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Prevalence of *Salmonella enterica* in Italian salami and evaluation of isolates survival in a human macrophage-like (U937) cell line.

Coordinatore:

Chiar.mo Prof. Attilio Corradi

Tutor:

Chiar.ma Prof.ssa Silvia Bonardi

Dottorando: Ilaria Bruini

Index

1. Abstract
 - 1.1 Riassunto
2. Introduction
3. The Genus *Salmonella*
 - 3.1 Biochemical characteristics
 - 3.2 Virulence factors
 - 3.3 Survival and replication within macrophages
4. *Salmonella enterica* serovars responsible for human infection
 - 4.1 Typhoidal Salmonellosis
 - 4.1.1 Epidemiology
 - 4.1.2 Risk factors and transmission
 - 4.1.3 Non-Typhoidal Salmonellosis (NTS)
 - 4.1.4 Epidemiology
 - 4.1.5 Risk factors and transmission
5. Pathogenesis and Clinical Manifestation
 - 5.1 Clinical Manifestation
6. Antimicrobial Resistance
7. The role of pig as reservoir
 - 7.1 Clinical Syndroms

8. Italian Salami

9. Materials and Method

9.1 Sample collection

9.2 *Salmonella* detection and typing

9.3 *Salmonella* enumeration

9.4 Measure of pH and a_w

9.5 Sodium chloride analysis

9.6 Macrophage experiment

9.7 Laboratory protocols

10. Results

11. Discussion

11.1 Influence of pH and a_w values

11.2 Influence of antimicrobial starter cultures

11.3 Analytical methods performance

11.4 Survival and replication within macrophages

12. Bibliography

1. Abstract

Salmonella is the second pathogen reported in the European Union (EU) as causative agents of human foodborne diseases (EFSA and ECDC, 2015). In 2014, *S. Enteritidis* and *S. Typhimurium* were the two more frequently isolated serovariants in confirmed human cases. The main source of contamination is food of animal origin, especially pig meat, which is largely consumed in EU (Devine, 2003); in Italy, in particular, the consumption of a wide range of salami produced with pork is very common. Pigs are important reservoir of infection for humans as they are asymptomatic carriers of broad host-range serovars of *Salmonella* (Fedorka-Cray *et al.*, 2000).

From April to December 2015, 155 ground pork and fat mixture samples (corresponding to 155 different batches) used for salami production were tested for *Salmonella*. All samples were provided by four production plants located in Emilia-Romagna region, northern Italy. Salami batches were tested only in case their ground raw mixture was positive for *Salmonella*. The detection of *Salmonella* was performed using a molecular method based on the Real-Time polymerase chain reaction (PCR) and positive samples were confirmed by the ISO 6579:2002 bacteriological standard method. The enumeration of *Salmonella* in the Real-Time PCR positive samples was performed following the miniaturized Most Probable Number (MPN) technique according to ISO 6579-2:2012. Since the ability of *Salmonella* to cause systemic disease has been assessed in mice according to their survival and growth in murine cell lines, we evaluated the capacity of some *Salmonella* isolates to survive and replicate within a human macrophages-like cell line (U937). This study was performed to investigate the potential invasiveness for humans of *Salmonella* strains found in ready-to-eat pork products.

Salmonella was isolated from 24/46 (52.2%) of raw mixture samples and from 16/59 (27.1%) of cured salami; eight *Salmonella* serovars were identified, with *S. Derby* (12/24; 50.0%) as the most common. The MPN enumeration of *Salmonella* in raw mixture ranged from 160 MPN/g to < 1.3 MPN/g, while in cured salami it ranged from 8.7 MPN/g to 1.3 MPN/g in seven samples (43.7%), and was < 1.3 MPN/g in the remaining nine (56.3%). Three of the four tested strains of *S. Typhimurium* 4,[5],12:i-

were able to survive and growth in the human macrophage-like (U937) cell line. On the contrary, one isolate of *S. Typhimurium* 4,[5],12:i:- and one strain of *S. Infantis* were not able to survive.

1.1 Riassunto

In Europa, *Salmonella* è il secondo patogeno causa di malattia di origine alimentare trasmissibili all'uomo (EFSA and ECDC, 2015). Nel 2014, *S. Enteritidis* e *S. Typhimurium* sono state le due siero varianti maggiormente isolati in casi confermati di salmonellosi umana. La principale fonte di contaminazione sono gli alimenti di origine animale, principalmente la carne di suino, largamente consumata in Europa (Devine, 2003); in Italia, in modo particolare, è molto diffuso il consumo di salami di vario tipo, prodotti con carne di suino. I suini rappresentano importanti serbatoi di infezione per l'uomo, dal momento che sono portatori asintomatici di un'ampia gamma di siero varianti di *Salmonella* (Fedorka-Cray *et al.*, 2000).

Nel periodo compreso fra aprile e dicembre 2015, 155 campioni di carne macinata e grasso (corrispondenti a 155 diversi lotti), utilizzati per la produzione di salami, sono stati testati per la presenza di *Salmonella*. Tutti i campioni sono stati forniti da quattro stabilimenti di produzione situati in Emilia-Romagna, nel nord Italia. I lotti di salami sono stati testati solo nel caso in cui la carne macinata, con cui erano stati prodotti, risultava essere positiva per la presenza di *Salmonella*. La ricerca di *Salmonella* è stata condotta utilizzando il metodo molecolare Real-Time polymerase chain reaction (PCR) ed i campioni positivi sono stati confermati col metodo batteriologico ISO 6579:2002, considerato lo standard di riferimento. Il conteggio di *Salmonella* nei campioni positivi alla Real-Time è stato eseguito seguendo il metodo miniaturizzato Most Probable Number (MPN), come previsto da Norma ISO 6579-2:2012. Dal momento che, nei topi, è stato dimostrato che *Salmonella* è in grado di causare infezioni sistemiche, grazie alla sua capacità di sopravvivere e crescere all'interno di linee cellulari murine, nel nostro studio, abbiamo valutato la capacità di alcuni isolati di *Salmonella*, di sopravvivere e replicare all'interno di una linea cellulare umana (U937). Questo studio è stato eseguito

per valutare la potenziale invasività, per l'uomo, di ceppi di *Salmonella* isolati da prodotti ready-to-eat a base di carne di suino.

Salmonella è stata isolata in 24/46 (52.2%) impasti di carne macinata ed in 16/59 (27.1%) di salami; sono state identificate otto siero varianti, di queste *S. Derby* è risultata essere la più frequente (12/24; 50%). Dal conteggio MPN eseguito sugli impasti abbiamo ottenuto un valore compreso fra 160 MPN/g e < 1.3 MPN/g, mentre il valore ottenuto dal conteggio nei salami era 8.7 MPN/g a 1.3 MPN/g in sette campioni (43.7%) e < 1.3 MPN/g nei restanti nove (56.3%). Tre dei quattro ceppi di *Salmonella Typhimurium* 4,[5],12:i:- testati sono stati in grado di sopravvivere e replicare all'interno della linea cellulare U937. Al contrario, un isolato di *S. Typhimurium* 4,[5],12:i:- ed uno di *S. Infantis* non sono stati in grado di sopravvivere.

2. Introduction

Salmonella is a Gram-negative bacterium, which is part of the family of the *Enterobacteriaceae*. This is a great importance pathogen, indeed it ranks second among pathogens reported in the European Union (EU) as causative agents of human foodborne diseases. In 2014, a total of 88,715 confirmed salmonellosis cases were reported by 28 EU Member States (MS), resulting in an EU notification rate of 23.4 cases per 100,000 population. This represented a 15.3% increase in the EU notification rate compared with 2013 (EFSA and ECDC, 2015). As in previous years, the two most commonly reported *Salmonella* serovars in 2014 were *S. Enteritidis* and *S. Typhimurium*, representing 44.4% and 17.4%, respectively, of all reported serovars in confirmed human cases. The proportion of *S. Enteritidis* increased compared with 2013. This increase was mainly attributed to increase in cases in one member state. *S. Typhimurium* cases, including the monophasic variant of *S. Typhimurium* 1,4,[5],12:i:-, decreased by 21.7% compared with 2013. Cases of *S. Infantis*, the fourth most common serovar, returned to the level of 2012 after the increase in 2013. Most MS reported data on *Salmonella* in food of animal origin, primarily pig meat, which is widely consumed in the European Union (Devine, 2003). In Italy, consumption of salami, produced with pork, was recently associated with both clustered and sporadic cases of salmonellosis due to *S. Goaldcost* (Scavia *et al.*, 2013). In fact, pigs are important reservoir of infection for humans as they are asymptomatic carriers of broad host-range serovars of *Salmonella*. They usually get infected through oral intake of the organism, and, after infection, they can become carriers in the tonsils, the intestine and the gut-associated lymphoid tissue (Fedorka-Cray *et al.*, 2000). Most of the time, carrier are not excreting the bacteria but under stressful conditions re-shedding may occur. In this way, carriers are a permanent potential source of infection for other animals, including humans (Rostagno *et al.*, 2010). The EU requires all of its MS to test pork and pork products for all *Salmonella* serotypes with public health significance (Reg.(EC) 2073/2005). Control of *Salmonella* infection in pigs is included in the Reg. (EC) No. 2160/2003 of the European Parliament. Article 4 of this regulation states that Community targets

shall be established for the reduction of the prevalence of *Salmonella* in herds of slaughter pigs (Anonymous, 2003).

The aims of the study were: *i)* to investigate the prevalence of *Salmonella* in minced pork meat and fat mixtures used for salami production, *ii)* to test the batches of salami, manufactured with the *Salmonella*-positive raw meat, at the end of their curing period to assess the antimicrobial effect of curing, *iii)* to count *Salmonella* in dry-cured salami to evaluate the risk for the consumer, *iv)* to evaluate the ability of isolates to survive in a human macrophage-like (U937) cell line.

3. The Genus *Salmonella*

The discovery of the genus *Salmonella* dates to 1885, when the American bacteriologist Daniel Elmer Salmon and his assistant Theobald Smith were searching for the cause of cholera in pigs. They proposed as causal agent a new species of bacteria isolated from ill pigs, previously called *S. cholerae-suis*. Afterwards it has been demonstrated that this microorganism seldom causes enteric symptoms in pigs and, therefore, was not the causal agent of cholera (Fàbrega and Vila, 2013).

The genus *Salmonella* is closely related to the genus *Escherichia* (Fàbrega and Vila, 2013); they diversified from a common ancestor 100 million years ago (Rychlik *et al.*, 2009).

Salmonella is a member of the family *Enterobacteriaceae*, which includes Gram-negative, rod-shaped, facultative anaerobic, non-spore forming, catalase-positive, oxidase-negative microorganisms; these bacteria usually show peritrichous motility and range in diameter from around 0.7 to 1.5 μm , with a length of 2 to 5 μm , generally producing colonies of 2-4 mm diameter (Löfström *et al.*, 2010).

The classification of this genus is based on the White-Kauffman-Le Minor scheme, which is considered by public health organizations worldwide the gold standard for *Salmonella* serotyping (Franklin *et al.*, 2011). The genus includes only two species called *S. enterica* and *S. bongori*. *S. enterica*, in turn, is divided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI) (EFSA, 2010). At first *S. bongori* was considered to be a different subspecies, but now is recognized apart from the rest of the *S. enterica* lineages (Fàbrega and Vila, 2013). *S. enterica* subspecies *enterica* (I) represent the vast majority of *Salmonella* strains isolated from warm-blooded animals and humans; the other subspecies and *S. bongori* are instead more typically isolated from cold-blooded animals (Desai *et al.*, 2013).

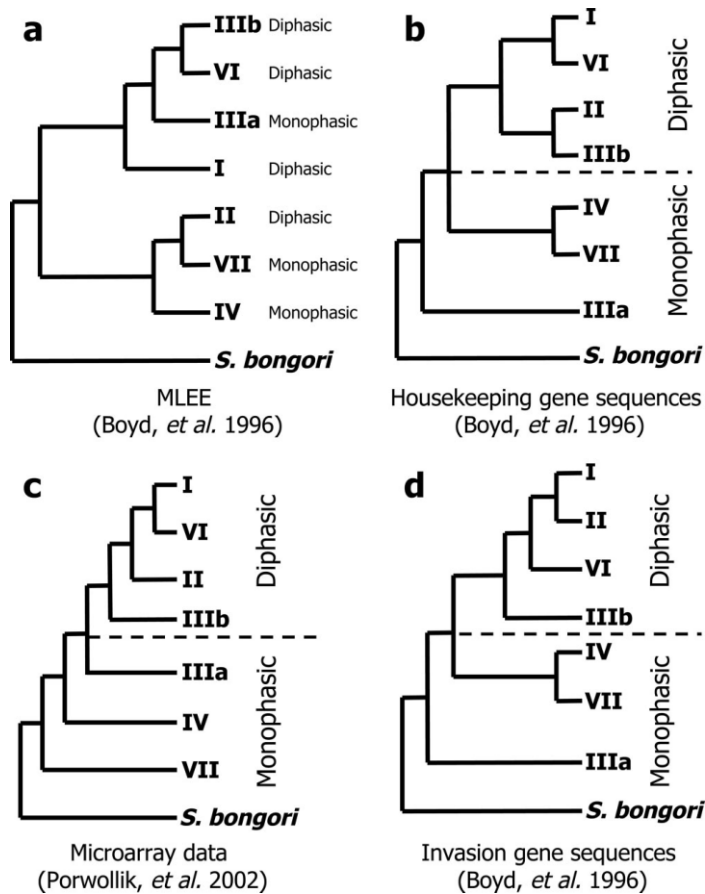


FIGURE 1. Summary of phylogenetic studies (McQuiston *et al.*, 2008).

According to the Kauffman-White-Le Minor scheme subspecies are further divided into serotypes on the basis of surface antigen. This identification uses polyclonal antiserum to determine O antigens (somatic), H antigens (flagellar) and Vi antigens (capsular polysaccharides) (Franklin *et al.*, 2011). Currently over 2.600 different serovars are known by this scheme (Gal Mor *et al.*, 2014), each of these having a unique combination of somatic O and flagellar H1 and H2 antigens (Chen *et al.*, 2013). *Salmonella* serovars may be found predominantly in one host, may be ubiquitous or may have an unknown host, therefore *Salmonella* serovars may be host-specific, host-restricted or ubiquitous (Löfström *et al.*, 2010).

The O antigen is the external component of the lipopolysaccharide (LPS) located in the cell membrane and is divided in two parts: the innermost is the same in all enterobacteria and is formed

by five carbohydrates; the outermost is formed by saccharide chains that contain a sequence of oligosaccharides and the position of these molecules in the chain determines different features of the somatic antigen (65 known). By convention bacteria containing structurally similar somatic antigen are brought together in serogroups (A, B, C) (EFSA, 2010).

The H antigen, the flagellar one, provides mobility to different serovars of *Salmonella* (except *S. Gallinarum* and *S. Pullorum* which are motionless). Flagella filaments consist of flagellin, a protein that can exist in two forms or phases, named H1 and H2, because it's encoded by two different genes. In fact it has been described, for the first time in *S. Typhimurium*, a phase shift through a mechanism of molecular switching of an invertible segment of DNA that contains a promoter. If the invertible segment of DNA is oriented in H2 phase, the promoter leads the expression of two open reading frame (ORF), one encoding for the H2 flagellin and the other one encoding for the H1 flagellin gene repressor, thus you will have only the expression of H2 flagellin. The invertible segment switching determines the opposite orientation of the promoter, which, in this way, can act on flagellin's genes inducing the only expression of H1 flagellin (Andrews and Bäumler, 2005).

The Vi antigen is the virulence capsular polysaccharide produced by *S. Typhi* and *S. Paratyphi*. It is a high-molecular-weight cell-surface polysaccharide that forms a hydrated layer called "capsule" (Whitfield C., 2006). The capsule is often the outermost structure of a bacterial cell and therefore is critical for interactions with the environment, it helps bacteria in resisting desiccation, colonizing host tissues, forming biofilm, resisting bacteriophages, reducing opsonophagocytosis and complement-mediated killing (Taylor and Roberts, 2005). It is also implicated in the evasion of the innate immune system (Keestra-Gounder *et al.*, 2015). The production of Vi antigen reduces serum complement binding/killing and promotes intracellular replication. Purified Vi antigen is currently used in parenteral vaccines (Liston *et al.*, 2016).

3.1 Biochemical characteristics

The biochemical characteristics of *Salmonella* are of practical importance for its identification and also may help to better understand the biology of this microorganism (Fierer and Fleming, 1983).

Biochemically, *Salmonella* shows a typical profile, which is characterized by a positive reaction for several tests, including:

- Nitrate reduction;
- Methyl Red;
- H₂S production;
- Maltose fermentation;
- Growth with NH₄ citrate;
- Sorbitol and mannitol fermentation;
- Glucose fermentation with gas production (Ewig, 1966; Traub *et al.*, 1970).

Salmonella shows, instead, negative reaction for the following tests:

- Indole production;
- Saccharose fermentation;
- Adonite and inosite fermentation;
- Urea hydrolysis;
- Voges-Proskauer;
- Lattose fermentation;
- Salicin fermentation;
- Jelly liquefaction;
- Growth with Potassium Cyanide (KCN) (Ewig *et al.*, 1960; Ewig and Ball, 1966).

3.2 Virulence factors

The most important virulence factors are encoded by genes located within highly conserved *Salmonella* pathogenicity islands (SPIs), whereas others are encoded by genes located on a virulence plasmid (pSLT) or in the chromosome. *Salmonella enterica* possesses five major pathogenicity islands (SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5) within its chromosome, but only four of them (SPI-2 is absent) are present in the chromosome of *Salmonella bongori* (Fàbrega and Vila, 2013).

The SPI-1 and SPI-2 genes code for proteins forming the Type Three Secretion System (T3SS) which enables the carriage of *S. enterica* proteins from the bacteria cell directly into the cytosol of host cells. T3SS requires five different kind of proteins including chaperone, translocator, effector, apparatus protein and transcriptional regulator to work properly. The structure of a T3S apparatus, called injectisome, is conserved among different pathogenic T3SSs and looks like flagellar T3SS (Galan and Colmer, 1999; Cornelis *et al.*, 2000). An injectisome is composed of a structurally conserved basal body, which contains two pairs of rings that span the inner and the outer membrane and is connected to cytoplasmatic ring. When, during infection, the injectisome of a pathogenic bacterium get in touch with a host cell, it extends its needle-like structure which protrudes outside the cell with a pore-forming protein (translocator) at the distal tip for delivery of effectors (Hueck, 1998).

The SPI-1 is a horizontally acquired chromosomal region of 40kb, located at 63 centisome (Mills *et al.*, 1995). The master regulator of SPI-1 genes is HilD, a family of transcription factors (AraC), encoded within SPI-1. HilD expression and activity are controlled by multiple pathways which sense the environmental cues associated with invasion. HilD activates transcription of several SPI-1 genes, including components of the T3SS, secreted effector proteins, and the TFs HilA and InvF. HilA and InvF, in turn, activate transcription of additional T3SS components and effector proteins. Approximately half of the known HilD-regulated genes are located outside SPI-1 (Rychlik *et al.*, 2009).

SPI-1 plays an important role in the intestine invasiveness (Mills *et al.*,1995). In fact it encoded T3SS, forming the needle-like complex, required for the transport of the bacterium proteins across cytoplasmic membrane of eukaryotic cells into their cytosol, where they elicit cytoskeletal and physiological changes, including actin rearrangement, leading to the uptake of *S. enterica* by non-phagocytic cells (Kaniga *et al.*,1995). The SPI-1 T3SS is a prerequisite for symptoms of both abdominal and systemic disease (Ellermeier and Schlauch, 2007). It has also been reported that SPI-1 genes induce macrophage cytotoxicity, regardless of cell invasion (Chen *et al.*, 1996).

Instead, the SPI-2 correspond to a 25 Kb fragment, located at 30 centisome, organized in four operons: regulatory, structural I, structural II and effector/chaperone. The two-component regulatory system SsrA/SsrB is responsible for the transcriptional regulation of SPI-2 operons as well as the regulation of effector genes located outside SPI-2 (Yoshida *et al.*, 2014). The T3SS needle complex comprises basal component proteins in the inner membrane (including SsaR), proto-channel proteins, outer membrane proteins, proteins forming a hollow tube generating the needle and outer ring proteins (including SseB) which forms the translocon that traverses the host vacuolar membrane. This SPI-2 T3SS injects at least 20 effector proteins across the vacuolar membrane into the host cytosol. These effectors interact with the host machinery and are necessary for modification of the vacuole and modulation of intracellular processes (Yoshida *et al.*, 2014), which will enable the bacteria to survive and replicate within a membrane-bound structure called the *Salmonella*-containing vacuole (Salcedo *et al.*, 2001).

Finally, SPI-2 encoded T3SS is required for the carriage of *S. enterica* proteins across the phagosomal membrane and increases bacterium survival within phagocytic cells, avoiding its suppression from NADPH oxidase (Mills *et al.*, 1995).

The functions of genes localized on the remaining SPIs are less characterized. It can be stated that SPI-3 genes, for example, are involved both in gut colonization and in intracellular survival, while SPI-4 genes encode for non-fimbrial adhesin and are required for the intestinal phase of disease. Finally, SPI-5 genes are co-regulated with either SPI-1 or SPI-2 genes, so they encode for effector proteins carried by either of these T3SSs (Rychlik *et al.*, 2009).

Some *Salmonella* serovars, such as Typhi, Paratyphi, Hadar and Infantis, do not possess any plasmids (Rychlik *et al.*, 2006), while serovars frequently associated with infections of human and farm animals, including Abortusovis, Choleraesuis, Dublin, Enteritidis, Gallinarum/Pullorum, Paratyphi C, Sendai and Typhimurium own them (De Moraes and Teplitski, 2015). Actually, it is quite difficult to find a field strain of these serovars which would be free of any plasmids; this can be explained by the presence of serovar specific virulence plasmids (Rychlik *et al.*, 2006). These plasmids contain virulence determinants that contribute to pathogenesis, especially during the systemic phase of the disease (Tezcan-Merdol *et al.*, 2001; De Moraes and Teplitski, 2015). They vary in constitution among the different serovars, for example their size can vary ranging from 50 kb (serovar Choleraesuis) to 285 kb (serovar Sendai) (Feng *et al.*, 2012). Despite several differences in composition, these plasmids carry in common some genetic elements which are highly conserved, notably the “*Salmonella* plasmid virulence” (*spv*) gene cluster (Beninger *et al.*, 1988; Gulig *et al.*, 1993). The *spv* gene cluster represents the main virulence determinant on the plasmid, in fact different studies demonstrate that mutational inactivation of its expression has the same effect on virulence as its loss (Gulig and Curtiss, 1988; Rhen *et al.*, 1988; Norel *et al.*, 1989). In more detail, the *spv* cluster includes five genes, *spv*RABCD, identified soon after the association of the *Salmonella* serovar specific plasmids with its virulence (Jones *et al.*, 1982; Norel *et al.*, 1989; Gulig *et al.*, 1992). The first gene, *spv*R, is a positive transcriptional regulator of the expression of *spv* genes and belongs to the Lys/MetR family of prokaryotic transcriptional regulators (Coynault *et al.*, 1992; Tezcan-Merdol *et al.*, 2001) whose activity is typically modulated through the presence of small-molecular-weight ligands, such as intermediates in amino acids biosynthesis pathways (Schell, 1993) or reactive substances such as hydrogen peroxide (Aslund *et al.*, 1999). For an efficient expression it is also required the starvation-associated sigma factor RpoS (Lesnick *et al.*, 2001; Tezcan-Merdol *et al.*, 2001). Signals for the expression of the *spv* locus are growth restriction, reduced nutrient supply or lowered pH (Guiney *et al.*, 1995). *Spv*B and *Spv*C are the central effector genes of this operon since the presence of *spv*BC only is enough to complement for the missing virulence plasmid in *S.*Typhimurium. In 2001, Lesnick *et al.* (2001) and Tezcan-Merdol

et al. (2001), demonstrated that SpvB ADP-ribosylates actin of the macrophage cell and destabilizes its cytoskeleton.

In conclusion we can say that the virulence plasmid makes an important contribution in *Salmonella* infection, even if it's not essential (Gulig *et al.*, 1988). In fact *in vivo* studies suggest that the *spv* genes act to enhance bacterial replication in macrophages (Fields *et al.*, 1986; Gulig and Doyle, 1993); furthermore, studies conducted in bovine monocyte-derived macrophages have indicated that the *spv* genes are required for bacterial growth (Libby *et al.*, 1997). In addition, in 2000, Libby *et al.* (2000) reported that the expression of the *spv* genes is also required to induce cytotoxicity in infected human monocyte-derived macrophages, characterized by cell detachment and eventual apoptosis.

Salmonella can also harbour other high molecular weight plasmids of up to 200 kb in size, which were observed in strains isolated between 1917 and 1950 (Jones and Stanley, 1992), before the so-called "antibiotic era". These plasmids belonged to IncI, IncX and IncF incompatibility groups and no functions could be attributed to their presence, including the resistance to antibiotics. Among *Salmonella* strains, plasmids of IncI, IncH and IncF incompatibility groups are the most frequently observed in *Salmonella*, followed by the IncN, IncP and IncQ. Since plasmids of similar incompatibility groups were found among plasmids from the pre-antibiotic era and current plasmids coding for antibiotic resistance, it can be assumed that the later evolved from the former ones by acquisition of new genetic elements (Rychlik *et al.*, 2006).

Another type of *Salmonella* plasmids are the low molecular weight plasmids, which are commonly isolated from Enterobacteriaceae. They are found only in about 10% of *Salmonella* field strains and their biological functions are widely unknown. Nevertheless, these plasmids have been largely used in differentiation and molecular typing of field strains (Threlfall *et al.*, 1994; Ridley *et al.*, 1996).

The virulence plasmid is observed more frequently among human extraintestinal isolates compared with environmental or faecal isolates (Fierer *et al.*, 1992; Guiney *et al.*, 1995). Therefore, the virulence plasmids, and consequently the *spv* genes, operate as virulence markers in human

salmonellosis, besides representing virulence factor in animal infections (Tezcan-Merdol *et al.*, 2001).

Gram-negative bacteria, such as *Salmonella enterica*, and *Escherichia coli* and *Shigella* spp., naturally release the so-called outer membrane vesicles (OMVs) (Liu *et al.*, 2016), which are produced by vesiculation, a secretory process ubiquitous to these microorganisms. It is believed that the process of vesiculation is linked to bacterial stress, with increases vesicle production occurring during conditions of high membrane stress (Macdonald and Kuehn, 2013).

These particles form during growth as the outer membrane blebs outwards and pinches off, resulting in nanoscale (20-250 nm) spheres of the outer membrane containing soluble periplasmic components trapped in their lumens (Lee *et al.*, 2007). Therefore, the composition of OMVs reflects components of the outer membrane and periplasm, for example soluble proteins, integral membrane proteins, lipoproteins and glycolipids (Baker *et al.*, 2014). In particular, the natural OMVs produced by *Salmonella* are heterogeneous complexes that contain pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoproteins and outer membrane proteins (OMPs), which are the major components of OMVs (Kuehn and Kesty, 2005). LPS, specifically, is essential for the biosynthesis and assembly of the bacterial outer membrane, and is composed of three parts, including conserved lipid A, core oligosaccharide and repeated O-antigen polysaccharide (Raetz and Whitfield, 2002). Natural OMVs play several important roles. For example, they can help to bacterial survival by decreasing levels of toxic compounds such as toluene, taking off stress products from the cell such as misfolded periplasmic proteins, neutralizing environmental agents that target the OM such as antimicrobial peptides, promoting the release of attacking phage or nucleating the formation of bacterial communities, such as biofilms (Baker *et al.*, 2014, Kulp and Kuehn, 2010).

OMVs released from the envelope of pathogenic bacteria play a key role in host-pathogen interactions, indeed they can protect infecting bacteria from the host immune response, allow bacterial survival in the stressful environment within the host during infection, and aid to acquire nutrients in an iron-and-nutrient-scarce host environment (Kulp and Kuehn, 2010).

OMVs have specific virulence-associated activities too, in fact they can contain active toxins which can be delivered into host cells. During infection, the stressful environment can cause OMV upregulation which determine increased production of these particles, accompanied by increased toxicity and activation of both innate and adaptive immune responses (Kesty *et al.*, 2004).

Fimbriae play a critical role in virulence by allowing bacteria to interact with host cells and other solid substrate (Low *et al.*, 1996); in more detail their task is to mediate the initial attachment of the bacterium to the host cell surface (Bäumler *et al.*, 1996).

Fimbriae are another important virulence factors, which comprise a class of non flagellar, proteinaceous filaments, highly organized, produced on the surface of a wide range of bacteria. They are polymers composed mainly of a single protein species named fimbrin, which usually forms filaments 2 to 7 nm wide. Two general groups of fimbriae are differentiated by the presence or absence of *N*-methylphenylalanine (N-MePhe) as the N-terminal amino acid residue on their respective fimbrins. *Salmonella* produces mannose-sensitive type 1 fimbriae, which are rigid and 6 to 7 nm wide; the mannose-sensitive adherence characteristic is conferred by an additional minor fimbria-associated adhesion protein. It is likely that the D-mannose forms the receptor of these type 1 fimbriae on eukaryotic cells, since the presence of the carbohydrate prevents the agglutination of erythrocytes fimbria-mediated. Type 2 fimbriae are non hemagglutinating and actually are type 1 fimbriae lacking their associated adhesin. Type 3 fimbriae, finally, observed in *Salmonella* spp. as well as in other *Enterobacteriaceae*, are thin filaments 2 to 4 nm in diameter and are notable for their ability to agglutinate tannic acid-treated erythrocytes.

Salmonella also produces a cholera toxin-related enterotoxin (Stn), a virulence factor and a causative agent of diarrhea in acute gastroenteritis (Nakano *et al.*, 2012), which participate in the pathogenesis of the intestinal phase. This enterotoxin is heat labile (100°C, 15 min) and shares antigenic determinants with cholera toxin. Like cholera enterotoxin, *Salmonella* enterotoxin elevates cyclic AMP (cAMP) levels of crypt epithelial cells in the small intestine (Reytmeier *et al.*, 1986). The increase in cAMP is accompanied by an elevation in the synthesis and release of prostaglandins, which also may contribute to the enterotoxic response (Chopra *et al.*, 1994).

Salmonella enterotoxin gene (*stn*) consisted of 749 bp and encoded a gene product (Stn) with a molecular mass of 29 kDa (Chopra *et al.*, 1999); it has been shown that the *stn* gene is distributed only in *Salmonella* species, irrespective of their serotypes. This finding indicates that the *stn* gene might be useful for the identification or detection of *Salmonella* and that Stn might be involved in functions unique to *Salmonella* (Nakano *et al.*, 2012).

Salmonella produces a heat-stable cytotoxic factor, which facilitates bacterial dissemination (Koo *et al.*, 1984; Solano *et al.*, 2001). Specifically, *Salmonella* cytotoxin is a component of the bacterial outer membrane which disrupts and detaches green monkey kidney (VERO) cells and Chinese hamster ovary (CHO) cell monolayers and affects the membrane conformation and shape. Because of these findings it is thought that this toxin is involved in facilitating entry into host cells and directly cause cytotoxicity; thus favouring bacterial dissemination (Reitmeyer *et al.*, 1986; Solano *et al.*, 2001).

Another main factor implied in *Salmonella* virulence is the development of antimicrobial resistance. Since the last 30 years there has been an increasing concern about the worldwide emergence of multidrug-resistant phenotypes among *Salmonella* serotypes such as *S. Typhimurium*, *S. Enteritidis* and *S. Newport*. A main problem is the emergence resistance to quinolones, fluoroquinolones or extended-spectrum cephalosporins such as ceftiofur and ceftriaxone. The development of resistance to antimicrobial agents by *Salmonella* is attributable to one of several mechanisms such as production of enzymes that inactivate antimicrobial agents through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of antimicrobial efflux pumps and modification of cellular drug targets (Abatcha *et al.*, 2014).

The acquisition of this ability takes place through the horizontal transfer of resistance genes, included in plasmids, transposons or integrons, between bacteria belonging to the same genus or to different genus. This is a very widespread phenomenon which allowed *Salmonella* to survive the treatment with different kind of antibiotics. The indiscriminate use of antibiotics in human and veterinary medicine has probably contributed to the establishment of this phenomenon. (Graziani *et al.*, 2008; Crump *et al.*, 2015).

Finally, the iron intake is essential for bacterial growth, but its finding is difficult since it is largely complexed to proteins (lactoferrin, transferrin, ferritin), so its unbound is very low. Then *Salmonella* processes two molecules, called siderophores, which chelate iron ions from the intestinal environment and from the serum with high affinity. Once produced and released by the bacterium, siderophores bind iron forming a complex which is then reintroduced in the bacterial cell thanks to special surface receptors. One of these molecules is named enterochelin or enterobactin while the other one is named aerobactin (Cox, 1999).

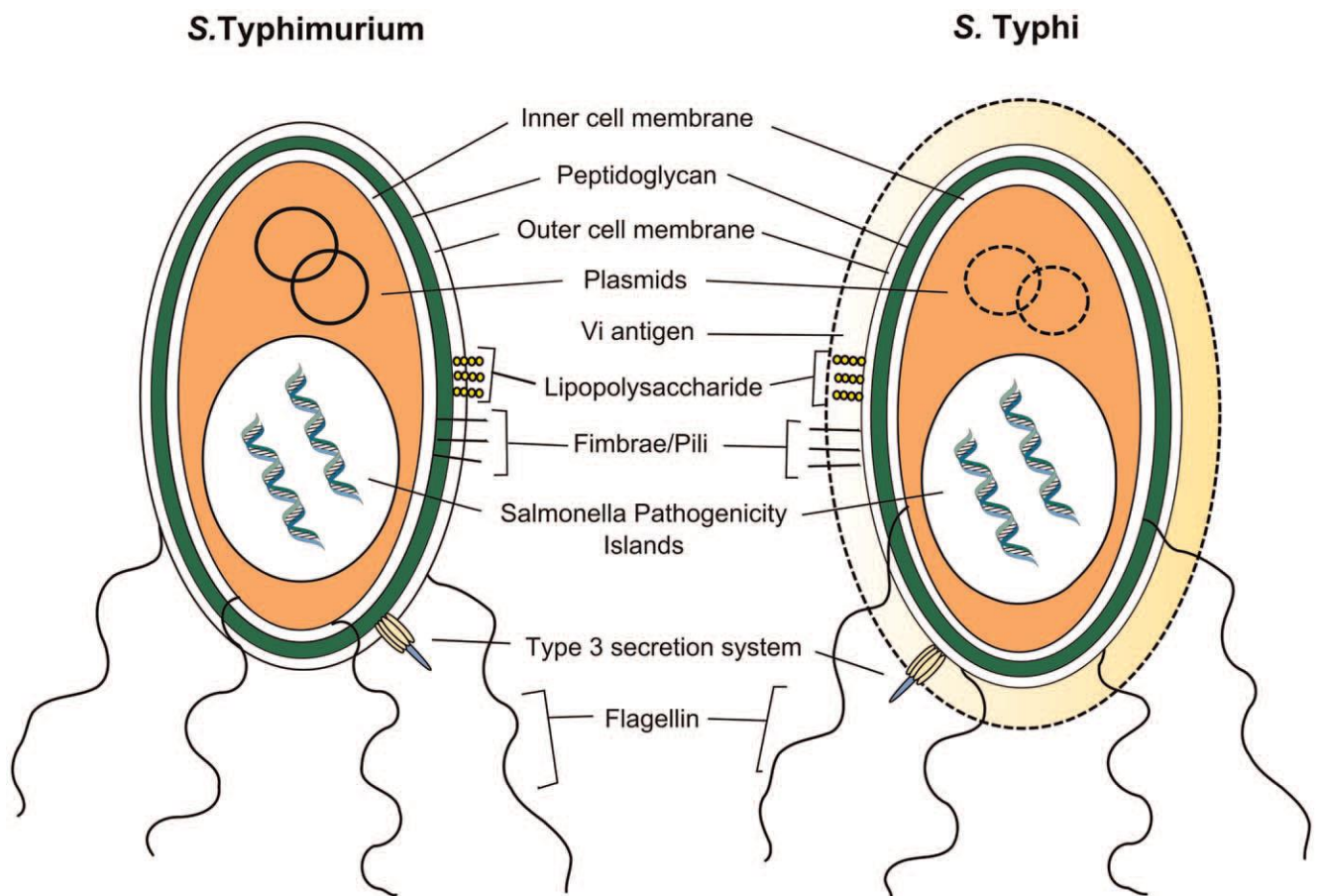


FIGURE 2: Virulence factors of *S. Typhimurium* and *S. Typhi* (De Jong *et al.*, 2012).

3.3 Survival and replication within macrophages

The ability of *Salmonella entericato* pass the intestinal barrier and spread in the body and, accordingly, to cause systemic disease depends on its capacity to invade, survive and replicate within cells of the granulocyte/monocyte lineage, such as macrophages. In more detail *S. Typhi* is adapted to grow and replicate in macrophages of human origin, while *S. Typhimurium* survives and replicates preferentially in murine macrophages (Schwan *et al.*, 2000).

The skills of these cells to engulf and kill bacteria is primarily determined by their activation state. Macrophages are originally classified into two different phenotypes known as “classically activated/M1” or “alternatively activated/M2”, according to their role in Th-1 and Th-2 driven immune responses respectively (Mills *et al.*, 2000). *In vitro* the M1 phenotype can be induced from exposure to the proinflammatory cytokine gamma interferon (IFN- γ) and the stimulation of a toll-like receptor (TLR), such as the stimulation of TLR4 by lipopolysaccharide (LPS). This cell, which is equipped with antimicrobial and proinflammatory features, is therefore indispensable for fighting bacterial infections (Lathrop *et al.*, 2015). The M2 designation comprises indeed several distinct noninflammatory macrophages phenotypes, which are induced *in vitro* by exposure to different agents. One of these, the product of exposure to the Th2 cytokine interleukine 4 (IL-4) (designated M2a), participates in traditional Th2-mediated immune responses, such as fighting extracellular parasites, and contributes to allergic reaction (Mantovani *et al.*, 2004).

Macrophages use several bactericidal and bacteriostatic processes that must be prevented, counteracted and resisted for intracellular *S. enterica* to survive and replicate. During the first few minutes of the phagocytosis NADPH oxidase produces reactive oxygen species which damage bacterial DNA, protein and membrane. Toxic reactive nitrogen species are instead produced later by inducible NO synthase (iNOS) (Thompson *et al.*, 2011).

In addition, a major process by which macrophages kill phagocytosed bacteria is the fusion of lysosomes to phagosomes to form the so-called phagolysosomes (Garin *et al.*, 2001). This fusion is the fulcrum of this process and results in the delivery of lysosomal hydrolases, or endosomal

proteases, which are active in the acidified (pH<5) lumen of the phagosome. Supplemented further by the activities of cationic proteins and antimicrobial peptides, such as the defensins, the mature phagosome is extremely bactericidal and, in some cell types, also functions as the source of antigenic peptides that are required for the development of an adaptive immune response against individual pathogens (Steele-Mortimer, 2008).

Biogenesis of the phagosome is a highly synchronous process, characterized by the sequential acquisition of specific cellular proteins delivered by fusion with different endosomal compartments ultimately resulting in the formation of the phagolysosome (Garin *et al.*, 2001).

Salmonella is able to elude phagocytosis, survive, replicate and spread across the host body through macrophages. In particular, *Salmonella* possesses two multi-functional virulence systems which contribute to its intramacrophage growth and virulence: the PhoP-PhoQ system and the SPI-2 type III secretion system (T3SS) (Thompson *et al.*, 2011).

PhoP-PhoQ is one of such two-component system that has been identified in several bacteria, including *Salmonella*, *Yersinia pestis* and *Pseudomonas aeruginosa* (Ren *et al.*, 2016). This is a two-component system that rules bacterial virulence, mediates the adaptation to Mg²⁺-limiting environments and regulates different cellular activities in several Gram-negatives species; it is compound of the inner membrane sensor kinase PhoQ and the cytoplasmic response regulator PhoP (Groisman, 2001). This system is encoded by the *phoP* genetic locus, whose name dates back to the first identification in *Salmonella* Typhimurium, while monitoring the expression of a nonspecific acid phosphatase (Kier *et al.*, 1979). The first evidence of the direct involvement of PhoP in *Salmonella* virulence was provided in 1986 by Fields *et al.* (1986) who have conducted a genetic screen in *phoP* mutant bacteria which had survival defect in macrophages (Ren *et al.*, 2016). In this study they developed an *in vitro* assay to identify 83 mutants of *S. Typhimurium* attenuated for survival within macrophages *in vitro*; they discovered that all mutants were less virulent than the parent strain *in vivo*, demonstrating that survival into macrophages is essential for virulence (Fields *et al.*, 1986).

The realization that PhoP-PhoQ plays a crucial role in the control of the virulence in *Salmonella* promoted new interest in the system and has led to a very good characterization of the PhoP regulon, rendering it one of the best characterized regulons in enteric bacteria (Groisman *et al.*, 2001).

The *phoP* regulatory locus is composed of two genes, *phoP* and *phoQ*. The PhoP (transcriptional activator) and PhoQ (sensor/kinase) proteins have aminoacids similarity to other bacterial regulatory proteins (two-component regulators) that control the synthesis of many proteins in response to environmental signals. The PhoP and PhoQ gene products are essential for the transcriptional activator of a number of unlinked *phoP*-activated genes, called *pag* loci (Groisman *et al.*, 1989). One *pag* locus is essential for full virulence and survival within macrophages (*pagC*); in fact *S. Typhimurium* strains with *pagC* mutations are not as attenuated in virulence as *phoP* or *phoQ* mutants, suggesting that other *pag* loci are essential for full virulence (Groisman *et al.*, 1989; Miller *et al.*, 1989).

When PhoQ senses the environmental signals such as low concentration of Mg^{2+} or acidic pH produced by neutrophils and macrophages, it is activated by autophosphorilation (Alpuche *et al.*, 1992; Bearson *et al.*, 1998). Subsequently, the phosphoryl of PhoQ is transferred to the conserved aspartyl of PhoP and then the phosphorylated PhoP activates the transcription of *phoP* itself and PhoP-regulated genes. Therefore, the crucial task of PhoP for bacterial virulence is mainly attributed to its central role in survival into the host (Oyston *et al.*, 2000).

The SPI-2 type III secretion system (T3SS) is mainly involved in the mechanism thanks to which *Salmonella* eludes phagocytosis; indeed it belongs to a group of microorganisms, which also includes *Leishmania* spp. and *Mycobacterium lepraemurium*, that resist to inactivation by lysosomal factors within the macrophage phagolysosomes compartment (Lowrie *et al.*, 1979; Buchmeier and Heffron, 1991). In fact, *Salmonella* owns the capacity to modify its phagosome, which is called “the *Salmonella*-containing vacuole” (SCV), so that it acquires some characteristics of late endosomes, but remains distinct from normal phagolysosomes (Thompson *et al.*, 2011).

The biogenesis of SCV can be separated into 3 stages: early (<30 min p.i.), intermediate (30 min-approx 5 h p.i.) and the late (>5 h p.i.), each of which is associated with specific sets or subsets of T3SS effectors. The best-documented aspect of SCV biogenesis is the sequential delivery of endolysosomal membrane proteins that defines the conversion of early to intermediate SCVs. Thus, the early SCV membrane is highly enriched in early endosome membrane markers, such as EEA1, rab5 and transferrin receptor, replaced within 20-40 min with late endosomal/lysosomal markers, such as Lamps and vATPase (Steele-Mortimer *et al.*, 1999; Smith *et al.*, 2005). This variation in membrane content is accompanied by a decrease in the luminal pH (pH_{scv}) to <4.5 and redistribution of the SCV to a predominantly juxtannuclear position near the microtubule organizing centre (MTOC) (Salcedo *et al.*, 2001; Steele-Mortimer, 2008).

The *Salmonella*-containing vacuoles are functionally separated from the normal degradative pathway of the macrophage host cell thanks to the ability of the microorganism to diverge from it. Indeed, intracellular *Salmonella* organisms reside within these vacuoles and are able to avoid the fusion with functional lysosomes (Rathman *et al.*, 1997).

Salmonella owns a third virulence system, the SPI-1 encoded T3SS1, which mediates interaction between the bacterium and host cells. Unlike the other two, this system is induced in extracellular bacteria and is essential for the bacterium-driven entry into nonphagocytic cells, such as intestinal epithelial cells (Lathrop *et al.*, 2015).

4. Salmonella enterica serovars responsible for human infection

Serovars of *S. enterica* can be divided into Typhoidal and Non-Typhoidal (NTS) (Hurley *et al.*, 2014). Although this two groups present likeness at the genetic level, they cause very different diseases and immune responses in human. Host specificity and/or clinical syndromes characterize different serovars and symptoms can range from asymptomatic carriage to invasive systemic disease. Most *S. enterica* serovars associated with disease in humans and in other warm blooded animals are part of subspecies I, which includes typhoidal and non-typhoidal serovars. Differences between these two sets arise in epidemiology, clinical manifestation and human host response (Gal-Mor *et al.*, 2014).

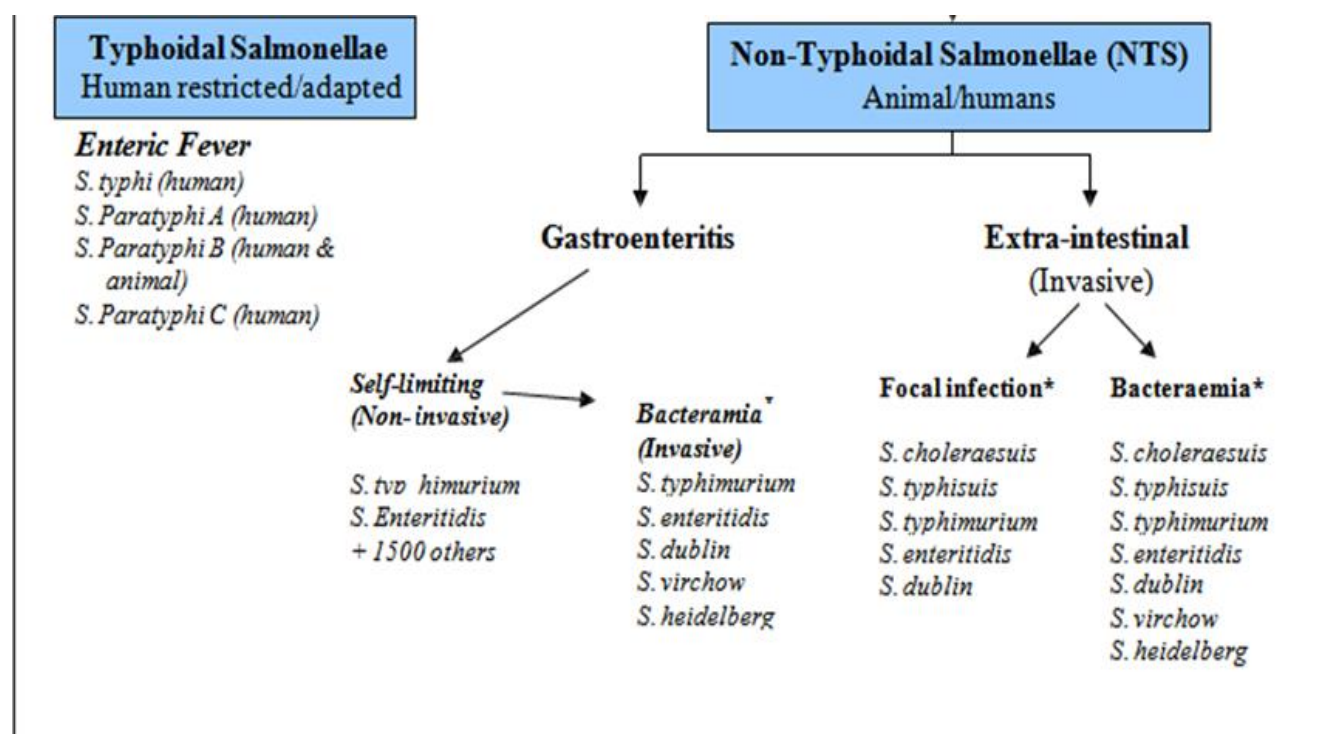


FIGURE 3. Classification of the genus *Salmonella* (Akyala *et al.*, 2015).

4.1 Typhoidal Salmonellosis

Typhoidal serovars of *Salmonella enterica* are responsible for the so-called typhoid fever, a systemic infection caused by *S. Typhi*, a highly adapted human-specific pathogen that evolved about 50.000 years ago. A very similar but often less severe disease is caused by *S. Paratyphi A, B* and sometimes *C* (Bhan *et al.*, 2005).

4.1.1 Epidemiology

Typhoid fever is a major public health problem worldwide, for this reason have been made several efforts to estimate its global burden (Mogasale *et al.*, 2014). In particular, in 2015, Crump and colleagues (2015) assessed 21.6 million cases in the year 2000 with the highest incidence in children in south and southeast Asia. For 2010, instead, Buckle and colleagues (2010) estimated 13.5 million cases of typhoid fever, but the rectified estimated calculating for the low sensitivity of blood cultures for isolation of the bacteria was 26.9 million cases, resulting in more than 200.000 deaths (Mogasale *et al.*, 2014; Gal-Mor *et al.*, 2014).

In the 19th century typhoid fever was an important cause of illness and death in Europe and United States because of overcrowded and unsanitary urban conditions (Parry *et al.*, 2002); then, during the 20th century, it became rare thanks to the development of industrialization and the improvement of sanitary conditions (Breiman *et al.*, 2012).

Today typhoid fever is endemic in the developing countries in which access to clean and safe water and adequate sanitary condition are still lacking (Gal-Mor *et al.*, 2014). The paucity of these basic services weighs heavily on public health, and typhoid fever is one of the most serious consequences (Mogasale *et al.*, 2014). Furthermore, in these areas many hospitals lack structures for blood cultures and up to 90 percent of patients with typhoid fever are treated as outpatients; for this reason reliable data to estimate the burden of the disease are difficult to obtain (Parry *et al.*, 2002).

Humans are the only known reservoir for typhoidal *Salmonella* (Bäumler and Fang, 2013); this exclusive adaptation to human host is the reason for which research on typhoidal *Salmonella* serovars is hampered (Kidgell *et al.*, 2002). Accordingly, most of the relevant research on typhoid fever is based on *in vitro* studies of human immune cell lines and on infection of laboratory animals with other serovars which, in contrast to *S. Typhi*, can infect animals as well as humans (Wain *et al.*, 2002). In fact murine infection with *S. Typhimurium* cause a disease similar to human typhoid fever and, therefore, it has been employed as a model (Santos *et al.*, 2003).

This troubles would be overtaken with the demonstration that one strain of *S. Typhi* was not restricted to humans. Such a strain is anticipated to have existed in ancient times. Many pseudogenes were generated by accumulation of mutations, insertions or deletions within the genome of modern-time *S. Typhi*, suggesting its recent evolutionary origin. Relying on molecular estimation for the accumulation of synonymous polymorphism, this ancestral strain was assumed to be approximately 50.000 years old (Kidgell *et al.*, 2002; Papagrigorakis *et al.*, 2007).

4.1.2 Risk factors and transmission

Transmission of typhoid fever occurs through the faecal-oral route and possesses a multifactorial nature. It depends primarily on direct contact with the stool of an infected individual (Akullian *et al.*, 2015), which can contaminate water and food; for this reason food handlers have been often implicated as source of infections (Sharma *et al.*, 2009).

Risk is highest in densely populated areas that lack proper sanitation and access to safe drinking water. In these endemic regions, house-hold level hygiene, food and water safety and handling practices, as well as close contact with a typhoid patient, are associated with the direct transmission of typhoidal salmonellosis (Liu *et al.*, 2011). Because typhoidal serovars of *S. enterica* are exclusively human host-adapted, reservoirs of infection exist solely within groups of infected

individuals, a small number of which (1-6%) develop a chronic state, which allows the disease to persist during inter-epidemic periods (Akullian *et al.*, 2015). As a consequence, latent infected people are the carriers for the spread of infection and disease (Monack, 2012).

Furthermore, consumption of raw vegetables and unwashed fruits is considered a risk factor, since these products have been associated with typhoid and salmonellosis. In Bangladesh, for example, fruits are often sold on the streets, where vendors have limited access to hand-washing facilities, and consumers cut and kept unrefrigerated products at home for hours (Ram *et al.*, 2006).

Consumption of butter and yoghurt is considered another risk factor strongly associated with typhoid. Contaminated dairy products, by dirty hands or unpotable water, may efficiently support pathogen growth, although dairy cattle do not carry the microorganism (Glynn and Bradley, 1992). In India it has been reported that many typhoid outbreaks have been transmitted through the consumption of milk and ice cream. In this country poor people buy cheap dairy products from local vendors who may handle them in a non-eligible way or, even, adulterate them with untreated water (Sharma *et al.*, 2009).

4.1.3. Non-Typhoidal Salmonellosis (NTS)

Non-typhoidal serovars of *Salmonella enterica* (NTS) are the most common pathogens causing gastrointestinal infections worldwide (Chen *et al.*, 2013).

NTS include several serovariants, each of which is adapted to one or more particular hosts. In particular, *S. Gallinarum* or *S. Abortusovis* are, respectively, highly host adapted to poultry and sheep, and may only cause very mild symptoms in humans; in contrast, *S. Choleraesuis*, host adapted to swine, may also induce severe systemic illness in human population (Chiu *et al.*, 2005; Liu *et al.*, 2009). In a similar way, *S. Dublin*, whose primary host is cattle, is mainly responsible for the systemic form of infection in humans. *S. Enteritidis* and *S. Typhimurium* are ubiquitous serovars and affect both man and animals; they also are able to cause typhoid-like infections in mice, which

are used as laboratory model of typhoid fever, and humans, or asymptomatic intestinal colonization in chickens (Su and Chiu, 2007).

4.1.4. Epidemiology

Accurate estimates of the burden of diarrheal diseases caused by *Salmonella* species, and other foodborne pathogens, are needed to effectively set public health goals and allocate resources to reduce disease burden. The World Health Organization (WHO) established the Foodborne Disease Burden Epidemiology Reference Group for the purpose of provide global foodborne disease estimates (Senior, 2009).

Nevertheless, these data underestimate the magnitude of the problem; in fact, to be ascertained in a laboratory-based surveillance system, an ill person must seek medical care, submit a specimen (generally stool), the laboratory must test for the pathogen and report positive outcomes; finally, the laboratory-confirmed infection must be ascertained by public health authorities. Therefore the cases which are reported in a laboratory-based surveillance represent only a small proportion of the total community cases (Majowicz *et al.*,2010).

Non-typhoidal salmonellosis occur worldwide. A total of 93.8 million cases of gastroenteritis due to NTS infection are estimated each year, resulting in approximately 155.000 deaths; of these, 80.3 million cases are foodborne. Despite global morbidity, mortality is primarily restricted to developing countries. (Majowicz *et al.*, 2010).

Invasive non-typhoidal salmonellae are among the most common isolates from febrile presentations in adults and children across sub-Saharan Africa, especially where the prevalence of HIV is high (Reddy *et al.*, 2010); they were first described in patients with HIV infection from Africa and United states in the mid-1980s (Gordon *et al.*, 2008). In this area NTS are the most common cause of bacteriemia, provoking more than 100.000 deaths per year (MacLennan, 2014). In more detail, in 1984 the first cases of non-typhoidal bacteraemia in US patients with AIDS was described, and

the first epidemiological link between invasive *Salmonella* infection and AIDS was made in New Jersey (Profeta *et al.*, 1985). Finally, after an age-stratified study in New York City, which showed that invasive NTS was over-represented in patients with AIDS, in 1990 non-typhoidal *Salmonella* has been confirmed as a common HIV-related pathogen in sub-Saharan African adults (Gilks *et al.*, 1990). Co-infection with malaria and malnutrition are other risk factors in addition to HIV.

4.1.5. Risk factors and transmission

The most common vehicles of NTS transmission are animal-derived food product, such as pork, poultry, eggs and dairy, which may be contaminated by *Salmonella* because animals are infected or because fecal contamination occurs during processing (Rabsh *et al.*, 2001). The transmission can also result from person to person contact or from direct contact with animals, such as pets/ruminants, pigs, birds, rodents, reptiles or amphibians (Haeusler and Curtis, 2013).

Another important source of infection is the consumption of contaminated products, including fruits, tomatoes, spinach, fresh sprouts, ice cream premix, juice and peanuts, which have all been associated with outbreaks in recent years (Gal-Mor *et al.*, 2014).

The majority of human cases are caused by only a few non-typhoidal serovars. In particular, *S. Enteritidis* and *S. Typhimurium* including the monophasic variant *S. Typhimurium* 1,4,[5],12:i:-, are the most commonly reported *Salmonella* serovars in confirmed human cases in 2014, followed by *S. Infantis*, which was the fourth most common serovar in human salmonellosis (Rabsh *et al.*, 2001; EFSA and ECDC, 2015).

5. Pathogenesis and Clinical Manifestation

The outcome of a *Salmonella* infection is determined by the status of the host and the status of the bacterium. Whereas the status of the bacterium is determined by virulence factors, the status of the host is determined by age, genetic and environmental factors (Van Asten and Van Dijk., 2005). Among host factors, achlorhydria as a result of aging, previous gastrectomy, treatments with histamine H₂ receptor antagonists, proton-pump inhibitors, or large amounts of antacids may lower the infective dose and favor the onset of complications (Parry *et al.*, 2002). In particular, studies on human volunteers revealed that the ingestion of small inocula ($\leq 10^3$ cells) may cause asymptomatic excretion, although even smaller inocula (5 to 100 microorganisms) may cause disease in susceptible hosts (Blaser and Newman, 1982).

In general, *Salmonella* enters a host through the ingestion of contaminated food or water (Löfström *et al.*, 2010) and reaches the stomach where the pH approaches the value of 1-2 (Rychlik and Barrow, 2005). The bacterium is relatively resistant to low pH values when in the stationary phase of growth. When in the exponential phase, however, it is less acid resistant and can survive exposure only to moderately low pH values between 4 and 5; in both cases *Salmonella* can become more resistant to acidity after a short period of adaptation at moderate pH (Lee *et al.*, 1994).

This phenomenon takes the name of acid tolerance response (ATR) (Foster, 1990; Foster, 1991) and provides for two adaptation steps: transient adaptation is achieved after 20 minutes of exposure to moderate pH, while the second step of sustained adaptation requires about 60 minutes of exposure (Rychlik and Barrow, 2005).

Leaving the stomach *Salmonella* becomes exposed to several external stress factors, including increase in osmotic pressure and decrease in oxygen concentration, that leads the pathogen to induce anaerobic respiration (Merrel *et al.*, 2002; Xu *et al.*, 2003).

Afterwards *Salmonella* reaches the small intestine, where other component of the normal microflora restrict its growth by creating a nutrient-depleted environment, releasing metabolic products such as propionate or butyrate, which are harmful for *Salmonella*, or producing bacteriocins. Thanks to the

communication pathway known as “quorum sensing” the pathogen is able to sense the presence of other bacterial species (Rychlik and Barrow., 2005).

In the small intestine the bacteria adhere to mucosal cells and then invade the mucosa (Parry *et al.*, 2002) using the fimbrial antigens. The M (microfold) cells are specialized epithelial cells located in the follicle-associated epithelium in the Peyer’s patches and are probably the primary intestinal sites targeted for invasion (Santos *et al.*, 2003). These cells are an important portal of entry of *Salmonella*, together with other pathogens, into the submucosal lymphoid system. They are highly endocytic and can rapidly transfer material from their luminal side to their basal side, where the T cells and antigen-presenting cells reside, ready to elicit an immune response. It has been suggested that the cells of the columnar epithelium may also be an important portal of entry, since they are more numerous than the M cells (Hughes and Galan, 2002)

From the infected tissues bacteria spread to the regional lymph nodes, where macrophages form a first effective barrier to prevent a further spread; if the pathogen is able to avoid them, systemic disease can occur (Löfström *et al.*, 2010). *Salmonella*, indeed, is able to elude phagocytosis, and remain within the modified phagosome called *Salmonella*-containing vacuole, in which it survive and replicate (Steele Mortimer, 2008). Type III secretion systems are used to translocate bacterial effector proteins into the host cells, mediating both invasion and vacuole biogenesis. The latter is a complex and dynamic process, involving extensive membrane remodeling, interactions with the endolysosomal pathway, actin rearrangements and microtubule-based movement and tubule extension (Steele-Mortimer, 2008). This case bacteria disseminate via the efferent lymphatic and the thoracic duct into vena cava, through which they reach spleen and liver, where replicate. From these organs microorganisms are released in large number to the blood stream; this happens at a critical point, which is probably determined by the number of bacteria, their virulence and the host response (Parry *et al.*, 2002).

The incubation period is between 7 and 14 days and, after that, *Salmonella* reaches other organs, widely disseminating the whole organism: this is known as bacteremic phase (Butler *et al.*, 1978; Wain *et al.*, 2001). Liver, spleen, bone marrow, gallbladder and Peyer’s patches of the terminal

ileum are the most common sites of secondary infection. Microorganisms may now reinvade the intestinal wall by retrograde spread from the bile (secondary infection) or may be naturally shed in the stool being, in this way, ready to infect other hosts (Smith *et al.*, 2016). Pathology in the Peyer's patches include hyperplasia of lymphoid follicles, necrosis of lymphoid follicles and ulceration in the long axis of the bowel, with the possibility of perforation and haemorrhage (Singh *et al.*, 2001).

5.1 Clinical manifestation

The clinical pattern of salmonellosis in humans can be divided into four different forms: enteric fever, gastroenteritis, bacteremia and other complications of non-typhoidal salmonellosis.

The enteric fever is caused by *S. Typhi*, whereas *S. Paratyphi A*, *B* and *C* cause paratyphoid fever with symptoms which are milder and a lower mortality rate. Both serotypes are solely human host adapted (Sherer and Miller, 2001).

Infection typically occurs due to ingestion of food or water contaminated with human waste. Roughly 10% of patients may relapse, die or encounter serious complications such as typhoid encephalopathy, gastrointestinal bleeding and intestinal perforation. Relapse is the most common occurrence, probably due to persisting organisms within reticuloendothelial system (RES). Often, typhoid encephalopathy is accompanied by shock associated with mortality. Slight gastrointestinal bleeding can be resolved without blood transfusion, but 1 to 2% of cases can be fatal if a large vessel is involved (Hu and Kopecko, 2003).

Intestinal perforation may present with abdominal pain, riding pulse and falling blood pressure in sick people; therefore, it is a very serious complication in 1 to 3% of hospitalized patients (Parry, 2006).

The gastrointestinal form is caused by non-typhoidal salmonellae, which include at least 150 serotypes with *S. Typhimurium* and *S. Enteritidis* being the most common (Gray and Fedorka-Cray, 2002).

It is usually a self-limiting infection (three to seven days) confined to the gastrointestinal system which manifests as nausea, vomiting and diarrhea occurring 6-48 hours after ingestion. Stool are usually non-bloody and of moderate volume, although this maybe variable; microscopy stool examination generally shows leukocytes and red blood cells (Arda *et al.*, 2001). Some cases of gastroenteritis require hospitalization due to the severity of the diarrhea and dehydration (Mead *et al.*, 1999).

Associated symptoms may include fever, chills, abdominal cramps, myalgias and headache. Severe right quadrant pain occurs sometimes mimicking appendicitis (Arda *et al.*, 2001).

Patients may continue to carry *Salmonella* in their gastrointestinal tracts after the acute infection for a mean duration of four weeks; neonates and children usually shed bacteria for longer periods (up to seven weeks) (Buchwald *et al.*, 1984). Furthermore, some patients may develop a chronic carrier state, defined as stool or urine positive for *Salmonella* 12 months later the onset of acute illness. Chronic carriage represents an important public health issue as a mechanism of transmission to other persons. Infants, women, persons with gallstones or kidney stones and immunocompromised people are at higher risk for chronic carriage (Shimoni *et al.*, 1999).

The most frequent complication of gastroenteritis is bacteremia, which occurs in 1-4% of immunocompetent patients (Buchwald *et al.*, 1984). Bacteremia is a serious condition in which bacteria enter the bloodstream after passing through the intestinal barrier. This condition it has been associated with highly invasive serotypes like *S. Choleraesuis* or *S. Dublin* (Shimoni *et al.*, 1999).

Several people are at increased risk for bacteremia; the category includes patients at the extreme of age, such as infants and the elderly, immunocompromised persons, such as pregnant women, acquired immunodeficiency syndrome (AIDS) people, transplant recipients and patients with malignancy or autoimmune diseases. HIV-infected persons have up to a 100-fold increased risk for salmonellosis compared to the normal populations. Recurrent bacteremia is classified as an AIDS-defining event (Celum *et al.*, 1987; Gordon *et al.*, 2008).

Moreover, in 10-25% of adults with bacteremia endovascular complications, including seeding of atherosclerotic plaques or aneurysms, especially in the aorta, may occur. Bacteremia may also lead

to localized infections in 5-10% of cases including meningitis, endocarditis, pneumonia, empyema, abscess formation, osteomyelitis or septic arthritis. Abscesses may develop in association with malignant tumor and patients with sickle cell disease have a prediction to develop *Salmonella* osteomyelitis. Arthritis can occur due to direct infection into the joint which is typically monoarticular. A reactive polyarthritis can also occur after the onset of gastroenteritis; however, this is an immune phenomenon rather than a dissemination infection (Cohen *et al.*, 1978).

Salmonellosis can be spread by chronic carriers who potentially infect many individuals. On average, *Salmonella* persists in the gastrointestinal tract from 6 weeks to 3 months, depending on the serotypes (Scherer and Miller, 2001).

Only about 0.1% of non-typhoidal *Salmonella* cases are shed in stool samples for periods exceeding one year. About 2 to 5% of untreated convalescent typhoid cases will excrete *S. Typhi* in feces for 1 to 3 months and between 1 and 4% become chronic carriers excreting the microorganism for more than one year (Parry, 2006).

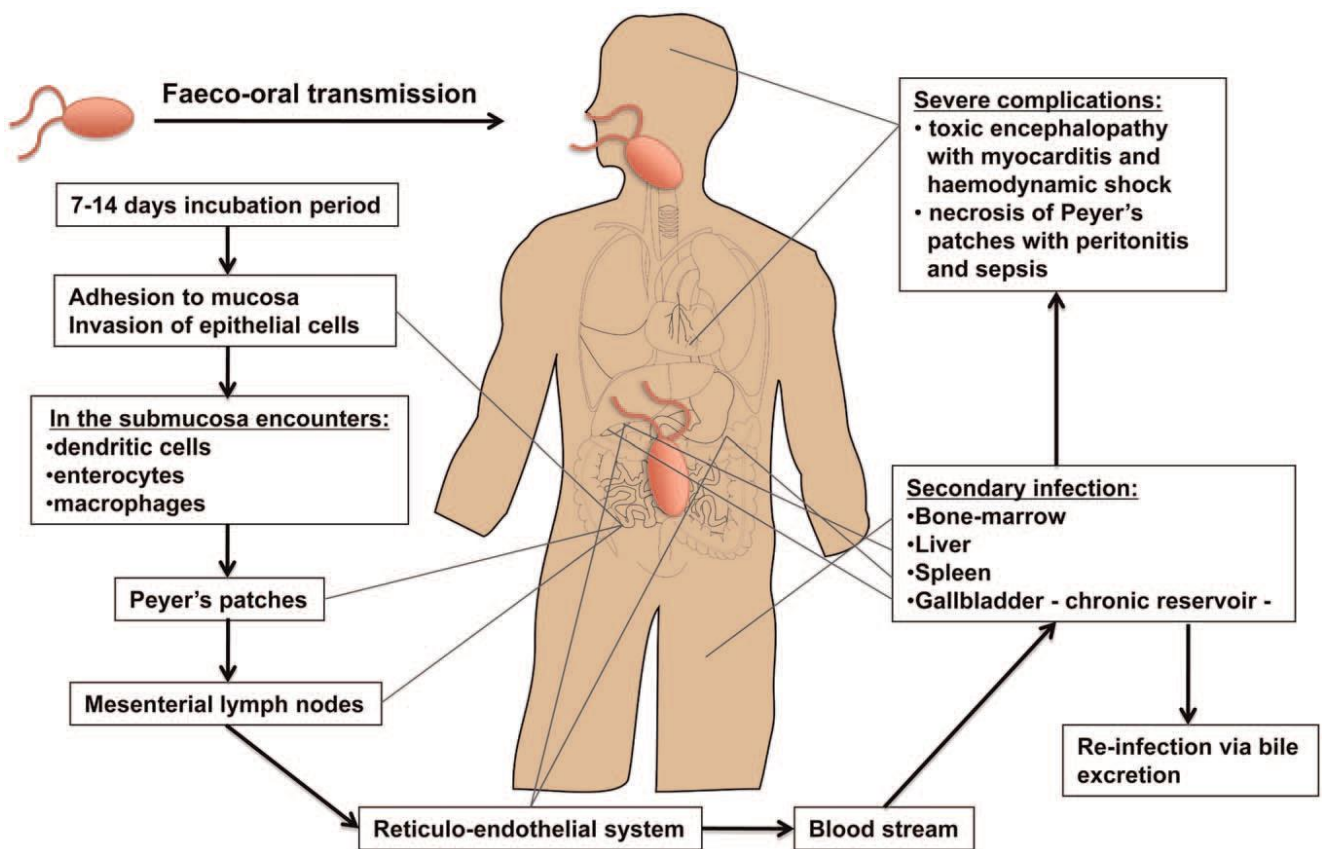


FIGURE 4: Dissemination of *Salmonella* in human (De Jong *et al.*, 2012).

6. Antimicrobial Resistance

Over the last decades an increasing prevalence of antimicrobial resistance has been observed in *Salmonella enterica* (Threlfall, 2002), causing a serious health problem worldwide. The development of Multi Drug Resistance (MDR) among isolates of *Salmonella* serovar Typhimurium, but also among other serovars, has been of overriding importance. The MDR phenotype in *Salmonella* Typhimurium began to appear in the early 1980s in the United Kingdom; the isolates displayed resistance to five antimicrobial agents, such as ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, constituting a resistance phenotype commonly referred to as R-type ACSSuT. By the 1990s, this phenotype had been reported from several other countries in Europe as well as in the United States, Canada, Israel, Turkey and Japan (Threlfall, 2002).

Another important resistance trend among *Salmonella* isolates has been the resistance to quinolones, such as nalidixic acid, and to fluoroquinolones, such as ciprofloxacin. Although nalidixic acid is not used for medical treatment, resistance to this antimicrobial agent is of clinical importance since it may be associated with reduced clinical effectiveness of fluoroquinolone treatments (Dimitrov *et al.*, 2007; Stevenson *et al.*, 2007).

The onset of resistant to extended-spectrum cephalosporins, such as ceftriaxone, represents another substantial public health concern; these drugs are, indeed, used for the treatment of invasive *Salmonella* infection, especially in children, since fluoroquinolones may be avoided. This kind of resistance has been recognized in *Salmonella* since the mid-1980s and is usually mediated through beta-lactamases of the ESBL or AmpC type (Miriagou *et al.*, 2004). In Europe, cephalosporin-resistant *Salmonella* was first detected in France and Italy in 1989 and 1990, respectively (Villa *et al.*, 2002).

Over recent years, it has been reported from several studies the emergence of extensively drug-resistant isolates of *Salmonella*. *Salmonella* serovar Typhimurium isolates resistant to 12 to 15 antimicrobial agents, including 6 or 7 CLSI drug classes, comprising cepheims, have been reported from Malaysia and Vietnam. In a similar way, *Salmonella* isolates resistant to 6 or 7 antimicrobial

agents, including 3 to 5 CLSI drug classes, have been detected in Thailand (Wannaprasat *et al.*, 2011).

Moreover, since 2009, the emergence of extended-spectrum cephalosporins and carbapenems resistant variants has been reported, especially in China, Columbia, Pakistan and United States (Le Hello *et al.*, 2013).

Among typhoidal *Salmonella* isolates, resistance to the traditional first-line antimicrobials ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole is generally caused by resistant determinants located on plasmids. Resistance to ampicillin is often mediated through beta-lactamases (i.e. *bla*_{PSE} and *bla*_{TEM}) whereas several mechanism may be associated with chloramphenicol resistance. Three types of chloramphenicol acetyltransferases (CATs, type I to III) have been described, but also genes involving non enzymatic resistance mechanisms have been detected, such as *cmlA* and *floR* (Arcangioli *et al.*, 2000).

In non-typhoidal *Salmonella*, instead, the genes mediating R-type ACSSuT are generally clustered together in chromosomal genetic element called *Salmonella* genomic island (SGI-1) where a 14-kb region bracketed by two integron structures contains the antimicrobial resistance genes contributing to its ACSSuT properties. In particular, *bla*_{PSE-1} conferring resistance to ampicillin, *floR* to chloramphenicol and florfenicol, *aadA2* to streptomycin, *sulI* to sulfonamides and *tetG* to tetracycline (Boyd *et al.*, 2002).

As a matter of fact, resistance to fluoroquinolone is determined by mutations in genes of the quinolone resistance-determining regions (QRDRs); these genes are *gyrA*, *gyrB*, *parC* and *parE*, which encode, respectively, for DNA gyrase and topoisomerase IV, the target enzyme of this class of antimicrobial agents (Hopkins *et al.*, 2005). In addition to the chromosomally encoded mechanisms, plasmid-mediated resistance mechanisms have been described. The first to be described in the late 1990s was *qnrA*, then several other mechanisms have been discovered, including different *qnr* variants, *aac(6')-Ib-cr*, *qepA* and *oqxAB* (Strahilevitz *et al.*, 2009).

Furthermore, resistance to cephalosporins, including extended-spectrum cephalosporins such as ceftriaxone, is commonly mediated through beta-lactamases, which are enzymes which inactivate

the drug by cleaving the beta-lactam ring. The beta-lactamases mediating resistance to extended-spectrum cephalosporins can be divided into three groups: extended-spectrum beta-lactamases (ESBLs), carbapenemases and AmpC-type beta-lactamases (Paterson and Bonomo, 2005). Among the AmpC plasmid-mediated beta-lactamases, cephamycinases (CMY), encoded by *bla_{CMY}* genes, are the predominant cause of cephalosporin resistance in nontyphoidal *Salmonella*. The genes encoding the beta-lactamase enzyme are generally located on mobile genetic elements such as plasmids, transposons and integrons. Therefore, resistance may spread horizontally between isolates, clones and serovars (Philippon *et al.*, 2002).

Finally, resistance to macrolides is determined by the action of a macrolide-2'-phosphotransferase encoded by the *mphA* gene; the mechanism have been detected in *Salmonella enterica* isolates with elevated azithromycin MICs, defined as MICs of >16 µl/ml. The first case of azithromycin treatment failure in a patient with invasive *Salmonella* infection was reported in 2010 (Molloy *et al.*, 2010).

7. The role of pig as reservoir

The transmission usually occurs through the fecal-oral route and, after infection, pigs can become healthy carriers, excreting *Salmonella* in their faeces, due to the permanence of the bacteria in the tonsils, the intestines and the gut-associated lymphoid tissue (Fedorka-Cray *et al.*, 2000). In this way, carriers are a perpetual potential source of infection for other animals including humans (De Busser *et al.*, 2011). In addition to this, *Salmonella* may persist in the environment for long periods, in particular *S. Choleraesuis* survives in dry faeces for at least 13 months post-shedding, demonstrating such persistence an important risk factor (Gray *et al.*, 1995).

The microorganism known as *S. Choleraesuis* was first isolated from pigs. This was the predominant serovar in pigs during the 1950s and 1960s, and in 1958 and 1968 it represented 90% and 74.2 of all *Salmonella* isolates from pigs, respectively. Afterwards, its prevalence has decreased until being considered an infrequent isolate (Sojka *et al.*, 1977).

The importance of pigs as a source of salmonellosis for humans has been shown in different investigations (Piras *et al.*, 2011) and pork contaminated by *Salmonella* is recognized as one of the most important causes of human foodborne salmonellosis (Swanenburg *et al.*, 2001).

Pigs can become infected with *Salmonella* at the breeding and/or fattening farm. However, from the moment the pigs leave the farm, there are also many opportunities to become infected during transport, lairage or slaughter. The stress associated with these phases is an important cause of infection, as well as grouping with other pigs, which can be infected or shedding carriers. Contact with a *Salmonella*-contaminated environment, as during lairage, may contribute to the dissemination of the infection (Swanenburg *et al.*, 2001). It has also been shown that the proportion of pigs in a herd that excrete *Salmonella* increased after transport (Isaacson, 1999). Moreover, Fedorka-Cray *et al.* (1995) showed that the microorganism could be isolated from mesenteric lymph nodes and caecal and rectal contents as soon as three hours after infection; hence, it can be asserted that it is possible for pigs to pick up *Salmonella* during transport or at lairage, and start excreting the microorganism before being slaughtered. Thus, they can infect other pigs, trucks and

lairage environment. Moreover, carcasses can become infected at the slaughterhouse by contaminated slaughter equipment or infected aerosols (Kotula and Emswiller-Rose, 1988; Sammarco *et al.*, 1997).

7.1 Clinical Syndroms

Clinical porcine salmonellosis can be divided in two syndroms: one involves *S. Typhimurium*, which is associated with enterocolitis, while the other one involves *S. Choleraesuis* and is generally associated with septicaemia (Wilcock and Schwartz, 1992).

Intensively reared, weaned pigs are most often affected by *Salmonella*, infection. In general, *S. Typhimurium* tends to cause disease in young pigs from 6 to 12 weeks of age. Disease from this serovar is rare in adult animals, but infection is frequent. *S. Choleraesuis* causes disease among pigs of a wider range of ages. Mortality tends to be higher in younger than in older pigs, while morbidity is often equal regardless of age. Disease from *S. Choleraesuis* in the adult is not a common occurrence. However, if susceptible population is exposed, the animals will be significantly affected (Funk and Gebreyes., 2004).

The occurrence of salmonellosis in suckling pigs is rare, presumably because of lactogenic immunity, but infection is not uncommon. However, neonatal pigs are susceptible to oral challenge with *Salmonella* and develop a disease similar to that observed in weaned pigs (Funk and Gebreyes, 2010).

The septicaemic form of porcine salmonellosis is usually caused by *S. Choleraesuis*. Affected pigs are inappetent, lethargic and febrile, with temperatures of up to 41.7°C. Respiratory signs may consist of a shallow, moist cough and diaphragmatic breathing. Clinical signs first appear after 24-36 h of infection (Reed *et al.*, 1986).

Usually, diarrhoea is not a characteristic of *S. Choleraesuis* infection until at least the fourth or fifth day; it may last from five to seven days after insurgence if chronic reinfection is not occurring.

It may appear gross lesions such as colitis, infarction of gastric mucosa, swollen mesenteric lymph nodes, splenomegaly, hepatomegaly and lung congestion. Random white foci of necrosis are often observed in the liver (Wilcock and Schwartz, 1992).

Instead, the paratyphoid nodule is the microscopic lesion which is most often associated with *S. Choleraesuis*. In the liver this type of lesion can be observed as clusters of histiocytes amid foci of acute coagulative hepatocellular necrosis and corresponds to the whiter foci seen grossly (Lawson and Dow, 1966).

Other lesions may include fibrinoid thrombi in venules of gastric mucosa and cyanotic skin and glomerular capillaries. It is often observed also swelling of histiocytes and epithelial cells, typical of Gram-negative sepsis, as well as hyperplasia of reticular cells of the spleen and lymph nodes (Wilcock *et al.*, 1976).

On the contrary, the enterocolitic form in pigs is typically associated with *S. Typhimurium* infection and, occasionally, with *S. Choleraesuis* infection. In contrast to the septicaemic disease, the initial sign of infection is often watery, yellow diarrhoea. Infected pigs are inappetent, febrile and lethargic. Mortality is usually low, however morbidity can be high within a few days of infection (Wilcock and Schwartz, 1992).

At necropsy the major gross lesion is focal or diffuse necrotic colitis and typhlitis. Mesenteric lymph nodes are greatly enlarged. Intestinal lesions develop as red, rough mucosal surfaces, which may also have grey-yellow debris. Colon and caecal contents are bile-stained and scant, often with black or sand-like gritty material on the surface. Intestinal necrosis may be seen as sharply delineated button ulcers, often associated with resolving lesions (Wood and Rose, 1992).

Local or diffuse necrosis of cryptic and surface enterocytes can be revealed by histopathological examination. The lamina propria and submucosa contain macrophages and lymphocytes, with neutrophils observed only in the very early stages of disease. It is also common to observe lymphoid atrophy or regenerative hyperplasia associated with this disease (Reed *et al.*, 1986).

8. Italian Salami

In recent years European consumers have gradually changed their behavior towards food. Higher dietary, hygienic and health in foodstuffs remain fundamentals standards, but nowadays consumers look more and more for certification and reassurance of product's origins and production methods. This heightened consumer awareness is reflected in the demand for products with individual characteristics, due to specific product methods, composition or origin (Anonymous, 2004).

Among traditional products, dry fermented sausages are very popular and widespread. In Europe, and in particular in Italy, these products have a long history and they have evolved over centuries into several types (Conter *et al.*, 2007). Dry fermented sausages produced in the Mediterranean countries are usually air dried, due to particular climate, and rarely smoked. Pork is the main meat used and fungal starter cultures may be used on the external surface imparting a complexity of flavor to the product (Talon *et al.*, 2004).

Dry fermented sausages, the so-called salami, that are produced in Italy differ in several aspects, such as raw materials, formulation and manufacturing process. About one hundred of salami are produced in the different regions of the country, although some of them are manufactured on a small scale (Zanardi *et al.*, 2010).

The salami manufactured in the Emilia-Romagna region of northern Italy are generally made exclusively with pork, have coarsely ground meat and 3-4 mm large cubes of fat. The fresh meat is carefully selected from deboned shoulder and belly. Fat used is normally pork backfat. The salami should contain 25-30 % fat and hard fat is preferred. After grounding, salt, whole peppercorns and garlic are added to the mixture. Some formulations comprise also white wine, sugars and starter bacterial cultures. The salami are then stuffed into a pork casing and aged for 25 to 40 days to obtain final product characterized by sweet taste and delicate aroma (Mataragas *et al.*, 2015).

The use of salt (sodium chloride, NaCl) is essential in dry fermented sausages, due to solubilization of proteins and emulsion of fat. Moreover, NaCl can control the growth of undesirable bacteria responsible for spoilage of meat and pathogenic bacteria (King *et al.*, 2016). The usual amount of

salt added is generally between 2-4 % by weight (Ockerman and Basu, 2007) and these values increase in final products due to drying process (Zanardi *et al.*, 2010).

In addition to NaCl, other salts can be added to the mixture. Nitrates (maximum 300 mg/kg) and nitrites (maximum 150 mg/kg) are normally added to inhibit *Clostridium botulinum* (Hospital *et al.*, 2016), Enterobacteriaceae and enterococci (Coloretti *et al.*, 2008) and to favour the cured red colour of the meat (Villaverde *et al.*, 2014). Ascorbate (maximum 1 g/kg) has important antioxidative action, may be required to compensate the pro-oxidant effect of nitrites on meat proteins and acts as enhancer of colour formation (Villaverde *et al.*, 2014).

Many salami formulations contain starter bacterial cultures and sugar. Starter cultures are composed of selected lactic acid bacteria (LAB), *i.e.* homofermentative lactobacilli and/or pediococci, and Gram-positive, catalase-positive cocci (GCC), *i.e.* nonpathogenic, coagulase-negative staphylococci and/or kocuriae. They improve the quality and safety of the final product to standardize the production process (Lücke, 1998, 2000). Nonetheless, traditional manufacturers prefer the spontaneous fermentation without added starter cultures. In this case, the required microorganisms originate from the meat itself or from the plant environment (Santos *et al.*, 1998).

9. Materials and Methods

9.1 Sample collection

From April to December 2015, 155 ground pork and fat mixture samples (corresponding to 155 different batches) were tested for *Salmonella*. Only pig meat and pig backfat were used for the salami manufacturing. When a mixture sample tested positive for *Salmonella*, the corresponding batch of salami was analyzed at the end of the curing period. Overall, 24 batches of salami were tested for *Salmonella* and analyzed for pH, a_w values and for NaCl content. All samples were provided by four production plants (here identified as: A, B, C, D) located in Emilia-Romagna region, northern Italy. The number of samples provided by the each plant was different, varying from 47 (A), to 27 (B), 11 (C) and 70 (D). In two plants (A and B; 74 samples) microbial starter cultures were added to the ground meat and fat mixtures to rapidly lower the pH value at the beginning of ripening. In two plants (C and D; 81 samples) microbial starters were not used. All producers added salt, nitrites and nitrates, ascorbates and black pepper to the ground mixtures before casing. To avoid interference with *Salmonella* growth, the mixture samples were collected before starter cultures or any ingredient was added.

Salami batches were tested only in case their mixture of origin was positive for *Salmonella*. According to Regulation EU 2073/2005, five units per batch were tested at the end of the curing period, which ranged from 20 to 48 days. Overall, 24 batches, corresponding to 120 salami, were initially tested but during the study a total of 160 salami were analyzed, because eight batches resulted to be contaminated by *Salmonella* despite prolonged curing. They were re-tested after an additional curing period, whose duration ranged from 21 to 38 days and was decided by the producers, until *Salmonella* was no more isolated.

9.2 Salmonella detection and typing

Detection of *Salmonella* was performed following the ISO 6579:2002 bacteriological standard method used in the European Union and a molecular method based on the real-time polymerase chain reaction (PCR). The first step was the pre-enrichment, as expected from ISO 6579:2002, developed suspending 25 g of each sample in 225 ml of BPW (Buffered Peptone Water, Oxoid, Basingstoke, UK) and homogenizing for 2 minutes in a Stomacher blender. After 18 ± 2 h at $37 \pm 1^\circ\text{C}$, the pre-enrichment cultures were analyzed by Real-Time PCR. DNA extraction was carried out using SureFood PREP *Salmonella* Kit (R-Biopharm, Darmstadt, Germany); the master-mix was prepared following SureFast *Salmonella* ONE Kit (R-Biopharm). The kit is based on the utilization of two Taqman probes: HEX, which amplifies a specific sequence of the beta actin protein, a structural protein found in all kinds of cells, and, for this reason, acts as internal control; FAM, which amplifies a specific sequence of the 16S ribosomal subunit of *Salmonella*. Chromosomal DNA of each sample was tested for the presence of *Salmonella* by Real-Time PCR with the instrument Mx3005P QPCR System (Agilent Technologies, Italy). The reaction was performed in a final volume of 25 μl containing 5 μl of template and 20 μl of master-mix. The following thermal program was applied: a single cycle of DNA polymerase activation of 5 min at 95°C followed by 45 amplification cycles of 15 s at 95°C (denaturing step) and 30 s at 60°C (annealing-extension step). The samples with a cycle threshold (CT) value lower than 40 were considered positive and analyzed by ISO 6579:2002 method. The samples showing a CT value equal or higher to 40 were considered negative for *Salmonella* and discharged.

At the beginning of the study, a 25 g-aliquot of the samples was used both for the molecular analyses and for ISO detection of *Salmonella*. Thereafter, since we found several positive results by PCR not confirmed by the ISO method, we preferred testing both 25 g- and 50 g- aliquots for the isolation of the microorganism. Then we can divide the study into two parts: *i*) part I: Real-Time PCR, ISO detection and MPN enumeration of *Salmonella* were performed analyzing 25 g of the samples; *ii*) part II: Real-Time PCR was performed from 25 g pre-enriched samples, but positive samples were tested by ISO 6579:2002 using both 25 g and 50 g of the samples. MPN enumeration was performed on 50 g

sample aliquots. The modification was introduced when 42 mixture samples and 25 salami had already been tested.

Aliquots of 1 ml and 0.1 ml of the pre-enrichment broths were seeded in 10 ml of Mueller-Kauffman Tetrathionate Broth (MKTB; Oxoid) and Rappaport-Vassiliadis Broth (RVB; Oxoid), respectively, and incubated at $37 \pm 1^\circ\text{C}$ for $24 \pm \text{h}$ (MKTB) and $41.5 \pm 1^\circ\text{C}$ for $24 \pm \text{h}$ (RVB). After incubation, a 10 μl loopful each was plated on XLD Agar (Oxoid) and *Salmonella* Chromogenic Agar (Oxoid). Plates were incubated at $37 \pm 1^\circ\text{C}$ for $24 \pm 3 \text{ h}$. *Salmonella* suspect colonies were biochemically tested seeding into Triple Sugar Iron Agar (Biolife, Milan, Italy), Lysine Iron Agar (Oxoid) and Christensen's Urea Agar (Biolife) and incubated at $37 \pm 1^\circ\text{C}$ for 20-24. Cultures showing positive reaction for *Salmonella* were assayed by slide agglutination with an O-omnivalent *Salmonella* serum (Denka Seiken, Tokyo, Japan). Biochemical identification to the genus was carried out with API® 20E system (bioMérieux, Marcy l'Etoile, France).

Salmonella serotyping was performed following the White-Kauffmann-Le Minor scheme by slide agglutination with O and H antigen specific sera (DID, Milan, Italy; Biogenetics, Padua, Italy).

Identification of *S. enterica* 4,[5],12:i:- and its differentiation from *S. Typhimurium* were phenotypically obtained repeating phase inversion at least three times without evidencing expression of phase-two flagellar antigens and genotypically with PCR (Barco *et al.*, 2011).

Salmonella PFGE typing was performed according to standard methods (Ribot *et al.*, 2006) with *Xba*I restriction of DNA.

9.3 *Salmonella* enumeration

In the Real-Time PCR positive samples, the enumeration of *Salmonella* was performed following the miniaturized Most Probable Number technique according to ISO 6579-2:2012. At the beginning of the study (part I), the enumeration was performed by testing a 25 g-aliquot of the PCR-positive samples.

In the second part of the study, MPN enumeration was performed testing 50 g of the samples (part II).

The modification was introduced when 42 mixture samples and 25 cured salami had already been tested.

A 25 or 50 g portion of the samples was diluted 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} in BPW in triplicate in 12 multi-well microtiter plates and incubated at $37 \pm 1^\circ\text{C}$ for 16-20 h. Thereafter, a 20 μl aliquot from each well was transferred in a 12 multi-well microtiter plate containing 2 ml of Modified Semi-Solid Rappaport-Vassiliadis (MSRV) per well. After incubation at $41.5 \pm 1^\circ\text{C}$ for $24 \pm 3\text{h}$, the wells showing a grey-white zone extending out of the inoculum drop were further tested by streaking a 10 μl loopful of the bacterial growth onto XLD (Oxoid) agar plates. Microtiter plates not showing bacterial growth were incubated for further $24 \text{h} \pm 3 \text{h}$, after that they were considered negative if no well showed a grey-white growth extending out of the inoculum. XLD agar plates were incubated at $37 \pm 1^\circ\text{C}$ for 24 h and suspect *Salmonella* colonies were selected and subjected to ISO 6579 confirmation tests. After biochemical and serological confirmation, the number of plates with confirmed *Salmonella* colonies was used to estimate the MPN *Salmonella*/ g.

9.4 Measure of pH and a_w

The pH value was measured on 5 g of salami homogenized in distilled water (10/1 water/sample, w/w) by the Crison micro pH 2001 instrument (Crison Instruments, Barcelona, Spain) equipped with a Xerolyt 52-02 electrode (Crison). The a_w value was measured at 25°C by an AquaLab series 4TE a_w meter (Decagon Devices, Inc., Pullman 99163, WA).

9.5 Sodium chloride analysis

Sodium chloride (NaCl) analysis was performed following the ISO 1841-1:1996 method for the

determination the chloride content of meat and meat products with NaCl contents equal to or greater than 1.0%.

9.6 Macrophage experiment

The macrophage experiment was performed following protocols obtained from different studies (Ekman *et al.*, 1999; Schwann *et al.*, 2000; Daigle *et al.*, 2001). We selected five strains of *Salmonella*, isolated from our samples: four strains of the monophasic variant of *S. Typhimurium* 1,4,[5],12:i:- and one strain of *S. Infantis*, which is the fourth most common serovar in human cases of salmonellosis in EU countries.

The ability of *S. Typhimurium* to cause systemic disease has been studied in mice, through survival and replication of the bacteria in murine macrophages. However, other studies are required to assess the ability of the microorganisms to survive and replicate in human macrophages. In our study we evaluated the capacity of *Salmonella* to survive and replicate within a human macrophages-like cell line (U937), to assess their virulence for humans.

9.7 Laboratory protocols

DNA extraction

1- Preparation of the basic material

Transfer 1.0 ml of the enrichment culture under sterile conditions into a 1.5 ml reaction tube.

Centrifuge for 5 min. at 12.000 rpm.

Discard the fluid and inactivate per sterilization.

2- Lysis of the basic material

Add 400 µl of Lysis Buffer, mix it briefly and close the reaction tube with caps.

Incubate under continuously shaking for 10 minutes at 99°C.

3- Setting of optimal binding conditions

Centrifuge the sample lysate for 1 minute at 12.000 rpm.

Transfer ca. 300 µl of the liquid supernatant into a new 1.5 ml reaction tube.

4- Binding of the nucleic acid on a Spin Filter

Add 200 µl of Binding Buffer to the filtrate and mix the sample.

Place a spin filter into a yellow 2.0 receiver tube; transfer the solution directly onto the filter and incubate at room temperature for 1 minute.

Centrifuge for 1 minute at 12.000 rpm.

Discard the filtrate and place the spin filter back into the yellow receiver tube.

5- Purification of the bound nucleic acids

Add 550 µl of Wash Buffer to the spin filter and centrifuge for 1 minute at 12.000 rpm; discard the filtrate and place the spin filter back into the receiver tube (perform two times).

6- Drying of the Spin Filter

Remove the residual ethanol by final centrifugation for 2 minutes at 12.000 rpm.

7- Elution of nucleic acids from the Spin Filter

Place the spin filter into a clear 1.5 ml receiver tube and add 100 µl of the preheated Elution Buffer directly onto the spin filter.

Incubate 3 minutes at room temperature and centrifugate for 1 minute at 10.000 rpm, after centrifugation discard the spin filter.

The eluted DNA is ready-to-use for the PCR. The DNA can be stored for up 24 hours at 4°C; for a storage time of more than 24 hours it should be kept at -20°C.

Real-Time PCR

1- Preparation of the master mix

Calculate the total number of reactions needed (samples and control reactions). Recommended control reactios: negative control, positive control and extraction control. The master-mix includes an internal amplification control (inhibition control) for each reaction.

It is also recommended to prepare the master-mix with 10% additional volume in order to compensate reagent loss. Allow the reagents to thaw, mix by vortexing and centrifuge before opening and use. The tube of the taq polymerase should be kept at -20°C and not be mixed by vortexing.

COMPONENTS FOR MASTER-MIX	AMOUNT PER REACTION	10 REACTION (WITH 10% EXCESS)
Reaction Mix	19.9 μ l	218.9 μ l
Taq polymerase	0.1 μ l	1.1 μ l
Total Volume	20 μl	220 μl

TABLE 1: Example for the calculation and preparation of 10 reactions.

Mix each master-mix well and centrifuge shortly before use.

2- Setup

Initial denaturation : 5 minutes at 95°C

Cycles : 45

Denaturation : 15 seconds at 95°C

Annealing/Extension : 30 seconds at 60°C

3- Preparation of the Real-Time PCR Mix

Pipette 20 μ l of the master-mix into appropriate tubes/wells or capillaries.

Close the tube of the negative control (the negative control consist of the master-mix).

Pipette 5 μ l of sample DNA into the designated tubes/wells or capillaries.

Pipette 5 μ l of the positive control into the designated tubes/wells or capillaries.

Centrifuge all tubes/ wells or capillaries shortly at low speed.

Place tubes/wells or capillaries into the Real-Time PCR instrument and start the run according to the setup.

ISO 6579:2002- Horizontal method for the detection of *Salmonella* spp.

1- Pre-enrichment in non-selective liquid medium

Buffered Peptone Water is inoculated at ambient temperature then incubated for 18 ± 2 hours at $37^\circ\text{C} \pm 1^\circ\text{C}$.

2- Enrichment in selective liquid media

Transfer 0.1 ml of the culture obtained to a tube containing 10 ml of Rappaport-Vassiliadis medium with soya (RVS broth); transfer 1 ml of the culture obtained to a tube containing 10 ml of Muller-Kauffman tetrathionate/novobiocin broth (MKTTn broth). The RVS broth is incubated at $41.5^\circ\text{C} \pm 1^\circ\text{C}$ for $24 \text{ h} \pm 3 \text{ h}$ and the MKTTn broth at $37^\circ\text{C} \pm 1^\circ\text{C}$ for $24 \text{ h} \pm 3 \text{ h}$.

3- Plating-out and identification

After incubation for $24 \text{ h} \pm 3 \text{ h}$, using the culture obtained in the RVS broth, inoculate by means of a loop the surface of one large-size Petri dish containing the first selective plating-out medium Xylose Lysine Deoxycholate agar (XLD), so that well-isolated colonies will be obtained.

Proceed in the same way with the second selective plating-out medium using a sterile loop and Petri dishes as above. The choice of the second appropriate medium is left to the discretion of the testing laboratory; in the present study *Salmonella* Chromogenic Agar Base was used.

After incubation for $24 \text{ h} \pm 3 \text{ h}$, using the culture obtained in the MKTTn broth repeat the procedure just described with the two selective plating-out media.

Invert the dishes so that the bottom is uppermost and place them in the incubator set at 37°C for both the first and the second plating-out medium.

After incubation for 24 h \pm 3 h, examine the plates for the presence of typical colonies of *Salmonella* and atypical colonies that may be *Salmonella*. Mark their position on the bottom of the dish.

Typical colonies of *Salmonella* grown on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE : *Salmonella* H₂S negative variants (i.e. *S. Paratyphi* A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening.

Typical colonies of *Salmonella* grown on *Salmonella* Chromogenic Agar Base are mauve.

4- Selection of colonies for confirmation

For confirmation, take from each plate of each selective medium at least one colony considered to be typical or suspect and further four colonies if the first is negative.

Streak the selected colonies onto the surface of pre-dried nutrient agar plates in a manner which will allow well isolated colonies to develop. Incubate the inoculated plates at 37°C \pm 1°C for 24 h \pm 3 h.

Use pure cultures for biochemical and serological confirmation.

5- Biochemical confirmation

Inoculate the following media with each of the cultures obtained from the colonies selected before.

Triple Sugar/Iron Agar (TSI Agar): streak the agar slant surface and stab the butt; incubate at 37°C \pm 1°C for 24 h \pm 3 h. Interpret the changes in the medium as follows.

Butt:

- yellow glucose positive (glucose used)
- red or unchanged glucose negative (glucose not used)

- black formation of hydrogen sulfide
- bubbles or cracks gas formation

Slant surface:

- yellow lactose and/or sucrose positive (lactose and/or sucrose used)
- red or unchanged lactose and sucrose negative (neither lactose nor sucrose used)

Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and (in about 90% of the cases) formation of hydrogen sulfide (blackening of the agar).

When a lactose-positive *Salmonella* is seeded, the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar test only.

Urea Agar (Christensen): streak the agar slant surface. Incubate at 37°C ±1°C for 24 h ± 3 and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenolred to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h.

L-Lysine Decarboxylation Medium: inoculate just below the surface of the liquid medium. Incubate at 37°C ±1°C for 24 h ± 3 h.

Turbidity and a purple colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

Detection of beta-galactosidase: suspend a loopful of the suspected colony in a tube containing 0.25 ml of the saline solution. Add 1 drop of toluene and shake the tube. Put the tube in a water bath set at 37°C and leave for several minutes (approximately 5). Add 0.25 ml of the reagent

for detection of beta-galactosidase and mix. Replace the tube in the water bath set at 37°C and leave for 24 h ± 3 h, examining the tube at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 minutes.

Medium for Voges-Proskauer (VP) reaction: suspend a loopful of the suspected colony in a sterile tube containing 3 ml of the VP medium. Incubate 37°C ±1°C for 24 h ± 3 h. After incubation, add two drops of the creatine solution, three drops of the ethanolic solution of 1-naphthol and then two drops of the potassium hydroxide solution; shake after the addition of each reagent.

The formation of a pink ring to bright red colour within 15 minutes indicates a positive reaction.

Medium for indole reaction: inoculate a tube containing 5 ml of the tryptone/tryptophan medium with the suspected colony. Incubate at 37°C ±1°C for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent. The formation of a red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

Biochemical identification of the isolates was performed by using the API 20E ® (bioMérieux, Marcy l'Etoile, France) microsubstrate system, which consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with the bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

6- Serological confirmation and serotyping

The detection of the presence of *Salmonella* O-, Vi- and H- antigens is tested by slide agglutination with the appropriate sera, from pure colonies and after auto-agglutinable strains have been eliminated.

Elimination of auto-agglutinable strains: place one drop of the saline solution onto a carefully cleaned glass slide. Disperse in the drop, by means of a loop, part of the colony to be tested, in order to obtain a homogeneous and turbid suspension. Rock the slide gently for 30 seconds to 60 seconds. Observe the result against a dark background, preferably with the aid of a magnifying glass.

If the bacteria have clumped into more or less distinct units, the strain is considered auto-agglutinable and shall not be submitted to the following tests as the detection of the antigens is not feasible.

Examination for O-antigens: using one non-autoagglutinating pure colony, proceed using one drop of the anti-O serum instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

Use the poly-and monovalent sera one after the other.

Examination for Vi-antigens: proceed using one drop of the anti-Vi serum instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

Examination for H-antigens: inoculate the semi-solid nutrient agar with a pure non-autoagglutinable colony. Incubate the medium at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for $24 \text{ h} \pm 3 \text{ h}$. Use this culture for examination for the H-antigens, proceeding using one drop of the anti-H serum instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

7- Definitive confirmation

Strains which are considered to be *Salmonella*, or which may be *Salmonella*, shall be sent to a recognized *Salmonella* reference centre for definitive typing.

8- Expression of results

In accordance with the results of the interpretation, indicate the presence or absence of *Salmonella* in a test portion of x or x ml of product (see ISO 7218).

ISO 6579:2002- Part 2: Enumeration by a miniaturized most probable number technique

The method is based on miniaturization of the dilution, pre-enrichment and selective enrichment steps. The selective enrichment medium, modified semi-solid Rappaport-Vassiliadis (MSRV) is intended for the detection of motile salmonellae and is not appropriate for the detection of non-motile salmonellae.

1- Test portion, initial suspension

Prepare an initial suspension by diluting the test portion 10-fold in BPW. For example, add 25 g of sample to 225 ml of BPW and homogenize in a stomacher for 1 minute.

2- Dilution and pre-enrichment in non-selective liquid medium

Take a 12-well microtiter plate with empty wells in the first row of three wells and containing 2 ml of BPW in the other wells (second, third and fourth row with each 3 wells).

Transfer to each well of the first (empty) row of three wells, using a pipette, 2.5 ml of the initial suspension.

Transfer 0.5 ml of each well from the first row into the 2 ml of BPW in the successive wells in the second row (first 5^{-1} dilution).

Transfer 0.5 ml of each well from the second row into the 2 ml of BPW in the successive wells in the third row (second 5^{-1} dilution).

Before transferring the 0.5 ml of the second row into the third row, mix the suspension in the wells by repeatedly (carefully) sucking up and blowing out of the suspension in the pipette and in the wells. Proceed in the same way for the other rows.

Incubate the 12-well microtitre plate at 37°C for $18\text{ h} \pm 2\text{ h}$.

3- Selective enrichment on a semi-solid medium

Allow the MSR/V in the 12-well microtiter plates to equilibrate at room temperature if they were stored at a lower temperature.

Inoculate each well containing 2 ml MSR/V with 20 μl of the BPW culture, i.e. using a multi-channel pipette. Use new tips for each row of three wells.

Place the 20 μl BPW culture at the margin of the well and on the surface of the medium.

When taking a subculture from BPW, try not to disturb particulate samples. Therefore, move the microtitre plates carefully. Avoid pipetting particulate matter on to the MSR/V plates.

Incubate the inoculated MSR/V plates at 41.5°C for $24\text{ h} \pm 3\text{ h}$. Do not invert the plates.

Suspect wells show a grey-white, turbid zone extending out from the inoculated drop. The turbid zone is characterized by a white halo with a clearly defined edge.

If the wells are negative after 24 h, reincubate for a further $24\text{ h} \pm 3\text{ h}$.

4- Selective plating

Subculture suspect MSR/V wells by dipping 1 μl loop just inside the border of the opaque growth and by inoculating this culture material on the surface of one normal size XLD plate, so that well-isolated colonies are obtained.

Subculture at least the highest dilutions: the ones which still show three suspect MSRv wells as well as the subsequent dilutions showing two or one suspect MSRv wells.

Incubate the inverted XLD plates at 37°C for 24 h ± 3 h.

Return negative MSRv plates to the 41.5 °C incubator and incubate for a further 24 h ± 3 h.

Perform the selective plating procedure if after 48 h of incubation additional wells become suspect.

Typical colonies of *Salmonella* spp. grown on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

5- Biochemical confirmation

Take from each XLD plate at least one colony considered to be typical or suspect.

If well-isolated colonies (of a pure culture) are available on the selective plating media, the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium, like nutrient agar can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium. Incubate inoculated nutrient agar plates at 37°C for 24 h ± 3 h.

Use pure cultures for biochemical and serological confirmation.

Macrophage experiment

1- Bacteria and culture conditions

Bacteria for infection assay were cultured overnight in BPW (Buffered Peptone Water, Oxoid, Basingstoke, UK) at 37°C for revitalization. Then they were plated on *Salmonella* Chromogenic Agar (Oxoid) and incubated overnight at 37°C. Afterwards one pure colony was selected and diluted in BPW (Oxoid) to obtain the necessary concentration.

2- U937 cell culture

U937 cells (human macrophage-like) were grown in RPMI 1640 medium (Euroclone, Milan, Italy) supplemented with glutamine and heat-treated (56°C, 30 min) 10% fetal calf serum (Euroclone) and incubated at 37°C under an atmosphere of 95% air-5% CO₂.

The U937 cells were seeded onto 24-well tissue culture plates and activated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) at a concentration of 10⁻⁸ 72 hours before infection. The PMA treatment has been shown to induce stable differentiation of U937 monocytes into macrophages without undesirable gene upregulation. This treatment cause the U937 cells to become adherent and activated.

Prior to infection, PMA-containing medium was removed and cells were gently washed with RPMI 1640 medium to remove residual PMA and replaced with 500 µl per well of fresh medium without PMA.

U937-macrophages were infected with a MOI (multiplicity of infection) of 10:1, centrifugated at 800 rpm for 5 minutes, to synchronize the phagocytosis and incubated for 45 minutes at 37°C with 5% CO₂(0-h time point).Then infected cell monolayers were washed three times with Phosphate-Buffered Saline (PBS) (Oxoid). RPMI 1640 containing 50 µg of gentamicin (Euroclone)per ml was added to kill any remaining extracellular bacteria. After 2 h of further incubation at 37°C, the medium in the 24-well plates was replaced again with RPMI containing 5µg of gentamicin (Euroclone) per ml. Host cells stayed in this medium for the remainder of the infection to prevent extracellular growth of any released bacteria.

For viable count determinationthe infected macrophages monolayers were washed three times with PBS and bacteria were harvested by adding 300 µgof 0.1 % Tryton X-100 (Sigma-Aldrich, Darmstadt,Germany) in distilled water to each well. After 3 min, cell lysates were collected and serially diluted 10-fold in PBS and aliquots were plated onto PCA (Plate Count Agar) (Oxoid) to assess bacterial CFU (colony forming unit). Bacteria survival and growth were evaluated at different time points post infection (p.i.), in particular at 24, 48 and 72 h p.i.

PFGE (Pulsed-Field Gel Electrophoresis)

The method for PFGE is described following Ribot *et al.*, 2006.

Day 1: Starting from isolated colonies, grown onto Blood Agar (Oxoid), the strain to be examined and the strain of *S. Braenderup* H9812, used as internal control and molecular weight marker, were plated onto Nutrient Agar (Oxoid) and incubated overnight at 37°C for 14-18 h.

Day 2: 1% Agarose (Seakem Gold) in TE 10-1 solution was prepared and maintained at 54-56°C in a double boiler.

Each bacterial culture to be examined was resuspended in 2 ml of CSB (Cell Suspension Buffer). The concentration of each suspension and of the control strain were fixed at 0.8-1 optical density using spectrophotometric at a wavelength of 610 nm.

For each bacterial culture and for the control strain, 10 µl of Proteinase K (PK) were aliquoted in microtubes.

200 µl of CBS suspension + bacterial cells were dispensed in microtubes with PK and 200 µl of Seakem Gold 1% Agarose was added to each microtube, mixing gently. Then the mixture was transferred in the plug stencil and allowed to cool for 10 min at 4°C.

5 ml/strain of CLB (Cell Lysis Buffer) were dispensed in 50 ml Falcon tubes with 50 µl of PK. The plugs of each strain were transferred in lysis buffer and incubated 2 h in a double boiler at 54-56°C with constant shaking at 150-175 rpm.

Bidistilled sterile water and TE solution were conditioning at 54-56°C.

The appropriate sieves were applied to the microtubes and CLB was eliminated. The plugs were washed two times with 10 ml of bidistilled sterile water at 54-56°C in a double boiler with constant shaking for 10 min for each washing, followed by three washing series with 10 ml of TE 10-1 solution. Store the plugs in microtubes containing 1.5 ml of TE 10-1 solution at 4°C.

RESTRICTION PHASE: each plug was cut in 3 parts of 2.5-3 mm and 1/3 was placed in a 2 ml microtube.

The restriction solution for the restriction enzyme XbaI was prepared calculating a final volume of 200 µl/sample, containing: the specific buffer for the enzyme at the final concentration of 1X, the

enzyme volume equivalent to 50 U/sample (brought to final volume with molecular biology water).

The incubation lasted 2-6 h, or alternatively, o.n., at 37°C.

Day 3: 2-2.2 L of TBE 0.5X running buffer was prepared and poured in the apparatus CHEF-DRII (BIO RAD) electrophoretic cell and allowed to cool at 14°C.

110 ml of 1% running gel (BIO RAD Pulsed Field Certified Agarose) was prepared in TBE 0.5X, melted in microwave, cooled at 54-56°C, poured in a special mold and left to solidify for 30 min.

For each sample, a plug was inserted in a well inside the gel, according to the default order. In wells number 1, 5, 10 and 15 a plug of *S. Braenderup* was charged. Wells were sealed with 1% Agarose in TE.

The gel was left to solidify for 10 min. and then it was placed in the electrophoretic cell to start the run with the following parameters: 6V/cm, switch time of 2.2s- 63.8s, 120° corner, running time 19-20 h.

Day 4: The gel was stained in a 1µg/ml ethidium bromide solution for 20 min. and then bleached in water for 60 min.

The image was acquired at transilluminator and stored as TIFF file.

10. Results

Detection and enumeration of *Salmonella* in raw mixture samples

The Real-Time PCR detected CT-values ranging from 24.78 to 39.99 in 46 out of 155 (29.7%) mixture samples. Six additional samples (3.9%) were characterized by CT-values higher than 40 and discharged. The positive samples by Real-Time PCR were tested by ISO 6579:2002 for *Salmonella* detection and by ISO TS 6579-2:2012 for *Salmonella* MPN enumeration. *Salmonella* was isolated from 24/46 (52.2%) Real-Time positive samples. Its prevalence in meat and fat mixture samples prior to the addition of additives, salt and starter cultures was 15.5% (95% CI: 10.6 – 22.0).

Eight *Salmonella* serovars were identified, with *S. Derby* (12/24; 50.0%) as the most common, followed by *S. Typhimurium* monophasic variant 4,[5],12:i:- (4/24; 16.7%), *S. London* (2/24; 8.3%) and *S. Stanley* (2/24; 8.3%). *S. Brandenburg*, *S. Goettingen*, *S. Infantis* and *S. Rissen* were identified in one sample each (1/24; 4.2%). Different *Xba*I PFGE profiles were found among the most common serovars, namely *S. Derby* and *S. enterica* 4,[5],12:i:-. Seven PFGE profiles were identified among *S. Derby* isolates (D1-D7) and four among the monophasic variant of *S. enterica* 4,[5],12:i:- (MT1-MT4). One PFGE type was identified for *S. London* (L1) and *S. Stanley* (S1).

In part II of the study, when ISO detection of *Salmonella* on both 25 and 50 g of the samples was performed, *Salmonella* was isolated from 20 samples. Twenty samples (100%) were positive by testing 50 g, 12 (60%) were positive by 25 g. The difference between the two procedures (ISO 50 g vs. ISO 25 g) was statistically significant (p value= 0.0033; Fisher's exact test).

The MPN enumeration of *Salmonella* ranged from 160 MPN/g to < 1.3 MPN/g (Table 2). In 19/24 (79.2%) samples it was <1.3 MPN/g, corresponding to the lowest detectable value.

Samples/Batch n°	MPN values
15 (A)	<i>S. London</i> (31/g)
78 (A)	<i>S. Stanley</i> (12/g)
79 (A)	<i>S. Stanley</i> (29/g)
96 (B)	<i>S. Derby</i> (< 1.3/g)
121 (B)	<i>S. Derby</i> (< 1.3/g)
140 (D)	<i>S. Goettingen</i> (< 1.3/g)

TABLE 2: *Salmonella* MPN values in ground meat positive samples.

Detection and enumeration of *Salmonella* in cured salami

Twenty-four batches of cured salami, manufactured with the mixture samples contaminated by *Salmonella*, were re-analyzed at the end of their curing time. A total of 160 salami were tested, because eight batches were still found positive despite prolonged curing and should be re-tested after additional curing time.

By Real-Time PCR 59/160 (36.9%) samples showed a CT-value ranging from 23.67 to 39.78.

Fourteen samples (8.8%) showed a CT-value higher than 40 and were discharged. The ISO 6579 method detected *Salmonella* in 16 salami, belonging to six different batches. The proportion of *Salmonella*-positive mixture samples, which ended up as contaminated salami, was therefore 25.0% (6/24). Since the *Salmonella*-positive salami belonged to 6 batches, the final prevalence of contaminated batches was 3.9% (6/155) (95% CI: 1.8-8.2). The proportion of *Salmonella*-positive salami was 10.0% (16/160) and the confirmation rate of the ISO method toward Real-Time PCR was

27.1% (16/59).

In part II of the study, when ISO detection of *Salmonella* on both 25 and 50 g of the samples was performed, *Salmonella* was isolated from 13 salami. Eleven samples (84.6%) were positive by testing 50 g, 8(61.5%) were positive by testing 25 g. The difference between the two procedures (ISO 50 g vs. ISO 25 g) was not statistically significant (p value= 0.378; Fisher's exact test). Two samples were negative with either ISO 50 g or ISO 25 g procedures, but were positive by 50 g - MPN enumeration.

Salmonella-positive salami were manufactured by three producers (A, B and D). The *Salmonella*-positive batches were tested again after an additional curing time of 21-38 days. However, one batch out of six (16.7%) was found to be still *Salmonella*-positive, and resulted negative after a curing period of 8 days more (total curing time: 57 days). This batch of salami was produced by plant A with the addition of culture starters to the ground meat.

Seventeen isolates were detected, as one sample was contaminated by two different *Salmonella* strains. Six *Salmonella* serovars were identified and *S. Derby* was the most prevalent (9/17; 52.9%), followed by *S. London* (2/17; 11.8%), *S. Panama* (2/17; 11.8%), *S. Branderup* (2/17; 11.8%), *S. Goldcoast* (1/17; 5.9%) and *S. Stanley* (1/17; 5.9%). Four PFGE profiles were identified among *S. Derby* isolates (D1, D4, D8, D9), one of which was found in the correspondent mixture sample. Two PFGE profiles were found between *S. Branderup* strains. One PFGE type was identified for *S. London* (L1) and *S. Stanley* (S1), identical to the genotypes detected in the correspondent mixture samples. For *S. Panama*, PFGE analysis could not identify a genomic profile, because of self-degrading DNA.

The enumeration of *Salmonella* ranged from 8.7 MPN/g to 1.3 MPN/g in seven samples (43.7%), and was < 1.3 MPN/g in the remaining nine (56.3%) (Table 3).

Samples	MPN values
PF-15/1	8.7 MPN/g
PF-15/2	1.4 MPN/g
PF-15/3	< 1.3 MPN/g
PF-15 II/1	< 1.3 MPN/g
PF 78/4	< 1.3 MPN/g (50)
PF 79/2	< 1.3 MPN/g (50)
PF 79/3	8.7 MPN/g (50)
PF 79/4	1.3 MPN/g (50)
PF 96/1	< 1.3 MPN/g (50)
PF 96/3	< 1.3 MPN/g (50)
PF 96/4	< 1.3 MPN/g (50)
PF 96/5	< 1.3 MPN/g (50)
PF 121/1	< 1.3 MPN/g (50)
PF 121/4	< 1.3 MPN/g (50)
PF 121/5	< 1.3 MPN/g (50)
PF 140/2	2.7 MPN/g (50)

TABLE 3: *Salmonella* MPN values in salami positive samples.

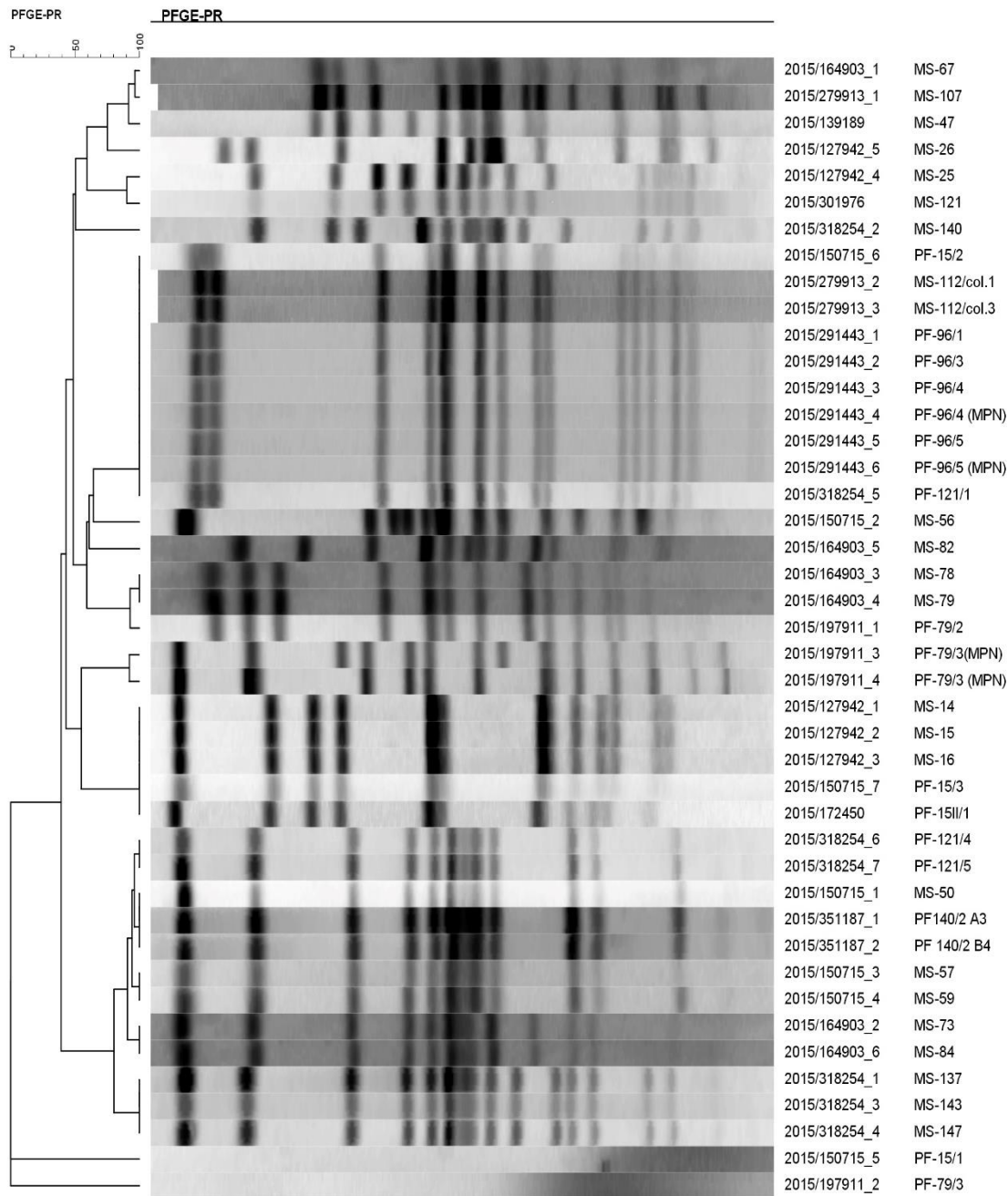


FIGURA 5: Similarities dendrograms elaborated by IZSLER-Parma.

Physico-chemical parameters: pH, a_w and NaCl content

The salami pH values ranged from 4.68 to 6.48 (average value: 5.89). pH values lower than 5.00 were observed in 5 samples only (3.1%) belonging to one batch of fine-grained salami added with starter cultures and characterized by a diameter of 12-15 cm.

In the *Salmonella*-positive salami the pH values ranged from 5.56 to 6.29 (average value: 5.92). The protective pH value for *Salmonella* growth was < 5.56 and was measured in 16 (10%) of the samples. No difference in the pH value was observed between the samples contaminated by the highest *Salmonella* levels (8.7 MPN/g, 2.7 MPN/g and 1.4 MPN/g) and the samples contaminated by 1.3 MPN/g or < 1.3 MPN/g.

The salami a_w values ranged from 0.8216 to 0.9561 (average value: 0.9048). In the samples contaminated by *Salmonella* a_w values from 0.8957 to 0.9512 (average value: 0.9177) were observed. The protective a_w value for *Salmonella* growth was < 0.8957 and was found in 32 (20%) of the samples.

No influence of the a_w value on *Salmonella* levels was observed, since in salami characterized by the highest a_w values (0.9201 to 0.9512) counts corresponding to 1.3 MPN/g and < 1.3 MPN/g were found.

The NaCl content of cured salami ranged from 2.25% to 4.80% (average value: 3.67%). In the *Salmonella*-positive salami NaCl was between 2.86% and 4.28% (average value: 3.50%). No salami characterized by NaCl $> 4.38\%$ were found to be positive for *Salmonella* and this sodium chloride level, together with low pH and a_w values, can probably contribute to prevent the growth of the microorganism. In 21 samples (13.2%) NaCl content was higher than 4.28, thus contributing to inhibit *Salmonella* growth. In the samples contaminated by the highest *Salmonella* counts (1.4 MPN/g to 8.7 MPN/g) NaCl was higher than 3.25% and reached 4.21% and 3.35% in the two highest contaminated products (8.7 MPN/g), respectively.

Salmonella load decreased in cured salami if compared to raw ground meat and fat mixtures. Counts as high as 160 MPN/g (*S. London*) found in a mixture sample were never detected in cured products, where the maximum load was 8.7 MPN/g. However, the cured batch of salami produced in plant B from the raw mixture contaminated by 160 MPN/g was negative for *Salmonella*, probably because of the starter cultures' activity.

Sample n°	pH	a _w	NaCl %
PF-15/1	6,25	0,9137	4,21
PF-15/2	6,01	0,9156	4,28
PF-15/3	6,19	0,918	3,66
PF-15 II/1	6,29	0,8963	3,89
PF-78/4	5,98	0,9017	3,72
PF-79/2	5,95	0,8957	3,57
PF-79/3	5,85	0,9036	3,35
PF-79/4	5,99	0,9015	3,31
PF-96/1	5,78	0,9463	2,86
PF-96/3	6,03	0,9263	3,64
PF-96/4	5,84	0,9482	2,94
PF-96/5	5,63	0,9512	2,98
PF-121/1	5,93	0,9201	3,44
PF-121/4	5,79	0,9077	3,47
PF-121/5	5,56	0,9201	3,49
PF-140/2	5,59	0,9182	3,24

TABLE 4: Physico-chemical parameters of *Salmonella*-positive salami.

Addition of microbial starter cultures

Overall, we tested 74 batches of salami produced using starter cultures and 81 batches produced traditionally without starter cultures. The proportion of *Salmonella*-positive batches was 6.8% (5/74) for the salami produced with microbial starters and 1.2% (1/81) for those produced without microbial cultures. Comparing the curing time of the salami belonging to the two groups, we observed an average time of 32.4 days for the first one and an average time of 38.7 days for the second one.

In salami produced by using starter cultures, *Salmonella* load always decreased during curing and lowered from 31 MPN/g (batch 15) and 29 MPN/g (batch 79) in the mixture samples to 8.7, 1.4 and < 1.3 MN/g (batch 15) and 8.7, 1.3 and < 1.3 MPN/g (batch 79) in the cured products. As shown in the previous paragraph, in one raw mixture *Salmonella* count reached 160 MPN/g but was no more isolated in the cured product. On the contrary, the only one positive batch of salami produced in plant D without starter cultures slightly increased its *Salmonella* load during curing, which was < 1.3MPN/g in the raw mixture and 2.7 MPN/g in the final product.

Batch n°	First sampling	Second sampling (curing time: 21-38 more days)	Third sampling (curing time: 8 more days)
15 (A)	1) <i>S. Goldcoast</i> (8.7/g) 2) <i>S. Derby</i> (1.4/g) 3) <i>S. London</i> (< 1.3/g)	<i>S. London</i> (<1.3/g)	Negative
78 (A)	1) <i>S. Panama</i> (< 1.3/g)	Negative	
79 (A)	1) <i>S. Stanley</i> (< 1.3/g) 2) <i>S. Panama</i> , <i>S. Branderup</i> (8.7/g) 3) <i>S. Branderup</i> (1.3/g)	Negative	
96 (B)	1) <i>S. Derby</i> (< 1.3/g) 2) <i>S. Derby</i> (< 1.3/g) 3) <i>S. Derby</i> (1.3/g) 4) <i>S. Derby</i> (1.3/g)	Negative	
121 (B)	1) <i>S. Derby</i> (< 1.3/g) 2) <i>S. Derby</i> (< 1.3/g) 3) <i>S. Derby</i> (< 1.3/g)	Negative	
140 (D)	1) <i>S. Derby</i> (2.7/g)	Negative	

TABLE 5: *Salmonella* MPN values in salami tested at different curing times.

Survival and replication within macrophages

Three of the four tested strains of *S. Typhimurium* 4,[5],12:i:- were able to survive and growth in the human macrophage-like (U937) cell line. One isolate of *S. Typhimurium* 4,[5],12:i:- and the strain of *S. Infantis* were not able to to survive. In further detail, one strain of *S. Typhimurium* 4,[5],12:i:- (MS 26) replicated in all evaluated three times post infection, that means 24 h, 48 h and 72 h post infection, while the other two strains (MS 67 and MS 107) replicated at 24h and 48 h post infection, but not at 72 h post infection. Moreover we observed that these two strains possess very similar PFGE profiles. For this reason we intend to proceed with their whole genome sequencing to better understand the genomic features which enable their survival and growth in human macrophages.

PFGE-PR

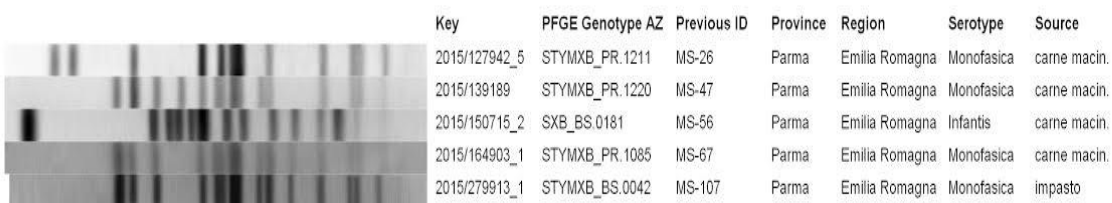


FIGURE 6: PFGE profiles of the analyzed strains for the ability to survive and growth in human macrophages.

11. Discussion

11.1 Influence of pH and a_w values

In our study on Italian salami we observed that *Salmonella* growth was absent in samples showing pH < 5.56, a_w < 0.8957 and NaCl > 4.28%. Considering that these physico-chemical parameters cooperate in reducing *Salmonella* vitality, they cannot be considered as single factors able to inhibit its growth.

During curing, progressive drying is responsible for NaCl increases and a_w lowering. In mixtures added with starter cultures pH lowers more rapidly than in samples produced without microbial starters, thus hindering the growth of acid-non-tolerant microorganisms (Zanardi *et al.*,2010).

In our study, *Salmonella* was detected in cured salami showing different physico-chemical features. In sample No 121, after only 20 days of curing, the pH reached 5.56 but the a_w was still 0.92. In sample No. 79 the very low a_w (0.8957) was balanced by a higher pH (5.95). In these samples *Salmonella* was below the minimum detectable value (< 1.3 MPN/g). In sample No. 15 the high NaCl content (4.28%), associated with low pH and a_w values, was able to maintain *Salmonella* load as low as 1.4 MPN/g. Thus we can say that our results confirm pH and a_w as the most important parameters able to prevent *Salmonella* growth in dry-cured Italian salami, which are normally characterized by low NaCl values. This finding was confirmed in the only one batch of salami (No. 15), which was positive for *Salmonella* after 28 and 49 days of curing and was found to be negative after 57 days. The a_w value dropped from 0.915 (28 days) to 0.8963 (49 days) and 0.8921 (57 days), but the NaCl showed little to no variations.

11.2 Influence of microbial starter cultures

In the study we observed *Salmonella* growth in salami added with microbial starter cultures mostly, if compared with the traditional salami produced without microbial cultures. This finding may be a

consequence of the shorter curing time of the first type of salami (average time of curing: 32.4 days) if compared to the second one (average time: 38.7 days). Anyway, other reasons could not be excluded, but we can say that, probably, the microbiology quality of raw pork and fat, used in the production of the different types of salami, or the hygienic measures at processing are of crucial importance to obtain a microbiologically safe finished product.

In the production of traditional Italian salami great attention is paid to hygienic measures and/or to pork and fat of higher microbiological quality. The production of pork salami is a multi-step process in which tradition (pork and fat of high quality, natural casing, long lasting curing time) and innovation (starter cultures, shorter curing time) are often mixed together. However, starter cultures seem to be not effective to overcome *Salmonella* growth unless curing time is sufficiently long and the a_w value drops below 0.8957. Nevertheless, a positive effect of starter microbial cultures can be found in the reduction of *Salmonella* load in contaminated salami, thus contributing to the reduction of the infectious dose for the consumers.

11.3 Analytical methods performance

The different results between Real-Time PCR and ISO 6579:2002 method can be attributed to several factors. First of all, the molecular PCR-based methods are equipped with a higher sensitivity (in particular, the kit used in this study has a limit of detection of ≤ 5 DNA copies) in respect to the cultural-based methods. A recent study demonstrated that a Real-Time PCR protocol was able to detect down to 2-4 *Salmonella* CFU in 25 g of different samples, including raw pork (Rodriguez-Lazaro *et al.*, 2014). A European study performed to validate a Real-Time PCR method to detect *Salmonella* in pork meat showed that it was an excellent alternative to the ISO 6579:2002 standard. The limit of detection was down to 10 CFU per 25 g, but previous testing showed that the method

detected *Salmonella* down to 1 CFU in 25 of different types of food, including pork, in less than 24 h (Delibato *et al.*, 2014).

As DNA is stable after the death of the microorganisms (Li *et al.*, 2013; Wolffs *et al.*, 2005) PCR methods are able to amplify DNA from both dead and viable bacteria; this can conduct to false-positive results. On the contrary cultural methods detect only viable cells, but are time-consuming and labour-intensive (Barbau-Piednoir *et al.*, 2014).

On the other hand, cultural methods are less sensitive and can be unable to detect very low numbers of *Salmonella* in naturally heavily contaminated samples, as ground raw pork. To increase the sensitivity of the cultural ISO 6579 method in case of very low *Salmonella* load (< 1 CFU/g), we suggest testing more than 25 g of the samples, since we obtained more correspondence between PCR results and the ISO cultural method testing 50 g of samples. In this study, the majority (79%) of the raw mixture samples were contaminated by a very few number of *Salmonella* per gram, which was <1.3 MPN/g. The limited number of *Salmonella* cells contaminating ground pork, associated with the high load of accessorial flora, represents an obstacle which can be get around increasing the sample weight. For this reason, we strongly suggest to test at least 50 g when non-target bacteria may overgrow a very limited number of *Salmonella* cells in naturally contaminated samples, even if the Regulation (EC) 2073/2005 laid down to detect *Salmonella* in 25 g of food samples.

In conclusion, our study underlines the usefulness of the tested Real-Time PCR method, since it allows rapid detection of *Salmonella* in raw pork and cured pork products, thanks to its speed of execution. This method could be associated with the ISO 6579 method; in this way it would be possible to reduce the time of execution required by the cultural method and to decrease the number of samples to be tested by the ISO 6579 method.

11.4 Survival and replication within macrophages

The survival and proliferation of pathogens within macrophages is critical for establishing systemic infections (Ge *et al.*, 2010). Our results suggest that there are differences in the intramacrophage survival ability of the *Salmonella* strains tested, that could influence the development of infection. For this reason, to better understand the virulence of the strains isolated from dry-cured salami, we will further investigate them by whole genome sequencing and compare them with other sequenced *Salmonella* human pathogenic strains.

12. Bibliography

- Abatcha M.G., Zakaria Z., Kaur D.G., Thong K.L. (2014). Review Article: A trends of *Salmonella* and antibiotic resistance. *Advances in Life Science and Technology*. **17**.
- Alpuche Aranda C.M., Swanson J.A., Loomis W.P., Miller S.I. (1992). *Salmonella* Typhimurium activates virulence gene transcription within acidified macrophages phagosomes. *Proceedings of the National Academy Science USA*. 89: 10079-10083.
- Akyala A.I. and Alsam S. (2015). Extended Spectrum Beta Lactamase Producing Strains of *Salmonella* species - A Systematic Review. *Journal of Microbiology Research*. **5**(2): 57-70
- Akullian A., Ng'eno E., Matheson A.I., Cosmas L., Macharia D., Fields B., Bigogo G., Mugoh M., John-Stewart G., Walson J.L., Wakefield J., Montgomery J.L. Environmental Transmission of Typhoid Fever in an Urban Slum. *Plos Negl Trop Dis* **9** (12).
- Andrews H.L., Baumler A.J. (2005). *Salmonella* species. In: *Foodborne Pathogens, Microbiology and Molecular Biology*. Fratamico P.M., Bhunia A.K., Smith J.L. (Eds). Caister Academic Press, Norfolk, UK, 327-334.
- Anonymous, 2003. Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents. *Official Journal of European Union* 325, 1-15.
- Anonymous, 2004. Protection of geographical indications, designations of origin and certificates of specific character for agricultural products and foodstuffs – Working document of the Commission Service. *Guide to Community Regulations*, 2nd Edition, August 2004.
- Anonymous, 2005. Commission Regulation (EC) No 2073/2005 of November 2005 on microbiological criteria for foodstuff. *Official Journal of the European Union* 338, 1-26.
- Arcangioli M.A., Leroy-Setrin S., Martel J.L, Chaslus-Dancla E. (2000). Evolution of chloramphenicol resistance, with emergence of cross-resistance to florfenicol, in bovine *Salmonella* Typhimurium strains implicates definitive phage type (DT) 104. *Journal of Medical Microbiology*. **49**:103-110.
- Arda I.S., Birgu F.E., Demirhan V.B., Aslan H., Ozyaylali I. (2001). Acute Abdomen Caused by *Salmonella* Typhimurium Infection in Children. *Journal of Pediatric Surgery*. **36**(12):1849-1852.
- Bai J., Kim S.I, Ryu S., Yoon H. (2014). Identification and characterization of outer membrane vesicle-associated proteins in *Salmonella enterica* serovar Typhimurium. *Infection and Immunity*. 82(10):4001-4010.
- Baker J.L., Chen L., Rosenthal J.A., Putnam D., DeLisa M.P. (2014). Microbial biosynthesis of designer outer membrane vesicles. *Current Opinion in Biotechnology*. **29**:76-84.

- Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Coucke, W., Roosens, N.H., Botteldoorn, N., 2014. Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis. *Journal of Microbiological Methods* **103**, 131-137.
- Bäumlér A., Tsolis R.M., Bowe F.A., Kusters J.G., Hoffman S., Heffron F. (1996). The *pef* Fimbrial Operon of *Salmonella typhimurium* Mediates Adhesion to Murine Small Intestine and Is Necessary for Fluid Accumulation in the Infant Mouse. *Infection and Immunity*. **64**(1):61–68.
- Bäumlér A. and Fang F.C. (2013) Host specificity of bacterial pathogens. *Cold Spring Harb. Perspect. Med.* **3**:a010041.
- Bearson B.L., Wilson L., Foster J.W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella Typhimurium* against inorganic acid stress. *Journal of Bacteriology*. **180**:2409-2417.
- Beninger P.R., Chikami G., Tanabe K., Roudier C., Fierer J. And guiney D.G. (1998). Physical and genetic mapping of the *Salmonella dublin* virulence plasmid pSDL2. Relationship to plasmids form other *Salmonella* strains. *The Journal of Clinical Investigation*. **81**:1341-1347.
- BhanM.K.,Bahl R., Bhatnagar S. (2005). Typhoid and paratyphoid fever. *Lancet*. **366**: 749–62.
- Blaser M.J. and Newman L.S. (1982). A Review of Human Salmonellosis: I. Infective Dose. *Clinical Infectious Diseases*. **4**:1096.
- Boyd D., Cloeckert A., Chaslus-Dancla E., Mulvey M.R. (2002).Characterization of Variant *Salmonella* Genomic Island 1 Multidrug Resistance Regions from Serovars Typhimurium DT104 and Agona. *Antimicrobial Agents and Chemotherapy*. **46**(6):1714–1722.
- Breiman R.F., Cosmas I., Njuguna H., Audi A., Olack B., Ochieng J.B., Wamola N., Bigogo G., Awiti G., Tabu C.W., Burke H., Williamson J., Oundo J.O., Mintz E.D., Feikin D.R. (2012). Population-Based Incidence of Typhoid Fever in an Urban Informal Settlement and a Rural Area in Kenya: Implications for Typhoid Vaccine Use in Africa. *Plos One*. **7** (1).
- Buchmeier N.A. and Heffron F. (1991). Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. *Infection and Immunity*. **59** (7): 2232-2238.
- Buchwald D.S. and M.J. Blaser (1984). A review of human salmonellosis: duration of excretion following infection with nontyphi *Salmonella*. *Reviews of Infectious Diseases*. **6**(3):345-356.
- Butler T., Bell W.R., Levin .J, Linh N.N.,Arnold K. (1978). Typhoid fever: studies of coagulation, bacteremia, and endotoxemia. *Archieves of Internal Medicine*. **138**:407-10.
- Celum C.L., Chaisson R.E., Rutherford G.W. *et al.* (1987). Incidence of salmonellosis in patients with AIDS. *The Journal of Infectious Diseases*. **156**:998-1002.

- Chen LM, Kaniga K, Galan JE (1996). *Salmonella* spp. are cytotoxic for cultured macrophages. *Molecular Microbiology*. **21**:1101-1115.
- Cheng H.M., Wang Y., Su L.H., Chiu C.H. (2013). Nontyphoid *Salmonella* Infection: Microbiology, Clinical Features and Antimicrobial Therapy. *Pediatrics and Neonatology*, **54**,147-152.
- Chiu CH, Su LH, Hung CC, Chen KL, Chu C. (2004). Prevalence and antimicrobial susceptibility of serogroup D Nontyphoidal *Salmonella* in a University Hospital in Taiwan. *Journal of Clinical Microbiology*. **42**:415-417.
- Cohen P.S., O'Brien T.F., Shoenbaum S.C. et al. (1978). The risk of endothelial infection in adults with *Salmonella* bacteremia. *Annals of Internal Medicine*. **89**:931-932.
- Collison S. K., Emody L., Muller K.H., Trust T.J., Kay W.W. (1991). Purification and Characterization of Thin, Aggregative Fimbriae from *Salmonella* enteritidis. *Journal of Bacteriology*. **173**(15):4773-4781.
- Coloretti, F., Chiavari C., Armaforte E., Carri, S., Castagnetti G.B., (2008). Combined use of starter cultures and preservatives to control production of biogenic amines and improve sensorial profile in low-acid salami. *Journal of Agricultural and Food Chemistry*. **56**:11238-11244.
- Conter, M., Zanardi, E., Ghidini, S., Pennisi, L., Vergara, A., Campanini, G., Ianieri, A., 2007. Survey on typology, PRPs and HACCP plan in dry fermented sausage sector of Northern Italy. *Food Control* **18**:650-655.
- Cornelis G.R., Van Gijsegem F. (2000). Assembly and function of type III secretory systems. *Annual review of Microbiology*. **52**:735-774.
- Cox J. (1999). *Salmonella*. In: *Encyclopedia of Food Microbiology*, vol III. Robinson R.K. (Ed.). Academic Press, London, pp. 1928-1937.
- Coynault, C., Robbe-Saule, V., Popoff, M.Y., and Norel, F. (1992) Growth phase and SpvR regulation of *Salmonella* Typhimurium spvABC virulence genes. *Microb Pathog* **13**:133-145.
- Crump J.A., Sjölund-Karlsson M., Gordon M.A., Parry C.M. (2015). Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive *Salmonella* Infections. *Clinical Microbiology Reviews*. **28**(4):901-937.
- Daigle F., Graham J.E., Curtiss R.III (2001). Identification of *Salmonella* Typhi genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). *Molecular Biology*. **41** (5):1211-1222.
- De Jong H. K., Parry C.M., Van der Poll T., Wiersing W.J. (2012). Host-Pathogen Interaction in Invasive Salmonellosis. *Plos Pathogens*. **8** (10): e1002933.
- Delibato, E., Rodriguez-Lazaro, D., Gianfranceschi, M., De Cesare, A., Comin, D., Gattuso, A., Hernandez, M., Sonnessa, M., Pasquali, F., Sreter-Lancz, Z., Saiz-Abajo, M.J., Pérez-De-Juan, J., Butrón, J., Prukner-Radovic, E. Horvatek Tomic, D., Johannessen, G.S., Jakočiūnė, D., Olsen, J.E., Chemaly, M., Le Gall, F., González-García, P., Lettini, A.A., Lukac, M., Quesne, S., Zampieron, C., De Santis, P., Lovari, S., Bertasi, B.,

- Pavoni, E., Proroga, Y.T., Capuano, F., Manfreda, G., De Medici, D., 2014. European validation of Real-Time PCR method for detection of *Salmonella* spp. in pork meat. *International Journal of Food Microbiology*. **184**:134-138.
- De Moraes M.H. and Teplitski M. (2015). Fast and efficient three-step target-specific curing of a virulence plasmid in *Salmonella enterica*. *AmbExpress*. **5**:52.
 - Desai P.T., Porwollik S., Long F., Cheng P., Wollam A., Clifton S.W., Weinstock G.M., McClelland M. (2013). Evolutionary Genomics of *Salmonella* Subspecies. *mBIO American Society for Microbiology*, **4**(2),1-12.
 - Devine R. (2003). Meat consumption trends in the world and in European Union. *INRA Production Animals*. **16**:325-327.
 - Dimitrov T., Udo, E.E., Albaksami O., Kilani A.A., Shehab E-D- M.R. (2007). Ciprofloxacin treatment failure in a case of typhoid fever caused by *Salmonella enterica* serotype Paratyphi A with reduced susceptibility to ciprofloxacin. *Journal of Medical Microbiology* .**56**: 277–279.
 - EFSA (European Food Safety Agency) 2010. Scientific Opinion on monitoring and assessment of the public health risk of “*Salmonella* Typhimurium- like” strains. *The EFSA Journal*, **8** (10):1826.
 - EFSA (European Food Safety Agency) 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *The EFSA Journal*, **13**(12):4329.
 - Ekman P., Saarinen M., He Q., Virtala M., Salmi M., Granfors K. (1999). Human monocytic U937 cells kills *Salmonella* in vitro by NO-independent mechanisms. *Infection and Immunity*. **67** (7):3670-3673.
 - Ellermeier Jeremy R., Slauch James M. (2007). Adaptation to the host environment: regulation of the SPII type III secretion system in *Salmonella enterica* serovar Typhimurium. *Current Opinion in Microbiology*. **10**:24-29.
 - Ewing, W. H., Davis B., Edwards P. (1960). The decarboxylase reactions of Enterobacteriaceae and their value in taxonomy. *Public Health Lab*. **18**:77-83.
 - Ewing, W. H., and M. M. Ball. (1966). The biochemical reactions of members of the genus *Salmonella*. National Communicable Disease Center, Atlanta, Ga.
 - Ewing, W. H. and Ball M.M. (1966). The biochemical reactions of members of the genus *Salmonella*. National Communicable Disease Center, Atlanta, Ga.
 - Fàbrega A., Vila J. (2013). *Salmonella enterica* Serovar Typhimurium Skills To Succeed in the Host: Virulence and Regulation. *Clinical Microbiology Reviews*, **26**(2), 308-341.
 - Fedorka-Cray P.J., Collins-Kelley L., Stabel L., Gray J.T., Laufer J.A. (1995). Alternate routes of invasion may affect pathogenesis of *Salmonella typhimurium* in swine. *Infection and Immunity*. **63**:2658-2664.

- Fedorka-Cray P.J., Gray J.T., Wray C. (2000). *Salmonella* infections in pigs. In: Wray C., Wray A. (Eds). *Salmonella* in Domestic Animals. CAB International, Wallingford, pp.191-207.
- Feng Y., Liu J., Li Y.-G., Cao F.-L., Johnston R.N., Zhou J., Liu G.-R., Liu S.-L. (2012). Inheritance of the *Salmonella* virulence plasmids: Mostly vertical and rarely horizontal. *Infection, Genetics and Evolution*. **12**:1058–1063.
- Fields P.I., Ronald V. S., Haidaris C.G., Heffron F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophages are avirulent. *Proceedings of the National Academy Science USA*. **83**:5189-5193.
- Fierer J. and Fleming W. (1983). Distinctive Biochemical Features of *Salmonella* Dublin Isolated in California. *Journal of Clinical Microbiology*. **17**(3):552-554.
- Fierer, J., Krause, M., Tauxe, R., Guiney, D.G. (1992). *Salmonella typhimurium* bacteremia: association with the virulence plasmid. *The Journal of Infectious Diseases*. **166**: 639-642.
- Foster, J.W. and Hall, H.K. (1990). Adaptive acidification tolerance response of *Salmonella Typhimurium*. *Journal of Bacteriology*. **172**:771–778.
- Foster, J.W. (1991). *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. *Journal of Bacteriology*. **173**:6896–6902.
- Franklin K., Lingohr E.J., Yoshida C., Anjum M., Bodrossy L., Clark C.G., Kropinski A. M., Karmali M. A. (2011). Rapid Genosertotyping Tool for Classification of *Salmonella* Serovars. *Journal of Clinical Microbiology*, **49**(8), 2954-2965.
- Funk J. and Gebreyes W.A. (2004). Risk factors associated with *Salmonella* prevalence on swine farms. *Journal of Swine Health and Production*. **12**(5):246–251.
- Funk J. and Gebreyes W.A. (2010). Risk factors associated with *Salmonella* on swine farms. *Journal of Swine Health and Production*. **19**(2): 156-168.
- Galan J.E. and Collmer A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*. **284**:1322-1328.
- Gal-Mor O., Boyle E.C. and Grassi G.A. (2014). Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Frontiers in Microbiology*. **5**, article 391.
- Garin J., Diez R., Kieffer S., Duclos S., Gagnon E., Sadoul R., Rondeau C, Desjardins M. (2001). The phagosome proteome: insight into phagosome functions. *The Journal of Cell Biology*. **152**:165-180.
- Ge S., Danino V., He Q., Hinton J. CD, Granfors K. (2010). Microarray analysis of response of *Salmonella* during infection of HLA-B27-transfected human macrophage-like U937 cells. *BMC Genomics*. **11**:456.

- Gilks CF, Brindle RJ, Otieno LS. (1990). Life-threatening bacteraemia in HIV-1 seropositive adults admitted to hospital in Nairobi, Kenya. *The Lancet*. **336**:545–549.
- Gordon Melita A. (2008). *Salmonella* infections in immunocompromised adults. *Journal of Infection*. **56**:413-422.
- Gray J.T., Fedorka-Cray P.J., Stable T.J. (1995). Influence of inoculation route on the carrier state of *Salmonella choleraesuis* in swine. *Veterinary Microbiology*. **47**:43-59.
- Graziani C., Busani L., Dionisi A.M., Lucarelli C., Owczarek S., Ricci A., Mancin M., Caprioli A., Luzzi I. (2008). Antimicrobial resistance in *Salmonella enterica* serovar Typhimurium from human and animal sources in Italy. *Veterinary Microbiology* **128**:414–418.
- Gray J. T. and Fedorka-Cray, P. J. 2002. *Salmonella*. In Cliver, D. O. and Riemann, H. P. (Eds.). *Foodborne diseases*, p. 55-68. San Diego: Academic Press.
- Groisman E.A., Chiao E., Lipps C.J., Heffron F. (1989). *Salmonella typhimurium* *phoP* virulence gene transcriptional regulator. *Proceedings of the National Academy Science USA*. **86**: 7077-7081.
- Groisman Eduardo A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. *Journal of Bacteriology*. **183**:1835-1842.
- Guiney D.G., Libby S., Fang F.C., Krause M., Fierer J. (1995). Growth-phase regulation of plasmid virulence genes in *Salmonella*. *Trends in Microbiology*. **3**: 275-279.
- Guiney, D.G., Fang, F., Krause, M., Libby, S., Buchmeier, N.A., Fierer, J. (1995). Biology and clinical significance of virulence plasmids in *Salmonella* serovars. *Clinical Infectious Diseases*. **21**:146-151.
- Gulig, P.A., and Curtiss, R., III. (1988). Cloning and transposon insertion mutagenesis of virulence genes of the 100 kilobase plasmid of *Salmonella typhimurium*. *Infection and Immunity*. **56**: 2891-2901.
- Gulig P.A., Caldwell A.L., Chiodo V.A. (1992). Identification, genetic analysis and DNA sequence of a 7.8-kilobase virulence region of the *Salmonella typhimurium* virulence plasmid. *Molecular Microbiology*. **6**:1395-1411.
- Gulig P.A., Danbara H., Guiney D.G., Lax A.J., Norel F. and Rhen M. (1993). Molecular analysis of *spv* virulence genes of the *Salmonella* virulence plasmid. *Molecular Microbiology*. **7**: 825-830.
- Gulig, P.A., and Doyle, T.J. (1993) The *Salmonella* Typhimurium virulence plasmid increases the growth rate of salmonellae in mice. *Infection and Immunity*. **61**:504-511.
- Gunn J.S., Ernst R.K., McCoy A.J., Miller S.I. (2000). Constitutive mutations of the *Salmonella enterica* Serovar Typhimurium Transcriptional Virulence regulator *phoP*. *Infection and Immunity*. **68**(6): 3758-3762.
- Haeusler G.M. and Curtis N. (2013). Non-typhoidal *Salmonella* in children: microbiology, epidemiology and treatment. *Advances in Experimental Medicine and Biology*. **764**: 13-26.

- Hopkins K.L., Davies R.H., Threlfall E.J. (2005). Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: Recent developments. *International Journal of Antimicrobial Agents* **25**:358–373.
- Hospital X.F., Hierro E., Stringer S., Fernández M. (2016). A study on the toxigenesis by *Clostridium botulinum* in nitrate and nitrite-reduced dry fermented sausages. *International Journal of Food Microbiology*. **218**:66-70.
- Hu L. and Kopecko D. J. (2003). Typhoid *Salmonella*. In Millotis, M. D. and Bier, J. W. (Eds.). *International handbook of foodborne pathogens*, p. 151-165. New York: Marcel Dekker, Inc.
- Hueck C.J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Review*. **62**:379-433.
- Hughes E.A. and Galan J.E. (2002). Immune Response to *Salmonella*: Minireview Location, Location, Location? *Immunity*. **16**:325–328.
- Hurley D., McCusker M.P., Fanning S., Martins M. (2014). *Salmonella*–host interactions – modulation of the host innate immune system. *Frontiers in Immunology*, **5**, Article 481.
- Isaacson R.E., Firkins L.D., Weigel R.M., Zuckermann F.A., DiPietro J.A. (1999). Effect of transportation and feed withdrawal on shedding of *Salmonella* Typhimurium among experimentally infected pigs. *American Journal of Veterinary Research*. **60**:1155-1158.
- Jones G.W., Rabert D.K., Svinarich D.M., Withfield H.J. (1982). Association of adhesive, invasive and virulent phenotypes of *Salmonella* Typhimurium with autonomous 60-megadalton plasmids. *Infection and Immunity*. **38**:476-486.
- Jones C. and Stanley, J. (1992). *Salmonella* plasmids of the pre-antibiotic era. *Journal of General Microbiology*. **138**:189–197.
- Kaniga K., Trollinger D., Gala J.E. (1995). Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella* Typhimurium that have homology to the *Shigella* IpaD and IpaA proteins. *Journal of Bacteriology*. **177**:7078-7085.
- Keestra-Gounder AM, Tsolis RM, Bäumler AJ. (2015). Now you see me, now you don't: The interaction of *Salmonella* with innate immune receptors. *Nat Rev Microbiol*, **13** (4):206-16.
- Kesty N.C., Mason K.M., Reedy M., Miller S.E., Kuehn M.J. (2004). Enterotoxigenic *Escherichia coli* vesicles target toxin delivery into mammalian cells. *The EMBO Journal*. **23**: 4538–4549.
- Kidgell C, Reichard U, Wain J, Linz B, Torpdahl M, Dougan G, Achtman M. (2002). *Salmonella* Typhi, the causative agent of typhoid fever, is approximately 50,000 years old. *Infect. Genet. Evol.* **2**:39-45.
- Kier L.D., Weppelman R.M., Ames B.N. (1979). Regulation of nonspecific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. *Journal of Bacteriology* **138**:155-161.

- King A.M., Glass K.A., Milkowski A.L., Seman D.L., Sindelar J.J.(2016). Modeling the impact of ingoing sodium nitrite, sodium ascorbate, and residual nitrite concentrations on growth parameters of *Listeria monocytogenes* in cooked, cured pork sausage. *Journal of Food Protection*. **79**:184-193.
- Kotula A.W. and Emswiler-Rose B.S. (1988). Airborne microorganisms in a pork processing establishment. *Journal of Food Protection*. **51**:935-937.
- Kuehn M.J. and Kesty N.C. (2005). Bacterial outer membrane vesicles and the host–pathogen interaction. *Genes and development*. **19**:2645–2655.
- Kulp A. and Kuehn M.J. (2010). Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annual review of Microbiology*. **64**: 163–184.
- Lathrop S.K., Binder K.A., Starr T., Cooper K.G., Chong A., Carmody A.B., (2015). Replication of *Salmonella enterica* serovar Typhimurium in human monocyte-derived macrophages. *Infection and Immunity*. **83**(7):2661-2671.
- Lawson G. and Dow C. (1966). Porcine salmonellosis. *Journal of Comparative pathology*. **76**:363-371.
- Lee I.S., Slonczewski, J.L. and Foster J.W. (1994). A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella* Typhimurium. *Journal of Bacteriology*. **176**:1422–1426.
- Lee E.Y., Bang J.Y., Park G.W., Choi D.S., Kang J.S., Kim H.J., Park K.S, Lee J.O., Kim Y.K., Kwon K.H., Kim K.P., Gho Y.S. (2007). Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics*. **7**:3143-3153.
- Le Hello S., Harrois D., Bouchrif B., Sontag L., Elhani D., Guibert V., Zerouali K., Weill F.X. (2013). Highly drug-resistant *Salmonella enterica* serotype Kentucky ST198-X1:a microbiological study. *The Lancet Infectious Diseases*. **13**:672-679.
- Lesnick M.L., Reiner N.E., Fierer J., Guiney D.G. (2001). The *Salmonella* spvB virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Molecular Microbiology*. **39**(6):1464-1470.
- Li, M., Zhao, G., Liu, J., Gao, X., Zhang, Q., 2013. Effect of different heat treatments on the degradation of *Salmonella* nucleic acid. *Journal of Food Safety* **33**, 536-544.
- Libby S., Adams L.G., Ficht T.A., Allen C., Whitford H.A., Buchmeyer N.A., Bossie S., Guiney D.G. (1997). The spv Genes on the *Salmonella* Dublin Virulence Plasmid Are Required for Severe Enteritis and Systemic Infection in the Natural Host. *Infection and Immunity*. **65**(5): 1786-1792.
- Libby, S.J., Lesnick, M., Hasegawa, P., Weidenhammer, E., Guiney, D.G. (2000). The *Salmonella* virulence plasmid spv genes are required for cytopathology in human monocyte-derived macrophages. *Cellular Microbiology*. **2**: 49-58.

- Liston Sean D., Ovchinnikova Olga G., Whitfield Chris, (2016). Unique lipid anchor attaches Vi antigen capsule to the surface of *Salmonella enterica* serovar Typhi. PNAS, **113**, 6719-6724.
- Liu WQ, Feng Y, Wang Y, Zou QH, Chen F, Guo JT, et al. (2009). *Salmonella* Paratyphi C: genetic divergence from *Salmonella* Choleraesuis and pathogenic convergence with *Salmonella* Typhi. PLoS One. 4:e4510.
- Liu A., Fong A., Becket E., Yuan J., Tamae C., Medrano L., Maiz M., Wahba C., Lee C., Lee K., Tran K.P., Yang H., Hoffman R.M., Salih A., Miller J.H. (2011). Selective Advantage of Resistant Strains at Trace Levels of Antibiotics: a Simple and Ultrasensitive Color Test for Detection of Antibiotics and Genotoxic Agents. Antimicrob. Agents Chemother. 1204-1210.
- Liu Q., Yi J., Liang K., Liu T., Roland K.L., Jiangd Y., Konga Q. (2016 in press). Outer membrane vesicles derived from *Salmonella* Typhimurium mutants with truncated LPS induce cross-protective immune responses against infection of *Salmonella enterica* serovars in the mouse model. International Journal of Medical Microbiology.
- Löfström C., Hoorfar J., Schelin J., Rådström P., Malorny B., (2010). *Salmonella*. In: Dongyou Liu (Eds) Molecular Detection of Foodborne Pathogens, CRC Press, Boca Raton. pp 447-458.
- Low d., Braaten B. and Van der Woude M. (1996). In *Escherichia coli* and *Salmonella* Typhimurium: Cellular and Molecular Biology. Ed. Neidhart F.C. (Am. Soc. Microbiol., Washington, DC) (1) 146-157.
- Lowrie D.B., Aber V.R., Jackett P.S. (1979). Phagosome-lysosome fusion and cyclic adenosine 3':5'-monophosphate in macrophages infected with *Mycobacterium microti*, *Mycobacterium bovis* BCG or *Mycobacterium lepraemurium*. Journal of General Microbiology. **110**:431-441.
- Lücke F.-K., (1998). Fermented sausages. In: Wood, B.J.B. (Ed.), Microbiology of fermented foods. Blackie Academic and Professional, London, pp. 441 – 483.
- Lücke F.-K., (2000). Utilization of microbes to process and preserve meat. Meat Science. **56**: 105–115.
- MacDonald I.A. and Kuehn M.J. (2013). Stress-Induced Outer Membrane Vesicle Production by *Pseudomonas aeruginosa*. Journal of Bacteriology. **195** (13):2971–2981.
- MacLennan C.A. (2014). Out of Africa: Links Between Invasive Nontyphoidal *Salmonella* Disease, Typhoid Fever, and Malaria. Clinical Infectious Diseases. **58**.
- Mantovani A., Sica A., Sozzani S., Allavena P., Vecchi A., Locati M. (2004). The chemokine system in diverse forms of macrophages activation and polarization. Trends in Immunology. **25**:677-686.
- Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., et al. (2010). International collaboration on enteric disease "Burden of Illness" studies. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clinical Infectious Disease. **50**:882–889.

- Mataragas M., Bellio A., Rovetto F., Astegiano S., Decastelli L., Cocolin L. (2015). Risk-based control of food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* in the Italian fermented sausages Cacciatore and Felino. *Meat Science*. **103**:39-45.
- McQuiston J. R., Herrera-Leon S., Wertheim B.C., Doyle J., Fields P.I., Tauxe R.V., Logsdon J.M. Jr. (2008). Molecular Phylogeny of the Salmonellae: Relationships among *Salmonella* Species and Subspecies Determined from Four Housekeeping Genes and Evidence of Lateral Gene Transfer Events. *Journal of Bacteriology*. **190** (21):7060–7067.
- Mead P.S., Slutsker L., Dietz V., McCaig L.F., Bresee J.S., Shapiro C., Griffin P.M., Tauxe V. (1999). Food-Related Illness and Death in the United States. *Emerging Infectious Diseases*. **5**(5):607-625.
- Merrell D.S., Butler S.M., Qadri F., Dolgano, N.A., Alam A., Cohen M.B., Calderwood S.B., Schoolnik G.K., Camilli A. (2002). Host-induced epidemic spread of the cholera bacterium. *Nature* **417**: 642–645.
- Miller S.I., Kukral A.M., Mekalanos J.J. (1989). A two-component regulatory system (*phoP* and *phoQ*) controls *Salmonella* Typhimurium virulence. *Proceedings of the National Academy of Science USA*. **86**:5054-5058.
- Miller S.I. and Mekalanos J.J. (1990). Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. *Journal of Bacteriology*. **172**: 2485-2490.
- Mills D.M., Bajas V., Lee C.A. (1995). A 40 Kb chromosomal fragment encoding *Salmonella* Typhimurium invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. *Molecular Microbiology*. **15**:749-759.
- Mills C.D., Kincaid K., Alt J.M., Heilman M.J., Hill A.M. (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *The Journal of Immunology*. **164**:6166-6173.
- Miriagou V., Tassios P.T., Legakis N.J., Tzouveleki L.S. (2004). Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. *International Journal of Antimicrobial Agents*. **23**:547-555.
- Mogasale V., Maskery B., Ochiai R.L., Lee J.S., Mogasale V.V., Ramani E., Kim Y.E., Park J.K., Wierzbica T.F. (2014). Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. *Lancet Glob Health*, **2**:570-580.
- Molloy A., Nair S., Cooke F.J., Wain J., Farrington M., Lehner P.J., Torok M.E. (2010). First Report of *Salmonella enterica* Serotype Paratyphi A Azithromycin Resistance Leading to Treatment Failure. *Journal of Clinical Microbiology*. **48**(12):4655–4657.
- Monack D.M. (2012). *Salmonella* persistence and transmission strategies. *Curr. Opin. Microbiol*. **15**:100-107.
- Norel F., Coynault C., Mras I., Hermant D., Popoff M.Y. (1989). Cloning and expression of plasmid DNA sequences involved in *Salmonella* serotype Typhimurium virulence. *Molecular Microbiology*. **3**:733-743.

- Ockerman H. W. Basu, L. (2007). Production and consumption of fermented meat products. In F. Toldrá (Ed.), Handbook of fermented meat and poultry (pp. 9–15). Iowa, USA: Blackwell Publishing.
- Oyston P.C., Dorrell N., Williams K., Li S.R., Green M., *et al.* (2000). The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis*. *Infection and Immunity*. **68**: 3419-3425.
- Papagrigorakis M.J., P. N.Synodinos, C. Yapijakis. (2007). Ancient typhoid epidemic reveals possible ancestral strain of *Salmonella enterica* serovar Typhi. *Infect Genet Evol* **7**: 126-127.
- Parry C.M., Hien T.T., Dougan G., White N.J., Farrar J.J. (2002). Typhoid Fever. *N.Engl.J.Med.* **347**(22):1770-1782.
- Parry C. M. (2006). Epidemiological and clinical aspects of human typhoid fever. In Matroeni, P. and Maskell, D. (Eds.). *Salmonella* infections: Clinical, immunological and molecular aspects, p. 1-18. New York: Cambridge University Press.
- Paterson D.L. and Bonomo R.A. (2005). Extended-Spectrum β -Lactamases: a Clinical Update. *Clinical Microbiology Reviews*. **18**(4):657–686.
- Philippon A., Arlet G. and Jacoby G.A. 2002). Plasmid-Determined AmpC-Type Lactamases. *Antimicrobial Agents and Chemotherapy*. **46**(1):1-11.
- Piras F., Brown D.J., Meloni D., Mureddu A., Mazzette R. (2011). Investigation of *Salmonella enterica* in Sardinia slaughter pigs: Prevalence, serotype and genotype characterization. *International Journal of Food Microbiology*. **151**:201-209.
- Profeta S, Forrester C, Eng RH. (1985).*Salmonella* infections in patients with acquired immunodeficiency syndrome. *Archives of Internal Medicine*.**145**:670–672
- Rabsch W., Tschäpe H., Bäuml A.J. (2001). Non-typhoidal salmonellosis: emerging problems. *Microbes and Infection*. **3**:237-247.
- Raetz C.R.H. and Whitfield C. (2002). Lipopolysaccharide Endotoxins. *Annual Review of Biochemistry*. **71**: 635–700.
- Rathman M., Barker L.P. and Falkow S. (1997). The unique trafficking pattern of *Salmonella typhimurium*-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry. *Infection and Immunity*. **65**:1475-1485.
- Reddy EA, Shaw AV, Crump JA. (2010). Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. *The Lancet Infectious Disease*. **10**:417–432.

- Reed W.M., Olander H.J. and Thacker H.L. (1986). Studies on the pathogenesis of *Salmonella typhimurium* and *Salmonella Choleraesuis* var *kunzendorf* infection in weanling pigs. American Journal of Veterinary Research **47**:75-83.
- Ren J., Sang Y., Tan Y., Tao J., Ni J., Liu S., Fan X., Zhao W., Lu J., Wu W., Yao Y.F. (2016). Acetylation of lysine 201 inhibits the DNA-binding ability of PhoP to regulate *Salmonella* virulence. Plos Pathogens. **12**(3):1-29.
- Rhen, M., Virtanen M., and Mäkelä P.H. (1988). Localization by insertion mutagenesis of a virulence-associated region on the *Salmonella* Typhimurium 96 kilobase pair plasmid. Microbial Pathogenesis **6**: 53-158.
- Ribot E.M., Fair M.A., Gautom R., Cameron D.N., Hunter S.B., Swaminathan B., Barret T.J. (2006). Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of *Escherichia coli* O157:H7, *Salmonella* and *Shigella* for PulseNet. Foodborne pathogens and Disease. **3**:59-67.
- Ridley, A.M., Punia, P., Ward, L.R., Rowe, B., Threlfall, E.J. (1996). Plasmid characterization and pulsed-field electrophoretic analysis demonstrate that ampicillin-resistant strains of *Salmonella enteritidis* phage type 6a are derived from *S. enteritidis* phage type 4. Journal of Applied Bacteriology. **81**.613–618.
- Rychlik I. and Barrow P.A. (2005). *Salmonella* stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS Microbiology Reviews **29** : 1021–1040.
- Rychlik I., Gregorova D., Hradrega H. (2006) Distribution and function of plasmids in *Salmonella enterica*. Veterinary Microbiology. **112**:1-10.
- Rychlik I., Karasova D., Sebkova A., Volf J., Sisak F., Havlickova H., Kummer V., Imre A., Szmolka A., Nagy B. (2009). Virulence potential of five major pathogenicity islands (SPI-1 to SPI-5) of *Salmonella enterica* serovar Enteritidis for chickens. BMC Microbiology, **9**:268.
- Rodriguez-Lazaro, D., Gonzalez-García, P., Delibato, E., De Medici, D., García-Gimeno, R.M., Valero, A., Hernandez, M., 2014. Next day *Salmonella* spp. detection method based on real-time PCR for meat, dairy and vegetable food products. International Journal of Food Microbiology **184**,113-120.
- Rostagno M.H., Eicher S.D., Lay Jr. D.C. (2010). Does pre-slaughter stress affect pork safety risk? Proceedings of the 21st IPVS Congress, Vancouver, Canada, July 18-21, 2010, p.176.
- Salcedo S.P., Noursadeghi M., Cohen J. and Holden D.W. (2001). Intracellular replication of *Salmonella typhimurium* strains in specific subsets of splenic macrophages in vivo. Cellular Microbiology. **3**(9):587-597.
- Sammarco M.L., Ripabelli G., Ruberto A., Iannitto G., Grasso G.M. (1997). Prevalence of *Salmonellae*, *Listeriae* and *Yersinia* in the slaughterhouse environment and on work surfaces, equipment and workers. Journal of Food Protection. **60**:367-371.

- Santos R.L., Tsolis R.M., Bäumlér A.J., Adams L.G. (2003). Pathogenesis of *Salmonella*-induced enteritis. Brazilian Journal of Medical and Biological Research. **36** (1): 3-12.
- Santos, E.M., González-Fernández, C., Jaime, I., Rovira, J., 1998. Comparative study of lactic acid bacteria house flora isolated in different varieties of “chorizo”. International Journal of Food Microbiology. **39**:123–128.
- Scavia G., Ciaravino G., Luzzi I., Lenglet A., Ricci A., Barco L., Pavan A., Zaffanella F., Dionisi A.M. (2013). A multistate epidemic outbreak of *Salmonella* Goldcoast infection in humans, June 2009 to March 2010: the investigation in Italy. Euro Surveill. **18**(11):pii=20424.
- Scherer C. A. and Miller S. I. (2001). Molecular pathogenesis of Salmonellae. In Groisman. E. A. (Ed.). Principles of bacterial pathogenesis, p. 265-316. United States of America: Academic Press.
- Schwann W.R., Huang X.Z., Hu L., Kopecko D.J. (2000). Differential bacterial survival, replication and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. Infection and Immunity. **68**:1005-1013.
- Senior K. (2009). Estimating the global burden of foodborne disease. The Lancet Infectious Disease. **9**:80–81.
- Sharma K. P., Ramakrishnan R., Hutin Y., Manickam P. and Gupte M.D. (2009). Risk factors for typhoid in Darjeeling, West Bengal, India:evidence for practical action. Tropical Medicine and International Health **14** (6):696–702.
- Shimoni Z., Pitlik S., Leibovici L., Samra Z., Konigsberger H., Drucker V.A., Ashkenazi S., Weinberger M. (1999). Nontyphoid *Salmonella* bacteremia: age-related differences in clinical presentation, bacteriology and outcome. Clinical Infectious Diseases **28**:822-827.
- Singh S. (2001). Pathogenesis and Laboratory Diagnosis. Journal, Indian Academy of Clinical Medicine. **2** No. 1 and 2.
- Smith A.C., Cirulis J.T., Casanova J.E., Scidmore M.A., Brumell J.H. (2005). Interaction of the *Salmonella*-containing vacuole with the endocytic recycling system. The Journal of Biological Chemistry. **280**:24634-24641.
- Smith C., Stringer A.M., Mao C., Palumbo M.J., Wadea J.T. (2016). Mapping the Regulatory Network for *Salmonella enterica* Serovar Typhimurium Invasion. American Society for Microbiology. **7** (5) e01024-16.
- Steele-Mortimer O., Meresse S., Gorvel J.P., Toh B.H., Finlay B.B. (1999). Biogenesis of *Salmonella* Typhimurium-containing vacuole in epithelial cells involves interaction with the early endocytic pathway. Cellular Microbiology. **1**:33-49.
- Steele-Mortimer (2008). The *Salmonella*-containing vacuole-Moving with the times. Current Opinion in Microbiology. **11**(1):38-45.

- Stevenson J.E., Gay K., Barrett T.J., Medalla F., Chiller M., Angulo F.J. (2007). Increase in Nalidixic Acid Resistance among Non-Typhi *Salmonella enterica* Isolates in the United States from 1996 to 2003. *Antimicrobial Agents and Chemotherapy*. **51**(1):195–197.
- Strahilevitz J., Jacoby G.A., Hooper D.C., Robicsek A. (2009). Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat. *Clinical Microbiology Reviews*. **22**(4):664-689.
- Su LH, Chiu CH. (2007). *Salmonella*: clinical importance and evolution of nomenclature. *Chang Gung Medical Journal*. **30**:210e9.
- Swanenburg M., Van der Wolf P.J., Urlings H.A.P., Snijders J.M.A., Van Knapen F. (2001a). *Salmonella* in the slaughter pigs: the effect of logistic slaughter procedures of pigs on the prevalence of salmonella in pork. *International Journal of Food Microbiology*. **70**:231-242.
- Swanenburg M., Urlings H.A.P., Keuzenkamp D.A., Snijders J.M.A. (2001b). *Salmonella* the lairage of pig slaughterhouses. *Journal of Food protection*. **64**:12-16.
- Talon, R., Leroy-Satrin, S., Fadda, S. (2004). Dry fermented sausages. In Y. H. Hui, L. M. Goddik, J. Josephsen, P. S. Stanfield, A. S. Hansen, W. K. Nip, F. Toldrá (Eds.), *Handbook of food and beverage fermentation technology* (pp. 397–416). New York, USA: Marcel Dekker Inc
- Taylor CM, Roberts IS (2005) Capsular polysaccharides and their role in virulence. *Contrib. Microbiol*. **12**:55-66.
- Tezcan-Merdol D, Nyman T., Lindberg U., Haaf F., Koch-Nolte F. and Rhen M. (2001). Actin is ADP-ribosylated by the *Salmonella enterica* virulence-associated protein SpvB. *Molecular Microbiology*. **39**(3):606-619.
- Threlfall, E.J., Hampton, M.D., Chart, H., Rowe, B. (1994). Use of plasmid profile typing for surveillance of *Salmonella enteritidis* phage type 4 from humans, poultry and eggs. *Epidemiology and Infection*. **112**:25–31.
- Threlfall E.J. (2002). Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. *FEMS Microbiology Reviews*. **26**:141-148.
- Thompson J.A., Liu M., Helaine S., Holden D.W. (2011). Contribution of the PhoP/Q regulon to survival and replication of *Salmonella enterica* serovar Typhimurium in macrophages. *Microbiology*. **157**: 2084-2093.
- Traub W.H., Raimond E.A., Linehani J. (1970). Identification of *Enterobacteriaceae* in the clinical microbiology laboratory. *Applied Microbiology*. **20** (3):303-308.
- Van Asten A.J.A.M. and Van Dijk J.E. (2005). Distribution of “classic” virulence factors among *Salmonella* spp. *FEMS Immunology and Medical Microbiology*. **44**:251–259.

- Villa L., Mammina C., Miriagou V., Tzouvelekis L.S., Tassios P.T., Nastasi A., Carattoli A. (2002). Multidrug and Broad-Spectrum Cephalosporin Resistance among *Salmonella enterica* Serotype Enteritidis Clinical Isolates in Southern Italy. *Journal of Clinical Microbiology*. **40**(7):2662–2665.
- Villaverde, A., Morcuende, D., Estévez, M., (2014). Effect of curing agents on the oxidative and nitrosative damage to meat proteins during processing of fermented sausages. *Journal of Food Science*. **79**:C1331-1342.
- Wain J, Bay VB, Ha V, *et al.* (2001). Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features. *Journal of Clinical Microbiology*. **39**:1571-6.
- Wain J., House D., Parkhill J., Parry C., Dougan G. (2002). Unlocking the genome of the human typhoid bacillus. *The Lancet Infectious Disease*, **2**: 163-170.
- Wannaprasat W., Padungtod P., Chuanchuen R. (2011). Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. *International Journal of Antimicrobial Agents*. **37**:457–461.
- Wilcock B.P., Armstrong C. and Olander H. (1976). The significance of the serovar in the clinical and pathologic features of naturally occurring porcine salmonellosis. *Canadian Journal of Comparative Medicine*. **40**:80-88.
- Wilcock B.P. and Schwartz K. (1992). Salmonellosis. In: Leman A.D., Straw B.E. Mengeling W.E., D’Allaire S. and Taylor D.J. (eds) *Diseases of swine*, 7th edn. Iowa State University Press, Ames, Iowa, pp. 570-583.
- Whitfield C., (2006). Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu. Rev. Biochem.* **75**:39-68.
- Wolffs, P., Norling, B., Radstrom, P., 2005. Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *Journal of Microbiological Methods* **60**, 315-323.
- Wood R.L. and Rose R. (1992). Population of *Salmonella* Typhimurium in internal organs of experimentally infected carrier swine. *American Journal of Veterinary Research*. **53**:653-657.
- Xu Q., Dziejman, M., Mekalanos J.J. (2003). Determination of the transcriptome of *Vibrio cholerae* during intrainestinal growth and midexponential phase in vitro. *Proceedings of the National Academy of Science. USA* **100**:1286–1291.
- Zanardi, E., Ghidini, S., Conter, M., Ianieri, A., (2010). Mineral composition of Italian salami and effect of NaCl partial replacement on compositional, physico-chemical and sensory parameters. *Meat Science* **86**, 742-747.