CEU CARDENAL HERRERA UNIVERSITY

FACULTY OF VETERINARY MEDICE

Contamination of *Salmonella* and *Campylobacter* during the broiler slaughter and in chicken fillet meat packaged under modified atmospheres

Thesis submitted to the CEU Cardenal Herrera University of Valencia in fulfilment of the requirements for degree of Doctor of Philosophy in the Faculty of Veterinary Medicine by:

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CERTIFICAN:

Que la memoria titulada **"Contamination of *Salmonella* and *Campylobacter* during the broiler slaughter and in chicken fillet meat packaged under modified atmospheres"**, que, para aspirar al grado de Doctor Internacional en Veterinaria presenta Dña. Sara González Bodí, realizada bajo nuestra dirección en la Universidad CEU Cardenal Herrera, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que,

AUTORIZAN:

A la interesada a su presentación en la Facultad de Veterinaria de la Universidad CEU Cardenal Herrera.

Y para que conste a los efectos oportunos, se presenta la referida memoria, firmando el presente certificado en Valencia a 17 de Noviembre de 2014.

Fdo. Dra. Clara Marín Orenga Fdo. Dra. Milagros Mateos Otero Fdo. Dr. Santiago Vega García

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AGRADECIMIENTOS

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
API	Identification system for *Enterobacteriaceae* and other Gram-negative rods
BPW	Buffered Peptone Water
º C	Celsius degrees
CDC	Center for Disease Control and Prevention
cfu	Colony-forming units
cm	Centimetre
DNA	Deoxyribonucleic acid
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ERIC	Enterobacterial Repetitive Intergenic Consensus
EU	European Union
***fla*A**	Flagellin gene A PCR/restriction fragment length polymorphism
g	Grams
GBS	Guillain-Barré syndrome
h	Hours
ISO	International Organization for Standardization
LB	Lauria-Bertoni
LPS	Lipopolysaccharide
M	Molar
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
mCCDA	Modified Cefoperazone Charcoal Deoxycholate agar
min	Minutes
mL	Millilitres
MLST	Multilocus Sequence Typing
MS	Member States
MSRV	Modified Semisolid Rappaport Vassiliadis
n	Number of observations
OD_{600}	Optical Density at 600 nm

P	Probability value
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RA	Reactive arthritis
RAPD	Radom Amplification of Polymorphic DNA
rep-PCR	Repetitive Extragenic Palindromic PCR
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
SE	Standard error
spp	Species (plural)
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VBNC	Viable but non-culturable
vol/vol	Volume to volume
vs	*Versus*
WHO	World Health Organisation
XLD	Xylose Lysine Deoxycholate

ABSTRACT.

Abstract

Salmonella and *Campylobacter* have been widely recognized as most important zoonotic pathogens with economic impact in animals and humans. Handling or consumption of contaminated poultry meat is considered the most common sources of both human infections. In this sense, the legislators have been working to limit *Salmonella* and *Campylobacter* presence; however, high number of salmonellosis and campylobacteriosis are continued declare. In order to lower the risk for human infections, most studies have been focused on the farm level to reduce the number of flocks colonized with these bacteria. Nevertheless, it has been reported that, after processing, carcasses are contaminated even when the flocks are *Salmonella* or *Campylobacter* negative at the farm. Therefore, it is necessary to introduce additional treatments to control or inhibit the survival and potential growth of both pathogens in chicken meat. In this context, the aim of this Thesis is to study the *Salmonella* and *Campylobacter* contamination of poultry carcasses during the slaughter process and evaluate different modified atmospheres packaging against the main strain isolated at slaughter level.

The objectives of the first experiment were to determine the status of *Campylobacter* and *Salmonella* flocks at the arrival of the live birds at slaughterhouse, the contamination of both pathogens on environmental surface of the slaughter line and the impact of different processing stages on the contamination of both pathogens on chicken carcasses throughout the process. Moreover, *Salmonella* and *Campylobacter* isolates were identified by serological methods and *Salmonella* strains were also characterized to investigate the genetic relation between the strains. A poultry processing plant located in the Valencia Region of an integrated broiler chicken company were visited 18 times during 9 months (from September to November 2011 and January to June 2012). At each visit, two flocks were studied during the working day of the slaughterhouse. At the arrival of the animals at the slaughterhouse, in order to determine the status of the flocks, two pooled faeces from the platform of the transporting truck and ten cloacal swabs were collected for *Salmonella* and *Campylobacter* detection, respectively. Then, before the processing of the flocks, environmental surface samples of 6 selected stages (exsanguination, scalding, de-feathering, evisceration, grading and air chilling stage) and foodstuff contact surfaces of clean transporting crates were collected to check the presence of both pathogens. Furthermore, during processing of the flock, 3 carcasses were taken from 3 select stages

of the processing line (after exsanguination, de-feathering and air chilling stage) to determine the degree of *Salmonella* and *Campylobacter* contamination. For *Salmonella* detection, samples were analysed according to ISO 6579:2002 (Annex D) and positive samples were serotyped by Kauffman-White-Le-Minor technique. Clonality among the *Salmonella* isolates was assessed by Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR). Representative isolates from the different ERIC-PCR patterns identified per sample were analysed by Pulsed-Field Gel Electrophoresis according to the PulseNet standardized protocol. For *Campylobacter* detection purpose, the swabs were tested by direct plating onto Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Preston Agar. Neck skin samples were analysed according to the ISO 10272-2:2006 (Annex E) for *Campylobacter* detection. Finally, Hippurate hydrolysis test was used for the speciation of *Campylobacter*. Our results showed that at the arrival of the slaughterhouse, the flocks were widely infected with both pathogens. According with the slaughterhouse environment, *Salmonella* contamination decreased after scalding and chilling stages. In contrast, *Salmonella* contamination increased after bleeding, de-feathering, grading and evisceration. Nevertheless, no *Campylobacter* spp. was isolated in any of the environmental samples analysed. The impact of different processing stages on the contamination of chicken carcasses showed that, *Salmonella* neck skin samples were higher contaminated at the de-feathering and exsanguination than at the air chilling stage. However, the internal surface cavity samples were higher *Salmonella* contaminated at the air chilling, and de-feathering than at the exsanguination stage. Moreover, samples from the neck skin were determined most frequently as *Salmonella* positive than those from internal surface cavities at the exsanguination and de-feathering stages. However, at the air chilling stage, samples from the internal cavity were more positive than those from neck skin. On the other hand, chicken carcasses were highly contaminated with *Campylobacter* throughout the processing, observing no differences among the different stages. The most prevalent strains isolated in this study were *S.* Enteritis and *C. jejuni*, the most prevalent strains involved in human outbreaks. Finally, the genetic relation study of *Salmonella* strains isolated revealed that, the same genetic pattern was found from all *Salmonella* isolates, suggesting a bacteria re-circulation across the poultry farms and slaughterhouse facilities.

The objective of the second experiment was to study the effect of the application of four modified atmospheres packaging conditions on chicken meat fillet (MAP-A:

50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) against *S.* Enteritidis and *C. jejuni* isolated from the slaughterhouse from the previous experiment. The enumeration of either *S.* Enteritidis or *C. jejuni* were done by subsequently serial-fold dilution and spread onto McConkey agar plate and onto mCCDA agar plate, respectability. A control group was used for physical-chemical and sensorial analysis. Our results showed that, application of 50% CO_2 MAP better controlled the *Salmonella* growth than MAP with lower (30%) or no presence of CO_2. *Campylobacter* growth was inhibited by the application of MAPs with high O_2 concentration (≥50%) and with Argon. However, packaging under MAP-A with anaerobic conditions, did not show effect at controlling *Campylobacter* growth. Regarding the physical-chemical analysis, chicken meat fillets packaged under standard poultry meat atmosphere showed significantly higher L* and lower a* values during the storage leading to a pale color of the chicken meat. The other MAPs studied did not show significantly differences on color parameters values among them during the storage. In the sensorial evaluation, judges preferred fillet samples packaged under MAP-D on the appearance and overall acceptability, and samples packaged under high CO_2 concentration (MAP-A) was evaluated as the best treatment on odor attribute among the MAPs studied. Generally, samples packaged under high oxygen concentration (MAP-B) had the lowest score of appearance, odor and overall acceptability.

Due to the above considerations, this Thesis showed that the slaughter operations contributed to increase *Salmonella* and *Campylobacter* contamination being necessary to control the whole processing line to control the bacteria from slaughter level. The control measures should be considered throughout the chain production. Therefore, biosecurity measures on farm level need to be accompanied by control measures at the slaughterhouse to reduce fecal contamination of broiler skin and to minimize cross-contamination of both pathogens. Furthermore, the right combination of different strategies such as modified atmosphere packaging could enhance the overall food safety and extend the shelf life of chicken meat.

CHAPTER I. LITERATURE REVIEW.

I. 1. European poultry meat production

Poultry meat developed its popularity as a preference food for the consumers being considered as lower in fat, healthier and cheaper than the other popular animal proteins like beef and lamb meat (Henchion *et al.,* 2014).

Since the 1960s, the global production of poultry meat has been growing faster than any other meat in both developed and developing countries. Between 1940 and 1960, advances in nutrition and genetic research allowed the actual modern broiler production to be able to produce 2 Kg of bird in 8 weeks instead of 16 weeks (Henchion *et al.,* 2014).

From 1940 to 1960, due to advances in production efficiency and refrigerated trucking, the markets expanded to a much larger geography, whereas the price of poultry dropped considerably. Therefore, since the price changed largely respect to other meat options, the demand for poultry meat increased drastically. During that period, production systems have been also changed from the seasonal small backyard sheds to large year-round naturally ventilated buildings. Actually, the modern poultry production occurs primarily in enclosed buildings to protect the birds from weather, predators, and the spread of diseases from wild birds. That allowed farmers to increase greatly the production and significantly reduce the amount of labor required (FAO, 2013).

Poultry industry represents almost 45% of world commerce (Henchion *et al.,* 2014). In 2013, the major broiler-producers countries in the world, in terms of volume were the United States, which contributed to a 20.06% of the world's total broiler production followed by China with 15.75%, Brazil with 14.96%, and EU-27 11.38% (Henchion *et al.,* 2014).

In 2013, the total poultry meat production in the EU-27 was 9.914 million tonnes leading to an increase of 14% compared to 2007. That is a result of the greater domestic demand for lower cost supplies of animal protein and reduced feed costs (EC, 2014). In Europe, there are five main producers of broiler meat. United Kingdom is responsible

for the largest share of the total EU production, accounting for 1.400 million tonnes, followed by Poland with 1.325 million tonnes, Germany with 1.150 million tonnes, France with 1.080 million tonnes and Spain with 1.063 million tonnes. The total value of the production at the primary farms, the slaughterhouses and the further processing of the poultry meat in the EU-27 in 2013 was €32bn. Additionally, the EU is an important player in the international trade of poultry meat. In 2013, the EU-27 exported 1.430 millions tonnes of poultry meat with a value of € 2.064bn. At the same time, the EU-27 imported 0.844 millions tonnes with a value of € 2.202bn.

In Spain, the poultry production is approximately € 2.557 millions, representing a 5.8% of the Final Agricultural Production and a 15.3% of the Final Livestock Production. Most of the poultry meat production is concentrated in four regions: Cataluña, with 28.7%, Valencia, with 16.9%, Andalucía, with 15.8%, and Galicia, with 13.1% of the total national production. The country has a total of 493 broiler farms throughout the territory, with 13 millions of fattening places (MAGRAMA, 2014).

I. 2. Foodborne pathogens

I. 2. 1 General aspect of *Salmonella*

I. 2. 1. 1. Historical context

Salmonella have been widely studied throughout the world since 1885, when Daniel Elmer Salmon and Theobald Smith (1886) (Figure 1), first isolated the organism from swine in association with "swine plague". Thomas Willis is considered as the pioneer in typhoid fever studies. Until his classic description in 1659 and its translation into English in 1684, few studies were carried out to separate this disease from similar diseases (Adelantado *et al.,* 2008). In 1826, Trousseau described typhoid mainly from the pathological point of view, and differentiated the real typhoid from the other gastro-intestinal infections. He described the classic inflammation of the Peyer glands and gave detailed description of the post-mortem appearances (Kelterborn, 1967). In 1829, Pierre Louis gave another classic description of typhoid and described the post-mortem findings in detail, especially the enlargement and ulceration of the Peyer patches. He

was also the first that used the word "Typhoid". However, he did not clearly differentiate between typhoid and typhus, which were often confused (Adelantado *et al.*, 2008). The first that distinguished typhoid from typhus fevers was Gerhard in 1837 (Adelantado *et al.*, 2008). Twenty years before that the bacterial origin of infectious diseases was discovered, William Budd stated that typhoid fever was an alimentary disease in which the infective material in faeces contaminated water, milk, and the hands of those who attended the sick (Moorehead, 2002). In 1888, *Bacterium enteritidis* was isolated from an outbreak of gastroenteritis that involved 58 persons with the consumption of beef. A. Gärtner isolated a bacterium from both red meat and spleen of one of the patients who died and he named the organism as *Bacillus enteritidis* (Kelterborn, 1967). Furthermore, Castellani and Chalmers (1919) renamed that organism as *Salmonella* Enteritidis. The first published table based on the terminology introduced by White (1929) and modified by Kauffmann, contained 20 serotypes. Nowadays, more than 2500 different serologically *Salmonella* serotypes have been recognized (OIE, 2010). Almost all of the 2500 *Salmonella* serovars are believed to be able to cause illness in humans. The epidemiology of human disease is dominated by only a few serovars. In the late 1970s, *S.* Typhimurium was the most common serovar and before this was *S.* Agona (Harbour *et al.*, 1977). Many of these peaks of infection have been associated with a particular food or animal vehicle, such as *S.* Enteritidis with poultry.

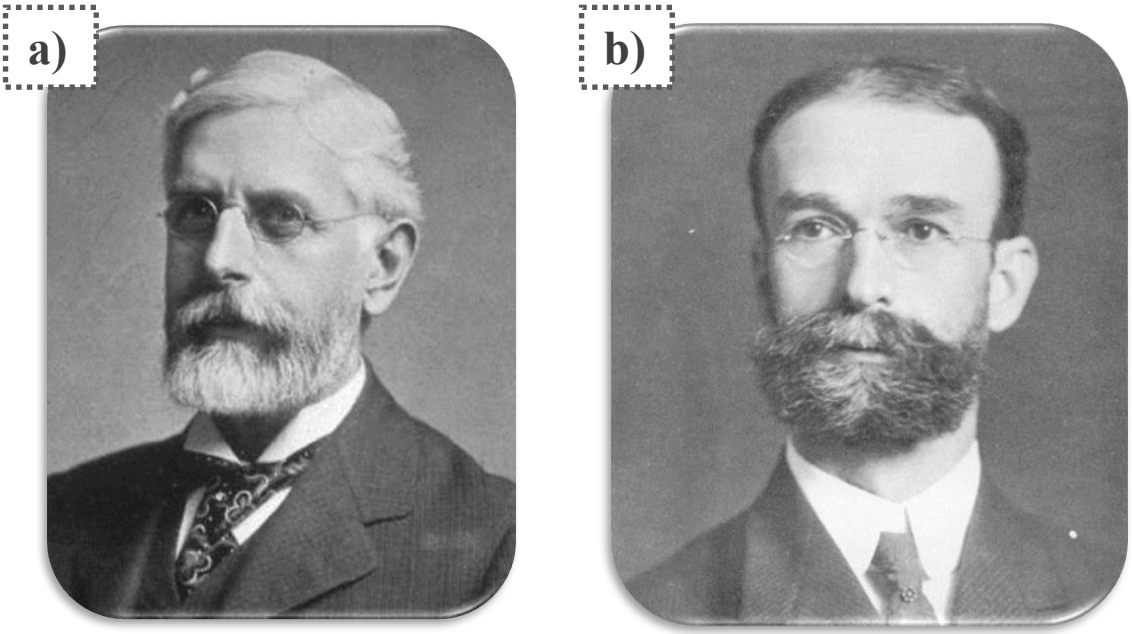


Figure 1. Daniel Elmer Salmon (a) and Theobald Smith(b).

I. 2. 1. 2 Taxonomy and characteristics

Salmonella genus is classified in the family of *Enterobacteriaceae*, which members are non-encapsulated, gram-negative bacilli. Almost all *Salmonella*s are motile since they have peritrichous flagella, with the exception of *S.* Gallinarum and *S.* Pullorum, (Tindall *et al.,* 2005). The organism is 0.7-1.5 μm wide and 2.0-5.0 μm length.

Salmonella is facultative anaerobic bacteria and use citrate as a sole carbon source (Meneses, 2010). The organism also produces hydrogen sulphide gas on triple-sugar iron agar and on decarboxylate lysine and ornithine test reduces nitrates to nitrites. *Salmonella* is negative on urease and indole test (Nikaido *et al.,* 2012).

Salmonella growth rate is dependent on several factors including temperature, pH, water activity and nutrients (Meneses, 2010). *Salmonella* grow at temperatures between 10 and 49 ºC, with an optimum of approximately 37 ºC (Zeirak *et al.,* 2012). At temperatures between 0 and 5 ºC, the organisms remain viable even though there is no growth (Zeraik *et al.,* 2012). Under freezing conditions and long-term frozen storage, there is a marked reduction in the number of *Salmonella*s, however not all of them are destroyed (Shah *et al.,* 2013). *Salmonella* are inactivated when exposed to temperatures of 55 ºC for one hour or 60 ºC for 15 to 20 minutes (Aljarallah *et al.,* 2007). *Salmonella* is eliminated during the cooking of food when the internal temperature of the food reaches 74 to 77 ºC. Nevertheless, household-cooking procedures used for eggs and egg-containing foods are frequently insufficient to ensure a safe meal (Coetzer and Tustin, 2004).

The optimal pH for *Salmonella* growth is between 6.5 and 7.5, with a growth in a pH range of 4.5 to 9.0 (Zeilani *et al.,* 2012). *Salmonella* grow at water activities above 0.93 (Beuchat *et al.,* 2010). The organism is resistant to drying and can survive for prolonged periods of storage at ambient temperature (Gruzdev *et al.,* 2011) and in faecal material, in slurry or on pasture (Kusar *et al.,* 2010). *Salmonella* is sensitive to gamma irradiation (Rodrigues *et al.,* 2011) and organic acids (Ávila *et al.,* 2013).

I. 2. 1 .3 *E*pidemiology in humans

Human clinical aspects

Salmonella is one of the major bacterial causes of gastroenteritis worldwide (EFSA, 2014). The organism may also be transmitted through direct contact with infected animals or faecal-contaminated environments and humans. The most common symptom of non-typhoidal *Salmonella* infection is acute gastroenteritis. Onset of intestinal salmonellosis occurs usually between a few hours and three to four days following ingestion of the infectious agent (Santana *et al.,* 2012). Human salmonellosis is usually characterized by acute fever, abdominal pain, nausea, and sometimes vomiting (EFSA, 2014). Symptoms are often mild and most infections are self-limiting, lasting few days (Scherer *et al.,* 2008). However, in some patients, the infection may be more serious and the dehydration can be life threatening. In these cases, as well as when *Salmonella* causes bloodstream infection, effective antimicrobials are essential for treatment. Salmonellosis has also been associated with long-term and sometimes chronic effects e.g. reactive arthritis (Colmegna *et al.*, 2004).

There are numerous food-borne sources of *Salmonella,* including a wide range of domestic and wild animals and a variety of foodstuffs covering both food of animal and plant origin. *Salmonella* infection occurs when organisms are introduced in food preparation areas and allowed to multiply on food, e.g. inadequate storage temperatures, inadequate cooking and cross contamination of ready-to-eat food (EFSA, 2014).

Salmonellosis in humans

Millions of human salmonellosis cases are reported worldwide every year and the disease results in thousands of deaths. Sundström *et al.* (2014) estimated that, it was an increase of the incidence of *Salmonella* in humans, with economic costs of € 5–55 million. Other increased costs were due to the higher incidences of sequelae € 3–49 million.

The European salmonellosis trend has been decreased for several years with a total of 92,916 confirmed cases of human salmonellosis reported by the European

Surveillance System in 2012 (27 EU Member States and three non-Member States, Figure 1) (EFSA, 2014). This represented a 4.7% of decrease in confirmed cases compared with 2011. However, in Spain there was an increasing trend of confirmed cases of human salmonellosis from 3,786 to 4,181 cases in 2011 and 2012, respectively. The proportion of domestic cases versus travel-associated cases varied markedly between countries, with >70% in the Nordic countries, Finland, Sweden and Norway (Figure 2).

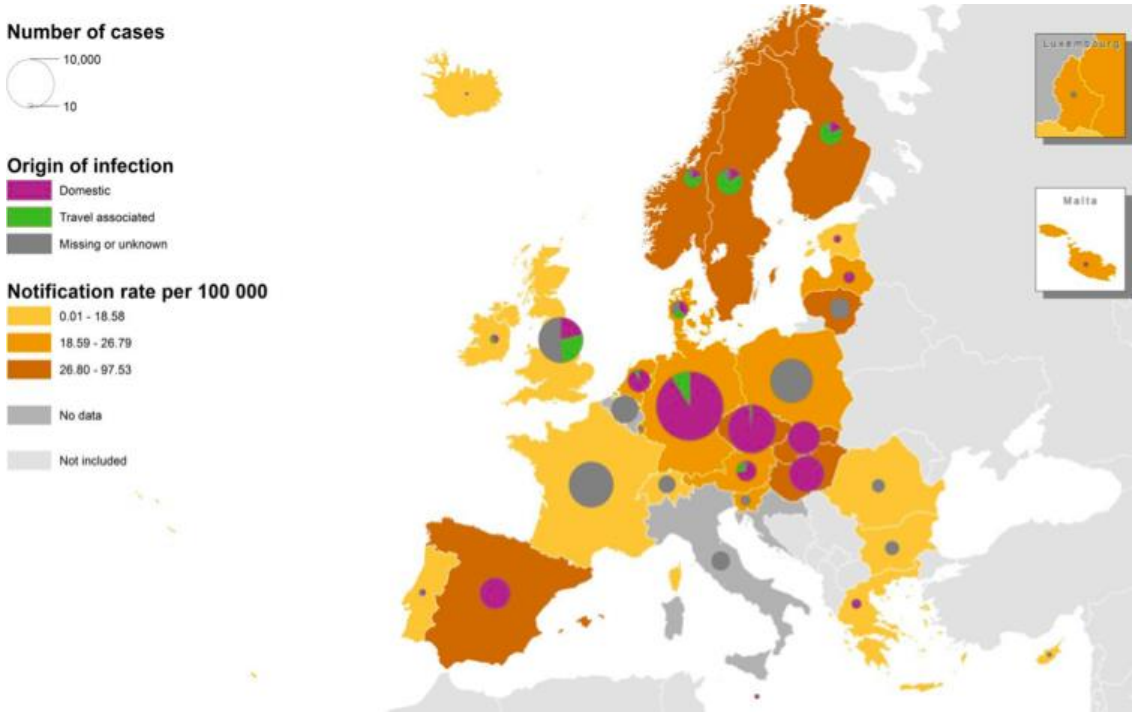


Figure 2. Salmonellosis notification rates and origin of infection in humans in the EU, 2012 (per population of 100,000; EFSA, 2014).

On average, 45.1% of the confirmed salmonellosis cases were hospitalised with 61 fatal cases. There was a clear seasonal trend in confirmed salmonellosis cases reported in the EU in 2008–2012, with the most cases during summer months. This pattern supports the influences of temperature and behaviour (i.e. food consumption habits such as barbequing) on *Salmonella* notification rates.

As in previous years, the two most commonly reported *Salmonella* serovars in human confirmed cases in 2012 were *S.* Enteritidis *and S.* Typhimurium, representing 41.3% and 22.1%, respectively (Table 1). The fewer cases of *S.* Enteritidis has been reduced a 5.8% from 2011 to 2012 reaching a total of 2,103 cases in the EU. Cases of *S.*

Typhimurium have been also decreased in 2012 compared with 2011. However, the monophasic *S.* Typhimurium 1,4,[5],12:i:- increased in 2012 with a total of 103 cases. *Salmonella* Infantis, the fourth most common serovar increase in 2012, by 14.5% (from 2.1 to 2.5%). The highest increase was observed in *S.* Stanley due to a multi-country outbreak, affecting at least seven MSs, and being linked to the turkey production chain. Among the top 10 serovars list, *S.* Thompson and *S.* Panama were related with 1,100 and with 706 cases, respectively (Table 1).

Table 1. Distribution of confirmed salmonellosis cases in humans by serovars (10 most common serovars, date 2011-2012), EFSA Journal, 2014.

2012			2011		
Serotype	N	%	Serotype	N	%
S. Enteritidis	34,019	41.3	*S.* Enteritidis	36,122	44.6
S. Typhimurium	18,248	22.1	*S.* Typhimurium	19,785	24.4
S. Typhimurium, monophasic 1,4,[5],12:i:-	5,932	7.2	*S.* Typhimurium, monophasic 1,4,[5],12:i:-	3,739	4.6
S. Infantis	2,021	2.5	*S.* Infantis	1,765	2.2
S. Stanley	1,128	1.4	*S.* Newport	813	1.0
S. Thompson	1,100	1.3	*S.* Derby	712	0.9
S. Newport	777	0.9	*S.* Kentucky	583	0.7
S. Derby	735	0.9	*S.* Poona	559	0.7
S. Panama	706	0.9	*S.* Stanley	526	0.6
S. Kentucky	651	0.8	*S.* Virchow	497	0.6
Other	17,092	20.7	Other	15,941	19.7
Total	**82,409**	**100**	**Total**	**81,042**	**100**

Source: 25 MSs and two non-MSs: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, and United Kingdom.

I. 2 .1 .4 Main sources

The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, which may result in a variety of foodstuffs of both animal and plant origin (Obukhovska, 2013). Studies reported that dairy products (Richter *et al.*, 2000), beef (Koohmaraie *et al.*, 2012), fish (Onyango *et al.*, 2009), pork (Meyer *et al.*, 2010), poultry meat and eggs (Fearnley *et al.*, 2011), fruits and vegetables (Gautam *et al.*, 2014) are vehicles for *Salmonella* transmission to humans. However, poultry meat and eggs are frequently considered to be the main vehicles for human infection (EFSA, 2014), due to the ability of *Salmonella* to proliferate in the gastrointestinal tract

of chickens (Ma *et al.*, 2014).

Cross-contamination in the kitchen can also be considered as a risk for *Salmonella* infection, where contamination occurs between raw chicken products to hands, kitchen utensils or other food. Poor sanitation practices, equipment design, and ingredient control are main factors involved with cross-contamination (Podolak *et al.*, 2010). A significant association were observed between *S.* Enteritidis infections and eating chicken outside the home and eating undercooked eggs inside the home (Marcus *et al.*, 2007).

In Spain, the largest outbreak of *Salmonella* infection was on 28 July 2005. Over 2000 cases of *Salmonella* gastroenteritis have been reported to the Centro Nacional de Epidemiología (National Centre for Epidemiology, CNE) in Spain. The outbreak was associated with consumption of pre-cooked, vacuum-packed roast chicken.

I. 2. 2. General aspects of *Campylobacter*

I. 2. 2. 1 Historical context

The first description of *Campylobacter* was in 1886. Theodor Escherish identified a spiral shaped bacteria in stool sample from neonates with diarrhoeal and fever by a filtration technique (Skirrow and Blaser, 1992) (Figure 3). *Campylobacter* were initially classified as a *Vibrio* spp. due to spiral morphology. However, Sebald and Véron in 1963 postulated it as new genus named *Campylobacter* (Sebald and Véron, 1963). The genus contains 18 species and 6 subspecies classified by comparison of 16S rRNA gene sequences. Among these, *C. jejuni subsp. jejuni*, *C. jejuni* subsp. doylei, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* are most commonly isolated from human and animal diarrheal specimens (EFSA, 2014). The *Campylobacter* genus belongs to the epsilon subdivision of the Proteobacteria classified based on characterization of 23S rRNA sequence (Vandamme *et al.*, 2005). Other members of this subdivision include Arcobacter, Helicobacter and Wolinella genera.

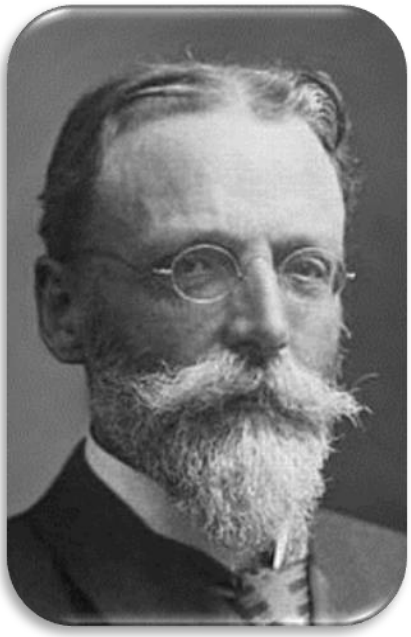


Figure 3. Theodor Escherish

I. 3. 2. 2 Taxonomy and characteristics

Campylobacter are Gram-negative curved or spiral shaped rods, 0.2 to 0.8 µm wide and 0.5 to 5µm length. The cells are highly motile with a polar flagellum at one or both ends of the bacterium. They are catalase and oxidase positive and urease test negative. Temperature range for growth is 30-44 °C (van Putten *et al.,* 2009), with an optimum temperature of 42 °C. In general, *Campylobacter* grow under micro-aerobic conditions with low levels of oxygen (3-15%) or microaerophilic environment. *Campylobacter* are sensitive to several environmental conditions like dry surfaces or osmotic stress and are generally less resistant to environmental stress than other foodborne pathogens such as *Salmonella* spp. (Silva *et al.,* 2011). *Campylobacter* is also sensitive to freezing and salinity conditions (Garénaux *et al.,* 2009).

Under unfavourable conditions, *Campylobacter* may form coccoid cells, which has been associated with a loss of culturability using traditional culture methods (Klančnik *et al.,* 2013). Rosenquist *et al.* (2006) related this coccoid form as a nonviable, degenerative form or a dormant state that is non-culturable with metabolically active and recoverable in suitable animal host (VBNC, Viable But Non-Culturable) (Figure 4).

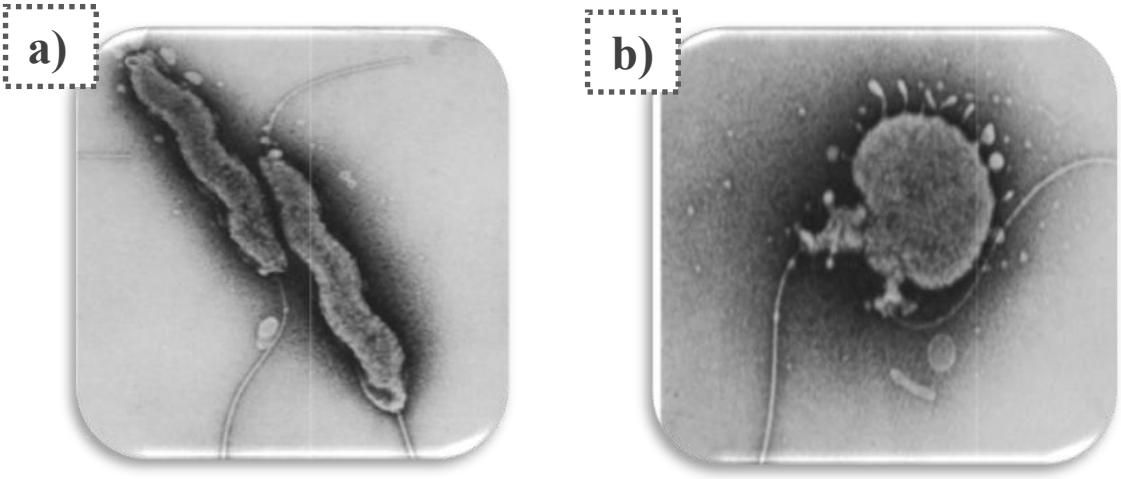


Figure 4. *Campylobacter jejuni* forms*: a)* spiral form. b) coccoid form. (Pead 1979).

I. 2. 2. 3 *E*pidemiology in humans

Human clinical aspects

Campylobacteriosis in humans is caused mainly by thermotolerant *Campylobacter* spp. Human infections are caused most commonly by *C. jejuni* followed by *C. coli* and *C. lari*. Other *Campylobacter* species, including the non-thermophilic *C.fetus*, are also known to occasionally cause human infection. The infective dose is generally low induced by 500-800 bacterium (Conlan *et al.*, 2011). Common symptoms of campylobacteriosis are acute gastroenteritis, cramping abdominal pain, fever vomiting and headaches (WHO, 2011). Diarrhoea occurs shortly after the onset of abdominal pain and varies from mild, non-inflammatory, watery symptoms to severe and bloody. The incubation period of *Campylobacter* is 3 days with a range of 18 hours to 8 days (Horn and Lake, 2013). Disease outcome mainly dependents on virulence of the infecting strain, however host response and host immune status are also related.

Campylobacteriosis is usually a self-limiting disease and antimicrobial treatment is not recommended for uncomplicated cases. In severe cases, macrolide antibiotic (erythromycin) or fluoroquinolones (ciprofloxacin) are the antimicrobial treatment (Mourand *et al.*, 2014). Patients continue to excrete *Campylobacter* in their feces for several weeks to months after recovery, although the infection has been treated with antibiotics. Mortality is low and usually related with elderly or suffering patients, which

present another disease (Zilbauer *et al.,* 2008).

Post-infectious complications of campylobacteriosis can occur, and Guillian Barré Syndrome (GBS) is the most commonly reported chronic sequelae (Zautner *et al.,* 2014). This complication is a demyelinating neuropathy (Rajabally *et al.,* 2014) and characterised by ascending paralysis (Zilbauer *et al.,* 2008). It is estimated that one on 1000 *Campylobacter* infections lead to GBS, leading to 2-3% fatal cases (Allos, 1997). The surface of the bacterium has lipooligosaccharides (LOS), which are important for the development of GBS. LOS stimulated the peripheral nerve gangliosides resulting in generation of autoreactive antibodies inflammation and tissue damage (Nyati and Nyati, 2013). Miller Fisher syndrome is a non-paralytic variant of GBS and causes inability to move the eyes and having non- reactive pupils (Mori *et al.,* 2012). Reactive arthritis is also associated with *Campylobacter* post-infection with 7 out of 100 cases (Ajene *et al.,* 2013). The reactive arthritis occurs mainly in joints, particularly knees and ankles (Ajene *et al.,* 2013). In more rare cases, *C. jejuni* has been associated with intestinal haemorrhage (Chamovitz *et al.,* 1983), toxic megacolon (McKinley *et al.,* 1980), haemolytic uraemic syndrome (Shulman and Moel, 1983) and bowel syndrome (Gradel *et al.,* 2009).

Campylobacteriosis in humans

According to several studies, *Campylobacter* is the most reported bacterial cause of human gastroenteritis in the industrialized world (Deogratias *et al.,* 2014; Duarte *et al.,* 2014; Sarkar *et al.,* 2014; Patrick *et al.,* 2013). The World Health Organization (WHO) estimated that around 1% of the European population is infected by *Campylobacter* each year. The European Surveillance System from 30 countries (27 EU Member States and three non-Member States) reported 214,268 confirmed cases of human *Campylobacter*iosis in EU in 2012 (Figure 5), with a decreased of 4.3% compared to 2011 (EFSA, 2014).

While the occurrence of salmonellosis appears to be receding, the number of *Campylobacter* infections has been increased from 2008 to 2012 (Hall *et al.,* 2005). This can be explained by various factors such as increased physician awareness, and improvement of the culture and detection methods at the laboratories (Friedman *et al.,*

2000). *Campylobacter* gastrointestinal illness rarely requires hospitalization, since the true incidence rate is likely substantially higher than reported cases (Friedman *et al.*, 2000). This is supported by data from England and Whales, where it was found that for each reported case there was nine others not reported cases (Wheeler *et al.*, 1999).

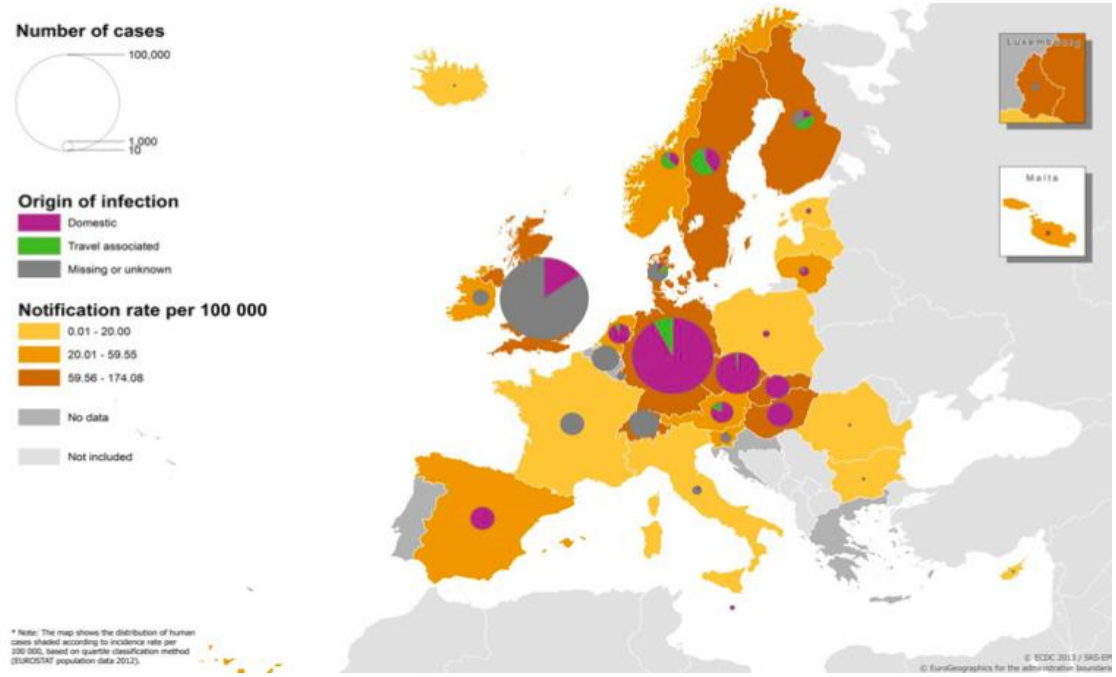


Figure 5. Campylobacteriosis notification rates and origin of infection in humans in the EU, 2012 (per population of 100,000; EFSA, 2014).

The mortality of *Campylobacter* infection in human is uncommon. In 2012, 31 deaths were reported by 14 MSs, with the United Kingdom accounting for 20 of these (EFSA, 2014). Children less than the age of one and young adults aged fifteen to twenty-five acquire more frequently the infection (Patrick *et al.*, 2013).

There was a clear seasonal trend in confirmed *Campylobacter*iosis cases during the summer months (EFSA, 2014). This could be explain because during this period there is a variation in human behaviour such as increased animal contact, eating barbecue meals, drinking or accidental ingestion of untreated water (Newell *et al.*, 2011). Moreover, during summer time, the fly population increases drastically being considered as a potential source of infection (Jonsson *et al.*, 2012)). The most commonly reported *Campylobacter* serovars in 2012 were *C. jejuni* representing 81.1% of confirmed cases reported in the EU.

I. 2. 4. 3 Main sources

There are numerous routes of *Campylobacter* infection including consumption of pig and cattle meat (Denis *et al.,* 2011), raw milk (Bianchini *et al.,* 2014) untreated water (Khan *et al.,* 2014) or rain water (Rechenburg and Kistemann, 2009). Additionally, traveling to foreign countries was associated with campylobacteriosis (Olson *et al.,* 2008).

Nevertheless, the avian species are the most common hosts for *Campylobacter* spp. due to their higher body temperature (Skirrow *et al.,* 2000). The intestinal tract of chicken, especially the cecum and colon, can harbour a large number of *Campylobacter* spp. During processing, the intestinal tract may escape or rupture and the contents are transferred to the skin of the carcass (Rosenquist *et al.,* 2006). *Campylobacter* spp. remain in a liquid film on the skin and become entrapped in its cervices and channels which provides a favourable environment for cross-contamination (Zbrun *et al.,* 2013). Persistence and survival of *Campylobacter* spp. is promoted by a suitable microenvironment of the skin (Chantarapanont *et al.,* 2003). Under frozen conditions or storage at 4°C, *Campylobacter* spp. are able to persist in the carcass (Eideh and Al-Qadiri, 2010). Most cases of infection are associated with handling, eating raw or undercooked poultry meat. Furthermore, cross-contamination can occur between raw contaminated meat and cooked foods (Hoelzl *et al.,* 2013).

A qualitative cross-contamination study showed that *Campylobacter* are easily transferred from raw chicken products to cutting boards, plates and especially to hands (Pouillot *et al.,* 2012). In addition, kitchen utensils such as stainless steel have an important role to cross-contamination (Kusumaningrum *et al.,* 2004).

The packaging can also be considered as a risk factor; in fact Harrison *et al.* (2001) demonstrated that 3% of the outer packaging from raw poultry products was by contaminated *Campylobacter*. Controlled atmosphere packaging can also improve the growth, increasing the risk for consumers if the contaminated chicken is not adequately stored or handled (Luber *et al.,* 2006).

I. 3. Foodborne pathogens in broiler meat production chain

I. 3.1 Primary Production

I. 3. 1. 1 Prevalence in broiler batches

Salmonella

Chickens are often unapparent carriers, which has contributed to hindering the diagnosis of *Salmonella* at farm level (EFSA, 2014). Nevertheless, *Salmonella* infection can be directly diagnosed at farm level or at the abattoir by isolating of the bacterium.

Young chickens (less than 2 weeks) are extremely susceptible to infection by *Salmonella* spp. (Gaffga *et al.*, 2012). When day-old-chick flocks are contaminated with the bacterium, there is a rapid spread of *Salmonella* throughout the house, and the rest of the birds ingest the bacteria, being infected in few days (Paião *et al.*, 2013). This behaviour may be explained because the enteric immune system does not fully mature until some weeks after hatching (Berghaus *et al.*, 2011). van Immerseel *et al.* (2004) reported that young chicks infected with *Salmonella* resulting in persistent excretion for at least 18 weeks of rearing period. In this study, faecal shedding of infected chickens, determined with cloacal swabs, was higher in the first weeks of rearing and then became negative a few weeks post infection. It has been supposed that birds are more resistant to *Salmonella* due to the presence of a more complex intestinal flora when the birds become older (Swaggerty *et al.*, 2014). Similar results were obtained by Beal *et al.* (2004), who reported that irrespective of age at exposure, *Salmonella* infection of young birds persists until between 10 and 12 weeks. It is unclear why the infection resulted in a persistent excretion of *Salmonella*, but this may be related to differences in development of the immunity after infection (Matulova *et al.*, 2013).

In 2013, the European Food Safety Authorities report the results of the evaluation of the impact of *Salmonella* control programmes in poultry production in 2011. Since 2009, all European Union Member States have been obliged to implement national control programmes for *Salmonella* in broiler flocks in accordance with

Regulation (EC) No 2160/2003. The Regulation developed a measures to prevent, detect and control *Salmonella* at all stages of production chain, in order to reduce *Salmonella* prevalence and the risk to public health.

The Regulation include the sampling of flocks within the three weeks before the birds are moved to the slaughterhouse, taking at least two pairs of boot/sock swabs per flock. The EU target for broiler flocks, referred to in Regulation (EC) No 160/2003, was set in Regulation (EC) No 646/2007 as a maximum percentage of broiler flocks remaining positive for *S.* Enteritidis and/or *S.* Typhimurium of 1% or less by 31 December 2011.

In 2011, the Community observed prevalence of *Salmonella*-positive flocks was 3.2%. 24 European Union Member States and three non European Union Member States met the target of 1 % or less of the broiler flocks positive for *S.* Enteritidis and/or *S.* Typhimurium. Three European Union Member States did not achieve the 2011 *Salmonella* reduction target, although it should be noted that two of them (the Czech Republic and Latvia) reported low prevalence (≤2.3%). Cyprus reported a higher prevalence (11.1%), but a small number of flocks were tested (Figure 6, EFSA, 2013).

Figure 6. *Salmonella* in broiler flocks of *Gallus gallus* before slaughter in countries running control programmes, 2011 (EFSA, 2013).

The reported prevalence of *S.* Enteritidis and *S.* Typhimurium in the EU has continued to decline from 0.7% in 2009 and 0.4% in 2010 to 0.3% in 2011. A decreasing trend in the reported prevalence has been observed in most European Union Member States.

In Spain, prevalence of *Salmonella*-positive flocks was 0.1% from target serotypes. The Regional Ministry of Environment and Rural and Marine Affairs of the Valencia Region reported a prevalence of 0.6% *Salmonella*-positive broiler flocks in 2013 from control programmes (MAGRAMA, 2013).

Campylobacter

Campylobacter species are rarely detected in commercial broiler flocks under the age of 2 - 3 weeks old, indicating that poultry flocks are free from *Campylobacter* at the beginning of the rearing period (Williams *et al.*, 2009). Several studies have shown that the presence of *Campylobacter*-specific maternal antibodies in young chicks or the presence of competitive microbiota in the intestinal tract especially in the cecum of these young chicks, may have an inhibitory effect on *Campylobacter* colonization during the first 2 weeks of life (Riazi *et al.*, 2013). The bacteria predominantly colonize the ceca, and reside in the intestinal mucous layer over the intestinal crypts of the villi (Beery *et al.*, 1988), and translocation to internal organs is commonly observed (Cox *et al.*, 2010). Once introduced, around 3 weeks, *Campylobacter* spread very quickly throughout the broiler house, probably via drinking water or feeders systems and coprophagic behaviour (Wassenaar, 2011) and the birds become colonized and the environmental sources are contaminated with *Campylobacter* spp. (van Gerwe *et al.*, 2009). The infected chickens are asymptomatic colonizers without any clinical signs or pathology lesions (Dhillon *et al.*, 2006).

The prevalence of *Campylobacter* in broiler flocks, batches or individual animals in 2010 were reported by the European Food Safety Authority in 2012. The Member States (21) and two non-Member States reported that the proportion of *Campylobacter*-positive broiler flocks varies among countries ranging from 0% to 92.9%. In two of three MSs reporting animal-based data, the prevalence was extremely high (>78%). In four of the Member states reporting flock/batch-based data, the

reported occurrences were extremely high (>72%), whereas low levels (<6%) were observed in Estonia, Finland and Norway. Denmark, Finland, Sweden, and Norway reported the highest number of broiler flocks tested (Table 2, EFSA, 2012). This variation may reflect, at least in part, differences in sampling and testing schemes, as well as to the impact of the season of sampling (EFSA, 2012). Furthermore, Nordic countries compared to the other European countries showed lower prevalence of *Campylobacter* colonized flocks. The reason for this is still unknown, however climatic conditions, the distance between farms, and less intensive rearing practices may influence the flock prevalence (Nadeau *et al*., 2002). Moreover, the poultry industry in the Nordic countries is more closely regulated than elsewhere in Europe (Ekdahl and Andersson, 2004).

In most cases, Member states reported the occurrence of *Campylobacter* in broilers or broiler flocks in 2010 at similar levels as in previous years. However, Spain reported a higher proportion of positive flocks in a national survey in 2010 (82.2%) than in 2009 (59.6 %) (Table 2, EFSA, 2012).

Table 2. *Campylobacter* in broilers 2008-2010, (EFSA, 2012).

Country	2010		2009		2008	
	N	% pos	N	% pos	N	% pos
Broilers (animal-based data)						
Czech Republic	-	-	-	-	422	69.9
France	196	78.1	191	80.6	-	-
Hungary[2]	439	66.5	713	78.0	325	54.2
Romania	51	100	104	100	-	-
Total animal-based (3 MSs in 2010)	**686**	**72.3**	**1,008**	**80.8**	**747**	**63.1**
Broilers (flock-based data)						
Austria[1]	394	46.7	326	55.5	-	-
Czech Republic[1]	134	72.4	-	-	422	61.1
Denmark[10]	3,132	16.5	4,591	29.4	4,912	25.9
Estonia[1]	47	0	48	0	-	-
Finland[1,6]	338	1.8	-	-	-	-
Finland[1,7]	1,409	6.0	1,720	4.8	1,276	6.5
Germany[2,4]	-	-	149	15.4	345	32.2
Germany[2,5]	-	-	332	10.2	-	-
Lithuania	-	-	-	-	374	42.0
Poland	-	-	-	-	420	79.0
Slovenia[1,8]	100	88.0	157	73.2	-	-
Slovenia[1,9]	99	92.9	149	83.9	-	-
Spain[1]	202	82.2	198	59.6	-	-
Sweden[1]	3,357	13.2	3,219	12.0	2,398	12.4
United Kingdom[1]	-	-	400	77.5	-	-
Total flock-based (8 MSs in 2010)	**9,212**	**18.2**	**11,289**	**24.1**	**10,147**	**24.7**
Norway[2,3]	2,170	5.1	1,924	6.1	4,675	4.1
Switzerland	400	33.0	442	44.3	-	-

I. 3. 1. 2 Risk factors in broiler farm

Many epidemiological studies have been undertaken to identify the most important risk factor associated with the presence of *Salmonella* and *Campylobacter* at the poultry farm.

Vertical transmission of *S.* Enteritidis and *S.* Typhimurium from the parent flock to day-old chicks has often been reported and implemented as a main controlling factor in eradication programmes (Zhang *et al.,* 2011). These strains can cause lifelong colonization of the peri-reproductive tissues of the hens from which the eggs can be colonized before the shell is formed (Liljebjelke *et al.,* 2005). Day-old chicks infected with *Salmonella* were reported as a major *Salmonella* contamination risk factor by several authors (Marin and Lainez, 2009; Le Bouquin *et al.,* 2010; Methner *et al.,* 2011). *Salmonella* can be transmitted vertically from infected breeders to the day-old chicks (Barua *et al.,* 2013) or transmitted horizontally during hatching, loading and transport to the farm (Webb *et al.*, 2014).

The theory of vertical transmission for *Campylobacter* has been a controversial issue. *Campylobacter* is present in reproductive organs and semen, which may lead to vertical transmission from the hen to the chick (Cox *et al.*, 2012). However, there is a delay of two to three weeks before the bird become colonized with *Campylobacter*. Sahin *et al.* (2003) suggest that small numbers of *Campylobacter* may be present in the hatching chick, but the grow is inhibited by maternal bodies. Furthermore, more sensitive molecular detection techniques than conventional culture with selective enrichment have detected bacterial DNA in the cecal contents of 18-days-old embryos and broilers less than three weeks old (Ridley *et al.* 2008). Despite these observations, there is no clear evidence that vertical transmission or horizontal hatchery transmission occur with *Campylobacter* (Callicott *et al.,* 2006).

Several authors have studied and identified the main risk factors involve to horizontal transmission of both pathogens and it seems to be the most likely mechanism by which *Campylobacter* is introduced into a flock, with rapid colonization of a flock (Cox *et al.,* 2012).

549 Inaccurate house cleaning and disinfection between consecutive flocks is related
550 as a risk factor for both pathogens (Marin and Lainez, 2009; Ellerbroek *et al.,* 2010
551 Chinivasagam *et al.,* 2010). Davies and Wray (1997) reported that *Salmonella* can
552 persist at the environment of the poultry building after cleaning and disinfection
553 procedures for at least 1 year in an empty farm. Cleaning is an essential stage for the
554 removal of organic and inorganic debris from the surfaces of the poultry farm. The
555 development of improper cleaning and disinfection measures such as over-dilution of
556 the product or inconsistent application of disinfectants resulting in an inadequate
557 management, which favour the infection between consecutive flocks (Cardoso *et al.,*
558 2008).
559
560 On the other hand, feed could also be an important source of *Salmonella*, since
561 feed remains in close contact with house environment leading to the proper environment
562 for *Salmonella* multiplication. Nevertheless, several authors have revealed that the
563 application of heat treatment (60-80 ºC) in pelleted poultry feed reduces the *Salmonella*
564 contamination rate of the feed (Andino *et al.,* 2014). Heyndrickx *et al.* (2002) also
565 reported a significant relation between the flock status and the high contamination level
566 of the feed from feeders of the chicken houses. In contrast, it is widely accepted that
567 feed is not a potential source of *Campylobacter* transmission to poultry. The dry
568 conditions of feed are considered lethal to *Campylobacter* (Zbrun *et al.,* 2013).
569
570 Furthermore, water is an important vehicle of enteric pathogens such as
571 *Campylobacter and Salmonella* (Bolton *et al.,* 2012; Ailes *et al.,* 2013). Different
572 authors described the importance of the spread of these bacteria at water pipes and water
573 drinkers (Cox and Pavic, 2010; Toth *et al.,* 2013; Bronowski *et al.,* 2014).
574
575 Farmer management is usually attributed as a factor of *Salmonella* and
576 *Campylobacter* persistence and recontamination of the houses (Fraser *et al.,* 2010). Wet
577 or dirty environment, poor bedding quality and dirty plumage may facilitate the growth
578 of the bacteria (Wei *et al.,* 2013; Torralbo *et al.,* 2014). Contaminated boots and
579 clothing are potential entering sources and allowed to recontaminate the broiler houses
580 by the farmers or outside visitors (Berghaus *et al.,* 2013). Additionally, farmers who
581 have a general sense of order and system are more successful in eliminating an
582 incoming these infections than the less-diligent farmers (Davis *et al.,* 2013; Torralbo *et*

583 *al.,* 2014). Likewise, thinning of the flock, which is reducing bird density within the
584 broiler house, is a common procedure in many European countries. During thinning, the
585 doors of the poultry house are opened and the catching crew and equipment enter to the
586 poultry house without any hygiene measures breaking biosecurity barriers and
587 favouring the introduction of the bacteria at poultry house (Newel *et al.,* 2011). In
588 addition, the stress that birds experience during thinning might assist the establishment
589 and spread of pathogens through the flock (Fraser *et al.,* 2010; Koolman *et al.,* 2014).
590
591 The flock size, number of houses located on the farm and the annual number of
592 productions within the house had no significant association with the risk of *Salmonella*
593 contamination (Ansari-Lari *et al.,* 2010). However, increasing farm size has been
594 considered as a *Campylobacter* risk factor on broiler farms (Lyngstad *et al.,* 2008).
595 Berntdson *et al.* (1996) found that the risk increased when the flock size was more than
596 25,000 birds. With every additional house there is an increased number of biosecurity
597 barrier passes. Additionally, the first house that becomes positive provides a massive
598 reservoir of infection via cross-contamination for all the remaining houses (Lyngstad *et*
599 *al.,* 2008).
600
601 The presence of farm pets was often considered as a key route for introducing
602 new pathogens (Silva *et al.,* 2012; Sarkar *et al.,* 2014). Several authors have reported
603 that the presence of rodents on farm can have a strong association with *Salmonella* or
604 *Campylobacter* positivity flock (Meerburg and Kijlstra *et al.,* 2007; Umali *et al.,* 2012).
605 Rodents can become *Salmonella* infected with at least 15 cells and may therefore be
606 responsible for amplifying environmental contamination and direct infection of poultry
607 (Umali *et al.,* 2012). Mice can also acquire the infection from inaccessible parts of the
608 house and then deposit contaminated droppings directly into feeding systems and onto
609 egg belts (Meerburg and Kijlstra *et al.,* 2007). Additionally, other common pests such as
610 flies can multiply in houses throughout the year and may be as an important vector of
611 *Salmonella* and *Campylobacter* transmission (Choo *et al.,* 2011, Marin *et al.,* 2011;
612 Sommer *et al.,* 2013). Rushton *et al.* (2009) reported that the natural ventilation is one
613 predictor of colonization by increasing the number of flies entering a poultry house as
614 forced ventilation might lead to higher mortality of flies. A further study (Hald *et al.,*
615 2008) estimated that 30,000 flies entered a house during one rearing cycle, thereby
616 constituting a high risk of transmission to poultry. However, in those countries with

high winter prevalence, the temperatures in winter would be too low to sustain fly populations and therefore the role of flies as a source in winter would be questionable. Wild birds, especially where there are large breeding populations with access to animal faecal waste, may also be reservoirs and vectors of these pathogens (Waldenström *et al.*, 2002). Furthermore, contaminated droppings from the wild birds colonized with both enteric pathogens can be brought into the house by footwear, clothing or material and resulting in infection of the broilers (Pennycott *et al.*, 2006).

A last risk that should be considered is season. Seasonality had been reported as an important risk factor for *Salmonella* and *Campylobacter* contamination (Nichols *et al.*, 2012). For *Salmonella*, broiler flocks that hatched in summer period showed a lower risk of infection, compared to chickens hatched in the early winter period (Ravel *et al.*, 2010; Varga *et al.*, 2013). This variation corresponds with seasonal patterns for *Salmonella* contamination in broilers, indicating a higher risk during wet and cold period (Varga *et al.*, 2013). In contrast, seasonal variation in *Campylobacter* prevalence in broilers has a peak during the summer (Jore *et al.*, 2010; Nichols *et al.*, 2012). It has been suggested that the *Campylobacter* seasonality could be related to the abundance of flies in the summer, which act as mechanical vectors (Hald *et al.*, 2008).

I. 3.2 Transport and slaughter of broiler flock

I. 3. 2. 1 Risk factors at transportation and before slaughter

At the age of approximately six weeks, broilers are loades in crates and transported to the slaughterhouse. During transportation, the animals are under high stress conditions such as crowding, motion, temperature fluctuations and feed and water deprivation (Mainali *et al.*, 2009). This stress causes a disturbance of intestinal functions and may lower the resistance of the live animal and increase spreading of intestinal bacteria (Klancnick *et al.*, 2013). Marin and Lainez (2009) reported that transport to the slaughterhouse increased the prevalence of *Salmonella* positive birds due to faecal contamination of skin and feathers by neighbouring infected birds during shipping. The same hypothesis is reported for Altekruse *et al.* (1999) showing 1,000-fold increase of *Campylobacter* counts during transportation. The inadequate cleaning

and disinfection of the crates that transport the animals may also introduce *Salmonella* and *Campylobacter* into the free flocks (Ridley *et al.,* 2011; Näther *et al.,* 2009). A survey of over 10,000 poultry companies of varying sizes conducted by Auburn University discovered that 80% of poultry growers do not sanitize their crates and only 18.3% sanitize their trucks and trailers properly (Fielding, 2012). One reason for the persistent of these bacteria at the crates after cleaning and disinfection is the remaining organic matter detected regularly on truck crates after washing which reduce the efficacy of the process (O'Mahony *et al.,* 2011). Schroeder *et al.* (2014) reported the concentration on these pathogens at the surface of the bird is related to the levels on fully processed carcasses. Therefore, bring down the farm prevalence of these pathogens and stress during transport are important strategies to lower the risk of contaminated meat products entering the food chain (McCrea *et al.,* 2006; O'Mahony *et al.,* 2011).

I.3.2.2 Prevalence on broiler carcasses

In 2011, the European Food Safety Authorities reported the results of the estimation the prevalence of *Salmonella* and *Campylobacter*-contaminated broiler carcasses, at Community level and for each European Union Member States in 2008. The survey took place in Europe between January and December 2008 and was conducted at broiler-batch level in slaughterhouses, focusing on birds entering the food chain. The estimation of the prevalence of *Salmonella* and *Campylobacter*-contaminated broiler carcasses, at country-specific level, was the proportion of carcasses contaminated with these pathogens out of the total number of carcasses examined, accounting for slaughterhouse clustering. The total number of sampled broiler batches in the EU was 9,249 and 9,324 for *Salmonella* and *Campylobacter* detection on carcass samples, respectively.

Salmonella

In 2008, the Community studied the prevalence of *Salmonella*-contaminated broiler carcasses at EU level, in each member state and in both non-Member States, the results are presented in in figure 7. The EU prevalence was 15.6%. *S.* Enteritidis and/or *S.* Typhimurium were detected on broiler carcasses in 17 Member States and in one

non- Member States. The EU prevalence was 3.6%. Other serovars than *S.* Enteritidis and/or *S.* Typhimurium were also detected on broiler carcasses in 21 Member States and in one non- Member States. The EU prevalence was 11.2%.

In Spain, the prevalence of *Salmonella*-contaminated broiler carcasses was 15.6% of the total of 389 carcasses analysed. The prevalence of *S.* Enteritidis and/or *S.* Typhimurium was 3.6%.

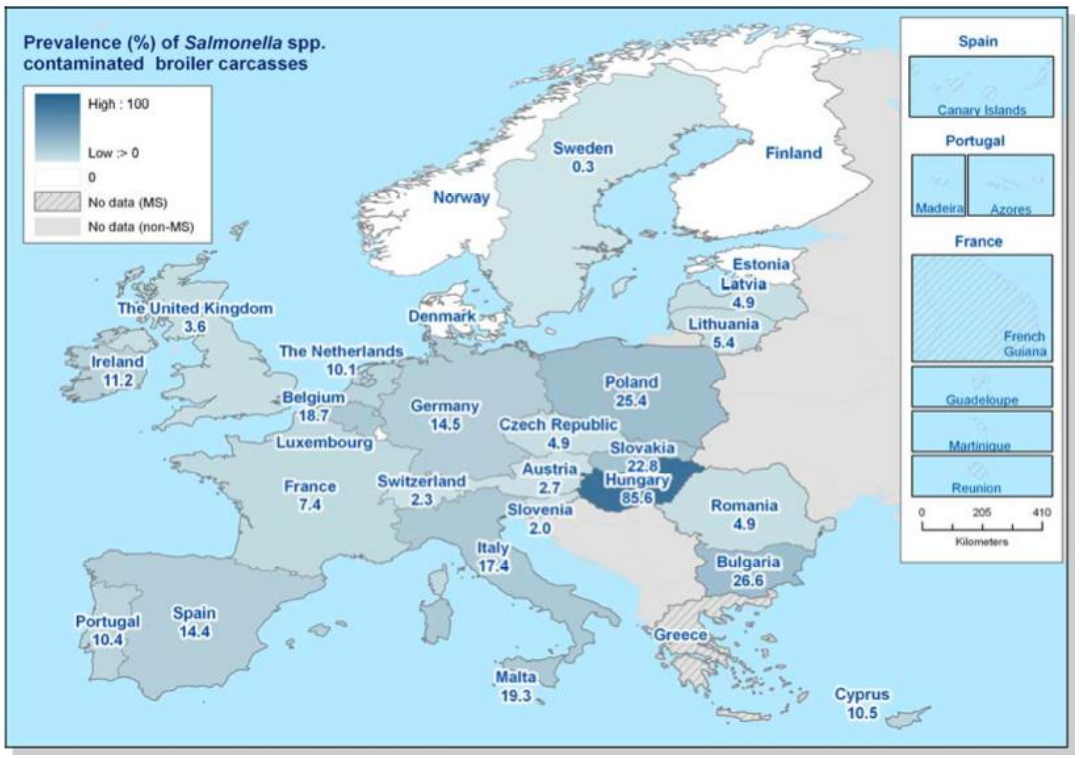


Figure 7. Prevalence of *Salmonella*-contaminated broiler carcasses in the EU, 2008, (EFSA 2011).

Campylobacter

The prevalence of *Campylobacter*-contaminated broiler carcasses at EU level, in each member state and in both non-Member States is presented in figure 8.

Campylobacter was detected on broiler carcasses in all participating European Union Member States and both non-Member States. The EU prevalence was 75.8%. *C. jejuni* was detected on broiler carcasses in all participating Member States and both non-Member States. *C. coli* was detected on broiler carcasses in most European Union Member States with the exception of Estonia, Finland and Sweden and of the non-Member States, Norway. *C. jejuni* was the most commonly reported species in 19

Member States and two non-Member States with up to 100% of this species identified among isolates in Estonia, Finland, Sweden and Norway. In contrast, *C. coli* was the most commonly isolated species in broiler batches in seven Member States (Bulgaria, Hungary, Italy, Luxembourg, Malta, Portugal and Spain).

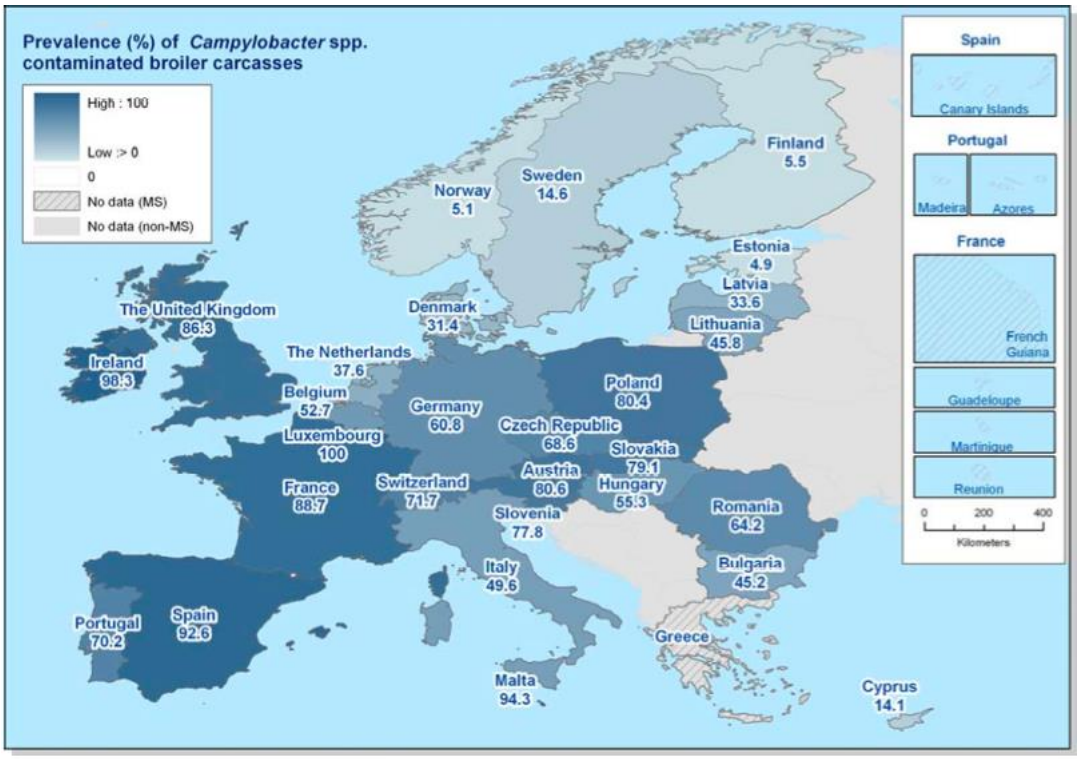


Figure 8. Prevalence of *Campylobacter*-contaminated broiler carcasses in the EU, 2008, EFSA 2011).

I.3.2.3 Risk factors during slaughter, dressing and processing

Broilers are slaughtered at an age of 35 to 40 days. Birds are delivered to the slaughterhouse in transport containers. Poultry slaughter and dressing may involve different technologies, dependent mainly on the commercial strategy of the company. In general, live birds are manually hanged by their legs on a moving processing line. They are stunned by electrical shock and killed by exsanguination. An alternative is to stun the broilers by CO_2 before hanging the birds on the slaughter line. During scalding, the feathers are loosened by submerging the carcasses in a water bath at a temperature of 54-56 ºC. The feathers are subsequently removed on a plucking machine by series of rotating discs, each with several rubber fingers. The head of the bird and the feet are removed before the carcasses are hung over on a second moving processing line. Then, different mechanical procedures by clamps remove crop, neck and internal organs of the carcasses. Along the line, water is used to wash both the carcasses and the equipment.

The most important washing point is immediately prior to chilling when the carcasses are washed inside and outside. Finally, carcasses are chilled by immersion in cold water, or most commonly in the EU in air chilling at 2 ºC for three hours. Water chilling is mainly used for frozen products while air chilling is used for refrigerated products. Cutting and packaging of broiler meat is also highly automated. Most of the broiler meat is sold as fresh product and about 80% of the products are marinated and packaged in a modified atmosphere (Björkroth *et al.*, 2005).

At the processing plant, it is widely acknowledged that contamination of the poultry carcasses and equipment with *Salmonella* and *Campylobacter* occurs during the slaughter process (Svobodová *et al.*, 2012; Zbrun *et al.*, 2013; Lehner *et al.*, 2014). Implementation of Hazard Analysis and Critical Control Points programmes (HACCP) to reduce contamination of carcasses with *Salmonella* is applied in EU, which separated the process of positive and negative poultry flock in order to prevent cross-contamination at slaughter (EFSA, 2014). At the present, no such control measure is implemented for *Campylobacter*.

Taking into consideration that after stun of the birds the anus sphincter is relaxed and massive contamination may occur with microorganisms from the digestive tract, including pathogens such as *Salmonella* spp. and *Campylobacter* spp. Therefore, it represents a high risk of contamination from the hygiene quality. It has been suggested that there are many stages during the poultry slaughtering process where contamination of both enteric pathogens can occur. During the slaughter process, any event but more particularly the stages of scalding, defeathering and evisceration, can lead to contamination of *Salmonella* (El-Alziz, 2013; Chokboonmongkol *et al.*, 2013). *Campylobacter* are present on the carcasses throughout the whole slaughter process, but the levels may decrease during scalding, chilling and freezing and may increase during defeathering and evisceration (Rejab *et al.*, 2012; Franz *et al.*, 2012; Sasaki *et al.*, 2013).

The scalding process is a major site of cross-contamination for *Salmonella*, but is less important in this respect for *Campylobacter*. Scalding water is shown to contaminate the surface of carcasses even if scalding reduces the total number of bacteria on the skin (Reiter *et al.*, 2007; Bily *et al.*, 2010; Lawes *et al.*, 2012).

Carramiñana *et al.* (2007) reported that immersion scalding has been shown to increase the level of *Salmonella* contamination in cases where the operating conditions are poor. This was probably caused by an accumulation of dirt and faeces in the scald water due to an inadequate flow of fresh water into the tank, making the scald tank a source of cross-contamination for subsequent carcasses (Li *et al.,* 2013; Choi *et al.,* 2014). Adams *et al.* (2013) explained that *Salmonella* could survive in scald water because of likely protection by faecal particles, feathers or even by carcass temperature, which does not reach the temperature of scalding water itself. Although, scalding reduces the numbers of *Campylobacter* present of the exterior of the birds, the survival of the bacterium in this stage, has also been reported (Rahimi *et al.,* 2010).

De-feathering stage is also considered for several authors an important site of *Salmonella* and *Campylobacter* contamination (Hue *et al.,* 2011; Goddard *et al.,* 2014; Duffy *et al.,* 2014). During de-feathering, the rubber fingers applied in the stage exert pressure on the carcasses, forcing potential contaminated fecal material out and spreading it on the carcasses and the slaughter equipment (Duffy *et al.,* 2014). Furthermore, the surface of the fingers becomes roughened with increasing use. This situation favours that the bacteria colonise crevices on the surface of the rubber fingers and multiply overnight if not disinfected properly. Then, during next de-feathering, bacteria are transferred from the rubber fingers to the carcasses producing cross-contamination between different flocks (Ellerbroek *et al.,* 2010).

Several authors have reveal that evisceration process lead to the considerably increase of cross-contamination of *Salmonella* and *Campylobacter*, especially since a lot of the times the perforation of the intestines cannot be avoided (Reiter *et al.,* 2007; Santos *et al.,* 2011; Elvers *et al.,* 2011). Ivanova *et al.* (2014) reported that cut and torn viscera encourage the contamination of both enteric pathogens onto exterior and interior of the carcasses.

Processing facilities can use two different methods to chill carcasses to reduce carcass temperature: immersion chilling or air chilling. Giombelli *et al.* (2013) reported immersion chilled carcasses were found to have significantly lower of *Salmonella* numbers per millilitre than air chilled carcasses. Some immersion-chill tanks use sanitizers such as chlorine (50 ppm maximum) to reduce other contaminants such as

blood and tissue fragments (Guerin *et al.*, 2010). The use of chlorine in the chill tank reduced *Campylobacter* numbers, but does not completely eliminate the bacterium (Berrang *et al.*, 2007). A study performed in Denmark has revealed that the air and water-chilling can lead to reductions of 0.8–1.0 $\log_{10}$cfu/g of *Campylobacter* concentration.

Finally, cross-contamination along the production line is an important risk factor that should be taken into consideration for both pathogens. The carcasses come into narrow contact with surfaces of the equipment that may develop residual tissue debris, which contain *Salmonella* and *Campylobacter* leading a contamination of the subsequent carcasses (Rasschaert *et al.*, 2008; Guerin *et al.*, 2010; Kudirkienė *et al.*, 2011). Furthermore, potential cross-contamination between carcasses occurs also when the external surface of the birds comes into contact with other carcasses, hands of the personnel, and trimming mesh gloves and knives (de Perio *et al.*, 2013). Once employees in the slaughter facility come into contact with livestock, they become the vehicle for the spread of both pathogens. Ellerbroek *et al.* (2010) also studied that processing equipment and workers were a source of cross contamination, reporting that staff's hands, slaughtering equipment, and transport boxes were contaminated by *Campylobacter*. Many authors have also shown a cross- contamination between batches from different flocks and the contamination of non-infected batches from previous slaughtered batches. Gloaguen *et al.* (2010) shown that *Campylobacter*-positive batches especially contaminate the first carcasses of subsequent negative batches. Rasschaert *et al.* (2008) demonstrated cross-contamination by *Salmonella* from flocks to the slaughter line and from the slaughter line to flocks, which were processed a few days later. Furthermore, the excessive use of water during the slaughter produces a lot of aerosols and droplets in the hanging, defeathering and evisceration stages which may also be a potential source of the cross-contamination (Peyrat *et al.*, 2008).

I.3.2.4 Methods and approach to reduce *Salmonella* and *Campylobacter* growth in

the raw chicken meat

In chicken processing companies, food safety management systems rely on a farm to fork approach and involve a range of good hygienic and manufacturing practices actions at each step of the food chain. In particular, under EU legislation, Good Hygiene Practice (GHP) and Hazard analysis and critical control points (HACCP) principles must be applied throughout the entire food chain as laid down by Regulation (EC) No 852/20044. There are different methods such as modified atmosphere packaging (Rajkovic *et al.*, 2010), high hydrostatic pressure treatment (Tananuwong *et al.*, 2012) and irradiation (Keklik *et al.*, 2012) among others methods, which could be applied to provide the required level of safety, and reduce of microorganisms that have contaminated the chicken meat. However, these antimicrobial technologies should be considered as supplementary methods of reducing microbial loads of foods of animal origin and should be part of an integrated program throughout the food chain.

Modified atmosphere packaging (MAP)

Over the last years, modified atmosphere packaging (MAP) has received increasing attention as a method of food preservation. MAP was originally created for extension of meat chicken shelf life by control spoilage microorganism (Fraqueza and Barreto, 2009). However, actually this method has been also developed for control of foodborne pathogenic bacteria (Melero *et al.*, 2012). This technique is invariably applied with refrigerated storage to improve quality and safety of meat and meat products.

MAP has been defined by Parry (1993) as the "enclosure of food products in high gas barrier materials, in which the gaseous environment has been changed or modified to slow respiration rates, reduce microbiological growth and retard enzymatic spoilage, with the intent of extending shelf life".

The gases most commonly used in chicken meat MAP are oxygen, carbon dioxide and nitrogen. These gases can be applied individually or in combination in order to obtain the cumulative effect of these gases, which have different purposes in food preservation (Mantilla *et al.*, 2012). However, trace gases, such as carbon monoxide,

nitrous oxide, sulphur dioxide, and argon, are commented as possible gases for MAP in meat (Kudra *et al.*, 2013; Herbert *et al.*, 2013).

Oxygen (O_2):

O_2 is important in the storage of fresh chicken meats as it maintains the meat pigment myoglobin in its oxygenated form, oxymyoglobin, which keeps the bright red colour of fresh meat, which consumers prefer (Vukasovič, 2014). Deterioration of colour is not a problem for most fresh poultry products, although a greying of poultry meat has been noted (Vukasovič, 2014). In general, O_2 promotes the growth of aerobic bacteria and inhibits the growth of facultative anaerobic or anaerobic bacteria. McMillin (2008) shown that, on meat and poultry products, *Salmonella* growth was effectively retarded by MAP with high and low O_2 concentration. Boysen *et al.* (2007) reported reductions in *Campylobacter* numbers of 2.0-2.6 $\log_{10}$cfu/g after eight days in atmosphere with a high percentage of O_2. Exposed to O_2, *C. jejuni* cells became slightly elongated and less coiled and they seem to lose their motility (Rajkovic *et al.*, 2010). In addition, loss of spiral morphology has been reported to be one of the stages before coccoid formation, which, among others, has been associated with oxidative stress and limited nutrients (Ikeda and Karlyshev, 2012).

Carbon dioxide (CO_2):

CO_2 has a strong bacteriostatic effect on aerobic microorganism and in some pathogens such as *S. enteritidis, C. botulinum* and *L. monocytogenes* (Shin *et al.*, 2010; Provincial *et al.*, 2013). It is soluble in both water and lipids. Devlieghere *et al.* (2001) have proposed the mechanisms of action attributed to CO_2, which are; lowering pH of the food, cellular penetration followed by decrease of cytoplasmic pH of the cell, specific actions on cytoplasmatic enzymes and specific actions on biological membranes.

The effect of CO_2 in MAP is dependent upon the dissolution of the gas into the packaged product, which has a number of consequences. The excessive absorption of CO_2 can cause pack collapse with some high moisture foods, such as meat, poultry and seafood and it can also cause increased drip in fresh meat, fluid release in ham, product

separation in cream, physiological damage to fruit and vegetables, and a sherbet-like taint in fatty fish (Kerry, 2012).

The bactericidal and bacteriostatic effects of CO_2 are temperature dependent. During storage at low temperature, CO_2 in MAP is capable of inhibiting some microorganisms; however, the lack of refrigeration at any time could allow or stimulate the growth of such organisms (Hotchkiss *et al.,* 2006). This situation should be concerned for some mesophilic pathogens, such as *Salmonella*, which cannot grow in modified atmospheres at refrigerated temperatures. However, when a temperature abuse occurs in the commercial chain of food handling, it may constitute a risk (Wen, 2010). In experiments performed on chicken breast having different pH (breast-low pH and thigh-high pH) inoculated with *S.* Enteritidis and stored in several atmospheres (vacuum, 100% CO_2, 100% N_2 and 20% / 80% CO_2/air), this bacterium survived, but did not grow at 3 °C (García de Fernando *et al.,* 1995). Nevertheless, at 10 °C, the numbers of *S.* Enteritidis increased rapidly in samples flushed with 100% nitrogen or with 20% / 80% CO_2, /air and to a lesser extent in vacuum-packaged samples.

In relation to the effect of CO_2 against *Campylobacter*, there are different authors who have reported that CO_2 has a protective effect with this bacterium, promoting it survival and protecting it from the negative effect of oxygen. (Rajkovic *et al.,* 2010; Byrd *et al.,* 2011; Meredith *et al.,* 2014).

Nitrogen (N_2):

N_2 is normally used to displace the O_2 in the packs and storage vessels to delay oxidative rancidity and inhibit the growth of aerobic microorganisms (McMillin, 2008). Because of its low solubility in water and lipids and its lack of taste, it also acts as a filler gas in MAP products to prevent the collapse of the pack containing high concentrations of CO_2.

Argon (Ar)

Argon is a noble gas, which is tasteless, odourless and more soluble in water and oil than nitrogen. It has been suggested that Ar can effectively inhibit enzymatic

activities, microbial growth and chemical spoilage reactions in perishable food (Herbert *et al.*, 2013). The polarizability and the higher ionization potential of Ar affect leading of gases to active sites, penetration of gases through membranes and developing biological reaction potentials (Morgan, 2007).

A study performed by Fraqueza and Barreto (2009) found that argon helped extend shelf life of uncooked turkey meat through control of oxidative reactions and microbial inhibition too. At the end of the 25 days of storage, turkey meat in the Ar-CO atmosphere had one log lower growth for psychrotrophic, total anaerobic counts. However, Tomankova *et al.* (2012) conflicts with the results of Fraqueza and Barreto (2009). Atmosphere of 70% O_2 and 30% CO_2 were compared with 70% Ar and 30% CO_2 for packaging poultry meat. The samples packaged with Ar had higher microbial content compared to O_2 atmosphere. Overall, it appears that there is not strong evidence in support of argon reducing microbial production. For this reason it is necessary to develop new studies to clarify this goal.

Irradiation

Irradiation has proven to be effective in eliminating bacteria from the surface of meat poultry. In the European Union (EU) food irradiation is regulated by Directive 1999/2/EC and Directive 1999/3/EC. The later Directive is the initial, and it is not yet completed positive list of food authorised for radiation treatment in the whole EU. Until the completion of this initial positive list, Member States may maintain national authorisations for irradiation food. In Spain, only dried aromatic herbs, spices and vegetable seasonings are authorized to irradiation treatment, with a maximum dose of 10 kGy. However, irradiation of other foodstuffs including poultry meat is temporarily permitted in some Member States like Czech Republic and The Netherlands, which are working with a maximum dose of 7 kGy for this product. In the United States food manufacturers are currently permitted to irradiate raw meat and poultry using gamma rays, X-rays, or electron beams. The maximum dose permitted is 3kGy and is sufficient to inactivate at least 99.9% of common food borne pathogens such as *Salmonella* and *Campylobacter* (Haughton *et al.*, 2011).

UV irradiation is believed to kill cells by causing mutations in DNA by cross-linkage of pyrimidines and cell death occurs when the threshold level of cross-linkages is exceeded (Yaun *et al.,* 2003). Sumner *et al.* (1996) demonstrated that UV irradiation is effective at reducing *Salmonella* on poultry carcasses up to 7 logs. Chun *et al.* (2009) investigated the applicability of UV-C irradiation on the inactivation of *C. jejuni* in ready-to-eat meat and poultry meat respectively. The results have clearly indicated that UV-C irradiation effectively decreased *C. jejuni* inoculated on meat during storage.

A major limitation disadvantage in the European Union of irradiation is that at present the use of irradiation for meat is strongly discouraged and it is not readily embraced by consumers due to potential health risks (Gyenis *et al.,* 2014). Consumers can reduce the risk of food borne illness by purchasing irradiated meat, but many are concerned about potential health risks (O'Bryan *et al.,* 2008).

High hydrostatic pressure

High pressure can damage bacterial cells through physical destruction of cells membranes and cause intracellular injury of cell components such as ATP, proteins and RNA. Additionally, the quality of the chicken meat is also affecting by high pressure by increasing water holding capacity, tenderizing, decreasing redness and inducing lipid oxidation (Tananuwong *et al.,* 2012). The limitations of this technology are the high cost of equipment, metal fatigue, long cycle times, and undesirable sensory changes in product (Tananuwong *et al.,* 2012).

Regarding the effectiveness in the control of the *Salmonella* and *Campylobacter*, treatment at 400 MPa for 2 min and 20 °C, resulted in an inactivation between 3.26 and 4.35 log of *Salmonella* in a chicken meat model system (Escriu and Mor-Mur, 2009). Furthermore, Morales *et al.* (2009) obtained a maximum of 4.21-$\log_{10}$ reductions in *S.* Enteritidis after 20- min high-pressure treatment of chicken breast fillets at 300 MPa and 12 ºC.

I. 4. Detection, identification and characterization

I.4.1 *Salmonella*

Bacteriological isolation and identification

Bacteriological isolation and subsequent confirmation by an appropriate biochemical and serological test are the traditional methods used in salmonellosis diagnostic (Mousing *et al.*, 1997). The bacteriological isolation of *Salmonella* requires four stages:

Pre-enrichment in non-selective liquid medium

Salmonella spp. has simple nutrient requirements. However, samples that may contain only low numbers of the bacteria, such as food and environmental samples usually include a pre-enrichment step, e.g. Buffered Peptone Water (van de Giessen, 1996).

Enrichment

This is a critical point in bacteria isolation, since the selective medium is involved in competitive flora elimination and allows *Salmonella* proliferation. The enrichment in selective medium demanded by ISO 6579:2002 (Annex D) is the Modified Semi-Solid Rappaport Vassiliadis (MSRV). This medium allows the motile *Salmonella*s to spread through the medium plate and be identified from other non-motile bacteria.

Plating-out and identification

These media are characterized by their "selectivity" and "differentiation". Selectivity means the addition of an inhibitory substance that prevents the growth of different Enterobacteria. Differentiation means the addition of a substance that allows us to characterize *Salmonella* against other bacteria (Mallison *et al.*, 2000). The main characters used in *Salmonella* identification are the production of

sulphydric acid and the inability to ferment glucose.

Confirmation

Colonies of presumptive *Salmonella* are subcultured then plated-out and their identity is confirmed by means of an appropriate biochemical and serological test (ISO 6579:2002). The most common biochemical test used to confirm presumptive *Salmonella* is the API-E20.

Phenotyping methods

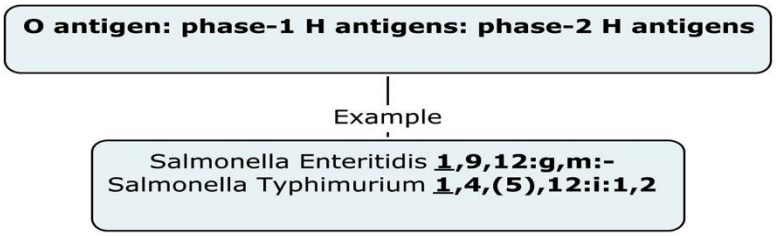
Serotyping

The members of the genus *Salmonella* are typed into serovars, which are differentiated from each other by the combinations of their somatic (O) and flagella (H) antigens and, to a lesser extent, by their biochemical reactions (Popoff and LeMinor, 1992; Popoff *et al.*, 1992).

The O antigen is part of the lipopolysaccharide component of the cell wall that also contains lipid A and a core portion. The O antigen or O-specific side chain consists of repetitive oligosaccharide units of which the type, order and repetition of sugar moieties differ between serovars. These differences and those in the flagella antigens are used to type *Salmonella* into serovars. At least 67 different O antigens are currently known and identified by the Arabic numerals from 1 to 67. Some of these occur singly (e.g. 11), whereas others occur in combination (e.g. 1, 4, 5, 12; and 6, 7).

The H antigens are heat-labile and form an integral part of the flagella in those serovars, which possess them. The antigens are designated by a combination of letters of the alphabet and numerals (e.g. a to z, z1 to z32 and 1 to 7). Two antigenic forms of the flagella may occur in culture. Therefore, a culture may contain cells in which the flagella are all in the same phase, or cells, which possess flagella of both phases. Most of the serovars contain flagella of two phases, but in some (e.g. *S.* Dublin) the flagella occur in only one phase.

In order to establish the complete antigenic composition of any *Salmonella* serovar, antigens of both flagella phases must be known, as well as the O antigens (Le Minor, 1984). This technique is perform by testing suspensions of the bacteria against antisera produced in rabbits against individual O and H antigens by means of a series of slide agglutination tests (Edwing, 1986). When a bacterial culture is mixed with a specific antiserum directed against bacterial surface components, the cells are bound together through antigen-antibody bonds to form aggregates (agglutination). This is usually visible to the naked eye as clumps in the suspension. By mixing specific antisera with a *Salmonella* culture, the O- and H antigens are determined. On the basis of the observed agglutination pattern, the serotype is determined using the Kauffmann-White Scheme.



Phage typing

Bacteriophage typing schemes for a number of important *Salmonella* serovars have been developed and are used internationally for epidemiological studies. The available information on the distribution of *Salmonella* serovar and phage types along the food chain varies greatly between countries. Phage typing of *S.* Enteritidis and *S.* Typhimurium is predominantly carried out according to the Colindale scheme.

In accordance with the standardized protocol, eighteen hours cultures on Blood agar plates were inoculated into 3 mL of a phage broth (double concentration nutrient broth with 0.85 % NaCl). After 1.5 h incubation with vigorous shaking, the broth was poured onto phage agar plates. After the removal of excess broth from the plates, 10 typing phages were spotted per plate using a micropipette. Dried plates were incubated overnight at 37º C, and the phage lysis pattern of each culture was compared with a published pattern list (Kim *et al.*, 2008).

Molecular techniques

Traditional methods for *Salmonella* isolation, confirmation and serotyping are too laborious and time consuming (van de Giessen, 1996). In recent years, several advances in diagnostic technology have been developed and mean an important advance in infectious disease studies. These molecular techniques are based on the Polymerase Chain Reaction and are characterized by being simple, rapid and discriminative (Fernandez-Cuenca, 2004).

Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) name derives from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified (Feder *et al.*, 2001). The use of PCR allows to amplify a single piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations.

Repetitive Sequence PCR-Based Microbial Typing

The repetitive extragenic palindromic REP sequences consist of 30-35 conserved bases and an extremely conserved inverted repeat (Stern *et al.*, 1995). An inverted repeat refers to close by sequences in DNA, which are the identical or nearly identical when read in the 5′ to 3′ direction on the opposite strands. *Escherichia coli* had 563 REP sequences in 295 clusters present throughout its genome (Lupski and Weinstock, 1992). A high degree of conservation is present among different bacterial species. These sequences could have a role in genome evolution (Gilson *et al.*, 1984). In *E .coli*, these palindromic units are present for the most part in extragenic areas either individually or in clusters (Gilson *et al.*, 1984). Additionally, REP sequences can be present in up to four tandem copies, which are always inverted. Repetitive extragenic palindromic PCR was optimized for subtyping *Salmonella* spp. from the different sources like chicken, mice, wild birds, a fly, pre- and post-transport crates, humans,

ducks, hogs, production facility, a turkey, and a calf (Franz *et al.*, 2012). The repetitive extragenic palindromic PCR separated the isolates "into spatially and temporally epidemiological relevant groups" (Hiett *et al.*, 2002).

The Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences are a second group of conserved repetitive DNA and can be found in several copies throughout many enterobacterial genomes (Hulton *et al.*, 1991). The ERIC consensus sequence is 126 bp long. These ERIC sequences have been found in *E. coli*, *S.* Typhimurium, *Yersinia pseudotuberculosis*, *Klebsiella pneumoniae*, and *Vibrio cholarae* (Hulton *et al.*, 1991). Every one of the ERIC sequences contains the same central core inverted repeat and is situated external to an open reading frame. The position of ERIC sequences varies among species. In *E. coli* and *S. typhimurium*, ERIC copy numbers have been proposed to be about 30 and 150, respectively (Hulton *et al.*, 1991).

Sequences for REP and ERIC have been found in genomes of several prokaryotes (Versalovic *et al.*, 1991). Bacterial genomic fingerprinting has been carried out by using PCR and consensus primers (outwardly directed) that specifically anneal to highly conserved REP and ERIC sequences (Versalovic *et al.*, 1994). PCR amplification of DNA between consensus repetitive element primers repetitive elements generates amplicons that differ in base pair length. Species-specific REP amplification patterns have been observed. There are REP and ERIC primer sets used in PCR, which have been validated to yield clear-cut species- and strain-specific genomic fingerprints (Versalovic *et al.*, 1994). This study by Versalovic *et al.* (1994) showed that REP- and ERIC-like sequences were found mainly in Gram-negative enteric and their close relatives in the same phyla. Versalovic *et al.* (1994) provided the first demonstration of ERIC-like sequences in eubacteria through techniques other than computer-assisted DNA sequence analysis. In addition, that study provided "the first documented use of extragenic repetitive sequences to directly fingerprint bacterial genomes". REP- and ERIC-PCR yield distinctive results among different bacterial species and strains that have these particular repetitive elements.

Genotyping methods

Pulse Field Gel Electrophoresis (PFGE)

Pulse Field Gel Electrophoresis (PFGE) is the gold standard for molecular typing of *Salmonella* (Tenover *et al.*, 1995). PFGE subtyping has been successfully applied to the subtyping of many pathogenic bacteria to establish the degree of genetic relatedness between isolates of the same species or serotype (Liebana, 2002). This methodology has been valuable in tracking sources of outbreaks in epidemiological studies. PFGE has been repeatedly shown to be more discriminating than other methods such as ribotyping for many bacteria (Olive and Bean, 1999).

Restriction enzymes that recognize few sites in the chromosome are used to generate large DNA fragments (Molbak *et al.*, 2006). These fragments are then separated by constantly changing the direction of the electrical field during electrophoresis (CDC, 2014). PFGE can separate DNA of different sizes using a determined switch time, which represents the duration of the alternating electric fields. There is a maximum size range related to each switch interval that does not allow further resolution (Birren and Lai, 1993).

Enzymes used for fingerprinting are chosen based on the length of recognition sequence of the enzyme and the GC content. For *Salmonella* fingerprinting XbaI (5′...T˅CTAGA...3′) is the enzyme of choice. When isolates require further characterization, BlnI nuclease enzyme is also considered (Levin, 2010). PFGE using these two enzymes have provided good discriminatory power to identify sources of contamination (Levin, 2010).

Kaldhone *et al.* (2008) applied PFGE to characterize turkey isolates collected from different sources. To fully evaluate the isolates, XbaI and BlnI were used and 55 different patterns were identified from 180 isolates. The authors emphasized the importance of using a combination of enzymes to distinguish among closely related serovars (Kadhone *et al.*, 2008).

PFGE with XbaI was useful to determine relatedness and genotypic changes of historic (1988-1995) and contemporary (1999-2001) isolates of *Salmonella* Newport (Berge, *et al.,* 2004). The same methodology was followed to identify the genomic DNA fingerprint profiles of *S.* Heidelberg isolated from retail meats (Zhao *et al.,* 2001). By using this technique, they found clones widely distributed in different types and brands of meats collected during 5 years from diverse retail stores (Zhao *et al.,* 2001).

PFGE is considered superior over other molecular typing methods (Leotta *et al.,* 2010). At each restriction site, 90% of the chromosome and approximately 0.05% of the genome is scanned, contributing to the high resolving power of the PFGE system (Michaud *et al.,* 2001).

The choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species (CDC, 2014). DNA restriction patterns generated by PFGE are stable and reproducible by different laboratories. The CDC provides nationally standardized procedures, proficiency testing programs, pattern databases and data sharing between State Federal and Labs via the Pulse–Net USA. PFGE was used in this project to generate a dendrogram that will make it possible to evaluate the genetic relatedness of serotypes obtained from different areas of the processing plant. In addition PFGE results would be used to determine genetic profiles.

I. 4. 2 *Campylobacter*

Bacteriological isolation and identification

The International Organization for Standardization, (ISO) has developed a standardized method for detection and enumeration of *Campylobacter* (ISO 10272-1: 2006; Part A: Detection and Part B: Colony-count technique. The bacteriological isolation of *Campylobacter* depends of the methods used:

Enrichment

Prior enrichment is developed which presumed to harbour low numbers of *Campylobacter*, or samples containing a relatively large fraction of injured cells due to

processing or unfavourable environmental conditions (Richardson *et al.*, 2009). Using an enrichment broth, prior to plating, usually provides better recovery when target cells are either low in number, injured, or stressed (Williams *et al.*, 2009). Some of the most frequently employed enrichment broth media for *Campylobacter* are Bolton, Preston, Park ‐ Sanders and Exeter. Since *Campylobacter* is sensitive towards peroxides, radical scavengers like horse/sheep blood and charcoal are often included in these enrichment broths, as well as growth promoting reagents like ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP). Enrichment broths operate with various selective systems to reduce growth of accompanying flora and combinations of cefoperazone, vancomycin, polymyxin B, amphotericin B, colistin, trimethoprim and rifampicin are employed. Furthermore, culturing is performed at 42 °C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2), (Potturi-Venkata *et al.*, 2007; Altekruse *et al.*, 1999). Bolton broth is currently recommended for enrichment in ISO 10272 ‐ 1 standards, and it was also proven to be superior to Preston and Mueller Hinton broth in supporting the growth of a test panel of *Campylobacter* strains of relevance to food safety.

Isolation and selection for confirmation

Following enrichment, or directly from samples with presumed high numbers of *Campylobacter*, the samples are spread onto selective agar plates. Again a high number of solid media exist for *Campylobacter*, and modifications to existing selective agars are numerous as well. Some of the most common ones are: modified charcoal cefoperazone deoxycholate (mCCDA), Skirrow, Karmali, Preston, Abeyta ‐ Hunt ‐ Bark (AHB), Campy ‐ cefex and Butzler. It is recommended to use two selective agars with different selective principles in parallel to increase the yield, but currently ISO 10272 ‐ 1 is the only standard culture method (Potturi-Venkata *et al.*, 2007). Solid media for enumeration of *Campylobacter* should always be dried to avoid excessive moist and thereby obtain single colonies.

Confirmation of *Campylobacter* presumptive colonies

It is performed by subculturing five colonies from selective media onto blood agar plate and examine for morphology and motility using a microscope. Furthermore, a number of tests can be performed in all cultures in which curved bacilli with a spiralling "corkscrew" motility are found to confirm the identification and determine the species: growth at 25 ºC (microaerobic) and at 41.5 ºC (aerobic), catalase, oxidase, glucose utilisation, hippurate hydrolysis, latex agglutination etc.

Phenotyping methods

Serotyping

Serotyping has a long history of use in the typing of *Campylobacter*. The two serotyping systems differ on the basis of either using of heat-labile (HL) (Lior *et al.*, 1982) or of soluble heat-stable (HS) antigens (Penner and Hennessy, 1980; Penner *et al.*, 1983). Schemes, according to Penner and Hennessy, (1980) are generally accepted and well evaluated. The major disadvantages of both of these techniques are the high number of untypeable strains, the time-consuming and the technically demanding requirements. Also antiserum reagents required for serotyping are not widely available (Wassenaar and Newell, 2000). Serotyping alone does not exhibit a high discriminatory power, but could be improved in combination with a DNA-based method (Fussing *et al.*, 2007).

Phage typing

Given the low resolving power of serotyping, phage typing has been employed as an extension to serotyping to further characterise *C. jejuni* and *C. coli*, and there are currently 76 recognised phage types (Hopkins *et al.*, 2004). This method makes use of a set of virulent phages that may or may not have specificity for cell-surface receptors on the bacterial host. If the bacteriophage is able to attach and infect, cell lysis will result, which can be seen as plaque formation on Petri dish cultures (Grajewsky *et al.*, 1985). The main limitations of phage typing, similarly to serotyping, include the occurrence of non- typeable strains and problems with cross-reactivity. Further, large panels of

specialised reagents and a high level of skill are required to perform phage typing, limiting the use of this method to reference laboratories (Sails *et al.,* 2003). Consequently, phage typing has largely been replaced by more rapid, sensitive and cost-effective genotyping methods.

Hippuricase speciation

The hippuricase biochemical test has been extensively used to differentiate *C. jejuni* from *C. coli* and *C. lari* (Nicholson and Patton, 1995). The basis, behind the test, lies in the specific capacity for *C. jejuni* to hydrolyse hippuric acid using N-benzoylglycine amidohydrolase (hippuricase), an enzyme encoded by the hipO gene (Hani and Chan, 1995). The hippuricase test has an approximately 90% success rate. Both false-negative atypical *C. jejuni* strains harbouring a truncated or lowly expressed hipO gene (Totten *et al.,* 1987) and non-*C. jejuni* false-positives have been documented (Nicholson and Patton, 1995). As with most phenotypic-based methods, the hippuricase test has been converted to PCR-based methods of speciation with higher success rates (Linton *et al.,* 1997; Bae *et al.,* 2005).

Molecular techniques

Polymerase Chain Reaction (PCR)

Conventional PCR, first developed for *Campylobacter jejuni* and coli in 1992, detects chromosomal gene sequences and is able to detect cells at low numbers (Moore *et al.,* 2005). This method detects DNA from live and dead bacteria that are multiplied and then visualized. Real-time PCR (rt-PCR) has also been investigated with *Campylobacter* and is based on mRNA or DNA as a target (Moore *et al.,* 2005). Hunter *et al.* (2009) analysed the short variable region (SVR) of the flagellin locus on carcasses at rehang and post chill by PCR. As carcasses moved through processing, genetic diversity of *Campylobacter* decreased; however, 1478 isolates were identified (Hunter *et al.,* 2009). Olsen *et al.* (2009) used rt-PCR to detect *Campylobacter* from airborne samples in the processing facility.

Genotyping methods

Pulsed-field gel electrophoresis (PGFE)

As mentioned earlier, PGFE is generally considered as the gold standard. Many epidemiological typing studies have successfully applied this method as a basis for identification of strains of *Campylobacter*. It has been used extensively for typing *Campylobacter* in studies associated with poultry (Posch *et al.,* 2006; Klein *et al.,* 2007; Lienau *et al.,* 2007). The disadvantages of PFGE consist of high costs and time requirement; it is also a technically demanding method. Comparison of PFGE profiles from different laboratories and between studies has also been difficult. Distinct electrophoretic conditions may influence obtained profiles, different restriction enzymes are used to digest DNA and furthermore some *Campylobacter* isolates cannot be typed by PFGE (Wassenaar and Newell, 2000). The widely-used restriction enzyme SmaI generates four to ten fragments. KpnI digest has more fragments than SmaI and is thus more discriminatory and it is often used as a secondary enzyme but has also been suggested as a primary choice for epidemiological studies (Michaud *et al.,* 2001).

5. Cornerstone study

Salmonella and *Campylobacter* have long been recognized as most important zoonotic pathogens of economic significance in animals and humans. There are numerous sources of human both pathogens, although consumption of poultry meat is considered the most common source of human infection (EFSA, 2014). In this sense, the legislators have been working to minimize *Salmonella* prevalence in poultry sectors with the introduction of a National Control Program to reduce the incidence of the bacteria in poultry flocks. The program for broiler flocks in Spain set measures to reduce the prevalence of *S.* Enteritidis and *S.* Typhimurium, the strains which pose the highest human health risk, to 1.0% or less for December 2011 (EC, 2007). Therefore, non-contaminated *Salmonella* broiler meat has been sold since 2011 for human consumption. On the other hand, Official National Program to control *Campylobacter* has not been yet implemented; although its implementation will be taken soon. Until now, the *Campylobacter* control strategies are based on the principles of food safety

risk analysis (FAO/WHO, 1995). Nevertheless, despite of the measures taken, high number of salmonellosis and campylobacteriosis are continued declare. Control strategies involve hygienic measures throughout all the production chain; during the rearing at the farm level, the transport to the slaughterhouse, the processing at the slaughter line and during the packaging of chicken meat. In this context, the studies of the present Thesis are focused on the processing and packaging stages. The first experiment was carried out in order to know the contamination of both pathogens during the process of the chicken carcasses and the effect of processing stages on the microbial contamination under Spanish conditions, for developing futures control strategies that reduce contamination of both pathogens. The second experiment was carried out with the aim to investigate and compare the effect of different modified atmospheres packaging with traditional gases (N_2, O_2 or CO_2) and innovative noble gas, Argon (Ar), on the survival of the most prevalent *Salmonella* and *Campylobacter* strains isolated from the slaughterhouse from the previous study.

CHAPTER II. OBJECTIVES OF THE STUDY.

The general aim of this Thesis was to study *Salmonella* and *Campylobacter* contamination of poultry carcasses during the slaughter process and evaluate the packaging under different modified atmospheres against the main strains isolated at slaughter level.

The specific aims of this Thesis were to:

1. Determine the status of *Salmonella* and *Campylobacter* flock at the arrival of the live birds at the slaughterhouse.

2. Evaluate the contamination of both pathogens on the environmental surface of different processing stages at the slaughter line.

3. Investigate the influence of different processing stages on the contamination of *Salmonella* and *Campylobacter* on chicken carcasses during the slaughter process.

4. Determine the main serotypes of *Salmonella* and *Campylobacter* involved in the slaughter process and the genetic relationship between *Salmonella* strains.

5. Study the effect of different modified atmospheres packaging against the most common *Salmonella* and *Campylobacter* strains isolated from the slaughterhouse.

6. Evaluate the effect of different modified atmospheres applied on the physical-chemical analysis of chicken meat fillets.

7. Evaluate the effect of different modified atmospheres applied on the sensory analysis of chicken meat fillets.

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CHAPTER III. MATERIAL AND METHODS.

III.1. Experiment 1: Epidemiology of *Salmonella* and *Campylobacter* at poultry slaughterhouse under Spanish standard commercial conditions

Study sample

This study was carried out in a commercial broiler poultry processing plant located in the Valencia Region (Spain) of an integrated broiler chicken company. The processing line was operating under standard commercial conditions. Over 9 months (from September to November 2011 and January to June 2012) a total of 18 visits were achieved at the processing plant. At each visit, two flocks were studied during the working day of the slaughterhouse (Figure 9). A flock was defined as a group of birds from the same hatchery, raised in a broiler house during the same period of time.

Moment of sampling for *Salmonella* and *Campylobacter* at the slaughterhouse

From each flock studied, samples were collected during different moments of the working day (at the arrival of the animals at the slaughterhouse, before slaughter of the flock and during the processing of the carcasses) to study *Salmonella* and *Campylobacter* contamination during the slaughter process (Figure 9).

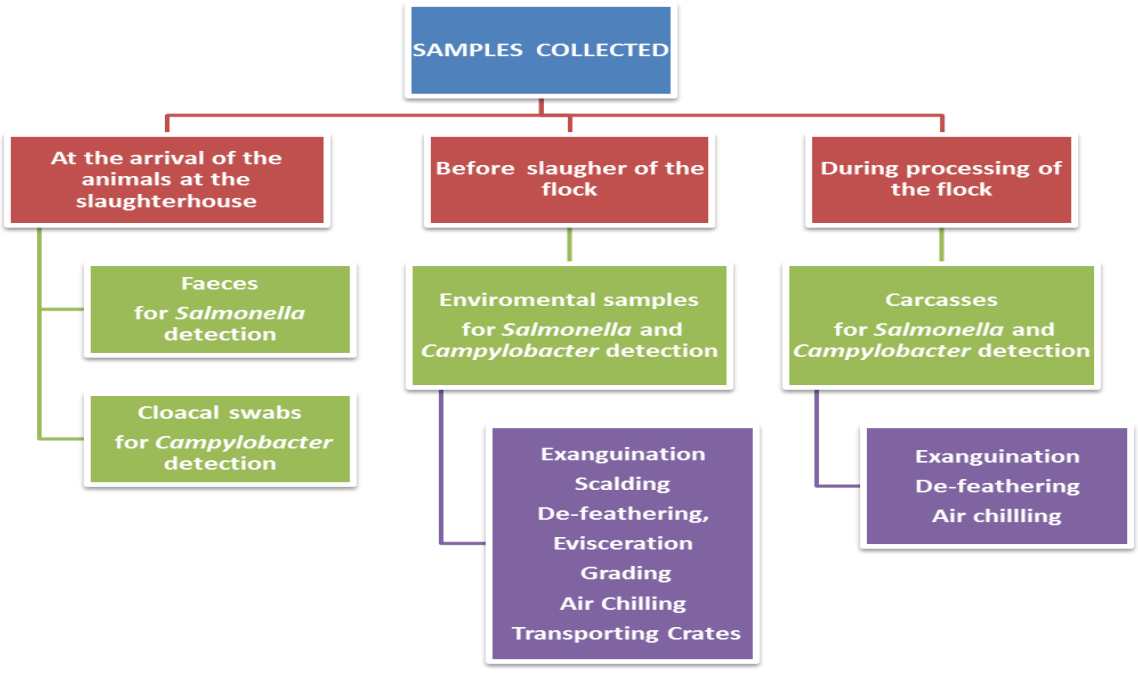

Figure 9. Samples collected during different moments of the working day of the slaughterhouse.

At the arrival of the animals at the slaughterhouse: In order to determine the status of the flock, two pooled faeces samples and cloacal swabs were collected for *Salmonella* and *Campylobacter* determination, respectively. For detecting of *Salmonella,* faeces samples were collected directly from the platform of the truck during the unloading at the slaughterhouse, as recommended by European Food Safety Authority (200-300 g each; EC, 2005) (Figure 10). For detecting of *Campylobacter*, ten cloacal swabs samples (Cary Blair sterile transport swabs, DELTALAB, Rubí, Spain) were collected when animals were hanged on the processing line (Figure 10). A flock was declared infected if at least one of the samples was tested as positive.

Figure 10. Samples collected to determine the *Salmonella* and *Campylobacter* status of infection of the flock. a) Pooled faeces samples collected directly from the truck at the arrival of the animals at the slaughterhouse. b) Cloacal swabs collected from hanged birds.

Before slaughter of the flock: To assess the *Salmonella* and *Campylobacter* status of the slaughterhouse facilities, environmental samples were taken before the processing of the carcasses. For this purpose, 10 cm^2 of surface samples were taken from equipment at 6 selected stages (exsanguination, scalding, de-feathering, evisceration, grading and air chilling stage) (Figure 11).

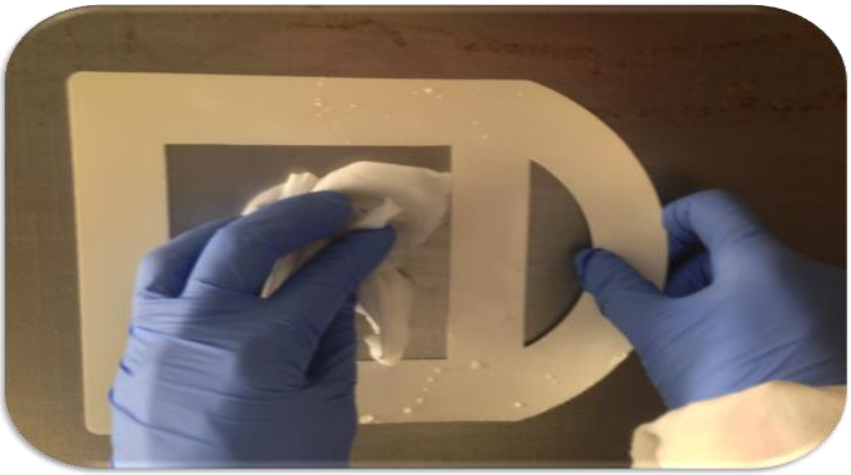

Figure 11. Environmental samples from equipment at selected stages of the processing line.

Foodstuff contact surfaces of clean transporting crates were also sampled. All environmental surface samples were collected with sterile wet gauze pads with disinfectant neutralizer (AES Laboratories®, Brux Cedex, France) and transported in sterile sample pots.

During processing of the flock: 3 carcasses were taken from 3 select stages of the processing line (after exsanguination, de-feathering, and air chilling as a finished product) to determine the degree of contamination of *Salmonella* and *Campylobacter* in the carcasses throughout the processing. Each carcass was removed by hand with aseptic conditions and placed into an individual sterile bag (Seward, Worthing, UK) (Figure 12). All the samples collected were transported in a cool box at ≤ 4 ºC to the laboratory for microbial analysis, and were analysed within 2 hours after sampling.

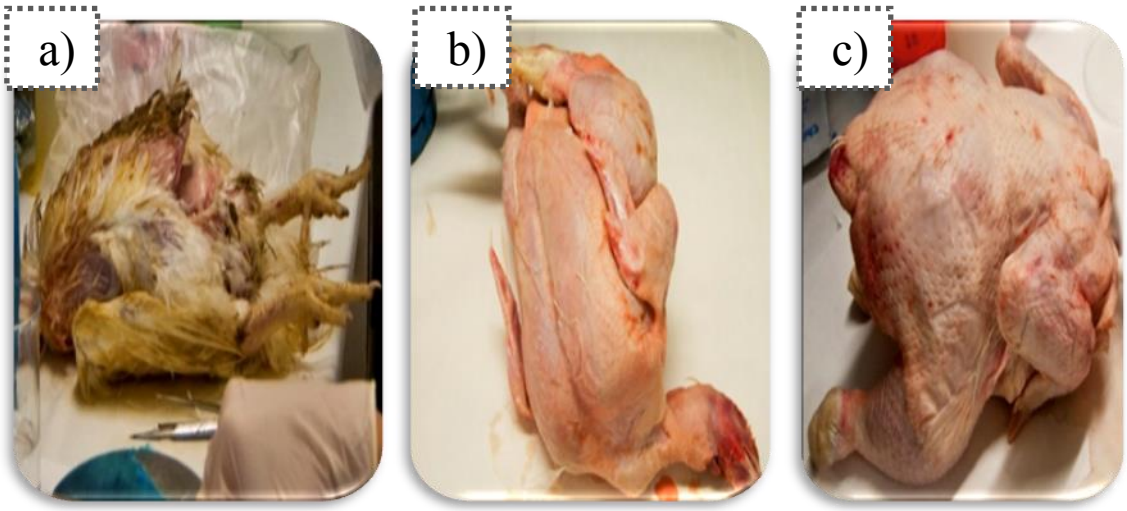

Figure 12. Carcasses collected from three selected stages: a) exsanguination, b) de-feathering and c) air chilling. All the carcasses were collected after the stage had completed and before the commencement of the next processing stage.

Bacteriological analysis

Salmonella isolation, serotyping and genotyping

For *Salmonella* detection, faeces (25g) and environmental wet gauze pads samples were analysed according to ISO 6579:2002 (Annex D) (Figure 13). For this purpose, firstly, the samples were pre-enriched in 1:10 vol/vol Buffered Peptone Water (BPW, Scharlau®, Barcelona, Spain) and incubated at 37±1 °C for 18±2 h. The pre-enriched samples were transferred onto Semi-Solid Rappaport Vassiliadis agar plate (MSRV, Difco®, Valencia, Spain), incubated at 41.5±1 °C for 24-48 h. Suspicious plates were transferred to two different agar plates, ASSAP (AES Laboratories®, Bruz Cedex, France) and Xylose Lysine Deoxycholate agar (XLD, Liofilchem®, Valencia, Spain) and incubated at 37±1 °C for 24±3 h. After the incubation period, 5 suspect colonies of *Salmonella* were selected, and transferred to a nutrient agar plate (Scharlab®, Barcelona, Spain) incubated at 37±1 °C for 24±3 h. Then, Urease test was performed 4 h at 37 °C. Finally, a biochemical test API-20E (API-20®, bioMerieux, Madrid, Spain) was done to confirm *Samonella* spp. Moreover, *Salmonella* strains isolated were serotyped in accordance with Kauffman-White-Le-Minor technique. According to this technique, each strain has to be mixed with polyvalent and monovalent antisera until the antigenic formula is determined. One drop of antisera has to be mixed with the strain in circular movements. If agglutination was not observed, the reaction was considered negative.

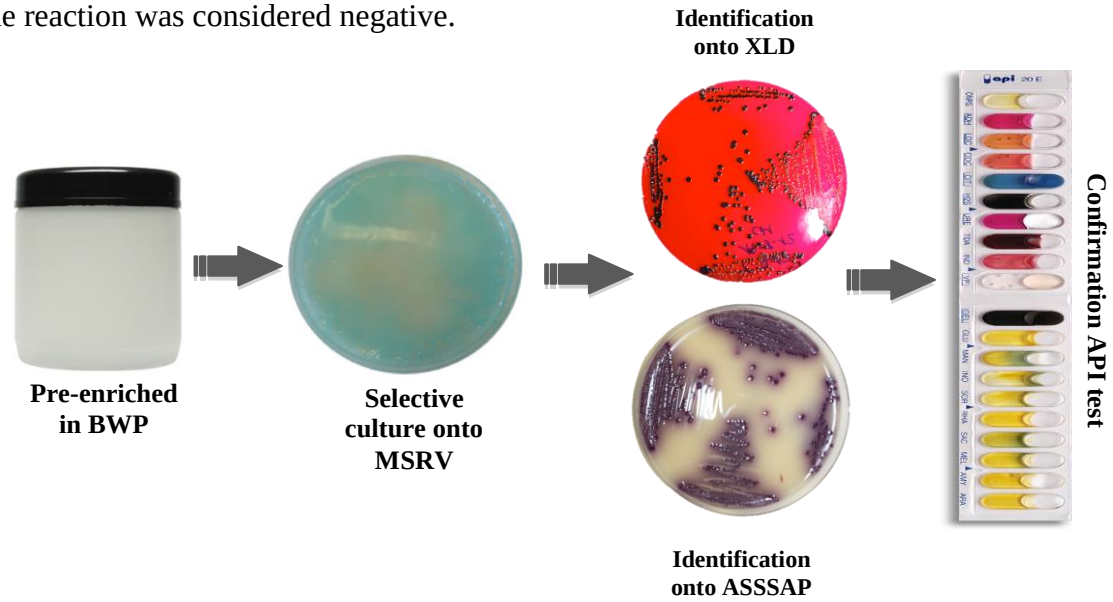

Figure 13. ISO 6579:2002 (Annex D) scheme for detection of *Salmonella spp*. BWP: Buffered Peptone Water. MSRV: Modified Semisolid Rappaport-Vassiliadis. XLD: Xlose-Lysine-Desoxicolate agar. ASSAP: ASSAP agar.

According with *Salmonella* detection on carcasses, neck skin and internal surfaces cavity samples were collected (Figure 14). On one hand, neck skin samples were obtained for each carcass by cutting a strip of skin from neck (25g) with a sterile scalpel and tweezers. The skin sample was put into a sterile bag and diluted at 1:10 vol/vol BPW. The mix was homogenized by stomaching at 230 rpm for 120 s (Stomacher®400 circulator, Seward Ltd., Worthing, UK). Then, 10 mL of the homogenate was use for *Salmonella* analysis and anothers 10 mL of the homogenate were use for future analysis with *Campylobacter*. On the other hand, samples of internal surface cavity of each carcass were also collected with sterile wet gauze pads (AES laboratories, Bruz Cedex, France). Sterile wet gauze was introduced into the internal cavity and then rubbed the entire internal surface to take the sample. Neck skin homogenates and internal surfaces cavity samples were analysed for *Salmonella* detection according with ISO 6579:2002 (Annex D), as described above. All *Salmonella* strains were maintained at -80 ºC for later studies.

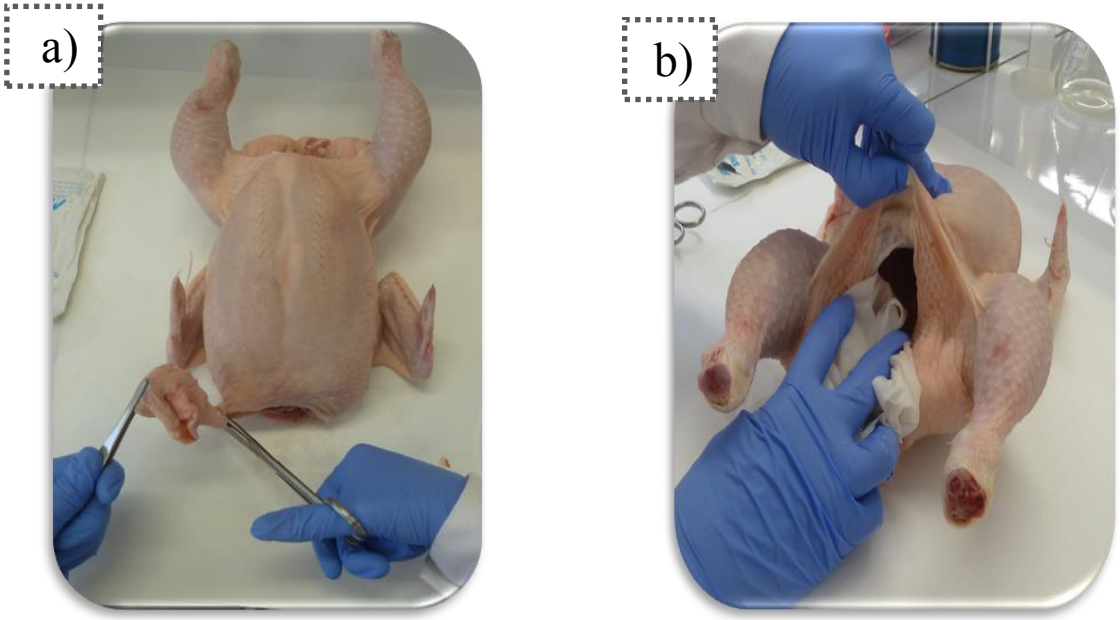

Figure 14. Carcasses sampling. a) Neck skin. b) Internal surfaces cavity.

Salmonella strains isolated were serotyped in accordance with Kauffman-White-Le-Minor technique. According to this technique, each strain has to be mixed with polyvalent and monovalent antisera until the antigenic formula is determined. One drop of antisera has to be mixed with the strain in circular movements. If an agglutination reaction was observed, the reaction was considered positive. If agglutination was not observed, the reaction was considered negative.

Clonality among the isolates of this study was assessed by enterobacterial repetitive Intergenic consensus (ERIC-PCR) as previously described by Rasschaert *et al.* (2005). ERIC-PCR was used as a screening tool and allowed to determine the genotypic diversity among *Salmonella* isolates within an individual host and within a gull colony. Representative isolates from the different ERIC-PCR patterns identified per sample were analysed by pulsed-field gel electrophoresis (PFGE).

PFGE was performed according to the PulseNet standardized protocol "Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia coli* non-O157 (STEC), *Salmonella* serotypes, *Shigella sonnei* and *Shigella flexneri*" (www.pulsenetinternational.org) (Figure 15). The isolates were analysed using XbaI restriction enzyme (Roche Applied Science, Indianapolis, IN), and the resulting PFGE patterns were analysed using the Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Similarity matrices were calculated using the Dice coefficient and cluster analysis was performed by the unweighted-pair group method with arithmetic mean (UPGMA). Isolates with a minimum level of similarity of 80 % were considered genetically similar or identical.

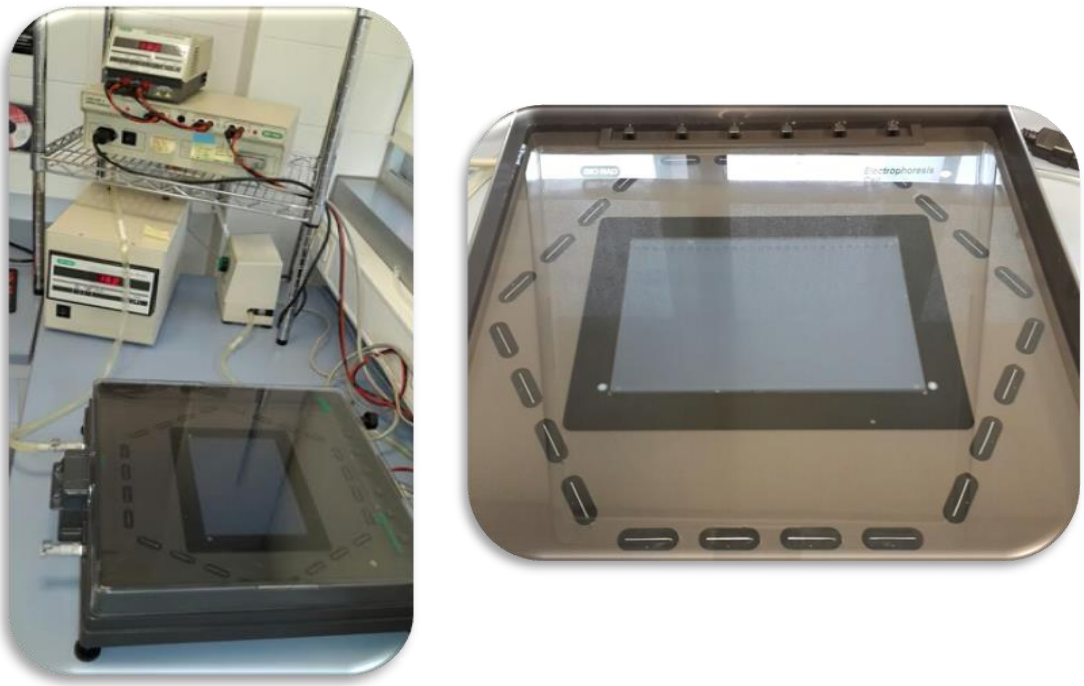

Figure 15. Pulsed-field gel electrophoresis (PFGE) analysis.

Campylobacter isolation and speciation

All the cloacal swabs collected were tested by direct plating onto a Modified Charcoal Cefoperazone Deoxycholate a gar (mCCDA, CM0739 and SR0155, OXOID, Dardilly, France) and Preston agar (CM0689, SR0117 and SR0048, OXOID, Dardilly, France). Then, the samples were incubated at 41.5±1 °C, in a microaerobic atmosphere (84% N_2, 10% CO_2, 6% O_2) (CampyGen, Oxoid) for 44±4 h. *Campylobacter*-like colonies were purified on blood agar (AES Laboratories®, Bruz Cedex, France) for further characterization. First, cellular morphology and motility under phase-contrast microscopy were evaluated followed of oxidase and catalase tests and planting at different temperatures and atmospheres onto Columbia blood agar (AES Laboratories®, Bruz Cedex, France). Finally, Hippurate hydrolysis test was used for the speciation of bacterium.

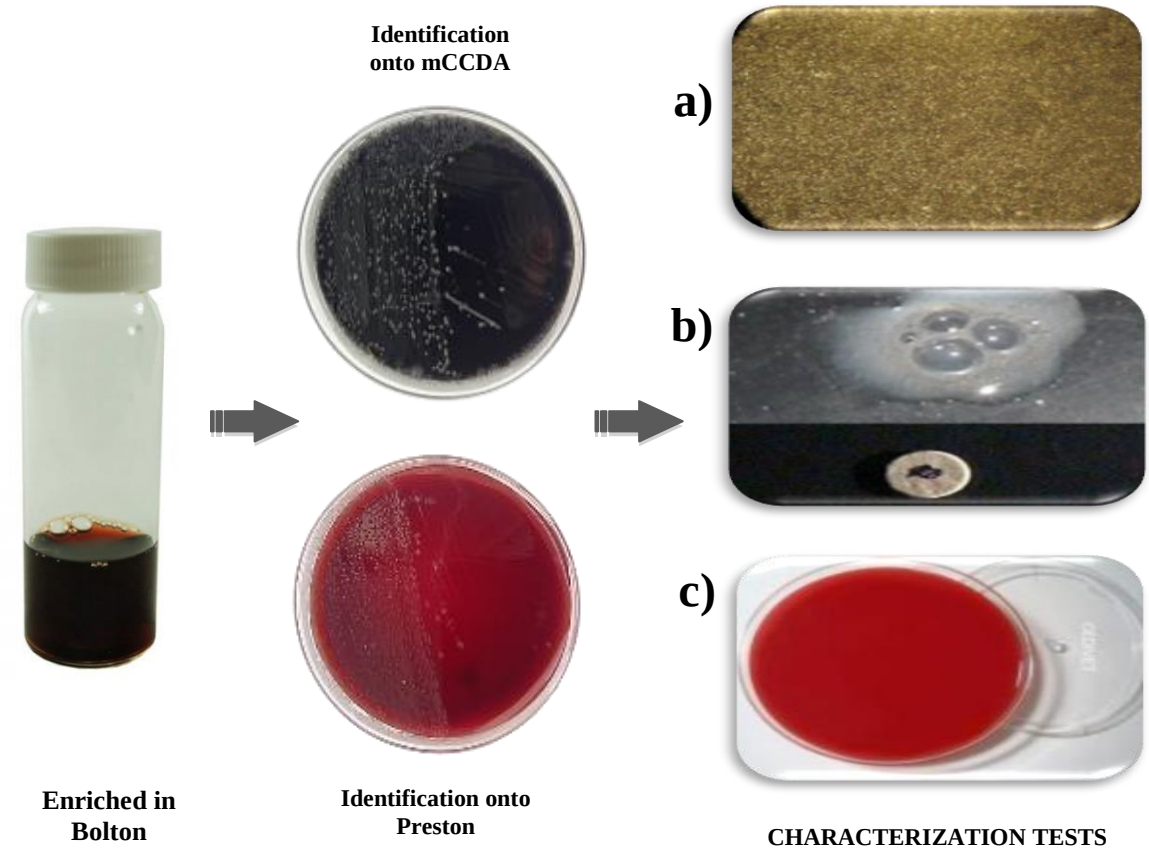

Figure 16. ISO 10272-2:2006 (Annex E) scheme for detection of *Campylobacter spp*. mCCDA: Modified Charcoal Cefoperazone Deoxycholate Agar. Preston: Preston agar. a) Cellular morphology and motility. b) Oxidase and catalase tests. c) Planting at different temperatures and atmospheres onto Columbia blood agar.

According with *Campylobacter* detection on environmental wet gauze pads samples and neck skin homogenates (10mL, as reported above), samples were analysed according to the ISO 10272-1:2006 (Annex E) (Figure 16). For detection purpose, samples were pre-enriched in 1:10 vol/vol Bolton Broth (CM0983, OXOID, Dardilly, France) and then pre-incubated at 37±1 ºC for 5±1 h. Finally, the pre-enriched broth was incubated at 41.5±1 °C for 43±1 h. Afterwards, 100 µl of the sample was cultured on the two selective agar plates (mCCDA and Preston agar) and incubated at 41.5±1 °C, in a microaerobic atmosphere for 44±4 h. *Campylobacter*-like colonies from homogenates neck skin samples were purified on blood agar and identified, as described above. *Campylobacter* strains isolated were maintained at -80 ºC for later studies.

Statistical analysis

The analysis of the *Salmonella* and *Campylobacter* results were carried out using a commercially available software program (SPSS 16.0 software package; SPSS Inc., Chicago, Illinois, USA, 2002). A generalised linear model, which assumed a binomial distribution for colonising of *Salmonella* and *Campylobacter*, was fitted to the data to determine the presence and diversity of both pathogens on live chicken birds at the arrival of the animals at the slaughterhouse, on the carcasses at different stages of the poultry slaughter process and the impact of each processing stage on microbiological contamination of chicken broiler carcasses. In addition, the association with the status of live broiler chicken flocks and *Salmonella* contaminated carcasses samples from exsanguination, de-feathering and air chilling stage was also studied. In all tests, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample was assigned a 1 if it had *Salmonella* or *Campylobacter* prevalence or a 0 if they had not. A *P* value of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error.

III.2. Experiment 2: Effect of modified atmosphere packaging against *Salmonella* and *Campylobacter*

Bacterial strains and culture preparation

The most frequently *Salmonella* and *Campylobacter* strains isolated from contaminated carcass finished product from the previous study (*S.* Enteritis and *C. jejuni*) were used to asses the effect of different modified atmosphere packaging, on the survival of these pathogens on skinless chicken meat fillets.

S. Enteritidis strain was activated by streaking on McConkey agar plate (AES Laboratoire, Combourg, France) followed by incubation for 24 h at 37 °C. Single colonies of *Salmonella* were inoculated into 200 mL Luria-Bertani (LB, Scharlau®, Barcelona, Spain) and incubated in a rotary shaker at 120 rpm at 37 °C for 24 h to enrich cell numbers.

C. jejuni strain were plated onto Columbia Blood agar plate supplemented with 5% horse blood (Oxoid, Barcelona, Spain) and incubated at 37 ºC for 48 h under microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) generated by CampyGen® (Oxoid, Barcelona, Spain). Single colonies of *Campylobacter* were transferred into brain heart infusion broth (BHI, Oxoid, Barcelona, Spain) and were incubated under microaerobic atmosphere as describe above to enrich cell numbers.

Following the incubation period of both isolated serovars, the cultures optical density was measured at 600 nm (OD_{600}) by spectrophotometer (UV-1, Thermo Electron Corporation, Cambridge, UK). The cultures were diluted using both fresh broths (LB and BHI) from each bacteria, to give a final OD_{600} = 0.2 (6 $\log_{10}$ CFU ml-1) and incubated under conditions as described above for *Salmonella* and *Campylobacter*. Then, the batches cultures were serially centrifuged in 50 mL falcon tubes at 3,000 rpm for 10 min. to recover the precipitated colonies. The cell pellets were washed with maximum recovery diluent (MRD, Liofilchem®, Barcelona, Spain) followed by centrifugation at 3,000 rpm for 10 min. The process was repeated 3 times for each pathogen. Finally, the cell pellets were resuspended in MRD to achieve the final

concentration of 6 $\log_{10}$ CFU ml-1 (OD_{600} = 0.2) for both isolated serovars.

Preparation of samples and modified atmosphere packaging (MAP)

Processed chicken carcasses from the same batch were provided by a local poultry slaughterhouse (Valencia, Spain) within 1 h after slaughter. A total of 288 pieces of chicken meat fillets were aseptically cut to have a standardized surface area and weight (25g). Then, samples were placed into polypropylene trays (Amcor Flexibles, Barcelona, Spain) to evaluate the effect of different modified atmospheres on the microbiological, physical-chemical and sensorial analysis of chicken meat fillets (Figure 17).

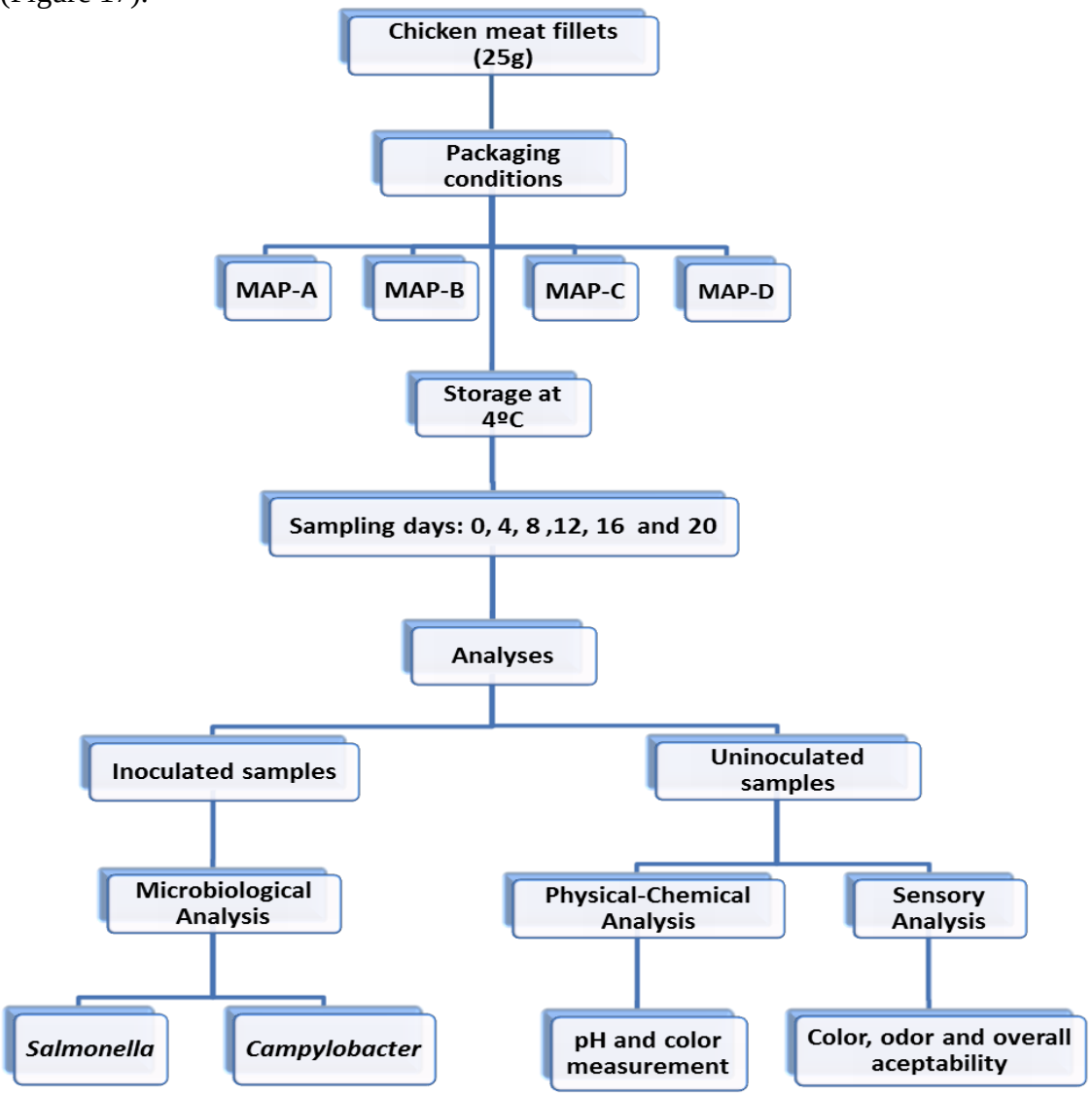

Figure. 17. Design of the experiment with modified atmospheres at meat chicken fillets. MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N2 / Ar.).

Modified atmosphere conditions were obtained by flushing the trays with three gas mixtures (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.). For each MAP conditions, a total of 72 chicken fillet meat samples were used. Thermosealing was done in an ULMA-Smart 300 packing machine (Oñati, Spain) using a polypropylene film (Amcor Flexibles, Barcelona, Spain) (Figure 18). The gas concentrations in all packages were measured by a gas analyser (Dansensor, Ringsted, Denmark).

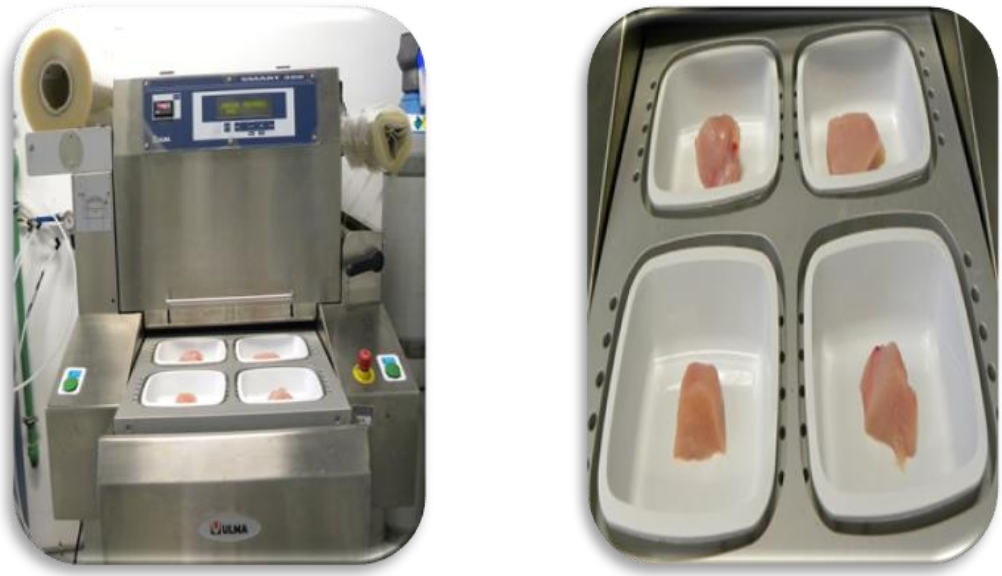

Figure 18. Modified atmosphere packaging of chicken fillet meat.

Inoculation of chicken fillet meat

Chicken fillet meat samples packaged in each MAP were divided in two groups. The first was used to study the effect of each MAP against *Salmonella* and *Campylobacter* culture (36 inoculated samples). Half of these samples were inoculated with *S.* Enteritidis culture and the other with *C. jejuni* culture. Previously, a septum was placed onto the film surface of each tray. Then, a syringe was introduced through the septum to spread 100 µl of inoculum onto the surface of each chicken fillet meat sample, to avoid inside MAP conditions (Figure 19). The initial concentration on the fillets was 6.5±0.2 $\log_{10}$ CFU/g. This relatively high concentration was chosen to be able to detect reductions of three to four logs units. Finally, samples were stored at 4 ºC without exposure to light.

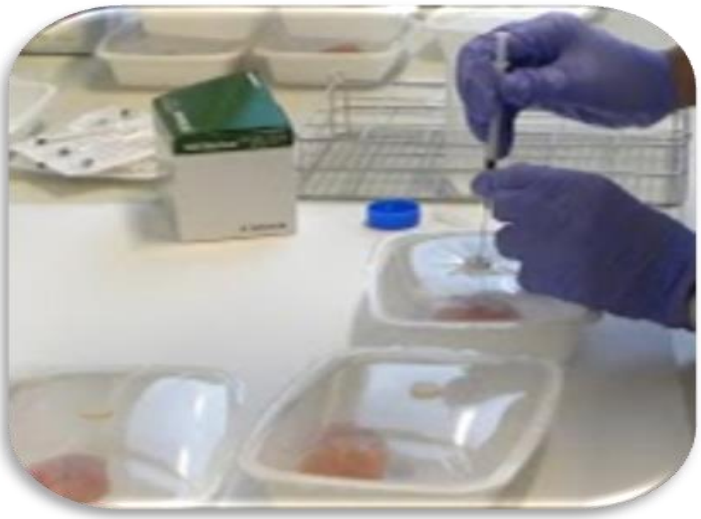

Figure 19. Inoculation method of chicken fillet meat.

Microbiological analysis

The enumeration of either *S.* Enteritidis or *C. jejuni* for the inoculated chicken meat fillets packaged was carried out at 0, 4, 8, 12, 16 and 20 days of storage (sampling days). The numbers of pathogens on three independent chicken meat fillet packaged were determined washing in 225 mL of 0.1% buffered peptone water (BWP, AES, Valencia, Spain) by stomaching the samples at 230 rpm for 120 s (Stomacher®400 circulator, Seward Ltd., Worthing, UK). Then, this initial dilution was subsequently serial-fold diluted at least six times, and 100 µl of each dilution was spread onto McConkey agar plate for *Salmonella* and onto mCCDA agar plate for *Campylobacter* and incubated as mentioned above. Numbers of *Salmonella* and *Campylobacter* were calculated according to the criteria described in ISO 6887-1.

Physical-Chemical Analysis

The other group of the chicken fillets meat samples packaged in each MAP (36 uninoculated samples) was use for physical-chemical analysis (Figure 20). After packaging, samples were stored at 4 ºC, and the analyses were carried out at the same sampling day of storage of inoculated samples.

pH determination

The pH values were determined with a portable pH meter equipped with a pH electrode (Thermo scientific) (Figure 20). The results were expressed as the mean of three values acquired on different area of three chicken meat fillet samples (n = 9) per MAP and sampling day.

Color measurement

Color measurements were determined with a Minolta (Model CR-300, Ramsey, N.Y., USA) (Figure 13) on the surface of three chicken meat fillets per MAP and sampling day, approximately 30 min after opening the package (Figure 20). The CIEL*a*b* color space was used, in which L* (±, lightness/ darkness), a* (±, red/green), and b* (±, yellow/blue) values were determined at three different area on each chicken meat fillet. Higher L* values describe samples having lighter color, whereas lower L* values those having darker color. Positive a* values were related with samples with redder color, whereas a negative a* values describe those with greener color. A standard white calibration plate was employed to calibrate the equipment.

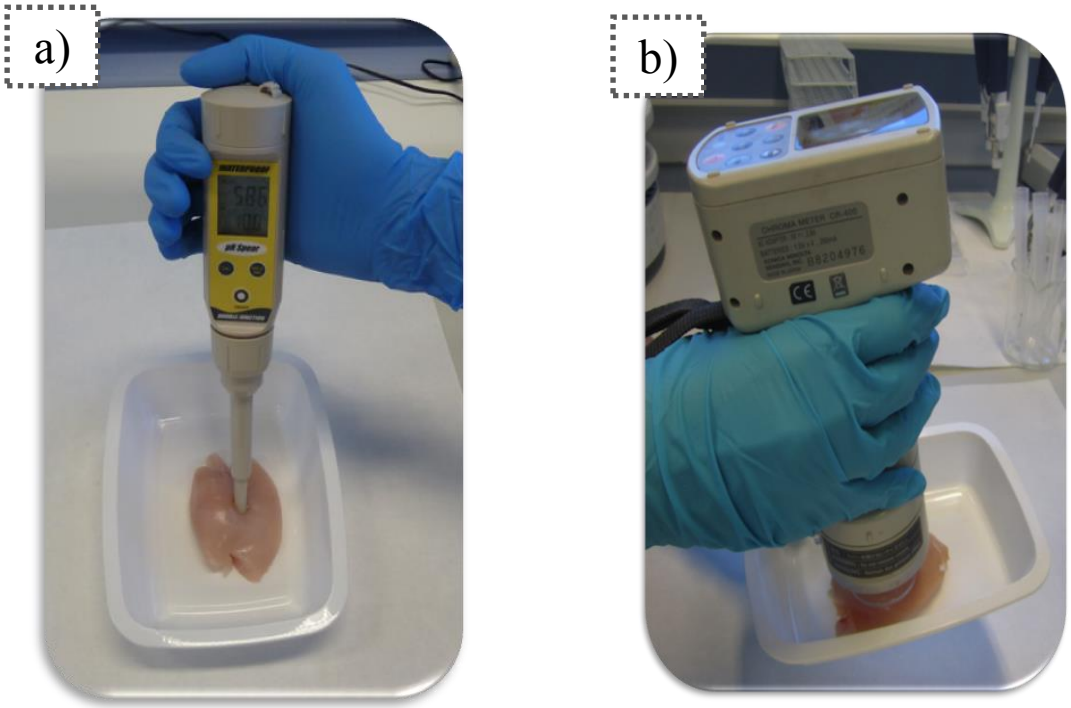

Figure 20. Physical-chemical analysis: a) pH determination with pH meter. b) Color measurement with Minolta system.

Sensory analysis

Acceptance test was used in the sensory evaluation. Visual quality of the chicken meat fillet samples was assessment by a total of 6 untrained judges per each MAP and sampling day. Each sample was coded, presented in random order and the attributes of appearance, odor and overall acceptability were evaluated using a five point hedonic scale as described in the table 3. At each sampling day, panelists were presented with freshly cut untreated chicken meat fillet as a reference.

Table 3. Hedonic scale for appearance, odor and overall acceptability for chicken meat fillets.

Measurement	Score	Quality description
Appearance	1	Dislike extremely; very poor, not usable.
	2	Dislike moderately; poor, excessive defects, limited marketability.
	3	Neither like nor dislike; borderline, fair, slightly to moderately objectionable defects, lower limit of appeal.
	4	Like moderately; good, minor defects, not objectionable.
	5	Like extremely; excellent, essentially free from defects, fresh-like and typical .
Odor	1	Dislike extremely.
	2	Unacceptable; poor, stale, musty, and mouldy.
	3	Fairly acceptable.
	4	Good; not objectionable, acceptable.
	5	Excellent; typical, very much acceptable.
Overall acceptability	1	Dislike extremely, very poor.
	2	Dislike moderately, poor.
	3	Neither like nor dislike; fair, limited marketability.
	4	Very good; will definitely buy .
	5	Extremely good; most definitely buy.

Statistical Analysis

Statistical analysis was performed using STATGRAPHICS Plus 4.1 (Manugistic Inc., Rockville, MD, USA). The specific differences between means were determined by least significant difference (LSD) applied after the analysis of variance (ANOVA). Significance differences were defined at $P \leq 0.05$.

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CHAPTER IV. RESULTS.

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IV.1. Experiment 1: Epidemiology of *Salmonella* and *Campylobacter* at poultry slaughterhouse under Spanish standard commercial conditions

Salmonella

In total, 504 samples were collected at the slaughterhouse level to evaluate the percentage of *Salmonella* contamination. The bacterium was isolated in 45.0% of the samples (n = 227), leading to 40.4% of the 252 environmental surfaces (n = 102), 69.4% of the 36 faeces (n = 25), 59.2% of the 108 neck skins (n = 64) and 33.3% of positive samples of the 108 internal surface cavities analysed (n = 36).

During this study a total of 36 broiler flocks were sampled for *Salmonella* at their arrival to the slaughterhouse, and 25 of them (69.4%), were positive for the bacterium in faeces samples.

Among all the environmental samples collected prior to the arrival of the flocks studied, *Salmonella* was isolated (from highest to lowest) in de-feathering (69.0%), grading (56.0%), evisceration (47.0%), transporting crates (47.0%), exsanguination (44.0%), air chilling (14.0%) and scalding stage (6.0%). Significant statistically differences were found between the different stages, being the de-feathering and grading the most contaminated stages at the slaughterhouse (P = 0.000, Table 4).

Table 4. Percentage of *Salmonella*-positive environmental surface samples collected before processing of the flocks.

Environmental samples	*Salmonella* (%)	S.E
Bleeding	44.0^{b}	8.3
Scalding	6.0^{a}	3.8
De-feathering	69.0^{c}	7.7
Evisceration	47.0^{b}	8.3
Grading	56.0^{cb}	8.3
Air chilling	14.0^{a}	5.8
Transporting crates	47.0^{b}	8.3

n: Number of samples collected. S.E: Standard error. *Salmonella* (%): Percentage of *Salmonella* positive samples from each stage. Percentages with different letter are significantly different ($P \leq 0.05$). From each stage, 36 samples were taken throughout the study.

Moreover, according with the samples collected from the carcasses at three selected stages, neck skin samples were contaminated (from highest to lowest) in 81.0% in de-feathering, 67.0% in exsanguination and 31.0% in air chilling stage. Otherwise, the internal surfaces cavities samples were contaminated (from highest to lowest) in 53.0% air chilling, 44.0% in de-feathering and 3.0% in exsanguination stage. Significant differences were found between *Salmonella* contamination of either neck skin or internal surfaces cavities samples and stage analysed (P = 0.000, Table 5) Moreover, samples from the skin neck were found most frequently positive than those from internal surfaces cavities for *Salmonella* at exsanguination and de-feathering stages (P = 0.000, Table 5). However, in the finished carcass product sampled at the air chilling stage, samples from internal cavities were more contaminated than those from neck skin (P = 0.000, Table 5).

Table 5. Percentage of *Salmonella*-positive samples collected from the chicken carcasses samples at exsanguination, de-feathering and air chilling stages.

Stage	Sample type			
	Neck skin		Internal surfaces cavities	
	***Salmonella* (%)**	**S.E**	***Salmonella* (%)**	**S.E**
Exsanguination	67.0a^{z}	7.9	3.0A^{y}	2.7
De-feathering	81.0a^{z}	6.6	44.0B^{y}	8.3
Air chilling	31.0b^{z}	7.7	53.0B^{y}	8.3

n: Number of samples collected. S.E: Standard error. *Salmonella* (%): Percentage of *Salmonella* positive samples from each stage. Means in column with different lower case and capital letters are significantly different ($P \leq 0.05$). Means rows with different superscript letters are significantly different ($P \leq 0.05$). From each stage, 36 neck skin samples were taken throughout the experiment.

According to the status of the broiler flocks at their arrival to the slaughterhouse, when the *Salmonella*-status of the live flocks were positive (n = 25), 5.0%, 76.0% and 78.0% of the chicken carcasses samples were *Salmonella* positive at stage of exsanguination, de-feathering and air chilling, respectively (Table 6). On the other hand, when the *Salmonella*-status of the live flocks was negative (n = 11), 48.0%, 56.0% and 50.0% of the chicken carcasses samples collected were *Salmonella* positive at stage of exsanguination, de-feathering and air chilling, respectively (Table 6). The chicken carcasses samples from exsanguination stage showed significant differences according to the live flock status (positive or negative) and respect to the different stages studied,

(P = 0.000, Table 6). However, significant differences were not found between *Salmonella* contaminated chicken carcasses samples of either de-feathering or air chilling stage and also among the stages considering the status of the life flock previous to the slaughter ($P \geq 0.05$, Table 6).

Table 6. Percentage of *Salmonella*-positive collected from the chicken carcasses samples (neck skin and internal cavity) at exsanguination, de-feathering and air chilling stages, according to *Salmonella* status of the live broiler chicken flocks.

Flock status	Chicken carcasses samples					
	Exsanguination stage		De-feathering stage		Air chilling stage	
	Salmonella (%)	S.E	*Salmonella* (%)	S.E	*Salmonella* (%)	S.E
Negative (n = 11)	5A[a]	4.4	77A[b]	8.9	80A[b]	
Positive (n = 25)	48B[a]	7.1	56A[a]	7.0	55A[a]	

n: Number of samples collected. S.E: Standard error. *Salmonella* (%): Percentage of *Samonella* positive samples from each stage. Means in column with different capital letters are significantly different ($P \leq 0.05$). Means rows with different superscript letters are significantly different ($P \leq 0.05$). From each stage, 36 neck skin samples were taken throughout the experiment.

In order to determine the characterization and genetic clonality, *Salmonella* isolates from environmental surfaces, faeces, neck skin and internal surfaces cavity samples of this study were serotyped and analysed by ERIC-PCR and PGFE. Firstly, the isolates were serotyped according to the Kauffmann-White scheme (Popoff and Le Minor, 1997). A total of two different serotypes were determined from the 227 isolates, 98.2% belonged to serotype Enteritidis (n = 223) and 1.8% to serotype Kentucky (n = 4). The latter serotype was found in faeces samples from the flock at the arrival to the slaugherthouse and in neck skin samples from exsanguination and de-feathering stages during the processing. From the environment was recovered only after processing of the *S.* Kentucky contaminated flock.

The study of genetic similarity showed that, *S.* Enteritidis strains collected from live birds (faeces samples) and from slaughter process (carcasses and environmental samples) had ≥ 85.0% genetic similarity according the analysis of XbaI PFGE pattern, confirming that these strains belonged to the identical pattern. The same genotype was

also identified from *S.* Kentucky strains with ≥ 85.0% genetic similarity according to analysis of XbaIPFGE pattern (Figure 21).

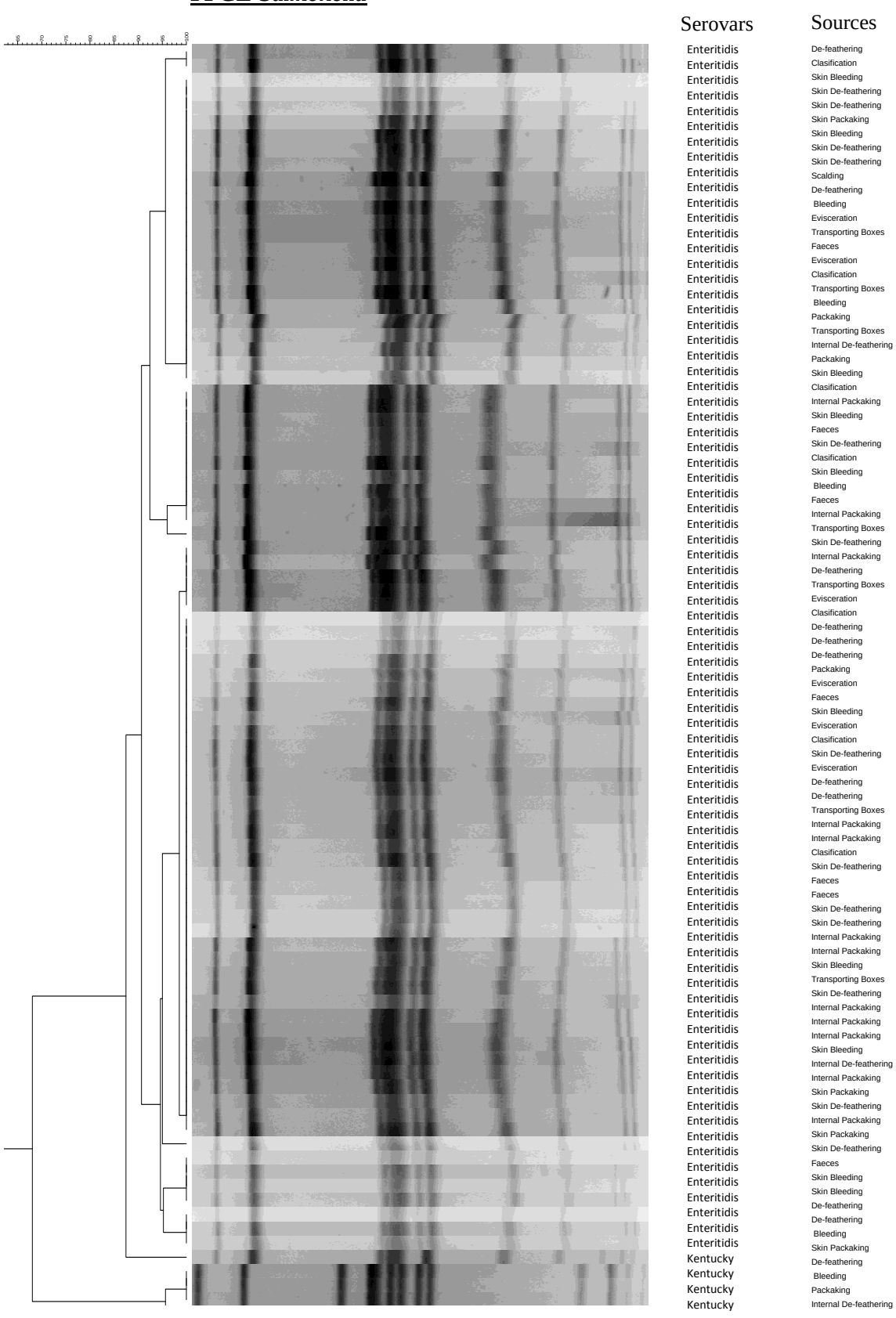

Figure 21. Two representative XBAI PGFE profiles of the 227 *Salmonella* isolates analysed and their similarity dendrogram (*S.* Braenderup H9812 was also restricted whit XbaI and used as a size standard).

Campylobacter

During this study, 720 samples were collected for the detection of *Campylobacter* and 49.8% were positive for the bacterium (n = 359).

Among the 36 flocks analysed, 84.2% were positive for the bacterium in cloacal swabs (n = 31). *C. jejuni* was detected as the most predominant specie, isolated in 55.3% of the positive cloacal samples.

A total of 252 environmental samples were collected from the different stages studies and no *Campylobacter* spp. was isolated in any of the stages studied.

Regarding the neck skin samples (n = 108) analysed at various stages of processing in the slaughterhouse (after exsanguination, after the defeathering and after air chilling), the presence of the bacteria was observed at 100% of the samples, independently of the processing stage. Moreover, *C. jejuni* was the most common serotype detected, isolated in 69.0% of the skin samples (n = 75).

IV.2. Experiment 2: Effect of modified atmosphere packaging against *Salmonella* and *Campylobacter*

The initial *Salmonella* and *Campylobacter* concentration (day 0) inoculated into the samples packed under the different MAPs studied was 6.5±0.2 log_{10} CFU/g.

Salmonella

After applying the different modified atmosphere conditions in inoculated chicken breast fillets packaged, significantly differences of *Salmonella* counts were observed among the different modified atmospheres within each sampling day (P = 0.000, Figure 22).

For samples packaged under MAP-A, the results of *Salmonella* counts at inoculated chicken breast fillets were 6.7 log_{10} CFU/g, 6.3 log_{10} CFU/g, 6.2 log_{10} CFU/g, 7.0 log_{10} CFU/g and 7.3 log10 CFU/g at 4, 8, 12, 16 and 20 sampling days, respectively. A significantly decrease of the *Salmonella* concentration was observed between 8 and 12 days of storage (P = 0.000), after that, the number of *Salmonella* colonies increased (Figure 22).

The application of MAP-B at inoculated chicken breast fillets showed that, during the storage, there were an increased of the *Salmonella* count by 7.8 log_{10} CFU/g, 8.9 log_{10} CFU/g, 9.3 log_{10} CFU/g, 10.2 log_{10} CFU/g and 10.8 log_{10} CFU/g at 4, 8, 12, 16 and 20 sampling days, respectively. Consequently, MAP-B did not control *Salmonella* growth in chicken breast fillets, since the numbers of the colonies increased constantly until the end of the storage (Figure 22).

According to the inoculated chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C), the results of *Salmonella* counts were 6.2 log_{10} CFU/g, 6.8 log_{10} CFU/g, 6.9 log_{10} CFU/g, 8.1 log_{10} CFU/g and 9.3 log_{10} CFU/g at 4, 8, 12, 16 and 20 sampling days, respectively. Significantly decrease of the *Salmonella* concentration was observed in samples packed under MAP-C at 4 day of storage (P = 0.001). After that, the number of *Salmonella* colonies increased until the end of storage

at 4 ºC (Figure 22).

For samples packaged under MAP-D, the results of *Salmonella* counts at inoculated chicken breast fillets were 7.6 log_{10} CFU/g, 8.2 log_{10} CFU/g, 8.7 log_{10} CFU/g, 10.7 log_{10} CFU/g and 10.2 log_{10} CFU/g at 4, 8, 12, 16 and 20 sampling days, respectively, resulting in a regularly increase (P = 0.000, Figure 22).

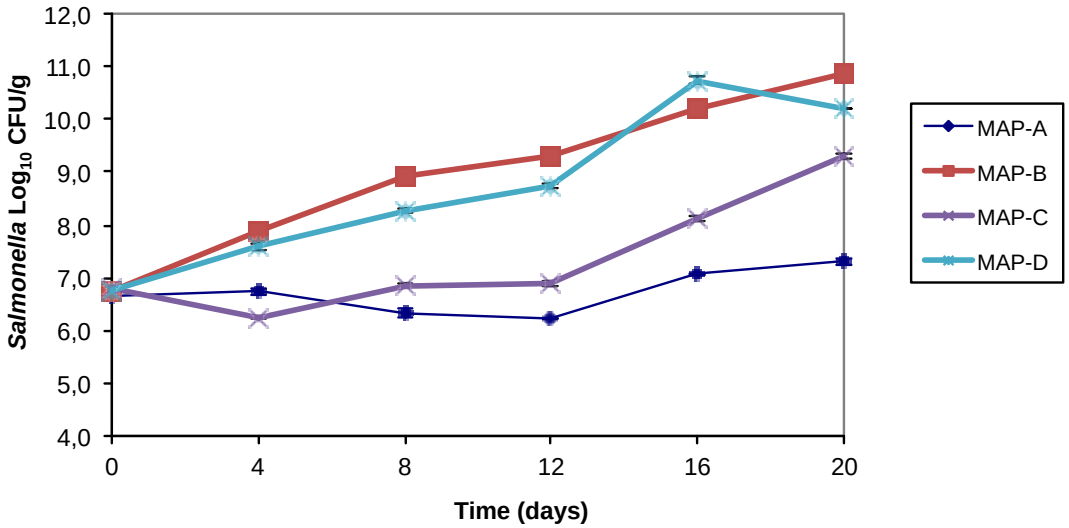

Figure 22. *Salmonella* growth (Log CFU/g ± S. D) at each sampling days, of inoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N*2* / Ar.) during 20 days of storage at 4 ºC. Vertical bars represent standard deviation.

Campylobacter

For samples packaged under MAP-A, the results of *Campylobacter* counts at inoculated chicken breast fillets were 6.2 log_{10} CFU/g, 6.4 log_{10} CFU/g, 5.9 log_{10} CFU/g, 5.8 log_{10} CFU/g and 5.9 log_{10} CFU/g at 4, 8, 12, 16 and 20 sampling days, respectively. The *Campylobacter* concentration slightly decreased in samples stored under MAP-A until 12 days of storage. After that, the *Campylobacter* counts maintained similar values, showing no significant differences at 16 and 20 days of storage at 4 ºC (P = 0.076, Figure 23).

From the beginning of the storage, application of MAP-B and standard poultry meat atmosphere (MAP-C) in chicken breast fillets helped at controlled *Campylobacter* growth. After 4 days of storage at 4 ºC, a total inhibition of *Campylobacter* was

observed in samples packaged under both modified atmosphere conditions (Figure 23).

For samples packaged under MAP-D, the *Campylobacter* concentration significantly decreased, reaching values of 4.3 log_{10} CFU/g, 3.5 log_{10} CFU/g, 3.6 log_{10} CFU/g at 4, 8 and 12 sampling days, respectively *(P* = 0.000). After that, a total inhibition of *Campylobacter* colonies was observed till the end of the storage at 4 ºC (Figure 23).

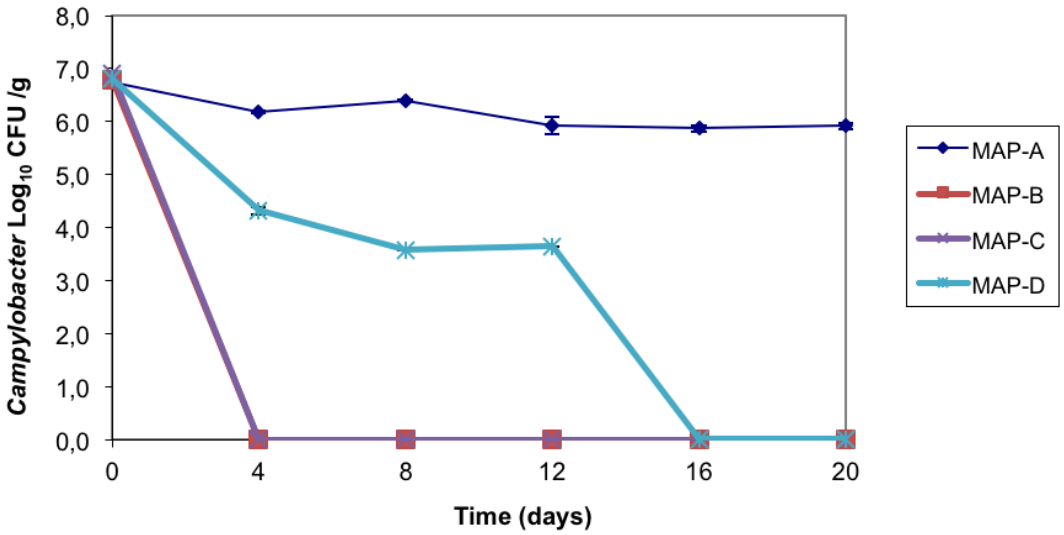

Figure 23. *Campylobacter* growth (Log CFU/g ± S. D) at each sampling days, of inoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N*2* / Ar.) during 20 days of storage at 4 ºC. Vertical bars represent standard deviation.

Physical-Chemical Analysis

pH determination

The initial pH (day 0) of chicken breast fillets stored under the different MAPs studied was 5.2 ± 0.2.

For samples packaged under MAP-A, the results of pH were 4.9, 5.3, 5.2, 5.2, and 5.1 at 4, 8, 12, 16 and 20 sampling days, respectively. Considering samples packaged under MAP-B the results of pH were 5.3, 5.3, 5.5, 5.6 and 5.6 at 4, 8, 12, 16 and 20 sampling day, respectively. According to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C), the results of pH were 5.2, 5.1, 5.3, 5.4, and 5.4 at 4, 8, 12, 16 and 20 sampling days, respectively. For samples packaged under MAP-D the results of were 5.1, 5.3, 5.6, 5.5, and 5.6 at 4, 8, 12, 16 and 20

sampling days, respectively. During the storage, significantly differences of pH values were not observed among the samples stored under different modified atmospheres (P > 0.050, Figure 24).

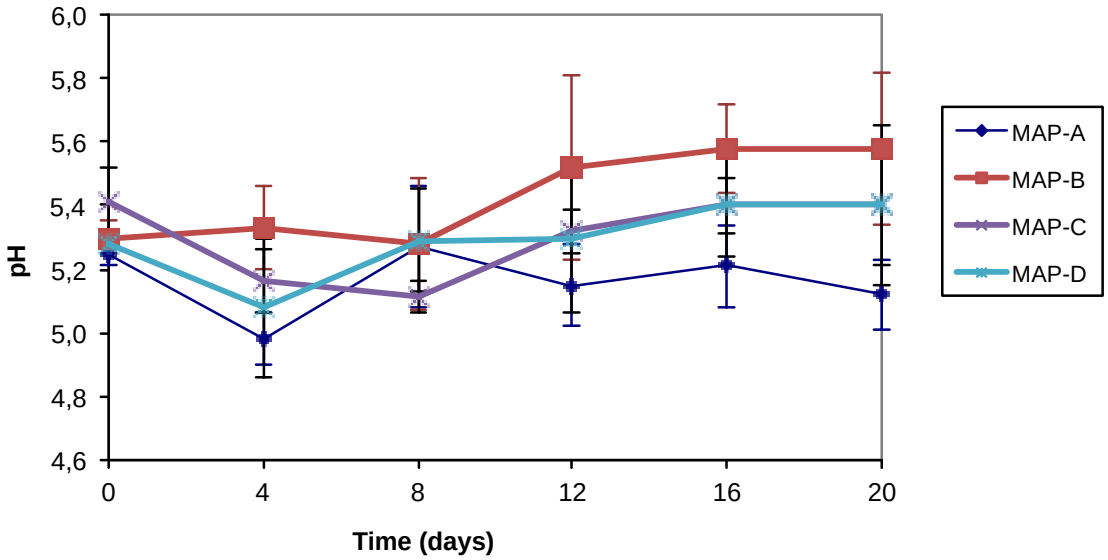

Figure 24. Changes in pH of uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. Vertical bars represent standard deviation.

Color measurement

The initial values of L*, a*, and b* parameters (day 0) of chicken breast fillets stored under MAP-A, MAP-B, MAP-C and MAP-D were 57.2±0.9, 0.6±0.2, 5.4±0.9, respectively.

For samples packaged under MAP-A, the values of L* were 59.5, 56.5, 60.2, 57.1 and 59.4 at 4, 8, 12, 16 and 20 sampling days, respectively. Similar trend was observed for samples packaged under MAP-B with values of 54.1, 55.8, 52.2, 55.8 and 54.7 at 4, 8, 12, 16 and 20 sampling days, respectively. According to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C) the values of L* were 58.3, 58.7, 63.6, 64.6 and 66.3 at 4, 8, 12, 16 and 20 sampling days, respectively. Considering samples packaged under MAP-D the results were 59.3, 53.9, 54.8, 56.8 and 54.5 at 4, 8, 12, 16 and 20 sampling days, respectively (Figure 25).

The L* value which refers to the lightness showed no statistical changes throughout the storage for samples packaged under MAP-A, MAP-B and MAP-D (P >

0.050). However, the L* values of the samples packaged under standard poultry meat MAP (MAP-C) increased progressively up to day 20 of the storage (P = 0.000, Figure 25).

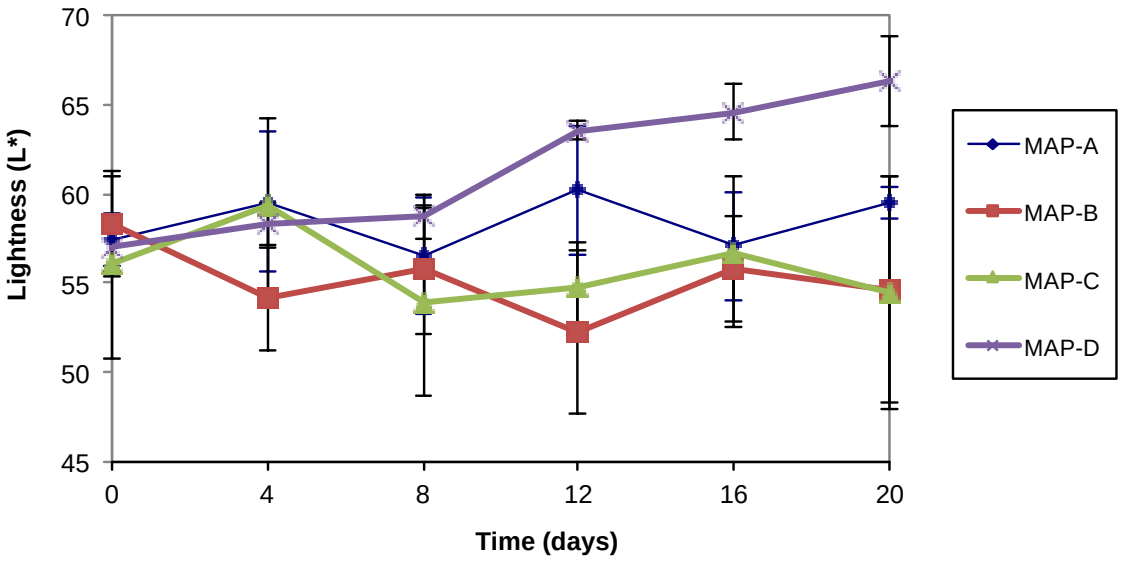

Figure 25. Color changes of L* values from uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. Vertical bars represent standard deviation.

For samples packaged under MAP-A, the values of a* were 0.4, 1.4, -0.1, 0.8 and -0.6 at 4, 8, 12, 16 and 20 sampling days, respectively. According to the chicken breast fillets packaged under MAP-B, the results were 1.4, 0.1, 0.3, -0.4 and 0.1 at 4, 8, 12, 16 and 20 sampling days, respectively. Regarding to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C) the values of a* were 0.9, 2.2, -0.4, -1.1 and -1.5 at 4, 8, 12, 16 and 20 sampling days, respectively. Considering samples packaged under MAP-D, the results were 0.3, 0.6, 1.1, 1.4 and 1.4 at 4, 8, 12, 16 and 20 sampling days, respectively (Figure 26).

The a* value which refers to the redness showed no statistical changes throughout the storage of samples packaged under MAP-A, MAP-B and MAP-D (P > 0.050, Figure 18). Nevertheless, chicken meat fillet samples packaged under standard poultry meat atmosphere (MAP-C) had a significant decrease during the storage, reaching lower a* values at the end of the storage (P = 0.023, Figure 26).

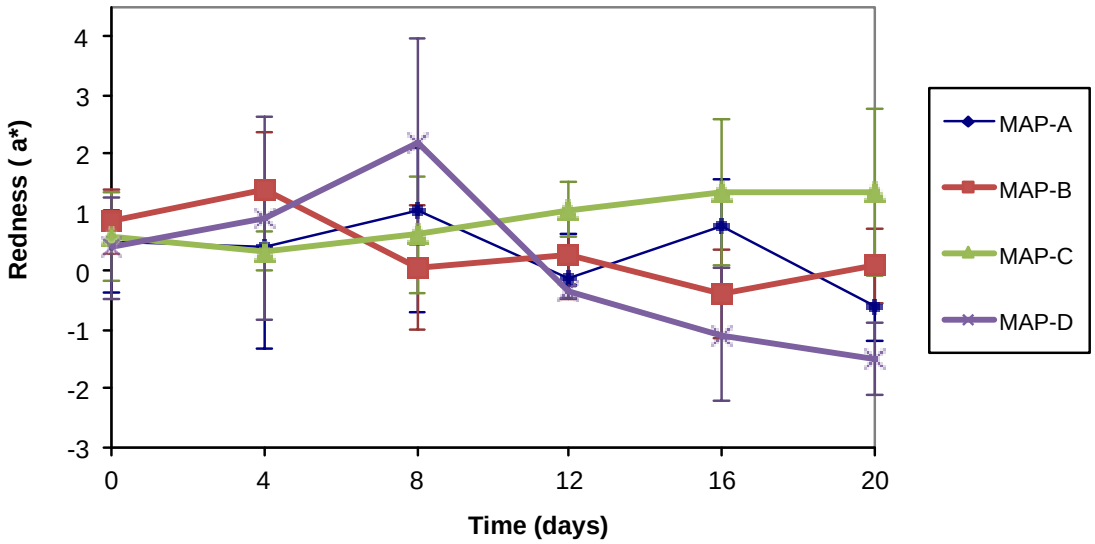

Figure 26. Color changes of a* values from uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. Vertical bars represent standard deviation.

For samples packaged under MAP-A, the b* values were 4.2, 5.9, 5.8, 5.9 and 5.7 at 4, 8, 12, 16 and 20 sampling days, respectively. According to the chicken breast fillets packaged under MAP-B, the results were 4.5, 6.5, 5.9, 6.3 and 6.6 at 4, 8, 12, 16 and 20 sampling days, respectively. Regarding to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C) the values of b* were 3.9, 6.4, 5.1, 5.7 and 4.5 at 4, 8, 12, 16 and 20 sampling days, respectively (Figure 27). Considering samples packaged under MAP-D, the results were 3.7, 4.6, 4.8, 3.7 and 4.5 at 4, 8, 12, 16 and 20 sampling days, respectively.

The b* value which refers to the yellowness showed no statistical changes throughout the storage of samples packaged under MAP´s studied ($P > 0.050$, Figure 27).

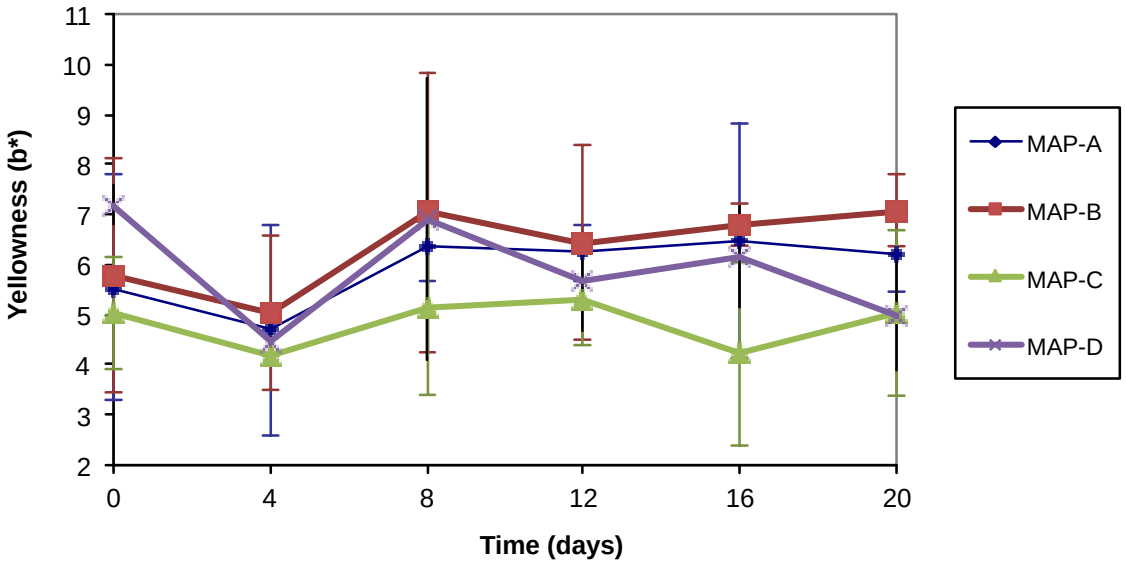

Figure 27. Color changes of b* values from uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. Vertical bars represent standard deviation.

Sensory analysis

Regarding to the sensory evaluation, the initial scores of appearance, odor and acceptability (day 0) of chicken breast fillets stored under the different MAPs studied was 5 (the highest score).

Appearance

For samples packaged under MAP-A, the results of appearance evaluation were 4.0, 4.0, 3.5, 2.5 and 1.5 at 4, 8, 12, 16 and 20 sampling days, respectively. Similar behaviour was observed for samples packaged under MAP-B being scored as 3.8, 2.0, 1.3, 1.0 and 1.0 at 4, 8, 12, 16 and 20 sampling days, respectively. According to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C) the results of appearance evaluation were 4.6, 3.5, 3.5, 2.8, and 1.8 at 4, 8, 12, 16 and 20 sampling days, respectively. Considering samples packaged under MAP-D the results were 4.5, 3.8, 4.3, 3.2, and 3.6 at 4, 8, 12, 16 and 20 sampling days, respectively (Figure 28).

Chicken breast fillets samples packaged under MAP-A, MAP-B, MAP-C were evaluated by the judges below the limit of acceptability (score as 3) after 12, 4 and 12 days of storage, respectively. On the other hand, samples stored under MAP-D were scored over the limit of acceptability throughout the storage. Significantly decrease of appearance evaluation was observed among the different modified atmospheres within each sampling day ($P < 0.05$, Figure 28).

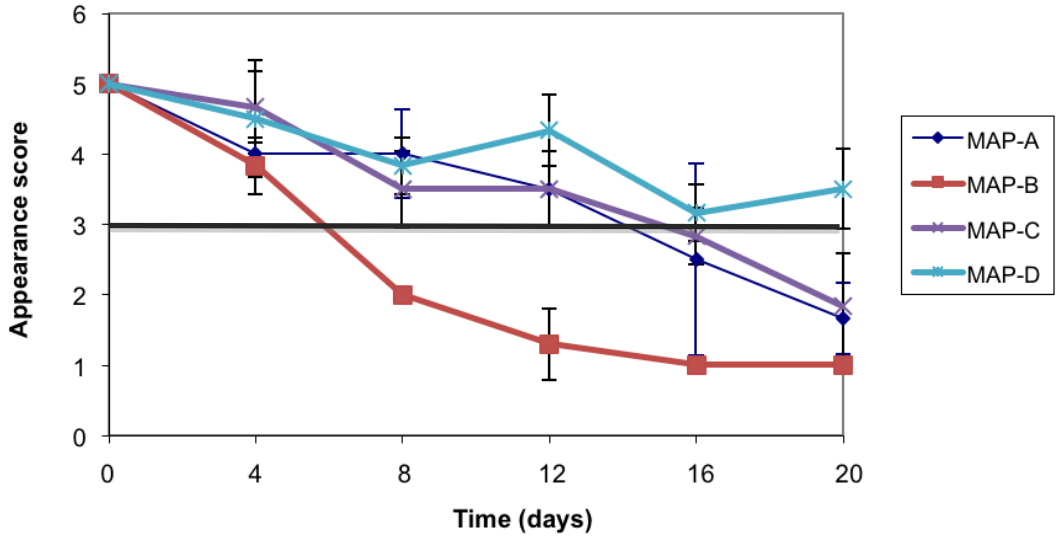

Figure 28. Appearance score of uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. The black line represented the limit of acceptability of appearance evaluation, scored as 3. Visual appearance was based on a visual scale (5 = Like extremely; 4 = like moderately, 3 = neither like; 2 = dislike moderately; and 1 = dislike extremely). Vertical bars represent standard deviation.

Odor

For samples packaged under MAP-A, the results of odor evaluation were 4.2, 4.3, 3.5, 2.3 and 1.0 at 4, 8, 12, 16 and 20 sampling days, respectively. According to the chicken breast fillets packaged under MAP-B, the results were 3.7, 1.7, 1.7, 1.0 and 1.0 at 4, 8, 12, 16 and 20 sampling days, respectively. Regarding to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C) the results of odor evaluation were 4.9, 4.2, 2.0, 1.0, and 1.0 at 4, 8, 12, 16 and 20 sampling days, respectively. Considering samples packaged under MAP-D, the results were 4.5, 3.5, 1.2, 1.0 and 1.0 at 4, 8, 12, 16 and 20 sampling days, respectively (Figure 29).

Chicken breast fillets samples packaged under MAP-A, MAP-B, MAP-C and MAP-D were evaluated by the judges below the limit of acceptability of odor (scored as 3) after 12, 4, 8 and 8 days of storage, respectively. The odor evaluation for all the samples packaged under MAPs studied decrease significantly during the storage ($P < 0.05$, Figure 29).

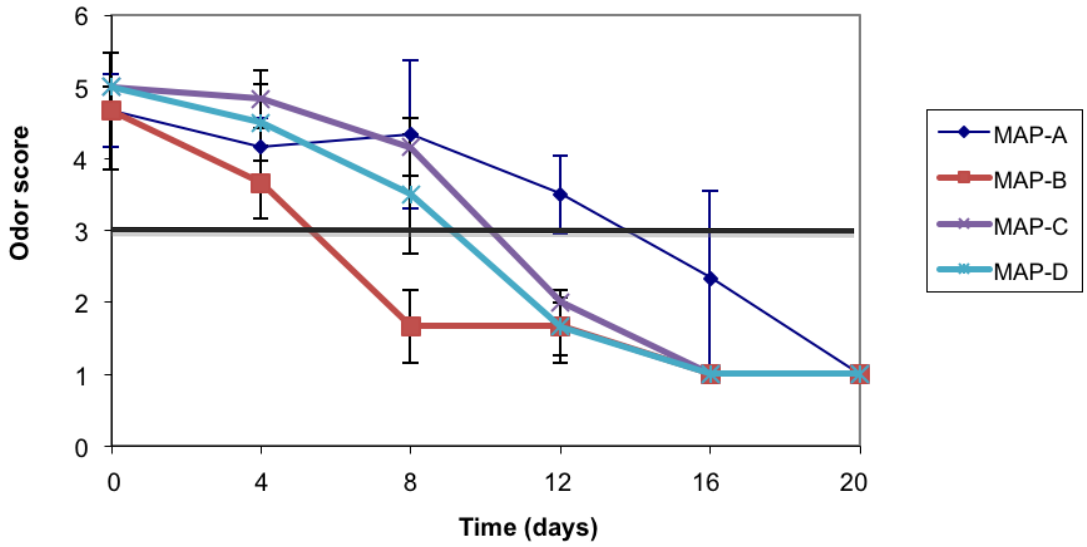

Figure 29. Odor score of uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. The black line represented the limit of acceptability of odor evaluation, scored as 3. Odor evaluation was based on a visual scale (5 = Excellent; 4 = good, 3 = fairly acceptable; 2 = unacceptable; and 1 = dislike). Vertical bars represent standard deviation.

Overall Acceptability

For samples packaged under MAP-A the results of overall acceptability evaluation were 3.8, 4.5, 4.2, 2.3 and 1.8 at 4, 8, 12, 16 and 20 sampling days, respectively. According to the chicken breast fillets packaged under MAP-B, the results were 3.8, 2.0, 1.7, 1.0 and 1.0 at 4, 8, 12, 16 and 20 sampling days. Regarding to the chicken breast fillets packaged under standard poultry meat atmosphere (MAP-C) the results were 4.8, 4.2, 2.7, 2.0, and 1.3 at 4, 8, 12, 16 and 20 sampling days, respectively. The results of overall acceptability evaluation for samples packaged under MAP-D were 4.2, 3.8, 3.2, 3.5, and 3.2 at 4, 8, 12, 16 and 20 sampling days, respectively.

Chicken breast fillets samples packaged under MAP-A, MAP-B and MAP-C were evaluated by the judges below the limit of overall acceptability (scored as 3) after

12, 4 and 8 days of storage, respectively. Whereas, samples stored under MAP-D were scored over the limit of acceptability of appearance throughout the storage. Significantly decrease of overall acceptability evaluation was observed among the different modified atmospheres within each sampling day ($P < 0.05$, Figure 30).

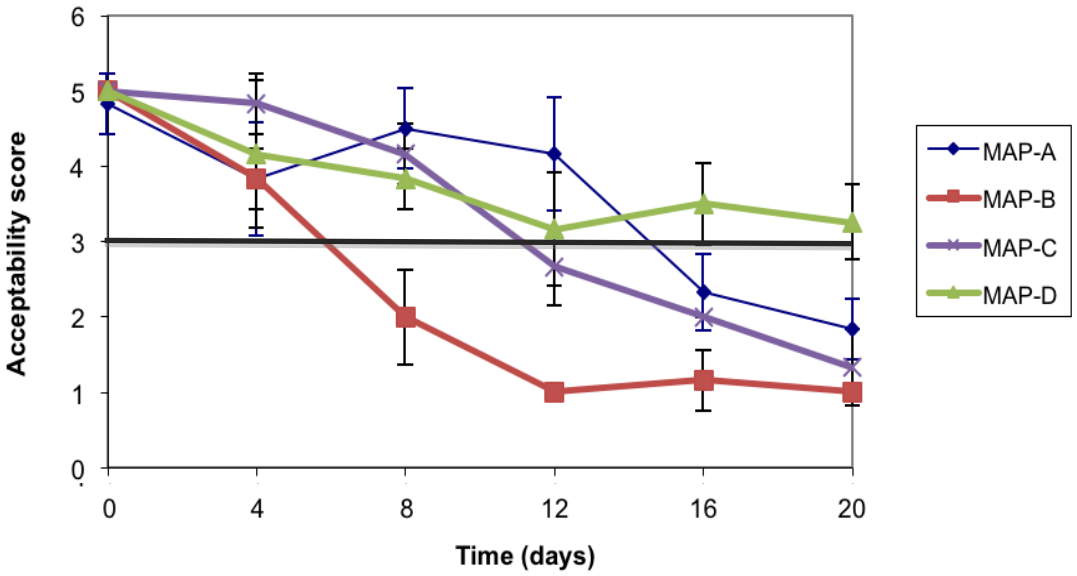

Figure 30. Overall acceptability score of uninoculated chicken breast fillets packaged under MAP conditions (MAP-A: 50%/50% N_2 / CO_2. MAP-B: 50%/50% N_2 / O_2. MAP-C: 30%/70% O_2 / CO_2. MAP-D: 50%/50% N_2 / Ar.) during 20 days of storage at 4 ºC. The black line represented the limit of acceptability of overall acceptability evaluation, scored as 3. Overall acceptability evaluation was based on a visual scale (5 = extremely good; 4 = very good, 3 = neither like; 2 = dislike moderately; and 1 = dislike extremely). Vertical bars represent standard deviation.

CHAPTER V. DICUSSION.

V.1 Experiment 1: Epidemiology of *Salmonella* and *Campylobacter* at poultry slaughterhouse under Spanish standard commercial conditions

Salmonella and *Campylobacter* are two of the most important causes of human bacterial gastroenteritis in the industrialized world (EFSA, 2014). Epidemiological studies identified that handling of the raw poultry and eating poultry products are the most common source of human infection for both pathogens (Laroche and Magras, 2013).

Salmonellosis and campylobacteriosis control are commonly focused on the importance of the status of the flock and the reduction of the occurrence of both pathogens at the farm level (Marin and Lainez, 2009; Berghaus *et al.,* 2013). Nevertheless, contamination of poultry and poultry meat may occur throughout the whole production chain from the rearing period at the poultry farm, to the processing line at the slaughterhouse (Rosenquist *et al.,* 2006).

The infection of the chickens at the farm level has been studied for several authors in different countries (Berghaus *et al.,* 2013 Barua *et al.,* 2013; Thakur *et al.,* 2013). Marin and Lainez (2009) studied the main sources for *Salmonella* contamination of broilers flocks, during the rearing period under Spanish conditions. The authors reported that the most contaminated samples during the rearing period were delivery-box liners, faeces, dust, farming boots and feed from feeders. Furthermore, the main risk factors of *Salmonella* at the end of the rearing were feed from feeders, *Salmonella* status of the house after cleaning and disinfection procedures and *Salmonella* status of day-old chicks. Other authors also emphasized on the importance of size of the farm (Heyndrickx *et al.,* 2002), rearing flocks in autumn and the presence of litter-beetle in the house (Chinivasagam *et al.,* 2010; Varga *et al.,* 2013) as *Salmonella* risk factors at the poultry farm. For *Campylobacter*, several pathways have been suggested to explain flock colonization during the rearing including vertical transmission, contamination from previous flock and exposure to potential source of the bacterium such as other animals on the farm, insects, rodents, environment, litter and drinking water (Toth *et al.,* 2013; Wassenaar *et al.,* 2011).

Moreover, holding and transportation from the poultry farm to slaughterhouse are known to be also risk factors for both pathogens contamination (Ellerbroek *et al.*, 2010; Marin and Lainez, 2009). These activities are stressful for poultry, causing a disturbance of intestinal functions and may lower the resistance of the live animal and increase spreading of intestinal bacteria (Rasschaert *et al.*, 2008; Scherer *et al.*, 2008). Reiter *et al.* (2007) reported that transport to the processing plant increased the *Salmonella* prevalence of positive birds due to faecal contamination of the skin and feathers by neighbouring infected birds during shipping. Related with this hypothesis, Ellerbroek *et al.* (2010) reported that prolonged crating of the animals was a contributor to the *Campylobacter* contamination of processed broiled carcasses. Moreover, the use of contaminated trucks during transport is a great concern as *Salmonella* could infect free flocks (Habib *et al.*, 2010, Marin and Lainez, 2009).

Consequently, the status of the flock at the arrival to the slaughterhouse is a challenge to avoid the cross-contamination during the processing (McCrea *et al.*, 2006). In this study, the results showed that the flocks were highly infected by both pathogens at the arrival to the slaughterhouse. The scientific report of EFSA, showed a prevalence of *Salmonella* on broiler chicken flocks ranging from 4.9 to 100% among the European Countries in 2012 (EFSA, 2014). Our results showed that 69.4% of the broiler flocks were colonized by *Salmonella* at their arrival to the poultry processing plant. A high *Salmonella* rate was also detected by Henry *et al.* (2012), where 65% of the broiler chicken flocks processed were *Salmonella* positive. Concerning to the *Campylobacter* status, 84.2% of the broiler flocks were positive according with several authors (Rosenquist *et al.*, 2006; Gloaguen *et al.*, 2010; Chokboonmongkol *et al.*, 2013). There are different *Salmonella* and *Campylobacter* control strategies at the farm level, trying to avoid the infection of the flock. The use of biosecurity measures to eliminate the bacteria from the flocks, include the insect control (Fraser *et al.*, 2010) and correct programs of cleaning and disinfection (Cardoso *et al.*, 2008). Furthermore, non-biosecurity-based approaches, such as antibacterial treatments, probiotics, or vaccination, which prevent the presence or reduce the concentration of bacteria, have been also developed to control both pathogens during the rearing period (Berghaus *et al.*, 2011; Rocha *et al.*, 2011).

Moreover, the control of these bacteria has to continue until the end of the processing chain, since the contamination of the product might be also affected by the degree of cross-contamination occurring during the slaughterhouse stages (Sequeira *et al.*, 2013). Several authors studied the different factors that influenced the contamination of the processing line (Hue *et al.*, 2011; Franz *et al.*, 2012). The results of the present study showed that, the surfaces of de-feathering and grading stages were the most *Salmonella* contaminated stages in the processing line. The stages of exsanguination, evisceration and transporting crates also presented high levels of *Salmonella* contamination. According with these results, several studies showed that the de-feathering stage consistently increased *Salmonella* contamination during this processing level (El-Alziz, 2013; Sasaki *et al.*, 2013). Generally, several authors reported that, in latter stages of the processing line, the overall contamination decreased (Fraqueza and Barreto *et al.*, 2009; Franz *et al.*, 2012). However, the results of the study showed that, the grading stage had a high level of *Salmonella* contamination. Choi *et al.* (2014) reported that, although the different washes carried out during the processing reduced the microbial load throughout the slaughter line, *Salmonella* had a high capacity to survive in the environment protected by the organic material, and located in the cracks and gaps of the surfaces. Evisceration stage is also considered an important source of contamination (Elvers *et al.*, 2011; Ivanova *et al.*, 2014). The automatic evisceration used in the most of the slaughterhouses, could not adapt itself to the natural size variation of carcasses, resulting in the rupture of internal organs and the liberation of faecal content (Goddard *et al.*, 2014). The transporting crates are also related with *Salmonella* contamination, since the 47.0% of these were positive. Henry *et al.* (2012) confirmed that poorly washed crates was an important source of *Salmonella*. On the other hand, air chilling and scalding stages showed the least *Salmonella* contamination rate. Scalding stage has been related with the *Salmonella* reduction, since the high temperatures in the tanks inhibit the bacterium spoilage (Berrang *et al.* 2011). However, several authors emphasised that *Salmonella* could survive in scald water likely protected by faecal particles and feathers (Rasschaert *et al.*, 2008; Henry *et al.*, 2012; Choi *et al.*, 2014). In order to reduce the microbiological contamination, the scalding water should be changed often, not only at the end of the working day (Bucher *et al.*, 2012). Moreover, decreases of *Salmonella* contamination are commonly associated with chilling conditions (Henry *et al.*, 2012; Giombelli *et al.*, 2013).

On the other hand, the results of *Campylobacter* contamination on the different stages studies showed that, no *Campylobacter* spp. was isolated in any of the environmental samples analysed. It has been reported that, the resistance of *Campylobacter* is reduced in the environment of the slaughterhouse and it would be unlikely that it persists in that after cleaning and disinfection procedures (Garénaux *et al.,* 2009). However, it has been also described that *Campylobacter* might enter in the state of viable but nonculturable bacteria after exposure to different stress factors, which can occur during the process of sampling and storing cells (Bronowski *et al.,* 2014). Nevertheless, the bacteria could survive and revert to culturable conditions when provided with appropriate conditions (Bronowski *et al.,* 2014). Since traditional standard culture methods are not able to detect low concentration of contamination and nonculturable *Campylobacter,* further studies are needed to understand the epidemiology of *Campylobacter* at the poultry slaughterhouses facilities (Kiess *et al.,* 2010).

Moreover, the results of *Salmonella* carcasses contamination on the neck skin and from internal cavity samples showed that, the external contamination decreased until the end of the process. However, the internal contamination of the carcasses increased during the slaughter. Otherwise, our results showed that high levels of *Campylobacter* contamination neck skin were detected in the three select stages and no differences were observed among them. At the processing, *Salmonella* and *Campylobacter* presence in the gastrointestinal tract of chickens could contaminate the external surface of the carcasses and the processing line, increasing the risk of the contamination of both pathogens (Franz *et al.,* 2012). The rapid rate of production, close proximity of the carcasses and limitations in the design of processing equipment, might difficult the right wash of the internal cavity, giving an ideal environment for the bacteria protection (Rosenquist *et al.,* 2006). Additionally, the results of the influence of different processing stages studied on the contamination of both pathogens on chicken carcasses during the slaughter process showed that, the carcasses from exsanguination and de-feathering stages presented high percentage of contaminated product. Hue *et al.* (2001) reported the importance of the exsanguination stage on *Campylobacter* contamination having high possibility of contact between intestinal material and carcasses. Furthermore, the contamination of carcasses of both pathogens increased during the de-feathering, due to the escape of fecal material through the cloaca by the

action of the picker fingers pressing abdomen and enhancing the contamination of external and internal surfaces of the carcasses (Rosenquist *et al.,* 2006; Chokboonmgkol *et al.,* 2013). In contrast, air chilling conditions are related with the reduction of the overall contamination of the carcasses (Giombelli *et al.,* 2013). It has been shown that, the numbers of contaminated carcasses might be reduced by controlling stressors such as drying of skin surface or cold conditions (Guerin *et al.,* 2010).

Furthermore, the status of the live flock was also considered as an important factor for safety quality of the finished product (Gloaguen *et al.,* 2011). The carcasses, belonged to positive *Salmonella* flock, were higher contaminated of *Salmonella* at the exsanguination stage than those from the negative flock. However, at the stages of the de-feathering and air chilling, no differences of *Salmonella* contamination were observed both between positive and negative *Salmonella* flocks and between the two stages, this fact demonstrate the cross-contamination between different flocks and slaughter surfaces (Gloaguen *et al.,* 2011). Generally, slaughterhouse operational hygiene and quality managements are close related with microbial carcasses status (Peyrat *et al.,* 2008; Choi *et al.,* 2014). Therefore, it has been reported that, the improvement of the processing line operations could reduce the contamination levels of the finished products (Hue *et al.,* 2001). Poor performance of slaughter operations, such as increase of temperature in the evisceration room and presence of visible dirty marks, increased the chances to have contaminated carcasses (Henry *et al.,* 2012). During the processing, poultry carcasses became contaminated manly with fecal contents, being a significant controllable food safety hazard for poultry processing (Kidie *et al.,* 2013). Therefore, preventive methods to reduce fecal contamination of the poultry carcasses have been carried out at the poultry farm and the slaughter plant. At farm level, measures like withdrawal of feed and water from animals for 8 to 12 h before slaughter processing, (Rosenquist *et al.,* 2006) and feed formulation that rapidly clear the digestive tract (Faber *et al.,* 2012) reduced the amount of fecal material at slaughter. At the slaughterhouse level, several studies reported that the application of new procedures to remove fecal material at early stages of processing such as, cloacal plugging and vent suturing, could minimize the risk of contamination and cross contamination (Burh *et al.,* 2003; Northcutt *et al.,* 2008). Washing procedures of the carcasses with rupture of the viscera (Guerin *et al.,* 2010), the application of multistage scalding and the use of approved chemicals (Rejab *et al.,* 2012), were also considered as potential management

options giving a reduction of the contamination. Moreover, the application of complementary and innovative post-slaughter operations like chemical treatments with ozone and peroxy acids, physical treatments with steam-ultrasound, forced air chilling, irradiation interventions and modified atmospheres packaging, may improve the safety and extend the shelf life of the finished product (Rajkovic *et al.,* 2010; Keklik *et al.*, 2012; Tananuwong *et al.*, 2012).

Two different serotypes of *Salmonella* were identified through the processing (*S.* Enteritidis and *S.* Kentucky), and *S.* Enteritidis was the most common one. This result is in agreement with other Spanish studies (Capita *et al.,* 2007, Marin and Lainez, 2009), in which *S.* Enteritidis was the most isolated serotype in Spanish broilers flocks at slaughterhouses and in human cases of salmonellosis over the last few years. Capita *et al.* (2007) used genotypical characterization and the same genetic pattern was found from all *S.* Enteritis and from *S.* Kentucky isolated in this study. Considering that the strains isolated from different farms, owned by the same integrated company, were grouped into the same cluster, suggested a strong genetic relationship among the *Salmonella* strains from broiler breeder. As reported before, the importance of vertical transmission has been demonstrated by several authors (Cox *et al.,* 2012; Zhang *et al.*, 2103) For this reason, all the breeding flocks that are positive for *Salmonella* infection have to be slaughtered under special conditions (Commission Decision 2013/22EU). Therefore, the processing of *Salmonella* positive flocks lead to a contamination of the slaughter line, a cross-contamination during the slaughter and a re-circulation of the bacteria at the farm by the transporting trucks and crates (Olsen *et al.,* 2003). Davies and Wray (1997) also observed the persistence of single common pattern of *S.* Enteritidis from integrated poultry organization in UK. The authors reported that *Salmonella* strain re-circulated across to the poultry flocks and re-entered in the slaughterhouse, surviving on the line. Other previous studies showed that some *Salmonella* serotypes, which were normally detected at the farm level, were also recovered in the environment of the slaughterhouse and in the finished products (McBride *et al.,* 1980; Lahellec and Collin 1985; Jones *et al.,* 1991). In the same way, *C. jejuni* was the most common strain isolated through the processing line and at the final product, which corresponded with the most common strain that causes human outbreaks in Europe (EFSA, 2014).

In conclusion, the status of *Salmonella* and *Campylobacter* flock at the arrival of the live birds at slaughterhouse showed high levels of contamination for both pathogens. According with the slaughterhouse environment, *Salmonella* contamination decreased after scalding and chilling stages. In contrast, *Salmonella* contamination increased after bleeding, de-feathering, grading and evisceration. Nevertheless, no *Campylobacter* spp. was isolated in any of the environmental samples analysed. The impact of different processing stages on the contamination of chicken carcasses showed that, *Salmonella* neck skin samples were higher contaminated at the de-feathering and exsanguination than at the air chilling stage. However, the internal surface cavity samples were higher *Salmonella* contaminated at the air chilling, and de-feathering than at the exsanguination stage. Moreover, samples from the neck skin were determined most frequently as *Salmonella* positive than those from internal surface cavities at the exsanguination and de-feathering stages. However, at the air chilling stage, samples from the internal cavity were more positive than those from neck skin. On the other hand, chicken carcasses were highly contaminated with *Campylobacter* throughout the processing, observing no differences among the different stages. The most prevalent strains isolated in this study were *S.* Enteritis and *C. jejuni*, the most prevalent strains involved in human outbreaks. Finally, the genetic relation study of *Salmonella* strains isolated revealed that, the same genetic pattern was found from all *Salmonella* isolates, suggesting a bacteria re-circulation across the poultry farms and slaughterhouse facilities.

V.2 Experiment 2: Effect of modified atmosphere packaging against *Salmonella* and *Campylobacter*

As previously described, chickens carry millions of bacteria internally and externally, being an excellent vehicle for foodborne pathogens, specially *Campylobacter* and *Salmonella* (Capita *et al.,* 2007). Both pathogens have been known to persist on chicken skin during poultry processing because of their ability to attach to the skin and become entrapped in deeper skin layers, crevices, or feather follicles (Gloaguen *et al.,* 2011). These sites may provide a suitable microenvironment for bacteria to lodge (Hardy *et al.,* 2013). Since the consumption of poultry meat increased in the last two decades due to its nutritional profile, versatility and low price, the control of the microbial quality of poultry meat is a crucial concern for the food industry (Henchion *et al.,* 2014). Chicken meat is a highly perishable product even when stored

in chilled conditions, and its shelf life is less than 5 days after slaughter (Buchr *et al.*, 2014). Consequently, a combination of different conservation techniques, such as chilled storage, modified atmosphere packaging (MAP), freezing and preservatives methods, are necessary to lower the potential of foodborne illness and to extend the product's shelf-life (Kozačinsk *et al.,* 2012). However, since the consumers are demanding for minimally processed foods, the most attractive technology is modified atmosphere packaging (Melero *et al.,* 2012). MAP contributes to microbial and lipid oxidation stability of poultry meat and prolong their shelf-life compared to those packaged in ambient conditions (Fraqueza and Barreto, 2009). Moreover, MAP is related with safety product leading to an effective reduction of pathogenic microorganisms like *Campylobacter* spp. and *Salmonella* spp (Boysen *et al.,* 2007; Kudra *et al.,* 2011).

The results of this study showed that, the application of atmosphere with anaerobic conditions was the most effective treatment among the gas mixtures studied to control *Salmonella* Enteritidis growth inoculated onto fresh chicken fillets stored at 4 ºC (MAP-A). CO_2 is widely used in gas mixtures for fresh meat products due to its antimicrobial properties. Carbon dioxide atmosphere packaging inhibited *Enterobacteriaceae* growth, including *Salmonella,* by prolongation of the lag phase and generation time during the logarithmical phase growth (Floros and Matsos, 2005). Gram-negative are generally more sensitive to CO_2 than Gram-positive bacteria (Church, 1994), since most of the second ones are facultative or strict anaerobes (Gill, 1988). McMillin (2008) reported that levels of 20–60% CO_2 were required to be effective against aerobic organisms by penetrating membranes and lowering intracellular pH, thus changing the cellular metabolic processes. Another reason of the inhibitory effect of CO_2 included the inhibition of substrate uptake by microorganisms (Farber, 1991), and the direct impact on enzymes as well as the alteration on the properties of proteins (Dixon and Kell, 1989). Khawla *et al.* (2005) studied the effect of high CO_2 MAP (70%/30% CO_2 / N_2) against *Salmonella* Infantis in chilled chicken breasts. The authors observed that *Salmonella* counts were reduced by 72% followed by stability, when stored at 7 °C. These results contrast with those found in our study, where *Salmonella* counts were reduced till 8 day of storage. After that, the MAP-A was not effective to control *Salmonella* growth, increasing the counts of the bacteria till the end of the storage. It is widely known that, the CO_2 effect on microorganisms depends

on several conditions such as concentration of the gas, the initial bacterial load, the temperature of storage and the type of food (Fernandes *et al.*, 2014). Oscar (2007) also reported that the initial concentration of *Salmonella* is an important factor in growth rate. Moreover, the results of another study suggested that the generation time of *S.* Enteritidis varied considerably at lower temperatures (Fehlhaber and Krüger, 1998).

The results of the standard poultry meat modified atmosphere (MAP-C) application showed a similar effectiveness as observed with MAP-A (anaerobic condition) against *Salmonella* at the beginning of the storage. However, after 4 days of storage, MAP-C was not able to control *Salmonella* growth. The effect of CO_2 against *Salmonella* was directly related with the gas concentration. As observed by Provincial *et al.* (2013), fresh sea bream fillets packaged under fewer than 60% of CO_2 showed lower inactivation rate of *Salmonella* compare to 70% and 80% of CO_2. Therefore, application of atmospheres with higher concentrations of CO_2 showed better inhibition/control of the *Salmonella* growth than those packed under lower or no presence of carbon dioxide (Provincial *et al.*, 2013).

Atmospheres with high concentration of O_2 and Argon did not controlled *Salmonella* growth, (MAP-B and MAP-D, respectively. Similar results were also reported by Hulánková *et al.* (2010). The authors observed that *S.* Enteritidis initial concentrations of 4.0, 2.5, 1.5 and 0.5 log were not significantly decreased by the application of 20%/80% CO_2 / O_2 MAP in chicken legs stored at 3 ºC. However, samples stored under 30%/70% CO_2 / N_2 showed a considerable decrease of *S.* Enteritidis only for those with an initial concentration of 4.0 and 2.5 log. Nychas and Tassou (1996) also studied the *S.* Enteritidis survive in chicken breast stored under high O_2 atmosphere (20%/80% CO_2/ O_2) at 3 and 10 ºC. At low temperature *S.* Enteritidis survived and no significantly growth was observed during the storage. However, at 10 ºC, the numbers of *S.* Enteritidis increased rapidly under high O_2 atmosphere. Moreover, according to our results, other studies described that mixtures of Ar with N_2 and O_2 were less effective against *Salmonella* than those based on CO_2 (Enomoto *et al.*, 1997; Debs-Louka *et al.*, 1999). Ruiz-Capillas and Jiménez-Colmenero (2010) reported that the use of Ar MAP for cooked sausages was not significantly effective on *Salmonella* growth. Similarly, Parra *et al.* (2010) indicated that gases mixture with Ar for packaged dry-cured ham did not control *Salmonella* growth during 120 days of storage.

Nevertheless, Fraqueza and Barreto (2009) observed a beneficial effect in uncooked turkey meat stored in MAP with Argon. In this study, Turkey breast samples were placed in high barrier bags containing four different gas flush combinations: 100% N_2; 50%/50% Ar / N_2; 50%/50% Ar / CO_2; or 50%/50% N_2 / CO_2 for up to 25 days of storage. At the end of the storage, turkey samples packaged under the 50%/50% Ar / CO_2 had one log lower growth for psychrotrophic, total anaerobic counts as well as for *Brochothrix thermosphacta*. Likewise, Curiel *et al.* (2011) suggested that different Ar concentrations could inhibit *Carnobacterium divergens* growth on fresh pork sausages.

Regarding the effect of the MAPs applied against *Campylobacter*, the application of gas mixtures with high concentration of oxygen showed a significant control of bacterium growth throughout the storage (MAP-B and MAP-D). On the other hand, *Campylobacter* growth was not affected by the application of anaerobic atmosphere inoculated onto fresh chicken fillets, leading to high microbiological counts throughout the storage (MAP-A). These findings are in agreement with other studies, which reported the effect of anaerobic atmospheres on the survival of *Campylobacter* in chicken meat. Boysen *et al.* (2007) observed that *C. jejuni*, inoculated onto chicken fillets significantly survived longer under anaerobic MAPs (100% N_2 and 70%/30% N_2/CO_2) than under aerobic poultry atmosphere (70/30% O_2/CO_2), confirming that CO_2 afforded protective effect on the survival of *Campylobacter*. A similar effect was also described by Wesley and Stadelman (1985) as the result of the reduction of the oxygen concentration in the ambient atmosphere. Furthermore, Rajkovic *et al.* (2010) showed that the survival of *C. jejuni* strains inoculated onto chicken legs was scarcely affected by the use of 80% CO_2 when compared to 80% O_2. The authors observed that, after the O_2 exposure (adverse environmental condition), *C. jejuni* cells became slightly elongated and less coiled, losing their spiral morphology resulting in coccoid formation (Non-Culturable form), affecting therefore their growth under selective media by absence of culturable cell numbers.

The effectiveness of the atmosphere with Argon (MAP-D) was lower compare to atmospheres with high O_2 concentration (MAP-B and MAP-C). However, at the end of the storage no significant differences were observed among these atmospheres that completely inhibited the *Campylobacter* growth. In literature no studies have been found on the effect of Ar MAP against *Campylobacter* strains. Other studies with fresh

cut-products have been reported that the beneficial effect of Ar is due to the formation of inert gas hydrate called clatharate which reduces the activity of intracellular water, thereby reducing the leaching of organic material and movement of the microorganism into deeper tissues (Wu, Zhang and Adhikari, 2012; Wu, Zhang and Wang, 2012). Nevertheless, further studies are needed to obtain more results about the effect of Argon gas against *Campylobacter* survives onto chicken meat.

Regarding the physical-chemical analysis, chicken meat fillets packaged under standard poultry meat atmosphere showed that, the pH values of the MAPs studied were generally similar during the storage. Several authors (Vongsawasdi *et al.,* 2008; Melero *et al.,* 2012; Herbert *et al.,* 2013) also reported no significant variations of pH values of meat chicken samples at the different gas mixture conditions studied along the storage.

According to the color analysis, samples packaged under standard poultry meat atmosphere showed significantly higher L* and lower a* values during the storage resulting in a pale hue of the chicken meat (MAP-C). Several authors indicated that pale hue of chicken meat is related with a higher lightness (L*), lower redness (a*) and not variations of the yellowness (b*) (Petracci *et al.,* 2004; van Laack *et al.,* 2000; Qiao *et al.,* 2001). Therefore, the meat paleness (L*) is not highly correlated with b*values as observed in previous studies carried out on pale broiler breast meat (Fletcher, 1999; van Laack *et al.,* 2000; Qiao *et al.,* 2001). Despite of our results, Boysen *et al.* (2007) observed that fillets chicken packaged under aerobic poultry meat atmosphere maintained the red hue throughout the self-life (7 days) compared with those packaged under anerobic conditions (70%/30% N_2/CO_2 and 100% N_2).

The other MAPs studied did not showed significantly differences on color (L*, a* and b*) during the storage, maintaining similar values throughout the storage. Similarly, Petracci *et al.* (2004) reported that the typical range of L* value at broiler meat is between 50 and 56. Moreover, higher variability was also observed for a* (range: 0 to 13) and b* (range: −3 to 12) on chicken meat samples.

In the sensorial evaluation, samples packaged under MAP-D were scored as the best treatment on the appearance and overall acceptability among the MAPs studied. In terms of the odor evaluation, judges preferred the MAP-A being evaluated as the best

treatment). Ruiz-Capillas and Jimenez-Colmenero (2010) observed a positive sensory acceptability by the judges for the fresh pork sausages packaged under 30%/70% CO_2 / Ar. Herbert *et al.* (2013) also reported a sensory benefit on chicken breast fillets stored in 15%/60%/25% Ar / O_2 / CO_2, resulting in a higher retention (preservation) of the natural pink color of samples studied. However, Tomankova *et al.* (2012) showed that poultry meat samples packaged under MAP 70%/30% Ar and O_2 MAP had an unpleasant aroma and lower acceptability compared those packaged under 70%/30% O_2/CO_2 MAP.

Contrarily to what observed in MAP-D, MAP-B was scored as the lowest treatment on appearance, odor and overall acceptability. The storage of the poultry meat under high-oxygen atmospheres has been found to cause loss of quality (Vukasovic, 2014). The increase of the oxygen concentration could induce lipid oxidation causing rancid off-flavours. The rancid aromas have often very low threshold values and could be easily detected by consumers (Campo *et al.*, 2003). High levels of oxygen could also cause intermolecular cross-linking, provoking tenderness, juiciness and the decrease of nutritional values of the meat due to the loss of essential amino acids and reduced digestibility (Vukasovic, 2014).

In conclusion, according to the results observed among the MAPs studied, samples packed under 50% CO_2 showed better control of the *Salmonella* growth than those packed under lower (30%) or no presence of carbon dioxide. *Campylobacter* growth was inhibited by the application of MAPs with high O_2 concentration (≥50%) and with Argon. Nevertheless, CO_2 packaging did not showed effect at controlling *Campylobacter* growth. Regarding the physical-chemical analysis, chicken meat fillets packaged under standard poultry meat atmosphere showed significantly higher L* and lower a* values during the storage resulting in a pale color of the chicken meat. The other MAPs studied did not show significantly differences on color values among them. In the sensorial evaluation, MAP-D was evaluated as the best on appearance and overall acceptability among the MAPs studied. In terms of the odor evaluation, judges preferred chicken meat fillet samples packaged under high CO_2 concentration (MAP-A). On the other hand, samples packaged under high oxygen concentration (MAP-B) had the lowest score of appearance, odor and overall acceptability. In this context, due to the different effects observed on MAPs applied against *Salmonella* and *Campylobacter*,

further studies will be considered to obtain the atmosphere which combining different percentages of the gases studied, maintaining and enhancing their inhibitory effect for both pathogens.

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CHAPTER VI. CONCLUSIONS.

1. The status of the flocks at the arrival of the live birds was high infected by both pathogens at the arrival to the slaughterhouse.

2. According to the slaughterhouse environment, *Salmonella* contamination decreased after scalding and chilling and increased after bleeding, de-feathering, grading and evisceration stages. Nevertheless, no *Campylobacter* spp. was isolated in any of the environmental samples analysed.

3. The contamination of poultry carcasses during the slaughter process showed that, *Salmonella* neck skin samples were higher contaminated at the de-feathering and exsanguination than at the air chilling stage. However, the internal surface cavity samples were higher contaminated of *Salmonella* at air chilling, and de-feathering than at the exsanguination stage. *Campylobacter* contamination on chicken carcasses showed high percentage in poultry carcasses from initial processing stages till the final product.

4. The most prevalent serovars isolated were *S.* Enteritidis and *C. jejuni*. *Salmonella* strains isolated showed the same pattern suggesting a re-circulation across to the poultry farms and slaughterhouse facilities.

5. Among the MAPs studied, samples packed under 50% of CO_2 showed better control of the *Salmonella* growth than those packed under lower (30%) or no presence of CO_2. However, *Campylobacter* growth was inhibited by application of MAPs with high O_2 concentration (≥50%) and MAP with Argon. Nevertheless, CO_2 packaging did not showed effect at controlling *Campylobacter* growth.

6. Chicken meat fillets packaged under standard poultry meat (MAP-C) atmosphere showed significantly higher L* and lower a* values during the storage resulting in a pale color of the chicken meat. The other MAPs studied did not show significantly differences on color parameters values among them during the storage. Concerning to the pH, significantly differences were not observed among the samples stored under different modified atmospheres studied.

7. MAP-D was evaluated by the judges as the best treatment on the appearance and overall acceptability. In terms of the odor, judges preferred the chicken meat fillet samples packaged under high CO_2 concentration (MAP-A). Generally, samples packaged under high oxygen concentration (MAP-B) had the lowest score of appearance, odor and overall acceptability.

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