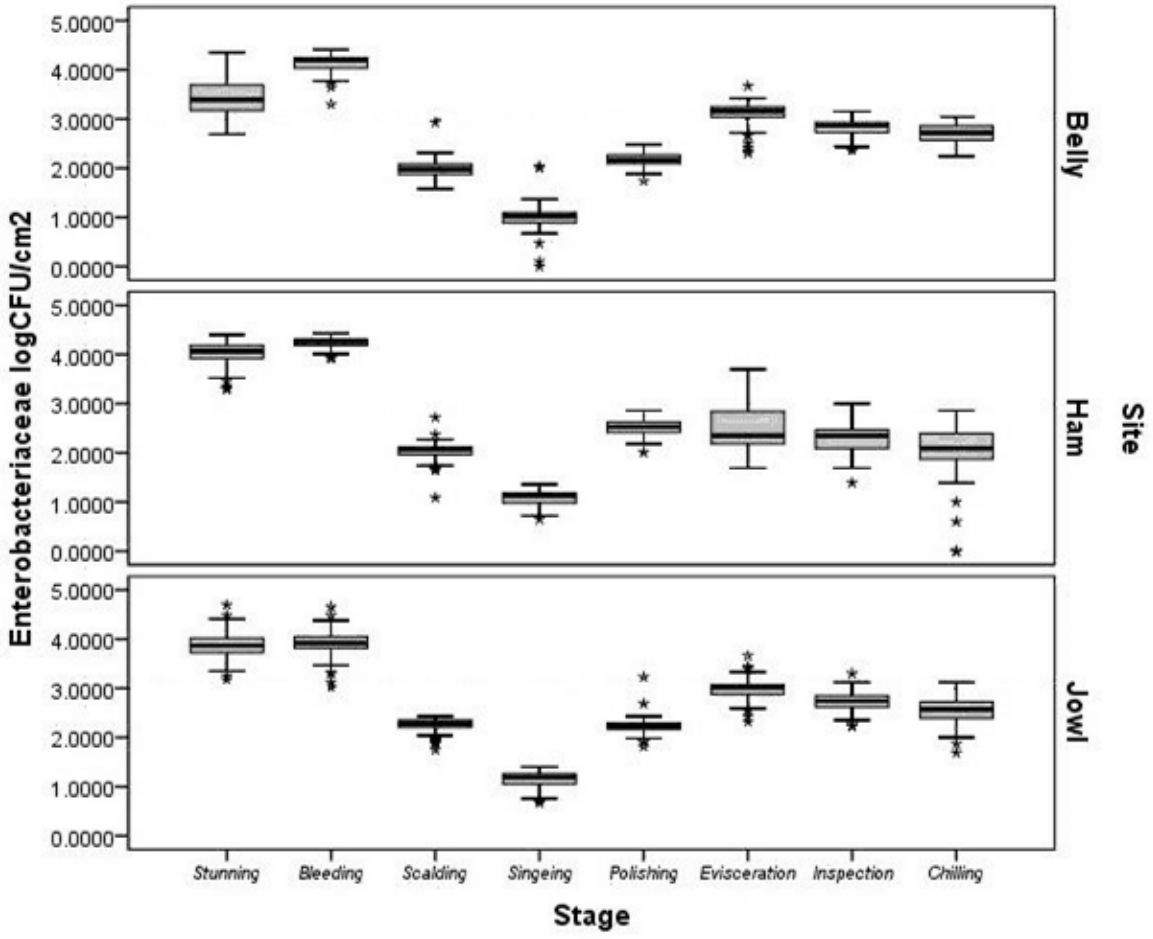


Figure 3: Mean Enterobacteriaceae counts (log cfu/cm²) on jowl, belly and ham at different stages of the slaughter process. (Wheatley *et al*, 2014)

1.2 Pig-associated zoonoses

Zoonoses are infections that can be transmitted directly or indirectly between animals and humans, for example by consuming contaminated food or through contact with infected animals. The severity of these diseases in humans varies from mild symptoms to life-threatening conditions. In the EU, over 320 000 human cases of zoonoses are reported annually, but the real number is probably much higher (EFSA & ECDC, 2015). The dominant pathogenic bacteria causing zoonoses in the EU are *Campylobacter* spp., *Salmonella* spp., pathogenic strains of *Escherichia coli*, *Yersinia* spp. and *Listeria* spp. (EFSA & ECDC, 2015).

The most important pig-related zoonotic disease-causing bacteria are *Salmonella* spp. and *Yersinia* spp. (EFSA & ECDC, 2015). These pathogenic bacteria are hosted in the gastro-intestinal tract of the pigs and can be excreted in the pig feces (Ghafir *et al*, 2008; Ray & Bhunia, 2008). Contamination of the pig carcasses with these bacteria can originate from either direct or indirect contact with feces from an infected pig or from contact with the microbial flora present in the slaughterhouse environment (Berends *et al*, 1997; Botteldoorn *et al*, 2003; Gürtler *et al*, 2005; Nesbakken *et al*, 2003; Van Damme *et al*, 2015).

After the slaughter process, pig carcasses are often contaminated with bacteria such as Enterobacteriaceae including *Salmonella* and *Yersinia*. Recent research demonstrated that respectively 60% and 53% of the Belgian pig carcasses are contaminated with *Salmonella* and *Yersinia enterocolitica* bioserotype 4/O:3 (Biasino *et al*, 2016; Van Damme *et al*, 2017). Moreover, large variations in contamination levels of the different carcass parts were observed. Especially the forelegs and heads were the parts with the highest degree of contamination. Approximately 28% of the samples taken from the head and 37% of the samples from the forelegs were positive for *Salmonella* (Biasino *et al*, 2016).

1.2.1 *Salmonella* spp.

Taxonomy

The classification of the *Salmonella* spp. has a complex history, but the most recent consensus recognizes two species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori* (**Figure 4**). *S. enterica* is further classified into six subspecies based on the Kauffmann-White scheme (Brenner *et al*, 2000; Grimont & Weill, 2007): Subspecies I, or *S. enterica* subsp. *enterica*; Subspecies II, or *S. enterica* subsp. *salamae*; Subspecies IIIa, or *S. enterica* subsp. *arizonae*; Subspecies IIIb, or *S. enterica* subsp. *diarizonae*; Subspecies IV, or *S. enterica* subsp. *houtenae*; and Subspecies VI, or *S. enterica* subsp. *indica* (Le Minor & Popoff, 1987; Tindall *et al*, 2005). A sequence analysis has determined the genetic relationships within this genus and has largely confirmed the above classification scheme (Edwards *et al*, 2002).

The genus is further subdivided into more than 2500 serotypes based on the serologic identification of O (somatic), H (flagellar) and VI (capsular) antigens. The O-antigen presents the type of saccharide sequence in the lipopolysaccharide (LPS). The most common O-antigen serogroups in the *S. enterica* subspecies are A, B, C1, C2, D and E. Strains in these serogroups cause approximately 99% of the salmonellosis cases in humans and warm-blooded animals (Popoff & Le Minor, 1997). The H-antigen represents the flagellin, which is the major protein of the flagellar complex. Some serotypes have two phases of H antigens and can switch between them (Grimont & Weill, 2007). The VI antigen is a superficial antigen overlying the O antigen and is only exposed by a few serotypes.

Serotype names designated by antigenic formulae comprise the following four parts: (1) subspecies designation (subspecies I to VI), (2) O antigens followed by a colon, (3) H antigens (phase 1) followed by a colon and (4) H antigens (phase 2, if present) (Brenner *et al*, 2000). For example the formula of *Salmonella enterica* serotype Derby is 1,4,5,12:fg:1,2. Beside the antigenic formulae names, the serotypes receive an easier name which usually refers to the geographic location where the serotype was first isolated, for example *S.* Derby and *S.* London (Grimont & Weill, 2007).

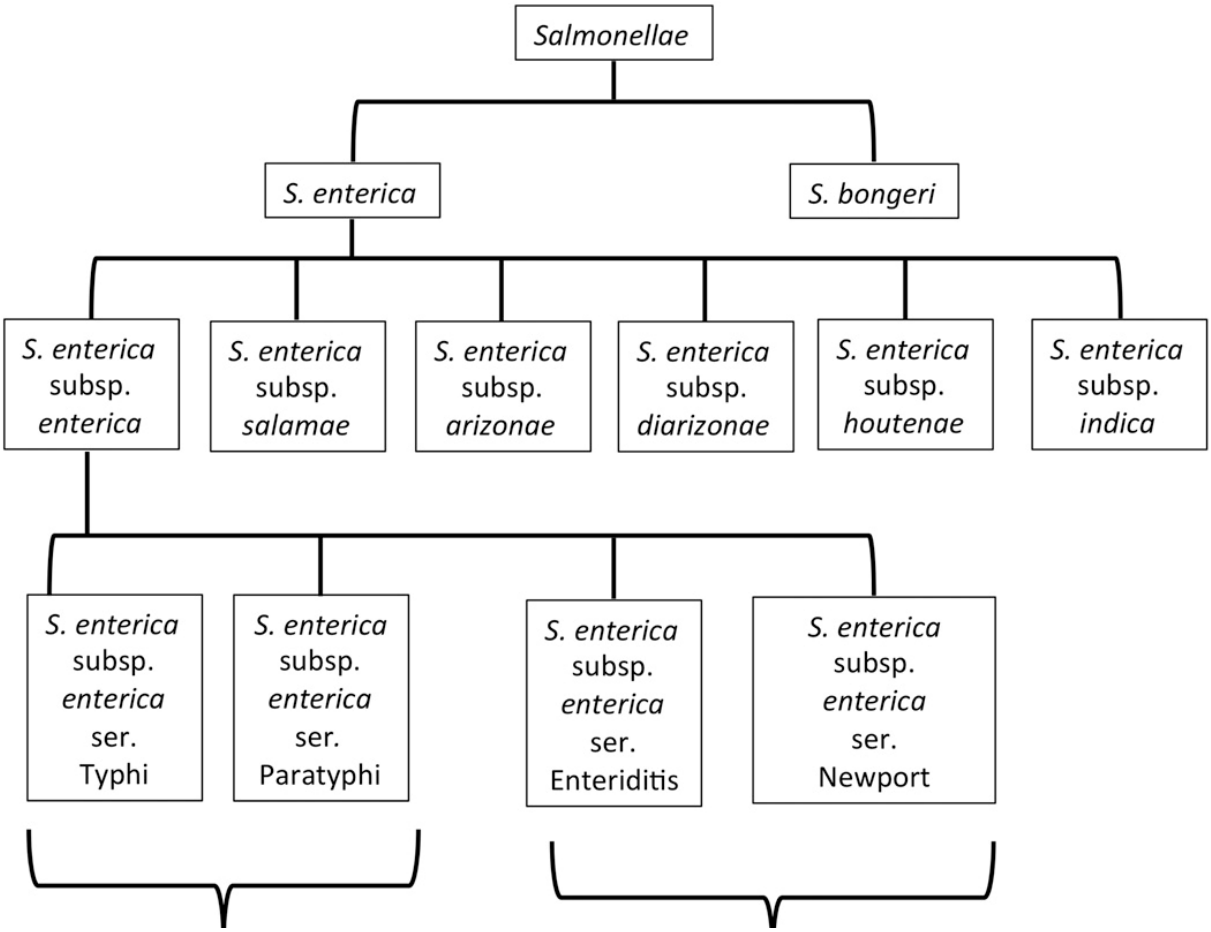


Figure 4: The *Salmonella* genus, including species, subspecies and serotype designations. Four representative serotypes are shown for *S. enterica* subsp. *enterica*. Only the most common typhyoidal serotypes and representative non-typhoidal serotypes are shown. (Dekker & Frank, 2015)

Growth conditions

Salmonella is a Gram-negative, non-spore forming, motile, facultative anaerobic bacillus and belongs to the family Enterobacteriaceae. Traditionally, they are characterized by their ability to metabolize citrate as a sole carbon source and use lysine as a nitrogen source. Another characteristic is their ability to produce hydrogen sulfide (Kaper *et al*, 2011). The classical biochemical testing alone cannot distinguish pathogenic from non-pathogenic *Salmonella* strains. Therefore, the classical biochemical testing is accompanied by serological tests and molecular methods such as PCR-based assays (Lee *et al*, 2015).

Salmonella spp. is a facultative anaerobe organism and can grow over a wide temperature range going from 5 to 45°C, with an optimum growth temperature ranging from 35°C to 43°C.

Growth is markedly slowed at temperatures below 15°C. At 8°C, the doubling times of *Salmonella* spp. were reported between 22 and 35h (Broughall *et al*, 1983; Gibson *et al*, 1988; Grau, 1987; Membré *et al*, 2005). Although the lowest temperature at which *Salmonella* may grow is approximately 5°C, most serotypes do not grow in food stored below 7°C (D'Aoust, 1991; International Commission on Microbiological Specifications for Foods, 2005). Interestingly, *Salmonella* is able to survive for extended periods in chilled and frozen foods (Dominguez & Schaffner, 2009). The pH growth range of *Salmonella* is from 4 to 9, but the optimum is 6.5-7.5 (Doyle & Cliver, 1990). Growth is also inhibited when the a_w is lower than 0.94 (Montville & Matthews, 2008). In addition, *Salmonella* spp. can survive for a long time in low moisture foods ($a_w < 0,70$) such as chocolate and peanut butter (Podolak *et al*, 2010).

Disease development

Members of the genus *Salmonella* cause salmonellosis in humans. This is a well-characterized spectrum of diseases in humans, ranging from asymptomatic carriage to fatal typhoidal fever. Mainly, there are two different disease courses, depending on whether the disease-causing *Salmonella* is a typhoidal or non-typhoidal serotype. Infected people with a non-typhoidal *Salmonella* develop diarrhea with fever and abdominal cramps between 12 and 72 hours after infection (Centers for Disease Control and Prevention, 2015). In healthy individuals, the infected person recovers after 4 to 7 days without a treatment. In susceptible hosts such as pregnant, elderly, infants and immune-compromised persons, certain non-typhoidal strains may spread from the intestines through the bloodstream to other sites in the body and trigger the inflammation system. This may result in death unless the patient is treated properly with antibiotics (Centers for Disease Control and Prevention, 2015).

Infection with a typhoidal *Salmonella* serotype (predominantly Typhi and Paratyphi) presents as a serious systemic disease. Potentially, the *Salmonella* can invade the intestinal mucosa through microfold cells. After invading the intestinal mucosa, the typhoidal strains disseminate through a transient primary bacteremia, which is initially a clinically undetectable infection. Thus, the pathogen is invasive but does not normally trigger a rapid inflammatory or diarrheal response (Dougan & Baker, 2014; Snyder *et al*, 1963). This is a key feature of *S.* Typhi infection and is distinct from nontyphoidal *Salmonella* serotypes infections. After infection, some individuals mostly infants, adolescents and elderly, will develop typhoid fever, which includes symptoms such as high body temperature (>39°C), coughing, vomiting, headache and a rapid pulse. Sometimes there are complications, which include neurologic involvement, intestinal perforation and death (Dougan & Baker, 2014; Leung *et al*, 2012; Lutterloh *et al*, 2012; Neil *et al*, 2012).

Serotype distribution

In 2015, the five most common serotypes among all serotyped isolates from all origins in the EU were *S.* Typhimurium, followed by *S.* Infantis, *S.* Enteritidis, *S.* Dublin and *S.* Derby. Among the serotypes isolated from pigs, *S.* Typhimurium was the most commonly reported (56.9% of the 2,401 isolates) in 2015. *S.* Typhimurium is widely spread in pig herds through all the reporting European member states. *S.* Derby was the second most common serotype, accounting for 13.7% of the isolates. Interestingly, it was the most common serotype isolated from pigs in Denmark (54.2% of serotyped isolates) and in Italy (47.2% of serotyped isolates) (EFSA & ECDC, 2015). Overall, 9.0% of serotyped isolates from pigs were reported as isolates belonging to the group of monophasic strains of *S.* Typhimurium (*S.*1,4,5,12:i:-, *S.*1,4,12:i:-,

S. monophasic Typhimurium). These strains were found in Italy and the United Kingdom. In these two countries, the *S.* monophasic Typhimurium accounted for a large proportion of pig isolates: 27.8% and 58.9% in Italy and the United Kingdom, respectively. Other reported serotypes, with a proportion below 5% of all pig-related isolates, were *S*. Goldcoast, *S.* Rissen and *S.* Infantis (EFSA & ECDC, 2015). The most important serotype of these three is *S*. Rissen. It accounted for 25.6% of serotyped pig isolates in Spain. Garcia-Fierro *et al* (2016) demonstrated a high frequency of resistance and multidrug-resistance among *S*. Rissen isolates obtained from different sources but mainly from pigs. Moreover, they described a strain of *S*. Rissen that is circulating among humans, pigs and other sources (García-Fierro *et al*, 2016).

On pig carcasses and in pork, *S*. Typhimurium, Derby and monophasic Typhimurium were also frequently reported in the EU in 2015, but the top three order differed from the order in pigs (**Figure 5**). *S.* Derby was the most commonly reported serotype (22.9%) from pork in 2015, followed by monophasic strains of *S.* Typhimurium (22.3%) and *S*. Typhimurium (20.6%). In Italy and the United Kingdom, monophasic strains of *S*. Typhimurium were the most abundant *Salmonella* isolates in pork (52.4%, and 55.6%, respectively). Interestingly, S. Derby was the most common serotype isolated in Denmark both from pig meat (42.1%) and pig herds (54.2%) (EFSA & ECDC, 2015). The difference in prevalence between pigs and pork suggests that some pig-adapted serotypes such as *S*. Derby could have developed adaptive mechanisms, which allow them to grow in less favorable environments, including pig carcasses (Hayward et al., 2016).

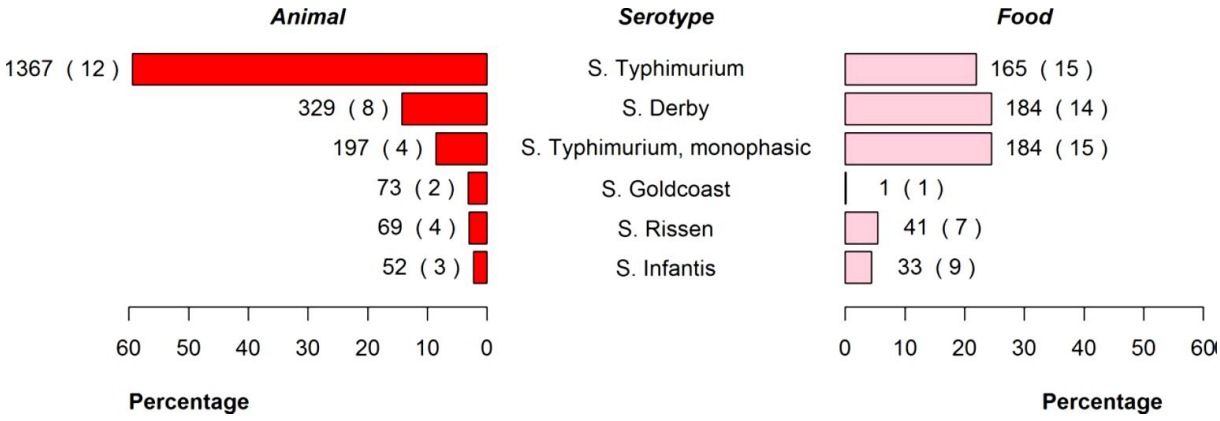


Figure 5: Population pyramid showing the distribution in the European Union of the most common *Salmonella* serotypes in pigs and pork in 2015. The percentages are calculated on the total number of isolates for the animal and food category. The values at the side of each bar present the number of isolates for each serotype and the values between parenthesis represent the number of reporting European Union member states. (EFSA & ECDC, 2015)

Epidemiology

In 2015, 94 625 confirmed salmonellosis cases were reported by 28 EU member states. This results in a notification rate of 21.2 cases per 100 000 persons. In addition, 126 fatal cases were reported in ten member states (EFSA & ECDC, 2015). In Belgium, 3170 cases of salmonellosis were reported to the EFSA in 2015 (EFSA & ECDC, 2015). Estimations indicate that 56.8% of the *Salmonella* infections are due to the consumption of contaminated pork meat (EFSA, 2012).

Salmonella spp. are also known to cause food-borne outbreaks. In 2015, 953 food-borne outbreaks caused by *Salmonella* were reported in the EU. This represents 21.8% of all outbreaks in the EU. Overall, the outbreaks involved 6616 cases, 1719 hospitalizations and 3 deaths. A total of 19.3% of the *Salmonella* outbreaks were reported with strong evidence (184 outbreaks). The most frequent food vehicles were eggs and egg products (21,2% of the reported strong-evidence outbreaks) and pig meat and pig meat products (13% of the strong-evidence outbreaks) (EFSA & ECDC, 2015).

S. Typhimurium, including the monophasic *S.* Typhimurium strains, was involved in 30 strong-evidence outbreaks (16.3%). Pork and derived products were the most common food vehicle and represented 40% of the food vehicle category associated with *S.* Typhimurium outbreaks (EFSA & ECDC, 2015).

1.2.2 Enteropathogenic *Yersinia* spp.

Taxonomy

Of the 17 *Yersinia* species, *Yersinia pestis, Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are known to cause diseases in humans (Winn & Koneman, 2006). *Y. pestis* is identified as the plague causing agent, but is not related to the consumption of pork, in contrast to *Y. enterocolitica*, and *Y. pseudotuberculosis* (Bottone, 1997; EFSA & ECDC, 2015).

Y. enterocolitica is subdivided in six biotypes 1A, 1B, 2, 3, 4 and 5 based on their results in biochemical tests such as Voges-Proskauer test, indole production, etc. The classification in serotypes of *Y. enterocolitica* is determined by the O-antigen (the type of LPS sugar chain) on the cell surface (Skurnik & Bengoechea, 2003). *Y. enterocolitica* can be separated into more than 70 serogroups (Wauters *et al*, 1987). Of these serogroups, the majority of infections worldwide are caused by serogroups O:3, O:9, O:5,27 and O:8 (Bottone, 1997). In addition, it is important to determine both serotype and biotype of a *Y. enterocolitica* isolate, since serotypes O:3 O:9, and O:8 can be identified from different biotypes (Aleksić, 1995). **Table 1** displays the combination of biotypes and serotypes and geographical distribution.

Table 1: Association of *Yersinia enterocolitica* with biotype, serotype and ecologic and geographic distribution. (Bottone, 1997)

Associated with human infections?	*Biogroup*	*Serogroup(s)*	*Ecologic/geographic distribution*
Yes	1B	O:8; O:4; O:13a,13b; O:18; O:20; O:21	Environment, pig (O:8), United States, Japan, Europe, The Netherlands (O:8-like)
	2	O:9; O:5,27	Pig, Europe (O:9), United States (O:5,27), Japan (O:5,27), Sweden, The Netherlands
	3	O:1,2,3; O:5,27	Chinchilla (O:1,2,3), pig (O:5,27)
	4	O:3	Pig, Europe, United States, Japan, South Africa, Scandinavia, Canada, The Netherlands
	5	O:2,3	Hare, Europe
No[a]	1A	O:5; O:6,30; O:7,8; O:18; O:46, nontypeable	Environment, pig, food, water, animal and human feces, global

[a] May be opportunist pathogens in patients with underlying disorders.

Based on their virulence potential, *Y. enterocolitica* can be divided into highly pathogenic biotype 1B strains, moderate-pathogenic biotype 2-5 strains and non-pathogenic biotype 1A strains (Bottone, 1999). *Y. enterocolitica* strains belonging to biotypes 1B and 2-5 carry the 70kb *Yersinia* virulence plasmid (pYV) that contains a type III secretion system that forms a structure on the bacterial surface for the injection of *Yersinia* outer membrane proteins (Yops) into target host cells. This plasmid is essential for full virulence expression and several typical chromosomally encoded virulence markers, including attachment-invasion locus (ail), invasion (inv) and *Yersinia* stable toxin-A (ystA) (Isberg, 1990). These chromosomal virulence determinants and the pYV are completely absent in the non-pathogenic biotype 1A (Miller *et al*, 1989). Interestingly, some scientists have proposed that biotype 1A strains may represent a potential group of emerging pathogens which share virulence-associated features with the pathogenic biotypes (Batzilla *et al*, 2011). In addition, biotype 1A strains have been identified in clinical specimens, but clear association with human disease has not been established (Batzilla *et al*, 2011).

Y. pseudotuberculosis strains are more homogenous than *Y. enterocolitica* strains. However, among *Y. pseudotuberculosis* strains, there is still some variation in biochemical reactions, which can be used to divide the species in four biotypes. There exist 15 different O serotypes (O:1 – O:15), which can be further divided in 10 sub-types (Tsubokura & Aleksić, 1995). All the *Y. pseudotuberculosis*, that are identified correctly, are considered as pathogenic if they carry the virulence plasmid pYV (Wunderink *et al*, 2014).

Growth conditions

Y. enterocolitica are Gram-negative rods with a pleomorphic shape ranging from small coccobacilli with rounded ends to more elongated bacilli. The microscopic morphology is determined by the medium in which the microorganism is grown. In addition, *Y. enterocolitica* is motile at 25°C, but at 37°C the flagellum genes are repressed resulting in loss of the motility (Bottone, 1977). *Y. enterocolitica* are facultative anaerobe, catalase positive, oxidase negative and have urease activity.

Y. enterocolitica is psychrotolerant and can grow at temperatures ranging from -2°C to 42°C, with optimal temperatures between 25-32°C (Gill & Reichel, 1989). At 30°C, *Y. enterocolitica* has a doubling time of approximately 30 min (Neuhaus *et al*, 2000). *Y. enterocolitica* can multiply in meat at low temperatures, even at 0°C (Lee *et al*, 1980). Some strains can survive freezing temperatures (Bergann *et al*, 1995; Schiemann, 1989). When *Y. enterocolitica* is stored at −20°C, most cells will be killed or damaged (Swaminathan *et al*, 1982). The ability of *Y. enterocolitica* to grow at low temperatures is of concern to food producers. The pH growth range is 4.1 to 10 with an optimum of 7.2-7.4 (Adams *et al*, 1991; Stern *et al*, 1980). The minimum a_w at which growth occurs is 0.96 (Stern *et al*, 1980).

Y. pseudotuberculosis is a pleomorphic bacillus as well, which can grow at temperatures ranging from 4°C to 43°C, with optimal temperatures between 25–28°C and has the same growth conditions as *Y. enterocolitica* (Wunderink *et al*, 2014).

Disease development

Y. enterocolitica causes yersiniosis in humans. Yersiniosis ranges from mild self-limiting diarrhea to acute mesenteric lymphadenitis (Fredriksson-Ahomaa *et al*, 2010). The symptoms of the infection depend on the bioserotype of the organism and the age, medical and physical state of the patient. A gastroenteritis is the most frequent form of yersiniosis. Symptoms typically develop 4-7 days after exposure and may last 1-3 weeks. It affects typically young children under 5 years (Bottone, 1999). In older children and young adults, acute yersiniosis can present as pseudoappendicular syndrome, which is sometimes confused with appendicitis (Chandler & Parisi, 1994). Some extra-intestinal long-term sequelae, including reactive arthritis, erythema nodosum, uveitis, glomerulonephritis and myocarditis have been reported (Bottone, 1997). *Y. enterocolitica* infections can possibly lead to sepsis, mostly in patients who have a predisposing underlying disease or are in an iron-overloaded state such as beta-thalassemia (Adamkiewicz *et al*, 1998; Bottone, 1997).

Y. enterocolitica usually causes a diarrheal disease, whereas *Y. pseudotuberculosis* causes mild enteric symptoms, which are usually self-limited. The infection can manifest as mesenteric lymphadenitis and may also be confused with appendicitis (Tertti *et al*, 1989). Septicemic illness is rare and if it occurs, it mostly does in patients with underlying disorders that increase susceptibility to severe infection (Crchova & Grondin, 1973; Ljungberg *et al*, 1995; Naiel & Raul, 1998).

Bioserotype distribution

In total, 99.5% of all the isolated *Yersinia* spp. in the EU belonged to *Y. enterocolitica*, which make it the most commonly reported species of *Yersinia*. Information about the *Y. enterocolitica* serotypes was provided for 2797 of confirmed *Y. enterocolitica* cases (EFSA & ECDC, 2015). The most common serotype was O:3 (82.2%), followed by O:9 (11.07%) and O:5,27 (1.6%). Biotype information was provided for 616 (8.6%) confirmed cases. In 2015, the most commonly reported biotypes were biotype 4 (45.5%) followed by biotype 1A (42.2%) and biotype 2 (11.2%). *Y. enterocolitica* biotype 1A was mainly (98.8%) reported by Denmark and included almost half (47.6%) of all *Y. enterocolitica* isolates reported by Denmark (EFSA & ECDC, 2015).

Y. pseudotuberculosis represented 0.5% of the cases reported. The United Kingdom and Ireland reported the highest proportion of *Y. pseudotuberculosis* infections, representing 6.8% and 7.7% of all their confirmed yersiniosis cases, respectively (EFSA & ECDC, 2015).

Only Germany and Spain reported positive findings of *Yersinia* spp. in pig herds. In total, 11% of the 2050 tested samples were positive for *Yersinia*. Serotype O:9 and O:3 were reported from pigs. Only very few European countries reported data concerning the surveillance of *Yersinia* in pig-carcasses and in pig meat products. Austria, Belgium, Germany, Italy and Spain reported data from 22 investigations on pork and derived products in different sampling stages. *Yersinia* was detected in 11.3% of the 952 units tested. Belgium, Germany and Spain reported findings of *Yersinia* ranging from 5.3% to 54.1%. The majority of the positive findings was found in minced meat. The remaining positive samples were fresh meat samples at retail and carcasses at slaughterhouse. *Y. enterocolitica* was identified in 98 out of 108 positive samples (EFSA & ECDC, 2015).

Epidemiology

In 2015, 7202 confirmed cases of yersiniosis were reported in the EU, making it the third most commonly reported zoonosis in the EU. The EU notification rate was 2.20 cases per 100 000 persons. No fatalities were reported among the confirmed yersiniosis cases. In Belgium, 350 cases of yersiniosis were reported to the EFSA, in 2015 (EFSA & ECDC, 2015). Estimations indicate that 77.3% of the *Yersinia* infections are due to the consumption of contaminated pork (Fosse *et al*, 2008). Especially, contaminated raw or undercooked pork is important in transmitting *Yersinia* to humans (Rosner *et al*, 2012).

In addition, 13 food-borne outbreaks in 2015 were caused by *Y. enterocolitica* comprising one strong-evidence outbreak and 12 weak-evidence outbreaks. The strong-evidence and two weak-evidence food-borne outbreaks were associated with the consumption of pork and derived products and were reported by Lithuania (1 strong- and 1 weak-evidence) and France (1 weak-evidence).

1.2.3 Regulations concerning zoonoses

The EU has imposed a food safety legislation, that applies to all member countries and third parties wishing to export food, including pork and pig-derived products, to the EU. The food hygiene regulations cover every level of the food chain and set down specific rules for foods of animal origin. Food operators are obliged to apply compulsory self-checking programs following the hazard analysis critical control point (HACCP) approach (Spescha *et al*, 2006). Bacterial hazards are a major issue in the production of food of animal origin. The identification of bacterial hazards, that may pose a health risk for the consumer, is an important aspect of HACCP (Rhodehamel, 1992).

Microbiological data on carcass contamination at each stage of slaughter are required to implement the HACCP system. The microbial data is very important, because carcasses can still be contaminated despite the absence of visible contamination (Bolton *et al*, 2002; Gill, 2009; Pearce *et al*, 2004). To measure the contamination on the carcass at the end of the slaughter line, indicator organisms such as total aerobic count and the number of Enterobacteriaceae are monitored (Ghafir *et al*, 2008). Regulation EC-2073/2005 stated that the total aerobic count, when analyzed with the destructive sampling method, may not exceed 5.0 $\log_{10}$ cfu/cm^2 (daily mean log), while the number of Enterobacteriaceae may not be higher than 3.0 $\log_{10}$ cfu/cm^2 (daily mean log) (European Commission, 2005). Furthermore, pathogenic bacteria such as *Salmonella* and *Yersinia enterocolitica* may not be present on the carcass. The Regulation EC-217/2014 imposes that a maximum of 3 out of 50 carcasses may test positive for the presence of *Salmonella* (European Commission, 2014). There are currently no European regulations concerning the presence of *Yersinia enterocolitica* on carcasses.

Slaughterhouses struggle to fulfil these requirements (Biasino *et al*, 2016). Consequently, the demand for other decontaminants is rising, especially for certain carcass parts that were of less value in the past, but are now gaining economic interest because of new export opportunities to other countries (i.e. the transport of legs and heads to China) (IATP, 2014).

1.3 Decontamination methods

In the past, adaptations of the slaughter process were investigated to reduce the microbial load on the carcasses (Goldbach & Alban, 2006). However, scientists as well as the pork producing industry has determined that a further reduction in the prevalence of pathogenic bacteria is best achieved by using decontamination technologies (Goldbach & Alban, 2006; Lawson *et al*, 2009). Although decontamination of the pig carcass is not common in the EU, the European Commission has made it possible through the Regulation of Hygiene in Animal Foods that physical methods such as hot water or steam are allowed, while chemical methods such as organic acids need approval from the European Commission after investigation by the European Food Safety Authority (Official Journal of the European Union, 2004).

1.3.1 Physical decontamination methods

During the slaughter process, the carcasses are routinely washed with cold or warm water, in particular at the end of the process. It is effective in removing visible contaminants such as soil, hairs and other debris (Hugas & Tsigarida, 2008). On the other hand, it must also be considered that cold or warm water spraying can distribute the bacteria on the carcass surfaces (Sofos & Smith, 1998). The highest reduction in microbial numbers is gained by increasing the temperature of the water (Sofos & Smith, 1998). For hot water treatments after evisceration with water of 80 to 85°C, an average of 2 $\log_{10}$ cfu/cm^2 and 1.4 $\log_{10}$ cfu/cm^2 reduction in *E. coli* and TAC were reported, respectively (Gill *et al*, 1998; Lawson *et al*, 2009). Increasing the water temperature above 85°C or an exposure time longer than 20s did not resulted in an increase of the reductions (Gill *et al*, 1995). Although hot water treatment resulted in bacterial reductions, potential adverse effects on the appearance and quality of carcasses must be taken in to account. There were no measurable changes in meat quality parameters detected, except on spots where muscle fibers are cut, color changes were observed (Bolton, 2001; Lawson *et al*, 2009; Pipek *et al*, 2005). Other disadvantages of the hot water method are the extensive use of water and energy cost (Lawson *et al*, 2009).

An alternative to the hot water treatment is the application of steam. Steam treatment at the end of slaughter process resulted in a reduction of TAC and Enterobacteriaceae by 1.8-2.3 and 1.3-1.9 $\log_{10}$ cfu/cm^2, respectively (Trivedi *et al*, 2007). The main advantage of using this decontamination method lies in the fact that steam at 100°C is able to penetrate cavities, crevices and hair follicles (James *et al*, 2007). However, the same adverse effects on the costs and appearance and quality of carcasses as mentioned for the hot water treatment apply for steam treatment (Bolton, 2001; Pipek *et al*, 2005).

In conclusion, the effectiveness of hot water and steam treatment as decontamination method is correlated with operational factors and factors related to the carcass. Operational factors are water temperature, pressure, flow rate, method of application and plant variation i.e. size and design of the plant, rate of slaughtering (Sofos & Smith, 1998). Factors related to the carcass are type of meat tissue, the initial microbial load, the type of the microbial ecology on the carcass and the time of exposure to contamination, which can affect bacterial attachment and biofilm formation (Hugas & Tsigarida, 2008).

1.3.2 Chemical decontamination methods

Chemical compounds used for the decontamination of carcasses basically comprise a wide variety of substances. Regarding pig carcasses, the chemical compounds are mainly restricted to low-molecule organic acids such as lactic, acetic, citric and fumaric acid. Other chemicals used for meat decontamination include chlorine, trisodium phosphate, acidified sodium chlorite and peroxyacids. Most of these substances are allowed in the USA but currently they are not or limitedly permitted in the EU (Official Journal of the European Union, 2004).

Organic acids have been traditionally used in food products to inhibit microbial growth and elongate the shelf life of the product by decreasing the pH. The pH has a great impact on the survival and growth of microorganisms in food products. In general, bacteria prefer a neutral pH (6.5-7.5), but most of them tolerate a pH range of 4 to 9. However, yeasts are more tolerant than bacteria to low pH values, whereas molds can grow in a wide pH range (Raybaudi-Massilia *et al*, 2009). One effective way to acidify the food products and subsequently limit the microbial growth is enhancing natural fermentation or adding organic acids to the products (Doores, 1993).

In solution, organic acids exist in a pH-dependent equilibrium between the non-dissociated and dissociated state, depending on the dissociation constant (pKa) of the acids (Beuchat, 2000). At a low pH, the majority of the organic acid portion favors the uncharged, non-dissociated state. This non-dissociated portion of the acids is primary responsible for the antimicrobial activity. It is freely permeable across the plasma membrane and thus can diffuse across the plasma membrane of microorganisms (Brul & Coote, 1999). Once inside the cell, the acid dissociates into a charged anion and proton due to the higher intracellular pH. These protons and anions can no longer diffuse through the membrane leading to a pH drop and an accumulation of toxic anions (Brul & Coote, 1999).

Figure 6 shows the mechanisms of action of organic acids inside a bacterial cell. The bacterial cell has to extrude the generated protons outside the cell to prevent conformational changes of the enzymes, cell structural proteins, phospholipids and nucleic acids (Batt & Tortorello, 2014). In addition, the high concentration of protons inside the cell inhibits glycolysis, affects cell signaling and inhibits active transport (Batt & Tortorello, 2014; Stratford & Eklund, 2003). Thus, these protons have to be transported outside the cell by a H^+ translocating adenosine triphosphate (ATP)-ase, but the transporter excessively consumes ATP, which will eventually leads to the depletion of the cellular energy (Davidson, 2001).

In addition, the accumulation of the anions possibly leads to osmotic stress, membrane disruption and inhibition of essential metabolic activity of other cellular anions such as glutamate and methionine (Bracey *et al*, 1998; Freese *et al*, 1973; Salmond *et al*, 1984). Organic acid anions interfere with membrane permeability through alteration of the structure of the cytoplasmic membrane due to an interaction with membrane proteins (Sheu & Freese, 1972).

Some organic acids with multiple carboxylic groups chelate metal ions in the medium and the ions embedded in the cell wall structure, resulting in a damaged cell wall. Citrate is one of these acids and can form stable complexes with the greatest affinity for transition ions such as Fe^{3+}. By removing these key ions from the medium and cell wall, the microbial growth is inhibited (Batt & Tortorello, 2014).

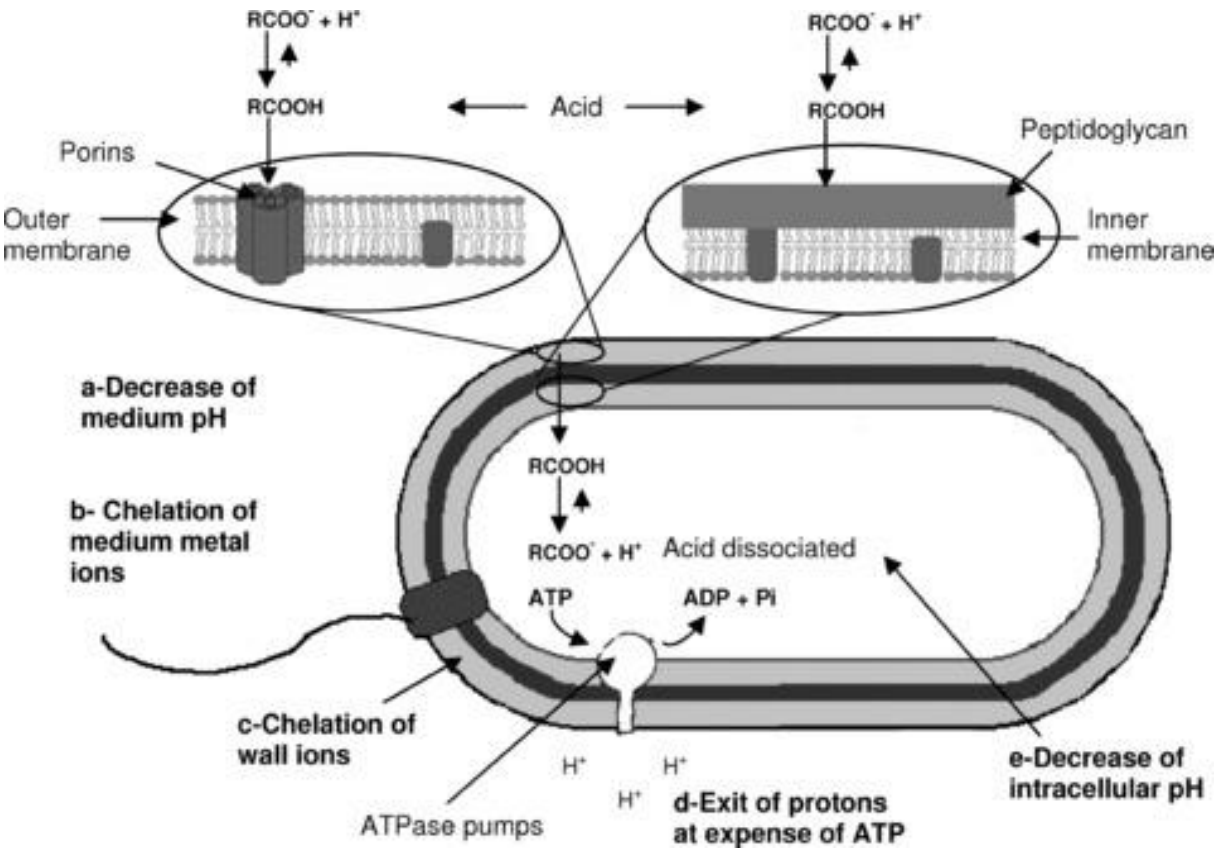


Figure 6: Mechanisms of action of organic acids in a bacterial cell (a-e). The left circle describes how the organic acids can pass through the outer membrane in Gram-negative bacteria, whereas the right circle illustrates how they can pass through the inner membrane in Gram-positive bacteria. (Raybaudi-Massilia *et al*, 2009)

Besides the proton pumps discussed in the previous section, some bacteria such as *Salmonella* and *E. coli* show another resistance mechanism against the high proton concentration. The acid tolerance response (ATR) is a genetically controlled process that allows the bacteria, when exposed to a mildly acid environment, to further protection from acid stress (Foster & Hall, 1990; Goodson & Rowbury, 1989). The ATR is triggered at pH values between 5.5 and 6.0 and protects cells against much stronger acids. The ATR results in the induction of enzymes responsible in raising the intracellular pH, the synthesis of acid shock proteins (ASPs) and the induction of alterations in membrane composition (Álvarez-Ordóñez *et al*, 2012).

The induced enzymes play a role in the lysine and arginine decarboxylase systems. These enzymes convert lysine to cadaverine and arginine to agmatine with consumption of a proton, thus increasing the intracellular pH (Lee *et al*, 2007; Kieboom & Abee, 2006). Besides the enzymatic control of the pH, ASPs are expressed to protect macromolecules from the low pH. The ferric uptake regulator (fur) gene is described as one of the key regulators of the ASPs induction. Mutations in this locus result in acid-sensitive phenotypes (Foster, 1993; Foster & Hall, 1991). There exists many ASPs with different functions, ranging from repair of macromolecular damage to regulation of cellular metabolism. The cells that are exposed to a low pH undergo a membrane adaptation. They decrease the unsaturated fatty acids to saturated fatty acids ratio and change the concentration of oleic or vaccenic acids and cyclic acids. These changes in membrane fatty acid composition were suggested to result in cells with decreased membrane fluidity, which showed a higher ability to survive at low pH (Foster, 2000).

Lactic acid is classified as an organic acid and exhibits beside the mechanisms mentioned above, additional ways to inactivate bacteria. First, it causes damage and permeabilization of the membrane and disruption of the membrane integrity (Wang *et al*, 2014, 2015). These membrane damage and morphological changes were confirmed by transmission electron microscopy (TEM). The TEM images showed a rough cell surface with ruptures or pores on the cell surface in cells treated with lactic acid, meanwhile the bacterial cells in the control maintained their smooth and compact cell surface (Wang *et al*, 2015). Through these pores intracellular K^+ ions and proteins can leak outside the cell (Wang *et al*, 2014, 2015). Eventually, the large leakage of K^+ and proteins lead to a great lack of intracellular K^+ and proteins, which influence the intracellular physiological functions and even result in growth inhibition and dead (Marklund *et al*, 2001).

Treatment with lactic acid is relatively cheap, simple and fast (Lawson *et al*, 2009; Rodríguez-Melcón *et al*, 2017). Furthermore, it is found naturally as a product of post-mortem glycolysis and has been designated by Food and Drug Administration (FDA) as generally recognized as safe (GRAS) for meat products (Mani-López *et al*, 2012; Pipek *et al*, 2005). Therefore, lactic acid is approved by the European Commission for use on bovine carcasses at pre- and post-chilling, offal and variety meats with a maximum concentration of 5% at temperatures not exceeding 55°C (EFSA, 2011; Official Journal of the European Union, 2013). The effect of the lactic acid treatment on bovine carcasses was investigated by different studies (Echeverry *et al*, 2009; Gill & Badoni, 2004; Gill, 2009; Rodríguez-Melcón *et al*, 2017). For example, it resulted in a 1-3 $\log_{10}$ cfu/cm^2 reduction of *E. coli* and *Salmonella* (Echeverry *et al*, 2009; Gill, 2009).

In contrast to bovine carcasses, it is still not allowed to use lactic acid as a decontaminant for pig carcasses in the EU (Official Journal of the European Union, 2004). In addition, there exist only a few studies about the decontaminating effects of lactic acid on pig-associated (pathogenic) bacteria. A study performed by Snijders *et al* (1985), reported a decrease in Enterobacteriaceae and TAC up to 3 $\log_{10}$ cfu/cm^2 by using 5% lactic acid solution (Snijders *et al*, 1985). Van Netten *et al* (1994) reported that a 2% and 5% lactic acid solution for 60s eliminated *S*. Typhimurium from pork carcasses inoculated with ca. 1 $\log_{10}$ cfu/cm^2, but not from those inoculated at ca. 2 $\log_{10}$ cfu/cm^2 (van Netten *et al*, 1995). Another study on fresh pork hams, showed on average a reduction of 1.9 $\log_{10}$ cfu/cm^2 of *Salmonella* Kentucky, Enteritidis and Montevideo by using 4% lactic acid solution at 25°C (DeGeer *et al*, 2016).

Sometimes, lactic acid is used in combination with other decontaminating techniques. The combination of steaming, followed by treatment with 2% lactic acid, was applied on the surface of carcasses at the end of the slaughter process and resulted in 1-3 $\log_{10}$ cfu/ml reduction of the mesophilic microflora (Pipek *et al*, 2006). Another study with a lactic acid spray (2%, 40 to 50°C) in combination with water spray on pork variety meats, resulted in a reduction of approximately 0.5 $\log_{10}$ cfu per sample of *Salmonella* and 0.8 $\log_{10}$ cfu per sample of *Y. enterocolitica* (King *et al*, 2012).

Despite the beneficial decontaminating effect, organic acids can cause adverse effects, including sensory properties, primarily meat color changes and odor. Because color is the main crucial quality trait affecting consumer decisions to purchase fresh meat, the meat industry pursues a stable carcass color (Canto *et al*, 2016). Organic acids leads to denaturation and oxidation of the haem pigments, which subsequently result in lower redness of the meat (Pipek *et al*, 2005; Smulders *et al*, 2011). For this reason, changes in surface color must be taken into account. Furthermore, concern has been raised that decontamination treatments may lead to increased tolerance and resistance of the microorganisms to further stresses, resulting in an increased survival of pathogens on the carcass. A study in beef, acid decontamination dripping wastes showed the survival of the pathogenic *E. coli* O157:H7 for several days in acidic water washings (Samelis *et al*, 2005). Van Netten *et al* (1997; 1998) described survival and growth of acid-adapted mesophilic pathogens, *Salmonella* Typhimurium and *Y. enterocolitica* on pork skin but they did not cause a health hazard during aerobic storage at 4°C.

2 Aim of the research project

After slaughter, *Salmonella* and *Yersinia enterocolitica* is often detected on Belgian pig carcasses (Biasino *et al*, 2016; Van Damme *et al*, 2017). *Salmonella* and *Yersinia* are human pathogenic bacteria and can cause gastroenteritis in humans and must be eliminated from the carcass (EFSA & ECDC, 2015). Currently, the decontaminating techniques are not sufficient to remove all *Salmonella* and *Yersinia enterocolitica* from the carcass. Therefore, the pig sector needs better decontaminating techniques. Lactic acid, which is currently allowed on bovine carcasses in the EU, is a potential decontaminant that can be applied during slaughter or after evisceration (Official Journal of the European Union, 2013). In contrast to bovine carcasses, it is still not allowed to use lactic acid on pig carcasses in the EU (Official Journal of the European Union, 2004). In addition, there is less data about the difference in decontaminating effects of lactic acid on specific serotypes of *Salmonella* and *Y. enterocolitica*.

Therefore, the aim of this research project is to assess the lactic acid sensitivity of specific strains and serotypes of *Salmonella* and *Y. enterocolitica* isolated from pig carcasses. In addition, the effect of lactic acid on different Enterobacteriaceae spp., which are currently used as an indicator for *Salmonella* contamination (Ghafir *et al*, 2008), will be tested. Another interesting aspect that will be investigated, is the difference in sensitivity towards lactic acid between bacteria that are coming from the carcass and the slaughterhouse environment.

In the first part of the study, different bacterial strains grown at 25°C and 37°C, will be exposed in vitro to lactic acid and subsequently stored for 48h at cooling temperatures. The effect of the treatment will be examined by measuring the difference of surviving cells between the treated and control cells at several timepoints before and after treatment. In the second part of the study, the lactic acid treatment will be further validated by artificially inoculating bacteria on pig skin. Subsequently, the skin will be treated with lactic acid and stored for 48h at 2°C. The effect of the treatment will be investigated by destructive sampling at several timepoints before and after the treatment.

3 Results

3.1 Lactic acid sensitivity tests in culture media

3.1.1 Lactic acid sensitivity of *Salmonella* serotypes

The data as means of the triplicate ± standard error (SE) of *Salmonella* Derby, Livingstone, Rissen, monophasic Typhimurium, Typhimurium, Bredeney, Brandenburg, Idikan and Infantis are shown in **tables 2-7**. The differences on XLD were more or less similar to the differences on PCA, therefore only the results of the selective plates are shown and discussed except when mentioned.

In general, the concentration of the treated cells of *S.* Derby, Livingstone, Rissen, monophasic Typhimurium and Typhimurium, that were grown at 25°C to stationary phase, varied more in comparison with the treated cells which were grown at 37°C (**Table 2-6**). For example, the treated *S.* Derby cells grown to stationary phase at 37°C, stored at 2°C for 48h and plated on XLD plates showed a concentration of 7.58±0.12, 7.47±0.23 and 7.50±0.18 $\log_{10}$ cfu/mL for strains S15, S416 and S383, respectively and the concentrations of the treated cells grown at 25°C were 6.50±0.16, 5.65±0.22 and 6.13±0.26 $\log_{10}$ cfu/mL for strains S15, S416 and S383, respectively (**Table 2**). In contrast, the concentrations of the control cells grown at 25°C and 37°C were stable between the three strains. For example, the control *S.* Rissen cells grown to stationary phase at 37°C, directly after treatment and plated on XLD plates showed a concentration of 8.34±0.08, 8.56±0.08 and 8.43±0.02 $\log_{10}$ cfu/mL for strains S83, S13.22A and S37, respectively, and the concentrations of the control cells grown at 25°C were 8.72±0.04, 8.68±0.07 and 8.68±0.06 $\log_{10}$ cfu/mL for strains S83, S13.22A and S37, respectively (**Table 4**).

To determine the percentage of stressed *Salmonella* cells, the percentage of sub-lethal injury was calculated (**Table 8**). The percentage of sub-lethality was greater, when the strains were grown at 25°C than at 37°C. The cells grown at 25°C displayed a sub-lethality in the range of 20.9±9.4% and 99.1±10.2%. The range of the cells grown at 37°C was 0.1±19.6% and 47.1±9.0%. Another interesting trend within the cells, grown at 37°C, was an increase in percentage of stressed cells between 24h and 48h in cooling. This trend applied to all serotypes except for Derby and Brandenburg. For example, the *S.* monophasic Typhimurium cells showed 24h and 48h after treatment a sub-lethality of 24.3±11.3% and 40.7±7.5%, respectively. Within all serotypes grown at 25°C, except for *S.* Idikan and Infantis, the percentage of sub-lethal injury increased as well between 24h and 48h. For example, *S.* Rissen cells showed 24h and 48h after treatment a sub-lethality of 76.4±7.3% and 85.7±4.5%, respectively.

If the means of all *Salmonella* serotypes, of which three strains were investigated, were compared with each other, more or less the same results were obtained (**Figure 7**). The control cells grown at 25°C and 37°C were stable during cooling and ranged between 8.02±0.12 and 8.82±0.04 $\log_{10}$ cfu/mL. In addition, the treated cells, which were grown at 37°C to stationary phase, were stable as well, the concentrations ranged between 7.06±0.17 and 7.60±0.08 $\log_{10}$ cfu/mL. The concentrations of the treated cells grown at 25°C were lower and differed more than at 37°C, furthermore a decreasing trend during cooling was visible. For example, the concentrations of treated *S.* Rissen grown at 25°C decreased from 6.25±0.18 $\log_{10}$ cfu/mL directly after treatment to 5.77±0.19 $\log_{10}$ cfu/mL after 24h in cooling and 5.20±0.24 $\log_{10}$ cfu/mL after 48h in cooling (**Figure 7 C**). When the differences between treated and control

Table 2: Lactic acid sensitivity of *Salmonella* Derby in culture media. Three strains of *Salmonella* Derby (S15, S416 and S383) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was compared with a control that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			S15				S416				S383				Mean			
			XLD		PCA		XLD		PCA		XLD		PCA		XLD		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	7.45	0.06	7.65	0.13	7.58	0.14	7.82	0.06	7.70	0.21	7.68	0.23	7.58	0.08	7.71	0.08
		Control	8.11	0.15	8.38	0.22	8.68	0.05	8.62	0.12	8.61	0.06	8.55	0.03	8.47	0.10	8.52	0.08
		Difference	0.66	0.12	0.73	0.15	1.10	0.11	0.80	0.06	0.92	0.15	0.87	0.20	0.89	0.09	0.80	0.08
	24h	Treated	7.55	0.12	7.62	0.06	7.56	0.16	7.75	0.12	7.69	0.18	7.72	0.22	7.60	0.08	7.70	0.08
		Control	8.45	0.04	8.38	0.15	8.60	0.06	8.62	0.04	8.49	0.08	8.54	0.05	8.51	0.04	8.51	0.06
		Difference	0.91	0.08	0.75	0.18	1.04	0.15	0.87	0.12	0.79	0.14	0.83	0.22	0.91	0.07	0.82	0.09
	48h	Treated	7.58	0.12	7.53	0.08	7.47	0.23	7.65	0.12	7.50	0.18	7.67	0.21	7.51	0.09	7.62	0.08
		Control	8.55	0.12	8.40	0.06	8.48	0.05	8.54	0.05	8.50	0.12	8.62	0.05	8.51	0.05	8.52	0.04
		Difference	0.98	0.04	0.87	0.12	1.01	0.28	0.89	0.16	1.00	0.10	0.95	0.15	1.00	0.09	0.91	0.07
25°C	0h	Treated	7.12	0.21	7.59	0.12	6.37	0.37	7.38	0.05	6.41	0.35	7.00	0.03	6.63	0.20	7.32	0.09
		Control	8.45	0.11	8.66	0.05	8.61	0.07	8.72	0.06	8.66	0.09	8.61	0.07	8.57	0.06	8.66	0.03
		Difference	1.33	0.29	1.07	0.09	2.25	0.39	1.35	0.08	2.26	0.38	1.62	0.06	1.94	0.24	1.34	0.09
	24h	Treated	6.93	0.15	7.49	0.08	6.05	0.39	7.27	0.08	6.15	0.24	6.99	0.05	6.38	0.20	7.25	0.08
		Control	8.09	0.20	8.24	0.09	8.32	0.25	8.63	0.06	8.59	0.09	8.58	0.07	8.34	0.12	8.48	0.07
		Difference	1.16	0.13	0.75	0.14	2.27	0.50	1.36	0.11	2.44	0.28	1.58	0.05	1.96	0.26	1.23	0.14
	48h	Treated	6.50	0.16	7.39	0.10	5.65	0.22	7.04	0.02	6.13	0.26	7.04	0.09	6.10	0.16	7.16	0.07
		Control	8.24	0.05	8.40	0.06	8.40	0.15	8.62	0.07	8.57	0.06	8.68	0.01	8.40	0.07	8.57	0.05
		Difference	1.74	0.13	1.01	0.05	2.74	0.36	1.58	0.09	2.44	0.27	1.64	0.09	2.31	0.20	1.41	0.11

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 3: Lactic acid sensitivity of *Salmonella* Livingstone in culture media. Three strains of *Salmonella* Livingstone (S66, S241 and S354) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			S66				S241				S354			
			XLD		PCA		XLD		PCA		XLD		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	7.72	0.30	7.85	0.15	7.54	0.26	7.86	0.11	7.43	0.33	7.74	0.06
		Control	8.79	0.05	8.75	0.08	8.63	0.04	8.55	0.12	8.58	0.04	8.60	0.05
		Difference	1.07	0.30	0.90	0.15	1.09	0.22	0.69	0.06	1.15	0.36	0.86	0.11
	24h	Treated	7.69	0.34	7.60	0.27	7.53	0.31	7.70	0.16	7.47	0.30	7.72	0.16
		Control	8.57	0.04	8.62	0.03	8.51	0.06	8.54	0.04	8.43	0.08	8.54	0.07
		Difference	0.88	0.36	1.02	0.29	0.99	0.28	0.84	0.14	0.96	0.37	0.82	0.21
	48h	Treated	7.65	0.31	7.65	0.20	7.41	0.31	7.62	0.17	7.21	0.47	7.66	0.17
		Control	8.52	0.08	8.61	0.07	8.55	0.13	8.50	0.05	8.44	0.05	8.53	0.03
		Difference	0.87	0.36	0.95	0.24	1.14	0.28	0.88	0.13	1.23	0.52	0.87	0.15
25°C	0h	Treated	7.35	0.33	7.84	0.23	6.12	0.28	6.99	0.50	7.05	0.20	7.44	0.08
		Control	8.82	0.09	8.90	0.10	8.81	0.04	8.77	0.01	8.68	0.03	8.78	0.03
		Difference	1.47	0.30	1.07	0.17	2.68	0.29	1.78	0.50	1.63	0.18	1.34	0.08
	24h	Treated	7.18	0.36	7.84	0.21	5.77	0.46	6.47	0.88	6.78	0.27	7.32	0.10
		Control	8.36	0.20	8.60	0.06	8.38	0.14	8.43	0.22	8.35	0.13	8.62	0.06
		Difference	1.18	0.52	0.75	0.26	2.60	0.42	1.96	0.67	1.57	0.34	1.30	0.10
	48h	Treated	6.67	0.40	7.58	0.18	5.49	0.76	6.44	0.89	6.28	0.27	7.04	0.04
		Control	8.46	0.11	8.83	0.31	8.38	0.17	8.67	0.03	7.83	0.26	8.60	0.04
		Difference	1.79	0.48	1.26	0.20	2.88	0.79	2.23	0.92	1.55	0.38	1.55	0.02

			Mean			
			XLD		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	7.56	0.15	7.82	0.06
		Control	8.67	0.04	8.63	0.05
		Difference	1.10	0.15	0.82	0.07
	24h	Treated	7.56	0.16	7.67	0.10
		Control	8.50	0.04	8.57	0.03
		Difference	0.94	0.17	0.89	0.12
	48h	Treated	7.42	0.20	7.64	0.09
		Control	8.50	0.05	8.55	0.03
		Difference	1.08	0.21	0.90	0.09
25°C	0h	Treated	6.84	0.23	7.42	0.20
		Control	8.77	0.04	8.82	0.04
		Difference	1.93	0.23	1.40	0.19
	24h	Treated	6.58	0.28	7.21	0.33
		Control	8.36	0.08	8.55	0.07
		Difference	1.78	0.30	1.34	0.27
	48h	Treated	6.15	0.31	7.02	0.31
		Control	8.22	0.14	8.70	0.10
		Difference	2.07	0.35	1.68	0.92

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 4: Lactic acid sensitivity of *Salmonella* Rissen in culture media. Three strains of *Salmonella* Rissen (S83, S13.22A and S37) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			S83				S13.22A				S37				Mean			
			XLD		PCA		XLD		PCA		XLD		PCA		XLD		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	6.81	0.10	7.00	0.18	7.31	0.33	7.44	0.31	7.31	0.30	7.25	0.23	7.14	0.16	7.23	0.14
		Control	8.34	0.08	8.38	0.06	8.56	0.08	8.53	0.04	8.43	0.02	8.40	0.04	8.44	0.05	8.44	0.03
		Difference	1.53	0.02	1.38	0.17	1.25	0.27	1.09	0.29	1.12	0.28	1.14	0.26	1.30	0.13	1.20	0.13
	24h	Treated	6.75	0.10	6.69	0.07	7.47	0.34	7.42	0.34	7.57	0.15	7.33	0.29	7.27	0.17	7.15	0.17
		Control	8.35	0.08	8.42	0.07	8.46	0.08	8.43	0.01	8.54	0.06	8.44	0.13	8.45	0.05	8.43	0.04
		Difference	1.60	0.05	1.72	0.15	0.98	0.36	1.02	0.35	0.97	0.09	1.11	0.17	1.18	0.15	1.28	0.16
	48h	Treated	6.61	0.12	7.08	0.38	7.31	0.33	7.31	0.19	7.27	0.21	7.24	0.10	7.06	0.17	7.21	0.13
		Control	8.10	0.12	8.43	0.06	8.45	0.09	8.45	0.07	8.40	0.12	8.44	0.07	8.32	0.07	8.44	0.03
		Difference	1.48	0.10	1.35	0.43	1.14	0.32	1.14	0.16	1.13	0.14	1.20	0.03	1.25	0.12	1.23	0.14
25°C	0h	Treated	6.75	0.17	6.81	0.16	5.71	0.00	5.97	0.14	6.31	0.30	6.38	0.20	6.25	0.18	6.39	0.15
		Control	8.72	0.04	8.78	0.08	8.68	0.07	8.68	0.02	8.68	0.06	8.65	0.07	8.69	0.03	8.71	0.04
		Difference	1.97	0.13	1.97	0.19	2.98	0.07	2.72	0.14	2.37	0.36	2.27	0.27	2.44	0.18	2.32	0.15
	24h	Treated	6.28	0.20	7.06	0.13	5.23	0.28	5.40	0.38	5.81	0.19	6.34	0.03	5.77	0.19	6.27	0.27
		Control	8.35	0.31	8.60	0.10	8.37	0.15	8.60	0.04	8.61	0.14	8.52	0.13	8.44	0.12	8.57	0.05
		Difference	2.06	0.24	1.54	0.20	3.14	0.13	3.20	0.34	2.81	0.33	2.18	0.16	2.67	0.20	2.31	0.27
	48h	Treated	5.91	0.10	6.76	0.19	4.76	0.55	5.55	0.21	4.92	0.14	5.64	0.30	5.20	0.24	5.98	0.23
		Control	8.16	0.14	8.52	0.04	8.18	0.17	8.61	0.02	8.41	0.11	8.33	0.17	8.25	0.08	8.49	0.07
		Difference	2.26	0.23	1.77	0.23	3.42	0.42	3.06	0.21	3.49	0.21	2.69	0.28	3.06	0.25	2.50	0.23

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 5: Lactic acid sensitivity of *Salmonella* Monophasic Typhimurium in culture media. Three strains of *Salmonella* Monophasic Typhimurium (S224, S141 and S60.18B) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			S224				S141				S60.18B				Mean			
			XLD		PCA		XLD		PCA		XLD		PCA		XLD		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	6.67	0.24	7.05	0.35	7.57	0.18	7.75	0.08	7.32	0.14	7.67	0.12	7.19	0.17	7.49	0.16
		Control	8.05	0.43	8.33	0.14	8.65	0.07	8.61	0.06	8.67	0.08	8.65	0.07	8.46	0.16	8.53	0.07
		Difference	1.38	0.61	1.28	0.48	1.08	0.11	0.86	0.03	1.35	0.14	0.98	0.11	1.27	0.19	1.04	0.16
	24h	Treated	6.93	0.34	7.00	0.39	7.63	0.25	7.81	0.10	7.40	0.27	7.61	0.18	7.32	0.18	7.47	0.18
		Control	8.45	0.04	8.54	0.02	8.54	0.06	8.54	0.04	8.56	0.04	8.52	0.08	8.52	0.03	8.53	0.03
		Difference	1.52	0.37	1.54	0.39	0.92	0.19	0.74	0.06	1.17	0.30	0.90	0.26	1.20	0.17	1.06	0.18
	48h	Treated	7.09	0.19	7.26	0.16	7.51	0.19	7.78	0.08	7.18	0.32	7.53	0.18	7.26	0.14	7.52	0.11
		Control	8.25	0.07	8.35	0.08	8.46	0.08	8.56	0.07	8.36	0.18	8.48	0.18	8.36	0.07	8.46	0.07
		Difference	1.16	0.15	1.10	0.11	0.95	0.11	0.79	0.05	1.18	0.46	0.95	0.32	1.10	0.15	0.94	0.11
25°C	0h	Treated	6.62	0.28	7.23	0.12	6.31	0.31	7.11	0.09	6.07	0.23	7.07	0.09	6.54	0.16	7.14	0.06
		Control	8.74	0.03	8.76	0.09	8.76	0.02	8.74	0.03	8.62	0.01	8.63	0.07	8.71	0.02	8.71	0.04
		Difference	2.12	0.28	1.53	0.13	2.44	0.30	1.63	0.12	2.55	0.24	1.57	0.11	2.18	0.15	1.58	0.06
	24h	Treated	6.68	0.34	7.23	0.18	6.47	0.39	7.50	0.44	5.73	0.15	6.92	0.08	6.48	0.21	7.22	0.16
		Control	8.44	0.09	8.55	0.04	8.37	0.20	8.57	0.08	8.24	0.18	8.45	0.07	8.39	0.09	8.52	0.04
		Difference	1.76	0.41	1.32	0.19	1.90	0.19	1.07	0.41	2.51	0.30	1.53	0.14	1.91	0.20	1.31	0.15
	48h	Treated	6.58	0.29	7.19	0.19	5.65	0.09	7.00	0.16	5.44	0.15	6.86	0.03	6.09	0.20	7.02	0.09
		Control	8.07	0.11	8.58	0.03	8.40	0.12	8.60	0.06	8.16	0.13	8.31	0.17	8.38	0.08	8.50	0.07
		Difference	1.49	0.38	1.39	0.16	2.74	0.20	1.59	0.22	2.72	0.12	1.45	0.14	2.28	0.24	1.48	0.09

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 6: Lactic acid sensitivity of *Salmonella* Typhimurium in culture media. Three strains of *Salmonella* Typhimurium (S319, S355 and S279) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			S319				S355				S279				Mean			
			XLD		PCA		XLD		PCA		XLD		PCA		XLD		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	7.24	0.20	7.22	0.21	7.74	0.26	7.93	0.17	7.47	0.10	7.72	0.05	7.49	0.12	7.62	0.13
		Control	8.21	0.12	8.35	0.05	8.68	0.07	8.67	0.06	8.57	0.09	8.44	0.07	8.49	0.09	8.49	0.06
		Difference	0.96	0.26	1.14	0.19	0.94	0.19	0.74	0.11	1.09	0.09	0.72	0.12	1.00	0.10	0.87	0.10
	24h	Treated	7.20	0.27	7.30	0.23	7.63	0.15	7.80	0.11	7.46	0.07	7.67	0.12	7.43	0.11	7.59	0.11
		Control	8.32	0.07	8.37	0.02	8.63	0.07	8.66	0.03	8.46	0.08	8.48	0.06	8.47	0.06	8.50	0.05
		Difference	1.12	0.33	1.08	0.23	1.00	0.11	0.86	0.11	1.01	0.15	0.81	0.17	1.04	0.11	0.91	0.10
	48h	Treated	6.87	0.30	7.14	0.24	7.53	0.15	7.82	0.14	7.20	0.22	7.58	0.04	7.20	0.15	7.51	0.13
		Control	8.26	0.07	8.38	0.09	8.45	0.11	8.58	0.04	8.46	0.03	8.66	0.06	8.39	0.05	8.54	0.05
		Difference	1.39	0.26	1.24	0.20	0.92	0.25	0.76	0.11	1.26	0.21	1.08	0.07	1.19	0.14	1.03	0.10
25°C	0h	Treated	6.04	0.20	7.18	0.28	6.24	0.32	6.79	0.11	5.63	0.07	6.44	0.21	5.97	0.14	6.80	0.15
		Control	8.61	0.05	8.57	0.10	8.62	0.06	8.68	0.04	8.67	0.07	8.69	0.09	8.63	0.03	8.65	0.04
		Difference	2.57	0.25	1.39	0.38	2.38	0.35	1.89	0.11	3.04	0.10	2.25	0.16	2.66	0.16	1.84	0.18
	24h	Treated	5.85	0.57	6.59	0.80	6.04	0.41	6.86	0.09	5.99	0.25	6.76	0.35	5.96	0.22	6.74	0.26
		Control	7.99	0.16	8.48	0.05	8.16	0.11	8.54	0.08	8.08	0.35	8.54	0.14	8.08	0.12	8.52	0.05
		Difference	2.15	0.60	1.89	0.81	2.13	0.48	1.68	0.12	2.09	0.17	1.78	0.21	2.12	0.23	1.78	0.25
	48h	Treated	5.34	0.38	6.32	0.51	5.72	0.44	6.74	0.15	4.87	0.08	6.07	0.40	5.31	0.21	6.38	0.22
		Control	7.84	0.13	8.65	0.09	8.11	0.11	8.39	0.08	8.07	0.33	8.59	0.14	8.01	0.12	8.55	0.07
		Difference	2.50	0.45	2.33	0.60	2.39	0.53	1.65	0.15	3.20	0.41	2.52	0.33	2.70	0.26	2.17	0.24

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 7: Lactic acid sensitivity of *Salmonella* Bredeney, Brandenburg, Idikan and Infantis in culture media. A strain of *Salmonella* Bredeney, Brandenburg, Idikan and Infantis were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			***S.* Bredeney**				***S.* Brandenburg**			
			XLD		**PCA**		**XLD**		**PCA**	
Time in cooling			**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**
37°C	**0h**	**Treated**	7.75	0.17	7.60	0.20	7.49	0.09	7.41	0.11
		Control	8.49	0.09	8.54	0.08	8.35	0.27	8.40	0.22
		Difference	0.74	0.08	0.95	0.15	0.86	0.36	0.99	0.33
	24h	**Treated**	7.76	0.06	7.74	0.07	7.38	0.17	7.34	0.26
		Control	8.69	0.03	8.74	0.04	8.66	0.06	8.81	0.16
		Difference	0.93	0.09	1.00	0.04	1.28	0.20	1.47	0.34
	48h	**Treated**	7.66	0.10	7.71	0.08	7.10	0.43	7.08	0.41
		Control	8.29	0.01	8.45	0.07	8.34	0.03	8.42	0.06
		Difference	0.63	0.09	0.74	0.12	1.24	0.41	1.34	0.37
25°C	**0h**	**Treated**	7.44	0.22	7.78	0.13	7.58	0.24	7.82	0.19
		Control	8.66	0.01	8.83	0.02	8.70	0.06	8.50	0.23
		Difference	1.22	0.23	1.05	0.12	1.12	0.24	0.69	0.12
	24h	**Treated**	7.23	0.23	7.76	0.12	7.46	0.13	7.88	0.17
		Control	8.33	0.12	8.49	0.02	8.32	0.03	8.57	0.05
		Difference	1.10	0.25	0.72	0.13	0.87	0.15	0.69	0.13
	48h	**Treated**	6.95	0.43	7.79	0.15	7.16	0.20	7.80	0.17
		Control	8.34	0.04	8.63	0.03	8.25	0.09	8.54	0.09
		Difference	1.40	0.46	0.84	0.16	1.08	0.19	0.74	0.20

			***Salmonella* Idikan**				***Salmonella* Infantis**			
			XLD		**PCA**		**XLD**		**PCA**	
Time in cooling			**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**
37°C	**0h**	**Treated**	7.20	0.27	7.17	0.32	7.47	0.25	7.62	0.22
		Control	8.52	0.13	8.50	0.10	8.52	0.17	8.60	0.04
		Difference	1.33	0.39	1.33	0.41	1.06	0.36	0.98	0.20
	24h	**Treated**	7.16	0.36	7.11	0.41	7.41	0.26	7.63	0.21
		Control	8.42	0.12	8.54	0.03	8.36	0.08	8.48	0.04
		Difference	1.26	0.48	1.44	0.45	0.95	0.32	0.85	0.23
	48h	**Treated**	7.10	0.31	7.23	0.32	7.38	0.39	7.67	0.34
		Control	8.40	0.07	8.48	0.11	8.31	0.06	8.54	0.03
		Difference	1.29	0.32	1.25	0.34	0.93	0.42	0.87	0.32
25°C	**0h**	**Treated**	6.58	0.39	7.57	0.10	7.11	0.20	7.73	0.17
		Control	8.75	0.05	8.87	0.07	8.64	0.05	8.68	0.04
		Difference	2.17	0.36	1.31	0.11	1.52	0.25	0.95	0.19
	24h	**Treated**	6.84	0.34	7.66	0.20	6.93	0.13	7.72	0.15
		Control	8.45	0.24	8.69	0.02	8.40	0.09	8.50	0.08
		Difference	1.61	0.57	1.03	0.21	1.46	0.09	0.78	0.07
	48h	**Treated**	6.18	0.76	7.12	0.47	6.66	0.51	7.61	0.17
		Control	8.22	0.05	8.62	0.07	8.12	0.03	8.31	0.02
		Difference	2.04	0.76	1.50	0.46	1.46	0.49	0.70	0.19

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 8: Sub-lethal injury of *Salmonella* serotypes in culture media. Different strains of *Salmonella* serotypes were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD) and plate count agar (PCA). The number of surviving cells was enumerated and the percentage of sub-lethal injury was calculated. Values are expressed as means of the triplicate (±SE). ~ negative percentage (mathematical concept).

		S. Derby		*S.* Livingstone		*S.* Rissen		*S.* Mo.Typhimurium		*S.* Typhimurium	
	Time in cooling	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**
37°C	**0h**	15.5	10.9	31.1	14.0	12.3	12.3	46.5	7.7	20.6	9.8
	24h	8.1	11.3	8.5	18.7	~		24.3	11.3	26.3	10.4
	48h	7.1	15.3	24.0	14.9	0.1	19.6	40.7	7.5	46.0	6.8
25°C	**0h**	73.3	12.9	28.8	61.8	20.9	9.4	79.3	4.8	78.5	6.6
	24h	92.1	12.8	55.2	20.5	31.3	41.7	81.3	6.7	76.4	7.3
	48h	99.1	10.2	80.8	6.8	72.3	10.0	87.7	4.4	85.7	4.5

		S. Bredeney		*S.* Brandenburg		*S.* Idikan		*S.* Infantis	
	Time in cooling	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**
37°C	**0h**	~		~		~		27.9	10.9
	24h	~		~		~		34.3	17.1
	48h	8.1	15.0	~		24.7	3.7	47.1	9.0
25°C	**0h**	52.4	9.7	41.2	6.9	83.5	10.5	75.8	2.6
	24h	67.5	10.3	61.5	4.0	83.4	5.4	83.5	2.6
	48h	79.6	10.3	76.6	3.1	83.3	7.0	77.6	1.6

[A] % sub-lethal injury

[B] standard error

cells grown at 25°C and 37°C were compared within each serotype at the three timepoints during cooling, they appeared to be not significantly different at each timepoint, except the difference of *S.* Rissen which were grown at 25°C at 48h after treatment was significantly higher than the difference at timepoint 0h (p=0.008). The differences of *S.* Derby grown at 37°C at timepoints 0h and 48h were significantly different (p=0.048) on the non-selective plates but not on the selective plates. In addition, when the differences within each serotype were compared between 25°C and 37°C, all the differences at 25°C of every serotype were significantly higher than at 37°C at all timepoints after treatment. Although all differences on the selective plates were significantly different between the two temperatures, the differences of *S.* Livingstone and monophasic Typhimurium at 24h were not significantly different between 25°C and 37°C on the non-selective plates.

The *Salmonella* serotypes Bredeney, Brandenburg, Idikan and Infantis showed more variability than the other serotypes (**Figure 8**). The differences between control and treated cells of serotypes Bredeney, Idikan and Infantis showed the same trend as the common serotypes i.e. the differences at 25°C were greater than the differences at 37°C. However, the effect of the treatment on *S.* Brandenburg showed no difference between the two temperatures.

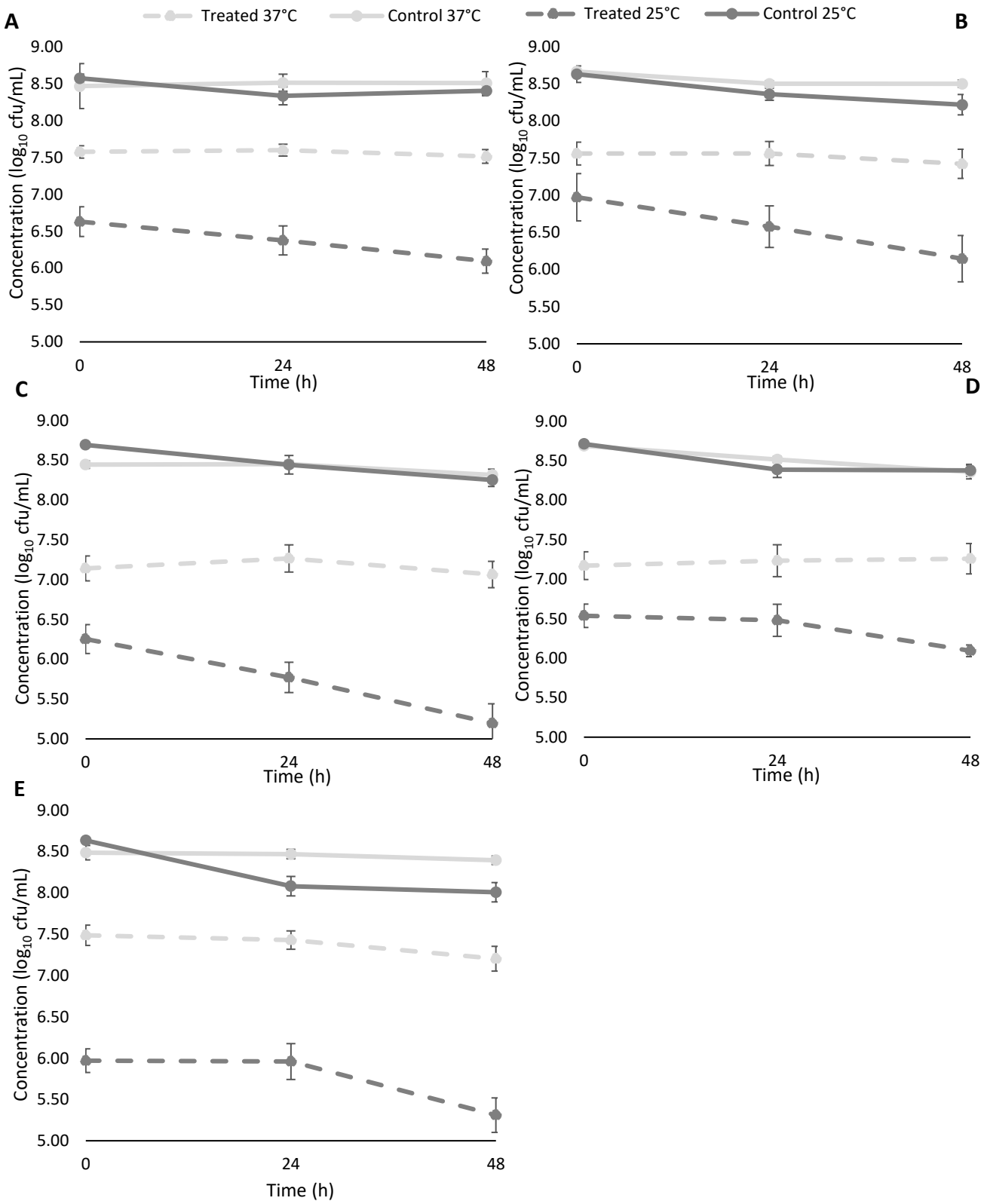


Figure 7: Lactic acid sensitivity of *Salmonella* Derby (A), Livingstone (B), Rissen (C), monophasic Typhimurium (D) and Typhimurium (E) in culture media. Three strains of *Salmonella* Derby, Livingstone, Rissen, monophasic Typhimurium and Typhimurium were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the strains (±SE).

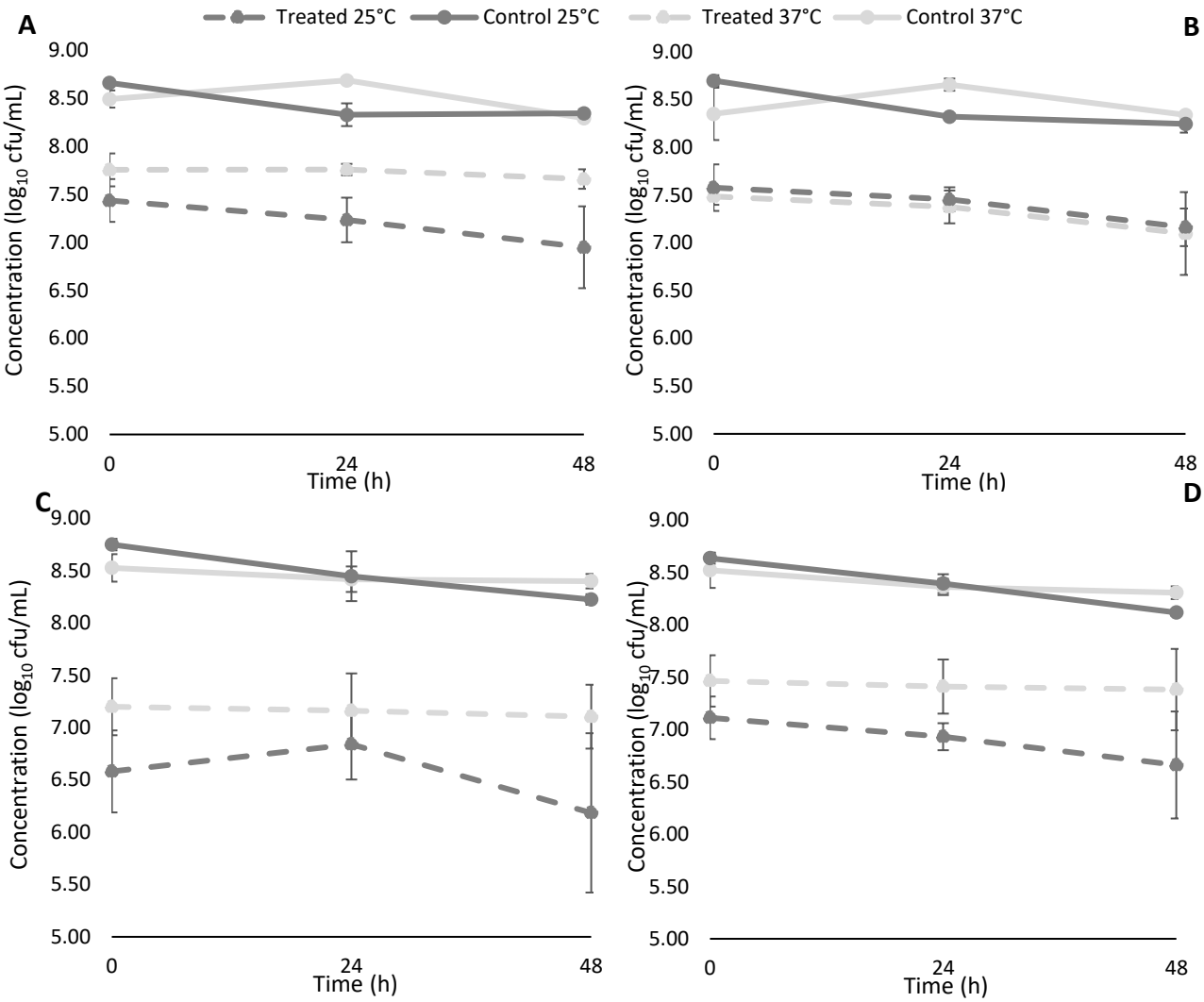


Figure 8: Lactic acid sensitivity of *Salmonella* Bredeney (A), Brandenburg (B), Idikan (C) and Infantis (D) in culture media. A strain of *Salmonella* Bredeney, Brandenburg, Idikan and Infantis were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the strains (±SE).

In **figure 9,** the differences between treated and control cells grown at 25°C and 37°C at the different timepoints are compared between *Salmonella* serotypes. When the cells were grown at 37°C, the differences of all the serotypes varied around 1.12 $\log_{10}$ cfu/mL at each timepoint (**Figure 9 B**). In contrast to the differences at 37°C, the difference at 25°C varied more (**Figure 9 A**). For example, the differences of *S.* Rissen grown at 25°C at 24h and 48h were higher than the differences of the other serotypes. In general, the mean difference was 2.32 $\log_{10}$ cfu/mL and was approximately 1.20 log higher than at 37°C. There were no significant variation found between the *Salmonella* serotypes at each timepoint and at both temperatures. However, on the non-selective plates at 25°C a significant difference directly after treatment between *S.* Rissen and *S.* Derby (p=0.001), *S.* Rissen and *S.* Livingstone (p=0.003) and *S.* Rissen and *S.* monophasic Typhimurium (p=0.045) was found. A significant difference after 48h in cooling between *S.* Rissen and *S.* Derby (p=0.015) and *S.* Rissen and *S.* monophasic Typhimurium (p=0.032) was found as well on the non-selective plates at 25°C.

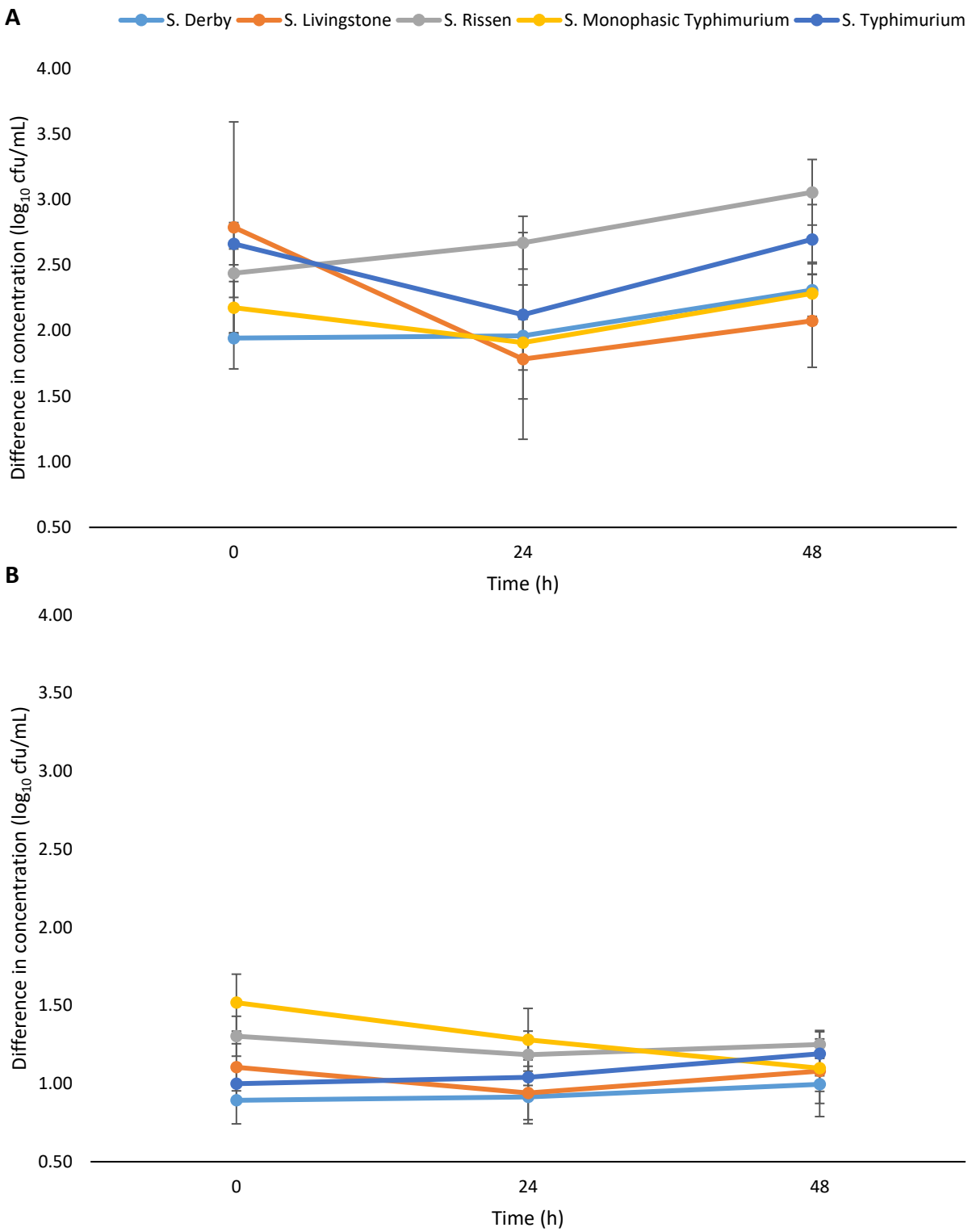


Figure 9: Comparison of the differences in lactic acid sensitivity of *Salmonella* serotypes in culture media. *Salmonella* serotypes Derby, Livingstone, Rissen, Monophasic Typhimurium and Typhimurium were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD). The mean differences between the treated and control, treated with 0.85% NaCl instead of lactic acid, of each serotype at the three timepoints is compared for the strains grown at 25°C (A) and 37°C (B). Differences are expressed as means of the serotype (±SE).

3.1.2 Lactic acid sensitivity of *Yersinia enterocolitica* bioserotypes

The data as means of the triplicate ± SE of *Yersinia enterocolitica* bioserotypes 4/O:3 and 2/O:9 are shown in **tables 9-10**. The differences on CIN were more or less similar to the differences on PCA, therefore only the results of the selective plates are shown and discussed, except when mentioned.

The concentrations of the treated cells of *Y. enterocolitica* bioserotype 4/O:3 and 2/O:9, that were grown at 37°C to stationary phase, varied more in comparison with the treated cells which were grown at 25°C (**Table 9-10**). For example, the treated *Y. enterocolitica* 4/O:3 cells grown to stationary phase at 37°C, stored at 2°C for 48h and plated on CIN plates showed a concentration of 5.76±0.98, 6.80±1.00 and 7.19±0.30 $\log_{10}$ cfu/mL for strains S277, S203 and S185, respectively and the concentrations of the treated cells grown at 25°C were 6.58±0.12, 6.60±0.34 and 6.67±0.27 $\log_{10}$ cfu/mL for strains S277, S203 and S185, respectively (**Table 9**). However, the concentrations of the control cells grown at 25°C and 37°C were stable between the three strains. For example, the control *Y. enterocolitica* 2/O:9 cells grown to stationary phase at 37°C, directly after treatment and plated on CIN plates showed a concentration of 7.27±0.36, 7.47±0.29 and 7.29±0.43 $\log_{10}$ cfu/mL for strains FAVV13523, Y47K and Y47C, respectively and the concentrations of the control cells grown at 25°C were 8.75±0.12, 8.84±0.05 and 8.87±0.01 $\log_{10}$ cfu/mL for strains FAVV13523, Y47K and Y47C, respectively (**Table 10**).

To determine the percentage of stressed *Y. enterocolitica* cells, the percentage of sub-lethal injury was calculated (**Table 11**). When grown at 25°C, *Y. enterocolitica* bioserotype 2/O:9 had a sub-lethal injury of 56.4±10.4% and 49.6±17.4% directly and 24h after treatment, respectively, in contrast to bioserotype 4/O:3, which showed no stressed cells. However, the sub-lethality after 48h in cooling of bioserotype 4/O:3 increased dramatically to 92.3±1.5%, which was even higher than bioserotype 2/O:9. Another interesting trend for both bioserotypes was a decrease in percentage of sub-lethality between directly and 48h after treatment when the strains were grown at 37°C and an increase when the cells were grown at 25°C.

In **figure 10**, the mean concentrations of each *Y. enterocolitica* bioserotype are shown. The control cells of bioserotype 4/O:3 grown at 25°C were stable during cooling and fluctuated around 8.81 $\log_{10}$ cfu/mL (**Figure 10 A**). The treated cells at 25°C stayed stable around 7.25 $\log_{10}$ cfu/mL up to 24h and decreased to 6.62±0.13 $\log_{10}$ cfu/mL after 48h. The cells grown at 37°C showed another trend. The control concentration increased during storage from 7.55±0.11 $\log_{10}$ cfu/mL at 0h to 8.20±0.18 $\log_{10}$ cfu/mL after 48h and the treated concentration increased with the same rate from 5.91±0.47 $\log_{10}$ cfu/mL at 0h to 6.59±0.47 $\log_{10}$ cfu/mL after 48h. When the differences between the treated and control cells were compared within bioserotype 4/O:3 at the different timepoints during cooling, there was no significant difference between the differences at each timepoint, except the difference of the cells which were grown at 25°C at 48h after treatment was significantly different from the difference at timepoint 0h ($p=0.033$) and between timepoint 24h and 48h there was a significant difference ($p=0.000$) on the non-selective plates but not on the selective plates. In addition, when the differences were compared between 25°C and 37°C, there was no significant difference at all timepoints between the differences.

In contrast to *Y. enterocolitica* bioserotype 4/O:3, the control cells of bioserotype 2/O:9 grown at 25°C were not stable during cooling (**Figure 10 B)**. First, the concentration decreased from 8.82±0.04 log_{10} cfu/mL directly after treatment to 7.70±0.13 log_{10} cfu/mL at 24h in cooling and subsequently increased to 8.36±0.09 log_{10} cfu/mL after 48h in cooling. The control cells grown at 37°C displayed the same trend as the control cells grown at 25°C. The treated cells grown at 25°C were stable around 7.10 log_{10} cfu/mL during cooling. Although the concentration of the treated cells grown at 37°C, were stable during the first 24h in cooling, it increased between 24h and 48h with 0.48 log_{10} cfu/mL. When the differences between the treated and control cells grown at 25°C were compared within bioserotype 2/O:9 at the different timepoints during cooling, there was a significant difference between all the timepoints (between 0h and 24h p=0.000, between 0h and 48h p=0.001 and between 24h and 48h p=0.000), but not when the cells were grown at 37°C. However, on the non-selective plates there was no significant difference at 25°C. In addition, when the differences were compared between 25°C and 37°C, there was no significant difference at all timepoints (p>0.05).

In **figure 11,** the differences between treated and control cells grown at 25°C and 37°C were compared between the two *Y. enterocolitica* bioserotypes. When the cells were grown at 25°C, the differences of bioserotype 4/O:3 at 24h and 48h after treatment was approximately 1 log_{10} cfu/mL higher than the differences of bioserotype 2/O:9 (**Figure 11 A**). When the cells were grown at 37°C, the differences of 4/O:3 fluctuated around 1.72 log_{10} cfu/mL at each timepoint in contrast to bioserotype 2/O:9 which were very variable (**Figure 11 B**). Although, the differences varied sometimes greatly, there was no significant difference found between *Y. enterocolitica* bioserotypes at each timepoint and at both temperatures.

Table 9: Lactic acid sensitivity of *Yersinia enterocolitica* bioserotype 4/O:3 in culture media. Three strains of *Yersinia enterocolitica* bioserotype 4/O:3 (Y277, Y203 and Y185) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on cefsulodin irgasan novobiocin agar (CIN) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			Y277				Y203				Y185				Mean			
			CIN		PCA		CIN		PCA		CIN		PCA		CIN		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	4.92	0.93	5.76	0.92	6.47	0.90	7.11	0.31	6.35	0.50	6.77	0.26	5.91	0.47	6.55	0.35
		Control	7.97	0.20	8.18	0.17	7.73	0.22	8.10	0.06	7.56	0.04	7.82	0.08	7.75	0.11	8.03	0.08
		Difference	3.04	0.78	2.42	0.74	1.26	0.69	0.98	0.33	1.21	0.49	1.05	0.19	1.84	0.45	1.48	0.34
	24h	Treated	5.37	1.01	6.08	0.95	6.47	0.93	7.30	0.39	7.00	0.18	7.12	0.25	6.28	0.47	6.83	0.36
		Control	8.12	0.28	8.23	0.28	8.15	0.12	8.36	0.10	7.70	0.37	7.93	0.25	7.99	0.16	8.17	0.13
		Difference	2.75	0.73	2.15	0.70	1.68	0.81	1.06	0.32	0.70	0.26	0.81	0.09	1.71	0.44	1.34	0.30
	48h	Treated	5.76	0.98	6.49	0.95	6.80	1.00	7.59	0.26	7.19	0.30	7.30	0.33	6.59	0.47	7.13	0.34
		Control	8.05	0.41	8.37	0.37	8.47	0.23	8.62	0.22	8.09	0.31	8.11	0.34	8.20	0.18	8.37	0.17
		Difference	2.28	0.58	1.88	0.58	1.66	0.77	1.03	0.05	0.90	0.06	0.82	0.12	1.61	0.34	1.24	0.24
25°C	0h	Treated	7.43	0.05	7.16	0.16	7.13	0.11	7.20	0.03	7.23	0.24	7.14	0.06	7.26	0.09	7.17	0.05
		Control	8.69	0.02	8.84	0.07	8.69	0.10	8.76	0.09	8.96	0.03	9.07	0.00	8.78	0.05	8.89	0.06
		Difference	1.25	0.05	1.68	0.16	1.57	0.10	1.56	0.08	1.73	0.24	1.93	0.06	1.52	0.10	1.72	0.08
	24h	Treated	7.69	0.04	7.10	0.28	6.86	0.09	7.16	0.14	7.19	0.20	7.21	0.04	7.24	0.14	7.16	0.09
		Control	8.95	0.03	8.96	0.11	8.81	0.13	8.96	0.11	8.70	0.27	9.37	0.07	8.82	0.09	9.10	0.08
		Difference	1.26	0.02	1.86	0.26	1.94	0.20	1.80	0.12	1.51	0.47	2.16	0.07	1.57	0.18	1.94	0.10
	48h	Treated	6.58	0.12	7.87	0.10	6.60	0.34	7.78	0.08	6.67	0.27	7.74	0.18	6.62	0.13	7.80	0.07
		Control	9.00	0.08	9.12	0.13	8.95	0.09	9.11	0.14	8.53	0.53	9.46	0.01	8.83	0.17	9.23	0.08
		Difference	2.42	0.17	1.25	0.14	2.36	0.31	1.33	0.06	1.86	0.78	1.72	0.19	2.21	0.26	1.43	0.10

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 10: Lactic acid sensitivity of *Yersinia enterocolitica* bioserotype 2/O:9 in culture media. Three strains of *Yersinia enterocolitica* bioserotype 2/O:9 (FAVV13523, Y47K and Y47C) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on cefsulodin irgasan novobiocin agar (CIN) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			FAVV13523				Y47K				Y47C				Mean			
			CIN		PCA		CIN		PCA		CIN		PCA		CIN		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	5.79	0.51	6.41	0.44	6.05	0.48	6.58	0.16	6.16	0.41	7.12	0.22	6.00	0.24	6.70	0.18
		Control	7.27	0.36	7.58	0.28	7.47	0.29	7.80	0.15	7.29	0.43	7.80	0.37	7.34	0.19	7.72	0.15
		Difference	1.47	0.15	1.17	0.18	1.42	0.43	1.22	0.09	1.13	0.53	0.67	0.56	1.34	0.21	1.02	0.19
	24h	Treated	5.84	0.81	6.45	0.52	5.78	0.71	6.66	0.40	6.28	0.53	6.93	0.40	5.97	0.36	6.68	0.23
		Control	6.69	0.36	7.68	0.30	6.77	0.46	8.06	0.12	7.25	0.33	8.20	0.15	6.91	0.21	7.98	0.13
		Difference	0.86	0.46	1.23	0.23	1.00	0.27	1.40	0.27	0.97	0.23	1.27	0.25	0.94	0.17	1.30	0.13
	48h	Treated	6.34	0.82	6.76	0.61	6.32	0.78	6.76	0.58	6.69	0.56	7.20	0.35	6.45	0.37	6.90	0.27
		Control	7.58	0.56	8.04	0.50	8.37	0.12	8.61	0.10	8.14	0.68	8.78	0.10	8.03	0.28	8.48	0.19
		Difference	1.24	0.42	1.28	0.27	2.05	0.66	1.85	0.48	1.45	0.42	1.59	0.30	1.58	0.28	1.57	0.20
25°C	0h	Treated	7.51	0.08	7.61	0.00	6.72	0.17	7.33	0.07	6.89	0.20	7.55	0.13	7.04	0.14	7.50	0.06
		Control	8.75	0.12	8.80	0.11	8.84	0.05	8.95	0.06	8.87	0.01	8.92	0.04	8.82	0.04	8.89	0.04
		Difference	1.25	0.06	1.18	0.11	2.13	0.13	1.62	0.03	1.98	0.21	1.37	0.16	1.79	0.15	1.39	0.09
	24h	Treated	7.60	0.15	7.64	0.04	6.76	0.10	7.39	0.06	7.02	0.21	7.66	0.09	7.13	0.15	7.57	0.05
		Control	7.65	0.24	9.08	0.16	7.71	0.26	9.15	0.05	7.74	0.29	9.09	0.12	7.70	0.13	9.11	0.06
		Difference	0.06	0.17	1.44	0.13	0.95	0.21	1.75	0.04	0.72	0.39	1.43	0.21	0.57	0.19	1.54	0.09
	48h	Treated	7.40	0.15	8.03	0.08	6.91	0.11	8.05	0.18	7.07	0.18	8.02	0.09	7.13	0.10	8.03	0.06
		Control	8.28	0.25	9.30	0.05	8.39	0.13	9.42	0.04	8.41	0.05	9.46	0.01	8.36	0.09	9.39	0.03
		Difference	0.88	0.16	1.27	0.12	1.48	0.21	1.38	0.22	1.34	0.17	1.43	0.09	1.23	0.13	1.36	0.08

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 11: Sub-lethal injury of *Yersinia enterocolitica* bioserotypes in culture media. Different strains of *Yersinia enterocolitica* bioserotypes were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on cefsulodin irgasan novobiocin agar (CIN) and plate count agar (PCA). The number of surviving cells was enumerated and the percentage of sub-lethal injury was calculated. Values are expressed as means of the triplicate (±SE). ~ negative percentage (mathematical concept).

		Y. 4/O:3		*Y.* 2/O:9	
	Time in cooling	**mean[A]**	**SE[B]**	**mean[A]**	**SE[B]**
37°C	**0h**	50.4	15.6	71.6	9.0
	24h	54.9	10.7	68.1	11.7
	48h	38.5	14.2	55.0	11.0
25°C	**0h**	~		56.4	10.4
	24h	~		49.6	17.4
	48h	92.3	1.5	84.6	3.8

[A] % sub-lethal injury

[B] standard error

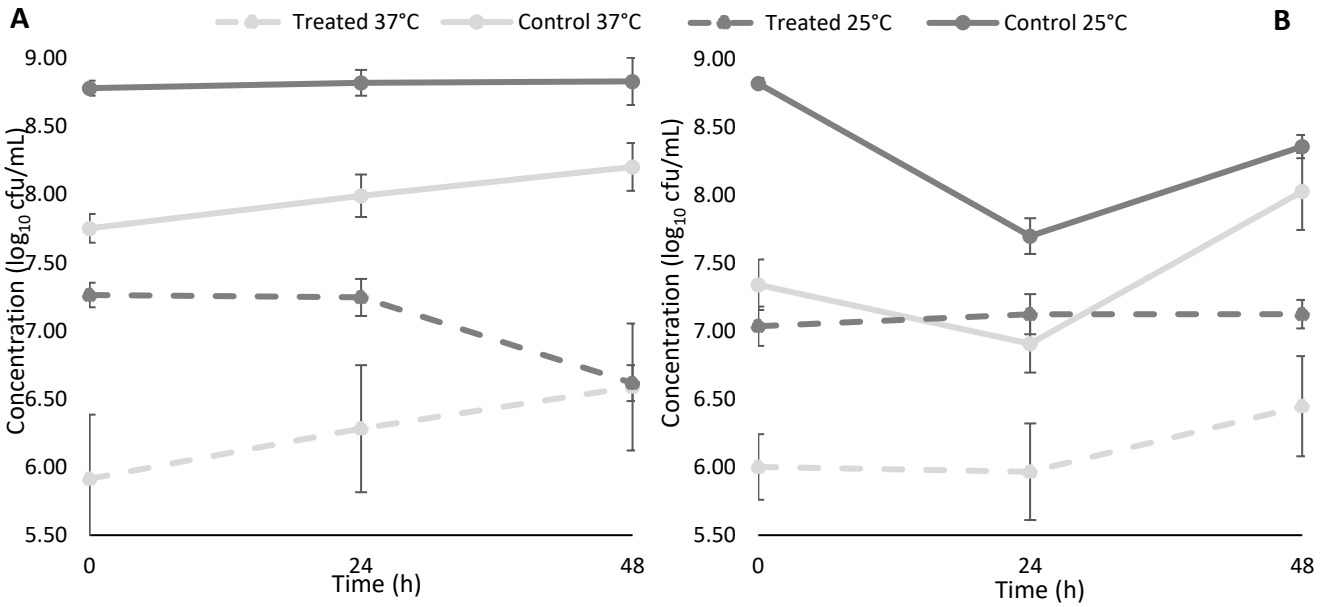


Figure 10: Lactic acid sensitivity of *Yersinia enterocolitica* bioserotype 4/O:3 (A) and 2/O:9 (B) in culture media. Three strains of *Yersinia enterocolitica* bioserotype 4/O:3 and 2/O:9 were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on cefsulodin irgasan novobiocin agar (CIN). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

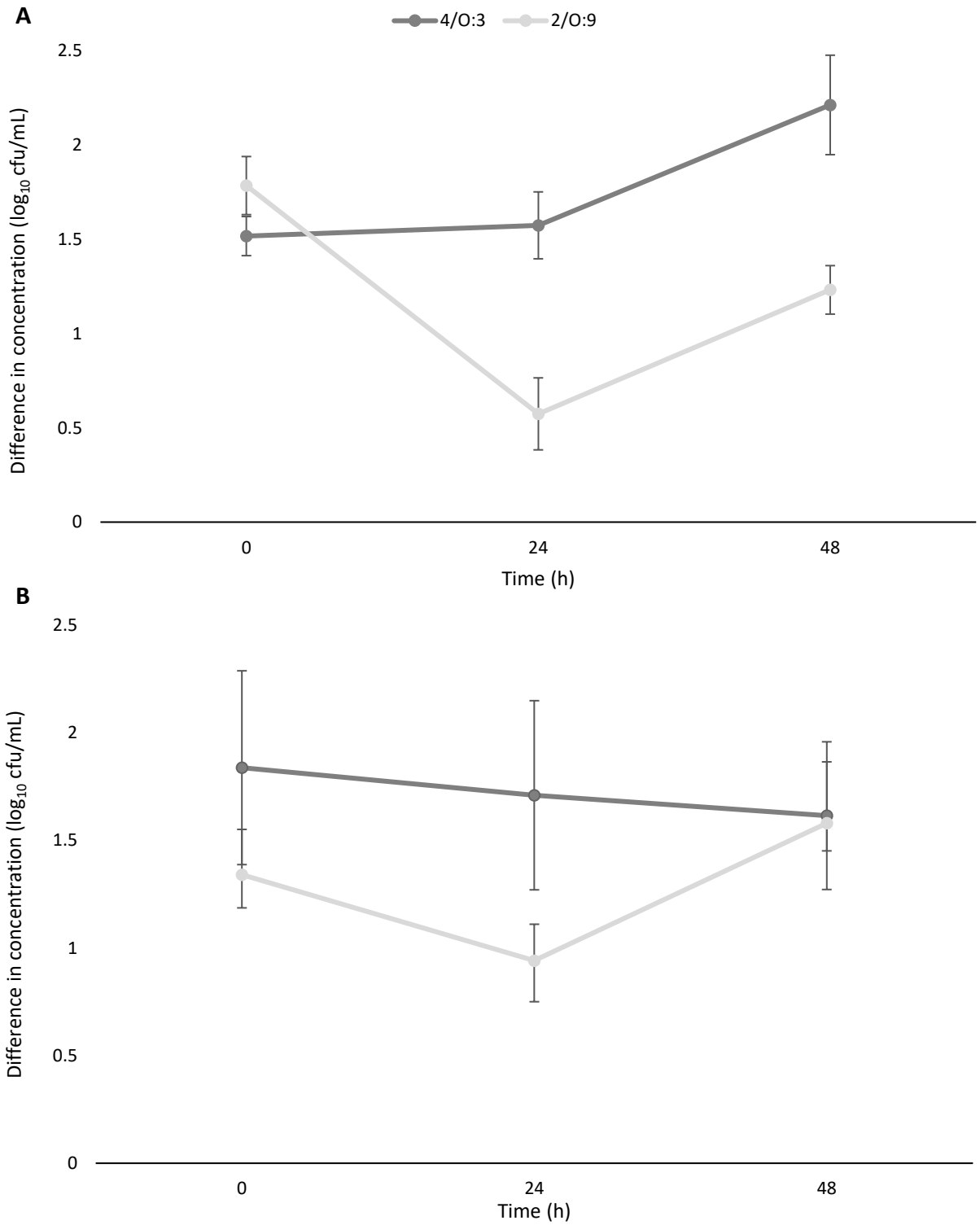


Figure 11: Comparison of the differences in lactic acid sensitivity of *Yersinia enterocolitica* bioserotypes in culture media. All *Yersinia enterocolitica* bioserotypes were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on cefsulodin irgasan novobiocin agar (CIN). The mean differences between the treated and control, treated with 0.85% NaCl instead of lactic acid, of each serotype at the three timepoints is compared for the strains grown at 25°C (A) and 37°C (B). Differences are expressed as means of the serotypes (±SE).

3.1.3 Pathogenicity test

To determine the pathogenicity of the cells, the cells were investigated for the potential loss of pYV. In order to do so, the different *Y. enterocolitica* strains, grown at 37°C and 25°C, were plated on Congo red magnesium oxalate agar (CRMOX) at the different timepoints after lactic acid exposure. The red colonies still possessed the pYV, in contrast to the white colonies, which had lost their pYV. After 48h at 37°C, the red and white colonies were enumerated and the percentage of red colonies was calculated. **Figure 11** shows that both bioserotypes displayed approximately the same trend. The percentage of pYV positive cells differed significantly (p=0.000) between 25°C and 37°C for both bioserotypes. The cells grown at 25°C showed between 82% and 100% of pYV positive cells, in contrast to the cells grown at 37°C, where the majority of the cells had lost their pYV.

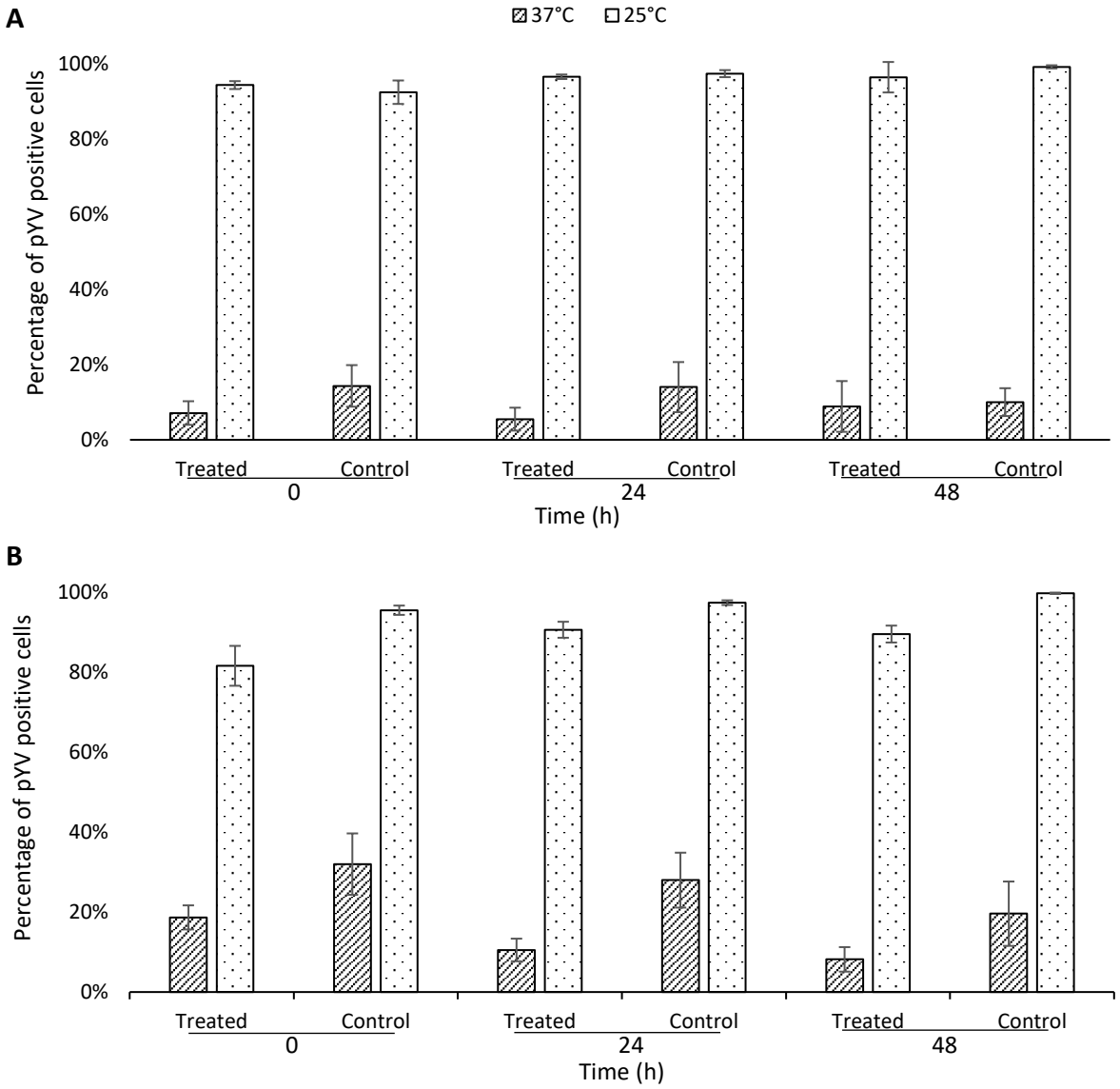


Figure 12: Pathogenicity test of *Yersinia enterocolitica* bioserotype 4/O:3 (A) and 2/O:9 (B). Three strains of *Yersinia enterocolitica* bioserotype 4:O:3 and 2/O:9 were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of *Yersinia* virulence plasmid (pYV) cells was determined by plating appropriate dilutions on Congo red magnesium oxalate agar (CRMOX) plates. The percentage of pYV positive cells was calculated. The control cells were treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

In addition, the percentage of pYV positive treated cells were lower as compared to the pYV positive control cells at the same timepoint. The differences between number of pYV positive control and treated cells of bioserotype 4/O:3 were not significant for both temperatures (**Figure 11 A**). However, the differences between number of pYV positive treated and control cells of bioserotype 2/O:9 were significantly different. For the cells grown at 37°C, directly, 24h and 48h after treatment the differences were 17.1% (p=0.005), 6.8% (p=0.005) and 10.2% (p=0.000), respectively. For the cells grown at 25°C, only after 24h there is a significant difference of 17.5% (p=0.001) between the treated and control cells (**Figure 11 B**).

3.1.4 Lactic acid sensitivity tests of *E. coli*, *K. pneumoniae*, *P. rustigianii* and *E. hermannii*

The data as means of the triplicate ± SE of *Escherichia coli, Klebsiella pneumoniae, Providencia rustigianii* and *Escherichia hermannii* are shown in **tables 12-14**. The differences on the selective plates were more or less similar to the differences on PCA, therefore only the results of the selective plates are shown and discussed except when mentioned.

The strain variability of *E. coli* was very low, except for the treated cells grown at 37°C, at 48h in cooling and plated on TBX. These concentrations were 7,90±0.71, 8.80±0.09 and 7.93±0.72 $\log_{10}$ cfu/mL for strains E1, E2 and E3, respectively (**Table 12**). The results of the *K. pneumoniae* strains varied more when the cells were grown at 25°C than at 37°C. For example, the treated cells grown to stationary phase at 37°C, stored at 2°C for 24h and plated on RAPID' En plates showed a concentration of 8.81±0.04, 8.71±0.04 and 8.75±0.02 $\log_{10}$ cfu/mL for strains K1, K2 and K3, respectively and the concentrations of the treated cells grown at 25°C were 7.80±0.24, 7.01±0.08 7.11±0.22 $\log_{10}$ cfu/mL for strains K1, K2 and K3, respectively (**Table 13**).

To determine the percentage of stressed *E. coli, K. pneumoniae, P. rustigianii* and *E. hermannii* cells, the percentage of sub-lethal injury was calculated (**Table 15**). *E. coli* showed no sub-lethality at both temperatures. Only the *K. pneumoniae* cells grown at 25°C showed a sub-lethality of 39.1±7.1%, 59.6±4.8% and 75.5±5.8% directly, 24h and 48h after treatment, respectively. The percentage of stressed cells of *P. rustigianii* and *E. hermannii* grown at 37°C at 48h after treatment increased during cooling to 78.7±4.8% and 82.9±1.9%, respectively.

In **figure 13**, the mean concentrations of each species are shown. When the *E. coli* cells were grown at 25°C and 37°C, the mean of the concentration of the treated and control cells were stable up to 24h after treatment (**Figure 13 A**). At 48h after treatment, the treated cells decreased to 7.61±0.12 and 8.84±0.10 $\log_{10}$ cfu/mL for 25°C and 37°C, respectively. When the differences between the treated and control cells were compared at the three timepoints during cooling, there was no significant difference between the timepoints, except the difference of the cells, which were grown at 25°C at 48h after treatment, was significant different from 0h (p=0.000) and 24h (p=0.000). Although the difference after 48h in cooling at 25°C (1.04±0.10 $\log_{10}$ cfu/mL) was greater than at 37°C (0.64±0.29 $\log_{10}$ cfu/mL), there was no significant difference at all timepoints.

When the *K. pneumoniae* cells were grown at 37°C, the mean of the concentration of the treated and control cells were almost similar and stable during cooling (**Figure 13 B**). Directly after treatment, there is a small difference of 0.38±0.05 $\log_{10}$ cfu/mL between the mean of the control and the treated cells, but after 24h and 48h in cooling the difference disappeared. However, when the *K. pneumoniae* cells were grown at 25°C, the mean of the concentration of the treated and control cells decreased during storage at 2°C from 7.38±0.13 to 6.20±0.24

Table 12: Lactic acid sensitivity of *Escherichia coli* in culture media. Three strains of *E. coli* (E1, E2 and E3) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on tryptone bile X-glucuronide agar (TBX) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			E1				E2				E3				Mean			
			TBX		PCA		TBX		PCA		TBX		PCA		TBX		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
		Treated	8.78	0.17	8.76	0.10	8.82	0.02	8.74	0.06	8.78	0.06	8.65	0.01	8.79	0.05	8.72	0.04
	0h	Control	9.21	0.05	9.10	0.07	9.22	0.10	9.12	0.12	9.00	0.07	9.07	0.06	9.14	0.05	9.10	0.04
		Difference	0.43	0.11	0.33	0.09	0.41	0.13	0.38	0.07	0.22	0.03	0.43	0.06	0.35	0.06	0.38	0.04
		Treated	8.81	0.04	8.73	0.04	8.97	0.04	8.92	0.04	8.63	0.04	8.51	0.07	8.81	0.05	8.72	0.07
37°C	24h	Control	8.98	0.02	8.99	0.03	9.16	0.02	9.08	0.04	8.78	0.03	8.64	0.12	8.97	0.06	8.91	0.08
		Difference	0.16	0.02	0.26	0.04	0.18	0.05	0.16	0.03	0.15	0.01	0.13	0.13	0.16	0.02	0.19	0.05
		Treated	7.90	0.71	7.99	0.69	8.80	0.09	8.67	0.16	7.93	0.72	7.91	0.58	8.21	0.33	8.19	0.29
	48h	Control	8.96	0.03	8.87	0.04	9.08	0.04	8.92	0.06	8.49	0.14	8.46	0.14	8.84	0.10	8.75	0.09
		Difference	1.06	0.68	0.88	0.72	0.28	0.07	0.25	0.10	0.56	0.63	0.55	0.52	0.64	0.29	0.56	0.27
		Treated	8.90	0.02	8.72	0.06	8.80	0.08	8.76	0.02	8.75	0.04	8.72	0.06	8.82	0.03	8.73	0.03
	0h	Control	9.04	0.05	8.97	0.02	9.09	0.04	9.07	0.01	9.03	0.05	8.95	0.03	9.05	0.02	9.00	0.02
		Difference	0.14	0.03	0.25	0.07	0.29	0.06	0.31	0.02	0.28	0.06	0.24	0.05	0.24	0.04	0.26	0.03
		Treated	8.72	0.03	8.62	0.14	8.85	0.02	8.71	0.06	8.70	0.04	8.52	0.04	8.76	0.03	8.62	0.05
25°C	24h	Control	8.87	0.02	8.86	0.07	9.00	0.03	8.91	0.06	8.68	0.09	8.53	0.10	8.85	0.05	8.77	0.07
		Difference	0.15	0.04	0.23	0.17	0.15	0.00	0.20	0.10	-0.01	0.10	0.01	0.11	0.10	0.04	0.15	0.07
		Treated	7.73	0.03	7.71	0.12	7.94	0.07	8.03	0.03	7.15	0.04	7.16	0.17	7.61	0.12	7.63	0.14
	48h	Control	8.47	0.07	8.61	0.10	9.01	0.13	9.02	0.04	8.45	0.09	8.02	0.12	8.64	0.10	8.55	0.15
		Difference	0.74	0.09	0.89	0.05	1.07	0.18	1.00	0.07	1.30	0.05	0.86	0.06	1.04	0.10	0.92	0.04

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 13: Lactic acid sensitivity of *Klebsiella pneumoniae* in culture media. Three strains of *Klebsiella pneumoniae* (K1, K2 and K3) were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on RAPID' Enterobacteriaceae agar (RAPID' En) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			K1				K2				K3				Mean			
			PAPID' En		PCA		PAPID' En		PCA		PAPID' En		PCA		PAPID' En		PCA	
Time in cooling			mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	8.62	0.06	8.51	0.02	8.65	0.05	8.46	0.03	8.64	0.04	8.49	0.03	8.64	0.03	8.49	0.02
		Control	9.03	0.07	8.77	0.11	8.97	0.04	8.84	0.05	9.05	0.04	8.89	0.08	9.01	0.03	8.83	0.05
		Difference	0.41	0.13	0.25	0.10	0.32	0.08	0.38	0.05	0.41	0.04	0.41	0.09	0.38	0.05	0.34	0.05
	24h	Treated	8.81	0.04	8.57	0.01	8.71	0.04	8.40	0.04	8.75	0.02	8.46	0.04	8.76	0.02	8.48	0.03
		Control	8.84	0.09	8.56	0.06	8.69	0.14	8.49	0.04	8.91	0.04	8.66	0.04	8.81	0.06	8.57	0.03
		Difference	0.03	0.07	-0.01	0.05	-0.02	0.17	0.09	0.08	0.16	0.06	0.20	0.01	0.06	0.06	0.09	0.04
	48h	Treated	8.77	0.05	8.71	0.06	8.69	0.02	8.62	0.07	8.73	0.04	8.60	0.03	8.73	0.02	8.64	0.03
		Control	8.66	0.11	8.69	0.08	8.71	0.02	8.62	0.05	8.77	0.10	8.55	0.02	8.71	0.05	8.62	0.03
		Difference	-0.11	0.12	-0.02	0.14	0.02	0.03	0.00	0.05	0.04	0.08	-0.05	0.04	-0.02	0.05	-0.02	0.04
25°C	0h	Treated	7.68	0.22	7.93	0.25	7.14	0.08	7.29	0.05	7.31	0.27	7.62	0.14	7.38	0.13	7.61	0.13
		Control	9.33	0.05	9.26	0.00	8.43	0.04	8.42	0.06	9.32	0.01	9.27	0.01	9.03	0.15	8.98	0.14
		Difference	1.65	0.17	1.32	0.25	1.30	0.13	1.13	0.10	2.00	0.26	1.65	0.14	1.65	0.14	1.37	0.12
	24h	Treated	7.80	0.24	8.11	0.29	7.01	0.08	7.40	0.03	7.11	0.22	7.65	0.16	7.30	0.16	7.72	0.14
		Control	8.58	0.07	8.71	0.10	7.93	0.07	7.98	0.02	8.75	0.05	8.78	0.03	8.42	0.13	8.49	0.13
		Difference	0.79	0.24	0.60	0.32	0.92	0.13	0.57	0.05	1.64	0.19	1.13	0.13	1.12	0.16	0.77	0.14
	48h	Treated	6.83	0.57	7.31	0.65	5.71	0.00	6.39	0.21	6.07	0.23	7.05	0.17	6.20	0.24	6.92	0.25
		Control	7.36	0.40	7.95	0.06	6.31	0.12	7.57	0.15	7.32	0.07	7.64	0.30	6.99	0.21	7.72	0.11
		Difference	0.53	0.17	0.64	0.59	0.60	0.12	1.19	0.32	1.25	0.22	0.59	0.32	0.79	0.14	0.80	0.24

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 14: Lactic acid sensitivity of *Providencia rustigianii* and *Escherichia hermannii* in culture media. A strain of *Providencia rustigianii* and *Escherichia hermannii* were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on RAPID' Enterobacteriaceae agar (RAPID' En) and plate count agar (PCA). The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

			Providencia rustigianii				*Eschericia hermannii*			
			RAPID' En		PCA		RAPID' En		PCA	
	Time in cooling		mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	Treated	7.59	0.24	7.80	0.08	7.92	0.17	8.34	0.04
		Control	8.33	0.01	8.36	0.02	8.79	0.05	8.73	0.03
		Difference	0.74	0.23	0.56	0.06	0.86	0.18	0.39	0.03
	24h	Treated	6.55	0.10	6.80	0.07	7.24	0.08	7.82	0.13
		Control	8.57	0.04	8.48	0.05	8.30	0.13	8.40	0.05
		Difference	2.03	0.07	1.68	0.08	1.06	0.20	0.58	0.16
	48h	Treated	5.97	0.14	6.66	0.19	7.21	0.05	7.99	0.04
		Control	8.46	0.02	8.28	0.03	8.14	0.06	8.21	0.04
		Difference	2.49	0.13	1.62	0.17	0.93	0.11	0.22	0.07
25°C	0h	Treated	5.01	0.17	5.64	0.32	6.56	0.28	6.58	0.20
		Control	8.73	0.05	8.82	0.21	8.85	0.03	8.86	0.03
		Difference	3.72	0.14	3.18	0.27	2.29	0.31	2.27	0.21
	24h	Treated	4.29	0.16	4.60	0.06	5.80	0.62	5.96	0.49
		Control	8.14	0.17	7.71	0.35	8.75	0.07	8.64	0.09
		Difference	3.86	0.07	3.11	0.32	2.95	0.68	2.67	0.59
	48h	Treated	4.46	0.27	4.76	0.32	5.31	0.60	5.76	0.66
		Control	8.29	0.02	8.36	0.02	8.17	0.10	8.32	0.09
		Difference	3.83	0.27	3.60	0.30	2.86	0.67	2.57	0.73

[A] $\log_{10}$ cfu/mL

[B] standard error

Table 15: Sub-lethal injury of *E. coli, K. pneumoniae, P. rustigianii* and *E. hermannii* in culture media. Different strains of *E. coli, K. pneumoniae, P. rustigianii* and *E. hermannii* were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on tryptone bile X-glucuronide agar (TBX) for the *E. coli* strains, RAPID' Enterobacteriaceae agar (RAPID' En) for the other strains and plate count agar (PCA). The number of surviving cells was enumerated and the percentage of sub-lethal injury was calculated. Values are expressed as means of the triplicate (±SE). ~ negative percentage (mathematical concept).

		E. coli		*K. pneumonia*		*P. rustigianii*		*E. hermannii*	
	Time in cooling	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]	mean[A]	SE[B]
37°C	0h	~		~		30.5	20.1	55.0	14.8
	24h	~		~		42.0	9.8	71.6	7.5
	48h	~		~		78.7	4.8	82.9	1.9
25°C	0h	~		39.1	7.1	72.5	11.4	1.6	20.4
	24h	~		59.6	4.8	48.1	13.4	22.8	22.5
	48h	~		75.5	5.8	50.0	5.8	63.2	6.9

[A] % sub-lethal injury

[B] standard error

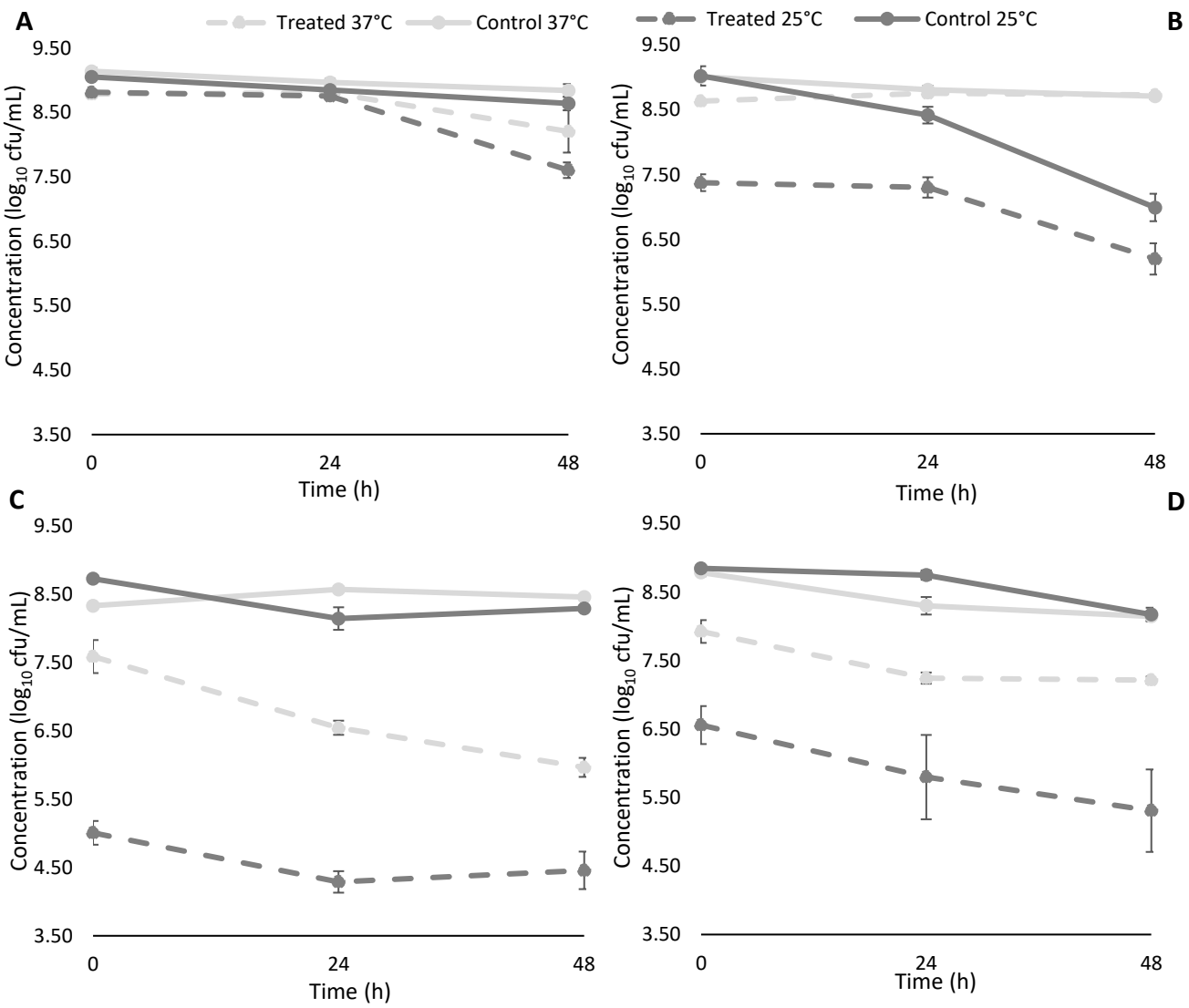


Figure 13: Lactic acid sensitivity of *Escherichia coli* (A), *Klebsiella pneumoniae* (B), *Providencia rustigianii* (C) and *Escherichia hermannii* (D) in culture media. Three strains of *E. coli* and *K. pneumoniae* and one strain of *P. rustigianii* and *E. hermannii* were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on tryptone bile X-glucuronide agar (TBX) for *E. coli* and RAPID' Enterobacteriaceae agar (RAPID' En) for the other Enterobacteriaceae spp. The number of surviving cells was compared with a control, that was treated with 0.85% NaCl instead of lactic acid. Values are expressed as means of the triplicate (±SE).

$\log_{10}$ cfu/mL and from 9.03±0.15 to 6.99±0.21 $\log_{10}$ cfu/mL, respectively. Although, the concentrations of the control and treated cells at 24h and 48h after treatment were not significantly different, the difference between both at 0h differed significantly (p=0.000) from the difference at 24h and 48h in cooling. In addition, the differences between treated and control grown at 25°C, were 1.27, 1.06 and 0.81 $\log_{10}$ cfu/mL higher than the differences at 37°C at timepoints 0h (p=0.000), 24h (p=0.000) and 48h (p=0.000), respectively.

The control cells grown at 25°C and 37°C of *P. rustigianii* were relatively stable during cooling and fluctuated around 8.5 $\log_{10}$ cfu/mL (**Figure 13 C**). The concentration of the treated cells grown at 25°C was lower at all timepoints than the concentration of the treated cells grown at 37°C. This resulted in a greater difference between treated and control concentration at 25°C than at 37°C. The control cells grown at 25°C and 37°C of *E. hermannii* were relatively stable as well during cooling but there was a small decreasing trend visible (**Figure 13 D**). The concentration of the treated cells grown at 25°C was lower at all timepoints than the concentration of the treated cells grown at 37°C. This resulted in a greater difference between treated and control at 25°C than at 37°C, but all differences at both temperatures were smaller than the differences of *P. rustigianii*.

3.1.5 Comparison of Enterobacteriaceae genera

Figure 14 shows the differences between control and treated cells of the Enterobacteriaceae genera *Salmonella*, *Yersinia*, *Escherichia* (including only species *E. coli*) and *Klebsiella*. Because there was no significant variation between the differences of the serotypes of *Salmonella*, one mean comprising al *Salmonella* serotypes except brandenburg was calculated. The same was done for bioserotypes of *Y. enterocolitica*. The differences on the selective plates were more or less similar to the differences on the non-selective plates, therefore only the results of the selective plates are shown and discussed except when mentioned.

First, the difference between the cells that were grown at 25°C are discussed (**Figure 14 A**). After lactic acid treatment, *Salmonella* showed the greatest reduction of all the genera followed by *Yersinia* and *Klebsiella*, in contrast to *Escherichia* which showed almost no effect during the first 24h in cooling. Therefore, directly after treatment, the difference of genera *Escherichia* was significantly lower than *Klebsiella* (p=0.026), *Salmonella* (p=0.000) and *Yersinia* (p=0.006). At 24h in cooling, the difference of *Salmonella* was significantly higher than *Escherichia* (p=0.000) and *Yersinia* (p=0.012), but no significant difference was found between *Salmonella* and *Yersinia* on the non-selective plates. In addition, a significant difference of 1.59 $\log_{10}$ cfu/mL (p=0.000) at 24h between *Escherichia* and *Yersinia* was found with the non-selective plates, but not with the selective plates. After 48h in cooling, the difference of *Salmonella* was significantly higher than *Klebsiella* (p=0.002) and *Escherichia* (p=0.013) but not from *Yersinia*.

Second, the difference between the cells that were grown at 37°C are discussed (**Figure 14 B**). In contrast to the findings at 25°C, *Yersinia* showed the greatest effect of the treatment, followed by *Salmonella*. The genera *Escherichia* and *Klebsiella* showed a small effect of the treatment. Directly after treatment and during the first 24h in cooling, the difference of genera *Salmonella* and *Yersinia* were significantly higher than *Escherichia* (p=0.006 and p=0.000) and *Klebsiella* (p=0.009 and p=0.000). In addition, directly after treatment, the difference of *Salmonella* was significantly lower than *Yersinia* on the selective plates but not significantly different on the non-selective plates. After 48h in cooling, the difference of *Escherichia* was significantly lower than *Yersinia* (p=0.001), the difference of *Klebsiella* was significantly lower than *Salmonella* (p=0.000) and *Yersinia* (p=0.000) and the difference of *Salmonella* was significantly lower than *Yersinia* (p=0.013).

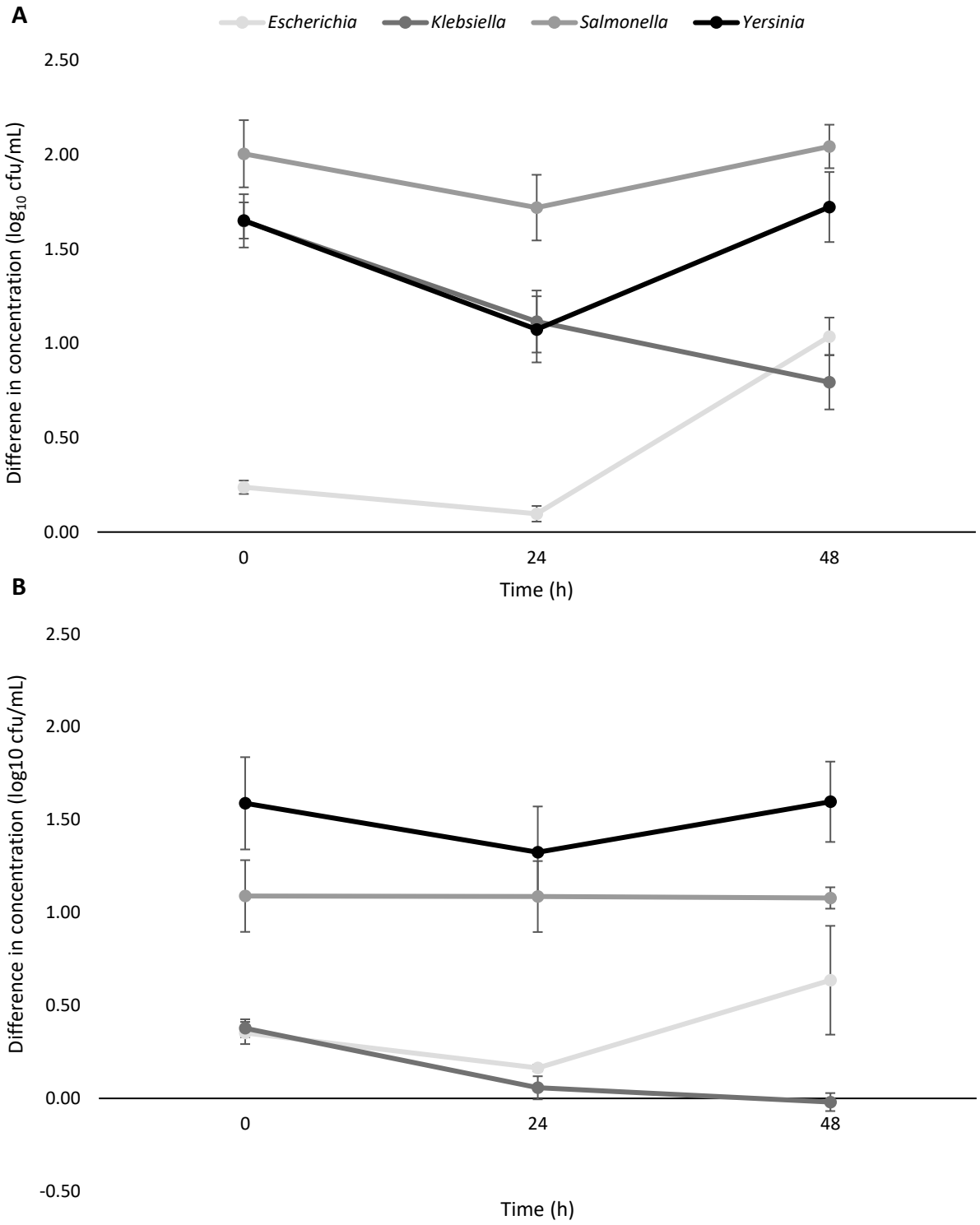


Figure 14: Comparison of the differences in lactic acid sensitivity of Enterobacteriaceae genera in culture media. All strains of the genera *Salmonella*, *Yersinia*, *Escherichia* (including only species *E. coli*) and *Klebsiella* were grown in tryptic soy broth (TSB) at 25°C and 37°C to stationary phase. Then, they were exposed to a 5% lactic acid solution, set at pH 4 at 25°C, in a 1/10 ratio for 10min and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on selective plates. The mean differences between the treated and control (treated with 0.85% NaCl instead of lactic acid) of each serotype at the three timepoints is compared for the strains grown at 25°C (A) and 37°C (B). Differences are expressed as means of the serotypes (±SE).

3.2 Preliminary lactic acid sensitivity study on pig skin

Figure 15 describes the surviving cells of *Salmonella* Derby and Typhimurium directly, 24h and 48h after treatment. Directly after treatment, both serotypes showed a reduced survival of the treated cells as compared with the control cells. At 24h and 48h after treatment, the effect of the lactic acid treatment disappeared and the treated *S.* Typhimurium count was even higher than the control at 24h after treatment. Overall, the number of treated and control cells decreased during cooling to approximately 4.8 $\log_{10}$ cfu/cm².

Figure 16 describes the surviving cells of *Y. enterocolitica* 4/O:3 and 2/O:9 directly, 24h and 48h after treatment. Directly after treatment, both serotypes showed a small reduction of about 0.3 $\log_{10}$ cfu/cm² between treated and control cells. *Y. enterocolitica* 4/O:3 treated and control counts decreased at the same rate during storage at 2°C. *Y. enterocolitica* 2/O:9 treated and control counts decreased in the first 24h at 2°C, but after 48h the numbers increased to almost 5 $\log_{10}$ cfu/cm².

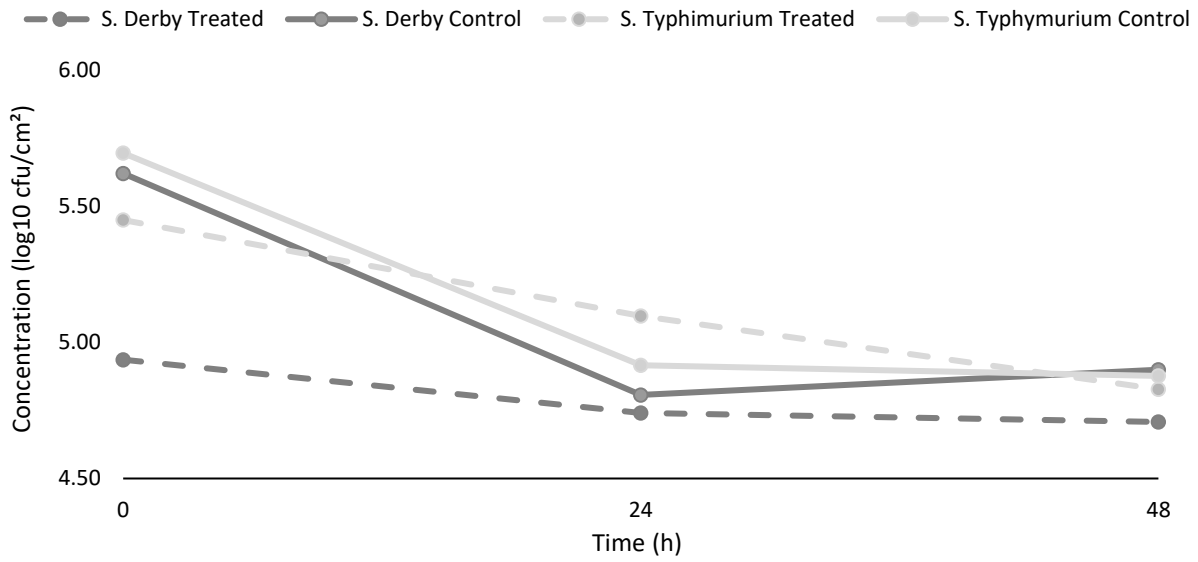


Figure 15: Preliminary lactic acid sensitivity tests of *Salmonella* Derby and Tyhpimurium on pig skin. A strain of *Salmonella* Derby (S416) and Typhimurium (S355) were grown in tryptic soy broth (TSB) 37°C to stationary phase and artificially inoculated on 100 cm² dorsal pig skin. Next, the skin pieces were dipped in a 5% lactic acid solution, set at pH 4 at 25°C and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on xylose lysine deoxycholate agar (XLD). The number of surviving cells was compared with a control, that was not treated with lactic acid.

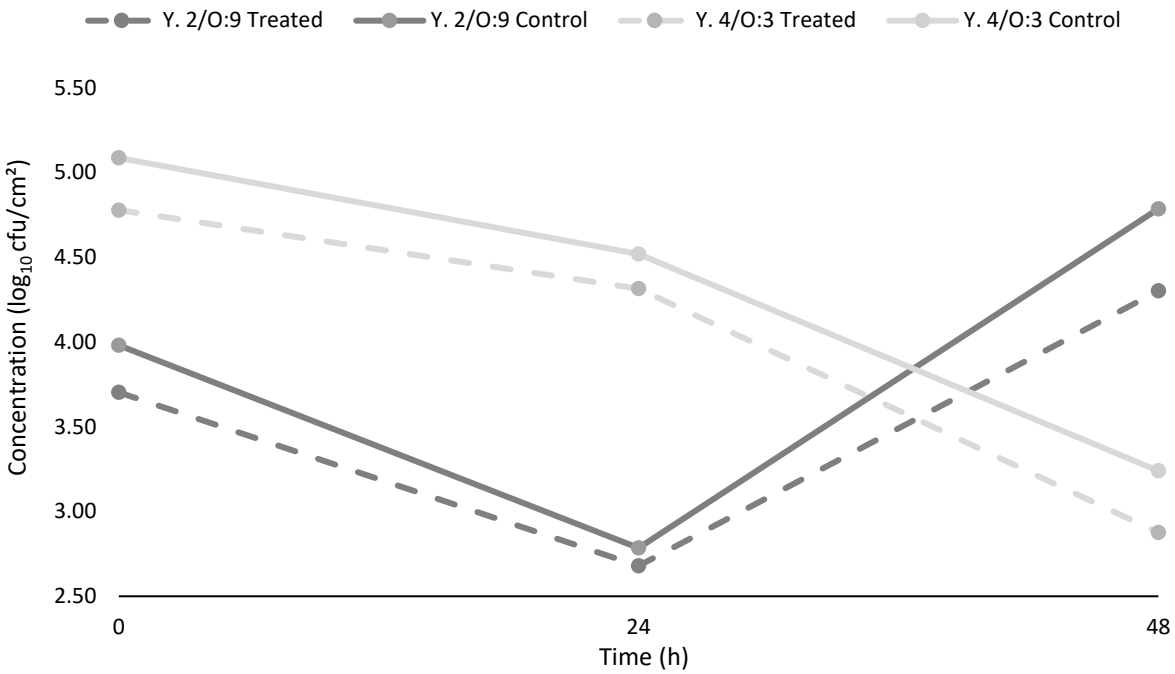


Figure 16: Preliminary lactic acid sensitivity tests of *Yersinia enterocolitica* bioserotype 4/O:3 and 2/O:9 on pig skin. A strain of *Yersinia enterocolitica* 4/O:3 (Y185) and 2/O:9 (Y47C) were grown in tryptic soy broth (TSB) 37°C to stationary phase and artificially inoculated on 100 cm² dorsal pig skin. Then, the skin pieces were dipped in a 5% lactic acid solution, set at pH 4 at 25°C and subsequently stored at 2°C. At 0h, 24h and 48h after lactic acid treatment, the number of surviving cells was determined by plating appropriate dilutions on cefsulodin irgasan novobiocin agar (CIN). The number of surviving cells was compared with a control, that was not treated with lactic acid.

4 Discussion

4.1 Lactic acid sensitivity of *Salmonella*

The results of the lactic acid sensitivity tests in culture media barely showed variation between the investigated serotypes of *Salmonella*. During cooling, the concentration of the control and treated cells grown at 37°C of all serotypes remained stable. However, the treated cells at 25°C displayed a decreasing trend. *S.* Derby, Livingstone, Rissen, monophasic Typhimurium, Typhimurium, Bredeney, Idikan and Infantis showed a reduction between the treated and control cells after exposure to lactic acid of 1.12 $\log_{10}$ cfu/mL when the cells were grown at 37°C and a reduction of 2.32 $\log_{10}$ cfu/mL at 25°C. Although, the effect of the treatment on these serotypes showed a 1.2 $\log_{10}$ cfu/mL difference between the reductions at the two growth temperatures, *S.* Brandenburg showed no difference between 25°C and 37°C. An important remark is that only one strain of *S.* Brandenburg was investigated. Therefore, to make appropriate conclusions about the lactic acid sensitivity of *S.* Brandenburg more strains must be included in the study.

Because there barely was variation between *Salmonella* serotypes (except *S.* Brandenburg), we can assume that these serotypes possibly did not have different acid and cold resistant mechanisms. Unfortunately, there is not much data about different resistant mechanisms between serotypes in the literature. The higher reduction at 25°C than at 37°C can be explained by the sub optimal growth temperature wherein the cells were grown before the lactic acid treatment (Gibson *et al*, 1988). When the *Salmonella* cells are grown at 25°C, they have to activate metabolic processes and alter membrane fluidity that aid them to grow in sub-optimal temperatures, but these changes make them probably more vulnerable for acid stress (Chang *et al*, 2003; Oscar, 1998).

The cells grown at 37°C were used to mimic the *Salmonella* contamination that comes from the intestines of the pig and the cells grown at 25°C were used to mimic the *Salmonella* contamination that comes from the slaughterhouse environment. From the results of this study, we can assume that *Salmonella* cells from the intestines are more resistant to the lactic acid treatment than when they come from the slaughterhouse environment. Although there was a difference in reductions at the two temperatures found, other parameters such as oxygen concentration, nutrient depletions, pH alterations, etc. have to be investigated as well to conclude if there was a difference between contamination of the intestines or slaughterhouse environment.

There is less information available on lactic acid sensitivity of *Salmonella* investigated in culture media, therefore, a direct comparison of our results with other studies is difficult. However, a study performed by Mikołajczyk & Radkowski in 2002 resulted in a reduction of 1 and 2 $\log_{10}$ cfu/mL when 0.05% and 0.03% lactic acid, respectively, was added to their agar media.

4.2 Lactic acid sensitivity of *Yersinia enterocolitica*

The results of the lactic acid sensitivity tests in culture media showed more variation between the two bioserotypes of *Yersinia enterocolitica*, than between the *Salmonella* serotypes. The cells of bioserotype 4/O:3 grown at 25°C were stable during the first 24h in cooling, but after 48h in cooling, the treated cells decreased in contrast to the control cells which remain stable. Furthermore, both treated and control cells grown at 37°C, displayed an increasing trend at the same rate during cooling. Bioserotype 2/O:9 showed a different trend. The concentration of the cells grown at 25°C and 37°C, except for the treated cells at 25°C, decreased during the first 24h and subsequently increased during the next 24h. The increase in concentration after 48h in cooling can be explained by the fact that *Yersinia enterocolitica* is a psychrotolerant bacteria and can grow at refrigerating temperatures (Bottone, 1997; Gill & Reichel, 1989; Lee *et al*, 1980).

Considering the high variability, there was no significant difference found between the differences of the two bioserotypes at each timepoint and at both temperatures. These results may imply that these bioserotypes possibly did not have different acid and cold resistant mechanisms. In addition, there is less data about different resistant mechanisms between *Y. enterocolitica* bioserotypes in the literature.

The cells grown at 37°C were used to mimic the *Y. enterocolitica* contamination that comes from the intestines of the pig and the cells grown at 25°C were used to mimic the *Y. enterocolitica* contamination that comes from the slaughterhouse environment. From the results of this study, we can suspect that there is no difference in lactic acid resistance between cells from the intestines and from the slaughterhouse environment. However, as mentioned above other parameters such as oxygen concentration, nutrient depletions, pH alterations, etc. have to be investigated.

There is not much information available on lactic acid sensitivity of *Y. enterocolitica* investigated at the conditions used in the experiments, therefore, a direct comparison of our results with other studies is difficult. However, a study performed by Janssen *et al* in 2006 and Vereecken *et al* in 2003 resulted in 1 log difference in concentration when *Y. enterocolitica* were cocultured with lactic acid bacterium *Lactobacillus sakei*.

The pathogenicity of the *Y. enterocolitica* bioserotypes was studied as well. In order to do so, the potential loss of pYV was investigated after lactic acid treatment and during cooling. At all timepoints, the percentage of pYV positive cells of both serotypes grown at 25°C was significantly higher than at 37°C. The cells grown at 25°C had barely lost their pYV, in contrast to 37°C where the majority of cells had lost their pYV. When grown at 37°C, it is known that *Y. enterocolitica* losses its pYV, in contrast to milder temperatures such as 25°C (Bottone, 1997). In addition, a trend was seen between the treated and control cells. The percentage of pYV positive treated cells were lower as compared to the pYV positive control cells at the same timepoint for both bioserotypes. A study by Wang *et al* (2014) on *E. coli* and *Salmonella* showed after exposure to lactic acid the formation of holes in the membrane of the bacteria, through which the pYV can possibly leave the cell.

4.3 Lactic acid sensitivity of *E. coli*, *K. pneumoniae*, *P. rustigianii* and *E. hermannii*

E. coli and Enterobacteriaceae counts on the carcass are used as an indicator for the presence of *Salmonella* (Ghafir *et al*, 2008), therefore *Escherichia coli, Klebsiella pneumoniae, Providencia rustigianii* and *Escherichia hermannii*, all belonging to the Enterobacteriaceae family, were included in the study. *E. coli* and *K. pneumoniae* are very common Enterobacteriaceae spp. in the intestine of the pig in contrast to *P. rustigianii* and *E. hermannii*, which are less abundant and rather rare species (Schierack *et al*, 2007).

When the *E. coli* cells were grown at 25°C and 37°C, the mean of the concentration of the treated and control cells differed barely from each other up to 24h after treatment. However, the concentrations of the treated cells were decreased at 48h after treatment. These results are in contrast to the findings of Rajkovic *et al* in 2009. They found a reduction of approximately 1.8 log cfu/mL after exposure to 8% lactic acid for *E. coli* O157:H7.

When the *K. pneumoniae* cells were grown at 37°C, the mean of the concentration of the treated and control cells were almost similar and stable during cooling so no reduction was seen. However, when the *K. pneumoniae* cells were grown at 25°C, the concentration of the treated and control cells decreased during cooling. A possible explanation for their low lactic acid sensitivity at 37°C could be that the cells were grown at their optimal growth temperature and therefore were possibly resistant to the lactic acid, in contrast to the cells that were grown at 25°C (Tripathy *et al*, 2014; Tsuji *et al*, 1982).

E. hermannii showed the same lactic acid sensitivity as *Salmonella*. When the bacteria were grown at 25°C and 37°C the reductions were approximately 2 and 1 $\log_{10}$ cfu/mL, respectively. The trends of *P. rustigianii* at 25°C and 37°C were similar to *E. hermannii* and *Salmonella* i.e. a greater reduction at 25°C than at 37°C, but the reductions were larger at both temperatures. In conclusion, *P. rustigianii* and *E. hermannii* had a greater lactic acid sensitivity than *E. coli* and *K. pneumoniae*.

4.4 Lactic acid sensitivity of Enterobacteriaceae genera

The reductions of *Salmonella* and *Y. enterocolitica* were compared with each other and with the genera *Klebsiella* and *Escherichia* (not including *E. hermannii*).

When the bacteria were grown at 25°C, *Salmonella* showed the greatest reduction of all, followed by *Yersinia* and *Klebsiella. Escherichia* showed a small effect during the first 24h in cooling, but after 48h in cooling the reduction increased. However, when the cells were grown at 37°C, *Yersinia* showed the greatest effect of the treatment, followed by *Salmonella*. In addition, *Escherichia* and *Klebsiella* showed a small effect of the treatment, but after 48h in cooling the reduction of *Escherichia* increased as observed at 25°C.

Klebsiella and *Escherichia* are currently used as indicator organism for *Salmonella* (Ghafir *et al*, 2008; Schierack *et al*, 2007). Interestingly, *Escherichia* and *Klebsiella* did not show the same lactic acid sensitivity as *Salmonella* and *Yersinia*, therefore the use of these indicator organisms has to be reconsidered when using lactic acid treatment.

4.5 Sub-lethal injury

Pathogenic bacteria can be injured under food processing-related stresses such as lactic acid treatment. Under favorable environmental conditions, these sub-lethally injured cells can repair sub-lethal damage and regain viability and pathogenicity (Liao & Fett, 2005; Wong *et al*, 1998). Therefore, the repair and recovery of injured cells in food systems have become an increasing concern on food safety (Xu *et al*, 2008).

Overall, the percentage of sub-lethality of the *Salmonella* serotypes was greater, when the strains were grown at 25°C than at 37°C. Another interesting trend was an increase in percentage of stressed cells between 24h and 48h in cooling at both temperatures. This trend applied to the majority of the serotypes, except for *S.* Derby, Brandenburg, Idikan and Infantis which stayed stable or decreased slightly. The cells grown at 25°C, which is not an optimal growth temperature for *Salmonella*, were probably already stressed before exposure to lactic acid (Gibson *et al*, 1988). During storage at 2°C, the cells were possibly additionally stressed by the low temperature, which resulted in an increase in sub-lethal cells. According to a study by Xu *et al* (2008), *Salmonella* showed after exposure to lactic acid a decreased growth on a selective plate in comparison with a non-selective plate, but a sub-lethality percentage was not calculated.

When grown at 25°C, *Yersinia enterocolitica* bioserotype 2/O:9 had a sub-lethal injury of approximately 50% directly and 24h after treatment, in contrast to bioserotype 4/O:3, which showed no stressed cells. Subsequently, during 48h in cooling, the sub-lethality increased dramatically for both bioserotypes. In addition, an interesting trend with both bioserotypes was a decrease in percentage of sub-lethality between directly and 48h after treatment when the strains were grown at 37°C. There is less data about the sub-lethality percentage of *Y. enterocolitica* after lactic acid stress. However, Vereecken *et al* (2003) described an inactivation phase of *Y. enterocolitica* during exposure to lactic acid.

E. coli showed no sub-lethality at both temperatures and can be explained by the high stress tolerance of *E. coli* (Richard & Foster, 2003). Only the *K. pneumoniae* cells grown at 25°C showed an increasing sub-lethality during cooling, however at 37°C there was no sub-lethal injury. A possible explanation for lack of sub-lethality at 37°C could be that the cells were grown at their optimal growth temperature and had no additionally stresses in advance, in contrast to the cells that were grown at 25°C (Tripathy *et al*, 2014; Tsuji *et al*, 1982). The strains of *P. rustigianii* and *E. hermannii* showed a relatively high sub-lethality at both temperatures.

4.6 Preliminary lactic acid sensitivity tests on pork skin

In the previous experiments, the effect of the lactic acid treatment was investigated in culture media i.e. optimal growth conditions for *Salmonella* and *Yersinia enterocolitica*. Further, the effect was investigated when the bacteria were grown on pig skin, which is not an optimal medium for *Salmonella* and *Yersinia enterocolitica* (Pin *et al*, 2011).

Directly after treatment, serotypes *S.* Derby and Typhimurium showed a reduced survival of the treated cells. Furthermore, the reduction of *S.* Derby and Typhimurium were 0.68 and 0.25 $\log_{10}$ cfu/mL, respectively, which was lower in comparison with the reductions found in culture media. However, at 24h and 48h after treatment, the effect of the lactic acid treatment disappeared. Overall, the number of treated and control cells decreased during cooling. A possible explanation for the smaller reduction on pork skin than in culture media, could be

that the pH of the lactic acid solution on the skin pieces increased rapidly after dipping from 4 to 5. This increase in pH causes more lactic acid molecules to dissociate and as a result fewer molecules can cross the membrane of the bacteria and perform their decontaminating actions (Brul & Coote, 1999). The relatively short exposure time can be pointed as a possible explanation for the reduced reduction. In this experiment, the skin pieces were dipped for 15s in the lactic acid solution in contrast to the in culture media experiments where the cells were exposed for 10min to lactic acid. However, after dipping a small film of lactic acid solution was present on the pig skin. Thus, the exposure time was longer than 15s, but due to the rapidly increasing pH, the effect can be minimalized. The decrease in concentration during cooling of *S.* Derby and Typhimurium can be explained by the effect of cooling, which is known to cause metabolic stress and membrane alterations (Chang *et al*, 2003). As reported in the literature, *Salmonella* serotypes do not grow in food stored below 7°C, but is able to survive during cooling (D'Aoust, 1991; Dominguez & Schaffner, 2009; International Commission on Microbiological Specifications for Foods, 2005).

A strain of *Y. enterocolitica* 4/O:3 and 2/O:9 were investigated as well. Directly after treatment, both serotypes showed a small reduction of about 0.3 $\log_{10}$ cfu/cm² between the treated and control concentration and the reduction stayed stable during cooling. This reduction was smaller in comparison with the reductions found in culture media. *Y. enterocolitica* 4/O:3 treated and control counts decreased at the same rate during cooling, which was in contrast with the increasing trend found in culture media. *Y. enterocolitica* 2/O:9 treated and control counts decreased during the first 24h at 2°C, but after 48h the numbers increased to a higher level than the initial inoculation. This trend of *Y. enterocolitica* 2/O:9 was similar to the trend found in culture media. The smaller reductions can be explained as well by the relatively high initial pH and short exposure time as described above. According to Bottone (1997), Gill *et al* (1989) and Lee *et al* (1980), *Y. enterocolitica* is a psychrotolerant bacteria and can grow at refrigerating temperatures, which confirms the increase in concentration of *Y. enterocolitica* 2/O:9 after 48h in cooling. The decrease in concentration of *Y. enterocolitica* 4/O:3 on pork skin during cooling is in contrast with the findings of Lee et al in 2008.

A study by King *et al* (2012), showed immediate reductions of approximately 0.5 log cfu per sample of *Salmonella* and 0.8 log cfu per sample of *Y. enterocolitica* when a lactic acid spray (2%, 40-50°C) was used on pork variety meats (King *et al*, 2012). The findings correspond with the results of this study, however the reductions of *Yersinia enterocolitica* were lower in this study. Other studies performed by van Netten *et al* (1991) and DeGeer *et al* (2016) resulted in reductions of *Salmonella* of 1 and 1.96 $\log_{10}$ cfu/cm², respectively, which were higher reductions than found in this study. Another study by Christiansen *et al* (2008) demonstrated reductions of 1.5 $\log_{10}$ cfu/cm^2 of *E. coli*, *Salmonella* Typhimurium and *Y. enterocolitica* on pork cheeks after lactic acid treatment, which are again higher than found in this study.

Besides the decontaminating effect, an efficient decontamination system should cause no adverse changes in color and smell of the product. Discoloration is one of the most problematic effect using organic acids (Canto *et al*, 2016). In these experiments on dorsal pig skin, there were no color changes visible between control and treated skin and no adverse smell was observed. In addition, the subcutaneous fat showed no discolorations as well. However, properly sensory testing should be performed and if a lower pH, other concentration of lactic acid or other type of matrix such as muscle tissue is used in a follow-up experiment, some adverse effects can emerge.

4.7 Conclusion and future perspectives

In conclusion, there were no differences in lactic acid sensitivity found between serotypes of *Salmonella* and between bioserotypes of *Yersinia enterocolitica*. The exposure of a 5% lactic acid solution to different *Salmonella* serotypes grown in culture media resulted in a reduction of 1 $\log_{10}$ cfu/mL when the strains were grown at 37°C and 2 $\log_{10}$ cfu/mL at 25°C. *Y. enterocolitica* showed a reduction of 1.5 $\log_{10}$ cfu/mL at both temperatures after exposure to lactic acid. *E. coli* and *K. pneumoniae* showed a lower lactic acid sensitivity than *Salmonella* and *Y. enterocolitica*. In addition, the preliminary experiments on pig skin showed a minor effect of the lactic acid treatment.

Slaughterhouses are able to use this knowledge to perform follow-up experiments which allow the slaughterhouses to implement a lactic acid treatment in a proper way. The implementation of the lactic acid decontaminating step can be after evisceration, but between slaughter steps as well, for example after the polishing step. In addition, these results can aid the government to make clear regulations concerning the implementation of the lactic acid treatment of pork carcasses in slaughterhouses, with the aim of decreasing the number of contaminated carcasses with pathogenic bacteria. These regulations will eventually result in on the one hand a decrease of human infections with pig-associated pathogens and on the other hand a reduction of the number of recalls of contaminated pork products, which leads to positive economic outcomes and a better image of the sector.

5 Materials and methods

5.1 Isolation and identification of pig-associated bacteria

5.1.1 Sample collection

Pig carcasses were sampled at different days, in different Belgian abattoirs and from several batches. After evisceration and trimming of the carcass, but before cooling, different parts of the carcass were sampled such as the head, throat, elbow, breast, inside of the ham and foreleg and the rectal content and tonsils were collected.

The different carcass parts, each of approximately 100 cm^2, were swabbed with a premoistered (with 25 mL of buffered peptone water (BPW; Bio-Rad, Marnes-la-Coquette, France)) cellulose sponge stick (3M™ Sponge-Stick SSL100; 3M, Diegem, Belgium). The whole intestinal tract was separated immediately after evisceration and the rectum was closed with threads, after which it was excised and collected in a sterile plastic bag. Tonsils were excised aseptically after removal of the pluck set and put in a sterile plastic bag.

Each sample was immediately placed in an insulated refrigerated box and transferred the same day to the laboratory were the isolation procedures immediately started.

5.1.2 Microbiological analysis of the samples

All the samples were analyzed for aerobic bacteria, *E. coli* and Enterobacteriaceae using direct plating and *Salmonella* spp. and pathogenic *Yersinia* spp. using selective enrichment. From the tonsils and rectal content samples, 10 g subsample was weighed aseptically and homogenized (Stomacher 400, Seward, Worthing West Sussex, UK) in 90 mL of BPW for 1 min. Swab sponges were stomached for 1 min just before starting the analyses. For direct plating, 100 µL of homogenate was plated onto plate count agar (PCA; Bio-Rad, Marnes-la-Coquette, France) using a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain) and incubated at 30°C for 48h before counting the colonies. To determine the Enterobacteriaceae count, 100 µL of homogenate was spiral plated onto a Rapid' Enterobacteriaceae agar (Bio-Rad, Marnes-la-Coquette, France) and red colonies with a diameter equal to or exceeding 0.5 mm were counted after incubation at 37°C for 24h. The total number of *E. coli* is determined by spiral plating 100 µL homogenate onto a tryptone bile X-glucuronide agar (TBX; Bio-Rad, Marnes-la-Coquette, France) and metallic green colonies were enumerated after incubation for 44°C for 21h. For selective *Yersinia* enrichment, 5 mL of BPW homogenate from tonsils/feces and carcass swabs were transferred into 45 mL of peptone sorbitol bile broth (PSB; Fluka, Steinheim, Germany) and incubated at 25°C for 48h. For selective *Salmonella* enrichment, an extra 25 mL of BPW is added to the swabs and incubated for 20h at 37°C.

5.1.3 *E. coli* and other Enterobacteriaceae spp. identification

Several *E. coli* and Enterobacteriaceae colonies were collected and subcultivated 3 times on PCA plates for identification with the matrix assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS; Bruker Daltonik). Direct transfer method was performed on all bacterial isolates. The isolated colonies were spotted on the steel MALDI-TOF MS 96-well target plate using sterile toothpicks. Then, the target plate was air-dried, followed by the addition of 1 µL of a matrix-organic solvent mixture (α-cyano-4-hydroxycinnamic acid (5 mg/mL) in a 50:48:2 acetonitrile: water: trifluoroacetic acid matrix solution) and allowed to

dry in air. All MALDI-TOF MS testing was performed in duplicate with the same isolate spotted onto two spots on the target plate.

Mass spectra profiles were acquired using a microflex LT MALDI-TOF MS following the manufacturer's recommendations. Spectra are recorded in the linear positive mode at a laser frequency of 60 Hz within a mass range of 2-20 kDa. All bacteria identifications were performed by MALDI-TOF Biotyper RTC and the Bruker MALDI Biotyper 3.1 software and library (Bruker Daltonics). Criteria used for microorganism analysis and identification were as stated by the manufacturer. A score of < 1.70 was interpreted as no identification, a score of 1.70-1.99 indicated identification to genus level and a score of ≥ 2.00 indicated identification to species level (Mestas *et al*, 2016).

To identify isolates, that were identified only to genus level, an API 20E (BioMérieux, Marcy l'Etoile, France) was performed according to the instructions of the manufacturer.

5.1.4 *Salmonella* spp. isolation, identification and characterization

Salmonella isolation was performed according to ISO 6579:2002 and ISO 6579:2002 Amd1:2007 (ISO, 2007). After the incubation of the pre-enrichment media, 3 drops (100 µL in total) of each culture medium were spotted onto a modified semi-solid Rappaport-Vassiliadis agar plate (MSRV; Lab M, Heywood, UK) and incubated at 41.5°C for 24h. Plates were examined for a whitish migration zone and if present a loopful from the edge of the migration zone was streaked onto a xylose lysine deoxycholate agar plate (XLD; Bio-Rad, Marnes-la-Coquette, France) which was incubated at 37°C for 24h. The XLD plates were examined for the presence of typical black colonies. Two presumptive colonies per XLD plate were selected and confirmed biochemically using triple sugar iron agar (TSI; Oxoid, Basingstoke, UK), tryptone broth (Lab M Limited, Heywood, UK), lysine decarboxylase broth (LB; Sigma-Aldrich, Saint Louis, Missouri, USA) and transferred into tryptone soya broth (TSB; Bio-Rad, Marnes-la-Coquette, France) for further molecular identification. Finally, 1 mL bacterial culture was stored in 2 mL glycerol at -20°C.

After re-cultivation on XLD, isolates were sub-cultured on PCA (37°C for 24h), after which one colony was suspended in 100 µL lysis buffer (Sodium Dodecyl Sulfate (SDS; Sigma-Aldrich, Saint Louis, Missouri, USA) / NaOH (Merck, Darmstadt, Germany), 50:50)) and subsequently lysed through heat treatment at 92°C for 17 min. All lysates were centrifuged at 12 000 rpm and stored at -20°C.

All *Salmonella* isolates were characterized by Enterobacterial Repetitive Intergenic Consensus sequence (ERIC)-PCRs, to select isolates for serotyping (Rasschaert *et al*, 2005; Versalovic *et al*, 1991). PCR assays were performed in 25 µL reactions containing 1 µL of crude cell lysate, 2 U GoTaq G2 Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA), 5× Gitschier buffer, 5 mM of each dNTP (Promega, Madison, Wisconsin, USA), 0.5% Tween (Sigma-Aldrich, Saint Louis, Missouri, USA), 0.01% gelatin (Sigma-Aldrich, Saint Louis, Missouri, USA) and 50 pmol of ERIC1 and ERIC2 primers (Versalovic *et al*, 1991).

The PCR amplification were performed in an automated Veriti 96 well Thermal cycler (Applied biosystems,) and the reaction consisted of one pre-denaturation at 95°C for 7 min, 30 cycles of denaturation at 90°C for 30s, primer annealing at 52°C for 1 min and extension at 65°C for 8 min, followed by a final extension at 65°C for 16 min. The PCR products were separated

based on size using a 1.5% agarose gel in tris-borate-EDTA (TBE) at 120V for 4h. The gels were stained with ethidium bromide and digitally captured under UV light.

ERIC PCR profiles were analyzed by GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient with 2.1% position tolerance. Based on these ERIC-PCR profiles, isolates were selected for serotyping. All these selected isolates were serotyped according to the Kauffmann-White scheme (Grimont & Weill, 2007) at the Scientific Institute of Public Health (WIV-ISP) by performing slide agglutination using antisera (Bio-Rad and SSI) for the different O- and H-serotypes.

5.1.5 *Yersinia* spp. isolation, identification and characterization

Yersinia isolation was performed according to ISO 10273:2003 (ISO, 2003). After enrichment, 0.5 mL of homogenate was exposed to 4.5 mL of 0.5% KOH (Merck, Darmstadt, Germany) in 0.5% NaCl (VWR, Leuven, Belgium) for 20s. Next, 100 µL of the alkali treated homogenate was streaked on cefsulodin Irgasan novobiocin agar (CIN; Oxoid, Basingstoke, UK) plates. The CIN agar plates were incubated at 30 °C for 24h for *Y. enterocolitica* and an additional 24h at room temperature for the detection of *Y. pseudotuberculosis*. Plates were examined for characteristic *Yersinia* colonies (**Figure 17**) using a stereo microscope with Henry illumination. Presumptive positive colonies were characterized biochemically using urea broth (Oxoid, Basingstoke, UK) and Kligler iron agar (KIA; Oxoid, Basingstoke, UK) and transferred into TSB for further molecular identification including virulence PCRs and serotyping (performed by lab-technicians).

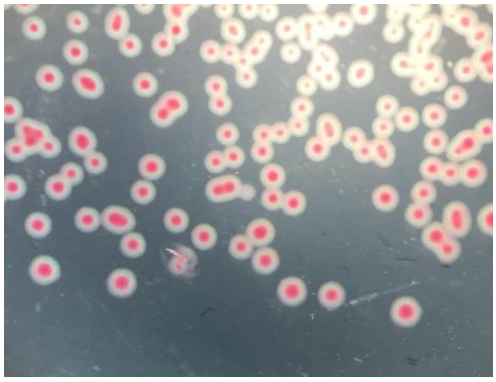


Figure 17: Pathogenic *Yersinia* colonies on cefsulodin Irgasan novobiocin agar (CIN) plate.

5.2 Lactic acid sensitivity tests in culture media

Different identified and characterized bacterial strains of *Salmonella* (**Table 16**), *Yersinia enterocolitica* (**Table 17**), *E. coli* and several Enterobacteriaceae spp. (**Table 18**). were tested for their sensitivity to lactic acid. The experimental set up was a modification of Van Houteghem *et al* performed in 2008.

The strains were grown in TSB and incubated at two different temperatures until they had reached their stationary phase. First, incubation at 37°C mimicked direct contamination from pig bacteria reservoirs. Second, incubation at 25°C was used to represent environmental contamination.

Table 16: *Salmonella* isolates

Serotype	Number	Abattoir	Isolation date	Carcass location
Derby	S15	A	03/11/2015	Belly
Derby	S383	B	15/02/2016	Throat
Derby	S416	C	09/02/2016	Dorsal
Livingstone	S66	B	05/11/2015	Dorsal
Livingstone	S241	D	30/11/2015	Head
Livingstone	S354	A	13/01/2016	Foreleg
Rissen	S37	B	10/11/2015	Foreleg
Rissen	S83	B	08/05/2014	Mouth
Rissen	13.22A	E	05/11/2015	Breast
Monophasic Typhimurium	S141	C	25/11/2015	Throat
Monophasic Typhimurium	S224	A	12/11/2015	Foreleg
Monophasic Typhimurium	60.18B	F	16/02/2015	Foreleg
Typhimurium	S279	E	07/01/2016	Head
Typhimurium	S319	G	13/01/2016	Foreleg
Typhimurium	S355	A	07/12/2015	Pelvic duct
Bredeney	S162	C	12/11/2015	Foreleg
Brandenburg	S216	A	25/11/2015	Breast
Idikan	72.22A	F	25/11/2015	Gut
Infantis	90.23A	H	07/01/2016	Mouth

Table 17: *Yersinia enterocolitica* isolates

Biotype	Serotype	Number	Carcass location
2	O:9	Y47C	/
2	O:9	Y47K	/
2	O:9	FAVV13523	/
4	O:3	Y185	Breast
4	O:3	Y203	Pelvic duct
4	O:3	Y277	Cheeks

Table 18: Enterobacteriaceae isolates

Species	Number	Carcass location
Escherichia coli	E1	Foreleg
Escherichia coli	E2	Foreleg
Escherichia coli	E3	Foreleg
Klebsiella pneumoniae	K1	Foreleg
Klebsiella pneumoniae	K2	Foreleg
Klebsiella pneumoniae	K3	Foreleg
Providencia rustigianii		Foreleg
Escherichia hermannii		Foreleg

A 5% solution of lactic acid (Fluka, Steinheim, Germany) was adjusted to pH 4.0 with 10 M NaOH at 25°C. Treatment of cells was performed by diluting the bacteria culture and a lactic acid solution in a 1/10 ratio. After a static treatment for 10 min in a heat block at 30°C, the treatment is stopped by rising the pH to 6.6 with 1 M NaOH, followed by 2 min centrifugation at 12,000 rounds per minute (rpm). The supernatant was removed and cells were resuspended in 1 mL TSB. The volume of 200 µL of surviving cells was transferred into a tube containing 10 mL of TSB and kept for 48h at 2°C.

The number of cells were enumerated before the treatment, immediately after the treatment, 24h and 48h at 2°C by plating appropriate dilutions using the spiral plate method on non-selective PCA plates and selective plates. The selective plates were XLD, TBX, RAPID' Enterobacteriaceae and CIN for *Salmonella*, *E. coli,* Enterobacteriaceae and *Yersinia* strains, respectively. In addition, to determine the percentage of *Yersinia* that have lost their virulence plasmid during the experiment, the *Yersinia* strains were plated as well on Congo-red magnesium oxalate agar (CRMOX), which existed of tryptic soy agar (TSA; Bio-Rad, Marnes-la-Coquette, France), 0.02 M sodium oxalate (Sigma-Aldrich, Saint Louis, Missouri, USA), 0.02 M $MgCl_2$ (Merck, Darmstadt, Germany), 0.20% D-galactose (Merck, Darmstadt, Germany) and 0.005% Congo red (Sigma-Aldrich, Saint Louis, Missouri, USA) (Riley & Toma, 1989). The small red colonies still possessed their pYV and the larger white colonies have lost the pYV (**Figure 18**). The percentage of pYV positive cells was calculated.

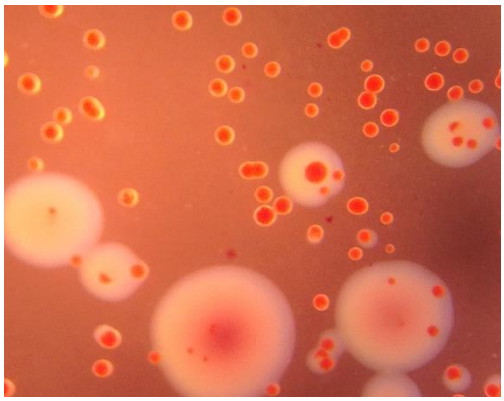


Figure 18: *Yersinia enterocolitica* colonies on Congo-red magnesium oxalate agar. The red colonies possess the *Yersinia* virulence plasmid (pYV). The white colonies have lost their pYV.

As a control, the same experimental set-up was used, but instead of lactic acid and NaOH, 0,85% NaCl solution was added to the cells. To examine the effect of the lactic acid treatment, the differences between the number of cells treated with the lactic acid and the control cells were calculated. The experiment was performed in triplicate and the averages were computed and compared. In addition, the sub-lethality percentage was calculated. A sub-lethally injured cell is defined as a cell that survives any lethal injury and is able to grow on a non-selective medium, but not on a selective medium. The sub-lethality percentage was calculated using following formula (Besse *et al*, 2000):

$$\%\text{sub-lethal injury} = \frac{\text{counts on PCA} - \text{counts on selctive plate}}{\text{counts on PCA}} \text{ x } 100\%$$

Quantitative test results were recorded in an Excel 2013 (Microsoft® Corporation, Redmond, Washington, USA) spreadsheet. Bacterial counts were $\log_{10}$-transformed prior to analysis. All analyses were done using Stata 14.1 (Stata Corporation, College Station, Texas, USA). For comparing the bacterial counts, linear regressions were used and normality of the residuals of the final models was checked using Shapiro-Wilk normality tests. The different strains were included as random effect to account for clustering per serotype. In addition, Bonferroni corrections were applied for multiple testing. A significance level of 5% was used. Statistical analysis was only performed on the (bio)serotypes whereof three strains were investigated. The other serotypes were only descriptive discussed.

5.3 Preliminary lactic acid sensitivity tests on pig skin

A preliminary study to assess the effect of lactic acid in vivo, was performed by artificially inoculate certain strains of *Salmonella* Derby (S416) and Typhimurium (S355) and *Yersinia enterocolitica* 4/O:3 (Y185) and 2/O:9 (Y47C), on dorsal pig skin, which was harvested of pig carcasses that were chilled for 24h. Subsequently, the skin was treated with lactic acid. The experimental set up was a modification of Greer and Dilts performed in 1995.

The strains were grown in TSB and incubated until they had reached their stationary phase at 37°C. This resulted in a microbial concentration of >8 $\log_{10}$ cfu/mL. From these cultures, 100 µL was inoculated on 6 times 100 cm^2 pig skin. Then, the skin pieces were allowed to air-dry. Tissues inoculated in this fashion were contaminated with about 6 $\log_{10}$ cfu/cm^2. Three of the pieces were dipped in a 5% lactic acid suspension (pH 4 at 25°C) for 15s and subsequently air-dried. One treated skin piece and an untreated piece were directly analyzed after treatment. The four other pieces were stored separately at 2°C in a petri dish. After 24h, the first 2 pieces (1 treated and 1 untreated piece) were analyzed and after 48h the 2 remaining pieces were analyzed. To determine the initial microbial concentration, a 100 cm^2 pig skin was analyzed before inoculation of the strains.

Analyzation was done by suspending each skin piece in 100 mL BPW in a sterile bag. Subsequently, the sample was homogenized with a stomacher and appropriate dilutions were plated on selective plates (XLD for *Salmonella* and CIN for *Yersinia enterocolitica*) by using the spiral plate method.

6 References

Adamkiewicz T V, Berkovitch M, Krishnan C, Polsinelli C, Kermack D & Olivieri NF (1998) Infection due to *Yersinia enterocolitica* in a series of patients with beta-thalassemia: incidence and predisposing factors. *Clin. Infect. Dis.* **27:** 1362–6

Adams MR, Little CL & Easter MC (1991) Modelling the effect of pH, acidulant and temperature on the growth rate of *Yersinia enterocolitica*. *J. Appl. Bacteriol.* **71:** 65–71

Alban L & Stärk KDC (2005) Where should the effort be put to reduce the *Salmonella* prevalence in the slaughtered swine carcass effectively? *Prev. Vet. Med.* **68:** 63–79

Aleksić S (1995) Occurrence of *Y. enterocolitica* antigens O:3, O:9 and O:8 in different *Yersinia* species, their corresponding H antigens and origin. *Contrib. Microbiol. Immunol.* **13:** 89–92

Algemene Directie Statistiek (2016) Aantal varkensslachtingen blijft stabiel op bijna 12 miljoen stuks per jaar. **32:** 2–4

Álvarez-Ordóñez A, Prieto M, Bernardo A, Hill C & López M (2012) The Acid Tolerance Response of *Salmonella* spp.: An adaptive strategy to survive in stressful environments prevailing in foods and the host. *Food Res. Int.* **45:** 482–492

Arguello H, Álvarez-Ordonez A, Carvajal A, Rubio P & Prieto M (2013) Role of Slaughtering in *Salmonella* Spreading and Control in Pork Production. *J. Food Prot.* **76:** 899–911

Batt CA & Tortorello M Lou (2014) Encyclopedia of food microbiology 2nd ed. London: Elsevier Inc

Batzilla J, Heesemann J & Rakin A (2011) The pathogenic potential of *Yersinia enterocolitica* 1A. *Int. J. Med. Microbiol.* **301:** 556–561

Belgian Meat Office (2015) Belgium reconfirmed as international pork supplier. Available at: https://www.freshfrombelgium.com/en/sector/belgian-meat-office/news-detail/15051/belgium-reconfirmed-as-international-pork-supplier [Accessed March 14, 2017]

Berends BR, Van Knapen F, Snijders JM & Mossel DA (1997) Identification and quantification of risk factors regarding *Salmonella* spp. on pork carcasses. *Int. J. Food Microbiol.* **36:** 199–206

Bergann T, Kleemann J & Sohr D (1995) Model investigations into psychrotrophic growth of *Yersinia enterocolitica*. *J Vet Med Ser B* **42:** 523–531

Besse GN, Brissonnet DF, Lafarge V & Leclerc V (2000) Effect of various environmental parameters on the recovery of sublethally salt-damaged and acid-damaged *Listeria monocytogenes*. *J. Appl. Microbiol.* **89:** 944–50

Beuchat L (2000) Use of sanitizers in raw fruit and vegetable processing. In *Minimally processed fruits and vegetables. Fundamental aspects and applications.*, Alzamora S Tapia M & L´opez-Malo A (eds) pp 63–78. Gaithersburg: Aspen Publishers Inc.

Biasino W, De Zutter L, Houf K & Van Damme I (2016) Distribution of *Salmonella*, ESBL/AmpC-producing *E. coli* and hygiene indicator bacteria on pig carcasses during slaughter. *Belgian Society for Food Microbiology Twenty-first Conference on Food Microbiology*, 12 - 13 September, 2016, Brussel

Bolton D (2001) Beef HACCP: intervention and non-intervention systems. *Int. J. Food Microbiol.* **66:** 119–129

Bolton DJ, Pearce RA, Sheridan JJ, Blair IS, McDowell DA & Harrington D (2002) Washing and chilling as critical control points in pork slaughter hazard analysis and critical control point (HACCP) systems. *J. Appl. Microbiol.* **92:** 893–902

Borch E, Nesbakken T & Christensen H (1996) Hazard identification in swine slaughter with respect to foodborne bacteria. *Int. J. Food Microbiol.* **30:** 9–25

Botteldoorn N, Heyndrickx M, Rijpens N, Grijspeerdt K & Herman L (2003) *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. *J. Appl. Microbiol.* **95:** 891–903

Bottone EJ (1977) *Yersinia enterocolitica*: a panoramic view of a charismatic microorganism. *CRC Crit. Rev. Microbiol.* **5:** 211–41

Bottone EJ (1997) *Yersinia enterocolitica*: the charisma continues. *Clin. Microbiol. Rev.* **10:** 257–76

Bottone EJ (1999) *Yersinia enterocolitica*: overview and epidemiologic correlates. *Microbes Infect.* **1:** 323–33

Bracey D, Holyoak CD & Coote PJ (1998) Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? *J. Appl. Microbiol.* **85:** 1056–66

Brenner FW, Villar RG, Angulo FJ, Tauxe R & Swaminathan B (2000) *Salmonella* nomenclature. *J. Clin. Microbiol.* **38:** 2465–7

Broughall JM, Anslow PA & Kilsby DC (1983) Hazard analysis applied to microbial growth in foods: development of mathematical models describing the effect of water activity. *J. Appl. Bacteriol.* **55:** 101–10

Brul S & Coote P (1999) Preservative agents in foods. Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol.* **50:** 1–17

Canto ACVCS, Costa-Lima BRC, Suman SP, Monteiro MLG, Viana FM, Salim APAA, Nair MN, Silva TJP & Conte-Junior CA (2016) Color attributes and oxidative stability of *longissimus lumborum* and *psoas* major muscles from Nellore bulls. *Meat Sci.* **121:** 19–26

Centers for Disease Control and Prevention (2015) Questions and Answers | *Salmonella* | CDC. Available at: https://www.cdc.gov/salmonella/general/ [Accessed March 16, 2017]

Chandler ND & Parisi MT (1994) *Yersinia enterocolitica* masquerading as appendicitis. *Arch. Pediatr. Adolesc. Med.* **148:** 527–8

Chang VP, Mills EW & Cutter CN (2003) Reduction of bacteria on pork carcasses associated with chilling method. *J. Food Prot.* **66:** 1019–24

Crchova V & Grondin C (1973) Urinary infection due to *Yersinia pseudotuberculosis*. *Vie Med. Can. Fr.* **2:** 3–5

D'Aoust JY (1991) Psychrotrophy and foodborne *Salmonella*. *Int. J. Food Microbiol.* **13:** 207–15

Van Damme I, Berkvens D, Vanantwerpen G, Baré J, Houf K, Wauters G & De Zutter L (2015) Contamination of freshly slaughtered pig carcasses with enteropathogenic *Yersinia* spp.: Distribution, quantification and identification of risk factors. *Int. J. Food Microbiol.* **204:** 33–40

Van Damme I, Mattheus W, Bertrand S & De Zutter L (2017) Quantification of hygiene indicators and *Salmonella* in the tonsils, oral cavity and rectal content samples of pigs during slaughter. *Food Microbiol.***:** Under Revision

Davidson P (2001) Chemical preservatives and natural antimicrobial compounds. In *Food microbiology: fundamentals and frontiers*, Doyle M Beuchat L & Montville T (eds) pp 593–627. Washington, D.C.: ASM Press.

DeGeer SL, Wang L, Hill GN, Singh M, Bilgili SF & Bratcher CL (2016) Optimizing application parameters for lactic acid and sodium metasilicate against pathogens on fresh beef, pork and deli meats. *Meat Sci.* **118:** 28–33

Dekker JP & Frank KM (2015) *Salmonella*, *Shigella*, and *Yersinia*. *Clin. Lab. Med.* **35:** 225–46

Dominguez SA & Schaffner DW (2009) Survival of *Salmonella* in processed chicken products during frozen storage. *J. Food Prot.* **72:** 2088–92

Doores S (1993) Organic acids. In *Antimicrobials in food.*, Davidson P & Branen A (eds) pp 95–136. New York: Marcel Dekker Inc.

Dougan G & Baker S (2014) *Salmonella enterica* Serovar Typhi and the Pathogenesis of Typhoid Fever. *Annu. Rev. Microbiol.* **68:** 317–336

Doyle M & Cliver D (1990) *Salmonella*. In *Foodborne diseases.* pp 185–204. San Diego, Calif. Academic Press Inc

Duggan SJ, Mannion C, Prendergast DM, Leonard N, Fanning S, Gonzales-Barron U, Egan J, Butler F & Duffy G (2010) Tracking the *Salmonella* status of pigs and pork from lairage through the slaughter process in the Republic of Ireland. *J. Food Prot.* **73:** 2148–60

Echeverry A, Brooks JC, Miller MF, Collins JA, Loneragan GH & Brashears MM (2009) Validation of intervention strategies to control *Escherichia coli* O157:H7 and *Salmonella* typhimurium DT 104 in mechanically tenderized and brine-enhanced beef. *J. Food Prot.* **72:** 1616–23

Edwards RA, Olsen GJ & Maloy SR (2002) Comparative genomics of closely related salmonellae. *Trends Microbiol.* **10:** 94–9

EFSA (2010) Scientific Opinion on a Quantitative Microbiological Risk Assessment of *Salmonella* in slaughter and breeder pigs. *EFSA J.* **8:** 1547

EFSA (2011) Scientific Opinion on the evaluation of the safety and efficacy of lactic acid for the removal of microbial surface contamination of beef carcasses, cuts and trimmings. *EFSA J.* **9:** 2317

EFSA (2012) Scientific Opinion on an estimation of the public health impact of setting a new target for the reduction of *Salmonella* in turkeys. *EFSA J.* **10:** 1–89

EFSA & ECDC (2015) The European Union summary report on trends and sources of zoonsoes, zoonotic agents and food-borne outbreaks in 2014. *EFSA J.* **13:**

European Commission (2005) Commission Regulation (EC) N0. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Communitites* **338:** 1–26

European Commission (2014) Commission Regulation (EU) No 217/2014 of 7 March 2014 amending Regulation (EC) No 2073/2005 as regards *Salmonella* in pig carcases. *Off. J. Eur. Communitites* **9:** 93–94

Fosse J, Seegers H & Magras C (2008) Foodborne zoonoses due to meat: a quantitative approach for a comparative risk assessment applied to pig slaughtering in Europe. *Vet. Res.* **39:** 1

Foster JW (1993) The acid tolerance response of *Salmonella* typhimurium involves transient synthesis of key acid shock proteins. *J. Bacteriol.* **175:** 1981–7

Foster JW (2000) Microbial responses to acid stress. In *Bacterial stress responses*, Storz G & Hengge-Aronis R (eds) pp 99–156. Washington: ASM Press

Foster JW & Hall HK (1990) Adaptive acidification tolerance response of *Salmonella* typhimurium. *J. Bacteriol.* **172:** 771–8

Foster JW & Hall HK (1991) Inducible pH homeostasis and the acid tolerance response of *Salmonella* typhimurium. *J. Bacteriol.* **173:** 5129–5135

Fredriksson-Ahomaa M, Meyer C, Bonke R, Stüber E & Wacheck S (2010) Characterization of *Yersinia enterocolitica* 4/O:3 isolates from tonsils of Bavarian slaughter pigs. *Lett. Appl. Microbiol.* **50:** 412–418

Freese E, Sheu CW & Galliers E (1973) Function of lipophilic acids as antimicrobial food additives. *Nature* **241:** 321–5

García-Fierro R, Montero I, Bances M, González-Hevia MÁ & Rodicio MR (2016) Antimicrobial Drug Resistance and Molecular Typing of *Salmonella enterica* Serovar Rissen from Different Sources. *Microb. Drug Resist.* **22:** 211–217

Ghafir Y, China B, Dierick K, De Zutter L & Daube G (2008) Hygiene indicator microorganisms for selected pathogens on beef, pork, and poultry meats in Belgium. *J. Food Prot.* **71:** 35–45

Gibson AM, Bratchell N & Roberts TA (1988) Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride and storage temperature. *Int. J. Food Microbiol.* **6:** 155–78

Gill C. & Badoni M (2004) Effects of peroxyacetic acid, acidified sodium chlorite or lactic acid solutions on the microflora of chilled beef carcasses. *Int. J. Food Microbiol.* **91:** 43–50

Gill CO (2009) Effects on the microbiological condition of product of decontaminating treatments routinely applied to carcasses at beef packing plants. *J. Food Prot.* **72:** 1790–801

Gill CO, Jones T & Badoni M (1998) The effects of hot water pasteurizing treatments on the microbiological conditions and appearances of pig and sheep carcasses. *Food Res. Int.* **31:** 273–278

Gill CO, McGinnis DS, Bryant J & Chabot B (1995) Decontamination of commercial, polished pig carcasses with hot water. *Food Microbiol.* **12:** 143–149

Gill CO & Reichel MP (1989) Growth of the cold-tolerant pathogens *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Listeria monocytogenes* on high-pH beef packaged under vacuum or carbon dioxide. *Food Microbiol.* **6:** 223–230

Goldbach SG & Alban L (2006) A cost–benefit analysis of *Salmonella*-control strategies in Danish pork production. *Prev. Vet. Med.* **77:** 1–14

Goodson M & Rowbury RJ (1989) Habituation to normally lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH value. *Lett. Appl. Microbiol.* **8:** 77–79

Grau FH (1987) Elimination of Pathogenic Organisms from Meat and Poultry. In, F. J. M. Smulders. (ed) pp 221–233. London : Elsevier

Greer GG & Dilts BD (1995) Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. *Int. J. Food Microbiol.* **25:** 141–51

Grimont P & Weill F (2007) Antigenic Formulae of the *Salmonella* serovars, 9th ed. *WHO Collab. Cent. Ref. Res. Salmonella, Geneva, Switzerland.*

Gürtler M, Alter T, Kasimir S, Linnebur M & Fehlhaber K (2005) Prevalence of *Yersinia enterocolitica* in fattening pigs. *J. Food Prot.* **68:** 850–4

Van Houteghem N, Devlieghere F, Rajkovic A, Gómez SMO, Uyttendaele M & Debevere J (2008) Effects of CO2 on the resuscitation of *Listeria monocytogenes* injured by various bactericidal treatments. *Int. J. Food Microbiol.* **123:** 67–73

Hugas M & Tsigarida E (2008) Pros and cons of carcass decontamination: The role of the European Food Safety Authority. *Meat Sci.* **78:** 43–52

IATP (2014) China's Pork Miracle? Agribusiness and Development in China's Pork Industry. *Glob. Meat Complex China Ser.*

International Commission on Microbiological Specifications for Foods (2005) Microbial ecology of food commodities Kluwer Academic/Plenum Publishers

Isberg RR (1990) Pathways for the penetration of enteroinvasive *Yersinia* into mammalian cells. *Mol. Biol. Med.* **7:** 73–82

ISO (2003) ISO 10273:2003. Microbiology of food and animal feeding stuffs - horizontal method for detection of presumptive pathogenic *Yersinia enterocolitica*. *Int. Organ. Stand. Geneva, Switz.*

ISO (2007) ISO 6579:2002/Amd. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Amendment 1: Annex D: Detection of *Salmonella* spp.in animal faeces and in environmental samples from the primary production stage. *Microbiol. food Anim. Feed.***:** 1–8

James C, James SJ, Hannay N, Purnell G, Barbedo-Pinto C, Yaman H, Araujo M, Gonzalez ML, Calvo J, Howell M & Corry JEL (2007) Decontamination of poultry carcasses using steam or hot water in combination with rapid cooling, chilling or freezing of carcass surfaces. *Int. J. Food Microbiol.* **114:** 195–203

Janssen M, Geeraerd AH, Logist F, De Visscher Y, Vereecken KM, Debevere J, Devlieghere F & Van Impe JF (2006) Modelling *Yersinia enterocolitica* inactivation in coculture experiments with *Lactobacillus sakei* as based on pH and lactic acid profiles. *Int. J. Food Microbiol.* **111:** 59–72

Kaper JB, Bopp CA, Strockbine NA, Fields PI & Nataro JP (2011) *Escherichia*, *Shigella*, and *Salmonella*. In *Manual of Clinical Microbiology, 10th Edition* pp 603–626. American Society of Microbiology

Kieboom J & Abee T (2006) Arginine-Dependent Acid Resistance in *Salmonella enterica* Serovar Typhimurium. *J. Bacteriol.* **188:** 5650–5653

King AM, Miller RK, Castillo A, Griffin DB & Hardin MD (2012) Effects of Lactic Acid and Commercial Chilling Processes on Survival of *Salmonella*, *Yersinia enterocolitica*, and *Campylobacter coli* in Pork Variety Meats. *J. Food Prot.* **75:** 1589–1594

Lawson LG, Jensen JD, Christiansen P & Lund M (2009) Cost-effectiveness of *Salmonella* reduction in Danish abattoirs. *Int. J. Food Microbiol.* **134:** 126–132

Lee KM, Runyon M, Herrman TJ, Phillips R & Hsieh J (2015) Review of *Salmonella* detection and identification methods: Aspects of rapid emergency response and food safety. *Food Control* **47:** 264–276

Lee WH, Harris ME, McClain D, Smith RE & Johnston RW (1980) Two modified selenite media for the recovery of *Yersinia enterocolitica* from meats. *Appl. Environ. Microbiol.* **39:** 205–9

Lee YH, Kim BH, Kim JH, Yoon WS, Bang SH & Park YK (2007) CadC Has a Global Translational Effect during Acid Adaptation in *Salmonella enterica* Serovar Typhimurium. *J. Bacteriol.* **189:** 2417–2425

Leung DT, Bogetz J, Itoh M, Ganapathi L, Pietroni MAC, Ryan ET & Chisti MJ (2012) Factors associated with encephalopathy in patients with *Salmonella enterica* serotype Typhi bacteremia presenting to a diarrheal hospital in Dhaka, Bangladesh. *Am. J. Trop. Med. Hyg.* **86:** 698–702

Liao C-H & Fett WF (2005) Resuscitation of acid-injured *Salmonella* in enrichment broth, in apple juice and on the surfaces of fresh-cut cucumber and apple*. *Lett. Appl. Microbiol.* **41:** 487–492

Ljungberg P, Valtonen M, Harjola VP, Kaukoranta-Tolvanen SS & Vaara M (1995) Report of four cases of *Yersinia pseudotuberculosis* septicemia and a literature review. *Eur. J. Clin. Microbiol. Infect. Dis.* **14:** 804–10

Lutterloh E, Likaka A, Sejvar J, Manda R, Naiene J, Monroe SS, Khaila T, Chilima B, Mallewa M, Kampondeni SD, Lowther SA, Capewell L, Date K, Townes D, Redwood Y, Schier JG, Nygren B, Tippett Barr B, Demby A, Phiri A, et al (2012) Multidrug-resistant typhoid fever with neurologic findings on the Malawi-Mozambique border. *Clin. Infect. Dis.* **54:** 1100–6

Mani-López E, García HS & López-Malo A (2012) Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Res. Int.* **45:** 713–721

Marklund L, Behnam-Motlagh P, Henriksson R & Grankvist K (2001) Bumetanide annihilation of amphotericin B-induced apoptosis and cytotoxicity is due to its effect on cellular K+ flux. *J. Antimicrob. Chemother.* **48:** 781–6

Membré J-M, Leporq B, Vialette M, Mettler E, Perrier L, Thuault D & Zwietering M (2005) Temperature effect on bacterial growth rate: quantitative microbiology approach including cardinal values and variability estimates to perform growth simulations on/in food. *Int. J. Food Microbiol.* **100:** 179–186

Mestas J, Quias T & Dien Bard J (2016) Direct Identification of Aerobic Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Is Accurate and Robust. *J. Clin. Lab. Anal.* **30:** 543–551

Mikołajczyk A & Radkowski M (2002) Elimination of *Salmonella* spp. by lactic acid. *Pol. J. Vet. Sci.* **5:** 139–43

Miller GY, Liu X, McNamara PE & Barber DA (2005) Influence of *Salmonella* in pigs preharvest and during pork processing on human health costs and risks from pork. *J. Food Prot.* **68:** 1788–98

Miller VL, Farmer JJ, Hill WE, Falkow S & Falkow S (1989) The ail locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infect. Immun.* **57:** 121–31

Le Minor L & Popoff MY (1987) Designation of *Salmonella enterica* sp. nov., nom. rev., as the Type and Only Species of the Genus *Salmonella*: Request for an Opinion. *Int. J. Syst. Bacteriol.* **37:** 465–468

Montville TJ & Matthews KR (2008) Food microbiology : an introduction ASM Press

Naiel B & Raul R (1998) Chronic prostatitis due to *Yersinia* pseudotuberculosis. *J. Clin. Microbiol.* **36:** 856

Neil KP, Sodha S V., Lukwago L, O-tipo S, Mikoleit M, Simington SD, Mukobi P, Balinandi S, Majalija S, Ayers J, Kagirita A, Wefula E, Asiimwe F, Kweyamba V, Talkington D, Shieh W-J, Adem P, Batten BC, Zaki SR & Mintz E (2012) A Large Outbreak of Typhoid Fever Associated With a High Rate of Intestinal Perforation in Kasese District, Uganda, 2008-2009. *Clin. Infect. Dis.* **54:** 1091–1099

Nesbakken T, Eckner K, Høidal HK & Røtterud O-J (2003) Occurrence of *Yersinia enterocolitica* and *Campylobacter* spp. in slaughter pigs and consequences for meat inspection, slaughtering, and dressing procedures. *Int. J. Food Microbiol.* **80:** 231–40

van Netten P, Mossel DAA & Veld JH In 't (1995) Lactic acid decontamination of fresh pork carcasses: a pilot plant study. *Int. J. Food Microbiol.* **25:** 1–9

Neuhaus K, Rapposch S, Francis KP & Scherer S (2000) Restart of exponential growth of cold-shocked *Yersinia enterocolitica* occurs after down-regulation of cspA1/A2 mRNA. *J. Bacteriol.* **182:** 3285–8

Notermans S, Zwietering MH & Mead GC (1994) The HACCP concept: identification of potentially hazardous micro-organisms. *Food Microbiol.* **11:** 203–214

Official Journal of the European Union (2004) Regulation (EC) No. 853/2004 of the European Parliament and of the Council of April 29, 2004 laying down specific hygiene rules for food of animal origin. *Off. J. Eur. Union***:** 22–82

Official Journal of the European Union (2013) Commission regulation (EU) no 101/2013 of 4 February 2013, concerning the use of lactic acid to reduce microbiological surface contamination on bovine carcasses. *Off. J. Eur. Union* **L34:** 1–3

Oscar TP (1998) Growth kinetics of Salmonella isolates in a laboratory medium as affected by isolate and holding temperature. *J. Food Prot.* **61:** 964–8

Pearce RA, Bolton DJ, Sheridan JJ, McDowell DA, Blair IS & Harrington D (2004) Studies to determine the critical control points in pork slaughter hazard analysis and critical control point systems. *Int. J. Food Microbiol.* **90:** 331–339

Pin C, Avendaño-Perez G, Cosciani-Cunico E, Gómez N, Gounadakic A, Nychas G-J, Skandamis P & Barker G (2011) Modelling *Salmonella* concentration throughout the pork supply chain by considering growth and survival in fluctuating conditions of temperature, pH and aw. *Int. J. Food Microbiol.* **145:** S96–S102

Pipek P, Houška M, Hoke K, Jeleníková J, Kýhos K & Šikulová M (2006) Decontamination of pork carcasses by steam and lactic acid. *J. Food Eng.* **74:** 224–231

Pipek P, Sikulová M, Jeleníková J & Izumimoto M (2005) Colour changes after carcasses decontamination by steam and lactic acid. *Meat Sci.* **69:** 673–80

Podolak R, Enache E, Stone W, Black DG & Elliott PH (2010) Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *J. Food Prot.* **73:** 1919–36

Popoff MY & Le Minor L (1997) Antigenic formulas of the *Salmonella* serovars, 7th revision World Health Organization Collaborating Centre for Reference and Research on *Salmonella*. Paris, France: Pasteur Institute

Rajkovic A, Smigic N, Uyttendaele M, Medic H, de Zutter L & Devlieghere F (2009) Resistance of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Campylobacter jejuni* after exposure to repetitive cycles of mild bactericidal treatments. *Food Microbiol.* **26:** 889–895

Rasschaert G, Houf K, Imberechts H, Grijspeerdt K, De Zutter L & Heyndrickx M (2005) Comparison of Five Repetitive-Sequence-Based PCR Typing Methods for Molecular Discrimination of *Salmonella enterica* Isolates. *J. Clin. Microbiol.* **43:** 3615–3623

Ray B & Bhunia AK (2008) Fundamental food microbiology thirth. CRC Press Boca Raton, Florida

Raybaudi-Massilia RM, Mosqueda-Melgar J, Soliva-Fortuny R & Martín-Belloso O (2009) Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials. *Compr. Rev. Food Sci. Food Saf.* **8:** 157–180

Rhodehamel EJ (1992) Overview of biological, chemical and physical hazards. In *HACCP Principles and Applications*, Pierson MD & Corlett DA (eds) pp 8–28. New York: Chapman & Hall

Richard HT & Foster JW (2003) Acid resistance in *Escherichia coli*. *Adv. Appl. Microbiol.* **52:** 167–86

Riley G & Toma S (1989) Detection of pathogenic *Yersinia enterocolitica* by using Congo red-magnesium oxalate agar medium. *J. Clin. Microbiol.* **27:** 213–214

Rodríguez-Melcón C, Alonso-Calleja C & Capita R (2017) Lactic acid concentrations that reduce microbial load yet minimally impact colour and sensory characteristics of beef. *Meat Sci.* **129:** 169–175

Rosner BM, Stark K, Höhle M & Werber D (2012) Risk factors for sporadic *Yersinia enterocolitica* infections, Germany 2009–2010. *Epidemiol. Infect.* **140:** 1738–1747

Salmond C V., Kroll RG & Booth IR (1984) The Effect of Food Preservatives on pH Homeostasis in *Escherichia coli*. *Microbiology* **130:** 2845–2850

Samelis J, Sofos JN, Kendall PA & Smith GC (2005) Survival or growth of *Escherichia coli* O157:H7 in a model system of fresh meat decontamination runoff waste fluids and its resistance to subsequent lactic acid stress. *Appl. Environ. Microbiol.* **71:** 6228–34

Schiemann DA (1989) *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. In *Foodborne bacterial pathogens.* pp 601–672. Marcel Dekker Inc.

Schierack P, Walk N, Reiter K, Weyrauch KD & Wieler LH (2007) Composition of intestinal Enterobacteriaceae populations of healthy domestic pigs. *Microbiology* **153:** 3830–3837

Sheu CW & Freese E (1972) Effects of fatty acids on growth and envelope proteins of *Bacillus subtilis*. *J. Bacteriol.* **111:** 516–24

Skurnik M & Bengoechea JA (2003) The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic Yersiniae. *Carbohydr. Res.* **338:** 2521–9

Smulders FJM, Wellm G, Hiesberger J, Rohrbacher I, Bauer A & Paulsen P (2011) Microbiological and Sensory Effects of the Combined Application of Hot–Cold Organic Acid Sprays and Steam Condensation at Subatmospheric Pressure for Decontamination of Inoculated Pig Tissue Surfaces. *J. Food Prot.* **74:** 1338–1344

Snijders JMA, van Logtestijn JG, Mossel DAA & Smulderst FJM (1985) Lactic acid as a decontaminant in slaughter and processing procedures. *Vet. Q.* **7:** 277–282

Snyder MJ, Hornick RB, Mccrumb FR, Morse LJ & Woodward TE (1963) Asymptomatic thyphoidal bacteremia in volunteers. *Antimicrob. Agents Chemother.* **161:** 604–7

Sofos JN & Smith GC (1998) Nonacid meat decontamination technologies: model studies and commercial applications. *Int. J. Food Microbiol.* **44:** 171–88

Spescha C, Stephan R & Zweifel C (2006) Microbiological contamination of pig carcasses at different stages of slaughter in two European Union-approved abattoirs. *J. Food Prot.* **69:** 2568–75

Stern NJ, Pierson MD & Kotula AW (1980) Effects ofpH and sodium chloride on *Yersinia enterocolitica* growth at room and refrigeration temperatures. *J. Food Sci.* **45:** 64–67

Stratford M & Eklund T (2003) Organic acids and esters. In *Food preservatives.*, Russell N & Gould G (eds) pp 48–84. London: Kluwer Academic/Plenum Publishers

Swaminathan B, Harmon MC & Mehlman IJ (1982) A Review: *Yersinia enterocolitica*. *J. Appl. Bacteriol.* **52:** 151–183

Swart AN, Evers EG, Simons RLL & Swanenburg M (2016) Modeling of *Salmonella* Contamination in the Pig Slaughterhouse. *Risk Anal.* **36:** 498–515

Tertti R, Vuento R, Mikkola P, Granfors K, Mäkelä AL & Toivanen A (1989) Clinical manifestations of *Yersinia pseudotuberculosis* infection in children. *Eur. J. Clin. Microbiol. Infect. Dis.* **8:** 587–91

Tindall BJ, Grimont PAD, Garrity GM & Euzéby JP (2005) Nomenclature and taxonomy of the genus *Salmonella*. *Int. J. Syst. Evol. Microbiol.* **55:** 521–524

Tompkin RB (1992) Corrective action procedures for deviations from the critical control point critical limits. In *HACCP Principles and Applications*, Pierson MD & Corlett A (eds) pp 72–82. New York: Chapman & Hall

Tripathy S, Sen R, Padhi SK, Mohanty S & Maiti NK (2014) Upregulation of transcripts for metabolism in diverse environments is a shared response associated with survival and adaptation of *Klebsiella pneumoniae* in response to temperature extremes. *Funct. Integr. Genomics* **14:** 591–601

Trivedi S, Reynolds AE & CHen J (2007) Use of a Commercial Household Steam Cleaning System To Decontaminate Beef and Hog Carcasses Processed by Four Small or Very Small Meat Processing Plants in Georgia. *J. Food Prot.* **70:** 635–640

Tsubokura M & Aleksić S (1995) A simplified antigenic scheme for serotyping of *Yersinia pseudotuberculosis*: phenotypic characterization of reference strains and preparation of O and H factor sera. *Contrib. Microbiol. Immunol.* **13:** 99–105

Tsuji A, Kaneko Y, Takahashi K, Ogawa M & Goto S (1982) The effects of temperature and pH on the growth of eight enteric and nine glucose non-fermenting species of gram-negative rods. *Microbiol. Immunol.* **26:** 15–24

Vereecken KM, Devlieghere F, Bockstaele A, Debevere J & Van Impe JF (2003) A model for lactic acid-induced inhibition of *Yersinia enterocolitica* in mono- and coculture with *Lactobacillus sakei*. *Food Microbiol.* **20:** 701–713

Versalovic J, Koeuth T & Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* **19:** 6823–31

Wang C, Chang T, Yang H & Cui M (2014) Surface physiological changes induced by lactic acid on pathogens in consideration of pKa and pH. *Food Control* **46:** 525–531

Wang C, Chang T, Yang H & Cui M (2015) Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella* Enteritidis, *Escherichia coli* and *Listeria monocytogenes*. *Food Control* **47:** 231–236

Wauters G, Kandolo K & Janssens M (1987) Revised biogrouping scheme of *Yersinia enterocolitica*. *Contrib. Microbiol. Immunol.* **9:** 14–21

Wheatley P, Giotis ES & McKevitt AI (2014) Effects of slaughtering operations on carcass contamination in an Irish pork production plant. *Ir. Vet. J.* **67:** 1

Winn WC & Koneman EW (2006) Koneman's color atlas and textbook of diagnostic microbiology. In pp 211–302. Baltimore: Lippincott Williams & Wilkins

Wong HC, Peng PY, Han JM, Chang CY & Lan SL (1998) Effect of mild acid treatment on the survival, enteropathogenicity, and protein production in vibrio parahaemolyticus. *Infect. Immun.* **66:** 3066–71

Wunderink HF, Oostvogel PM, Frénay IHME, Notermans DW, Fruth A & Kuijper EJ (2014) Difficulties in diagnosing terminal ileitis due to *Yersinia pseudotuberculosis*. *Eur. J. Clin. Microbiol. Infect. Dis.* **33:** 197–200

Xu H, Lee HY & Ahn J (2008) Cross-protective effect of acid-adapted *Salmonella enterica* on resistance to lethal acid and cold stress conditions. *Lett. Appl. Microbiol.* **47:** 290–7

7 Addenda

7.1 Microbial isolation and identification

7.1.1 The sampling procedure

Every sampling starts with a new batch.

Person 1 indicates the carcasses and collects the guts of the indicated carcasses.

Person 2 starts to sample the elbow of the carcass.

Person 3 samples the breast.

After evisceration, person 4 samples the elbow of the indicated carcasses.

Person 5 samples the throat.

The carcasses whereof the guts are taken, will be taken off the line and the tonsils will be collected.

From the guts, only the rectum with feces is collected and closed with dreads.

Swabbing protocol: Sponge-Stick 3M^{TM}

Before use, add 25 mL buffered peptone water (BPW) to the bag with the sponge.

Swabsurface: 100cm^2 (1ml = 4cm^2)

3M

Issue Date: 2013-03

Product Instructions

Sponge-Stick

PRODUCT DESCRIPTION

Dry or Pre-hydrated and biocide-free 3.8 x 7.6 cm (1.5 x 3 in.) sponge in sample bag. Pre-hydrated with specified agent. Various bag sizes. With or without gloves.

For Laboratory Use Only. Material Safety Data Sheet available.

SAFETY

The user should read, understand, and follow all safety information in the instructions. Retain the safety instructions for future reference.

⚠ **WARNING** Indicates a hazardous situation, which, if not avoided, could result in death or serious injury and/or property damage.

NOTICE Indicates a potentially hazardous situation which, if not avoided, could result in property damage.

⚠ WARNING

To reduce the risks associated with environmental contamination:
- Follow current industry standards and local regulations for disposal of contaminated waste.

To reduce the risks associated with exposure to biohazards:
- Dispose of samples according to all applicable government regulations and applicable laboratory procedures.

To reduce the risk associated with false negatives resulting in the use of contaminated carcass or environmental surfaces for food or beverage products:
- Always reference package label for storage instruction and expiration date.
- Always reference product instruction for usage.

To reduce the risks associated with exposure to chemicals and biohazards:
- Always follow standard good laboratory safety practices (GLP[1] or ISO 17025[2]), including proper containment procedures, wearing appropriate protective apparel while handling testing materials and test samples.

NOTICE

To reduce the risk of false-positive results due to cross contaminated carcass or environmental surfaces for food or beverage products that may result in re-testing or the rejection of food or beverage product:
- Do not touch the sponge or sponge stick to any unintended surface.
- Do not break the sponge from the sponge stick while sampling.
- Do not reach into the sponge bag.

To reduce the risk of cross contamination from reuse of sample handling device:
- Do not use the same sponge stick more than once.

USER RESPONSIBILITY

Users are responsible for familiarizing themselves with product instructions and information. Visit our website at **www.3M.com/foodsafety,** or contact your local 3M representative or distributor for more information.

When selecting a test method, it is important to recognize that external factors such as sampling methods, testing protocols, sample preparation, handling, and laboratory technique may influence results.

It is the user's responsibility in selecting any test method or product to evaluate a sufficient number of samples with the appropriate matrices and microbial challenges to satisfy the user that the chosen test method meets the user's criteria.

It is also the user's responsibility to determine that any test methods and results meet its customers' and suppliers' requirements.

As with any test method, results obtained from use of any 3M Food Safety product do not constitute a guarantee of the quality of the matrices or processes tested.

LIMITATION OF WARRANTIES / LIMITED REMEDY

EXCEPT AS EXPRESSLY STATED IN A LIMITED WARRANTY SECTION OF INDIVIDUAL PRODUCT PACKAGING, 3M DISCLAIMS ALL EXPRESS AND IMPLIED WARRANTIES, INCLUDING BUT NOT LIMITED TO, ANY WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR USE. If any 3M Food Safety Product is defective, 3M or its authorized distributor will, at its option, replace or refund the purchase price of the product. These are your exclusive remedies. You must promptly notify 3M within sixty days of discovery of any suspected defects in a product and return it to 3M. Please call Customer Service (1-800-328-1671 in the U.S.) or your official 3M Food Safety representative for a Returned Goods Authorization.

LIMITATION OF 3M LIABILITY

3M WILL NOT BE LIABLE FOR ANY LOSS OR DAMAGES, WHETHER DIRECT, INDIRECT, SPECIAL, INCIDENTAL OR CONSEQUENTIAL DAMAGES, INCLUDING BUT NOT LIMITED TO LOST PROFITS. In no event shall 3M's liability under any legal theory exceed the purchase price of the product alleged to be defective.

STORAGE AND DISPOSAL

Storage: Refer to package label for storage information.

Disposal: Follow current industry standards and local regulations for disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information.

Product Description	Volume	REF	Volume of Bag	Gloves
3M™ Sponge-Stick	0 mL	SSL100	0.89 L (30 oz.)	0
3M™ Sponge-Stick with Buffered Peptone Water Broth	10 mL	SSL10BPW	0.89 L (30 oz.)	0
3M™ Sponge-Stick with Letheen Broth	10 mL	SSL10LET	0.89 L (30 oz.)	0
3M™ Sponge-Stick with Letheen Broth, 2 Gloves	10 mL	SSL10LET2G	0.89 L (30 oz.)	2
3M™ Sponge-Stick with Neutralizing Buffer	10 mL	SSL10NB	0.89 L (30 oz.)	0
3M™ Sponge-Stick with Neutralizing Buffer, 2 Gloves	10 mL	SSL10NB2G	0.89 L (30 oz.)	2
3M™ Sponge-Stick with Neutralizing Buffer	10 mL	SSL10NB6X9	6" x 9" bag	0
3M™ Sponge-Stick with D/E Broth	10 mL	SSL10DE	0.89 L (30 oz.)	0

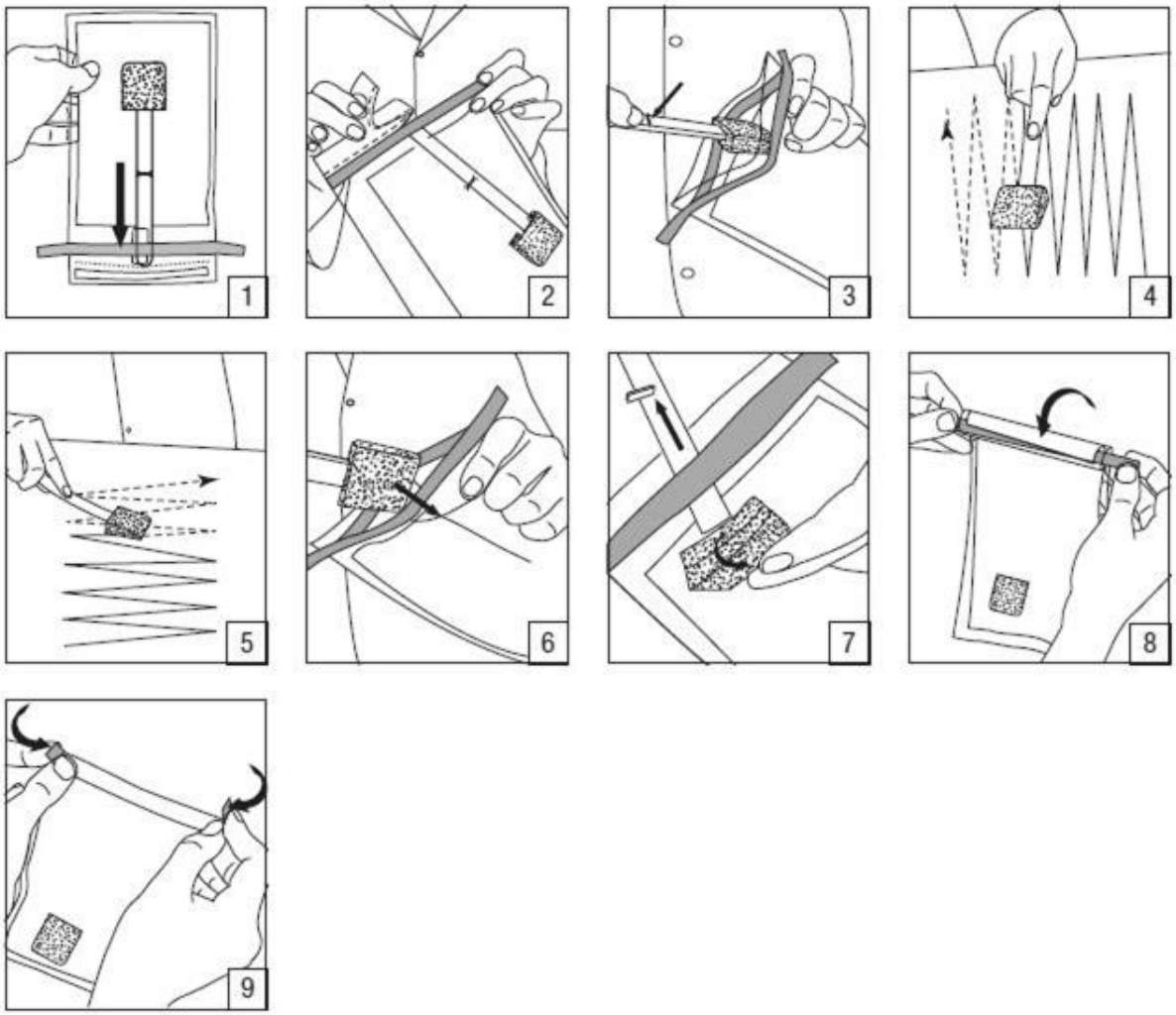
INSTRUCTIONS FOR USE

1. Shake stick to end of bag.
2. Tear bag open.
   NOTE: Add sterile diluent or broth to dry sponge.
3. Squeeze bag to open. Aseptically grasp the stick above the thumb-stop line to remove the sponge.
4. Aseptically swab across the entire sampling surface[3,4,5,6].
5. Turn sponge over. Change direction 90°. Aseptically swab the same sampled surface.
6. Aseptically place sponge into bag up to the thumb-stop.
7. Hold sponge in place inside bag. Bend stick to break. Allow sponge to drop in bag. Discard stick.
8. Fold bag to close.
9. Fold ends of blue wires inward.
10. Following user established procedures, remove any remaining enrichment broth or neutralizing solution residue from the sampled surface.

REFERENCES

1. U.S. Food and Drug Administration. Code of Federal Regulations, Title 21, Part 58. Good Laboratory Practice for Nonclinical Laboratory Studies.
2. ISO/IEC 17025. General requirements for the competence of testing and calibration laboratories.
3. American Public Health Association - Compendium of Methods for the Microbiological Examination of Foods - Chapter 3: Microbiological Monitoring of the Food Processing Environment, 4th edition.
4. US Food and Drug Administration - Bacteriological Analytical Method (available online at http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.html).
5. United States Department of Agriculture - Microbiological Lab Guidebook - Chapters 4.04, 5.04, and 8.07.
6. American Public Health Association - Standard Methods for the Examination of Dairy Products - Chapter 13: Microbiological Tests for Equipment, Containers, Water, and Air, 17th edition.

Refer to the current version of the standard methods listed above.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7.1.2 Microbial isolation

Per collected tonsils, cut off 10g of the tonsils and add 90mL BPW.

Per collected rectum take 10g feces of the rectum and add 90mL BPW.

Stomach the swab-, tonsil- and feces-bags for one minute.

Quantitative analysis of aerobic cfu for each sample except the tonsils and feces:

Plate 100µL on a plate count agar (PCA)-plate with a spiral plater.

Incubate the PCA-plates at 30°C for 48u ±3h.

Count the number of cfu on the plates.

Quantitative analysis of Enterobacteriaceae for each sample except the tonsils and feces:

Plate 100µL on a Rapid' Enterobacteriaceae / Agar-plate with a spiral plater.

Pour a second layer of Rapid' Enterobacteriaceae / Agar (2mm thick, ±10mL)

Incubate the Rapid' Enterobacteriaceae / Agar-plates at 37°C for 24u ±2h.

Count the number red colonies with a diameter equal to or exceeding 0.5mm on the Rapid' Enterobacteriaceae / Agar-plates.

Quantitative analysis of *E. coli* for each sample:

Plate 100µL on a tryptone bile x-glucuronide (TBX)-plate with a spiral plater.

Incubate the TBX-plates at 44°C for 21u ±3h.

Count the number of cfu with a metallic green color and a white border.

Quantitative and qualitative analysis of *Yersinia enterocolitica*:

Plate 2x 0.5mL of the tonsil and feces samples on a *Yersinia* selective plate (Cefsulodin-Irgasan-Novobiocin Agar (CIN)) with a Drigalski spatula.

Incubate the CIN-plates at 30°C for 24u ±2h.

Enrich 5mL of all the samples in 45mL peptone sorbitol bile broth (PSB) at 25°C for 48 ±2h.

Transfer 0.5mL in 4.5mL 0.5% KOH and 0.5% NaCl solution for 20 ±5s.

Plate 1öse or 100µL on CIN plates.

Incubate the CIN-plates at 30°C for 24u ±2h.

Inoculate five positive colonies (small colonies with a bull's eye morphology and a smooth border) on a PCA-plate at 30°C for 24h or 48-72h at room temperature.

Inoculate each colony in TSB.

Perform a Kligler iron agar (KIA)-test on the positive colonies (30 ±1°C, 24h). (Glucose: +, Lactose: - and H_2S: -)

Perform an urease-test on the positive colonies (30 ±1°C, 24h). (Urea: +)

Inoculate each colony on esculine plate (30°C, 24h) (no black color)

Suspend 1mL of the TSB in 2ml glycerol and store it at -20°C.

Qualitative analysis of *Salmonella*:

Enrich all the samples in ±25mL extra BPW at 37°C for 16-20h.

Plate 3 droplets on a modified semi-solid Rappaport Vassiliadis (MSRV)-plate at 41,5°C for 24h.

If there is growth, pick bacteria at the edge of the growth area and plate them on a Xylose-lysine-deoxycholate (XLD)-plate at 37°C for 24h. (Black colonies with a white border on a red background)

Pick one colony of a positive plate and transfer it to TSB.

Perform a Triple sugar iron agar (TSI)-test on the colonies (37 ±1°C, 24h). (Glucose: +, Lactose: -, gas out of glucose: +, sucrose: - and H_2S: +)

Perform a lysine decarboxylase-test on the colonies (37 ±1°C, 24h). (Lysine: +)

Perform an indole-test on the colonies (37 ±1°C, 24h). (Indole: -)

Suspend 1ml of the TSB in 2mL glycerol and store it at -20°C.

7.1.3 Kligler Iron Agar Test

Procedure:

Allow KIA to warm to room temperature prior to inoculation.

Obtain a pure culture of the organism to be tested. Select well-isolated colonies.

With an inoculating needle, pick the center of well-isolated colonies obtained from solid culture media.

Stab the center of the medium into the deep of the tube to within 3-5mm from the bottom.

Withdraw the inoculating needle and streak the surface of the slant.

Loosen closure on the tube before incubating.

Incubate aerobically at 30ºC. for 24hours.

Read tubes for acid production of the slant/butt, gas, and hydrogen sulfide reactions.

Interpretation of results:

An alkaline slant-acid butt (red/yellow) indicates fermentation of dextrose only.

An acid slant-butt (yellow/yellow) indicates fermentation of dextrose and lactose.

An alkaline slant-alkaline butt (red/red) indicates that neither dextrose nor lactose was fermented (non-fermenter).

Cracks, splits, or bubbles in the medium indicates gas production.

A black precipitate in the butt indicates hydrogen sulfide production.

7.1.4 Urease Test

Use a heavy inoculum from an 18- to 24-hour pure culture to inoculate the broth.

Shake the tube gently to suspend the bacteria.

Incubate tubes with loosened caps at 30°C.

Observe the broth for a color change at 24 hours.

Urease production is indicated by a bright pink (fuchsia) color throughout the broth.

7.1.5 Triple Sugar Iron Agar Test

Procedure:

1. With a sterilized straight inoculation needle touch the top of a well-isolated colony.

2. Inoculate TSI Agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant.

3. Leave the cap on loosely and incubate the tube at 37°C in ambient air for 24 hours.

Interpretation of results:

If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in butt and in the slant. Some organisms generate gases, which produces bubbles/cracks on the medium.

If lactose is not fermented but the small amount of glucose is, the oxygen deficient butt will be yellow (remember that butt comparatively have more glucose compared to slant i.e. more media more glucose), but on the slant the acid (less acid as media in slant is very less) will be oxidized to CO_2 and water by the organism and the slant will be red (alkaline or neutral pH).

If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids (remember peptone is a major constituent of TSI Agar).

If H_2S is produced, the black color of ferrous sulfide is seen.

7.1.6 Lysine Decarboxylation Test

Procedure:

1. Inoculate a nutrient broth to which 0.5% lysine is added.

2. Overlay the medium with a paraffin layer.

3. Incubate the tube at 37°C in ambient air for 24 hours.

Interpretation of results:

Purple color-Positive Decarboxylation.

Yellow color-Negative i.e. No decarboxylation.

7.1.7 Indole Test

Procedure:

1. Inoculate the tryptophan broth with broth culture.

2. Incubate at 37°C for 24 hours in ambient air.

3. Add 0.5 mL of Kovac's reagent to the broth culture.

Interpretation of results:

Positive: Pink colored rink after addition of the Kovac's reagent.

Negative: No color change even after the addition of the Kovac's reagent.

7.1.8 API 20E

API (Analytical Profile Index) 20E presented is a biochemical panel for identification and differentiation of members of the family Enterobacteriaceae.

In API 20E for identification of members of the family Enterobacteriaceae, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically-defined compositions for each test.

ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside.

ADH: decarboxylation of the amino acid arginine by arginine dihydrolase.

LDC: decarboxylations of the amino acid lysine by lysine decarboxylase.

ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase.

CIT: utilization of citrate as only carbon source.

H_2S: production of hydrogen sulfide.

URE: test for the enzyme urease.

TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent to put- Ferric Chloride.

IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase. Reagent- *Indole is detected by addition of Kovac's reagent.*

VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway.

GEL: test for the production of the enzyme gelatinase which liquefies gelatin.

GLU: fermentation of glucose (hexose sugar)

MAN: fermentation of mannose (hexose sugar)

INO: fermentation of inositol (cyclic polyalcohol)

SOR: fermentation of sorbitol (alcohol sugar)

RHA: fermentation of rhamnose (methyl pentose sugar)

SAC: fermentation of sucrose (disaccharide)

MEL: fermentation of melibiose (disaccharide)

AMY: fermentation of amygdalin (glycoside)

ARA: fermentation of arabinose (pentose sugar)

Setting up an API 20E Biochemical Test Strip

Pick up a single isolated colony (from a pure culture) and make a suspension of it in sterile distilled water.

Take the API20E Biochemical Test Strip which contains dehydrated bacterial media/bio-chemical reagents in 20 separate compartments. API20E Biochemical Test Strip is commercially available. (Bacteria will react with them and will give different colors which will help to identify bacteria to the species level).

Take a Pasteur-pipette and fill up (up to the brim) these compartments with the bacterial suspension.

Add sterile oil into the ADH, LDC, ODC, H2S and URE compartments.

Put some drops of water in the tray and put the API Test strip and close the tray.

Mark the tray with identification number (Patient ID or Organism ID), date and your initials.

Incubate the tray at 37^{o}C for 18 to 24 hours.

Results and Interpretation

For some of the compartments, you can just read the change in color straightway after 24 hours but for some you have to put reagents before reading.

Add following reagents to these specific compartments:

TDA: Put one drop of ferric chloride.

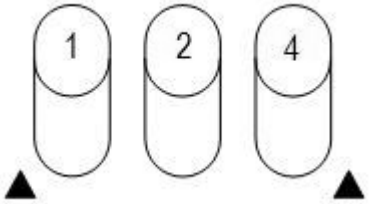
IND: Put one drop of Kovacs reagent.

VP: Put one drop of 40 % KOH (VP reagent 1) & One drop of VP Reagent 2 (α-Naphthol) (you have to wait for 10 minutes before telling it negative).

Get the API Reading Scale (color chart).

Mark each test as positive or negative on the lid of the tray.

The wells are marked off into triplets by black triangles, for which scores are allocated as follows:



Add up the scores for the positive wells only in each triplet. Supplementary tests, e.g.: oxidase may also be included in the profile. The highest score possible for a triplet is 7 (the sum of 1, 2 and 4) and the lowest is 0.

Triad	I			II			III			IV			V			VI			VII		
Tube	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	oxidase
Reaction	+	+	+	-	-	-	+	+	-	+	-	-	-	+	+	-	-	+	+	-	+
Point	1	2	4	0	0	0	1	2	0	1	0	0	0	2	4	0	0	4	1	0	4
Add	7			0			3			1			6			4			5		
7-digital Code	7031645																				

For example the profile for this combination of reactions is 7031645 (7 digit code).

Identify the organism by using API catalog or apiweb.

7.1.9 Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik)

Required Chemicals

- Deionized water
- Acetonitrile (ACN)
- Trifluoroacetic acid (TFA)
- Matrix HCCA (Referred to as HCCA)
- Standard solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) from Sigma-Aldrich
- Wooden application sticks, pipet tips or plastic inoculation loop to apply material
- 50 - 1000 µL pipet tips and a suitable pipet
- 2 - 200 µL pipet tips and a suitable pipet
- 0.5 - 10 µL pipet tips and a suitable pipet
- MALDI target plate

General cultivation conditions

Cultivation conditions have little effect on the identification. Media such as Columbia-Blood-Agar, Chocolate-Agar, or others can be used regardless of different growth phases or temperatures.

These different cultivation conditions produce only very small variations in observed peaks. Nearly all peaks are reproducible. Nevertheless, the same medium and growth conditions should always be used for the cultivation of microorganisms.

If possible freshly grown material (grown overnight) should be used, or in the case of slow-growing bacteria, grown for several days. If cultivation plates are stored at 4°C the quality of spectra deteriorates relatively quickly (within a couple of days). Storing the plates at room temperature for several days is acceptable.

To prepare the HCCA matrix solution

Add 250 µL standard solvent to a tube of HCCA.

Dissolve the HCCA by vortexing at room temperature until the solution is clear.

Direct transfer method

Smear biological material (single colony) as a thin film directly onto a spot on a MALDI target plate.

Note: Use only a small amount of material for direct transfer. Even if only a little biological material can be observed on the target, this is sufficient for measurement.

Overlay the spot with 1 µL of HCCA solution within 1 hour and allow to dry at room temperature.

Generation and analyzation of the spectra

Put the plate in a microflex LT MALDI-TOF MS and follow the manufacturer's instructions.

Record the spectra in the linear positive mode at a laser frequency of 60 Hz within a mass range of 2-20 kDa.

Perform all bacteria identifications by MALDI-TOF Biotyper RTC and the Bruker MALDI Biotyper 3.1 software and library (Bruker Daltonik).

A score of < 1.70 is interpreted as no identification, a score of 1.70-1.99 indicates identification to genus level and a score of ≥ 2.00 indicates identification to species level (Mestas *et al*, 2016).

7.2 Characterization of *Salmonella* isolates

7.2.1 Enterobacterial Repetitive Intergenic Consensus sequence-based PCR for confirmation of *Salmonella*

DNA preparation:

Add to an epp 50µl SDS, 50µL NaOH and sample picked from a colony on a XLD-plate.

Incubate the epp for 17minutes at 92°C and store the lysate at -20°C.

PCR-mix:

5µl 5x Gitschier buffer for 200ml

(16.6mL 1M $(NH_4)_2SO_4$, 67mL 1M Tris-HCl (pH 8.8), 6.7mL 1M $MgCl_2$, 1.3mL 0.005M EDTA (pH 8.8), 2.08mL 14.4M β-mercapto-ethanol and add H_2O until 200ml)

0.2µL BSA (20mg/ml)

2.5µL DMSO 100% (10mg/ml)

7.65µL UP

1.25µL dNTP

(1:1:1:1 mixture of the 4 nucleotides (dATP, dGTP, dTTP and dCTP) 100mM)

0.4µL Taq-polymerase (5U/µl)

Primers (Eric 1 and Eric 2, 0.3µg/µL (50 pmol), Versalovic 1991)

1µL Eric 1: 5'-ATGTAAGCTCCTGGGGATTCAC-3'

1µL Eric 2: 5'-(GA)CG(CT)CTTATC(CA)GGCCTAC-3'

2.5µL Tween (5%)

2.5µL Gelatine (0,1%)

1µL Lysate

Vortex + shortspin the PCR mix

PCR run:

7min 95°C

30sec 90°C |

1min 52°C | 30x

8min 65°C |

16 min 65°C

Agarose gel electrophoresis:

Make 10x TBE-buffer: 108g Tris, 55g H_2BO_3, 40mL 0.5M Na_2EDTA and add H_2O until 1litre. Dilute the TBE-buffer 10x.

Dissolve 3.15g agarose in 210mL 1x TBE buffer in a 500ml bottle.

Heat the gel in the microwave and put the bottle in a warm water bath.

Pour the agarose (50-70°C) and put the comb with 20 teeth in the gel.

Add 5 µL 6x loading buffer (30% glycerol, 0.125% bromophenol blue and 20mM Tris-HCl (pH 8)) to the PCR-mix and load 10 µL of the PCR-mix on the gel.

Load 6µL ladder (6:4:2 ratio 100bp:500bp:loading buffer).

Run the gel for 4h with 120V.

Incubate the gel for 30-45min in ethidium bromide.

Take a picture.

Analyze the ERIC PCR profiles with GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) and use the Dice coefficient with 2.1% position tolerance

7.2.2 *Salmonella* serotyping

Identification of *Salmonella* by Serotyping

Salmonellae are Gram-negative, flagellated, facultative anaerobic bacilli possessing three major antigens: H or flagellar antigen, O or somatic antigen, and Vi (capsular) antigen (possessed by only a few serovars). The different species are serotyped according to these three different antigens.

- **H (flagellar) antigen** may occur in either or both of two forms, phase 1 and phase 2. There are over 1800 known serovars which current classification considers being separate species. The organisms tend to change from one phase to the other.
- **O (somatic) antigens** occur on the surface of the outer membrane and are determined by specific sugar sequences on the cell surface.
- **Vi (capsular) antigen** is a superficial antigen overlying the O antigen (additional surface antigen). It is present in a few serovars, the most important being *Salmonella* Typhi, but also present in *Salmonella* Paratyphi C and *Salmonella* Dublin.

Once the O, H-phase 1 and H-phase 2 are identified, the antigenic formula can be used to identify the serotype by referring to a **Kauffman-White reference catalog.**

- The formula gives the O antigen(s) first followed by the H antigen(s). O antigens, Vi (when present), H antigens phase 1, H antigens phase 2 (when present).
- Colons separate the major antigens and commas separate the components of the antigens.
- Further conventions:
  - Underlined O factor is encoded by a bacteriophage (lysogenic strain)
  - [ ]: square bracketed factor may or may not be present (not phage-encoded)
  - [ ]: curly bracketed factor never coexists with others (exclusive)
  - ( ): parenthesis around a factor indicate weakly agglutinable factor

Examples

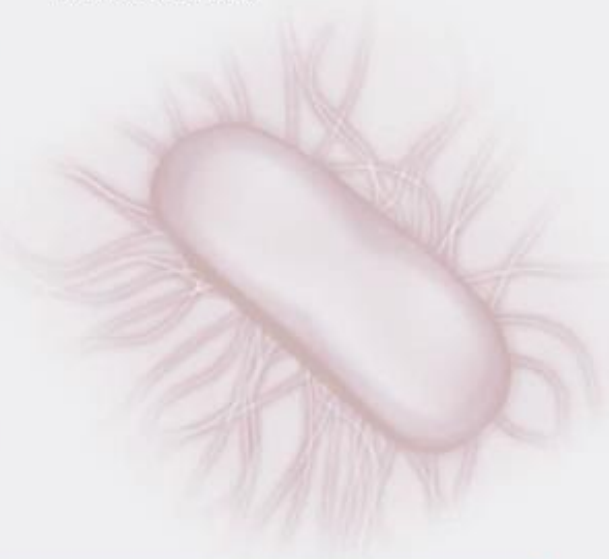
***Salmonella* enterica serotype Typhimurium: 1,4,[5],12:i:1,2**

This strain has the O antigen factors 1, 4, [5], and 12, the flagellar H antigen i (1st phase) and the flagellar H antigens 1 and 2 (2nd phase).

***Salmonella* enterica serotype Lagos: 1,4,[5],12:i:1,5**
This strain has the O antigen factors 1, 4, [5], and 12, the flagellar H antigen i (1st phase) and the flagellar H antigens 1 and 5 (2nd phase).

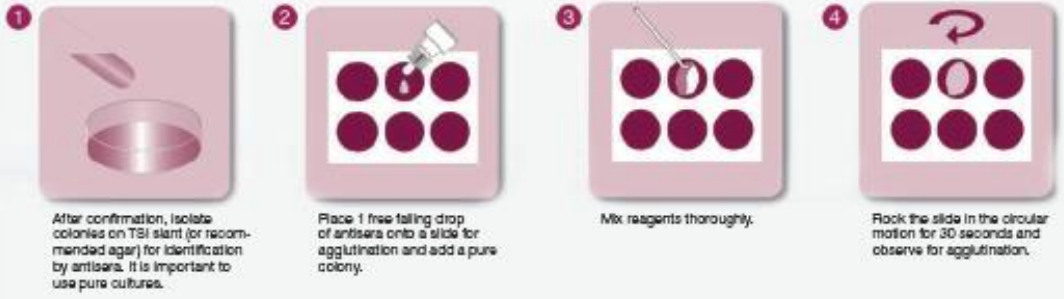
***Salmonella* enterica serotype Virchow: 6,7,14:r:1,2**
This strain has the O antigen factors 6, 7 and 14; the flagellar H antigen r (1st phase) and the flagellar H antigens 1, 2 (2nd phase).

For more examples, please refer to our Quick Guide (#xxx) including a table with some typical *Salmonella* serotypes isolated in food products.



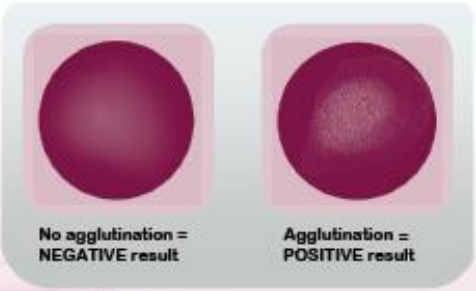
How to Perform *Salmonella* Serotyping

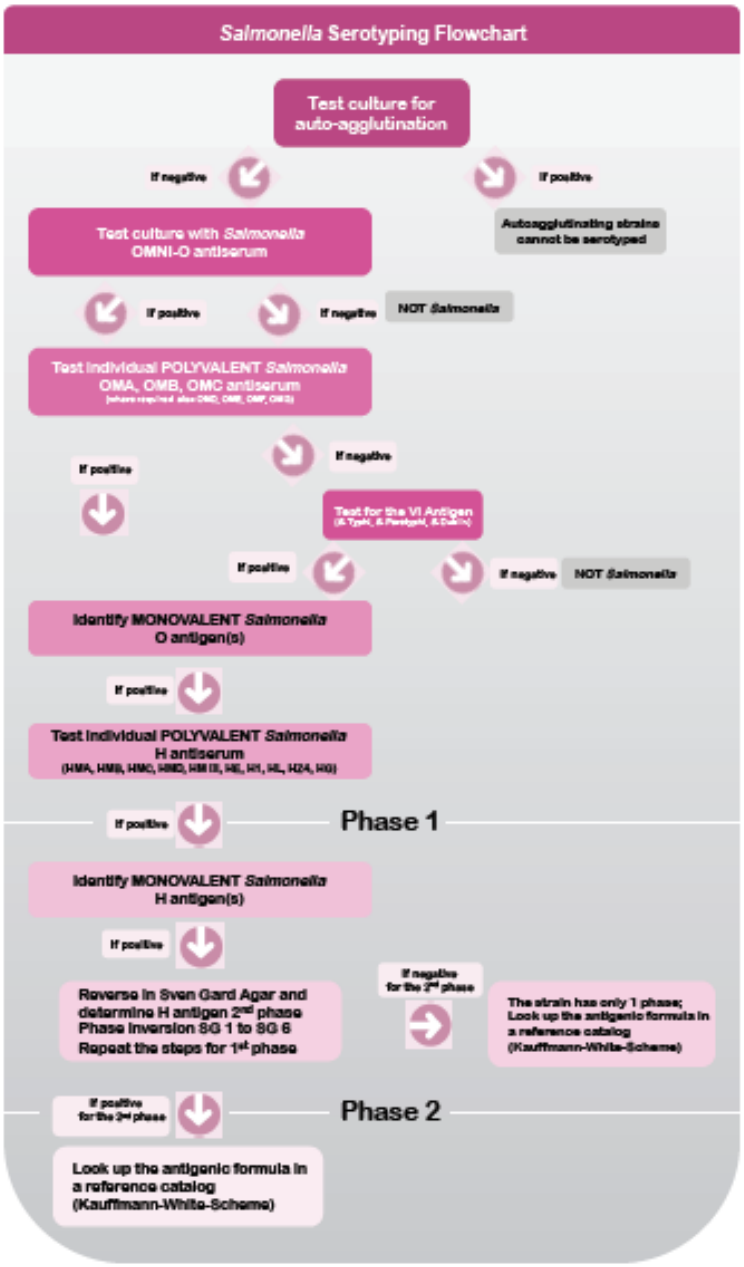
A Simple Protocol



- Serotyping is performed after identification of the species on a fresh, pure culture of *Salmonella* isolated on a non-selective agar medium. There are several media recommended for use, including Müller-Hinton or Nutrient Agar, TSI (Triple Sugar Iron) and/or LIA (Lysine Iron Agar) slants or TSA (Tryptic Soy Agar).
- Where polyvalent and monovalent antisera are available, start by testing agglutination with polyvalent sera, then with the specific monovalent sera corresponding to the mixture giving marked agglutination.
- Agglutination should appear between 1–10 seconds. If agglutination occurs > 60 sec, the antigens can not be identified correctly.

Easy Interpretation





1. Test the culture for auto-agglutination

- Test the culture first in Physiological Water/Saline; strains that produce auto-agglutination cannot be serotyped.
- In addition, all strains should be tested with the *Salmonella* Omni-O antiserum which contains antigens A – 60 for the presumptive identification of O-agglutinable strains of Salmonellae.
- Agglutination of a strain of *Salmonella* with Omni-O antiserum indicates that the strain is O-agglutinable and can be serotyped with specific sera.

2. Test for the O antigens

- Begin by testing the isolate with polyvalent O antiserum. The majority (about 98%) of *Salmonella* encountered in warm-blooded animals possess an O antigen corresponding to the agglutinins contained in OMA, OMB and OMC sera.
- When agglutination occurs with one of these 3 groups, the isolate is positive for that group.
- The individual monovalent O antisera are used to identify the O antigen(s).
- Repeat the agglutination step by testing the isolate in each monovalent O antiserum present in the group.

Example: The polyvalent O antiserum OMA shows agglutination therefore the following monovalent O antisera must be tested: O:1,2; O:4,5; O:9, O:46; O:3,10,15; O:1,3,19

- When a strain does not agglutinate the OMA, OMB or OMC polyvalent sera, it is recommended to test this strain with Vi serum and the other polyvalent O sera.
- If a Vi positive reaction is observed, the bacterial suspension must then be heated to 100°C for 30 minutes, before repeating the test with polyvalent OMA, OMB and OMC sera and the corresponding monovalent sera to define the O antigen.

3. Test for the H Antigen — Phase 1

- Begin by testing the isolate with a polyvalent H antiserum (HMA – HG). When agglutination occurs with one of these groups, the isolate is positive for that group.
- The individual monovalent H antiserum is used to identify the H antigen.
- Repeat the agglutination step by testing the isolate in each monovalent H antiserum present in the group.

Example: The polyvalent H Antiserum HMA shows agglutination therefore the following monovalent H antisera must be tested: a; c; d; l; z10.

Once the first H antigen is identified, a phase inversion on the isolate must be performed to force the organism to repress its dominant H phase and grow in the second phase.

4. Phase Inversion – Sven Gard Method

Sven Gard medium is used during serotyping of *Salmonella* to demonstrate the inapparent H antigen phase of biphasic *Salmonella* (Sven Gard method). Sven Gard agar should be used with the following *Salmonella* antisera: SG 1 to SG 6.

Example:

H:1,2 antigens were identified in a culture → SG6 (1,2 + 1,5 + 1,7 + z6) serum is used for phase inversion.

H:r antigen is identified in a culture → SG4 (r + z) antiserum is used for phase inversion.

5. Test for the H Antigen — Phase 2

- A culture at the periphery of the invasion zone of the Sven Gard agar should be taken.
- Start testing again by using the H polyvalent antisera (HMA – HG). If there is no agglutination, this serotype contains only one phase.
- If one of these groups shows agglutination, define the specific H phase by using the relevant H monovalent antisera.

As the antigenic formula with O, H – phase 1 and H – phase 2 are identified, the serotype is now specified by referring to a reference catalog, such as the Kauffman-White scheme.

Serotype	O Antigens	H Antigens Phase 1	H Antigens Phase 2
Agona	1, 4, [5], 12	f, g, s	[1, 2]
Anatum	3, [10] {15} {15, 34}	e, h	1, 6
Bareilly	6, 7, 14	y	1, 5
Blockley	6, 8	k	1, 5
Bovis Morbificans	6, 8 ,20	r, [i]	1, 5
Brandenburg	1, 4, 12	e, h	e, n, z15
Bredeney	1, 4, 12, 27	l, v	1, 7
Chester	1, 4, [5], 12	e, h	e, n, x
Derby	1, 4, [5], 12	f, g	[1, 2]
Dublin	1, 9, 12, [Vi]	g, p	-
Enteritidis	1, 9, 12	[f], g, m, [p]	[1, 7]
Gallinarium	1, 9, 12	-	-
Glouchester	1, 4, 12, 27	i	l, w
Hadar	6, 8	z10	e, n, x
Heidelberg	1, 4, [5], 12	R	1, 2
Indiana	1, 4, 12	z	1, 7
Infantis	6, 7, 14	R	1, 5
Javiana	1, 9, 12	l, z28	1, 5
Kentucky	8, 20	i	z6
Kottbus	6, 8	e, h	1, 5
Lagos	1, 4, [5], 12	i	1, 5
Lille	6, 7, 14	z38	-
Livingstone	6, 7, 14	d	l, w
Mbandaka	6, 7, 14	z10	e, n, z15
Meleagridis	3, [10] {15} {15, 34}	e, h	l, w
Montevideo	6, 7, 14	g, m, [p], s	[1, 2, 7]
Muenchen	6, 8	d	1, 2
Newport	6, 8, 20	e, h	1, 2
Orion	3, [10] {15} {15, 34}	y	1, 5
Paratyphi B	1, 4, [5], 12	b	1, 2
Saintpaul	1, 4, [5], 12	e, h	1, 2
Senftenberg	1, 3, 19	g, [s], t	-
Stanley	1, 4, [5], 12, [27]	d	1, 2
Thomson	6, 7,14	k	1, 5
Typhimurium	1, 4, [5], 12	i	1, 2
Virchow	6, 7	r	1, 2
Weltervreden	3, [10] {15}	r	z6

7.3 Lactic acid sensitivity tests in culture media

Day 1

Transfer one loopful of the isolate to 10m TSB (37°C ON or 25°C 24h).

Prepare all plates with stickers.

Prepare dilution tubes (TSB) with stickers.

Day 2

Put 5% lactic acid solution and the 0,85% NaCl in hot water bath at 25°C.

Put 2 times 100µl bacterial suspension in an eppendorf and label the eppendorfs.

Put the 2 eppendorfs in 30°C heath block.

Check pH of lactic acid solution: pH=4 at 25°C (adjust with 10M NaOH).

Start the treatment in the eppendorfs at 30°C.

Add 900µL lactic acid solution to the Eppendorf.

Invert the Eppendorf.

Start 10min.

After 10min: add 1M NaOH to increase the pH to 6.6.

Invert.

Take the eppendorf out of the heath block.

FOR THE CONTROL: ADD 0,85% NaCl instead of 1M NaOH (in the same amount).

After the treatment of all eppendorfs: centrifugation for 2min at 12000 rpm.

Remove the supernatant of all eppendorfs.

Resuspend the pellets in 1ml TSB.

Transfer 100µl of each eppendorf to 5ml TSB (for plating just after treatment).

Transfer 200µl of each eppendorf to 10ml TSB and incubate at 2°C.

Dilute the original bacterial suspensions (10µl in 10ml H_2O and again 10µl of the first dilution in 10 mL H_2O) and spiral plate on PCA and selective plate(s).

Dilute the 5ml TSB tubes (with 100µl bacteria suspension, just after treatment), 40µl in 4ml H_2O and plate on PCA and selective plate(s).

Day 3

Dilute TSB tubes (with 200µL bacteria suspension), Dilute 40µl of the bacterial suspensions from the 2°C in 4ml H_2O and plate on PCA and selective plate(s).

Read plates (plated on Day 2).

Day 4

Dilute TSB tubes (with 200µL bacteria suspension), Dilute 40µl of the bacterial suspensions from the 2°C in 4ml H_2O and plate on PCA and selective plate(s).

Read plates (plated on Day 3).

Day 5

Read plates (plated on Day 4).

Bacteria	**Plates and incubation conditions**		
Salmonella strains	PCA 37°C ON	XLD 37°C ON	
Yersinia strains	PCA 30°C 48h	CIN 30°C 48h	CRMOX 37°C 48h
E. coli isolates	PCA 37°C ON	TBX 37°C ON	
Enterobacteriaceae isolates	PCA 37°C ON	RAPID' Enterobacteriaceae 37°C ON	

7.4 Preliminary lactic acid sensitivity tests on pig skin

Day 1

Grow bacterial strains at 37°C on TSB for 24h.

Day 2

Collect dorsal pig skin of at least 700 cm^2 of a pig carcass that is cooled at 4°C for 24h.

Cut the pig skin in 7 pieces of 100 cm^2.

Take a first piece of 100 cm^2 tissue and put in 100 mL BPW.

Stomach and spiral plate appropriate dilutions on selective plate.

Put by spreading out 100 µL of bacterial culture on each of the 6 remaining 100 cm^2 tissues.

Allow to air-dry for 10min.

(100 µL 10^9 = 10^8, diluted 100 times (100 cm^2) = 6 $\log_{10}$ cfu / cm^2)

Dip 3 of the 6 tissues in 5% lactic acid set at pH 4 with 10M NaOH at 25°C for 15s.

Allow to air-dry for 10min.

Take 100 cm^2 lactic acid treated and 100 cm^2 untreated tissue, put each separately in 100 mL BPW.

Stomach and spiral plate appropriate dilutions on selective plate.

Put the four remaining tissues (2 lactic acid treated, 2 untreated) in a sterile bag and incubate at 2°C.

Day 3

Take 100 cm^2 treated and 100 cm^2 untreated tissue, put each separately in 100 mL BPW.

Stomach and spiral plate appropriate dilutions on selective plate.

Day 4

Take 100 cm^2 treated and 100 cm^2 untreated tissue, put each separately in 100 mL BPW.

Stomach and spiral plate appropriate dilutions on selective plate.

7.5 Composition of the media

7.5.1 Buffered peptone water

Typical Formula	g/liter
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5

pH 7.2 ± 0.2 @ 25°C

Add 20g to 1 liter of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes. It is extremely important that the distilled water used is of a high quality with a low mineral content/conductivity.

7.5.2 Peptone sorbitol bile broth

Typical Formula	g/liter
Bile salt mixture	1.5
Disodium phosphate	8.23
Monosodium phosphate	1.2
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Sorbitol	10.0

pH 7.6 ± 0.2 @ 25°C

Add 30,93g to 1 liter of water (purified as required), mix well and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.

7.5.3 Tryptone soya broth

Typical Formula	g/liter
Pancreatic digest of casein	17.0
Enzymatic digest of soya bean	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

pH 7.3 ± 0.2 @ 25°C

Add 30g to 1 liter of water (purified as required), mix well and distribute into final containers. sterilize by autoclaving at 121°C for 15 minutes.

7.5.4 Plate count agar

Typical Formula	g/liter
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar	9.0

pH 7.0 ± 0.2 @ 25°C

Add 17.5g to 1 liter of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.

7.5.5 Tryptone bile X-glucuronide agar

Typical Formula	g/liter
Tryptone	20.0
Bile Salts No. 3	1.5
Agar	15.0
X-glucuronide	0.075

pH 7.2 ± 0.2 @ 25°C

Suspend 36.6g of TBX Medium in 1 liter of distilled water. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and pour the medium into sterile Petri dishes.

7.5.6 Rapid' Enterobacteriaceae agar

Typical Formula	g/liter
Nutritive mix	17.3
Glucose	9.0
Color indicators	0.07
Selective agent	0.7
Agar	11.0

pH 7.4 ± 0.2 @ 25°C

Suspend 38.0g of Rapid' Enterobacteriaceae agar in 1 liter of distilled water. Bring to the boil with frequent agitation. **DO NOT AUTOCLAVE**. Cool to 50°C and pour into sterile Petri dishes.

7.5.7 Cefsulodin irgasan novobiocin agar

Typical Formula	**g/liter**
Special peptone	20.0
Yeast extract	2.0
Mannitol	20.0
Sodium pyruvate	2.0
Sodium chloride	1.0
Magnesium sulphate	0.01
Sodium desoxycholate	0.5
Neutral red	0.03
Crystal violet	0.001
Agar	12.5
pH 7.4 ± 0.2 @ 25°C	

<u>*Yersinia* selective supplement</u>

Code: SR0109

Vial contents (each vial is sufficient for 500 ml of medium)	**per vial**	**per liter**
Cefsulodin	7.5mg	15mg
Irgasan	2.0mg	4.0mg
Novobiocin	1.25mg	2.5mg

Suspend 29g in 500ml of distilled water and bring gently to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Allow to cool to approximately 50°C and aseptically add the contents of one vial of Yersinia Selective Supplement SR0109 reconstituted as directed in the instructions for use that accompany the product. Mix gently and pour into sterile Petri dishes.

7.5.8 Kligler iron agar

Typical Formula	**g/liter**
`Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.05
Agar	12.0
pH 7.4 ± 0.2 @ 25°C	

Suspend 55g in 1 liter of distilled water. Bring to the boil to dissolve completely. Mix well and distribute into containers. Sterilize by autoclaving at 121°C for 15 minutes. Allow to set as slopes with 1 inch butts.

7.5.9 Urea broth

Typical Formula	g/liter
Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.004
pH 6.8 ± 0.2	

Add 0.9 g to 95 ml of distilled water. Sterilize by autoclaving at 115°C for 20 minutes. Cool to 55°C and aseptically introduce 5 ml of sterile 40% Urea Solution SR0020. Mix well and distribute 10 ml amounts into sterile containers.

7.5.10 Esculin agar

Typical Formula	g/liter
Tryptone	15.0
Esculin	1.0
Ferric citrate	0.5
Bacteriological agar	12.0
pH 6.8 ± 0.2	

Mix 15g Tryptone, 1g esculin, 0.5g ferric citrate and 12g bacteriological agar in 1litre of distilled water and autoclave for 15 min at 121°C. Cool to 55°C and dispense into plastic Petri dishes.

7.5.11 Congo-red magnesium oxalate agar

Typical Formula	g/liter
Pancreatic digest of casein	15.0
Enzymatic* digest of soya bean	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.3 ± 0.2 @ 25°C	

Mix 40g Tryptic soy agar in 825 ml of distilled water and autoclave for 15 min at 121°C. Cool to 55°C and add the following solutions: 80 ml of 0.25 M sodium oxalate, 80 ml of 0.25 M magnesium chloride, 10 ml of 20% D-galactose and 5 ml of 1% Congo red. The galactose solution is sterilized by autoclaving for 10 min at 115°C. All other solutions are autoclaved for 15 min at 121°C. Mix the medium thoroughly and dispense into plastic Petri dishes.

Strains are CRMOX negative (CRMOX-) if only large colorless colonies are present. Positive strains (CRMOX+) always produce small red colonies (CRMOX+) and large colorless colonies (CRMOX-). The presence of CRMOX- colonies was due to a rapid loss of plasmid in vitro. Consequently, subculture of CRMOX+ colonies onto CRMOX will consistently yield both CRMOX+ and CRMOX- colonies. Subculture of CRMOX- colonies from CRMOX+ strains will grow only CRMOX- colonies (Riley & Toma, 1989).

7.5.12 Modified semi-solid Rappaport Vassiliadis agar

Typical Formula	**g/liter**
Tryptone	2.3
Meat peptone	2.3
Casein hydrolysate	4.7
Sodium chloride	7.3
Potassium dihydrogen phosphate	1.5
Magnesium chloride (anhydrous)	10.9
Malachite green oxalate	0.037
Agar	2.7
pH 5.2 ± 0.2 @ 25°C	

MSRV selective supplement

Code: SR0161

Vial contents (each vial is sufficient for 500ml of medium)	**per vial**	**per liter**
Novobiocin	10.0mg	20.0mg

Suspend 15.8g of MSRV Medium Base in 500ml of distilled water. Bring to the boil with frequent agitation. **DO NOT AUTOCLAVE**. Cool to 50°C and aseptically add the contents of 1 vial of MSRV Selective Supplement SR0161E reconstituted with 2ml of sterile distilled water. Mix well and pour into sterile Petri dishes. Air dry at room temperature for at least one hour. (Plates may be air-dried overnight prior to storage at 2-8°C.)

7.5.13 Xylose lysine deoxycholate agar

Typical Formula	g/liter
Yeast extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5

pH 7.4 ± 0.2 @ 25°C

Suspend 53g in 1 liter of distilled water. Heat with frequent agitation until the medium boils. **DO NOT OVERHEAT**. Transfer immediately to a water bath at 50°C. Pour into sterile Petri dishes as soon as the medium has cooled.

7.5.14 Lysine decarboxylation broth

Typical Formula	g/litre
Peptic digest of animal tissue	5.0
Beef extract	5.0
Dextrose	0.5
L-lysine	10.0
Bromocresol purple/red	0.016

pH 6.1 ± 0.2 @ 25°C

Take 5ml of the broth and put it in a 2ml tubes. Sterilize by autoclaving at 121°C for 15 minutes.

7.5.15 Triple sugar iron agar

Typical Formula	g/litre
`Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

pH 7.4 ± 0.2 @ 25°C

Suspend 65g in 1 liter of distilled water. Bring to the boil to dissolve completely. Mix well and distribute. Sterilize by autoclaving at 121°C for 15 minutes. Allow the medium to set in sloped form with a butt about 1 inch deep.

7.5.16 Indole broth

Typical Formula	g/litre
Tryptone	10.0
Sodium chloride	5.0
Tryptophan	3.0

pH 7.5 ± 0.2 @ 25°C

Dissolve 18g in 1 liter of distilled water and distribute into 2ml tubes. Sterilize by autoclaving at 121°C for 15 minutes.

Kovac's reagent:

p dimethylamino benzaldehyde	5 grams
amyl alcohol	75ml
concentrated hydrochloric acid	25ml