AGRICULTURAL UNIVERSITY OF ATHENS
DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION
LABORATORY OF FOOD QUALITY CONTROL AND HYGIENE

PhD Thesis

Ecophysiology of *Salmonella* stress response

Alkmini K. Gavriil

Supervisor:
Panagiotis N. Skandamis, Professor, AUA

Advisory Committee:
Panagiotis N. Skandamis, Professor, AUA
Eleftherios Drosinos, Professor, AUA
Ilias Giannenas, Assistant Professor, AUT

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"Οικο-φυσιολογία των αποκρίσεων του οργανισμού *Salmonella* σε συνθήκες καταπόνησης"

Alkmini K. Gavriil

Examining committee:
Panagiotis N. Skandamis, Professor, AUA (supervisor)
Eleftherios Drosinos, Professor, AUA
Ilias Giannenas, Assistant Professor, AUT
Konstantinos Koutsoumanis, Professor, AUT
Efstathios Panagou, Associate Professor, AUA
Effie Tsakalidou, Professor, AUA
Evangelos Oikonomou, Assistant Professor, AUT

Ecophysiology of *Salmonella* stress response

Department of Food Science and Human Nutrition
Laboratory of Food Quality Control and Hygiene

Summary

Salmonella spp. is a ubiquitous foodborne pathogen, with significant economical losses worldwide. With more than 2500 serotypes, this bacterium has evolved to survive under different environments and across multiple hosts. The ability of this pathogen to induce acid resistance mechanisms that help them better withstand severe acid stress following preculture to mild or moderate acid conditions can pose a major public threat, especially for ready-to-eat acid foods such as mayonnaise or deli-type salads. Apart from increased acid resistance, stimulation of acid resistance mechanisms may also result in increased virulence. Although almost any food product can be contaminated with this pathogen, products of animal origin have been highly associated with the presence of *Salmonella* spp., with *S.* Typhimurium and *S.* Enteritidis being the most common serovars isolated from pig meat and eggs, respectively. The risk of contamination with *Salmonella* during all the farm-to-fork stages alongside with the emergence of resistance or multi-drug resistance pathogens raises considerable concerns, particularly in an era were the public demand for more natural foods with fewer chemical additives is increasingly growing.

The main objectives of this thesis were (i) to screen the *in vitro* contribution of different levels of undissociated acetic acid over a range of different pH values in inducing acid resistance in *S.* Enteritidis and to further examine whether the *in vitro* results could be extrapolated in a food matrix, with regard to intrinsic and extrinsic food related factors (chapter 2), (ii) to assess the *in situ* adaptive responses of six *Salmonella* spp. strains in mayonnaise following adaptation to three acid conditions tested in chapter 2 and to further elucidate the role of pH and undissociated acetic acid in the phenotypic and transcriptomic adaptive profiles of two *S.* Enteritidis strains having the most prominent adaptive potential (chapter 3) and (iii) to assess the *in vitro* and *in situ* antimicrobial potential of plant aqueous extracts collected as by-products of essential oil production against three stains of *S.* Typhimurium (chapter 4).

The results of chapter 2 clearly established a link between *in vitro* acid resistance and undissociated acetic acid concentrations following acid exposure to pH 2.5, though the pH of the adaptation medium seemed also to affect the induction of acid resistance. In addition, it was shown that factors prevailing on the subsequent *in vitro*

(pH, temperature) or *in situ* (unspecified intrinsic food related factors) acid challenge may collectively determine the acid resistance phenotypes of the pathogen (chapter 2). In chapter 3, strain-dependent differences were found regarding the innate and adaptive responses of six *Salmonella* spp. strains inoculated in mayonnaise at 5ºC. Differences were also found in the contribution of pH and undissociated acetic acid on the phenotypic and transcriptomic profiles of the two *S.* Enteritidis strains. With regard to the contamination potential of pork meat with *S.* Typhimurium, in chapter 4 the *in vitro* antimicrobial effect of aqueous plant by-products was screened against three strains of *S.* Typhimurium in pork meat. The antimicrobial activity was mainly plant and temperature dependent. *In situ* application of oregano aqueous plant extracts effectively reduced the levels of S. Typhimurium FS8 populations in pork meat stored at 4ºC.

In conclusion, induction of acid resistance mechanisms can potentially protect *Salmonella* against lethal acid environments, with regard to strain variations and factors prevailing during acid challenge and acid exposure. Natural antimicrobials can provide an effective, alternative, economical and eco-friendly way for ensuring meat safety.

Scientific area: Food safety

Key words: *Salmonella*; acid adaptation; undissociated acetic acid; pH; acid resistance; transcriptomic profile; strain variability; pork meat; by-products; natural antimicrobials

Περίληψη

Το βακτήριο *Salmonella* spp. είναι τροφιμογενές παθογόνο, με ευρεία παρουσία στο περιβάλλον. Οι οικονομικές συνέπειες και οι επιπτώσεις στην υγεία λόγω των τροφιμογενών λοιμώξεων από *Salmonella* spp. Είναι παγκοσμίως σημαντικές. Το γένος *Salmonella* spp. περιλαμβάνει πάνω από 2500 οροτύπους και έχει τη δυνατότητα να επιβιώνει σε διαφορετικά περιβάλλοντα, προσβάλλοντας ένα μεγάλο εύρος ξενιστών. Η ικανότητα του παθογόνου *Salmonella* spp. να επάγει μηχανισμούς οξεοανθεκτικότητας μετά από προσαρμογή του σε ήπια ή μέτρια όξινες συνθήκες του επιτρέπουν να επιβιώσει σε ισχυρά όξινα περιβάλλοντα (στρες), διακυβεύοντας με τον τρόπο αυτό τη δημόσια υγεία. Ο κίνδυνος είναι ακόμα μεγαλύτερος όσον αφορά τα έτοιμα-προς κατανάλωση όξινα τρόφιμα, τα οποία δεν επιδέχονται κάποιο μεταχείριση πριν την κατανάλωση τους. Η προσαρμογή σε όξινες συνθήκες είναι ένα ιδιαίτερα περίπλοκο φαινόμενο που, εκτός από αυξημένη ανθεκτικότητα, προσδίδει στο βακτήριο και μεγαλύτερη παθογένεια. Αν και πρακτικά όλα τα τρόφιμα μπορούν να επιμολυνθούν από *Salmonella*, τα τρόφιμα ζωικής προέλευσης αποτελούν την κυριότερη αιτία σαλμονέλλωσης. Ο ορότυπος *S.* Typhimurium αποτελεί ένα από τους πιο συχνά απαντώμενους ορότυπους στο χοίρειο κρέας, ενώ η παρουσία του οροτύπου *S.* Enteritidis έχει συσχετισθεί κυρίως με αυγά ή προϊόντα με βάση τα αυγά. Η επιμόλυνση των τροφίμων με *Salmonella* spp. μπορεί να γίνει σε όλα τα στάδια της διαδικασίας παραγωγής και επεξεργασίας μέχρι το τραπέζι του καταναλωτή (from farm to fork). Το γεγονός αυτό, σε συνδυασμό με την αύξηση των μικροοργανισμών ανθεκτικών σε ένα ή και περισσότερα αντιβιοτικά, δημιουργεί ισχυρές πιέσεις στη βιομηχανία τροφίμων, ιδιαίτερα σε μία εποχή όπου οι απαιτήσεις των καταναλωτών για πιο φυσικά προϊόντα με λιγότερα χημικά συντηρητικά ολοένα και μεγαλώνουν.

Με βάση τα παραπάνω, οι κύριο στόχοι της παρούσας διδακτορικής διατριβής ήταν: (i) να αξιολογήσει την *in vitro* επίδραση διαφορετικών συγκεντρώσεων αδιάστατου οξικού οξέος προστιθέμενο σε ένα εύρος διαφορετικών τιμών pH στην επαγωγή όξινης ανθεκτικότητας του παθογόνου *S.* Enteritidis και να εξετάσει κατά πόσο τα αποτελέσματα από τα *in vitro* πειράματα μπορούν να επιβεβαιωθούν σε ένα τρόφιμο χαμηλού pH, λαμβάνοντας υπ' όψιν παράγοντες που σχετίζονται με το εσωτερικό και εξωτερικό περιβάλλον του τρόφιμου (κεφάλαιο 2), (ii) να αξιολογήσει

τους *in situ* αποκρίσεις έξι διαφορετικών στελεχών *Salmonella* spp. στη μαγιονέζα συντηρούμενη σε συνθήκες ψύξης (5 ^{o}C), μετά από την προσαρμογή τους σε τρεις διαφορετικές όξινες συνθήκες που επιλέχθηκαν με βάση τα *in vitro* αποτελέσματα του κεφαλαίου 2 και να προσδιορίσει το ρόλο του pH και του αδιάστατου οξικού οξέος στα φαινοτυπικά και μεταγραφικά προφίλ δύο επιλεγμένων στελεχών *S.*Enteritidis με το πιο αξιοσημείωτο δυναμικό προσαρμογής (κεφάλαιο 3) και, τέλος, (iii) να αξιολογήσει *in vitro* και *in situ* το αντιμικροβιακό δυναμικό διάφορων φυτικών υδατικών φάσεων, οι οποίες συλλέχθηκαν ως παραπροϊόντα της παραγωγής αιθέριων ελαίων, εναντίον τριών στελεχών *S.* Typhimurium.

Τα αποτελέσματα του κεφαλαίου 2 έδειξαν ότι υπάρχει σχέση μεταξύ της *in vitro* οξεοανθεκτικότητας του παθογόνου *S.* Enteritidis και των συγκεντρώσεων του αδιάστατου οξικού οξέος κατά την έκθεση του μικροοργανισμού σε pH 2.5, με το pH του μέσου προσαρμογής να επηρεάζει επίσης την επαγωγή ή μη αυξημένης ικανότητας επιβίωσης. Επιπλέον, βρέθηκε ότι οι παράγοντες που επικρατούν στη μετέπειτα *in vitro* (pH, θερμοκρασία επώασης) ή *in situ* (μη καθορισμένοι παράγοντες του εσωτερικού περιβάλλοντος του τρόφιμου) καταπόνηση του παθογόνου μπορεί επιπρόσθετα να επηρεάσουν τις φαινοτυπικές αποκρίσεις. Στο κεφάλαιο 3, διαστελεχιακές διαφορές επηρέασαν την επαγόμενη (induced) και μη επαγόμενη (innate) ανθεκτικότητα έξι διαφορετικών στελεχών *Salmonella* spp. σε μαγιονέζα συντηρούμενη στους 5^{o}C. Επιπλέον, βρέθηκε ότι το pH και το αδιάστατο οξικό οξύ επηρεάζουν διαφορετικά το φαινοτυπικό και μεταγραφικό προφίλ των δύο εξεταζόμενων στελεχών S. Enteritidis. Δεδομένου ότι η επιμόλυνση του χοίρειου κρέατος με *S.* Typhimurium είναι μεγάλη, στο κεφάλαιο 4 εξετάστηκε η *in vitro* αντιμικροβιακή δράση φυτικών υδατικών παραπροϊόντων σε τρία διαφορετικά στελέχη *S.* Typhimurium σε χοίρειο κρέας. Το αντιμικροβιακό δυναμικό των εξεταζόμενων φάσεων επηρεάστηκε κυρίως από τη θερμοκρασία επώασης και το είδος της υδατικής φάσεως που εξετάστηκε. Η *in situ* εφαρμογή των υδατικών φάσεων ρίγανης μείωσε αποτελεσματικά τους πληθυσμούς του παθογόνου S. Typhimurium FS8 σε χοίρειο κρέας συντηρούμενο στους 4^{o}C.

Συμπερασματικά, η επαγωγή των όξινων μηχανισμών ανθεκτικότητας μπορεί να προστατέψει το παθογόνο βακτήριο *Salmonella* spp. από τη θανατηφόρο επίδραση ισχυρά όξινων συνθηκών. Ωστόσο, η ενεργοποίηση ή όχι μηχανισμών επιβίωσης επηρεάζεται από τη διαστελεχιακή παραλλακτικότητα και από παράγοντες που επικρατούν τόσο κατά τη διάρκεια της διαδικασίας προσαρμογής όσο και κατά τη διάρκεια της όξινης καταπόνησης. Επιπλέον, οι φυσικές αντιμικροβιακές ενώσεις

δύνανται να παρέχουν μια αποτελεσματική, οικονομική και οικολογική λύση για τη βελτίωση και διασφάλιση της ασφάλειας του κρέατος.

Επιστημονική περιοχή: Ασφάλεια τροφίμων

Λέξεις κλειδιά: *Salmonella*; όξινη προσαρμογή; αδιάστατο οξικό οξύ; pH; όξινη ανθεκτικότητα; μεταγραφικό προφίλ; διαστελεχιακή παραλλακτικότητα; χοίρειο κρέας; παραπροϊόντα; φυσικά αντιμικροβιακά

Thesis committee

Thesis supervisor

Dr. Panagiotis Skandamis

Professor, Laboratory of Food Quality Control and Hygiene

Department of Food Science and Human Nutrition, Agricultural University of Athens

Thesis co-supervisors

Dr. Eleftherios Drosinos

Professor, Laboratory of Food Quality Control and Hygiene

Department of Food Science and Human Nutrition, Agricultural University of Athens

Dr. Ilias Giannenas

Assistant Professor, Laboratory of Nutrition

Department of Veterinary Medicine, Aristotle University of Thessaloniki

Other members

Dr. Konstantinos Koutsoumanis

Professor, Laboratory of Food Microbiology and Hygiene

Department of Food Science and Technology, Aristotle University of Thessaloniki

Dr. Efstathios Panagou

Associate Professor, Laboratory of Food Microbiology and Biotechnology

Department of Food Science and Human Nutrition, Agricultural University of Athens

Dr. Effie Tsakalidou

Professor, Laboratory of Dairy Research

Department of Food Science and Human Nutrition, Agricultural University of Athens

Dr. Evangelos Oikonomou

Assistant Professor, Laboratory of Hygiene of Food of Animal Origin – Veterinary Public Health

Department of Veterinary Medicine, Aristotle University of Thessaloniki

Στην οικογένεια μου…..

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CHAPTER 1

General introduction and Objectives of the thesis

1.1 General information: introducing the Thesis

Safer foods save lives. With every bite consumed, people are potentially exposed to either microbiological or chemical hazards (World Health Organization, 2015).

Food can contain several microorganisms, such as spoilage microbiota (e.g. yeasts and moulds) or pathogenic bacteria (e.g. *Salmonella* spp., *E. coli*). The presence of microorganisms can affect both safety and quality of food products (Everis, 2001). Microorganisms including pathogens can be introduced to the foods at any step along the food chain, including pre-harvest/slaughter, during harvest and slaughter and post-harvest/slaughter processing. Pre-harvest or pre-slaughter contamination is performed in the primary production through contaminated water, manure applied as fertilizer, feeding of infected foodstuffs, unhygienic human practices of worker in the field, farm equipment, footwear, work clothes and even rodents, insects, cats and dogs. During harvest, contamination may occur through contaminated harvest equipment, unwashed hand of field workers, whereas animal skin and hides and the environment are the major sources of meat or poultry contamination during slaughter. Finally, processes such as trimming, slicing, milling, shredding, peeling, mechanical abrasions and various methods of disintegration performed with contaminated equipment or contact with other equipment such as conveyor belts, filters, wooden surfaces etc. may also introduce unfavorable microorganisms at the post-harvest/slaughter stage. Infected food handles and their unhygienic practices, existence of biofilm commodities in the processing environment and use of poor water in fresh produce processing may also determine the spoilage and pathogenic microflora introduced in the food product. Contamination during post-harvest/slaughter may also be performed during distribution and storage (Alum et al., 2016).

Microbiological food safety is a global issue with significant implications for human health (Hintz et al., 2015) and a major concern for food industry, regulatory agencies and consumers (Khan et al., 2017; Wu, 2008). Foodborne diseases are a major cause of morbidity and mortality worldwide (World Health Organization, 2015). According to World Health Organization, foodborne diseases can be defined as "*any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water*".

The economical and medical burden of foodborne diseases is substantial. According to WHO, 600 million cases of foodborne diseases and 420.000 deaths

(125.000 corresponding to 30 % among children under 5 years old) are caused each year worldwide due to contamination of unsafe food. The annual economic losses in productivity and medical expenses in low-and middle-income countries are calculated to $110 US billions (https://www.who.int/activities/estimating-the-burden-of-foodborne-diseases).

At the same time, the food industry is ever-evolving to meet the seemingly contradictory trends and demands of the market. Consumer seek for healthier and more traditional food products with fewer additives, upgraded sensory quality, functionality and nutritional properties but at the same time safer with a longer shelf life and convenience in preparation and use (Nychas et al., 2008). Nonetheless, food contamination and spoilage is a problem that has yet to be controlled effectively (Khan et al., 2017). Towards these challenges, a number of traditional and novel food preservation processes, such as heating, freezing, drying, chilling, irradiation, high pressure, water activity reduction, acidification, nutrient restriction, or addition of natural/chemical antimicrobials have been used in order to control foodborne pathogens and increase products shelf life by inactivating microorganisms or reducing their growth rate (Everis, 2001; Wu, 2008). Nonetheless, some of these techniques may cause undesirable organoleptic effects, reducing consumer acceptability (Khan et al., 2017). Modern trends rely on combinations of mild preservation treatments and exposure of cells to several stress factors for controlling the spoilage and pathogenic microbiota. In the concept of this approach, known as hurdle technology, two or more hurdles intelligently applied at suboptimal levels can be more effective in controlling the microbial contaminants in foods than each hurdle applied individually at optimal levels (Lianou et al., 2012). Apart from improving the microbial stability of foods, hurdles deliberately combined can also enhance their sensory quality as well as their nutritional and economic properties (Leistner, 2000). However, low (sublethal) levels of a stress may strengthen the cells, increasing their resistance against subsequent lethal stresses (Alvarez-Ordóñez et al., 2015; Wesche et al., 2009).

Naturally derived antimicrobial compounds are increasingly gaining scientific and commercial attention as label-friendly alternatives to synthetic food preservatives (Davidson et al., 2013). This can be ascribed to the continuously growing public awareness of the harmful effects caused by overexposure to synthetic chemicals (Moreno et al., 2006) alongside with the emergence of resistant or multi-drug resistant pathogens (Bouarab-Chibane et al., 2019; Y. Liu et al., 2017), hard to control with

conventional antimicrobials (Frieri et al., 2017). Typical example of a multi-drug resistance pathogen virulent both to human and animals is *Salmonella* Typhimurium DT104, the 2nd most common isolated *Salmonella* species during 1990s after *S.* Enteritidis Phage Type 4 (Poppe et al., 1998). The commercial exploitation of naturally derived compounds such as organic acids, plant extracts, essential oils and bacteriocins could provide a promising, alternative, eco-friendly and often economical way to ensure food safety.

1.2 *Salmonella* spp.: General information and characteristics of an invisible enemy

1.2.1 General characteristics of Salmonella spp.

Salmonella spp. is a medically important pathogen for both animals and humans. They are facultative anaerobic, nonspore-forming, rod-shaped Gram-negative bacteria 2-5 μm x 0.5-1.5 μm in size, motile by peritrichous flagella belonging to Enterobacteriaceae family. It is a diverse and ubiquitous group of foodborne pathogens with more than 2500 distinct serotypes worldwide, showing medical and economical significance (Andino and Hannin, 2015; Uzzau 2000). Growth is observed in temperatures just above 5 and up to 47°C, with an optimum of 37°C. *Salmonella* are heat sensitive and they are destroyed with pasteurization. They are neutralophilic bacteria and can grow within a pH range 4-9, with an optimum of 6.5-7. The minimum pH varies with the type of acidulant. They require high water activity, between 0.99 and 0.94, yet they can still survive well in dried foods. They are not fastidious microorganisms and can multiply under various environmental conditions outside the living hosts (Adams and Moss, 2008; Pui et al., 2011).

Salmonellosis is the second most commonly reported zoonosis after campylobacteriosis with 91.662 human reported cases in all Member States of EU (19.7 cases per 100.000 population). In 2017, 1241 foodborne *Salmonella* outbreaks affecting 9.600 people were identified in EU, accounting for 24.4 % of the total number of outbreaks (EFSA and ECDC, 2018). In USA, salmonellosis is one of the most common foodborne infections, resulting in an estimated 1.2 million human cases and $365 million in direct medical costs annually (https://www.cdc.gov/training/SIC_CaseStudy/Infection_Salmonella_ptversion.pdf).

1.2.2 Taxonomy

The nomenclature of *Salmonella* spp. is complex and ever-evolving. The current taxonomy used by the Centers for Diseases Control and Prevention (CDC) is based on recommendations from the World Health Organization Collaborating Centre. According to the CDC system, the genus *Salmonella* is composed of two species, *S. enterica* and *bongori*. Each species contains several serovars. *S. enterica* is further subdivided to six subspecies, which are referred to with a Roman numeral and a name: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*. *S. enterica* subspecies are differentiated based on biochemical and genomic modifications (Brenner et al., 2000). More than 99% of serovars involved in human infections and warm-blooded animals belong to *S. enterica* sub. *enterica.* Serovars belonging to the other subspecies and to *S. bongori* are isolated from mainly from cold-blooded animals and environment but rarely from humans and account for less than 1% of clinical isolates (Pui et al., 2011). Within the subspecies *enterica*, the five most common isolated serovars are with decreasing order, Enteritidis, Typhimurium, monophasic Typhimurium, Infantis and Derby (EFSA and ECDC, 2018).

Kauffman-White serotyping scheme is used for the serological classification within the genus according to three major antigenic determinants, flagella H, somatic O and capsular (Vi) antigens (Pui et al., 2011; Ryan et al., 2017). More than 2.600 serovars are recognized based on their antigenic composition. Differentiation of isolates of the same serovar can also be performed by phage typing, which is based on the susceptibility of isolates to different selected bacteriophages. Phage typing is generally employed for epidemiological purposes, when the origin and characteristics of an outbreak needs to be determined (Pui et al., 2011).

Epidemiological classification of *Salmonella* serotypes is based on their host preferences. Three different groups are identified: host-restricted serovars are those almost exclusively related to one species (i.e. Typhi, Paratyphi, Gallinarium); serovars which are prevalent to one particular host species but can also cause disease in other host species (i.e Dublin, Cholerasuis) are recognized as host-adapted; finally, ubiquitous serovars are those capable of infecting a broad range of host species (i.e Typhimurium, Enteritidis, Newport) (Uzzau et al., 2000).

1.2.3. Transmission

Salmonella spp. has evolved to survive in a wide spectrum of environments and across multiple hosts (Foley et al., 2013). It is widely distributed in nature and can survive in the environment for long periods of time (Pui et al., 2011). The main niche of this pathogen is the intestinal tract of human and farm animals , although it can be present in the intestinal tract of wild birds, reptiles and occasionally insects (Andino and Hanning, 2015). *Salmonella* spp. spread through the fecal-oral route. The primary and most common source of transmission is through contaminated food or water. Food of animal origin, such as beef, poultry, milk and eggs are those more often implicated to *Salmonella* infections. Nonetheless, almost any food can be contaminated with this pathogen (Cianflone 2008; https://www.cdc.gov/training/SIC_CaseStudy/Infection_Salmonella_ptversion.pdf). For instance, a wide variety of products of vegetable origin have been linked to salmonellosis outbreaks, with the incidence of foodborne cases due to the consumption of contaminated fresh fruits and vegetables showing an increasing trend in the industrialized countries over the last years (Pui et al., 2011). Contamination of food with *Salmonella* can be performed at any point of the food chain, starting from livestock feed, through food manufacturing, processing and retailing as well as catering and food preparation in the home (Pui et al., 2011). Other sources of transmission include contamination through direct animal contact (e.g. turtles, iguanas, snakes, cats, dogs and rodents) if careful hand hygiene is not followed and more rarely through person to person contact (Cianflone 2008; https://www.cdc.gov/training/SIC_CaseStudy/Infection_Salmonella_ptversion.pdf). According to EFSA and ECDC (2018), the most commonly isolated serovars in 2017– in decreasing order - were Enteritidis, Typhimurium, monophasic Typhimurium (1,4,[5],12:i:-), Infantis, Newport, Agona, Kentucky and Derby. The three first serovars (Enteritidis, Typhimurium and monophasic Typhimurium) are the most dominant ones, representing the 70.5% of the 78.949 confirmed human salmonellosis with known serovars during this period.

1.2.4. The disease

Clinical manifestations of salmonellosis are gastroenteritis, enteric (typhoid) fever and bacteremia. Typhoid fever is caused by the host-restricted serovars Typhi and Paratyphi and it is mainly located in Southern and Central Asia, where it is endemic

(Andino and Hanning, 2015). Non-typhoidal *Salmonella* (NTS) are responsible for gastrointestinal infection (gastroenteritis). Among other foodborne pathogens, non-typhoidal salmonellosis is the leading cause of deaths and hospitalizations, accounting for 28% of deaths and 35% of hospitalizations in the US (Scallan et al., 2011). This disease is predominantly linked to serovars occurring widely in humans and animals, with Enteritidis and Typhimurium being the most prevalent ones (Adams and Moss, 2008). Incubation period is 12-72 h (usually 12-36 h). The principal symptoms are mild fever, diarrhea, abnormal cramps, nausea and vomiting. The disease is usually self-limiting, and the symptoms last from 4 to 7 days. A low percentage of patients suffering from gastrointestinal illness will develop bacteremia, a serious condition in which bacteria enter the bloodstream after passing through the intestinal barrier. Bacteremia is the most common complication of *Salmonella* gastroenteritis, with infants elderly and immunocompromised being at increased risk. It has been highly associated with invasive serotypes, such as Cholerasuis and Dublin (Cianflone, 2008; Pui et al., 2011). Persistence carriage of *Salmonella* spp. in the feces or urine of patients for more than 12 months leads to a chronic carrier state that may employ an important public health issue, since infected persons can serve as reservoirs for transmission to others (Cianflone, 2008).

1.2.5 Pathogenesis

The severity of *Salmonella* infections on humans vary, depending on the serotype implicated and the health status of the human host. People belonging to susceptible groups, such as immunocompromised, elderly and children below the age of 5 are more vulnerable compared to healthy population (Eng et al., 2015).

Once ingested, *Salmonella* bacteria have to pass through the alimentary system and into the stomach, where they have to endure the acidic conditions. Those cells that survive passage through the low pH environment, proceed to the lumen of gastrointestinal track organs (Foley et al., 2013) and must cross the intestinal epithelium in order to successfully colonize the host (Hume et al., 2017). *Salmonella* can invade the intestinal epithelium through three different routes: through invasion of non-phagocytic enterocytes (epithelial cells), specialized epithelial M cells or dendritic cells that come in between epithelial cells (Cota García, 2016; Hume et al., 2017). The first two routes are mediated by the Type 3 Secretion System encoded on *Salmonella* Pathogenicity Island - 1 (Cota García, 2016).

Epithelial and immune cells provide the initial protective barrier of the gastrointestinal tract organs (Foley et al., 2013). *Salmonella* cells compete with the gut microflora and adhere to the enterocytes or M cells (Foley et al., 2013), with M cells being the predominant target of invasion (Cota García, 2016). The process is facilitated by fimbriae and flagella present on the pathogen bacterial surface (Foley et al., 2013). Once attached to the epithelium, this pathogen displays a remarkable characteristic, by inducing its own phagocytosis in order to gain access to the host cells (Eng et al., 2015). This is performed by expression of a Type 3 Secretion System (T3SS), a multiprotein complex that acts like a 'molecular syringe' and allows *Salmonella* to transport toxins and other effector proteins into intestinal cells (Foley et al., 2013). T3SS is encoded by *Salmonella* Pathogenicity Island -1 (SPI-1), a chromosomal DNA region (Eng et al., 2015) related to *Salmonella* invasion of host cells (Foley et al., 2013). Effector proteins manipulate the cell physiology, by triggering the reconstitution of actin cytoskeleton, leading in the outward extension of the host cell membrane, a process known as membrane ruffling. This process ultimately leads to engulfment of *Salmonella* cells by the host cell membrane and internalization of the pathogen in the form of a membrane-bound compartment, called *Salmonella*-containing vacuole (SCV). Inside the cytoplasm, they express a second T3SS located on SPI-2, that has a crucial role in causing systemic infections and intracellular pathogenesis (Foley et al., 2013). Internalized SCV migrates from the luminal border to the basal membrane avoiding the destruction by the phagolysosomal processing pathways (Foley et al., 2013). One across the epithelium, *Salmonella* can invade further epithelial cells through the basolateral side or pass to underlying macrophages (Hume et al., 2017).

After invasion of the epithelium, *Salmonella* cells can either remain localized in the intestine, where they induce an inflammatory reaction in the intestinal mucosa (gastroenteritis) that leads to diarrhea due to accumulation of liquid in the intestinal lumen or cause systemic infection (Cota García, 2016). In the case of gastroenteritis, only a small portion of the ingested bacteria manage to invade the epithelium. The majority of the pathogenic population remains in the lumen of the intestine, where they gain bias over the native intestinal microbiota due to the host's inflammatory response to the small invading sub-population. The invading sub-populations will eventually be cleared (Hume et al., 2017). In systemic infection, *Salmonella* invade dendritic cells or macrophages, where they can survive or even multiply (in the case of macrophages) (Foley et al., 2013) and disseminates through the lymphatic system to other organs,

particularly the spleen, the liver, the gall bladder and the bone marrow (Cota García, 2016).

1.3 Stress response

During their life cycle, bacteria can encounter a number of different stresses all along the farm-to-folk chain. Stress refers to any deleterious factor or condition that adversely affects microbial growth and survival (Begley and Hill, 2015). These stresses may occur to the natural environmental niches of the microorganisms, inside the food ecosystem due to stressful conditions prevailing during food processing and storage and finally after consumption of the food due to the endogenous host defense systems (Álvarez-Ordóñez et al., 2012; Begley and Hill, 2015). These include adverse pH, low nutrient availability, oxidation and extreme temperatures (Alvarez-Ordóñez et al., 2015). Many of these stresses encountered in the food and natural ecosystem are similar to the *in vivo* stresses found as part of the host defense system (Begley and Hill, 2015; Wesche et al., 2009).

Based on their intensity, there are different levels of stress that determine the extent of the bacterial survival. For instance, lower levels of stressful agent may activate transient adaptive responses (Wesche et al., 2009), a process that is usually named as stress adaptation of stress hardening. Lethal levels of the stressful agent, on the other hand, may result in the death of a portion or entire the bacterial population (Wesche et al., 2009).

Foodborne bacteria are able to sense and react to the environmental challenges by activating complex, inducible adaptive mechanisms as a result of gene expression modifications. These mechanisms are induced during adaptation to mild (sublethal) levels of a given stress and can offer protection during subsequent exposure to normally lethal levels of the same or different (cross protection) type of stresses (Alvarez-Ordóñez et al., 2015; Begley and Hill, 2015). Nonetheless, this induced resistance is transient, meaning that once the stressor is removed, the cells return to their conventional tolerance against the specific stress (Alvarez-Ordóñez et al., 2015).

Salmonella spp. possess several mechanisms that enable them to survive under dynamic and hostile conditions. Among stresses commonly encountered by this pathogen, acid stress is one the most common and important stresses *Salmonella* may come across during its lifecycle (Spector and Kenyon, 2012).

1.4 Acid adaptive response

Acid stress can be defined as the combined biological effect of low pH and weak (organic) acids present in the environment (Bearson et al., 1997). It is a very important obstacle, frequently encountered naturally in many foods, such as fruit juices, or as a result of the addition of organic acids as food preservatives. Foodborne pathogens must also survive the highly acidic pH of the stomach in order to proliferate and colonize the small intestines or colon. Especially for *Salmonella* spp., its life cycle inside the human host includes acidification of the phago-lysosome, creating, therefore, another obstacle that has to be discarded (Spector and Kenyon, 2012). Therefore, the ability of this pathogen to sense, respond and adapt to acid environments is crucial for its epidemiology and virulence (Spector and Kenyon, 2012). In addition, it is widely accepted that the ability of the pathogens to adapt and survive is linked to their disease-causing ability (Audia et al., 2001). For instance, Berk et al. (2005) reported a positive correlation between acid resistance of *Salmonella* spp. DT104 and pathogenicity, with high acid resistance strains being isolated from humans.

Among potential acid stresses frequently encountered by *Salmonella* spp. is the presence of weak organic acids (Kwon and Ricke, 1998). Organic acids have long been established as food additives and preservatives for preventing food deterioration and extending shelf life (Ricke, 2003). For instance, they are used as preservatives in salad dressings and mayonnaise (Kwon et al., 2000; Kwon and Ricke, 1998) and as decontamination agents in meat carcasses (Dorsa, 1997). In addition, these molecules are widely distributed in nature, since they are often naturally present in plants and animal tissues (Theron and Lues, 2011) or they can accumulate in fermented products due to the metabolic activity of starter or indigenous microflora (Ricke, 2003). Finally, they are present in the gastrointestinal human and animal track (Kwon et al., 2000; Kwon and Ricke, 1998; Lachica et al., 2018). The interest regarding the application of organic acids can be ascribed to their antimicrobial activity and their "generally recognized as safe" (GRAS) status (Theron and Lues, 2011). Organic acids exhibit both bacteriostatic and bactericidal activities (Ricke, 2003) They are more effective than mineral acids, such as HCl in terms of antimicrobial activity (Cherrington et al., 1991). For instance, several authors have reported that lower growth limits were required for *Salmonella* and *Escherichia coli* when HCl was used as acidulant compared to acetic acid (Álvarez-Ordóñez et al., 2009a, 2010a; McKellar and Knight, 1999).

The mode of action of organic acids is complex and has yet to be elucidated (Theron and Lues, 2011). The antibacterial activity of organic acids has traditionally been attributed to the undissociated form of weak acids. Conditions of low pH favor the uncharged undissociated form: the lower the pH, the higher concentration of undissociated acid will be available, based on the pk_a, to cross the cytoplasmic membrane. This can be explained by the fact that undissociated molecules are, in contrast to the charged anions, lipid soluble and can easily penetrate through the membrane by simple (passive) diffusion and insert into the cytoplasm. Inside the cells, the higher pH of the cytoplasm results in the dissociation of the acid molecules into charged anions and protons, which cannot cross the cell membrane and, thus, they accumulate inside the cell. Diffusion takes places until equilibrium is reached in accordance to the pH gradient across the membrane, resulting in acidification of intracellular pH and disruption of the proton motive force. Based on the above procedure, which is widely known as the "classical weak-acid theory", the antimicrobial effectiveness of an organic acid is determined by its pk_a and the pH of the external medium (Brul and Coote, 1999; Hirshfield et al., 2003; Lianou et al., 2012; Stratford et al., 2013). In addition, a growing theory suggests that accumulation of acid anions may also be toxic for the cells, potentially inhibiting cellular activity by several mechanisms, e.g. due to increased osmolarity and inhibition of important metabolic pathways (Carpenter and Broadbent, 2009) (Figure 1.1).

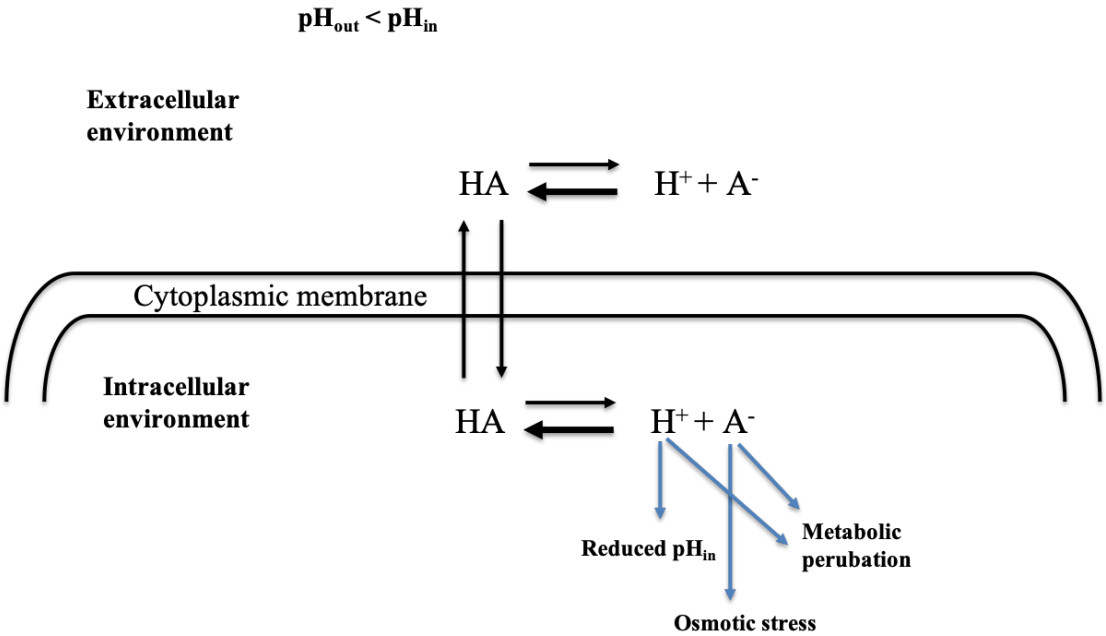

Figure 1.1. Antimicrobial mode of action of weak organic acids. The undissociated form of the weak acid (HA) accumulates insides the cell until the external and internal concentrations are equal (equilibrium). Dissociation of the undissociated form inside the cell results in a proton H^+ and an anion A^-, based on the pK_a of the acid and the local pH, resulting in acidification of intracellular pH and accumulation of weak acid anions. Based on Hirshfield et al. (2003)

Some organic acids such as acetic, lactic, benzoic and sorbic are well established preservatives agents (Lianou et al., 2012). For instance, acetic acid is widely employed as a food acidulant for hindering microbial growth and extending the shelf life of the products (Xu et al., 2008). Nonetheless, the risk that might be associated with the use of organic acid in food industry is related to potential induction of acid adaptation.

Under laboratory conditions, different protocols are used so far for the induction of acid resistance. For instance, short- or long- (following growth) term pre-exposure of cells to laboratory media acidified with HCl or organic acids (Álvarez-Ordóñez et al., 2009a, 2009b, 2010a; Foster and Hall, 1991; Greenacre et al., 2003; Koutsoumanis and Sofos, 2004; Yang et al., 2014a; Ye et al., 2019; Yuk and Marshall, 2004, 2005) or the glucose supplementation method, relying on exposure of cells to gradually decreasing pH due to fermentation of glucose (Buchanan and Edelson, 1996; Lianou et al., 2017; Malheiros et al., 2009; Samelis et al., 2003). Factors, that prevail during acid adaptation and acid (challenge) exposure, such as the growth phase of the pathogen, adaptation and challenge temperature, composition of the adaptation and challenge medium may drastically affect the final adaptive responses (reviewed by Álvarez-Ordóñez et al. 2012).

Apart from the traditional housekeeping mechanisms, the main induced acid resistance mechanisms utilized by *Salmonella* against extracellular pH perturbations include inducible pH homeostasis systems, which act to raise the intracellular pH, synthesis of Acid Shock Proteins (ASP) which aim to counter the potentially lethal effect of by acid stress and induction of membrane modifications (Alvarez-Ordóñez et al., 2015; Álvarez-Ordóñez et al., 2012; Audia et al., 2001; Bearson et al., 1997) (Figure 1.2).

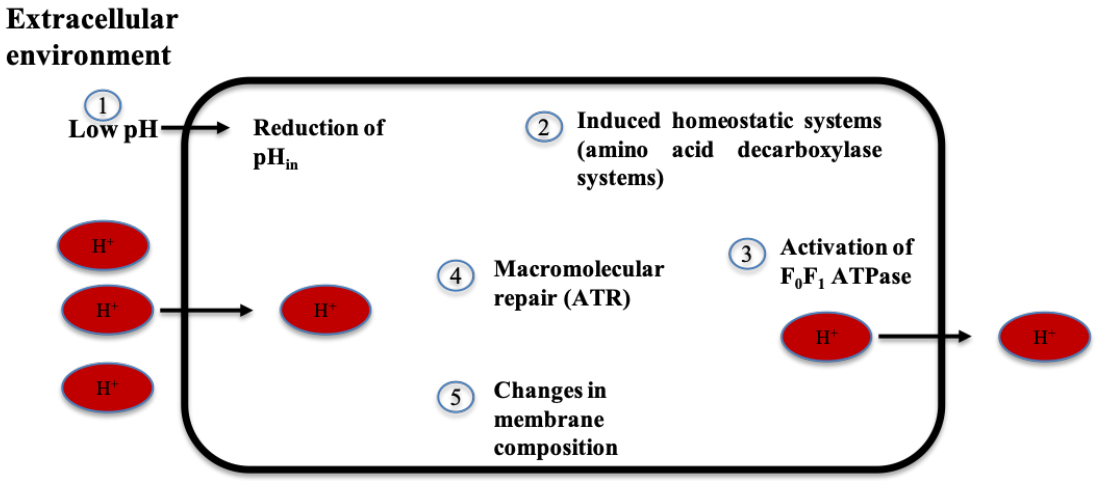

Figure 1.2. Cellular mechanisms of *S.* Typhimurium in response to acid stress. (1) Acid extracellular environments result in a decrease of intracellular pH. (2) The low pH activates amino acid decarboxylase systems that consumes intracellular pool of protons in order to maintain intracellular pH at constant levels. (3) Housekeeping cellular pumps (potassium-proton antiporters and sodium-proton antiporters) act by extruding protons from the cytoplasm with the same aim to maintain intracellular pH. (4) Several response regulators triggering the synthesis of ASP are also activated. (5) Membrane fatty acid composition is modulated leading to decreased membrane fluidity. Based on Alvarez-Ordóñez et al. (2015)

1.5 Molecular mechanisms involved in the inducible acid resistance of *Salmonella* spp.

1.5.1 Induced pH homeostasis mechanisms

pH homeostasis is the innate function of the cell that allows to maintain the intracellular pH relative stable over a broad range of extracellular pH values (Audia et al., 2001). Neutralophilic bacteria, such as *Salmonella* spp. prefer to grow at neutral pH values. Nonetheless, they have the ability to grow over a broad range of extracellular pH_o, ranging from 5 to 9. The housekeeping pH homeostatic mechanisms are typically used by Gram$^-$ bacteria to the control the intracellular pH. These mechanisms involve membrane-bound proton pumps which include K^+/H^+ and Na^+/H^+ antiporters, responsible for exchanging protons between the intracellular and extracellular environments. Housekeeping pH homeostasis system will protect the cell at external pH's above 4, but begin to fail below pH_o 4.5. Nonetheless, exposure to mild acid

conditions may activate emergency pH Acid Resistance (AR) systems that function to alkalize the intracellular pH when cells encounter a severe extracellular pH (Álvarez-Ordóñez et al., 2012; Audia et al., 2001; Bearson et al., 1997; Moat et al., 2002). These induced systems include amino acid decarboxylases, enzymes that replace the *a*-carboxyl group of the cognate amino acid by consuming one proton from the cytoplasm, according to the Equation *(1)*. The product of the reaction is highly basic, which is then secreted to the extracellular environment by the corresponding antiporters and exchanged for another amino acid substrate (Álvarez-Ordóñez et al., 2012; Viala et al., 2011).

$$NH_3^+\text{-}RCH\text{-}COO^- + H^- \longrightarrow NH_3^+\text{-}RCH_2 + CO_2. \qquad \textit{Equation (1)}$$

Consumption of intracellular protons can enhance pH homeostasis and allow cells to maintain an internal pH compatible with viability (Richard and Foster, 2004). Indeed, induction of these systems can increase the intracellular pH (Richard and Foster, 2004; Viala et al., 2011). In addition, Richard and Foster (2004) reported that amino-acids decarboxylase systems were also able to reverse the transmembrane potential under extreme acid conditions, thus mimicking the acidophiles strategy.

Salmonella spp. possess three induced amino acid decarboxylase systems: lysine/cadaverine, arginine/agmatine and ornithine/putrescine. Among them, it has been widely reported that lysine/cadaverine and arginine/agmatine contribute to the acid tolerance of *Salmonella* spp. following pre-exposure to acidic conditions (Álvarez-Ordóñez et al., 2010b; Díaz and Ricke, 2004; Greenacre et al., 2006; Kieboom and Abee, 2006; J. Liu et al., 2017; Park et al., 1996). These enzymes convert lysine and arginine to cadaverine and agmatine, respectively, which are subsequently transported extracellularly via the CadB and AdiY antiporters (Álvarez-Ordóñez et al., 2012; Bearson et al., 1997) (Figure 1.3). According to Foster and Hall (1991), adapted cells were able to maintain their intracellular pH_i by 0.5-0.9 units higher compared to those of unadapted. In addition, lysine/cadaverine decarboxylase system was found to have a growth improvement effect of *Salmonella* spp. and *E. coli* at low pH values (Viala et al., 2011; Vivijs et al., 2016). The contribution of both systems depends on the availability of the associated amino acids (Álvarez-Ordóñez et al., 2010b; de Jonge et al., 2003; Kieboom and Abee, 2006; J. Liu et al., 2017; Viala et al., 2011), the pH

optima of the decarboxylases and the oxygen availability (Kieboom and Abee, 2006; Viala et al., 2011; Vivijs et al., 2016).

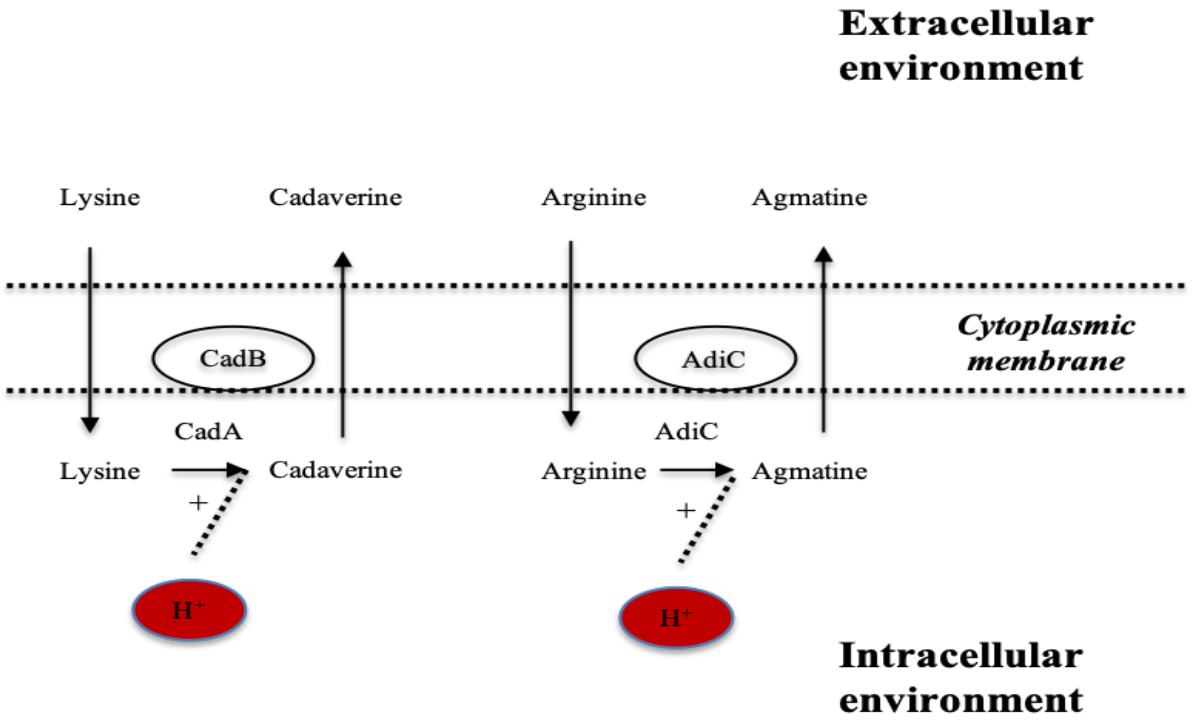

Figure 1.3. Inducible lysine/cadaverine and arginine/agmatine decarboxylase systems in *Salmonella* spp.. Based on Álvarez-Ordóñez et al. (2012)

Among the three systems, ornithine/putrescine decarboxylase system is the least one studied. This system converts ornithine to putrescine *via* the SpeF ornithine decarboxylase. Studies have reported that this system can improve growth of *S.* Typhimurium at moderately acidic pH under acidic conditions, but will modestly contribute to the survival at extreme pH (2.3) (Viala et al., 2011).

Given that the cognate amino acids are available, AR mechanisms will protect cells exposed to pH 2.5 or 2.3 (Álvarez-Ordóñez et al., 2010b; Kieboom and Abee, 2006; Spector and Kenyon, 2012; Viala et al., 2011). Nonetheless, these mechanisms aim to neutralize pH rather than tolerate acid stress (Park et al., 1996). Therefore, their function alone does not effectively protect cells against acid stress. Instead, the induction of the ASP is absolutely required for the acquisition of tolerance against stress. In fact, these two mechanisms will collaborate for the maximum acid resistance (Bearson et al., 1997; Park et al., 1996). Induction of emergency pH-homeostasis systems will allow ASP synthesis in lower extracellular pH values (pH_o 3.3) by keeping

the intracellular pH in a value more suitable for protein synthesis (Foster, 1991; Park et al., 1996).

1.5.2 Synthesis of Acid Shock Proteins

Pre-exposure to acid conditions may induce the productions of Acid Shock Proteins (ASP) as part of the Acid Tolerance Response (ATR) mechanism. As Acid Tolerance Response can be defined the induced acid resistance to lethal low pH following pre-exposure to mild acid conditions or growth at moderately low pH (Álvarez-Ordóñez et al., 2012). These ASPs aim to counteract the lethal effects of extreme acid stress by preventing or repairing macromolecule damage (Audia et al., 2001; Foster, 1993) rather than maintain internal pH. Adaptation to inorganic acid can protect cells against exposure to weak organic acids and *vice versa*, adaptation to weak organic acids can provide protection against subsequent inorganic stress (Álvarez-Ordóñez et al., 2009b; Arvizu-Medrano and Escartín, 2005; Greenacre et al., 2003; Kwon et al., 2000; Kwon and Ricke, 1998). ATR will protect cells against exposure to pH 3.0, but have no effect below pH 3.0 (Foster, 2001).

Salmonella spp. possess two distinct ATR systems, based on the physiological status of the cell at the time of acid exposure, logarithmic and stationary phase ATR. Each system requires different sets of ASPs which are controlled by distinct regulators (Foster, 2001).

1.5.2.1 Log-phase ATR

Several studies have demonstrated that pre-exposure of logarithmically growing cells to sublethal acid conditions enhanced their survival when exposed to acid stress (Foster and Hall, 1991; Greenacre et al., 2003; Kwon et al., 2000; Kwon and Ricke, 1998). Additionally, 60 different ASPs were recorded following log-phase adaptation (Foster, 2001).

Acid-inducible log-phase ATR is mediated by ferric uptake regulator (Fur), two-component regulatory system PhoPQ and alternative RNA polymerase σ factor (σ^s) (Álvarez-Ordóñez et al., 2012; Audia et al., 2001; Bearson et al., 1997; Foster, 2001). The iron-regulatory protein Fur is linked to the iron sensing mechanism and also governs the production of several ASP during log-phase ATR in an iron-independent manner (Hall and Foster, 1996); PhoPQ is induced under conditions of low Mg^{2+} or mild to moderate acid conditions (Bearson et al., 1998), whereas RpoS is a master

regulator linked to the general stress responses, triggered by various stress signals such as low pH, heat and high osmolarity (Hengge-Aronis, 2002).

1.5.2.2. Stationary phase ATR

Exposure of stationary phase cells to acid conditions results in acid induced stationary phase ATR. Its regulation is controlled by the master regulator OmpR. OmpR is a DNA-binding protein, part of the two-component regulatory system OmpR/EnvZ, where EnvZ consists a membrane-bound sensor kinase and OmpR the response regulator. OmpR autoinduces *ompR/envZ* operon in response to low pH. EnvZ is activated by autophosphorylation when sensing an environmental signal, such as high osmolarity and pH. It then transfers the phosphate to aspartate 55 of OmpR. Envz also possesses a phosphatase that removes phosphate form OmpR-P. Phosphorylation of OmpR can be alternatively performed through other phosphodonors, such as acetyl phosphate. OmpR-P is the active form that induces acid tolerance (Bang et al., 2002, 2000). OmpR/EnvZ system is best known for regulating the expression of two membrane porins, ompC and ompF in response to osmotic and pH stress (Chakraborty and Kenney, 2018). Nonetheless, a number of various genes with very different functions is also controlled by ompR (Bang et al., 2000).

OmpR possess also a key role in the virulence of *Salmonella* spp. by controlling the acidification of the cytoplasm, a process absolutely required for a successful invasion. Upon entry to macrophages in the form of SCV, *Salmonella* cytoplasm acidifies in response to the acidified macrophage vacuole. This acidification is dependent on the OmpR, which represses the *cadC/BA* operon. The latter encodes the lysine/cadaverine mechanism aiming to neutralize pH. Acidification is required for the secretion of virulence factors (Chakraborty et al., 2015).

Apart from the two above acid induced resistance systems, *Salmonella* spp. possess a pH-independent acid resistance mechanism, part of the general stress resistance expressed with the entry of cells into stationary phase of growth. This system does not require previous acid pre-exposure of cells and is regulated by the global stress regulator RpoS (Lee et al., 1994).

1.5.3 Modifications of membrane composition

Cytoplasmic membranes are composed of structured lipids, mainly phospholipids and integrated proteins (Beney and Gervais, 2001; Strahl and Errington,

2017). Cytoplasmic membranes have been charged with two main functions: they form a protective but permeable barrier outlining the cell cytoplasm and they regulate the passage of solutes between intracellular and extracellular environment (Yuk and Marshall, 2005). Membrane fluidity is crucial for cells, affecting membrane functions such as biochemical reactions, transport systems, and protein secretion (Yuk and Marshall, 2004).

Bacteria can alter their lipid composition in response to environmental stresses in order to maintain a degree of membrane fluidity compatible with life (Álvarez-Ordóñez et al., 2012). Several studies have reported that acid adaptation caused a decrease of the unsaturated fatty acids (UFA) to saturated fatty acids (SFA) (Álvarez-Ordóñez et al., 2009c; Yang et al., 2014a; Yuk and Marshall, 2005, 2004) and an increase in the cyclic fatty acids (Álvarez-Ordóñez et al., 2009c; Yang et al., 2014a). These changes in the membrane fatty acid composition have been linked to decreased membrane fluidity, that generally is more resistant against lethal acid stress (Álvarez-Ordóñez et al., 2012). Decreased membrane fluidity under acidic conditions may be associated with the ability of adapted cells to limit proton flux into the cells compared to non-adapted cells (Yuk and Marshall, 2004).

1.6 *Salmonella* and food safety

The ability of *Salmonella* to induce acid resistance mechanisms under acidic conditions has received wide attention over the last decades. This can be attributed to the perceived ability of the acid tolerant strains to endure lethal acid stress alongside with their higher pathogenicity potential. Starting with the pioneer studies of Foster and Hall (Foster, 1991; Foster and Hall, 1991, 1990) with *Salmonella* Typhimurium in the early 1990's, several studies have further established the ability of sublethal acid conditions to promote the survival of this pathogen. Although HCl was initially used in the acid adaptation protocols, organic acids were quickly introduced as acidulant agents, since they are widely encountered in food products. Acetic, citric and lactic are the most common organic acids employed in the adaptation treatments. Among them, acetic acid naturally found in vinegar, is the predominant acid utilized in several foods, such as mayonnaise and salad dressings (Kwon and Ricke, 1998). The ability of weak organic acids to increase acid resistance in *Salmonella* spp. has been well documented under different experimental conditions (Álvarez-Ordóñez et al., 2009b, 2010a; Arvizu-Medrano and Escartín, 2005; Greenacre et al., 2003). These acids can induce acid

resistance in *Salmonella* and *E. coli* even when present in neutral pH (Guilfoyle and Hirshfield, 1996; Kwon and Ricke, 1998). Acetate has been found to induce RpoS, one of the regulators that controls induction of acid resistance in log phase cells (Arnold et al., 2001; Schellhorn and Stones, 1992). Lee et al. (1995) suggested that acetate induces RpoS indirectly, by lowering the intracellular pH_i. Nonetheless, little is known regarding the effect of undissociated acid -the drastic form of organic acid which can enter the cell and dissociate intracellularly, thus resulting in reduction of pH_i- to induce acid resistance mechanisms.

Under real-world conditions, induction of acid resistance mechanisms can be performed either from adaptation to acids intrinsically encountered in foods or from some food industry interventions (Begley and Hill, 2015), e.g., in environments with sublethal acid levels due to dilution of acid concentration with water, etc.. From that point of view, ready-to-eat acid foods, such as mayonnaise or deli-type salads, can be contaminated with stress tolerant cells or even promote acid resistance, e.g. with the addition of contaminated particles of animal or vegetable origin that significantly raise the local pH (i.e., pH near the added particles), thus, resulting in different acid micro-environments that can induce stress hardening of pathogenic cells. This is something that should not be underestimated, especially when taking into consideration the marked increase in the consumption of the so-called delicatessen salads, containing mayonnaise or vinaigrette type base, both in Europe and USA the last two decades (Tassou et al., 2009).

Salmonella spp. can, also, be frequently found in food products of animal origin, with *S.* Typhimurium being one of the most common serovars isolated from pig meat (EFSA and ECDC, 2018). In this sense, meat can also pose a significant risk for public health, especially when taking into consideration the dramatical increase the last decades of resistant or multi-drug resistance pathogens, hard to control with conventional antimicrobials (Frieri et al., 2017). The rising consumer awareness regarding the negative effects of chemical preservatives on human health versus the perceived health benefits of natural antimicrobials (Pisochi et al., 2017) have increased their scientific and commercial attention as label-free alternatives to the synthetic antimicrobials used so far by the food industry. Therefore, identification and utilization of natural antimicrobials and their characterization with respect to the activity and

efficacy towards foodborne pathogens are key objectives for food safety research (Pisochi et al., 2017)..

1.7 Natural antimicrobials

Naturally occurring antimicrobial compounds are abundant in the environment. Based on their source of productions, they are classified as plant, animal or microbial origin antimicrobial agents. Plant derived antimicrobials include essential oils and other plant extracts; animal sources antimicrobials include enzymes (e.g. lysozyme, lactoperoxidase), proteins (e.g. lactoferrin, ovotransferrin, mild-derived peptides) and polysaccharides (chitosan); microbial origin antimicrobials compounds are produced during bacterial metabolism, including fermentation end products (e.g. organic acid, hydrogen peroxide and diacetyl), bacteriocins and other antagonistic compounds such as reuterin (Aloui and Khwaldia, 2016; Davidson et al., 2013; Gyawali and Ibrahim, 2014; Nazir et al., 2017). Other microbial sources include antagonistic organisms, mainly Lactic Acid Bacteria as well as yeasts (Aloui and Khwaldia, 2016) and bacteriophages (Davidson et al., 2013). Finally, algae and mushrooms are also recently used as natural sources of bioactive compounds (Gyawali and Ibrahim, 2014).

In recent years, plant derived metabolites have gained increasing interest since they are easily decomposed, they are environmental friendly and non phytotoxic, whereas many of them have a GRAS status (Cabral et al., 2013). Their use in foods addresses consumer demands for minimally processed natural products and at the same time they provide extra benefits to both food and consumers (Everis, 2001). Essential oils and other plant extracts are the compounds principally responsible for antimicrobial activities in plants, herbs and spices (Tajkarimi et al., 2010). Regarding herbs and spices, they are utilized since ancient times as flavoring agents, food preservatives and also in the concept of traditional (folk) medicine (Gyawali and Ibrahim, 2014; Shan et al., 2007).

The choice of the extraction procedure directly depends on the type of compounds aimed to extract (Radulovic et al., 2013). Essential oils are collected using hydrodistillation or steam distillation (Radulovic et al., 2013). Organic solvents or mixtures with water are the most common techniques applied to extract phenolic compounds from herb and spices. Common solvents used are water, acetone, ethyl acetate, alcohols (methanol, ethanol, propanol) and their mixtures. Ethyl acetate and acetone are used to extract the non-polar compounds such as flavonoids aglycones

(quercetin, luteolin, apigenin, eriodictyol) and terpenoids (carnosol, thymol, carvacrol), whereas alcohols, water and hydro-alcoholic mixtures are good solvents for phenolic mixtures (caffeic, rosmarinic, salvianoilc) and flavonoid glycosides (rutin, apigenin glycoside) (Ganiari et al., 2017).

Essential oils are one of the most studied plant derived antimicrobial compounds (Tornuk et al., 2011). Nonetheless, their utilization in food industry is restricted due to several limitations they present. These substances are active in higher concentrations in foods than *in vitro*, possibly due to their interactions with several major food components, such as lipids and proteins (Aloui and Khwaldia, 2016; Burt, 2004) and the lower water activity of foods compared to the laboratory media (Burt, 2004). The high concentration of essential oils required for antimicrobial activity may also limit the consumer acceptability beyond the threshold of acceptable (Nazer et al., 2005). In addition, high levels of essential oils may affect human health by inducing several disorders, such as intoxications, teratogenic effects, etc.(Aloui and Khwaldia, 2016). Finally, the essential oils have higher cost due to their lower extractability and yield (Aloui and Khwaldia, 2016; Bouarab-Chibane et al., 2019) and low water solubility (Bouarab-Chibane et al., 2019).

The antimicrobial activity of plant extracts other than essential oils has been generally ascribed to their phenolic composition (Chun et al., 2005; Poimenidou et al., 2016; Shan et al., 2007; Taguri et al., 2006). Phenolics are a diverse group of secondary plant metabolites (Gyawali and Ibrahim, 2014), with approximately 8000 structures currently known (Goleniowski et al., 2013). They include simple phenols, phenolic acid, flavonoids, silbeness, hydrolysable and condensed tannins, lignans and lignins (Figure 1.4) (Goleniowski et al., 2013). Apart from their antimicrobial activity, they also possess a variety of other physicochemical properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Balasundram et al., 2006). Due to their high structural diversity and variations in chemical composition, phenolic compounds differ in their effectiveness against pathogens (Nazir et al., 2017).

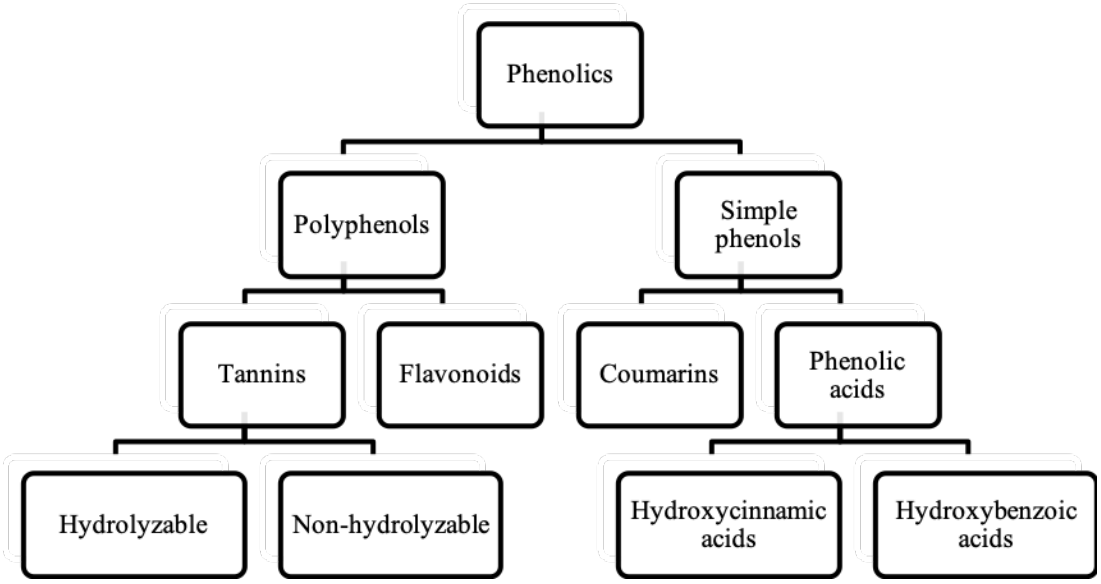

Figure 1.4**.** Schematic representation of phenolic compounds diversity. Based on Goleniowski et al. (2013)

The antimicrobial activity of phenolic compounds is largely related to their -OH groups. These groups interact with the cell membrane by hydrogen bonding (Bouarab-Chibane et al., 2019). The position (Gyawali and Ibrahim, 2014) and the number (Lai and Roy, 2004) of -OH groups has been reported to influence phenolics antibacterial activity. The exact mode of action of phenolic compounds has not been fully deciphered, although more than one mechanisms may be involved at cellular level (Bouarab-Chibane et al., 2019). In general, the mechanisms of plant secondary metabolites in which phenolic compounds belong include disruption of the cell membrane function and structure, inhibition of the DNA/RNA synthesis and function, involvement with intermediate metabolism, induction of coagulation of cytoplasmic constituents and interruption of cell signal communication (Radulovic et al., 2013). The order of events in the antibacterial action usually includes interaction of the antimicrobial compounds with the cytoplasmic membrane, diffusion of the compounds through the membrane and penetration into the intracellular environment and, finally, interaction with intracellular constituents or processes (Radulovic et al., 2013).

The antimicrobial effectiveness of plant extracts depends on several factors. The type of the target cells (e.g Gram staining, with $Gram^+$ bacteria being more susceptible compared to $Gram^-$ due to their differences in cell wall structures) (Kannat et al., 2010; Kim et al., 2013), storage temperature (Hayrapetyan et al., 2012; Iturriaga et al., 2012; Kim et al., 2001; Stojković et al., 2013) and the type (Fernández-López et al., 2005;

Gonelimali et al., 2018; Iturriaga et al., 2012; Moreno et al., 2006) and concentration (Gonelimali et al., 2018) of extracts can collectively affect the antimicrobial potential of plant extracts. In addition, the type of the food matrix can dramaticallly impact the extend of bacterial inhibition, with complex foods hindering the antimicrobial activity of phenolics due to their interactions with major food components such as lipids and proteins (Del Campo et al., 2000; Kim et al., 2004; Miceli et al., 2014; Uhart et al., 2006).

Most of their applications of phenolic-rich plant extracts other than essential oils in food safety are related to their antioxidant activity (Bouarab et al., 2018). Nonetheless, several studies have demonstrated a correlation between antioxidant activity and total phenolic content with antimicrobial activity (Chun et al., 2005; Shan et al., 2007). By products of plant food processing such as peels, seeds, husks and kernels, typically considered as waste may be used as a good source of phenolic compounds (polyphenols, tannins and flavonoids). Several phenolics have been identified in agricultural by-products, such as fruits (Fattouch et al., 2007; Hayrapetyan et al., 2012; Kannat et al., 2010), olive (Friedman et al., 2013) and grape (Sagdic et al., 2011) pomace (for more details see Balasundram et al. 2006; Gyawali and Ibrahim 2014)). In addition, by-products of purification essential oil procedure may also contain phenolics (Mandalari et al., 2007; Poimenidou et al., 2016). Nonetheless, investigation of agricultural by-products as sources of antibacterial activity is very limited (Gyawali and Ibrahim, 2014). Given that the waste and the economic burden of agroindustrial by-products is substantial (Oreopoulou and Tzia, 2007), their commercial exploitation as major sources of phenolic compounds could be an economically attractive way to enhance food safety (Gyawali and Ibrahim, 2014).

The *in situ* antimicrobial effectiveness of phenolics has been examined in a lesser extent compared to the *in vitro* results. This can be mainly ascribed to the reduced effectiveness of the extracts in real foods (Bouarab-Chibane et al., 2019; Negi, 2012). The complex and multicomponent environment of food systems cannot ensure the extrapolation of the *in vitro* results to food products (Negi, 2012). In fact, it has been demonstrated that the effectiveness of phenolics is diminished when incorporated into foods (Davidson et al., 2013; Negi, 2012; Shelef, 1983; Uhart et al., 2006) and higher concentrations are required for the antimicrobial activity to be manifested (Miceli et al., 2014; Owen and Palombo, 2007; Piskernik et al., 2011). Therefore, the *in situ* evaluation of the antimicrobial profile is crucial for the determination of the

effectiveness of phenolic compounds when applied in food matrices. Combinations of natural antimicrobials with another or with other processing technologies in a multi-hurdle preservation system may improve their efficacy (Careaga et al., 2003; Lin et al., 2005; Negi, 2012). Lin et al. (2005) reported that a certain combination of oregano, cranberry and lactic acid extract had better results compared to the effect of the single component itself. Similarly, Careaga et al. (2003) reported that a combination of a pepper extract and 1 % sodium chloride had better bactericidal results against *Pseudomonas aeruginosa*, but not *S.* Typhimurium.

Incorporation of phenolic compounds into the food bulk can be performed either with direct contact or through films and coatings (Gywali and Imbrahim, 2014).

1.6.1 Direct application

Antimicrobial compounds can be directly applied in food matrices in the form of power (Kim and Fung, 2004; Stojković et al., 2013; Uhart et al., 2006) or liquid (Careaga et al., 2003; Hayrapetyan et al., 2012).

Marination is a food preparation process that -in addition to improving flavor, texture and juiciness of meat (Yusop et al., 2012)- may also contribute to its microbiological profile either by extending their shelf life (Alakomi et al., 2017; Lytou et al., 2017) or by improving their safety against foodborne pathogens, such as *Salmonella* spp. (Lytou et al., 2019). Marinades can be supplemented with several sources of natural antimicrobials, such as herbal and berry extracts (Alakomi et al., 2017) and pomegranate juice (Lytou et al., 2018, 2017).

Nonetheless, the direct application of natural antimicrobials has several limitations. The rapid diffusion of these substances into the food bulk alongside with possible interactions of the bioactive compounds with major food substances, such as fat and proteins, may reduce their efficacy during storage and thus limit their application in the food industry (Aloui and Khwaldia, 2016).

1.6.2 Edible films and coatings

The last years, a promising trend involves incorporation of natural extracts (essential oils, plant extracts and their constituents) into edible films and coatings (Iturriaga et al., 2012).

Edible coatings are defined as a thin layer of edible material formed on a food product, whereas edible films as a preformed thin layer made of edible materials that

once formed can be placed on or between food components (McHugh, 2000). They consist an eco-friendly technology that permits the reduction of synthetic packaging material waste (Silva-Weiss et al., 2013) and fulfills the consumer expectations for more natural fresh-like products and environmental protection (Campos et al., 2011). Albeit they cannot replace traditional non-edible packaging still needed for hygienic reasons (Parreidt et al., 2018), they consist an additional stress factor that contribute to the preservation of food product (Campos et al., 2011).

Biopolymers used for the production of edible films and coatings include hydrocolloids (proteins and polysaccharides) and lipids. Proteins used include collagen, gelatin, caseins, whey protein, corn zein, wheat gluten, soy protein, egg white protein, myofibrillar protein, quinoa protein and keratin; polysaccharides include starch, cellulose and its derivatives, pectin, chitosan, alginate, carrageenan, pullulan and gellan gum. Lipids, on the other hand, include waxes, fatty waxes and paraffins, fatty acids and alcohols, acetylated glyceride, shellac resins, cocoa-based compounds and their derivatives. Mixtures of lipids with hydrocolloids, called composites can also be used. Plasticizers, such as glycerol, sorbitol, monoglycerides, polyethylene glycol, glucose are often added to increase flexibility and elasticity of the materials (Galus and Kadzinska, 2015; Parreidt et al., 2018; Pavli et al., 2018). Dependent on its composition, the forming matrices have different functionalities, since each biomaterial offers different properties (Diab et al., 2001). For instance, films and coatings made of polysaccharides are considered good oxygen, odor and oil barriers, but have high moisture permeability, due to their hydrophilic character (Pavli et al., 2018). Protein-forming films and coatings have also poor water resistance, but they exhibit better mechanical and barrier properties than polysaccharides (Pavli et al., 2018). Finally, lipid-based films and coatings have good water vapor barrier properties, but exhibit inflexibility, poor resistance to mechanical stress, sensitivity to rancidity (oxidation) and decreased organoleptical quality (Diab et al., 2001; Pavli et al., 2018).

Edible films and coating can improve storage, mainly by acting as water barriers, preventing dehydration and oxygen, and light barriers, retarding lipid oxidation (Gómez-Estaca et al., 2009a). In addition, these materials can enclose bioactive substances that confer antimicrobial/antioxidant properties, and thus, extending shelf life and improving safety (Choulitoudi et al., 2016; Gómez-Estaca et al., 2007; Iturriaga et al., 2012; Kraśniewska et al., 2015; Ponce et al., 2008). In this case, films and coatings are defined as active packaging. In active packaging, bioactive

compounds are gradually released by diffusion onto food surfaces, maintaining their concentrations in higher levels over longer periods of storage (Gyawali and Ibrahim, 2014). Nonetheless, interactions of the forming biopolymers with the bioactive compounds may alter the mechanical and barrier properties of the edible matrix (Gómez-Estaca et al., 2009b; Gómez-Guillén et al., 2007) and affect the release of active components (Gómez-Estaca et al., 2009a), depending on the type of the forming material and the type of the extract used.

1.7 Strain variability in stress responses

Inherent differences among strains of the same species identically treated under the conditions specified in the study is known as strain variability and constitutes a major source of variation in microbiological studies (Whiting and Golden, 2002). This means that the results coming from one microbial strain cannot be extrapolated to other strains of the same species (Lianou and Koutsoumanis, 2013a). In fact, different strains of a foodborne pathogen can vary in their tolerance to an applied stress (Sherry et al., 2004). Variations among strains with regard to their resistance to food preservation methodologies or to environmental stresses commonly encountered in food chain have been reported (Álvarez-Ordóñez et al., 2013). For instance, heterogeneity to their resistance phenotypes have been reported for *Salmonella* spp. strains under conditions of heat (Lianou and Koutsoumanis, 2013b; Sherry et al., 2004) or acid stress (Humphrey et al., 1995; Jørgensen et al., 2000; Lianou and Koutsoumanis, 2013b; Samelis et al., 2003). Strain-dependent effects has also been reported for a number of microorganisms under several environmental conditions, such as biofilm formation, virulence, acid and heat inactivation, growth capability (reviewed by Lianou and Koutsoumanis (2013b)), irradiaton (Sherry et al., 2004) and high-hydrostatic pressure (Álvarez-Ordóñez et al., 2013; Sherry et al., 2004). Adaptation to sublethal acid conditions may also promote phenotypic differences in the acid tolerance among *Salmonella* spp. strains (Berk et al., 2005; de Jonge et al., 2003; Lianou et al., 2017; Malheiros et al., 2009). Berry and Cutter (2000) classified three *E. coli* strains in acid resistance, acid adaptable and acid sensitive, based on the ability of non-adapted and adapted cells to endure extreme acid stress.

The intra-species variability among strains of foodborne pathogens is extensive (Lianou and Koutsoumanis, 2013a) and may highly impact the accuracy of microbial risk assessment (Delignette-Muller and Rosso, 2000).Therefore, it should be

systematically taken under consideration (Delignette-Muller and Rosso, 2000). Strain selection, thus, consists a crucial decision when designing and conducting challenge studies that aim to assess the behavior of foodborne pathogens in food products or simulating food- related environments (Lianou and Koutsoumanis, 2013a). According to NACMCF (2005), the selection of the strains should be based on the history and origin of the strains and their behavior in foods, whereas using at least 3-5 strains (individually or in combination) is recommended.

The diversity within stains has been generally linked to the presence or absence of specific genetic loci for several microorganisms (Begley and Hill, 2015). Acid resistance of *Salmonella* spp. strains has been generally correlated to σ^s levels. According to Jørgensen et al. (2000), differences in the acid resistance among different *Salmonella* spp. could be attributed to mutations of *rpos* gene or reduced levels of RpoS-dependent genes, with the latter being possibly attributed to mutations affecting the translational processing of the RpoS protein or, alternatively, to protein instability. Berk et al. (2005), on the other hand, reported that no mutations were detected in *rpos* gene of *S.* Typhimurium DT104 strains showing differences in their acid resistance phenotypic profile.

1.8 Objectives of the PhD thesis

As the food industry is ever-evolving to address new needs and demands, food safety remains an increasingly international concern due to its great impact on public health. In spite of the considerable amount of work delivered so far, the resistance of major foodborne pathogens, such as *Salmonella* spp., in foods or food-related systems continuous to attract scientific attention. Therefore, reports pertaining the behavior of this pathogen under various stressful environments are always considered as valuable information in the field of food safety**.**

The ability of acid stress tolerant cells to endure lethal acid environments can render the safety of food products questionable. The presence of organic acids in acid food matrices can increase the risk of contamination of these products with stress tolerant cells, due to, for instance, failures in sanitation treatments (e.g. dilution of organic acids in water). Nevertheless, food industries must always ensure the safety of their final products. Natural antimicrobials could provide an effective and up-to-date solution towards this direction. Essentials oils have been widely investigated as antimicrobial agents; however, they are usually active in high concentrations that

exceeds the sensory threshold of acceptance from consumers. Therefore, seeking for natural antimicrobials other than essential oils that can improve food safety without compromising the sensory properties of the food products is of utmost importance. In Greece, there is a huge production of essential oils from aromatic plants. The water that remains from this production is rich is phenolic compounds that have antimicrobial and antioxidant activity.

In light of the aforementioned, this PhD thesis aimed to investigate the ability of *Salmonella* spp. strains to survive in different food matrices and under different stresses, taking into consideration inter-strain variations. With regard to the fact that stress tolerant cells represent the worst-case scenario, **chapters 2** & **3** aimed primarily to fill the gaps in the existence literature regarding the effect of acid adaptation on the survival on this pathogen both *in vitro* and in acid food matrices. **Chapter 4** aimed to assess the *in vitro* and *in situ* antimicrobial activity of natural plant extracts in pork meat, a food product of animal origin commonly associated with *Salmonella* spp. contamination.

The main objectives of this thesis per chapter were:

1) (i) To examine the *in vitro* contribution of undissociated acetic acid over a range of different pH values in inducing acid resistance phenotypes of *Salmonella enterica* spp. *enterica* serovar Enteritidis Phage Type 4 and (ii) to evaluate the transferability of the *in vitro* results to *in situ* conditions, using a traditional Greek ready-to-eat acid appetizer stored under refrigeration, with regard to intrinsic and extrinsic food related factors (**chapter 2**)
2) (i) to assess the *in situ* adaptive responses of six *Salmonella* spp. strains in mayonnaise stored under refrigeration following adaptation to three acid conditions tested in chapter 2 and (ii) to further elucidate the individual effect of pH and undissociated acetic acid adaptation in the phenotypic and transcriptomic profiles of two of the above selected strains having the most notable adaptive potential (**chapter 3**)
3) (i) to screen the antimicrobial effect of ten laboratory (hydro-distilled) and one industrial (steam-distilled hydrolate) plant aqueous extracts acquired as by-products of essential oil production against three strains of *Salmonella* spp. and (ii) to evaluate the *in situ* antimicrobial activity of hydro-distilled oregano extract and industrial oregano hydrolate on improving the safety of pork meat against *Salmonella* strains (**chapter 4**).

CHAPTER 2

Sublethal concentrations of undissociated acetic acid may not always stimulate acid resistance in *Salmonella enterica* sub. *enterica* serovar Enteritidis Phage Type 4: implications of challenge substrate associated factors

Alkmini Gavriil, Athina Thanasoulia, Panagiotis N. Skandamis

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Abstract

Acid adaptation enhances survival of foodborne pathogens under lethal acid conditions that prevail in several food-related ecosystems. In the present study, the role of undissociated acetic acid in inducing acid resistance of *Salmonella* Enteritidis Phage Type 4 both in laboratory media and in an acid food matrix was investigated. Several combinations of acetic acid (0, 15, 25, 35 and 45 mM) and pH values (4.0, 4.5, 5.0, 5.5, 6.0) were screened for their ability to activate acid resistance mechanisms of pathogen exposed to pH 2.5 (screening assay). Increased survival was observed when increasing undissociated acetic acid within a range of sublethal concentrations (1.9-5.4 mM), but only at pH 5.5 and 6.0. No effect was observed at lower pH values, regardless of the undissociated acetic acid levels. Three combinations (15mM/pH5.0, 35mM/pH5.5, 45mM/pH6.0) were selected and further used for adaptation prior to inoculation in commercial tarama (fish roe) salad, i.e., an acid spread (pH 4.35 ± 0.02), stored at 5^{o}C. Surprisingly and contrary to the results of the screening assay, none of the acid adaptation treatments enhanced survival of *Salmonella* Enteritidis in the food matrix, as compared to non-adapted cells (control). Further examination of the food pH value, acidulant and storage (challenge) temperature on the responses of the pathogen adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0 was performed in culture media. Cells adapted to 35mM/pH5.5 were unable to induce acid resistance when exposed to pH 4.35 (tarama salad pH value) at 37^{o}C and 5^{o}C, whereas incubation under refrigeration (5^{o}C) at pH 4.35 sensitized 45mM/pH6.0 adapted cells against the subsequent acid and cold stress. In conclusion, pre-exposure to undissociated acetic acid affected the adaptive responses of *Salmonella* Enteritidis Phage Type 4 in a concentration- and pH-dependent manner, with regard to conditions prevailing during acid challenge.

Introduction

It is now well documented that pre-exposure of microorganisms to sublethal stress conditions may induce adaptive responses that enhance resistance to subsequent lethal factors (Wesche et al., 2009) of the same or multiple stresses (cross protection; (Chen, 2017)). Exposure to acidic environments is frequently encountered by microorganisms during their route from the food chain to the human host. Among the foodborne pathogens, *Salmonella* spp., is able to induce resistance mechanisms as a result of adaptation to mild or moderate acid stress (Alvarez-Ordóñez et al., 2015; Álvarez-Ordóñez et al., 2012). The acid induced phenotypic responses are highly affected by factors such as the selected strain (Berk et al., 2005; Lianou et al., 2017) and conditions prevailing during adaptive and subsequent lethal challenge treatments, e.g., acidulant, temperature and composition (Álvarez-Ordóñez et al., 2012). So far, different adaptation protocols are used in order to formulate suitable sublethal acid conditions. For instance, acid adaptation can be achieved by supplementation of growth media with glucose (Kim et al., 2015; Lianou et al., 2017) or by long- or short-term pre-exposure of the cells to various organic or inorganic acidulants (Álvarez-Ordóñez et al., 2009b, 2010a; Koutsoumanis and Sofos, 2004; Kwon et al., 2000; Usaga et al., 2014; Yang et al., 2014a). Nevertheless, it has been demonstrated that different protocols used to stimulate acid resistance can diversify the survival of *Escherichia coli* in apple juice stored under refrigeration (Usaga et al., 2014).

Organic acids such as acetic can form acid stress conditions frequently encountered by *Salmonella* spp., as they are widely applied to the food industry. They are common preservatives in foods, such as mayonnaise and salad dressings, carcass decontamination treatment agents, whereas they can also accumulate in fermentable products as the result of indigenous or starter cultures activity. Finally, they are naturally present inside the gastrointestinal human or animal track due to the metabolic activity of endogenous microflora (Kwon et al., 2000; Lachica et al., 2018; Lianou et al., 2012; Ricke, 2003; Shen et al., 2019; Smittle, 2000). The antimicrobial activity of organic acids has been traditionally attributed to their undissociated molecules (Ricke, 2003). Despite the antibacterial efficiency of organic acids, their application might also pose considerable risk associated with potential induction of acid resistance. Thus, apart from adaptation to acids intrinsically encountered in foods, some food industry interventions may also promote induction of acid resistance (Begley and Hill, 2015), e.g., in environments with sublethal acid levels due to dilution of acid concentration

with water, etc.. Stimulation of acid resistance mechanisms may result in increased likelihood of disease. A positive correlation between acid resistance and pathogenicity (Berk et al., 2005) has been found, alongside with evidence that acid adaptation increases virulence (Abdelwaheb and Ahmed, 2009). In addition, several regulators involved in the induced acid resistance of *Salmonella* spp. also control the expression of genes required for virulence (Fang et al., 2016).

So far, numerous studies have dealt with the responses of foodborne pathogens following adaptation to organic acids under different experimental conditions. Nevertheless, investigation pertaining the role of undissociated acid to the induction of acid resistance is limited. In addition, considering the protective effect of some food matrices on the ability of bacterial cells to tolerate lethal stresses (Álvarez-Ordóñez et al., 2009b; Waterman and Small, 1998), it is important to compare the results from experiments in laboratory media to those from food related environments.

Given the above, this study was conducted in order to examine the contribution of undissociated acetic acid over a range of different pH values to the induction of acid resistance in *Salmonella* Enteritidis (*S.* Enteritidis) in laboratory media. The second part of this study aimed to evaluate whether the results from the broth media could be extrapolated in foods, particularly in an acid food matrix (tarama salad containing citric acid as acidulant), stored under refrigeration.

Materials and Methods

Bacteria strain and growth conditions

Salmonella enterica ssp. *enterica* serovar Enteritidis (*S.* Enteritidis) P167807 Phage Type 4 (PT4), a food (beef) isolate reported in Boziaris et al. (Boziaris et al., 1998) was provided by the Laboratory of Food Microbiology and Biotechnology, Agricultural University of Athens, Greece.

Cells were monthly subcultured in Tryptone Soy Agar (TSA, Lab M Limited, Lancashire, UK) from stock cultures (-20^{o}C) and maintained at 4^{o}C. Prior to each experimentation, one single colony was transferred to 10 ml of Tryptone Soy Broth without dextrose (TSBG(-), Lab M Limited, Lancashire, UK) and incubated at 37^{o}C for 24 h. Subsequently, 100 μl of the 24-h cultures were transferred to 10 ml of the same medium and incubated at 37^{o}C for another 18 h, in order to collect stationary phase cells.

Minimum Inhibitory Concentration (MIC) determination

Stationary phase cultures were washed twice with ¼ Ringer solution (Lab M Limited, Lancashire, UK) (2709 X g, 10min, 4ºC) and resuspended in the appropriate medium. For the preparation of the media, Tryptone Soy Broth (Lab M Limited, Lancashire, UK) was supplemented with several concentrations (10, 20, 30, 40, 50, 75, 100, 150 and 200 mM) of acetic acid (Panreac, Barcelona, Spain) and then the pH was adjusted to 5.0 using HCl 6 N (Merck, Darmstadt, Germany) or NaOH 10 N (Panreac, Barcelona, Spain). After autoclave, the pH value of each acid concentration was confirmed with a digital pH-meter (pH 526, Metrohm Ltd, Switzerland) and differences (maximum ± 0.2) -if evident- were taken into consideration in the final assay. Samples were inoculated with approximately 5.0 log CFU/ml and incubated at 37ºC for up to 10 days to assess the growth responses. Sampling was performed on day 0 and after 5 and 10 days of storage by plating 0.1 ml of the appropriate dilution on TSA plates. The experiment was conducted four independent times with duplicate samples per trial.

Preparation and inoculation of adaptation media and acid challenge assays

Adaptation media were prepared by combining different concentrations of total acetic acid and pH values. More specifically, appropriate volumes of acetic acid (1 M) were added to 100 ml of TSBG(-). The media were then autoclaved, adjusted to the desired pH values with HCl 6 N or NaOH 10 N, in order to create different concentrations of undissociated acetic acid (UAA) and filtered-sterilized (0.2 μm, LLG Labware, USA). In all assays, adaptation was performed for 90 minutes at 37ºC to a preheated water bath. Enumeration of the initial adapted populations was carried out at the end of adaptation period by transferring 100 μl in 900 μl of ¼ Ringer and plating the appropriate dilution on TSA plates. Non-adapted (NA) cells grown at neutral pH (7.00) without being subjected to any pH adjustment or acetic acid pre-exposure were also used as controls in all experiments.

Adaptation and exposure to TSB adjusted to pH 2.5 (screening assay)

For screening assay, four different concentrations of total acetic acid (15, 25, 35 and 45 mM) were combined with pH adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 (± 0.05), as described above. Cells adapted to pH in the absence of acetic acid (pH-adapted cells; 0mM acetic acid) were used as 'positive' controls by adjusting the pH of the broth

medium to the same values as those mentioned above using only HCl 6 N. For the preparation of adapted cultures, stationary phase cells were centrifuged (2709 X g, 10 min, room temperature) and resuspended to the appropriate adaptation medium at a final concentration of approximately 6.5-7.0 log CFU/ml. Following adaptation, cells were harvested by centrifugation (2709 X g, 5 min, 37ºC) and immediately resuspended to TSB adjusted to pH 2.5 with HCl 6 N ($TSB_{2.5}$) at a final concentration of ~ 5.0 CFU/ml. Non-adapted (control) inocula were resuspended to $TSB_{2.5}$ without prior adaptation. Acid challenge was performed at 37ºC for 30 minutes. Samplings were carried out at 0, 2.5, 5 and 7.5 minutes of acid exposure by plating 0.1 ml of the appropriate dilution on TSA (detection limit of 1.0 log CFU/ml) and at 10, 15 and 30 minutes of exposure, by plating 1 ml of the challenged broth into three TSA petri dishes (detection limit of 0 log CFU/ml). Experiments were conducted in triplicate with duplicate samples per independent trial.

Impact of adaptive responses in tarama salad or TSB adjusted to pH 4.35

Based on the results of the screening assay ($TSB_{2.5}$), three acetic acid/pH combinations i.e. 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0, were selected. These treatments were further tested for their ability to induce acid resistance of *S.* Enteritidis at commercial tarama salad stored at 5ºC and at TSB adjusted to pH 4.35 ($TSB_{4.35}$) with HCl 6 N or citric acid 6 M (AnalaR, Dublin, Ireland) and incubated at 37ºC or 5ºC. Exposure of *S.* Enteritidis to $TSB_{4.35}$ incubated at two temperatures was performed in order to isolate the impact of food matrix on pathogen survival at the same pH value, acidulant and challenge temperature.

Preparation of adapted cultures inoculated in broth medium ($TSB_{4.35}$) was performed as described above. Briefly, stationary phase cells were centrifuged (2709 X g, 10 min, room temperature) and resuspended to each of the above adaptation media (i.e 15mM/pH5.0, 35mM/pH5.5 or 45mM/pH6.0) at a final concentration of approximately 6.5-7.0 log CFU/ml. Following adaptation, cells were harvested by centrifugation (2709 X g, 5 min, 37ºC) and resuspended to $TSB_{4.35}$ adjusted either with HCl (to stimulate food matrix pH) or citric acid (to stimulate food matrix acidulant), at a final concentration of ~ 5.0 CFU/ml. Non-adapted (control) inocula were resuspended to $TSB_{4.35}$ without prior adaptation. Samples were stored at 5ºC for 60 days or at 37ºC for 96 hours (when citric acid was used for pH adjustment) or 7 days (when HCl was

used for pH adjustment). Samplings were performed at different time intervals, depending on storage temperature and the use of HCl or citric acid for lowering the pH. Enumeration was carried out by plating 0.1 ml of the appropriate dilution on TSA plates containing 0.1 % sodium pyruvate (Applichem, Darmstadt, Germany) (TSA/SP) (detection limit of 1.3 log CFU/ml). This medium was selected for enabling the maximum recovery of injured cells (McDonald et al., 1983).

Experiments were conducted four independent times with duplicate samples per replicate.

For the inactivation experiments in the acid food matrix, commercial tarama salad packages of a Greek food industry were purchased from a local supermarket and transferred to the laboratory within 20 minutes. Tarama salad is a traditional Greek fish roe appetizer (spread) stored under refrigeration. Acidification is performed using citric acid. Apart from fish roe and citric acid, other ingredients used for the preparation of the product according to the labelling were mashed potatoes, vegetative oil, salt, pigments, flavorings, condenser and chemical preservative (sodium benzoate, sorbic acid). This product was selected since it is a domestically widespread acid food with very low to undetectable initial microbial load. Prior to inoculation, levels of indigenous microbiota of commercial packages was determined by diluting 10 g of each uninoculated package to 90 ml of ¼ Ringer and plated on TSA/SP plates. The pH of commercial tarama salad was also measured using a digital pHmeter.

For the preparation of adapted cultures inoculated in tarama salad, stationary phase cells were centrifuged (2709 X g, 10 min, 4^{o}C), washed twice with ¼ Ringer and then resuspended to each of the above adaptation media (i.e. 15mM/pH5.0, 35mM/pH5.5 or 45mM/pH6.0) at a final concentration of approximately 8.5-9.0 log CFU/ml. Following adaptation, cells were harvested by centrifugation (2709 X g, 5 min, 37^{o}C) and resuspended to 4 ml of diluted tarama salad prepared by mixing10 g of tarama salad with 30 ml ¼ Ringer. This was performed in order to acclimatize inocula in a medium similar to the subsequent food substrate. The suspension was vortexed for 1 minute and aliquots (0.8 ml) were added to 80 g of commercial tarama salad pre-weighed in sterilized containers. Non-adapted inocula were resuspended to 4 ml of diluted tarama salad but without prior adaptation. The initial inoculated population was approximately 6.5-7.0 log CFU/g. Samples were stored at 5^{o}C for 37 days. Samplings were conducted by transferring 10 g of inoculated tarama salad in 90 ml ¼ Ringer solution and homogenized in a stomacher apparatus (Seward, London, UK). Then, 0.1

ml of the appropriate dilution was spread on TSA/SP plates (detection limit of 2 log CFU/g). The selective Xylose Decarboxylase medium (XLD) was not used since no enumerated microflora was detected in the food matrix. In addition, preliminary trials showed that the recovery of *Salmonella* survivors in this medium was significantly lower compared to the non-selective one (TSA/SP). In any case, for TSA/SP counts lower than 3-3.5 log(cfu/g), populations were streaked to XLD to confirm they are *Salmonella* colonies. pH changes during storage were determined using a digital pHmeter. Experiments were conducted four independent times with duplicate samples per replicate.

Determination of 4D inactivation parameters

The time needed for a 4 log reduction (4D) of the microbial population was calculated by fitting the log transformed inactivation data collected from the screening assay at $TSB_{2.5}$ to Weibull with tail (Albert) model according to the equation $\log10(N)=\log10[(N(0)-N_{res}) \times 10^{(-(t/\delta)\hat{}p)} + N_{res}]$ (Albert and Mafart, 2005), whereas the log transformed data of tarama salad inactivation curves were fitted in Weibull model according to the equation $\log10(N)=\log10(N(0))-(t/\delta)^p$ (Mafart et al., 2002), where N_o the population at time t_o, N the population at time t, N_{res} the residual bacterial concentration log CFU/g at the end of microbial inactivation, δ the time needed for the first decimal reduction and *p* a shape parameter corresponding to different concavities; downward concave survival curves for p>1, upward concave survival curves for p<1 or linear curves for p=1. GinaFit, a freeware Add-in for Microsoft ®Excel (Geeraerd et al., 2005) available at https://cit.kuleuven.be/biotec/software/GinaFit was used for data fitting. In total, six curves per experimental case were fitted.

Statistical analysis

Log transformed inactivation data and 4D inactivation estimates were used for statistical analysis. Analysis of variance (SPSS 22.0 for Mac) was performed among cell populations (log CFU/ml or g) for each time point during all stresses and among 4D values calculated from the screening assay ($TSB_{2.5}$) and 'tarama' salad inactivation. Means were compared using Tukey's Honestly Significant Difference (HSD) test and were considered significant at 95% level. Comparison between NA and pH-adapted cells was performed using *t-test* of Microsoft® Excel 16 for Mac.

Results

MIC determination

The MIC of UAA for *S.* Enteritidis ranged between 5.2 and 7.2 mM. At concentrations of UAA ≤ 5.2 mM growth was observed at the 5th day of storage, while concentrations ≥ 7.2 mM displayed a bactericidal effect.

Screening assay at $TSB_{2.5}$

Populations at the end of adaptation period (90 min) ranged from 6.1 to 6.9 CFU/ml. The effect of adaptation to pH (i.e., 0mM/pH 6.0, 5.5, 5.0, 4.5 and 4.0; no added acetic acid) on *S.* Enteritidis acid resistance was evaluated comparing bacterial populations of pH-adapted and NA inocula (Figure 2.1). Adaptation to pH 6.0 did not increase acid resistance, since no marked differences (P>0.05) were observed during 2.5-15 minutes of acid exposure. In contrast, significantly higher ($P < 0.05$) survivors were recovered at the lower pH values 4.0-5.5 compared to NA cells. Nonetheless, the magnitude of the observed differences was pH-dependent. Log differences following adaptation to pH 5.5 and 5.0, albeit statistically significant ($P < 0.05$), were rather low (0.7-1.4 log CFU/ml). Adaptation to pH 4.5 and 4.0, on the other hand, clearly enhanced resistance: 1.3-2.5 and 0.6-1.9 log CFU/ml higher survivors were enumerated following adaptation to pH 4.5 and 4.0, respectively, compared to control (NA) (Figure 2.1). The ratio of $4D_{pH}/4D_{NA}$ (Figure 2.2) was used to characterize the pH induced acid resistance, where $4D_{pH}$ and $4D_{NA}$ the 4D parameters of the pH adapted and NA inocula, respectively, calculated by Weibull with tail (Albert) model. The ratio was calculated by dividing $4D_{pH}$ of each replicate with an average value of $4D_{NA}$. A clear difference ($P < 0.05$) was observed between treatments adapted to pH 6.0 and 4.5, with higher values, i.e., suggesting longer survival, obtained at the lower pH (4.5). In summary, adaptation over a range of different pH values enhanced survival, with maximum acid resistance induced by lower pH values.

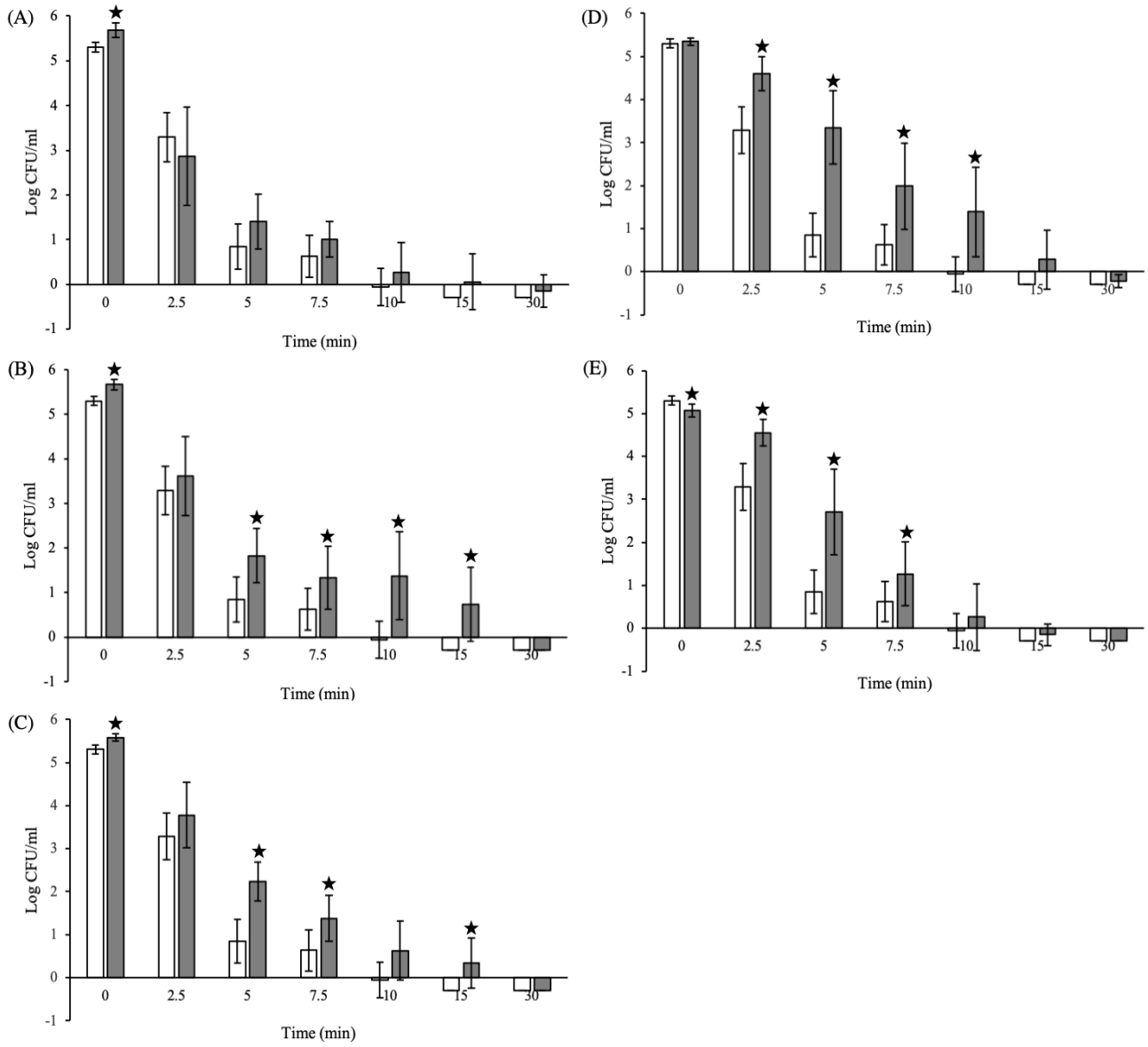

Figure 2.1. Inactivation of NA and pH-adapted cells of *S.* Enteritidis at pH 2.5 ($TSB_{2.5}$). Adaptation was performed to pH values (A) 6.0, (B) 5.5, (C) 5.0, (D) 4.5 and (E) 4.0. Reduction of the pH of the adaptation medium from 6.0 to 4.0 increased the enumerated survivors compared to control (NA), with maximum differences found for cells adapted to pH 4.0 and 4.5. White and grey bars represent NA and adapted populations, respectively. Each bar is an average of six replicates (± standard deviation). Stars indicate significant differences between two treatments for each time point according to *t-test*.

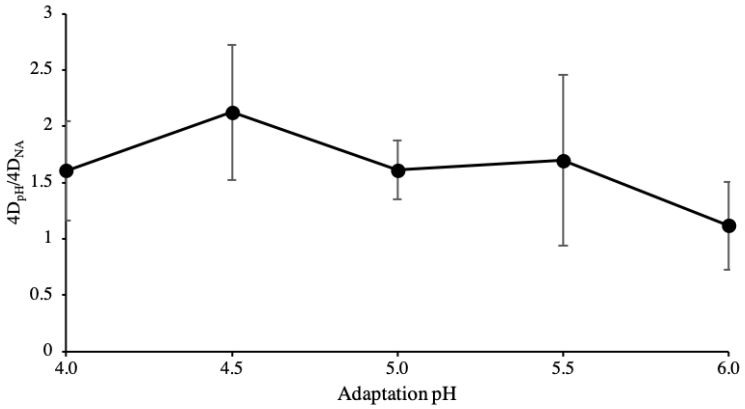

Figure 2.2. Induced acid resistance of *S.* Enteritidis following adaptation to different pH values. Each data point represents a mean ratio $4D_{pH}/4D_{NA}$ (± standard deviation), where $4D_{pH}$ and $4D_{NA}$ the 4D's for pH adapted and NA inocula, respectively, resulting from dividing $4D_{pH}$ of each replicate with an average value of $4D_{NA}$. Lower pH value 4.5 induced a higher ratio and thus an increased survival compared to higher pH value 6.0.

Adaptation to acetic acid enhanced acid resistance in a pH- and UAA concentration-dependent manner. Regarding cultures adapted to pH 6.0, a gradual increase in the ability of the pathogen to endure severe acid stress was observed with increasing concentrations of UAA. Adding 1.9 (35mM/pH6.0) and 2.4 (45mM/pH6.0) mM UAA acid resulted in up to 1.5–2.0 log units ($P < 0.05$) higher counts compared to cultures adapted to treatments with lower levels or without UAA (i.e., 0, 0.8 and 1.4 mM corresponding to 0mM/pH6.0, 15mM/pH6.0 and 25mM/pH6.0, respectively) (Figure 2.3A; Table 2.1). This trend was also confirmed by comparing the inactivation kinetics (Table 2.1). The addition of 1.9 (35mM/pH6.0) and 2.4 (45mM/pH6.0) mM UAA increased the time needed for a four-log reduction (4D) by more than two-fold, i.e. from 5.0 minutes required for control cells (0mM/pH6.0) to 10 ($P < 0.05$) and 11.6 min ($P < 0.05$), respectively (Table 2.1).

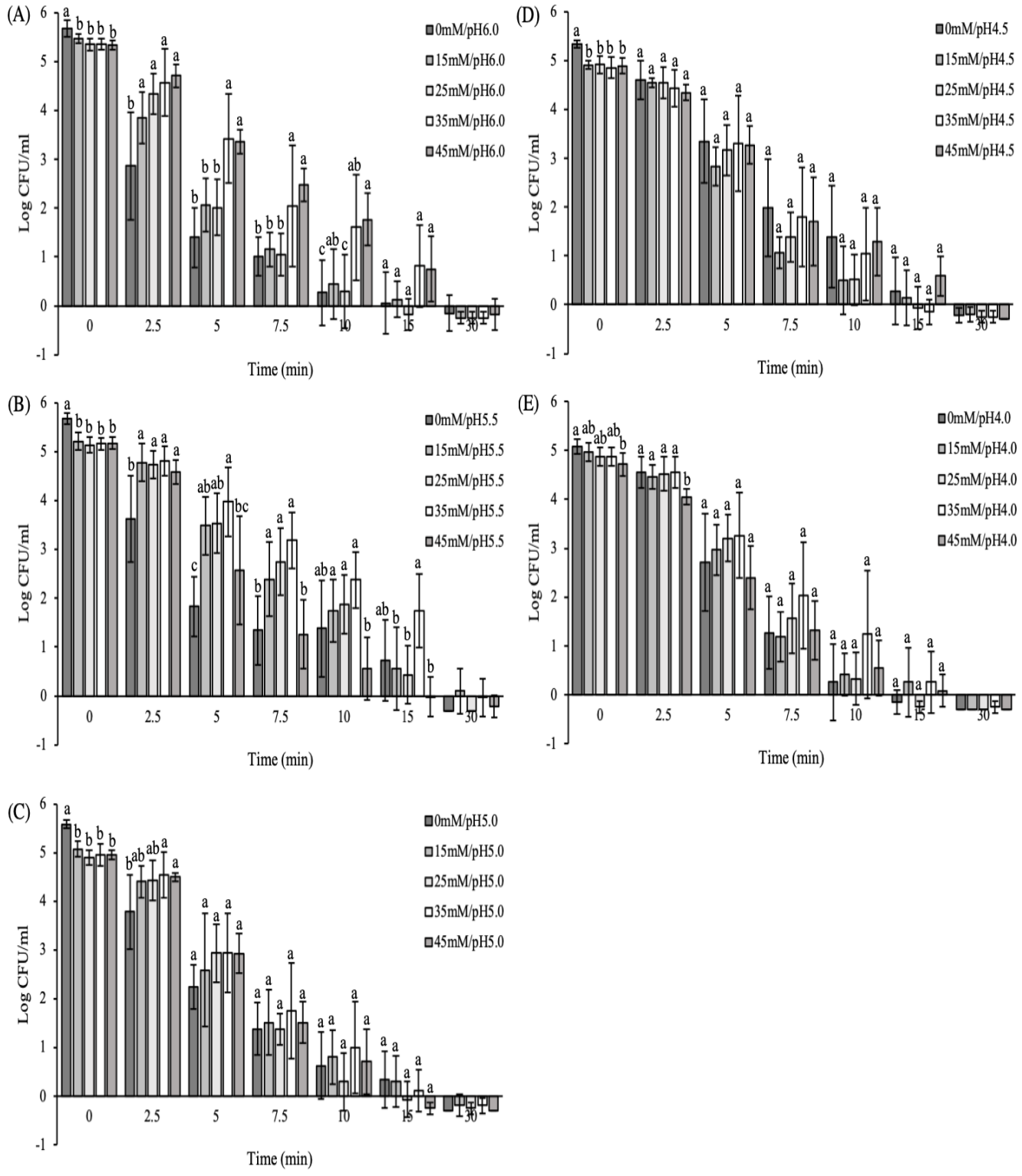

Figure 2.3. Inactivation of adapted *S.* Enteritidis cells at pH 2.5 ($TSB_{2.5}$) (screening assay). Diagrams represent treatments adapted to pH (A) 6.0, (B) 5.5, (C) 5.0, (D) 4.5 and (E) 4.0, whereas bars within each diagram different (undissociated) acetic acid concentrations. Adaptation to pH 6.0 and 5.5 within a range of increasing concentrations of UAA (1.9-5.4 mM) induced elevated acid resistance compared to the

individual effect of pH. Adaptation to lower pH values (5.0, 4.5 and 4.0) had no effect, regardless of the amount of UAA. Each bar is an average of six replicates (± (± standard deviation). Different letters indicate significant differences among treatments of the same time interval according to Tukey's HSD test.

Table 2.1. Adaptive treatments without (pH adapted cells) or in the presence of UAA used in the current study and their kinetic parameter estimates during acid inactivation at $TSB_{2.5}$.

pH adjusted with HCl/NaOH	Total AA[a] (mM)	UAA[b] (mM)	4D	RMSE[c]	R^{2} [d]
pH 6.0	0	0.0	5.0 ± 1.7 **(a)**	0.2092-0.6124	0.9788 ± 0.0116
	15	0.8	6.7 ± 1.3 **(ab)**	0.1499-0.522	0.9787 ± 0.0177
	25	1.4	6.4 ± 1.4 **(ab)**	0.2746-0.5035	0.9850 ± 0.0060
	35	1.9	10.0 ± 3.8 **(bc)**	0.1742-0.4406	0.9840 ± 0.0086
	45	2.4	11.6 ± 2.1 **(c)**	0.1029-0.3306	0.9903 ± 0.0082
pH 5.5	0	0.0	7.6 ± 3.5 **(a)**	0.2046-0.7463	0.9567 ± 0.0309
	15	2.3	10.9 ± 2.8 **(a)**	0.2023-0.3894	0.9823 ± 0.0076
	25	3.8	11.2 ± 1.5 **(a)**	0.1239-0.4631	0.9843 ± 0.0151
	35	5.4	16.0 ± 3.7 **(b)**	0.1603-0.3591	0.9827 ± 0.085
	45	6.9	7.7 ± 1.9 **(a)**	0.1824-0.6411	0.9830 ± 0.0128
pH 5.0	0	0.0	7.2 ± 1.2 **(a)**	0.0718-0.5381	0.9782 ± 0.0187
	15	5.5	8.4 ± 1.7 **(a)**	0.1628-0.6340	0.9701 ± 0.0215
	25	9.1	8.4 ± 0.6 **(a)**	0.0974-0.5299	0.9842 ± 0.0156
	35	12.8	8.9 ±2.3 **(a)**	0.1442-0.6641	0.9801 ± 0.0130
	45	16.4	8.9 ±1.0 **(a)**	0.1716-0.5709	0.9826 ± 0.0156
pH 4.5	0	0.0	9.7 ± 2.8 **(a)**	0.1087-0.6569	0.9873 ± 0.0199
	15	9.7	7.5 ±0.7 **(a)**	0.2253-0.5100	0.9735 ± 0.0201
	25	16.1	8.5 ± 0.7 **(a)**	0.1204-0.5669	0.9798 ± 0.0236
	35	22.6	9.2 ± 1.7 **(a)**	0.1613-0.456	0.9877 ± 0.0102
	45	29.0	10.8 ± 2.8 **(a)**	0.1876-0.3979	0.9819 ± 0.0101
pH 4.0	0	0.0	7.2 ± 2.0 **(a)**	0.1821-0.4894	0.9786 ± 0.0112
	15	12.8	7.9 ± 0.9 **(a)**	0.2534-0.6946	0.9758 ± 0.0250
	25	21.3	8.4 ± 1.1 **(a)**	0.0855-0.4374	0.9923 ±0.0078
	35	29.8	10.1 ± 3.1 **(a)**	0.2043-0.4317	0.9856 ± 0.0091

	45	38.3	8.6 ± 1.6 **(a)**	0.1485-0.3170	0.9831 ± 0.0187

Weibull with tail (Albert) model was used for calculation of 4D, RMSE and R^2 inactivation estimates. Values represent mean (± standard deviation) of six replicates. Different letters among treatments adapted at a given pH indicate significant differences of 4D values according to Tukey's HSD test.

[a] AA: Acetic acid

[b] Theoretical undissociated acetic acid was calculated according to Henderson–Hasselbalch equation

[c] RMSE: Root Mean Square Error

[d] R^2: regression coefficient

A similar trend was found for cells adapted to treatments with a lower final pH 5.5, though higher amounts of UAA were required to induce acid resistance at pH 5.5 compared to pH 6.0. For instance, adding 2.3 mM UAA (15mM/pH5.5), a similar concentration of UAA as in the case of 45mM/pH6.0, as well as 3.8 mM UAA (25mM/pH5.5) resulted in 1.0-1.7 log units ($P < 0.05$) higher survivors compared to the individual effect of pH 5.5 (no added UAA, 0mM/pH5.5), but did not have any impact on the 4D estimates ($P>0.05$) (Figure 2.3B, Table 2.1). Therefore, the log differences were not taken into consideration, since they were considered marginal. An additional increase in UAA concentrations to 5.4 mM (35mM/pH5.5) resulted in up to 2.2 log CFU/ml higher survivors compared to control (0mM/pH5.5) (Figure 2.3B) and the highest ($P < 0.05$) 4D value (16.0 min; Table 2.1). Further increase of UAA (6.9 mM, 45mM/pH5.5) limited survival and decreased 4D values to similar levels as in control (0mM/pH5.5) (Figure 2.3B, Table 2.1).

Contrary to the above results, no effect on the acid resistance of *S.* Enteritidis was observed when cells were previously exposed to acetic acid at pH values equal to or lower than 5.0 (Figure 2.3C-E, Table 2.1). This was evident even in concentrations of UAA that induced acid resistance at higher pH values (i.e. 5.5). For these treatments, similar log counts ($P>0.05$) and 4D estimates ($P>0.05$) were obtained for all UAA concentrations (Figure 2.3C-E, Table 2.1).

Overall, the induced acid resistance of *S.* Enteritidis PT4 against subsequent acid exposure to pH 2.5 ($TSB_{2.5}$) was affected by both pH and UAA concentration of the adaptation medium. Adaptation to pH 6.0 and 5.5 within a range of increasing

concentrations of UAA (1.9-5.4 mM) successfully protected pathogen against the subsequent severe acid stress.

Exposure to 'tarama' salad

In order to examine whether the results from the laboratory media could be extrapolated in an acid food matrix stored under refrigeration, three adaptation treatments (15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0) were selected for culture preparation prior to inoculation in tarama salad. The selection of the treatments was based on the concentration of UAA and their adaptive responses at $TSB_{2.5}$, as follows: 45mM/pH6.0 and 35mM/pH5.5 had different concentrations of theoretical UAA (2.4 mM and 5.4 mM, respectively) (Table 2.1) but both increased acid resistance of *S.* Enteritidis (Figure 2.3A and 2.3B). On the other hand, 15mM/pH5.0 contained approximately the same amount of UAA as 35mM/pH5.5 (5.5 and 5.4 mM, respectively) (Table 2.1), but had no effect on the subsequent acid resistance of the pathogen (Figure 2.3B-C). Non-adapted inocula were also used as control.

A total of 21 commercial tarama salad packages were used throughout the study. The initial pH of the salad was 4.35 ± 0.02. The levels of indigenous microflora were not quantifiable in the packages tested. No marked differences ($P>0.05$) were observed among enumerated populations and 4D values following inoculation in tarama salad at 5^{o}C, irrespectively of the preceded adaptation treatment or the control (NA) (Figure 2.4, Table 2.2). This is in contrast to the results of the screening assay, where adaptation to 35mM/pH5.5 and 45mM/pH6.0 adequately strengthened the cells against the subsequent severe acid stress (Figure 2.3A and 2.3B).

pH values remained unchanged throughout the storage period ($P>0.05$) (Figure 2.5).

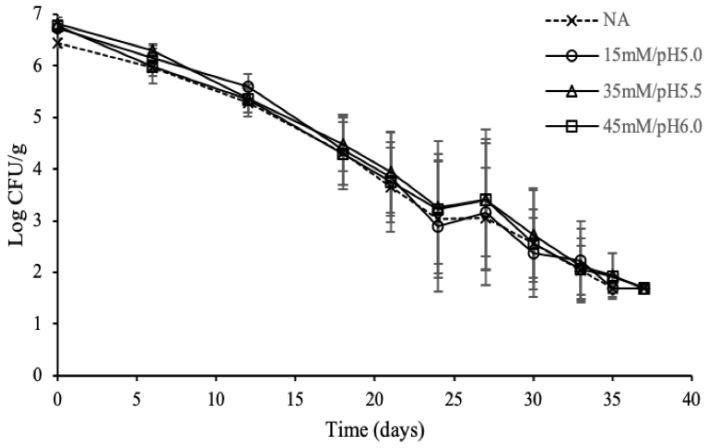

Figure 2.4 Acid adapted *S.* Enteritidis cells were not protected against tarama salad stored at 5°C**.** Each data point represents an average of six to eight replicates (± standard deviation).

Table 2.2. Kinetic parameter estimates of Weibull model during inactivation in tarama salad.

Treatment	4D	RMSE[a]	R^{2} [b]
NA	28.0 3 ± 5.32 **(a)**	0.0607-0.3426	0.9838 ± 0.0109
15mM/pH5.0	27.16.7 ± 5.6 **(a)**	0.0855-0.5095	0.9760 ± 0.0144
35mM/pH5.5	28.1± 6.0 **(a)**	0.1996-0.3776	0.9779 ± 0.0070
45mM/pH6.0	28.75 ± 6.12 **(a)**	0.1721-0.3650	0.9758 ± 0.0161

Values represent mean (± standard deviation) of six to eight replicates. Different letters indicate significant differences among 4D values according to Tukey's HSD test.

[a] RMSE: Root Mean Square Error

[b] R^{2}: regression coefficient

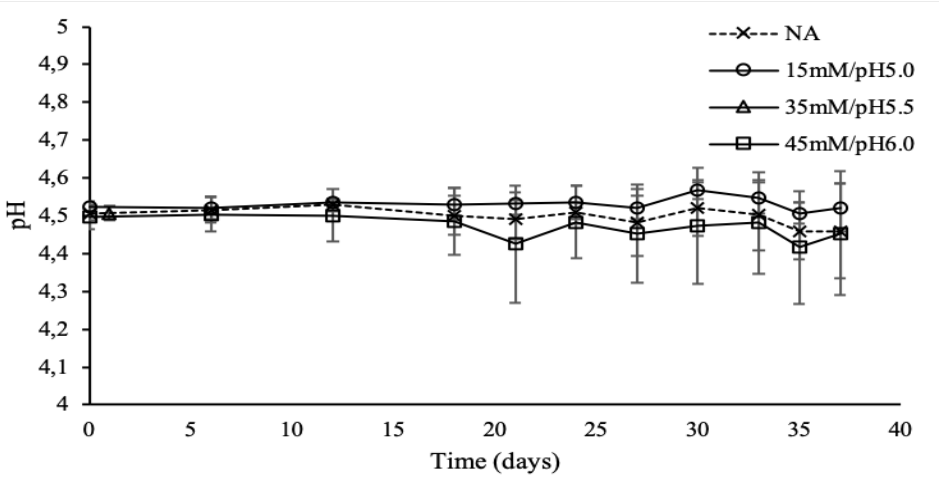

Figure 2.5. pH changes during storage of adapted and NA cells of *S.* Enteritidis in tarama salad stored at 5ºC.

Exposure to pH 4.35 using HCl and citric acid

Since acid adaptive responses following exposure to $TSB_{2.5}$ at 37ºC were different from those obtained in tarama salad stored under refrigeration (5ºC), an effort was made to examine the reasons underpinning these discrepancies. Thus, the effect of key individual factors for *S.* Enteritidis inactivation that may be of relevance to an acid food matrix (e.g., pH value, acidulant agent) and the effect of challenge temperature (5ºC, 37ºC) was further examined. Cells were first adapted to the selected treatments (15mM/pH5.0, 35mM/pH5.5 or 45mM/pH6.0), inoculated in $TSB_{4.35}$ (pH of the commercial tarama salad) using either HCl or citric acid (acidulant of tarama salad) and incubated at 37 or 5ºC (temperature effect).

Adaptation to 35mM/pH5.5 failed to protect the pathogen against the subsequent lethal stress of pH 4.35 ($TSB_{4.35}$) at 37 and 5ºC, regardless of the acidulant used (Figures 2.6 and 2.7). Even though this observation is not in line with the results obtained following $TSB_{2.5}$ inactivation at 37ºC (Figure 2.3B), it may explain the inability of these culture types to tolerate the lethal environment of tarama salad stored at 5ºC (Figure 2.4). On the other hand, adaptation to 45mM/pH6.0 successfully prolonged the survival of pathogen in $TSB_{4.35}$ at 37ºC for both acidulants used (Figure 2.6). The ability of 45mM/pH6.0 adapted cells to survive lethal acid stress at 37ºC was also observed following exposure to $TSB_{2.5}$ (Figure 2.3A), though this trend was not confirmed in tarama salad stored at 5ºC (Figure 2.4). More specifically, exposure to

$TSB_{4.35}$ adjusted with citric acid following adaptation to 45mM/pH6.0 resulted in up to 1.8 log units ($P < 0.05$) higher populations during 32-96 hours of incubation compared to the rest treated and untreated cultures (i.e., control and 15mM/pH5.0, 35mM/pH5.5) (Figure 2.6A). Exposure to HCl at 37ºC had a bactericidal effect for cells adapted to 15mM/pH5.0 and 35mM/pH5.5 as well as NA inocula, reducing the microbial load until the 5-7th day of storage (Figure 2.6B). Nonetheless, cells adapted to 45mM/pH6.0 exhibited a low reduction up to 1 log CFU/ml followed by an increase to their initial levels at the 3rd day of storage (Figure 2.6B). On the contrary, a shift in the incubation temperature from 37ºC to 5ºC sensitized 45mM/pH6.0 inocula (1.0-1.5 log CFU/ml lower counts) ($P < 0.05$) against the subsequent acid and cold stress compared to 15mM/pH5.0, 35mM/pH5.5 and control (NA) cells (Figure 2.7).

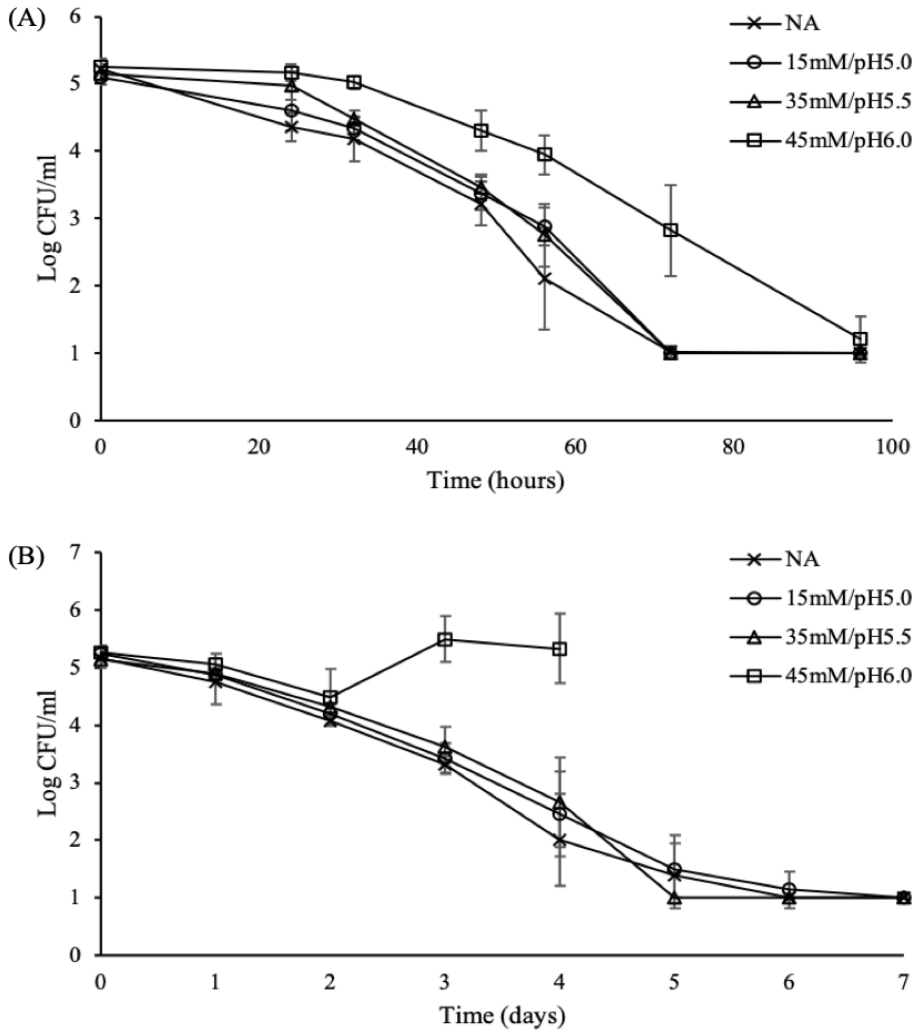

Figure 2.6. Effect of food matrix (pH, acidulant) on the responses of *S.* Enteritidis at 37ºC. Cells were exposed to $TSB_{4.35}$ at 37ºC adjusted either with citric acid (A) or HCl (B) following adaptation to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0. Each data point is an average of eight replicates (± standard deviation).

In line with the results from the screening assay and tarama salad inactivation, inoculation of 15mM/pH5.0 adapted cells in $TSB_{4.35}$ did not affect their survival, irrespectively of the incubation temperature or the acidulant used (Figures 2.6 and 2.7).

Overall, the pH value of the challenge medium as well as the incubation (challenge) temperature determined the responses of *S.* Enteritidis following adaptation to 35mM/pH5.5 and 45mM/pH6.0, respectively, but not to 15mM/pH5.0. On the contrary, similar trends were found when different acidulants were used for lowering the pH.

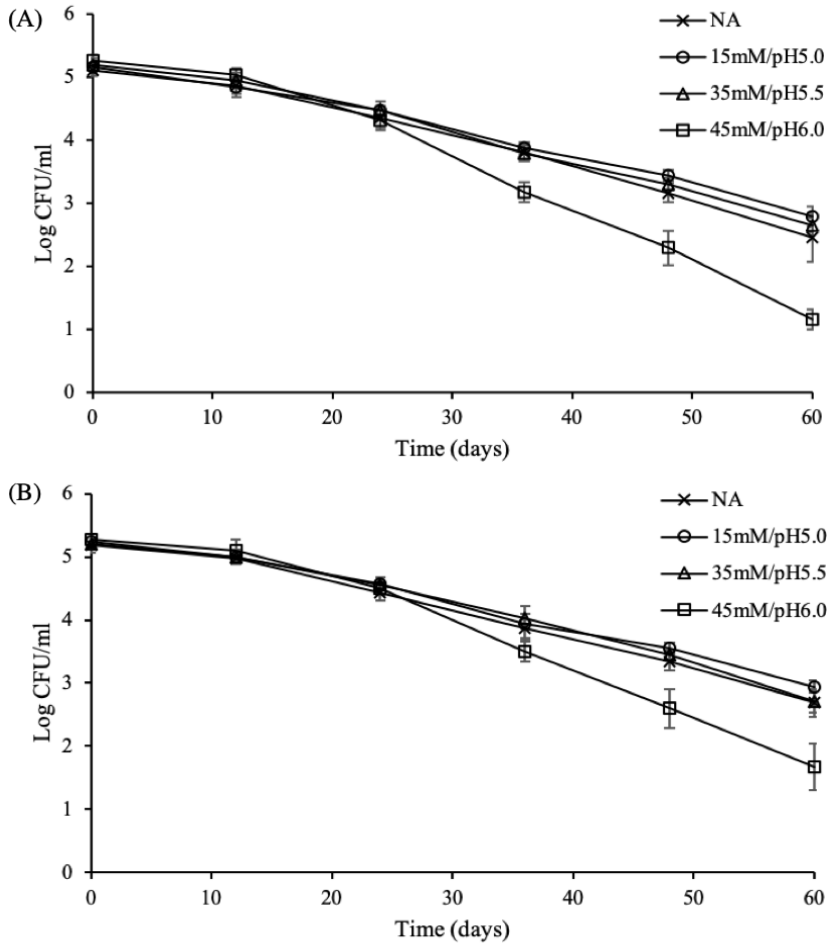

Figure 2.7. Effect of food matrix (pH, acidulant) and refrigeration temperature (5ºC) on the responses of *S*. Enteritidis. Cells were exposed to $TSB_{4.35}$ at 5ºC adjusted either with citric acid (A) or HCl (B) following adaptation to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0. Adaptation to 45mM/pH6.0 sensitized pathogen against the subsequent acid and cold stress. Each data point is an average of eight replicates (± standard deviation).

Discussion

In the present study, the effect of adaptation to UAA and pH on the subsequent acid adaptive responses of *S.* Enteritidis was examined in laboratory media and in an acid food matrix. Acetic acid was used as the adaptation agent, since it is the predominant acid in many foods, such as mayonnaise, salad dressings and sauces (Smittle, 2000). The amount of undissociated acetic acid in the adaptation treatments was estimated according to Henderson-Hasselbalch equation.

Experimental design covered a range of different combinations of pH values and concentrations of acetic acid *Salmonella* spp. may encounter in food-related ecosystems. *Salmonella* Enteritidis serotype was chosen as it was the most prevalent in Europe (EFSA and ECDC, 2018).

The individual effect of pH in the induced acid resistance of the pathogen was examined by adjusting the pH of the adaptation medium (TSBG(-)) with hydrochloric acid. This inorganic acid was used because it is completely dissociated in aqueous environments and therefore itself is not toxic for the cells (Presser et al., 1997). In line with previous reports, adaptation to moderate pH values (4.0-5.5) protected cells against subsequent acid exposure, with higher resistance observed at the lower pH values (4.0 and 4.5) (Humphrey et al., 1993; Lee et al., 1994; Ye et al., 2019; Yuk and Marshall, 2004). Nonetheless, the pH values required for the induction of acid resistance in pH adapted cells were lower compared to the milder pH values (5.5-6.0) required in the presence of acetic acid.

The addition of acetic acid to the adaptation media also enhanced survival of the pathogen following adaptation to some of the treatments tested. In general, the ability of weak organic acids to activate acid resistance mechanisms has previously been reported (Álvarez-Ordóñez et al., 2009a, 2009b, 2010a; Greenacre et al., 2003; Koutsoumanis and Sofos, 2004; Kwon et al., 2000; J. Liu et al., 2017; Yang et al., 2014a; Yuk and Marshall, 2005). Adaptation to juices, natural sources of organic acids,

was also found to elicit a protective effect in *Salmonella* spp. and *E. coli* (Kim et al., 2016; Yuk and Schneider, 2006). According to Yuk and Marshall (Yuk and Marshall, 2005) and Yuk and Schneider (Yuk and Schneider, 2006), the differences observed in the phenotypic responses of individual strains of *E. coli* and *Salmonella* spp. adapted to organic acids and juices, respectively, can be ascribed to the different amounts of undissociated acid present in the adaptation media. Acetate has been found to activate *rpoS* in log phase cells (Arnold et al., 2001). This gene is the master regulator of the general stress response in many Gram$^-$ bacteria (Lago et al., 2017) induced upon entry of cells into stationary phase of growth and under a variety of unfavorable conditions, such as low pH (Battesti et al., 2011). Lee et al. (Lee et al., 1995) has reported that induction of *rpoS* by acetate is performed indirectly, through the reduction of intracellular pH.

Nevertheless, the impact of different levels of undissociated acid in the induced acid resistance of *Salmonella* has not been widely investigated. Therefore, this chapter tries to establish a link between the levels of theoretical UAA calculated according to Henderson-Hasselbalch equation and the induced acid resistance of *S.* Enteritidis PT4 under extreme acid conditions. Based on the results of the broth screening, previous exposure of the pathogen to acetic acid increased its acid resistance in a UAA concentration- and pH-dependent manner. In mild pH values (5.5-6.0), increasing the amount of UAA within a range (1.9-5.4 mM) protected the pathogen against the detrimental effect of severe pH (2.5), even though higher concentrations of UAA were required at pH 5.5 compared to pH 6.0 for the induced resistance to be manifested. The concentrations of UAA that induced acid resistance were in each case lower, or at least close to the lowest limit of the calculated MIC range (5.2-7.2 mM), indicating that levels below the growth/no growth limit were needed for the stimulation of the protective effect. Adaptation to UAA close to the upper limit of the MIC (6.9 mM UAA, treatment 45mM/pH5.5) had no effect on acid tolerance of *S.* Enteritidis. On the other hand, addition of UAA in lower pH values (5.0-4.0), where a higher concentration of protons was present, had no effect on the acid phenotypic responses, regardless of the concentrations of theoretical UAA that were added.

Alterations in factors prevailing in the challenge substrate or during challenge drastically affected the ability of the pathogen to tolerate stress in culture media. Interestingly, increasing the pH of the challenge medium (TSB) from 2.5 to 4.35 clearly suppressed the induced resistance of cells adapted to 35mM/pH5.5 at both incubation

temperatures (5 and 37ºC) and acidulants (HCl, citric acid) tested. Although the exact reason for this shift is not known, different mechanisms triggered to support survival under extreme acid conditions may be significantly affected by conditions prevailing at the challenge substrate. For instance, the so-called Acid Tolerance Response (ATR) may protect stationary phase cells at external pH 3.0 (Lee et al., 1994), but will not provide significant protection at pH 2.5 (Spector and Kenyon, 2012). Amino acids-dependent pH homeostatic mechanisms, on the other hand, may enhance resistance at pH 2.5 (Álvarez-Ordóñez et al., 2010b) or pH 2.3 (Viala et al., 2011), provided that the cognate amino acids are available in the substrate. TSB used in the broth experiments is a rich medium, containing tryptone and soy peptone as protein sources, therefore providing the necessary amino acids. Contrary to the responses of 35mM/pH5.5 adapted cells, acid resistance of 45mM/pH6.0 inocula adapted to lower concentrations of UAA, but higher pH was affected only by incubation temperature. These inocula were sensitized when exposed to pH 4.35 ($TSB_{5.35}$) at 5ºC but not at 37ºC, regardless of the acidulant used. This result is in line with those reported from Tiwari et al. (Tiwari et al., 2004),who also found that prolonged exposure of acid adapted *Salmonella* cells to acidic conditions at 4ºC was more detrimental (e.g. had higher reductions) compared to the control samples stored at pH 7.3 at the same temperature. Exposure of *S.* Seftenberg non-adapted and acid-adapted cells to acid and cold stress altered the membrane fatty acid composition of both inocula, with higher changes found in acid adapted cultures. These alterations resulted in increased bacterial membrane fluidity (Álvarez-Ordóñez et al., 2009c). It is generally believed that a lower membrane fluidity correlates well with higher acid resistance of bacterial cells (Yang et al., 2014b; Yuk and Marshall, 2005, 2004). As demonstrated before, a shift in the storage temperature can affect the acid resistance phenotypes. Shen, Yu and Chou (Shen et al., 2007) reported that whereas no differences were observed between acid adapted and non-adapted cells of *S.* Typhimurium inoculated in skim milk and treated fermented milk stored at 37ºC, acid adaptation, in addition to promoting acid resistance, decreased the susceptibility of the pathogen to refrigeration (5ºC). Nonetheless, it is not the first time that the effects of acid adaptation are counteracted by subsequent stressors, increasing the sensitivity of acid adapted cells to lethal stresses compared to their non-adapted counterparts (Greenacre et al., 2006; Greenacre and Brocklehurst, 2006; Ye et al., 2019).

When tarama salad was used instead of $TSB_{4.35}$ at 5ºC, survival of all adapted cells was similar to the non-adapted cultures. Notably, enhanced resistance of acid adapted *S.* Typimurium was reported in fermented milk products stored at 5ºC (Shen et al., 2007). The increased sensitivity of 45mM/pH6.0 adapted cells at $TSB_{4.35}$ at 5ºC but not at tarama salad at 5ºC suggests a protective effect of food matrix on these inocula. This result indicates that intrinsic factors of tarama salad other than the pH value and the acidulant *per se* may also determine the resistance phenotypes. It has been found that a combination of organic acids and NaCl may elicit a protective effect against *Salmonella*, *E. coli* and *Shigella flexneri* (Chapman and Ross, 2009; Lee and Kang, 2016; Yoon et al., 2014), principally due to a raise in the intracellular pH (Lee and Kang, 2016). In addition, certain food ecosystems may help bacterial cells tolerate lethal acid environments (Álvarez-Ordóñez et al., 2009b; Waterman and Small, 1998). For instance, Alvarez et al. (Álvarez-Ordóñez et al., 2009b) reported that acid adapted cells challenge to Meat Extract at pH 3.0 exhibited higher resistance compared to those challenged to Brain Heart Infusion adjusted to the same pH value. Similarly, Waterman and Small (Waterman and Small, 1998) also manifested that *S.* Typhimurium was protected when inoculated into ground beef and boiled egg white but not when rice was used. In general, discrepancies between phenotypes in culture media and food matrices has been found by other authors as well (Vivijs et al., 2016).

Conclusions

In conclusion, pre-exposure of *S.* Enteritidis PT4 to organic (acetic acid) or inorganic (HCl) mild treatments may stimulate acid resistance mechanisms against subsequent extreme acid stress. Nevertheless, this effect cannot be directly extrapolated to acid foods, where other convoluted factors compromise the enhanced acid resistance phenotype. More specifically, the composition of adaptation medium (concentration of UAA, pH) and factors prevailing on the subsequent acid challenge (pH, temperature and other intrinsic but unspecified factors), may collectively determine the acid adaptive response of *Salmonella* in foods and thus, alter the resistance phenotypes. Further work is required in order to elucidate the effect of the food compounds in the total acquired acid resistance. In addition, given that strain variations can dramatically affect the acid resistance phenotypes, experimental assays expanded to include more strains adapted under the condition employed in the present study should be prompted.

Supplementary data

S2.1 Table. Inactivation of NA and adapted *S.* Enteritidis PT4 cells at $TSB_{2.5}$.

Treatments	Time (min)						
	0	2.5	5	7.5	10	15	30
NA	5.3 ± 0.1	3.3 ± 0.5	0.8 ± 0.5	0.6 ± 0.5	-0.1 ± 0.4	-0.3 ± 0.0	-0.3 ± 0.0
0mM/pH6.0	5.7 ± 0.2 **(a)**	2.9 ± 1.1**(b)**	1.4 ± 0.6 **(b)**	1.0 ± 0.4 **(b)**	0.3 ± 0.7 **(c)**	0.1 ± 0.6 **(a)**	-0.2 ± 0.1
15mM/pH6.0	5.5 ± 0.1 **(b)**	3.8 ± 0.5 **(a)**	2.1 ± 0.5 **(b)**	1.2 ± 0.3 **(b)**	0.4 ± 0.7 **(ab)**	0.1 ± 0.4 **(a)**	-0.3 ± 0.1
25mM/pH6.0	5.4 ± 0.1 **(b)**	4.3 ± 0.4 **(a)**	2.0 ± 0.6 **(b)**	1.0 ± 0.4 **(b)**	0.3 ± 0.8 **(c)**	-0.2 ± 0.3 **(a)**	-0.3 ± 0.1
35mM/pH6.0	5.4 ± 0.1 **(b)**	4.6 ± 0.7 **(a)**	3.4 ± 0.9 **(a)**	2.0 ± 1.2 **(a)**	1.6 ± 1.1 **(ab)**	0.8 ± 0.8 **(a)**	-0.3 ± 0.1
45mM/pH6.0	5.3 ± 0.1 **(b)**	4.7 ± 0.2 **(a)**	3.4 ± 0.2 **(a)**	2.5 ± 0.3 **(a)**	1.8 ± 0.5 **(a)**	0.8 ± 0.7 **(a)**	-0.2 ± 0.3
0mM/pH5.5	5.7 ± 0.1 **(a)**	3.6 ± 0.9 **(b)**	1.8 ± 0.6 **(c)**	1.3 ± 0.7 **(b)**	1.4 ± 1.0 **(ab)**	0.7 ± 0.8 **(ab)**	-0,3 ± 0.0
15mM/pH5.5	5.2 ± 0.2 **(b)**	4.8 ± 0.4 **(a)**	3.5 ± 0.6 **(ab)**	2.4 ± 0.8 **(a)**	1.7 ± 0.6 **(a)**	0.6 ± 0.9 **(b)**	0.1 ± 0.5
25mM/pH5.5	5.1 ± 0.2 **(b)**	4.7 ± 0.3 **(a)**	3.5 ± 0.6 **(ab)**	2.7 ± 0.7 **(a)**	1.9 ± 0.6 **(a)**	0.4 ± 0.6 **(b)**	-0.3 ± 0.0
35mM/pH5.5	5.2 ± 0.1 **(b)**	4.8 ± 0.3 **(a)**	4.0 ± 0.7 **(a)**	3.2 ± 0.6 **(a)**	2.4 ± 0.6 **(a)**	1.7 ± 0.7 **(a)**	0.0 ± 0.4
45mM/pH5.5	5.2 ± 0.1 **(b)**	4.6 ± 0.2 **(a)**	2.6 ± 1.1 **(bc)**	1.3 ± 0.7 **(b)**	0.6 ± 0.6 **(b)**	0.0 ± 0.4 **(a)**	-0.2 ± 0.2
0mM/pH5.0	5.6 ± 0.1 **(a)**	3.8 ± 0.8 **(b)**	2.2 ± 0.5 **(a)**	1.4 ± 0.5 **(a)**	0.6 ± 0.7 **(a)**	0.3 ± 0.6 **(a)**	-0,3 ± 0.0
15mM/pH5.0	5.1 ± 0.2 **(b)**	4.4 ± 0.3 **(ab)**	2.6 ± 1.2 **(a)**	1.5 ± 0.7 **(a)**	0.8 ± 0.6 **(a)**	0.3 ± 0.5 **(a)**	-0,2 ± 0.2
25mM/pH5.0	4.9 ± 0.2 **(b)**	4.4 ± 0.4 **(ab)**	2.9 ± 0.6 **(a)**	1.4 ± 0.3 **(a)**	0.3 ± 0.6 **(a)**	-0.1 ± 0.4 **(a)**	-0.3 ± 0.1
35mM/pH5.0	5.0 ± 0.2 **(b)**	4.5 ± 0.5 **(a)**	2.9 ± 0.8 **(a)**	1.8 ± 1.0 **(a)**	1.0 ± 0.9 **(a)**	0.1 ± 0.4 **(a)**	-0.2 ± 0.2
45mM/pH5.0	5.0 ± 0.1 **(b)**	4.5 ± 0.1 **(a)**	2.9 ± 0.4 **(a)**	1.5 ± 0.4 **(a)**	07 ± 0.7 **(a)**	-0.3 ± 0.1 **(a)**	-0.3 ± 0.0
0mM/pH4.5	5.3 ± 0.1 **(a)**	4.6 ± 0.4 **(a)**	3.4 ± 0.9 **(a)**	2.0± 1.0 **(a)**	1.4 ± 1.0 **(a)**	0.3 ± 0.7 **(a)**	-0.2 ± 0.1
15mM/pH4.5	4.9 ± 0.1 **(b)**	4.6 ± 0.1 **(a)**	2.8 ± 0.4 **(a)**	1.1 ± 0.3 **(a)**	0.5 ± 0.7 **(a)**	0.1 ± 0.6 **(a)**	-0.2 ± 0.2
25mM/pH4.5	4.9 ± 0.2 **(b)**	4.6 ± 0.3 **(a)**	3.2 ± 0.5 **(a)**	1.4 ± 0.5 **(a)**	0.5 ± 0.5 **(a)**	-0.1 ± 0.4 **(a)**	-0.3 ± 0.1
35mM/pH4.5	4.9 ± 0.2 **(b)**	4.4 ± 0.4 **(a)**	3.3 ± 1.0 **(a)**	1.8 ± 1.0 **(a)**	1.0 ± 1.0 **(a)**	-0.2 ± 0.3 **(a)**	-0.3 ± 0.1
45mM/pH4.5	3.9 ± 0.2 **(b)**	4.4 ± 0.2 **(a)**	3.3 ± 0.4 **(a)**	1.7 ± 0.9 **(a)**	1.3 ± 0.7 **(a)**	0.6 ± 0.4 **(a)**	-0.3 ± 0.0
0mM/pH4.0	5.1 ± 0.2 **(a)**	4.6 ± 0.3 **(a)**	2.7 ± 1.0 **(a)**	1.3 ± 0.7 **(a)**	0.3 ± 0.8 **(a)**	-0.2 ± 0.3 **(a)**	-0.3 ± 0.0
15mM/pH4.0	5.0 ± 0.2 **(ab)**	4.5 ± 0.2 **(a)**	3.0 ± 0.5 **(a)**	1.2 ± 0.5 **(a)**	0.4 ± 0.4 **(a)**	0.3 ± 0.7 **(a)**	-0.3 ± 0.0
25mM/pH4.0	4.9 ± 0.2 **(ab)**	4.5 ± 0.4 **(a)**	3.2 ± 0.5 **(a)**	1.6 ± 0.7 **(a)**	0.3 ± 0.5 **(a)**	-0.3 ± 0.1 **(a)**	-0.3 ± 0.0
35mM/pH4.0	4.9 ± 0.2 **(ab)**	4.6 ± 0.3 **(a)**	3.3 ± 0.9 **(a)**	2.0 ± 1.1 **(a)**	1.2 ± 1.3 **(a)**	0.3 ± 0.6 **(a)**	-0.3 ± 0.1
45mM/pH4.0	4.9 ± 0.2 **(b)**	4.1 ± 0.2 **(b)**	2.4 ± 0.7 **(a)**	1.3 ± 0.6 **(a)**	0.5 ± 0.6 **(a)**	0.1 ± 0.3 **(a)**	-0.3 ± 0.0

Different letters within columns at a given pH value indicate statistical differences among treatments at the same time intervals according to Tukey's HSD test.

S2.2 Table. Exposure to $TSB_{4.35}$ at 37ºC adjusted with citric acid.

	Treatment (log CFU/ml ± SD)			
Time (h)	Non-adapted	15mM/pH5.0	35mM/pH5.5	45mM/pH6.0
0	5.2 ± 0.1 **(b)**	5.1 ± 0.1 **(ab)**	5.2 ± 0.1 **(ab)**	5.3 ± 0.1 **(a)**
24	4.4 ± 0.2 **(b)**	4.6 ± 0.3 **(b)**	5.0 ± 0.2 **(a)**	5.2 ± 0.1 **(a)**
32	4.2 ± 0.3 **(c)**	4.3 ± 0.2 **(bc)**	4.5± 0.1 **(b)**	5.0 ± 0.1 **(a)**
48	3.2 ± 0.3 **(b)**	3.4 ± 0.3 **(b)**	3.5 ± 0.2 **(b)**	4.3 ± 0.3 **(a)**
56	2.1 ± 0.8 **(c)**	2.9 ± 0.3 **(b)**	2.8 ± 0.5 **(b)**	4.0 ± 0.3 **(a)**
72	1.0 ± 0.1 **(b)**	1.0 ± 0.0 **(b)**	1.0 ± 0.9 **(b)**	2.8 ± 0.7 **(a)**
96	1.0 ± 0.0 **(b)**	1.9 ± 0.0 **(b)**	1.0 ± 0.0 **(b)**	1.2 ± 0.3 **(a)**

Different letters within the same row indicate statistical differences according to Tukey's HSD test.

S2.3 Table. Exposure to $TSB_{4.35}$ at 37ºC adjusted with HCl.

	Treatment (log CFU/ml ± SD)			
Time (days)	Non-adapted	15mM/pH5.0	35mM/pH5.5	45mM/pH6.0
0	5.2 ± 0.1 **(a)**	5.2 ± 0.1 **(a)**	5.1 ± 0.1 **(a)**	5.3 ± 0.1 **(a)**
1	4.8 ± 0.4 **(b)**	4.9 ± 0.1 **(b)**	4.9 ± 0.1 **(b)**	5.1 ± 0.2 **(a)**
2	4.1 ± 0.1 **(b)**	4.2 ± 0.1 **(ab)**	4.3 ± 0.2 **(ab)**	4.5 ± 0.5 **(a)**
3	3.3 ± 0.1 **(c)**	3.4 ± 0.3 **(bc)**	3.6 ± 0.3 **(b)**	5.5 ± 0.4 **(a)**
4	2.0 ± 0.8 **(b)**	2.5 ± 0.7 **(b)**	2.7 ± 0.8 **(b)**	5.3 ± 0.6 **(a)**
5	1.4 ± 0.6 **(ab)**	1.5 ± 0.6 **(a)**	1.0 ± 0.0 **(b)**	
6	1.0 ± 0.0 **(a)**	1.1 ± 0.3 **(a)**	1.0 ± 0.0 **(a)**	
7	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	

Different letters within the same row indicate statistical differences according to Tukey's HSD test.

S2.4 Table. Exposure to $TSB_{4.35}$ at 5ºC adjusted with citric acid.

	Treatment (log CFU/ml ± SD)			
Time (days)	Non-adapted	15mM/pH5.0	35mM/pH5.5	45mM/pH6.0
0	5.1 ± 0.1 **(b)**	5.2 ± 0.1 **(b)**	5.2 ± 0.1 **(ab)**	5.3 ± 0.1 **(a)**
12	4.9 ± 0.1 **(a)**	4.8 ± 0.2 **(a)**	5.0 ± 0.1 **(a)**	5.0 ± 0.1 **(a)**
24	4.3 ± 0.2 **(ab)**	4.5 ± 0.1 **(a)**	4.5 ± 0.1 **(a)**	4.3 ± 0.2 **(b)**
36	3.8 ± 0.1 **(a)**	3.9 ± 0.1 **(a)**	3.8 ± 0.1 **(a)**	3.2 ± 0.2 **(b)**
48	3.2 ± 0.2 **(b)**	3.4 ± 0.1 **(a)**	3.3 ± 0.1 **(a)**	2.3 ± 0.3 **(c)**
60	2.5 ± 0.4 **(b)**	2.8 ± 0.2 **(a)**	2.7 ± 0.1 **(ab)**	1.2 ± 0.2 **(c)**

Different letters within the same row indicate statistical differences according to Tukey's HSD test.

S2.5 Table. Exposure to $TSB_{4.35}$ at 5ºC adjusted with HCl.

	Treatment (log CFU/ml ± SD)			
Time (days)	Non-adapted	15mM/pH5.0	35mM/pH5.5	45mM/pH6.0
0	5.2 ± 0.1 **(a)**	5.2 ± 0.1 **(a)**	5.2 ± 0.1 **(a)**	5.3 ± 0.1 **(a)**
12	5.0 ± 0.1 **(a)**	5.0 ± 0.1 **(a)**	5.0 ± 0.1 **(a)**	5.1 ± 0.2 **(a)**
24	4.4 ± 0.1 **(b)**	4.6 ± 0.1 **(a)**	4.6 ± 0.1 **(a)**	4.5 ± 0.1 **(ab)**
36	3.9 ± 0.2 **(a)**	3.9 ± 0.3 **(a)**	4.0 ± 0.1 **(a)**	3.5 ± 0.1 **(b)**
48	3.3 ± 0.1 **(b)**	3.6 ± 0.1 **(a)**	3.4 ± 0.1 **(ab)**	2.6 ± 0.3 **(c)**
60	2.7 ± 0.2 **(a)**	2.9 ± 0.1 **(a)**	2.7 ± 0.2 **(a)**	1.7 ± 0.4 **(b)**

Different letters within the same row indicate statistical differences according to Tukey's HSD test.

S2.6 Table. Exposure to tarama salad stored at 5ºC.

	Treatment (log CFU/ml ± SD)			
Time (days)	Non-adapted	15mM/pH5.0	35mM/pH5.5	45mM/pH6.0
0	6.4 ± 0.1 **(b)**	6.7 ± 0.1 **(a)**	6.8 ± 0.1 **(a)**	6.8 ± 0.1 **(a)**
6	6.0 ± 0.2 **(b)**	6.2 ± 0.2 **(ab)**	6.3 ± 0.1 **(a)**	6.0 ± 0.3 **(b)**
12	5.3 ± 0.3 **(b)**	5.6 ± 0.3 **(a)**	5.4 ± 0.3 **(ab)**	5.3 ± 0.2 **(ab)**
18	4.3 ± 0.7 **(a)**	4.4 ± 0.7 **(a)**	4.5 ± 0.5 **(a)**	4.3 ± 0.6 **(a)**
21	3.5 ± 0.9 **(a)**	3.8 ± 0.9 **(a)**	3.9 ± 0.8 **(a)**	3.8 ± 0.7 **(a)**
24	3.0 ± 1.1 **(a)**	2.9 ± 1.3 **(a)**	3.3 ± 1.3 **(a)**	3.2 ±1.1 **(a)**
27	3.0 ± 1.0 **(a)**	3.2 ± 1.4 **(a)**	3.4 ± 1.1 **(a)**	3.4 ± 1.4 **(a)**
30	2.6 ± 0.7 **(a)**	2.4 ± 0.7 **(a)**	2.7 ± 0.9 **(a)**	2.6 ± 1.0 **(a)**
33	2.0 ± 0.5 **(a)**	2.2 ± 0.8 **(a)**	2.1 ± 0.7 **(a)**	2.1 ± 0.6 **(a)**
35	1.7 ± 0.0 **(a)**	1.7 ± 0.0 **(a)**	1.9 ± 0.4 **(a)**	1.9 ± 0.4 **(a)**
37	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0

Different letters within the same row indicate statistical differences according to Tukey's HSD test.

CHAPTER 3

Prior exposure to different combinations of pH and undissociated acetic acid can affect the induced resistance of *Salmonella* spp. strains in mayonnaise stored under refrigeration and the regulation of acid-resistance related genes

Alkmini Gavriil, Spiros Paramithiotis, Asimina Skordaki, Eleni Tsiripov, Adamantia Papaioannou, Panagiotis N. Skandamis

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Abstract

The innate and inducible resistance of six *Salmonella* strains (4/74, FS8, FS115, P167807, ATCC 13076, WT) in mayonnaise at 5^{o}C following adaptation to different pH/undissociated acetic acid (UAA) combinations (15mM/pH5.0, 35mM/pH5.5, 45mM/pH6.0) was investigated. The inherent and acid-induced responses were strain-dependent. Two strains (ATCC 13076, WT), albeit not the most resistant innately, exhibited the most prominent adaptive potential. Limited/no adaptability was observed regarding the rest strains, though being more resistant inherently. The individual effect of pH and UAA adaptation in the phenotypic and transcriptomic profiles of ATCC 13076 and WT was further examined. The type (pH, UAA) and magnitude of stress intensity affected their responses. Variations in the type and magnitude of stress intensity also determined the relative gene expression of four genes (*adiA*, *cadB*, *rpoS*, *ompR*) implicated in *Salmonella* acid resistance mechanisms. *adiA* and *cadB* were overexpressed following adaptation to some treatments; *rpoS* and *ompR* were downregulated following adaptation to 15mM/pH5.0 and 35mM/pH5.5, respectively. Nonetheless, the transcriptomic profiles did not always correlate with the corresponding phenotypes. In conclusion, strain variations in *Salmonella* are extensive. The ability of the strains to adapt and induce resistant phenotypes and acid resistance-related genes is affected by the type and magnitude of the stress applied during adaptation.

Keywords: acid adaptation, *Salmonella* spp., strain variability, undissociated acetic acid, mayonnaise, amino acid decarboxylase systems, master stress regulators

Introduction

Salmonella spp. is a diverse group of zoonotic bacteria, with more than 2500 distinct serovars, the most prevalent ones belonging to Enteritidis and Typhimurium (EFSA and ECDC, 2018). Adaptation to mild acidic conditions can enhance pathogen acid resistance both *in vitro* (Álvarez-Ordóñez et al., 2009b, 2010a; Gavriil et al., 2020; J. Liu et al., 2017; Ye et al., 2019) and in acid food matrices (Álvarez-Ordóñez et al., 2009a). Main acid resistance mechanisms utilized by *Salmonella* against extracellular pH perturbations include inducible pH homeostasis systems and synthesis of Acid Shock Proteins (ASP). The first system relies on amino acid decarboxylase systems to neutralize intracellular pH. Arginine/agmatine and lysine/cadaverine systems contribute to the acid adaptive responses of *Salmonella* (de Jonge et al., 2003; Díaz and Ricke, 2004; Kieboom and Abee, 2006; Park et al., 1996; Viala et al., 2011) by decarboxylating arginine (*adiA*) to agmatine and lysine (*cadA*) to cadaverine *via* the corresponding antiporters (*adiC* and *cadB*, respectively) with a simultaneous proton consumption from the cytoplasm (Álvarez-Ordóñez et al., 2012). Synthesis of ASP during Acid Tolerance Response (ATR) aims to counter the potentially lethal effect of acid stress (Audia et al., 2001). Depending on the growth phase of the cells, separate regulators are required (Foster, 2001). RpoS (σ^s), Fur and the two-component regulatory system PhoPQ are implicated in log-phase ATR (Foster, 2001); two-component regulatory system OmpR/EnvZ triggers the acid induced stationary phase acid tolerance (Bang et al., 2000). *rpoS*-dependent system can also contribute to the overall induced acid resistance of stationary phase cells exposed to lethal pH (Lee et al., 1994). Pre-treatment of *Salmonella* under acid conditions is necessary for the induction of the above systems. Thus, examining the expression of these genes in response to different acid environments would provide valuable information regarding the conditions that favor stimulation of each system.

Mayonnaise is one of the most widely consumed condiments (Zhu et al., 2012), containing organic acids, most commonly acetic, as acidulants (Smittle, 2000). Although the intrinsic properties of commercial mayonnaise ensures its microbial safety (Smittle, 2000), homemade mayonnaise has been implicated in several outbreaks (Geimba et al., 2004; Ortega-Benito and Langridge, 1992). Unpasteurized eggs are considered as the principal vehicle of infection (Radford and Board, 1993). Both commercial and homemade mayonnaise can be supplemented with particles of animal/vegetable origin. This can significantly raise the local pH (i.e., pH near the

added particles) (Tassou et al., 2009), thus, resulting in different acid micro-environments that can induce stress hardening of pathogenic cells. Addition of contaminated ingredients will, therefore, compromise the safety of the final products (Vermeulen, 2008). Apart from conditions intrinsically encountered in foods, food industry interventions may also induce stress resistance mechanisms (Begley and Hill, 2015), e.g. in environments with sublethal acid levels due to dilution of acid concentration with water, etc. (Gavriil et al., 2020).

Inherent differences among strains of the same species subjected to the same treatments, are an important source of variability in microbiological studies (Whiting and Golden, 2002). This means that the results coming from one strain cannot reliably be extrapolated to others (Lianou and Koutsoumanis, 2013a). Inter-strain variations can highly affect the accuracy of exposure assessments and, as such, should systematically be taken under consideration (Delignette-Muller and Rosso, 2000).

Based on the above, this study aims (i) to assess the *in situ* acid adaptive responses of six *Salmonella* spp. strains in mayonnaise stored under refrigeration and (ii) to further elucidate the individual effect of pH and undissociated acetic acid adaptation in the phenotypic and transcriptomic responses of two of the above strains having the most notable adaptive potential.

Materials and Methods

Bacterial strains and growth media

Three strains of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) and three strains of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*Salmonella* Enteritidis) were used (Table 3.1). Frozen cultures were kept at -20^{o}C in Tryptone Soy Broth without Dextrose (TSBG(-), Lab M Limited, Lancashire, UK) supplemented with 20% glycerol and were subcultured monthly on Tryptone Soy Agar (TSA, Lab M Limited, Lancashire, UK) kept at 0-4^{o}C. Prior to each experiment, one single colony was transferred to TSBG(-) for 24 h at 37^{o}C. Then 0.1 ml of the above culture was transferred to 10 ml of fresh TSBG(-) and incubated for another 18 h at 37^{o}C in order to collect stationary phase cells.

Table 3.1. Strains used in the current study and their origin

Strains	Origin/Isolation source
S. Typhimurium 4/74	Calf bowel
S. Typhimurium FS8	Pork minced meat isolate (food industry)
S. Typhimurium FS115	Chicken meat isolate (food industry)
S. Enteritidis P167807 Phage Type 4	Division of Enteric Pathogens, Central Public Health Laboratory, London, UK
S. Enteritidis ATCC 13076	American Type Culture Collection
S. Enteritidis WT	Food isolate

Initial microbial load and pH of commercial mayonnaise packages

The pH of the commercial mayonnaise packages was measured using a digital pH meter (pH 526, Metrohm Ltd, Switzerland). The indigenous microbiota was determined by transferring 10 g of product from each uninoculated package to 90 ml sterile ¼ Ringer solution (Lab M Limited, Lancashire, UK) and homogenized in a stomacher apparatus (Seward, London, UK). 0.1ml of the appropriate serial dilution was then plated on TSA supplemented with 0.1% sodium pyruvate (TSA/SP) (Applichem, Darmstadt, Germany). Plates were incubated for 24 h at 37^{o}C.

Selection and preparation of acid adaptation media

The combined effect of pH and acetic acid adaptation was tested. Three different acid adaptive treatments (15mM/pH5.0, 35mM/pH5.5, 45mM/pH6.0) corresponding to two different levels of theoretical undissociated acetic acid (UAA) (Table 3.2) were screened for their potential to induce *in situ* adaptive responses to each of the six *Salmonella* strains inoculated in mayonnaise at 5^{o}C. The acid adaptive treatments were selected on the basis of theoretical UAA they contained, calculated according to Henderson– Hasselbalch equation (Table 3.2), and *in vitro* previous published results (Gavriil et al., 2020), as follows: treatments 45mM/pH6.0 and 35mM/pH5.5 had different concentrations of UAA (2.4 mM and 5.4 mM, respectively) but both increased resistance of *S*. Enteritidis PT4 P167807 challenged to pH 2.5. Conversely, treatments 35mM/pH5.5 and 15mM/pH5.0 contained approximately the same amount of UAA

(5.4 and 5.5 mM, respectively) but had different effect on the subsequent resistance of the pathogen challenged to pH 2.5. For the preparation of adaptation media, 15, 35 and 45 mM of total acetic acid were transferred to TSBG (-). The medium was autoclaved, cooled to room temperature and, then, the pH was adjusted to 5.0, 5.5 and 6.0, respectively, using 6N HCl (Merck, Darmstadt, Germany) or 10N NaOH (Panreac, Barcelona, Spain), in order to derive different concentrations of UAA (Table 3.2). All the adaptation media were finally filter sterilized (0.2 nm, LLG Labware, USA).

In order to further elucidate the contribution of pH *vs* UAA adaptation in the subsequent induced resistance, a second set of experimental assays was conducted using two of the six *Salmonella* strains (ATCC 13076 and WT) having the most prominent acid adaptive potential. The adaptation-inducing potential of pH was assessed by adapting cultures to the desired pH values (5.0, 5.5 or 6.0) using only 6N HCl (pH adapted cultures); addition of acetic acid at the same pH value would therefore be responsible for any differences observed between pH adapted cultures and those adapted to the acid combination. The treatments were selected as follows: adaptation to 45mM/pH6.0 and 0mM/pH6.0 were tested for both strains. Strain WT was further adapted to 0 and 35 mM of total acetic acid both at pH 5.5 (0mM/pH5.5 and 35mM/pH5.5, respectively), as well as at 0 and 15 mM of total acetic acid at pH 5.0 (0mM/pH5.0 and 15mM/pH5.0, respectively). Non-adapted (NA) cells without any pH adjustment or addition of acetic acid were also used as controls in all experimentations. Adapted and NA cultures were inoculated in mayonnaise, as described in above subsection.

Table 3.2. Acid adaptive treatments and their theoretical concentration in undissociated acetic acid calculated according to Henderson– Hasselbalch equation

Treatment	Total acetic acid (mM)	pH adjusted (HCl/NaOH)	Undissociated acetic acid (mM)
15mM/pH5.0	15	5.0	5.5
35mM/pH5.5	35	5.5	5.4
45mM/pH6.0	45	6.0	2.4

Preparation of acid adapted and non-adapted cultures and inoculation in mayonnaise

For the preparation of acid adapted inocula, the procedure described by Gavriil et al., (2020) was followed. Briefly, stationary phase cells were centrifuged (2709 X g, 10 min, 4ºC), washed twice with sterile ¼ Ringer solution and resuspended in the appropriate adaptation medium (15mM/pH5.0, 35mM/pH5.5 or 45mM/pH6.0) in a concentration of ~8.5-9.0 log CFU/ml. Adaptation was performed by placing cells for 90 min in a water bath preheated at 37ºC. Initial adaptive populations were enumerated at the end of adaptation period by plating 0.1 ml of the appropriate dilution in TSA/SP plates incubated at 37ºC for 24 h. Following adaptation, acid adaptive cells were harvested by centrifugation (2709 X g, 5 min, 37ºC) and resuspended in 4 ml of a diluted mayonnaise suspension. The mayonnaise-based suspension was prepared by mixing 10 g mayonnaise with 30 ml sterile ¼ Ringer solution. The inoculated suspension was then vortexed for 1 min and aliquots (0.8 ml) were added in commercial mayonnaise pre-weighed in sterile containers (80 g). The initial adapted populations inoculated in mayonnaise were approximately 6.0-7.0 log CFU/g. Non-adapted inocula were directly resuspended to 4 ml of the mayonnaise-based suspension without prior adaptation. Samples were stored at 5ºC and samplings were performed every few hours, depending on the strain tested. Enumeration of *Salmonella* survivors was performed as previously described in 2.2 subsection. Plates were incubated for 24 h at 37ºC in TSA/SP medium. The selective Xylose Decarboxylase medium (XLD) was not used since no enumerated microflora was detected in the food matrix. In addition, preliminary trials showed that the recovery of *Salmonella* survivors in this medium was significantly lower compared to the non-selective one (TSA/SP). In any case, for TSA/SP counts lower than 3-3.5 log(cfu/g), populations were streaked to XLD to confirm they are *Salmonella* colonies. pH changes during storage were also determined. Experiments were conducted at least in triplicate with duplicate samples per trial.

In vitro gene expression assay

Transcriptomic profiles of four genes (*adiA*, *cadB*, *rpoS*, *ompR*) as affected by strain, pH or UAA variations were investigated using Reverse Transcription PCR. Gene expression was assessed as follows: cultures of ATCC 13076 adapted to 45mM/pH6.0 and 0mM/pH6.0, and of WT adapted to 15mM/pH5.0, 0mM/pH5.0, 35mM/pH5.5, 0mM/pH5.5, 45mM/pH6.0 and 0mM/pH6.0 were centrifuged and the biomass was

mixed with 200 µl of RNAlater® solution (Ambion, Whaltham, MA, USA). Cultures of the same strains treated under identical conditions (37ºC, 90 min.) in TSBG(-) without any pH adjustment or addition of acetic acid were also used for normalization of the data. Untreated cells (e.g. cells collected immediately after cleaning, not treated in TSBG(-) with or without acetic acid or pH adjustment) were also collected and used for the normalization of the data from TSBG(-) treated cells (e.g. cells treated for 90 min at 37ºC in TSBG (-) without acetic acid or pH adjustment) (data not shown). This was performed in order to ensure that the latter treatment did not affect the transcript levels of the target genes. Samples were stored at -20ºC until use.

RNA extraction and cDNA synthesis were performed using Nucleospin RNA kit (Macherey-Nagel, Duren, Germany) and PrimerScript™ RT reagent Kit (Takara, Shiga, Japan), respectively, according to manufacturer instructions. Amplification of cDNA was performed by Real Time qPCR using KAPA SYBR FAST Master Mix (2x) Universal for ABI Prism (Kappa Biosystems, Boston, MA, USA). The primers concentration was 1 µM. Primers used and PCR conditions are listed in Table 3.3. *gmk*, *rpoD* and *gyrB* were evaluated as reference genes; normalization of the data was performed using *gmk* gene. Experiments were conducted twice in two independent biological replicates. In total, eight measurements were obtained per treatment per strain.

Relative expression and $\log_2$ (fold changes) values of the target genes were calculated according to the procedure of Hadjilouka et al. (2016).

Table 3.3 Genes and their function, primer sequences, amplicon sizes and PCR conditions of the *in vitro* gene expression assay

Genes	Function	Sequences	Product size (bp)	PCR Efficiency	Reference
adiA	Arginine decarboxylase	F: AACAGTCCGCAGGTGGGTTA R: AATTTCCGTGCCTTCGGTTT	90	1.97	Álvarez-Ordóñez et al., 2010b
cadB	Lysine/cadaverine antiporter	F: TCCAGACCGGCGTTCTTTAT R: ATGCCGGGAAGAACGTAGAA	96	2.01	Álvarez-Ordóñez et al., 2010b

rpoS	General stress regulator	F: GAATCTGACGAACACGCTCA R: CCACGCAAGATGACGATATG	171	2.07	Yang et al., 2014a
ompR	Response regulator of the OmpR-EnvZ regulatory system	F: ATCGTCTGCTGACCCGTGAATCTT R: TTACTTTGACTACGCAGGCGACGA	103	1.97	Cameron and Dorman, 2012
gmk	Housekeeping	F: TTGGCAGGGAGGCGTTT R: GCGCGAAGTGCCGTAGTAAT	62	1.95	Botteldoorn et al., 2006
rpoD	Housekeeping	F: ACATGGGTATTCAGGTAATGGAAGA R: CGGTGCTGGTGGTATTTTCA	75	2.01	Botteldoorn et al., 2006
gyrb	Housekeeping	F: GCGTGAACTGTCATTCCTGA R: TACCGTCTTTTTCGGTGGAG	176	2.00	Deekshit et al., 2015

Denaturation of cDNA was initiated at 95°C for 20 s followed by 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s. Melting curve analysis conditions were: 95°C for 15 sec, 60°C for 1min and finally raise to 95°C at 0.3°C/s

Determination of inactivation parameters

Determination of the time needed for a 4-log reduction (4D) was conducted by fitting the log transformed inactivation data to the Weibull with tail (Albert) model according to the equation: $\log_{10}(N)=\log_{10}[(N_0-N_{res})\bullet 10^{(-(t/\delta)\hat{}p)} + N_{res}]$ (Albert and Mafart, 2005), where *N* is the populations log CFU/g at time t, N_o is the initial bacterial concentration log(CFU/g) at time 0, N_{res} is the residual bacterial concentration log CFU/g at the end of microbial inactivation, δ is the time needed for the first decimal reduction and *p* is a shape parameter corresponding to downward concave survival curves (p>1), upward concave survival curves (p<1) or linear curves (p=1). Data fitting was performed using the software GInaFiT, a freeware Add-in for Microsoft ®Excel (Geeraerd et al., 2005). Goodness of the fitting was evaluated using regression coefficient (R^2) and root mean square error (RMSE). In total, at least six curves per treatment per strain were obtained.

Statistical analysis

Log transformed inactivation data and estimates of time to 4D inactivation were statistically compared using Analysis of Variance (ANOVA) (SPSS Statistics 23.0 for Mac). Mean values were evaluated using Tukey' s Honestly Significant Differences

(HSD) test and were considered significant for $P < 0.05$. Transcriptional data were compared using one-sample one-tailed t-test (SPSS Statistics 23.0 for Mac). Upregulation or downregulation of genes was thought to be significant ($P < 0.05$) for $\log_2$ (fold change) values greater than 2 or lower than -2, respectively.

Results

Physicochemical and microbiological characteristics of commercial mayonnaise packages

A total of 64 commercial mayonnaise packages containing acetic and citric acid, according to the labeling, were examined for their physicochemical properties and their initial microbial load. The pH value was 3.89 ± 0.05. For the majority of the packages, the initial microbiota was non-enumerable, except for seven packages in which, populations of up to ~3 log CFU/g were recovered.

Innate resistance

The innate resistance of NA phenotypes of the six *Salmonella* strains exposed to mayonnaise at 5^{o}C was strain-dependent. The time needed for their inactivation below the enumeration limit (i.e., 2 log CFU/g) ranged from some hours to several days. Based on the 4D estimates, their resistance with decreasing order was 4/74, FS8 ≥ P167807 > FS115 > ATCC 13076, WT (Table 4). Strains FS8, 4/74 and P167807 were approximately 4-5 times more resistant than ATCC 13076 and WT, remaining countable for up to 93-165 hours of storage. ATCC 13076 and WT, on the other hand, were undetectable by the time they reached 32-33 hours of storage. Strain FS115, was inactivated by the 93rd hour of storage, forming an intermediate category (Table 3.4, Figure 3.1).

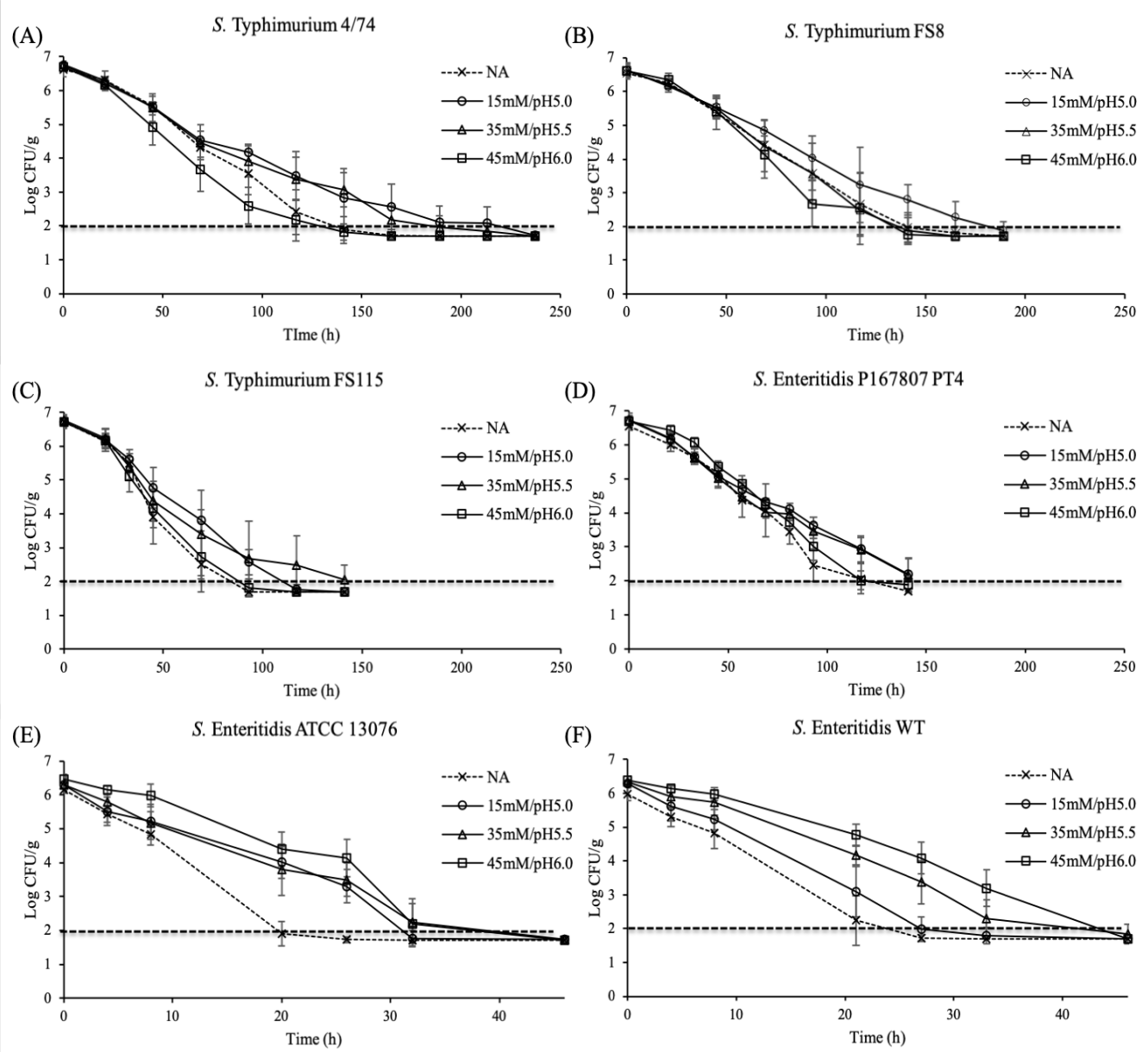

Figure 3.1. Inactivation of *Salmonella* 4/74 (A), FS8 (B), FS115 (C), P167807 (D), ATCC 13076 (E) and WT (F) in mayonnaise stored at 5°C following adaptation (90 minutes, 37°C) to different combinations of pH and UAA concentrations (15mM/pH5.0, 35mM/pH5.5, 45mM/pH6.0). Non-adapted cells were used as control. Each data point represents a mean of at least 6 replicates (± standard deviation). Dashed line indicates detection limit (2 log CFU/g).

Effect of adaptation on the survival of Salmonella strains in mayonnaise

The ability of the six *Salmonella* strains to induce resistance mechanisms following adaptation to three combinations of pH/UAA adaptation was examined. At

the end of adaptation period, initial populations in adaptation treatments involving addition of acetic acid were up to 0.5 log CFU/ml ($P < 0.05$) lower compared to the initial inoculum. No reductions ($P > 0.05$) were observed for cultures adapted to different pH values without the addition of acetic acid (i.e., 0mM/pH6.0, 0mM/pH5.5 and 0mM/pH5.0).

Strains 4/74, FS8, FS115 and P167807 exhibited slight/no adaptability to the selected treatments compared to their non-adapted counterparts. The differences in the average log counts between treated and untreated samples did not exceed 1.0-1.3 log CFU/g throughout the storage period, indicating marginal differences in their responses (Figure 3.1A-D). Calculated 4D estimates are presented in Table 3.4.

Contrary to the aforementioned strains marginally affected, pre-treatment of ATCC 13076 and WT strains to all/most of the acid conditions, markedly increased their survival in mayonnaise at 5^{o}C, compared to control (NA) (Figure 3.1E-F). Higher bacterial levels (up to 2.5 logs for 45mM/pH6.0 and ~2.0 logs for both 15mM/pH5.0 and 35mM/pH5.5) ($P < 0.05$) and higher 4D values ($P < 0.05$) were recorded for adapted cells compared to the untreated ones (NA) (Figure 3.1E; Table 3.4). Adaptation to pH 6.0 (0mM/pH6.0) only partially contributed to the induced resistance (0.5-1.8 higher logs compared to NA, 0-32 h) ($P < 0.05$), whereas addition of 2.4 mM at the same pH (45mM/pH6.0) maximized the protective effect by 0.6-1.7 (20-46 h) and 0.5-2.7 (0-46 h) logs compared to 0mM/pH6.0 and NA cultures, respectively ($P < 0.05$) (Figure 3.2A).

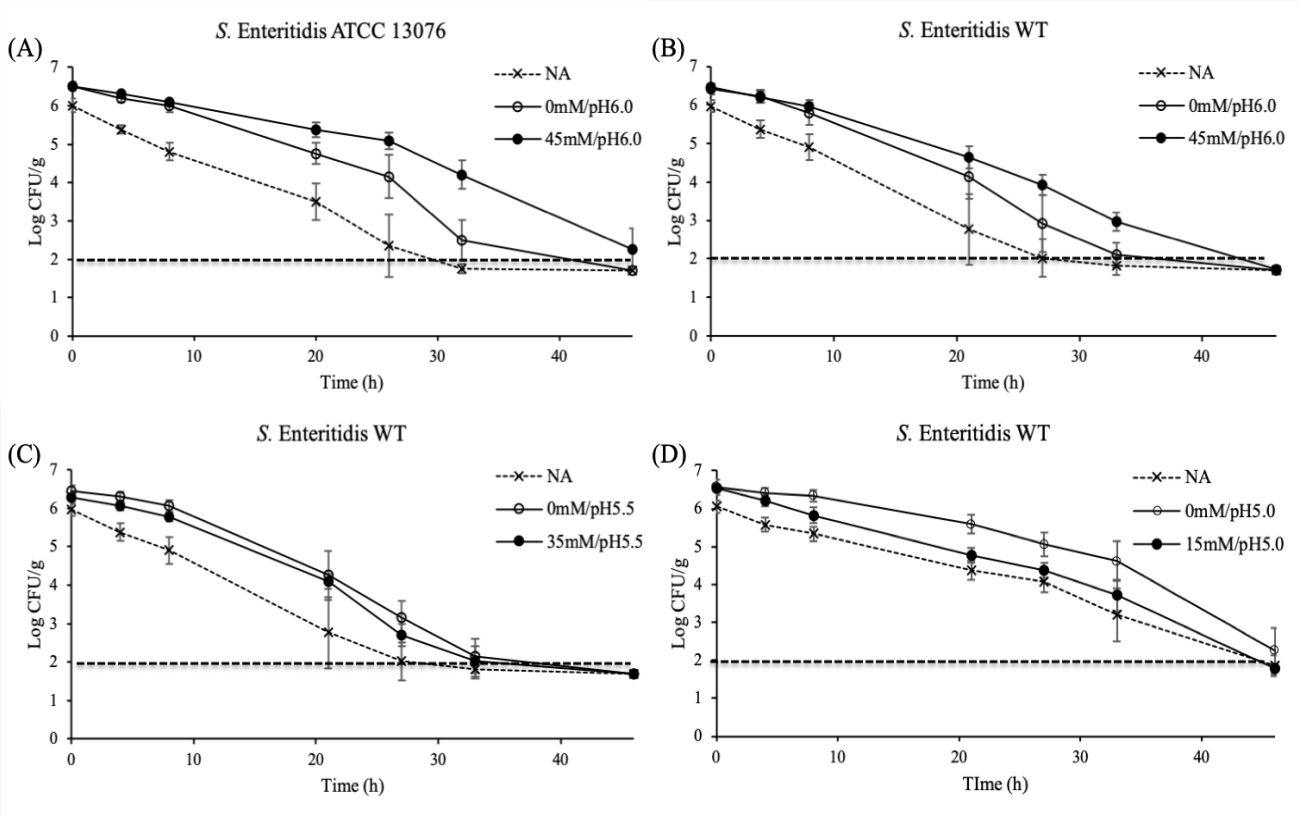

Figure 3.2. Inactivation of *Salmonella* Enteritidis ATCC 13076 following adaptation (90 minutes, 37ºC) to 0mM/pH6.0 and 45mM/pH6.0 (A) and WT to 0mM/pH6.0 and 45mM/pH6.0 (B), 0mM/pH5.5 and 35mM/pH5.5 (C) and 0mM/pH5.0 and 15mM/pH5.0 (D) in mayonnaise at 5ºC. Non-adapted cells were used as control. Each data point represents a mean of at least 6 replicates (± standard deviation). Dashed line indicates detection limit (2 log CFU/g).

Similarly, adaptation to 35mM/pH5.5 and 45mM/pH6.0 enhanced the survival of WT strain: higher populations [up to 2.0 (0-46 h) and 2.6 (0-33 h) logs, respectively] ($P < 0.05$) and higher 4D estimates ($P < 0.05$) were observed compared to NA cells (Figure 3.1F; Table 4). On the contrary, no impact was observed for 15mM/pH5.0 inocula containing the same level of UAA as 35mM/pH5.5, but different pH value (Figure 3.1F; Table 3.2; Table 3.4). Both the type (pH, UAA) and magnitude of the stress intensity determined the fate of the strain: the lower the pH or the UAA concentration during adaptation, the higher the subsequent survival in mayonnaise (Figure 2B-D). Adaptation of WT to pH 6.0 without (0mM/pH6.0), or in the presence of 2.4 mM UAA (45mM/pH6.0) promoted its survival, albeit the contribution of each stress to the total induced resistance was different compared to ATCC 13076 (Figure

3.2B). Both treatments resulted in increased *Salmonella* populations ($P < 0.05$) compared to their untreated counterparts (NA). Nonetheless, marginal differences were observed between 0mM/pH6.0 and 45mM/pH6.0 inocula, with the latter treatment promoting only up to 1.0 log CFU/g higher counts (27-33 h) ($P < 0.05$) compared to the former one (Figure 3.2B). As the pH of the adaptation medium decreased from 6.0 to 5.5 and finally to 5.0, while the concentration of UAA increased from 2.4 mM (45mM/pH6.0) to 5.4 (35mM/pH5.5) and 5.5 mM (15mM/pH5.0), the impact of pH seemed to gradually increase the subsequent survival of WT strain in mayonnaise compared to the effect of the UAA (Figure 3.2B-D). Marginal differences (up to 0.5 log CFU/g, 0-8 h and 27 h) ($P < 0.05$) were found between 0mM/pH5.5 (higher counts) and 35mM/pH5.5 (lower counts) inocula, (Figure 3.2C). Adaptation to lower pH value 5.0 (0mM/pH5.0) increased resistance by ~1.0 log CFU/g (4-46 h) ($P < 0.05$) compared to 15mM/pH5.0 treated cells (Figure 3.2D).

In all cases, no pH changes were recorded throughout the storage period (Figures 3.3 and 3.4).

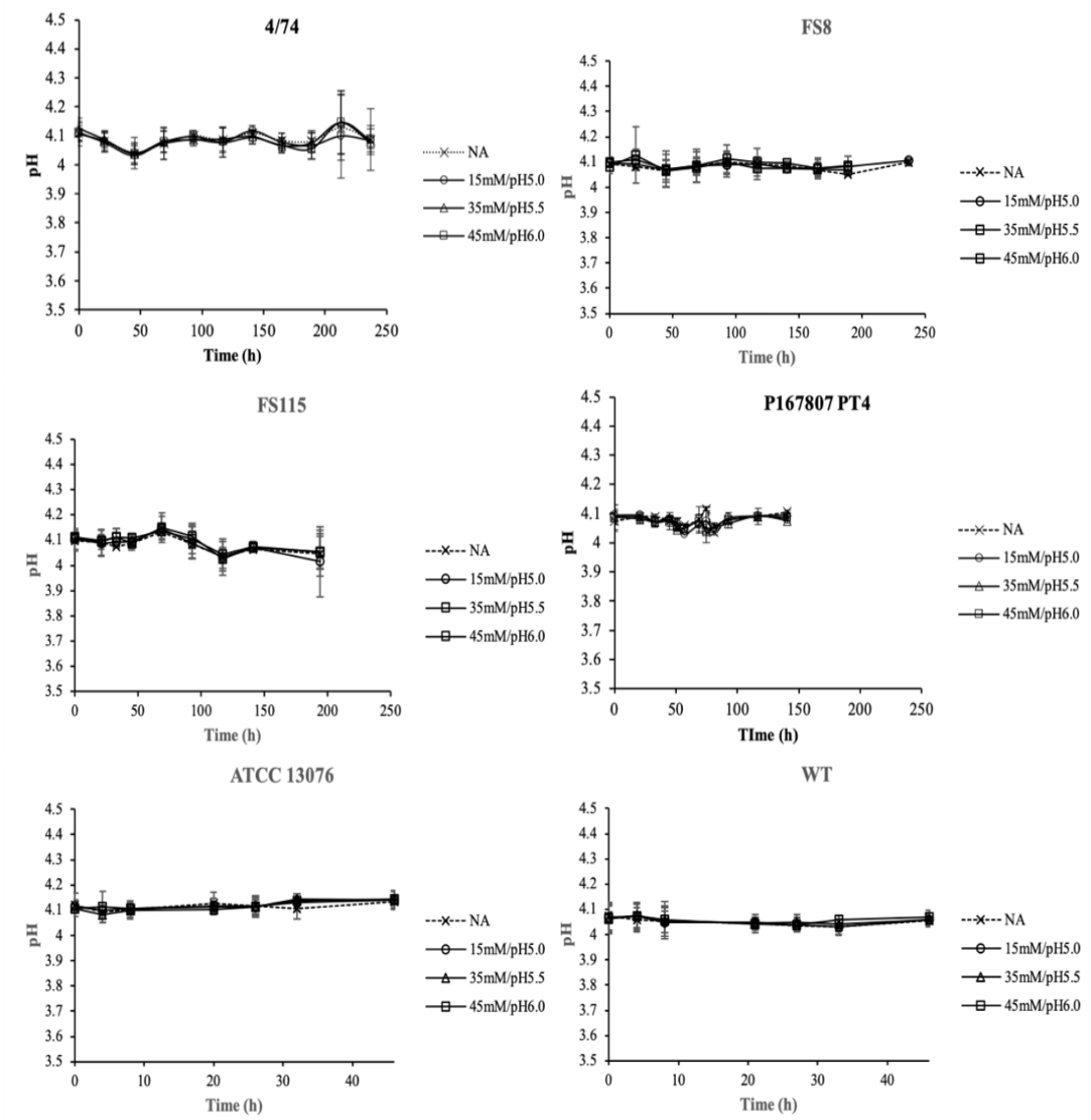

Figure 3.3. pH changes during storage of *Salmonella* strains in mayonnaise stored at 5ºC

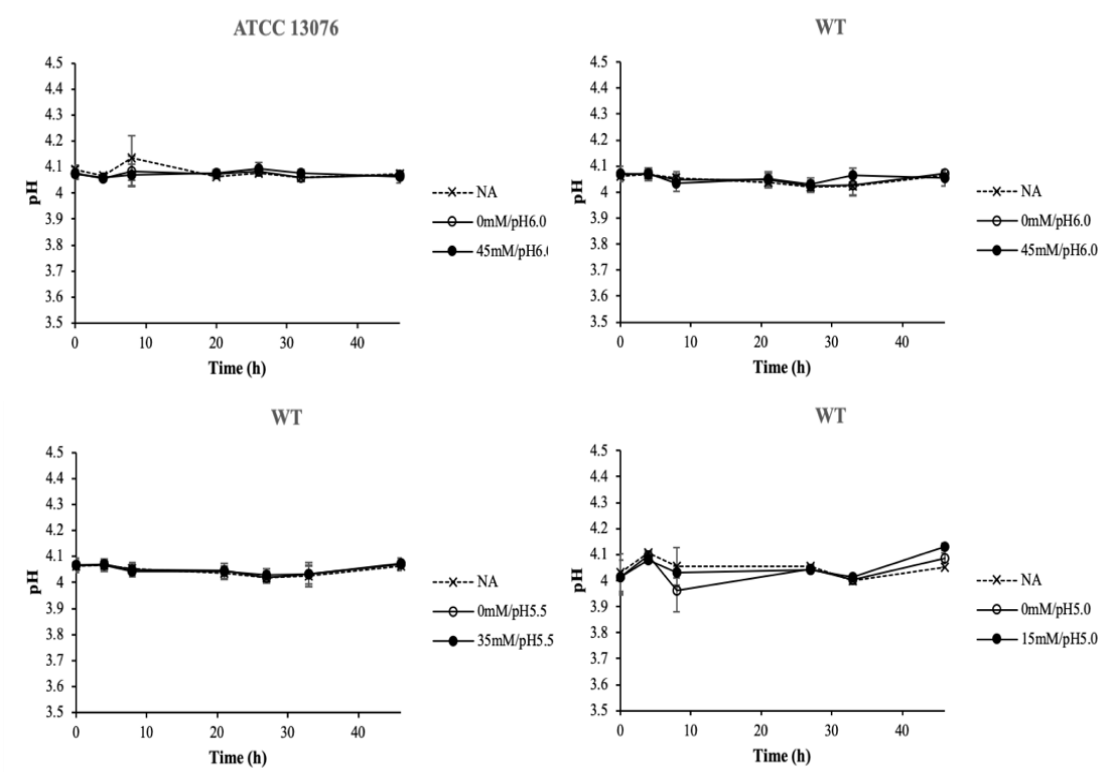

Figure 3.4. pH changes during storage of *Salmonella* ATCC 13076 and WT strains in mayonnaise stored at 5ºC

Overall, acid adaptation to all or most of the conditions tested, enhanced resistance of ATCC 13076 and WT strains, but had slight/no impact on the rest strains. The induced resistance of the two former strains was affected by the type and magnitude of acid stress intensity during adaptation.

Table 3.4. Mean (± standard deviation) values of time to 4D values, RMSE and R^2 as calculated by fitting the log-transformed inactivation data to Weibull with tail (Albert) model of GInaFit. Each value is the average of at least six measurements.

	Treatments											
	NA			**15mM/pH5.0**			**35mM/pH5.5**			**45mM/pH6.0**		
Strain	**4D**	**RMSE[a]**	**R^2 [b]**	**4D**	**RMSE[a]**	**R^2 [b]**	**4D**	**RMSE[a]**	**R^2 [b]**	**4D**	**RMSE[a]**	**R^2 [b]**
4/74	114.08 **BC, ab** (12.08)	0.2059 (0.0567)	0.9901 (0.0060)	152.64 **A, a** (31.37)	0.2241 (0.0452)	0.9843 (0.0083)	138.20 **AB, a** (19.30)	0.2559 (0.0696)	0.9803 (0.0090)	93.11 **C, a** (21.20)	0.2505 (0.0399)	0.9869 (0.0061)
FS8	117.26 **AB, a** (21.67)	0.3193 (0.1376)	0.9775 (0.0181)	141.40 **A, ab** (32.14)	0.2737 (0.0906)	0.9829 (0.0065)	115.92 **AB, ab** (15.34)	0.2879 (0.1177)	0.9854 (0.0065)	92.67 **B, a** (12.32)	0.2813 (0.0685)	0.9849 (0.0083)
FS115	66.96 **A, c** (3.11)	0.1500 (0.1005)	0.9955 (0.0049)	87.47 **A, c** (3.92)	0.2073 (0.0651)	0.9901 (0.0068)	88.76 **A, b** (33.58)	0.2215 (0.0439)	0.9881 (0.0075)	65.46 **A, b** (12.94)	0.2432 (0.1464)	0.9853 (0.0141)
P167807	95.08 **B, b** (9.70)	0.2417 (0.0986)	0.9800 (0.0143)	119.85 **A, b** (5.20)	0.2498 (0.0484)	0.9716 (0.0124)	109.98 **A, ab** (8.69)	0.2708 (0.0539)	0.9683 (0.0126)	98.23 **B, a** (3.75)	0.1932 (0.0372)	0.9894 (0.0042)
ATCC 13076	20.92 **B, d** (3.22)	0.1372 (0.1139)	0.9961 (0.0066)	29.87 **A, d** (2.19)	0.2928 (0.0783)	0.9813 (0.0107)	32.52 **A, c** (6.45)	0.2234 (0.1094)	0.9884 (0.0093)	31.50 **A, c** (4.20)	0.1430 (0.1013)	0.9948 (0.0052)
WT	23.52 **B, d** (3.76)	0.2197 (0.0971)	0.9877 (0.0141)	24.99 **B, d** (3.88)	0.2546 (0.1787)	0.9859 (0.0193)	34.50 **A, c** (3.51)	0.2045 (0.0965)	0.9879 (0.0130)	38.93 **A, c** (3.77)	0.1517 (0.0596)	0.9939 (0.0054)

Different capital letters within the same row indicate significant differences ($P < 0.05$) among different treatments of a single strain according to Tukey's HSD. Different lowercase letters within the same column indicate significant differences ($P < 0.05$) among different strains adapted to the same treatment according to Tukey's HSD. [a]RMSE: Root Mean Square Error. [b]R^2: Regression coefficient

Transcriptomic changes following adaptation

For the *in vitro* gene expression assay, samples were collected after adaptation to the selected treatments and gene expression was assayed in relation to the strain type and the preceded exposure to different pH/UAA combinations.

Similar transcriptomic profiles were obtained between the two strains (ATCC 13076 and WT) following adaptation to 0mM/pH6.0 or 45mM/pH6.0 (Figure 3.5). The overall trend was that pre-exposure to acetic acid (45mM/pH6.0) resulted in 4-5-$\log_2$(FC) upregulation of *adiA* ($P < 0.05$) in both strains. On the contrary, variations in the pH and UAA levels resulted in differences in the relative gene expression of WT strain (Figure 3.5). Adaptation to lower pH or UAA concentrations significantly increased the transcript levels of arginine decarboxylase (*adiA*). A 4-5-$\log_2$(FC) upregulation of *adiA* ($P < 0.05$) was observed only in the presence of 2.4 mM UAA (45mM/pH6.0) but not at higher UAA levels (5.4 and 5.5 mM UAA, 35mM/pH5.5 and 15mM/pH5.0, respectively). Similarly, a 4-$\log_2$(FC) ($P < 0.05$) up-regulation of this gene was observed following pH 5.0 and 5.5 adaptation (0mM/pH5.0 and 0mM/pH5.5, respectively), but not at the higher pH value (i.e. 0mM/pH6.0). The relative expression of *cadB* was only UAA-dependent. Higher concentrations of UAA (i.e. 5.4 and 5.5 mM UAA, 15mM/pH5.0 and 35mM/pH5.5, respectively) triggered an ~8-fold change induction of this gene, whereas no effect was observed in the presence of lower UAA levels (i.e 2.4 mM UAA, 45mM/pH6.0) or following pH adaptation (0mM/pH6.0, 0mM/pH5.5 and 0mM/pH5.0) (Figure 3.5).

Contrary to the above results, both master stress regulators (*rpoS*, *ompR*) were 2-3-fold change downregulated following adaptation to 15mM/pH5.0 and 35mM/pH5.5, respectively, containing approximately the same level of UAA (Figure 3.5; Table 3.2).

In summary, the type and magnitude of stress applied during adaption determined the transcriptional levels of the target genes. *adiA* and *cadB* were overexpressed in some of the treatments employed, showing higher susceptibility to variations in the acid adaptive conditions, whereas *ompR* and *rpoS* regulation were affected to a lesser extent.

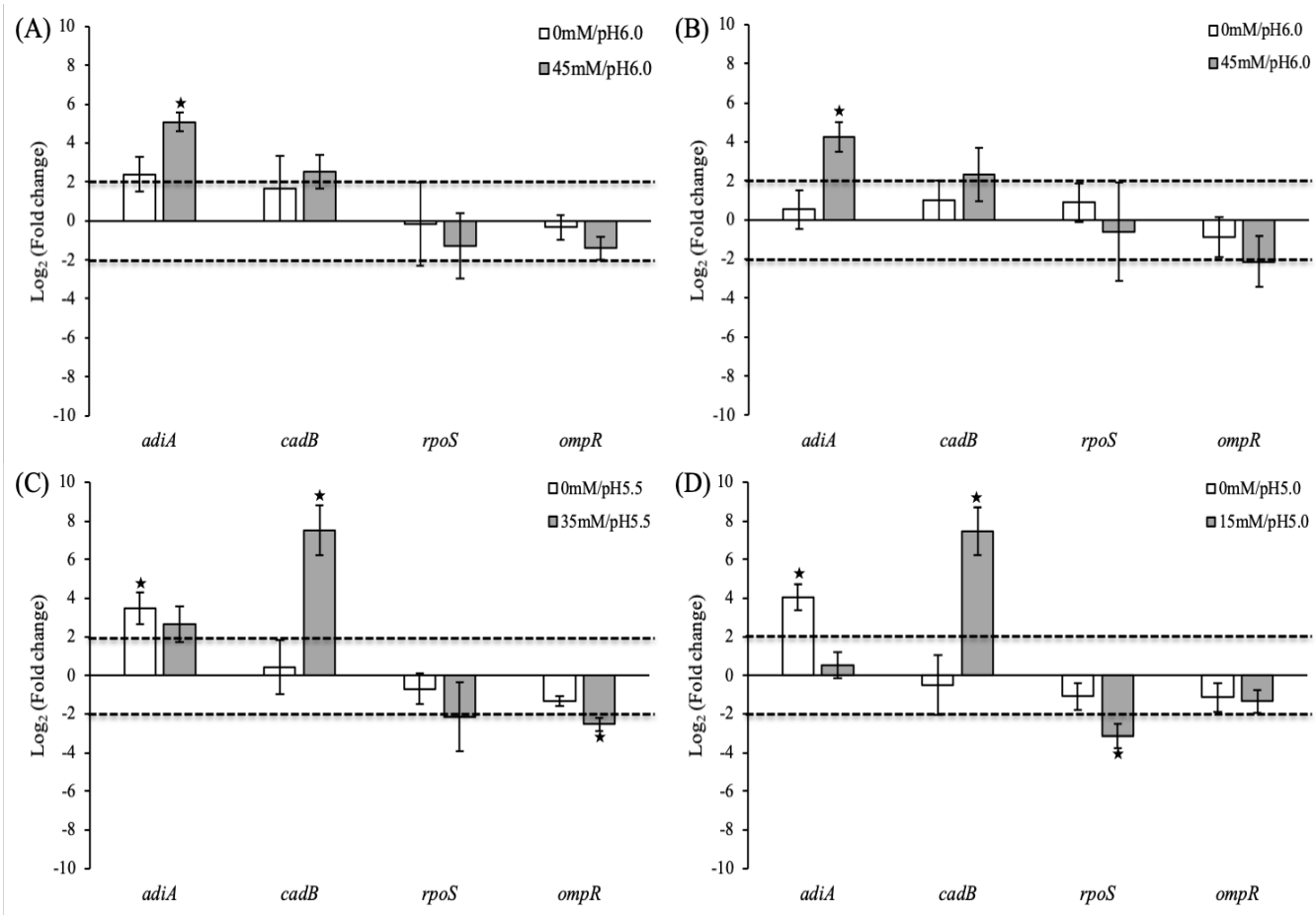

Figure 3.5. Fold changes in mRNA levels of *adiA, cadB, rpoS* and *ompR* following adaptation (90 minutes, 37ºC) of ATCC 13076 strain to 0mM/pH6.0 and 45mM/pH6.0 (A) and WT strain to 0mM/pH6.0 and 45mM/pH6.0 (B), 0mM/pH5.5 and 35mM/pH5.5 (C) and 0mM/pH5.0 and 15mM/pH5.0 (D). Normalization of data was performed using *gmk* as reference gene and samples collected at TSBG(-) treated for 90 minutes at 37ºC without addition of acetic acid or adjustment of pH as reference conditions. Each bar is a mean of eight replicates. Error bars represent the standard deviation of each mean. Star above bars indicates significant up/down-regulation of genes.

Discussion

In the current study, differences in the phenotypic responses of six *Salmonella* strains in mayonnaise stored under refrigeration (5ºC) were assessed and characterized. TSBG(-) was used for cell adaptation, since it is a medium rich in nitrogen sources (it contains tryptone and soy peptone), albeit not containing carbon sources, that may lead to gradual pH reduction due to metabolic products of fermentative metabolism. As such, the acid adaptation of strains was left to take place by subsequent exposure to different

pH and acetic acid levels following growth, without being affected by prior pH-dependent adaptation during growth to stationary phase. Nonetheless, it has been reported that complex media can promote higher acid resistance compared to minimal media (Álvarez-Ordóñez et al., 2009b; Tiwari et al., 2004).

A remarkable variability was observed regarding the innate (i.e., without induction of acid resistance) survival potential of the strains, with non-adapted 4/74 and FS8 and P167807 strains being more resistant compared to FS115, ATCC 13076 and WT. Variations among *Salmonella* strains with regard to their acid resistance have been documented (Humphrey et al., 1995; Jørgensen et al., 2000; Lianou and Koutsoumanis, 2013b). Regarding the effect of refrigeration, it has been shown that cold storage (Álvarez-Ordóñez et al., 2009c) or growth under low (10^{o}C) temperature (Yang et al., 2014b) can increase the membrane fluidity due to changes in membrane fatty acid composition. Decreased membrane fluidity has been linked to higher acid resistance (Yang et al., 2014b). Nevertheless, the results of this study improve our understanding regarding the survival of different *Salmonella* strains in mayonnaise stored under refrigeration. Given that a positive correlation between acid resistance and pathogenicity has been found (Berk et al., 2005), an underestimation of the survival of *Salmonella* in chilled stored mayonnaise may raise food safety issues.

Strain variations in the acid adaptive responses of *Salmonella* have also been found (Berk et al., 2005; de Jonge et al., 2003; Lianou et al., 2017; Malheiros et al., 2009). Nevertheless, the glucose supplementation method (Lianou et al., 2017; Malheiros et al., 2009) or long-time pre-exposure to a given pH value (Berk et al., 2005; de Jonge et al., 2003) were mainly used in order to test the adaptability among strains. In the current study, the ability of six *Salmonella* strains to activate acid defensive mechanisms was initially investigated following short-time pre-exposure to three different pH/UAA combinations. Their *in situ* acid adaptive responses were strain-dependent, with some strains being more adaptable compared to some others. A strain-dependent effect was also observed regarding treatment 15mM/pH5.0. It has been found that adaptation of two *E. coli* strains to lactic, acetic acid and hydrochloric acid, apart from a universal and acidulant-specific response, also promoted a strain specific response in their transcriptomic profiles (King et al., 2010). Additionally, (Melo et al., 2013) reported that three strains of *Listeria monocytogenes* adapted to a cheese stimulated medium, albeit showing no phenotypic differences, had a unique proteomic profile when exposed to gastric stress. Nonetheless, despite the increased adaptability

of ATCC 13076 and WT strains, their survival following acid adaptation was lower compared to the adapted cultures of 4/74, FS8, FS115 and P167807.

In a next step, an effort was made to disentangle the individual effects of pH and UAA adaptation on the subsequent resistance and assess them separately. The treatments were selected based on the prior indication that exposure to 45mM/pH6.0 enhanced survival of both strains in mayonnaise and on the different phenotypic responses observed following adaptation of WT to 15mM/pH5.0 (no impact) and 35mM/pH5.5 (increased survival), containing similar levels of UAA (~5.5 mM). It was shown that the type (pH, UAA) and the magnitude of the stress intensity during adaptation can determine the adaptive responses. Indeed, different acids can promote different extent of acid resistance (Álvarez-Ordóñez et al., 2009b, 2010a). In addition, different stress intensities during adaptation can account for differences in the induced resistance. pH values below 5.0 can maximize *Salmonella* acid resistance (Gavriil et al., 2020; Humphrey et al., 1993; Lee et al., 1994; Ye et al., 2019), whereas induced resistance of *S.* Enteritidis was observed only within a range of sublethal UAA concentrations, but not in higher or lower amounts (Gavriil et al., 2020).

Assessment of mRNA levels of *adiA*, *cadB*, *ompR* and *rpoS* genes was examined in order to test the impact of acid adaptation on the expression of genes with an established key role in the induced acid resistance of *Salmonella* with regard to strain, pH or UAA variations and to assess whether this disposition as expressed *via* gene expression could lead in increased resistance during exposure in mayonnaise at 5^{o}C. Overexpression was observed only in the amino acid decarboxylase-systems related genes (*adiA*, *cadB*). Indeed, pre-exposure to acid conditions can effectively trigger these systems in *Salmonella* and *E. coli* (Álvarez-Ordóñez et al., 2010b; Díaz and Ricke, 2004; Greenacre et al., 2006; Kieboom and Abee, 2006; Kim et al., 2016; J. Liu et al., 2017). Variations in the type and the magnitude of the stress intensity during adaptation determined the relative expression of the target genes, and especially of *adiA* and *cadB*: lower pH or UAA levels were required for the induction of *adiA*, whereas *cadB* regulation was favored in the presence of higher UAA concentrations. Differences in the acid environments can promote different extent of gene expression of the amino acid decarboxylase systems, as shown elsewhere (Díaz and Ricke, 2004; J. Liu et al., 2017). Nonetheless, the transcriptomic profiles did not always correlate with the corresponding phenotypes. For instance, an ~8-fold upregulation of *cadB* was observed

following adaptation to 15mM/pH5.0 (no induced resistance) but not following 0mM/pH5.0 adaptation (induced resistance). In addition, difference in the levels of *cadB* was reported between 35mM/pH5.5 (~8-fold upregulation) and 0mM/pH5.5 (no gene induction) treatments having similar resistance phenotypes. The contribution of arginine/agmatine and lysine/cadaverine systems to the survival against lethal acidity has been primarily linked to the presence of the cognate amino acids in the challenge medium (Álvarez-Ordóñez et al., 2010b; de Jonge et al., 2003; Kieboom and Abee, 2006), that may be absent in mayonnaise, since it is considered a poor nutrient substrate. Other factors include the pH optimum of the decarboxylases *adiA* and *cadA* (Viala et al., 2011; Vivijs et al., 2016) and the oxygen availability during growth (Kieboom and Abee, 2006; Viala et al., 2011). The expression of master stress regulators *ompR* and *rpoS*, on the other hand, were less affected by variations of the acid adaptive treatments. No induction of *ompR* and *rpoS* was observed for the majority of the acid treatments tested, even for those where induced resistance was recorded. According to Chakraborty and Kenney (2018), who also found similar levels both in *ompR* transcripts and OmpR molecules during acid stress at pH 5.6 in *S.* Typhimurium and *E. coli*, the low pH increases the OmpR binding affinity for DNA rather than the number of molecules. Cellular numbers of RpoS, on the contrary, are regulated at multiple levels (transcription, translation, degradation and regulation activity) (Battesti et al., 2011). Adaptation of WT strain in the presence of approximately 5.5 mM UAA downregulated both *rpoS* and *ompR*, though the combination of pH/UAA (15mM/pH5.0 for *rpoS*, 35mM/pH5.5 for *ompR*) required for the mRNA reduction to be manifested and the obtained phenotypes (no induction in 15mM/pH5.0, induced resistance in 35mM/pH5.5) were different in each case. The exact reason for the downregulation of *rpoS* and *ompR* is not known. In general, contradictory results are available pertaining the effect of acid adaptation on the relative gene expression of these genes. For instance, upregulation of *ompR* following pH adaptation has been reported by some authors (Bang et al., 2000; Quinn et al., 2014), whereas constant (Chakraborty and Kenney, 2018) or decreased (Stincone et al., 2011) transcripts have been documented by some others. Similarly, *rpoS* was downregulated in *E. coli* K-12 following acetic and lactic acid adaptation (King et al., 2010), whereas an upregulation of this gene was observed following adaptation to pineapple juice at 4 and 20ºC, with low temperature stimulating higher expression (Kim et al., 2016). In the current study, the relative levels of *rpoS*

and *ompR* were measured only at the end of adaptation period, and, therefore, no data associated with their transcriptional amounts are available during previous time-points. In order to holistically elucidate the role of adaptation conditions on the regulation of these genes, further analysis is required involving the assessment of global gene expression as well as changes in translational (protein) level. This is imperative because the cellular concentrations of RpoS molecules are regulated in multiple levels, as reported previously.

In conclusion, the present study demonstrates strain-dependent variations in the innate and adaptive responses of *Salmonella* spp. that do not allow results obtained from one strain to be universally applied to others. The ability of the strains to adapt and to induce resistance phenotypic responses and acid-resistance related genes can be affected by the type (pH, UAA) and the magnitude of the stress applied during adaptation, even though no direct correlations can always be established between the phenotypic and transcriptomic profiles. Future work could include more strains and focus on assembling their proteomic responses with regard to strain, type and magnitude variations. In addition, given that the time of acid pre-exposure and the type of the acidulant used can highly differentiate the phenotypic responses of *Salmonella*, their effect on the subsequent acid resistance of multiple *Salmonella* strains could also be investigated.

Supplementary data

S3.1 Table. Average log counts (± standard deviation) of *Salmonella* Typhimurium 4/74 non-adapted and adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0 inocula

	Treatment			
Time (h)	**Non-adapted**	**15mM/pH5.0**	**35mM/pH5.5**	**45mM/pH6.0**
0	6.6 ± 0.2 **(a)**	6.7 ± 0.1 **(a)**	6.8 ± 0.1 **(a)**	6.7 ± 0.1 **(a)**
21	6.3 ± 0.2 **(a)**	6.2 ± 0.1 **(ab)**	6.3 ± 0.1 **(ab)**	6.1 ± 0.2 **(b)**
45	5.5 ± 0.3 **(a)**	5.5 ± 0.1 **(a)**	5.5 ± 0.4 **(a)**	4.9 ± 0.5 **(b)**
69	4.3 ± 0.3 **(a)**	4.5 ± 0.3 **(a)**	4.5 ± 0.5 **(a)**	3.7 ± 0.7 **(b)**
93	3.4 ± 0.5 **(b)**	4.2 ± 0.2 **(a)**	3.9 ± 0.4 **(a)**	2.7 ± 0.6 **(c)**
117	2.4 ± 0.7 **(b)**	3.5 ± 0.7 **(a)**	3.4 ± 0.6 **(a)**	2.2 ± 0.6 **(b)**
141	1.9 ± 0.4 **(b)**	2.8 ± 0.9 **(a)**	3.1 ± 0.5 **(a)**	1.8 ± 0.2 **(b)**
165	1.7 ± 0.1 **(bc)**	2.6 ± 0.7 **(a)**	2.2 ± 0.4 **(ab)**	1.7 ± 0.0 **(c)**
189	1.7 ± 0.0 **(b)**	2.1 ± 0.5 **(a)**	2.0 ± 0.3 **(ab)**	1.7 ± 0.0 **(b)**
213	1.7 ± 0.0 **(b)**	2.1 ± 0.5 **(a)**	1.8 ± 0.2 **(ab)**	1.7 ± 0.0 **(b)**
237	1.7 ± 0.0 **(a)**	1.7 ± 0.1 **(a)**	1.7 ± 0.0 **(a)**	1.7 ± 0.0 **(a)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.2 Table. Average log counts (± standard deviation) of *Salmonella* Typhimurium FS8 non-adapted and adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0 inocula

	Treatment			
Time (h)	**Non-adapted**	**15mM/pH5.0**	**35mM/pH5.5**	**45mM/pH6.0**
0	6.5 ± 0.1 **(a)**	6.6 ± 0.2 **(a)**	6.6 ± .2 **(a)**	6.6 ± 0.2 **(a)**
21	6.3 ± 0.1 **(ab)**	6.1 ± 0.2 **(b)**	6.2 ± 0.2 **(ab)**	6.3 ± 0.2 **(a)**
45	5.4 ± 0.5 **(a)**	5.5 ± 0.3 **(a)**	5.5 ± 0.3 **(a)**	5.4 ± 0.2 **(a)**
69	4.4 ± 0.3 **(a)**	4.8 ± 0.3 **(a)**	4.4 ± 0.8 **(a)**	4.1 ± 0.7 **(a)**
93	3.6 ± 0.9 **(a)**	4.0 ± 0.6 **(a)**	3.6 ± 0.5 **(a)**	2.7 ± 0.7 **(b)**
117	2.7 ± 0.9 **(a)**	3.2 ± 1.1 **(a)**	2.5 ± 0.8 **(a)**	2.3 ± 0.7 **(a)**
141	2.0 ± 0.5 **(b)**	2.8 ± 0.5 **(a)**	1.9 ± 0.4 **(b)**	1.8 ± 0.2 **(b)**
165	1.8 ± 0.2 **(b)**	2.3 ± 0.5 **(a)**	1.7 ± 0.0 **(b)**	1.7 ± 0.0 **(b)**
189	1.7 ± 0.0 **(b)**	1.9 ± 0.3 **(a)**	1.7 ± 0.1 **(b)**	1.7 ± 0.0 **(b)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.3 Table. Average log counts (± standard deviation) of *Salmonella* Typhimurium FS115 non-adapted and adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0 inocula

	Treatment			
Time (h)	**Non-adapted**	**15mM/pH5.0**	**35mM/pH5.5**	**45mM/pH6.0**
0	6.7 ± 0.1 **(a)**	6.7 ± 0.2 **(a)**	6.8 ± 0.2 **(a)**	6.7 ± 0.2 **(a)**
21	6.1 ± 0.2 **(a)**	6.2 ± 0.3 **(a)**	6.2 ± 0.3 **(a)**	6.2 ± 0.2 **(a)**
33	5.5 ± 0.2 **(a)**	5.6 ± 0.3 **(ab)**	5.4 ± 0.4 **(ab)**	5.1 ± 0.5 **(b)**
45	3.9 ± 0.8 **(b)**	4.8 ± 0.6 **(a)**	4.4 ± 0.6 **(ab)**	4.2 ± 0.6 **(ab)**
69	2.5 ± 0.3 **(b)**	3.8 ± 0.3 **(a)**	3.4 ± 1.2 **(ab)**	2.7 ±1.0 **(b)**
93	1.7 ± 0.0 **(b)**	2.6 ± 0.4 **(a)**	2.7 ± 1.1 **(a)**	1.8 ± 0.3 **(b)**
117	1.7 ± 0.0 **(b)**	1.8 ± 0.1 **(b)**	2.5 ± 0.9 **(a)**	1.7 ± 0.0 **(b)**
141	1.7 ± 0.0 **(b)**	1.7 ± 0.9 **(b)**	2.1 ± 0.4 **(a)**	1.7 ± 0.0 **(b)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.4 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis P167807 PT4 non-adapted and adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0 inocula

	Treatment			
Time (h)	**Non-adapted**	**15mM/pH5.0**	**35mM/pH5.5**	**45mM/pH6.0**
0	6.6 ± 0.1 **(a)**	6.7 ± 0.2 **(a)**	6.7 ± 0.1 **(a)**	6.7 ± 0.1 **(a)**
21	6.0 ± 0.2 **(c)**	6.2 ± 0.2**(b)**	6.2 ± 0.2**(b)**	6.4 ± 0.1 **(a)**
33	5.6 ± 0.1 **(b)**	5.6 ± 0.2 **(b)**	5.6 ± 0.2 **(b)**	6.1 ± 0.1 **(a)**
45	5.2 ± 0.2 **(ab)**	5.1 ± 0.3 **(b)**	5.0 ± 0.3 **(b)**	5.3 ± 0.2 **(a)**
57	4.4 ± 0.4 **(b)**	4.7 ± 0.2 **(ab)**	4.5 ± 0.1 **(b)**	4.9 ± 0.2 **(a)**
69	4.1 ± 0.8 **(b)**	4.3 ± 0.1 **(a)**	4.0 ± 0.2 **(b)**	4.2 ± 0.1 **(ab)**
81	3.4 ± 0.3 **(c)**	4.1 ± 0.2 **(a)**	4.0 ± 0.1 **(bc)**	3.7 ± 0.2 **(b)**
93	2.5 ± 0.5 **(c)**	3.6 ± 0.3 **(a)**	3.5 ± 0.2 **(a)**	3.0 ± 0.4 **(b)**
117	2.1 ± 0.4 **(b)**	2.9 ± 0.4 **(a)**	2.9 ± 0.4 **(a)**	2.0 ± 0.3 **(b)**
141	1.7 ± 0.0 **(b)**	2.2 ± 0.5 **(a)**	2.2 ± 0.5 **(a)**	1.9 ± 0.2 **(ab)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.5 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis ATCC 13076 non-adapted and adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0

	Treatment			
Time (h)	**Non-adapted**	**15mM/pH5.0**	**35mM/pH5.5**	**45mM/pH6.0**
0	6.2 ± 0.1 **(b)**	6.3 ± 0.2 **(ab)**	6.3 ± 0.2 **(ab)**	6.5 ± 0.2 **(a)**
4	5.4 ± 0.2 **(b)**	5.5 ± 0.4 **(b)**	5.8 ± 0.2 **(ab)**	6.1 ± 0.1 **(a)**
8	4.8 ± 0.3 **(b)**	5.2 ± 0.5 **(b)**	5.2 ± 0.4 **(b)**	6.0 ± 0.3 **(a)**
20	1.9 ± 0.4 **(b)**	3.8 ± 0.8 **(a)**	3.7 ± 0.7 **(a)**	4.4 ± 0.5 **(a)**
26	1.7 ± 0.1 **(b)**	3.3 ± 0.5 **(a)**	3.5 ± 0.5 **(a)**	4.1 ± 0.6 **(a)**
32	1.7 ± 0.0 **(b)**	1.8 ± 0.2 **(ab)**	2.3 ± 0.8 **(a)**	2.3 ± 0.7 **(a)**
46	1.7 ± 0.0 **(a)**	1.7 ± 0.1 **(a)**	1.7 ± 0.1 **(a)**	1.7 ± 0.0 **(a)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.6 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis WT non-adapted and adapted to 15mM/pH5.0, 35mM/pH5.5 and 45mM/pH6.0

	Treatment			
Time (h)	Non-adapted	15mM/pH5.0	35mM/pH5.5	45mM/pH6.0
0	6.0 ± 0.2 **(b)**	6.3 ± 0.1 **(a)**	6.3 ± 0.2 **(a)**	6.4 ± 0.1 **(a)**
4	5.3 ± 0.3 **(c)**	5.6 ± 0.4 **(bc)**	5.9 ± 0.3 **(ab)**	6.1 ± 0.1 **(a)**
8	4.8 ± 0.5 **(c)**	5.2 ± 0.3 **(b)**	5.7 ± 0.4 **(a)**	6.0 ± 0.2 **(a)**
21	2.2 ± 0.7 **(d)**	3.1 ± 0.7 **(c)**	4.2 ± 0.3 **(b)**	4.8 ± 0.3 **(a)**
27	1.7 ± 0.1 **(c)**	2.0 ± 0.4 **(c)**	3.4 ± 0.6 **(b)**	4.1 ± 0.5 **(a)**
33	1.7 ± 0.0 **(c)**	1.8 ± 0.2 **(c)**	2.3 ± 0.6 **(b)**	3.2 ± 0.5 **(a)**
46	1.7 ± 0.0 **(b)**	1.7 ± 0.0 **(b)**	1.8 ± 0.3 **(a)**	1.7 ± 0.5 **(b)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.7 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis ATCC 13076 non-adapted and adapted to 0mM/pH6.0 and 45mM/pH6.0

	Treatment		
Time (h)	**Non-adapted**	**0mM/pH6.0**	**45mM/pH6.0**
0	6.0 ± 0.2 **(b)**	6.5 ± 0.1 **(a)**	6.5 ± 0.1 **(a)**
4	5.4 ± 0.1 **(b)**	6.2 ± 0.1 **(a)**	6.3 ± 0.1 **(a)**
8	4.8 ± 0.2 **(b)**	6.0 ± 0.2 **(a)**	6.1 ± 0.1 **(a)**
20	3.5 ± 0.5 **(c)**	4.8 ± 0.3 **(b)**	5.4 ± 0.2 **(a)**
26	2.4 ± 0.8 **(c)**	4.2 ± 0.6 **(b)**	5.1 ± 0.2 **(a)**
32	1.7 ± 0.1 **(c)**	2.5 ± 0.5 **(b)**	4.2 ± 0.4 **(a)**
46	1.7 ± 0.0 **(b)**	1.7 ± 0.0 **(b)**	2.3 ± 0.5 **(a)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.8 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis WT non-adapted and adapted to 0mM/pH6.0 and 45mM/pH6.0 inocula

	Treatment		
Time (h)	**Non-adapted**	**0mM/pH6.0**	**45mM/pH6.0**
0	6.0 ± 0.2 **(b)**	6.4 ± 0.1 **(a)**	6.5 ± 0.1 **(a)**
4	5.4 ± 0.2 **(b)**	6.2 ± 0.2 **(a)**	6.2 ± 0.2 **(a)**
8	4.9 ± 0.3 **(b)**	5.8 ± 0.3 **(a)**	6.0 ± 0.1 **(a)**
21	2.8 ± 0.9 **(b)**	4.1 ± 0.6 **(a)**	4.7 ± 0.3 **(a)**
27	2.0 ± 0.5 **(c)**	2.9 ± 0.7 **(b)**	3.9 ± 0.3 **(a)**
33	1.8 ± 0.2 **(c)**	2.1 ± 0.3 **(b)**	3.0 ± 0.2 **(a)**
46	1.7 ± 0.0 **(a)**	1.7 ± 0.0 **(a)**	1.7 ± 0.1 **(a)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.9 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis WT non-adapted and adapted to 0mM/pH5.5 and 35mM/pH5.5

	Treatment		
Time (h)	**Non-adapted**	**0mM/pH5.5**	**35mM/pH5.5**
0	6.0 ± 0.2 **(c)**	6.4 ± 0.2 **(a)**	6.3 ± 0.1 **(b)**
4	5.4 ± 0.2 **(c)**	6.3 ± 0.1 **(a)**	6.1 ± 0.1 **(b)**
8	4.9 ± 0.3 **(c)**	6.1 ± 0.2 **(a)**	5.8 ± 0.1 **(b)**
21	3.0 ± 0.9 **(b)**	4.3 ± 0.6 **(a)**	4.1 ± 0.2 **(a)**
27	2.0 ± 0.5 **(c)**	3.2 ± 0.4 **(a)**	2.7 ± 0.3 **(b)**
33	1.8 ± 0.2 **(a)**	2.2 ± 0.5 **(a)**	2.0 ± 0.4 **(a)**
46	1.7 ± 0.0 **(a)**	1.7 ± 0.0 **(a)**	1.7 ± 0.0 **(a)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD test

S3.10 Table. Average log counts (± standard deviation) of *Salmonella* Enteritidis WT non-adapted and adapted to 05mM/pH5.0 and 15mM/pH5.0 inocula

	Treatment		
Time (h)	**Non-adapted**	**0mM/pH5.0**	**15mM/pH5.0**
0	6.1 ± 0.2 **(b)**	6.6 ± 0.2 **(a)**	6.5 ± 0.1 **(a)**
4	5.6 ± 0.2 **(c)**	6.4 ± 0.1 **(a)**	6.2 ± 0.2 **(b)**
8	5.3 ± 0.2 **(c)**	6.3 ± 0.2 **(a)**	5.8 ± 0.2 **(b)**
21	4.4 ± 0.2 **(c)**	5.6 ± 0.2 **(a)**	4.8 ± 0.2 **(b)**
27	4.1 ± 0.3 **(c)**	5.1 ± 0.3 **(a)**	4.4 ± 0.2 **(b)**
33	3.2 ± 0.7 **(b)**	4.6 ± 0.5 **(a)**	3.7 ± 0.4 **(b)**
46	1.9 ± 0.3 **(b)**	2.3 ± 0.6 **(a)**	1.8 ± 0.1 **(b)**

Different letters within the same row indicate statistical differences ($P < 0.05$) among different treatments of the same time interval according to Tukey's HSD tes

CHAPTER 4

Evaluation of antimicrobial activities of plant aqueous extracts against *Salmonella* Typhimurium and their application to improve safety of pork meat

Gavriil Alkmini, Zilelidou Evangelia, Papadopoulos Aggelis-Evangelos, Siderakou Danae, Kasiotis M. Konstantinos, Haroutounian A. Serkos, Gardeli Chryssavgi, Giannenas Ilias, Skandamis N. Panagiotis

Abstract

Ten laboratory-collected hydro-distilled aqueous extracts (basil, calendula, corn silk, centrifuged oregano, garlic, laurel, oregano, rosemary, spearmint, thyme) and one industrial steam-distilled oregano hydrolate acquired as by-products of essential oils purification were evaluated for their *in vitro* antimicrobial activity against three *Salmonella* Typhimurium strains (4/74, FS8, FS115) at 4 and 37ºC. Susceptibility to the extracts was mainly plant- and temperature-dependent. Industrial oregano hydrolate eliminated strains immediately after inoculation, exhibiting the highest antimicrobial potency. Hydro-distilled extracts totally eliminated *Salmonella* at 4°C, apart from basil and garlic. At 37°C, oregano, centrifuged oregano, thyme, calendula and basil were bactericidal; garlic, spearmint, rosemary and corn silk were bacteriostatic. A strain-dependent effect was observed for laurel. The individual or combined effect of marinades and edible coatings prepared of hydro-distilled oregano extract and industrial oregano hydrolate with or without oregano essential oil (OEO) was tested in pork meat at 4°C inoculated with FS8 strain. Lower *in situ* activity was observed compared to *in vitro* assays. Marinades and edible coatings prepared of industrial oregano hydrolate + OEO were the most efficient in inhibiting pathogen. Marination in oregano extract and subsequent coating with either 50% oregano extract + OEO or water + OEO enhanced the performance of oregano extract. In conclusion, by-products of oregano essential oil purification may be promising alternative antimicrobials to pork meat stored under refrigeration when applied in the context of multiple hurdle approach.

Keywords: plant extracts, phenolics, natural antimicrobials, *Salmonella*, marination, edible coatings

Introduction

Naturally derived antimicrobial compounds are increasingly gaining commercial attention as label-friendly alternatives to synthetic food preservatives (Davidson et al., 2013). Among them, phenolic compounds, a diverse group of plant secondary metabolites, exhibit a wide range of physiological properties, including antimicrobial activity against a broad spectrum of pathogenic and spoilage bacteria (Giannenas et al., 2020; Nazir et al., 2017). Phenolic compounds are ubiquitous in plants (Balasundram et al., 2006), with aromatic plants such as herbs and spices being especially rich in their phenolic content (Chun et al., 2005). By-products of plant origin foods (Balasundram et al., 2006; Gyawali and Ibrahim, 2014) and essential oil purification (Poimenidou et al., 2016) are also good sources of phenolics. Therefore, their commercial exploitation can provide an economical and environmentally-friendly way to enhance food safety (Gyawali and Ibrahim, 2014; Nazir et al., 2017), especially when considering that the volume and economic burden of agro-industrial by-products processing are substantial (Oreopoulou and Tzia, 2007). Nevertheless, limited research is available regarding their potential antibacterial activity (Gyawali and Ibrahim, 2014; Nazir et al., 2017).

The *in vitro* antimicrobial effect of plant extracts has been widely documented. However, fewer studies are available pertaining their *in situ* efficacy, probably due to the reduced effectiveness of plant extracts in food products (Negi, 2012). Indeed, due to the complex and diverse nature of food environments, the extrapolation of the *in vitro* results to food products cannot be ensured (Negi, 2012) at concentrations that maintain antimicrobial potential without compromising the sensory properties. Therefore, the *in situ* evaluation of their antimicrobial profile, as well as seeking alternative ways to optimize their efficacy, such as combining different treatments, are of utmost important for the systematic application in food matrices.

Apart from their application in extending the shelf life of food products (Choulitoudi et al., 2016; Shahamirian et al., 2019), phenolic compounds have been used for improving food safety, e.g., by promoting inactivation or growth inhibition of foodborne pathogens (Hayrapetyan et al., 2012; Poimenidou et al., 2016). Among foodborne pathogens, *Salmonella* spp. is the second most common cause of reported zoonosis in Europe (EFSA and ECDC, 2018) and the leading cause of hospitalization in the States (Scallan et al., 2011). With more than 2500 distinct serotypes, this pathogen consists a major health problem worldwide (Pui et al., 2011), evolved to

survive in a wide range of environments and across multiple hosts (Foley et al., 2013). Its prevalence has been highly associated with products of animal origin, such as eggs, meat and poultry (EFSA and ECDC, 2018). *S.* Typhimurium is one of the most common serovars isolated from pig meat (EFSA and ECDC, 2018).

Differences in the innate characteristics among strains of the same species identically treated consist a major source of variation in microbiological studies, referred to as strain variability (Whiting and Golden, 2002). Differences in the phenotypic responses among stains of foodborne pathogens with regard to their inactivation potential can be extensive and therefore, should systematically be taken under consideration (Lianou and Koutsoumanis, 2013a).

Considering the above, the current study aimed primarily to screen the *in vitro* antimicrobial effect of ten laboratory (hydro-distilled) and one industrial (steam-distilled hydrolate) plant aqueous extracts acquired as by-products of essential oil purification procedure, against three strains of *Salmonella* spp. The second part aimed to evaluate the *in situ* antimicrobial activity of hydro-distilled oregano extract and industrial oregano hydrolate on improving the safety of pork meat against *Salmonella*.

Materials and Methods

Bacterial strains and inoculum preparation

Eleven plant aqueous extracts were screened for their *in vitro* antimicrobial profile against three different strains of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (Table 1). Stock cultures were maintained in Tryptone Soy Broth (TSB, Lab M Limited, Lancashire, UK) at -20ºC supplemented with 20% glycerol and were monthly subcultured to Tryptic Soy Agar (TSA, Lab M Limited, Lancashire, UK) stored at 0-4ºC. Before experiments, one colony from each strain was transferred into 10 ml TSB (37 ºC, 24 h), followed by a second transfer of 100 μl from 24-h cultures to 10 ml of fresh TSB (37 ºC, 18h). Before their use, activated cells were centrifuged (2709 X g, 10 min, 4 ºC), washed twice with ¼ Ringer solution (Lab M Limited, Lancashire, UK) and resuspended in 10 ml of the appropriate medium (aqueous extracts, de-ionized sterile water, TSB, TSB adjusted to pH 5.5 or 3.5, hydro-distilled oregano extract adjusted to pH 3.5). Antimicrobial activity of hydro-distilled oregano extract and industrial oregano hydrolate in pork meat was evaluated using only FS8

strain. The strain was prepared as described above and resuspended in 10 ml of ¼ Ringer solution.

Table 4.1: Strains used in the current study and their origin

Strains	Origin/Isolation source
Salmonella Typhimurium 4/74	Calf bowel
Salmonella Typhimurium FS8	Pork minced meat (food industry)
Salmonella Typhimurium FS115	Chicken meat (food industry)

Extraction of aqueous plant extracts

Oregano, thyme, calendula, basil, laurel, rosemary, spearmint, corn silk, and garlic were purchased in a dried form from a local supermarket. Each plant was mixed with de-ionized water at a 1:10 ratio and hydro-distilled in a Clevenger apparatus for 3 hours, according to Gardeli et al. (2008). After the end of hydro-distillation, both essential oil and hydrolate were discarded, whereas the remaining liquid waste where the plant was boiled was collected by removing the solid retentate. Centrifuged oregano extract was collected by centrifuging the hydro-distilled oregano extract (2709 X g, 5 min, 4 °C) and using the supernatant as a new antimicrobial aqueous phase. All the aqueous fractions were covered with silver foil and stored at 4 °C for 48 hours before further use.

An industrially acquired oregano hydrolate collected as by-product of oregano essential oil steam distillation was also used throughout this study. The aqueous extract was purchased by an industry in Northern Greece and was transferred to the laboratory. The extract was covered in silver foil and stored at 4 °C until use.

In vitro antimicrobial activity of plant extracts

The activated cultures of *Salmonella* strains were serially diluted and inoculated in the appropriate medium (aqueous extracts, de-ionized sterile water, TSB, TSB adjusted to pH 5.5 or 3.5, hydro-distilled oregano extract adjusted to pH 3.5) at a final concentration of approximately 10^6 CFU/ml. De-ionized water and TSB were used as controls. Inoculation in TSB adjusted to pH 5.5 or 3.5 (HCl 6 N) (Merck, Darmstadt, Germany) was performed in order to simulate the lower pH values of hydro-distilled

extracts (5.2-5.6) and industrial oregano hydrolate (3.5), respectively. The impact of different pH values between the hydro-distilled oregano extract and industrial oregano hydrolate was also investigated by adjusting the pH of the former extract at 3.5 (HCl 6 N). The inoculated solutions were incubated at either 37 or 4 °C; sampling was performed at 0, 3, 6, 9 and 24 hours or at regular time intervals for up to 44 days (depending on the plant extract), respectively. Enumeration was carried out by plating 50 µl of the appropriate dilution on TSA supplemented with 0.1 % sodium pyruvate (AppliChem, Lot 600104 67) (TSA/SP). The detection limit was set at 1.3 log CFU/ml. Petri dishes were incubated at 37 °C for 48 hours.

All experiments were conducted in triplicate in two independent replicates.

Preparation of antimicrobial edible coatings

Edible coatings made of sodium alginate 1.5 % w/v were prepared using water, hydro-distilled oregano extract and industrial oregano hydrolate. For the preparation of the coatings, appropriate quantity of the sodium alginate base was added to the extracts, previously heated at 50-60ºC, and stirred until its total dissolution. Coatings prepared of hydro-distilled oregano extract did not form the film matrix when immersed to $CaCl_2$. Therefore, preparation of these coatings was performed either by mixing the extract with de-ionized sterile water in a ratio 1:1 (50% oregano), by using centrifuged oregano extract as the film forming material or by using their combination (i.e centrifuged oregano extract diluted 1:1 in de-ionized sterile water, 50% centrifuged oregano). Industrial oregano hydrolate was also diluted 1:1 in de-ionized water (50% industrial) in order to further compare their activity. Coatings prepared of de-ionized water were used as control. All coatings were supplemented with or without 0.2 or 0.5 % v/v oregano essential oil (OEO). Incorporation of OEO into the extract alginate solutions was performed using an Ultra Turrax (5 min, 26000 rpm) under aseptic conditions. In summary, the coatings used were the followings: de-ionized sterile water, de-ionized sterile water + OEO, 50% oregano, 50% oregano + OEO, centrifuged oregano, centrifuged oregano + OEO, 50% centrifuged oregano, 50% centrifuged + OEO, industrial oregano, industrial oregano + OEO, 50% industrial oregano, 50% industrial oregano + OEO.

Meat preparation and inoculation

Pork meat thigh was purchased from a local supermarket and transferred to the laboratory within 20 minutes. Pieces of 10 ± 0.2 g were aseptically cut and stored at 4 °C until their use. For their inoculation, 100 μl of the culture were gently spread on the entire upper surface of the sample and allowed to adhere for 15 minutes at 4 ºC. The same process was carried out for the bottom surface of the samples, followed again by a fifteen-minute adhesion of the inoculum. The final cell concentration of *Salmonella* on the meat was approximately 10^6 CFU/g.

Evaluation of oregano aqueous extracts antimicrobial activity against S. Typhimurium FS8 on pork meat:

After their inoculation with *S.* Typhimurium FS8, pork meat samples were subjected to three types of antimicrobial treatments, all based on hydro-distilled oregano extract and industrial oregano hydrolate: a) marination, b) edible coatings, c) combined marination and edible coatings.

a) *Evaluation of marination antimicrobial activity against S. Typhimurium FS8 on pork meat*

Hydro-distilled oregano extract, industrial oregano hydrolate and de-ionized sterile water (control) supplemented with or without 0.2 % oregano essential oil were used as marinating solutions and tested for their antimicrobial activity against FS8 strain. Inoculated pork samples were immerged in 300 ml of each solution and marinated for 3 h at 25 °C (room temperature). Samples were placed in capped petri dishes and stored at 4 °C for four days. Sampling was performed immediately after treatment and after four days of storage.

b) *Evaluation of edible coatings antimicrobial activity against S. Typhimurium FS8 on pork meat*

Sodium alginate (1.5% w/v) edible coatings of de-ionized sterile water, de-ionized sterile water + 0.5% OEO, 50% oregano, 50% oregano + 0.5% OEO, centrifuged oregano, centrifuged oregano + 0.5% OEO, 50% centrifuged oregano, 50% centrifuged + 0.5% OEO, industrial oregano, industrial oregano + 0.5% OEO, 50% industrial oregano, 50% industrial oregano + 0.5% OEO were prepared as described in subsection 2.4. Inoculated pork samples were first immersed in the coating solution for 2 minutes, allowed to drain for 2-3 seconds and then dipped into calcium chloride

($CaCl_2$) solution (2% w/v) for 3 minutes. Finally, they were placed in capped petri dishes and stored at 4 °C for five days. Sampling was performed immediately after coating and once per day for up to 5 days of storage.

c) *Evaluation of combined marination and edible coatings antimicrobial activity against S. Typhimurium FS8 on pork meat*

Inoculated pork meat samples were marinated with hydro-distilled oregano extract, industrial oregano hydrolate or de-ionized sterile water supplemented with or without 0.2% OEO as described in (a) and then covered with sodium alginate (1.5% w/v) edible coatings made of de-ionized sterile water, de-ionized sterile water + 0.2% OEO, 50% oregano, 50% oregano + 0.2% OEO, industrial oregano, industrial oregano + 0.2% OEO, as described in (b) and subsection 2.4. Samples were finally placed in capped petri dishes and stored at 4 °C for four days. Sampling was performed immediately after marination and on the 2nd and 4th day of storage.

In each of the above experiments, uninoculated untreated samples and inoculated untreated samples were used as controls. Experiments were performed in duplicate in three independent replicates.

Microbiological analysis

Microbiological analysis of pork samples was performed by homogenizing each sample (10 ± 0.2 g) with 90 ml ¼ Ringer solution in a stomacher apparatus (Seward, London, UK) for 60 seconds. Enumeration of surviving *Salmonella* populations was performed by plating 100 μl of the appropriate dilution in Xylose Lysine Decarboxylase (XLD; Lab M Limited, Lancashire, UK). Plates were incubated for 24 h at 37 °C.

pH measurement

Changes in the pH of pork samples were determined at the end of microbiological analysis by immersing the electrode of a digital pHmeter (pH 526, Metrohm Ltd, Switzerland) in the homogenized pork samples.

Composition of the aqueous extracts

Chemical standards and solvents

Chemical standards were purchased from Extrasynthese (Genay Cedec, France), Sigma-Aldrich (St. Louis, MO, USA), Alfa Aesar (Haverhill, MA, USA) and Fluka Chemie (Buchs, Switzerland) with purity above 90%. LC-MS grade solvents and

analytical grade ethanol were obtained from Merck (Darmstadt, Germany). For the chromatographic analysis HPLC-grade water was prepared using a Milli-Q system (Merck Millipore, Burlington, MA, USA). Prior to use, all solvents (except acetonitrile) were filtered through cellulose acetate membranes of 0.45 μm pore size.

Stock Solutions

The stock solutions of samples and analytical standards were prepared by dissolution in methanol at concentration of 1000 μg/ml (in limited cases the solubility was enhanced with the addition of low amount of dimethylsulfoxide, DMSO) and maintained at -18 °C in the absence of light. These solutions were diluted and utilized for the construction of calibration curves immediately prior to the performance of the analysis. For each sample, duplicate analyses were carried out by direct injection into the analytical instruments.

Preparation of hydro-distilled aqueous extracts

The hydro-distilled aqueous extracts, obtained as described in 2.2 subsection, were freeze-dried (Scientz-18N, Freeze dryer, Ningbo Scientz Biotechnology Co., Ltd., China) to provide the respective samples as the dry powders that were subsequently subjected to chemical analysis.

Preparation of steam-distilled aqueous extract (industrial oregano hydrolate)

The sample of steam-distilled industrial oregano hydrolate, was prepared by mixing 35 ml of the extract with an equal quantity of pentane (Carlo Ebra Reagents, Val de Reuil, France) and agitated until all gases were removed. This procedure was repeated twice and the combined organic phases were removed using a rotary evaporator (Rotavapor R-210, Büchi, Flawil, Switzerland).

Analytical Method Validation

The validation of the analytical method developed was performed through the construction of calibration curves utilizing at least 6 points (with 3 replicates per level) for every analyte. The linear calibration curves in the studied concentration range of 10 to 2000 ng/ml, demonstrated acceptable correlation coefficient values ($r^2 \geq 0.99$) for every analyte. Recovery of the investigated compounds (as a criterion of the trueness of the method) was evaluated at two concentration levels (40 and 400 ppb) by the

addition of mixed solutions of the standards into the respective extract and were within the acceptable range of 80%−120%. Considering that the sampled plant material (oregano, thyme, rosemary etc.) contains numerous of the studied analytes, the method validation was verified by utilizing the standard addition procedure. Precision values were always acceptable with percent Relative Standard Deviation (RSD%) < 14%.

Determination of bioactive phytochemicals content of hydro-distilled aqueous extracts using UPLC-HESI-MS/MS

The Ultra High-Performance Liquid Chromatography Heated Electrospray Ionization - Tandem Mass Spectrometry (UPLC-HESI-MS/MS) analysis were carried out on an Accela Ultra High-Performance Liquid Chromatography system (Thermo Fisher Scientific, Waltham, MA, USA), coupled with a TSQ Quantum Access triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode and equipped with an autosampler (Thermo Fischer Scientific, San Jose, CA, USA). Mass spectrometric analysis was conducted using heated electrospray ionization (HESI), operated in two complementary modes (positive and negative). Additionally, the Selected Ion Monitoring (SIM) mode was adequate and used to confirm the presence of several analytes. Ion source and vacuum parameters of the mass spectrometer, precursor and product ions and the collision activated ionization for the target analytes, were obtained-optimized by the direct infusion in full scan mode of their standard solutions. The spray voltage was set at 2700 V, sheath gas (nitrogen) and auxiliary gas (argon) pressures were set at 25 and 10 arbitrary units, respectively. Capillary temperature was set at 320 °C and collision pressure at 1.5 mTorr. The monitoring ion transitions are presented in Table 4.2. Phytochemicals were separated on a Hypersil Gold 3 μm, 100×2.1mm i.d. chromatographic column (Thermo Fischer Scientific, San Jos, CA), using a a flow rate of 300μl/min, and a mobile phase consisting of: water (A) and acetonitrile (B), both containing formic acid (0.1%). The gradient program was: 0.0–1.0 min: 10% B, 1.0–12.0 min from 10% B to 100%, 12.0-12.1 min 10%B, and 12.1–14.0 min 10% B. The injection volume was 10 μL, maintaining a column temperature at 35 °C.

Table 4.2. MRM transitions and the utilized respective parameters

Compound	Precursor ion (m/z)	Quantitation ion, m/z (collision energy, eV)	Confirmation ion(s), m/z (collision energy, eV)	Ionization mode
Rutin	609	301 (15)	271 (21)	(-)
Quercetin	300,9	179 (20)	151 (20)	(-)
Chlorogenic acid	353.1	191 (15)		(-)
Syringic acid	197.1	179 (18)	135 (15)	(-)
Naringenin	271	151 (21)	253 (20)	(-)
Ellagic acid	300.8	283.8 (30)	228.9 (28)	(-)
Caffeic acid	181	178 (12)	134 (14)	(+)
Rosmarinic acid	359	161 (18)	197 (15)	(-)
Luteolin	285	151 (24)	199 (20), 132,8 (27)	(-)
Apigenin	269	117 (30)	151 (30)	(-)
Hesperidin	610.9	303.1 (21)	465.1 (17)	(+)
Kaempferol	285	255 (28)	117 (28)	(-)
Gallic acid	168.9	125.2 (12)	79.2 (17)	(-)
Pinocembrin	256.1	152.9 (21)		(+)
p-Coumaric acid	163	119 (20)	93 (22)	(-)
Myricetin	317	179.2 (23)	151.3 (24)	(-)
Orientin	447.2	327 (19)		(-)
Catechin	288.3	109 (25)		(-)
Vitexin	431	311 (18)		(-)
Hyperoside	463.1	300.1 (16)		(-)
Diosmin	609	463 (21)		(+)
Phloridzin	435	167 (28)		(-)
Adipic acid	145.1	101.1 (20)		(-)

HPLC-DAD method for the determination of steam-distilled industrial hydrolate

The carvacrol and thymol content of industrial oregano hydrolate was determined by High Performance Liquid Chromatography coupled to Diode Array detector (HPLC-DAD). The analysis was performed on an Agilent 1100 instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode-array detector (measuring absorbance over the full wavelength range during the entire run). The column used was a Kromasil C18 column (250mm x 4.6mm, particle size 5 μm), thermostated at 30 °C, and connected with a guard column of the same material (8mm x 4 mm). Injection was performed through a Rheodyne injection valve (model 7725I) with a 20 μl fixed loop. For the chromatographic analysis HPLC-grade water was prepared using a Milli-Q system (Merck Millipore, Burlington, MA, USA), whereas all HPLC grade solvents (except acetonitrile) were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size. The mobile phase consisted of solvent A (obtained by the addition of 3% acetic acid in 2 mM sodium acetate aqueous solution) and solvent B (acetonitrile). Run time was set at 70 min with a constant flow rate at 1.0 ml/min in accordance with the following gradient: 0 min, 95% A and 5% B; after 45 min, the pumps were adjusted to 85% A and 15% B; at 60 min, 65% A and 35% B; at 65 min, 50% A and 50% B; and finally, at 70 min, 100% B. This routine was followed by a 30 min equilibration period. Peaks were identified by comparing their retention times and UV–vis spectra with the reference compounds, and data were quantitated using the calibration curves obtained for the reference analytes. HP ChemStation 5.01 software package system (Hewlett Packard, Palo Alto, CA, USA) was used for data acquisition and analysis.

Determination of inactivation parameters

Determination of the time needed for a 4 log reduction (4D) for the microbial populations incubated at 4°C was calculated by fitting the log transformed inactivation data to Weibull model according to the equation: $\log N/N_o = -(t/\delta)^p$ (Mafart et al., 2002), where N_o the population at time t_o, N the population at time t, N_{res} the residual bacterial concentration log CFU, δ the time needed for the first decimal reduction and p a shape parameter corresponding to different concavities (downward concave survival curves for p>1, upward concave survival curves for p<1 or linear curves for p=1). GinaFit, a

freeware Add-in for Microsoft ®Excel (Geeraerd et al., 2005) available at https://cit.kuleuven.be/biotec/software/GinaFit was used for data fitting. In total, six curves per experimental case were fitted

Statistical analysis

Analysis of Variance (SPSS 22.0 for Mac) was used for data analysis of the log transformed cell populations collected from the *in vitro* and *in situ* treatments. Means were compared using Tukey's Honestly Significant Test (HSD) and differences were considered significant at 95% level.

Results

In vitro antimicrobial activity

Eleven plant aqueous extracts acquired as by-products of essential oil production, either hydro-or steam-distilled, were screened for their *in vitro* antibacterial profiles against three strains of *S.* Typhimurium at 4 and 37 °C. The chemical composition of the extracts is presented in Table 4.3. Hydro-distilled aqueous extracts were consisted mainly of phenolic compounds, such as flavonoids and phenolic acids (e.g. hesperidin, luteolin, rosmarinic acid, chlorogenic acid). On the other hand, industrial oregano hydrolate collected by steam-distillation was composed of carvacrol (92.3 %) and to a lesser concentration of thymol (7.1 %).

Table 4.3: Composition of hydro-and steam-distilled aqueous extracts based on UPLC-HESI-MS/MS and HPLC-DAD analysis, respectively. The main compound of each extract is indicated in bold.

Compound	Basil	Calendula	Corn silk	Centrifuged Oregano	Garlic	Laurel	Oregano	Rosemary	Spearmint	Thyme	Industrial oregano
Adipic acid[1]	nd	32250.0 ± 500.3	nd	nd	nd	nd	nd	nd	nd	nd	nd
Apigenin[1]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Caffeic acid[1]	nd	nd	nd		61.8 ± 9.4		nd	nd	nd	171.7 ± 20.5	nd
Catechin[1]	nd	nd	nd	2449.1 ± 442.3	**163768.8 ± 7732.5**	**15496.6 ± 2137.3**	nd	nd	nd	nd	nd
Chlorogenic acid[1]	38.9 ± 5.7	85.7 ± 10.2	nd	74.1 ± 9.7	nd	nd	nd	30.3 ± 4.8	1604.0 ± 71.2	nd	nd
Diosmin[1]	nd		nd	49.8 ± 12.6	nd	nd	nd	nd	795.0 ± 90.2	nd	nd
Ellagic acid[1]	nd	298.2 ± 17.5	nd	nd	nd	nd	nd	nd	nd	nd	nd
Gallic acid[1]	nd	nd	nd	nd	nd	479.0 ± 29.9	nd	nd	911.6 ± 34.9	nd	nd
Hesperidin[1]	nd	nd	nd	nd	nd	nd	273.7 ± 37.6	nd	6576.1 ± 430.3	**2087.0 ± 315.6**	nd
Hyperoside[1]	nd	nd	nd	nd	nd	nd	123.8 ± 20.8	nd	nd	nd	nd
Luteolin[1]	nd	**396.7 ± 31.0**	393.4 ± 44.1	nd	nd	nd	nd	nd	404.6 ± 59.5	nd	nd
Myricetin[1]	nd	nd	nd	nd	nd	nd	nd	87.1 ± 7.2	133.4 ± 15.0	nd	nd

Naringenin[1]	nd	nd	nd	nd	nd	nd	27.5 ± 5.0	nd	nd	nd	nd
Orientin[1]	nd	nd	579.4 ± 59.4	45.8 ± 8.0	nd	nd	nd	nd	4559.9 ± 235.5	nd	nd
Phloridzin[1]	nd	nd	nd	nd	nd	nd	180.1 ± 17.2	nd	nd	nd	nd
Pinocembrin[1]	nd	nd	nd	nd	nd	nd	nd	nd	34.7 ±5.4	nd	nd
Protocatechuic acid[1]	nd	nd	nd	**8759.5 ± 87.2**	nd	nd	nd	nd	nd	nd	nd
Quercetin[1]	nd	nd	233.2 ± 39.2	nd	nd	nd	nd	nd	nd	nd	nd
Rosmarinic acid[1]	**12164.7 ± 435.6**	nd	**3451.3 ± 120.3**	1610.6 ± 90.8	nd	nd	**2730.3 ± 195.5**	**1783.6 ± 170.0**	**54977.7 ± 2301.8**	212.4 ± 40.4	nd
Rutin[1]	nd	nd	nd	nd	nd	1961.2 ± 203.8	382.0 ± 41.4	nd	1961.2 ± 69.9	nd	nd
Syringic acid[1]	nd	nd	nd	344.6 ± 46.8	nd	nd	nd	nd	31994.2 ± 1002.6	nd	nd
Carvacrol[2]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	92.3
Thymol[2]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	7.1

[1]: ng/g

[2]: %

nd: not detected

A marked variability was observed in their antimicrobial potential of the extracts. Their antimicrobial activity was mainly dependent on plant and incubation temperature. Industrial oregano hydrolate had the highest bactericidal potency, irrespectively of the incubation temperature. It reduced strains cell densities below the threshold of detection (1.3 log CFU/ml) immediately after inoculation. Industrial oregano hydrolate also reduced pathogen more rapidly compared to TSB and hydro-distilled oregano extract both adjusted to pH 3.5, indicating a higher antimicrobial potency of this extract compared to pH controls (Figures 4.1 & 4.2).

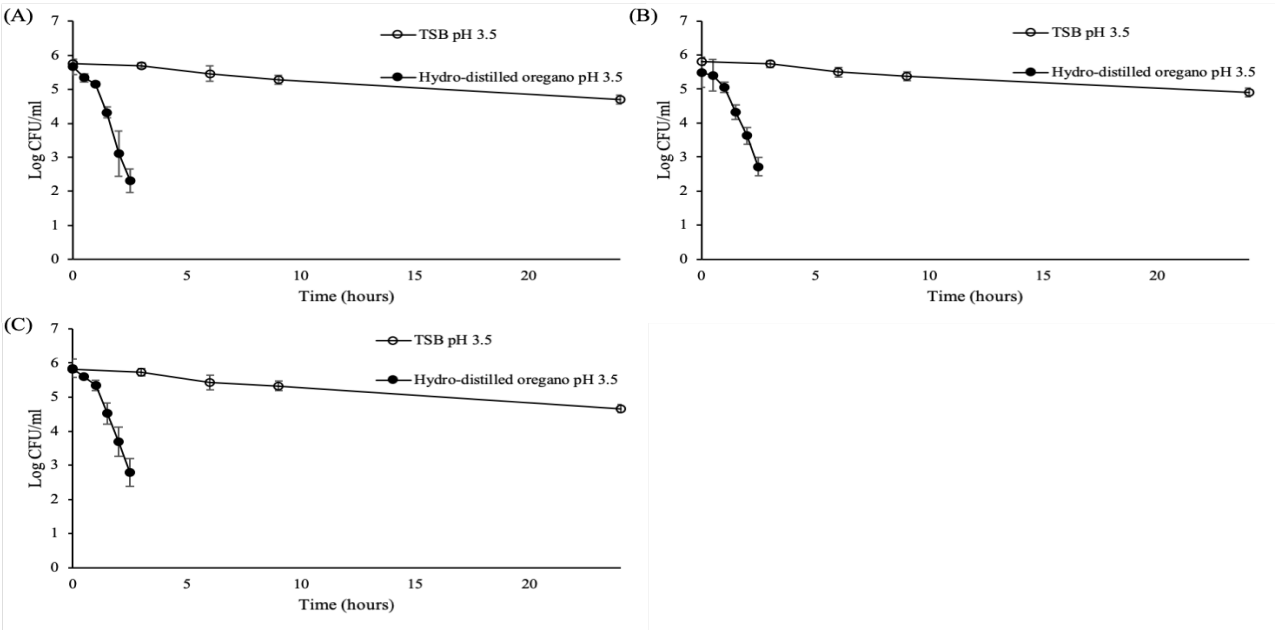

Figure 4.1: *In vitro* inactivation of *S.* Typhimurium (A) 4/74, (B) FS8 and (C) FS115 in TSB and hydro-distilled oregano extract, both adjusted to pH 3.5 with HCl 6 N, at 4 ^{o}C. Each data point is a mean of 6 replicates (± standard deviation).

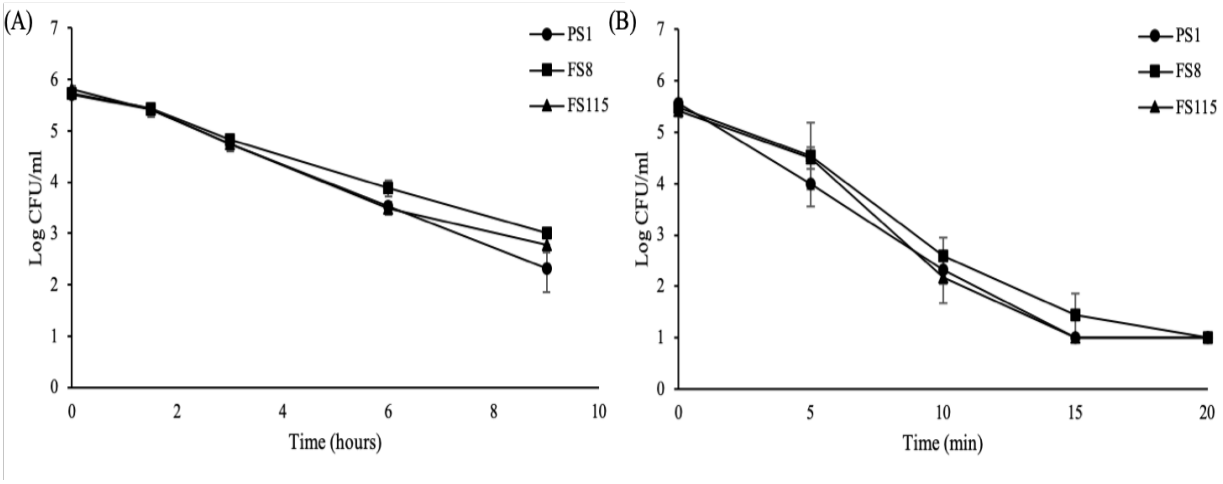

Figure 4.2: *In vitro* inactivation of *S.* Typhimurium 4/74, FS8 and FS115 in (A) TSB and (B) hydro-distilled oregano extract, both adjusted to pH 3.5 with HCl 6 N, at 37 ºC. Each data point is a mean of 6 replicates (± standard deviation).

Among the hydro-distilled aqueous extracts, oregano exhibited the strongest antimicrobial activity in both temperatures, followed by thyme, calendula and centrifuged oregano extracts with activities that varied dependent on the temperature and the strain used (Figures 4.3 & 4.44). On the other hand, corn silk and garlic had the lowest/no impact on the survival of *Salmonella* strains within the incubation period at both temperatures (Figures 4.3 and 4.4). At 4 °C, all extracts effectively reduced pathogen levels, apart from garlic, which did not cause any decline to the initial population during incubation period (Figure 4.3). The time needed for a four-log reduction (4D) during incubation at 4ºC is presented in Table 4.4 and the order of antimicrobial potential based on the 4D's estimates in Table 4.5. No reduction was observed when cultures were inoculated to TSB and TSB adjusted to pH 5.5 (controls). On the other hand, a 2.0 log CFU/ml reduction was observed during incubation to de-ionized water, although this reduction was lower compared to the effect of the extracts (Figure 4.3). No strain-dependent effect was evident for the majority of extracts, even though significantly ($P < 0.05$) higher populations of approximately 1.0 log CFU/ml were enumerated for strain FS8 in calendula extract at 7th and 8th day of incubation compared to FS8 and FS115 strains.

At 37 °C, oregano, centrifuged oregano, thyme, calendula and basil decreased pathogen population within 24 hours of incubation (Figure 4.4). Oregano almost

eliminated initial populations within the first nine hours of incubation. Basil, calendula, centrifuged oregano and thyme extracts, also reduced/inactivated *Salmonella* within 24 hours of incubation, though at a lower rate compared to oregano (Figure 4.4). On the other hand, garlic, corn silk, spearmint and rosemary had only a bacteriostatic effect, permitting pathogen growth in a lower growth rate and up to lower final populations ($P < 0.05$) compared to the controls (TSB and TSB adjusted to pH 5.5) (Figure 4.4). The highest number of survivors were enumerated in garlic and the lowest in rosemary (Figure 4.4). A strain-dependent effect was observed for laurel aqueous extract: FS8 strain was increased by 1.3 log units, while strains 4/74 and FS115 were reduced by 2.2 and 1.5 log units, respectively (Figure 4.4).

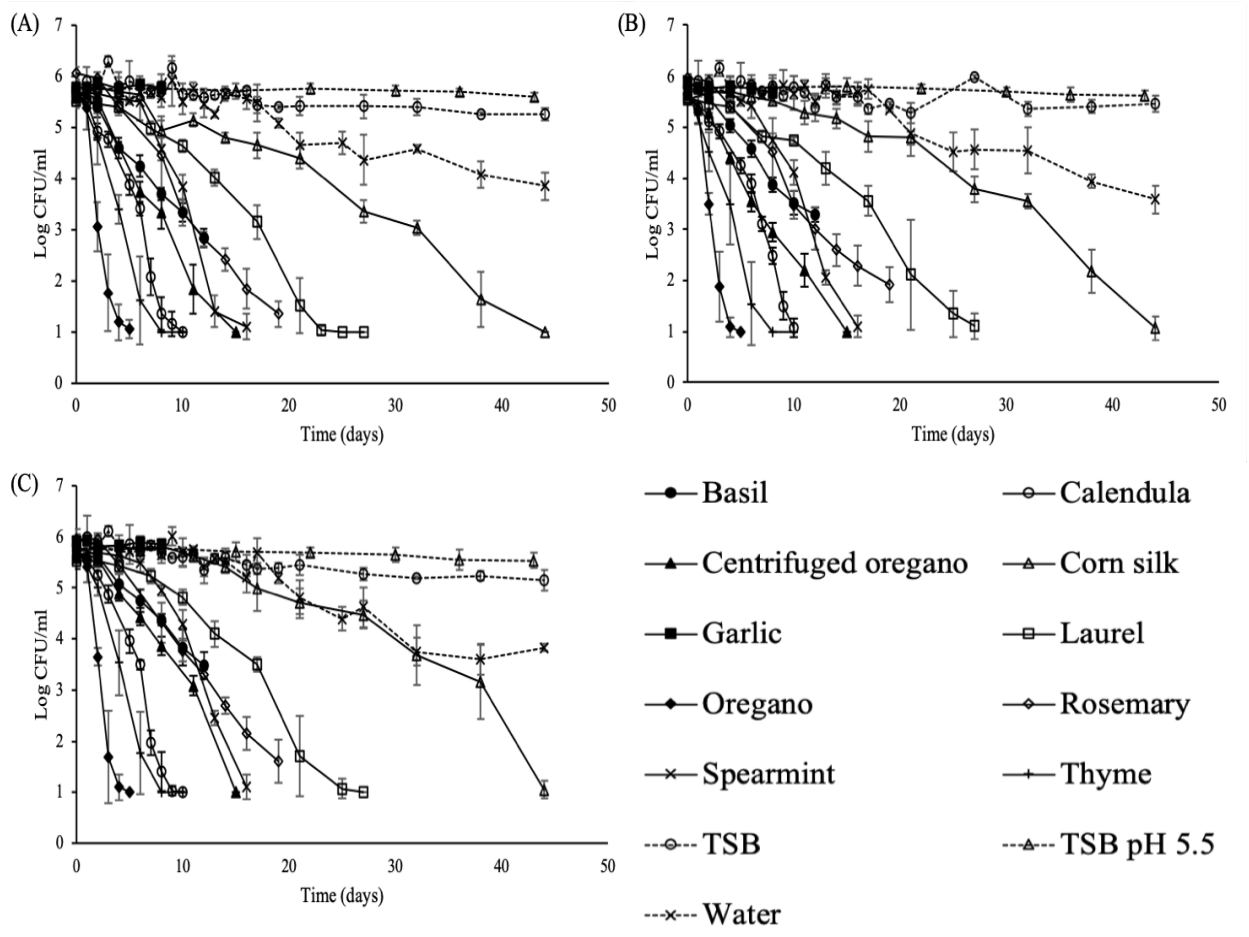

Figure 4.3: Inactivation kinetics of *S.* Typhimurium (A) 4/74, (B) FS8 and (C) FS115 in ten different plant aqueous extracts incubated at 4 ^{o}C. Extraction was carried out by hydro-distillation. TSB, TSB adjusted to pH 5.5 and de-ionized sterile water were used as controls. Each data point is a mean of at least 6 replicates (± standard deviation).

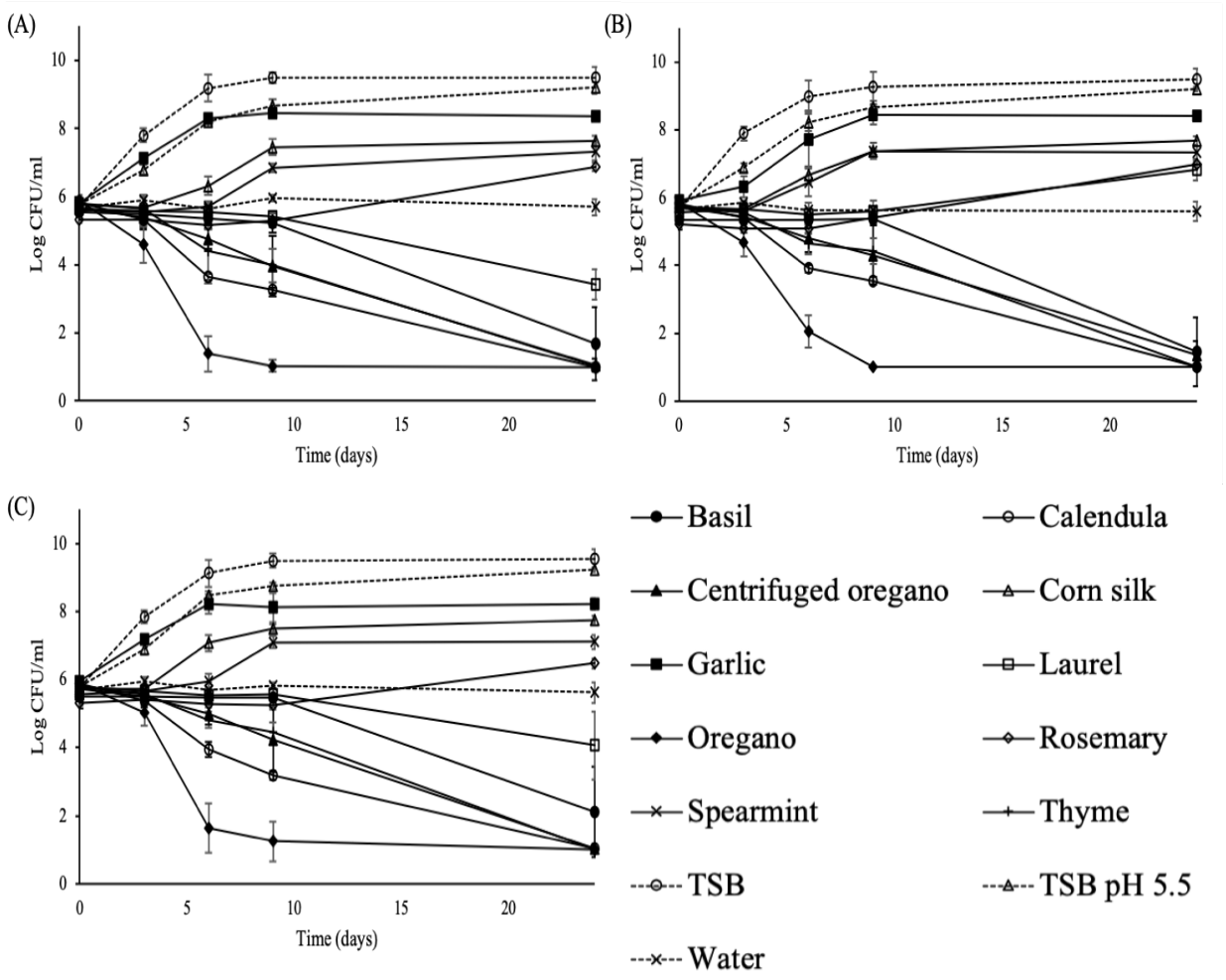

Figure 4.4: Inactivation or growth inhibition kinetics of *S.* Typhimurium (A) 4/74, (B) FS8 and (C) FS115 in ten different plant aqueous extracts incubated at 37 ºC. Extraction was carried out by hydro-distillation. TSB, TSB adjusted to pH 5.5 and de-ionized water were used as controls. Each data point is a mean of at least 6 replicates (± standard deviation).

Table 4.4: *In vitro* inactivation 4D estimates of strains 4/74, FS8 and FS115 at 4 ºC.**.** 4D ‘s were calculated by fitting the log-transformed data to the Weibull model. Each value is a mean of at least 6 replicates ± standard deviation. Goodness of the fitting was evaluated using regression coefficient and (R^2) and root-mean square error (RMSE)

Strain	Plant Extract	4D (days)	RMSE	R^2
	Basil	16.23 ± 076 **(f)**	0.1286 ± 0.0216	0.9860 ± 0.055
	Calendula	8.22 ± 0.21 **(c)**	0.3102 ± 0.0483	0.9727 ± 0.079
	Corn silk	39.38 ± 1.47 **(h)**	0.2421 ± 0.0590	0.9793 ± 0.076
	C. Oregano	11.86 ± 0.89 **(d)**	0.2429 ± 0.0557	0.9834 ± 0.0063
4/74	Laurel	21.27 ± 1.37 **(g)**	0.1481 ± 0.0429	0.9936 ± 0.0035
	Oregano	3.09 ± 0.62 **(a)**	0.3468 ± 0.0618	0.9806 ± 0.0081
	Rosemary	14.54 ± 0.90 **(e)**	0.3367 ± 0.0677	0.9671 ± 0.0123
	Spearmint	14.56 ± 0.88 **(e)**	0.2726 ± 0.1579	0.9748 ± 0.207
	Thyme	6.26 ± 0.79 **(b)**	0.2725 ± 0.1493	0.9837 ± 0.0189
	Basil	16.95 ± 0.90 **(f)**	0.1901 ± 0.0405	0.9688 ± 0.0117
	Calendula	9.29 ± 0.26 **(c)**	0.1929 ± 0.0567	0.9858 ± 0.0091
	Corn silk	41.58 ± 1.07 **(h)**	0.2284 ± 0.0305	0.9794 ± 0.0063
	C. Oregano	12.48 ± 0.38 **(d)**	0.2468 ± 0.0575	0.9811 ± 0.0080
FS8	Laurel	24.12 ± 2.68 **(g)**	0.2780 ± 0.0287	0.9750 ± 0.0070
	Oregano	3.24 ± 0.32 **(a)**	0.3722 ± 0.1488	0.9729 ± 0.0154
	Rosemary	16.56 ± 1.14 **(ef)**	0.3136 ± 0.0414	0.9677 ± 0.0057
	Spearmint	14.83 ± 0.41 **(e)**	0.2369 ± 0.1351	0.9789 ± 0.200
	Thyme	6.15 ± 1.04 **(b)**	0.1829 ± 0.304	0.9936 ± 0.0018
	Basil	18.69 ± 1.43 **(e)**	0.1166 ± 0.0361	0.9863 ± 0.0073
	Calendula	7.91 ± 0.23 **(b)**	0.3424 ± 0.0714	0.9708 ± 0.0121
	Corn silk	43.39 ± 1.71 **(g)**	0.2025 ± 0.0347	0.9832 ± 0.0059
	C. Oregano	13.90± 0.18 **(c)**	0.2025 ± 0.0521	0.9847 ± 0.0080
FS115	Laurel	22.69 ± 2.01 **(f)**	0.2396 ± 0.1165	0.9811 ± 0.0175
	Oregano	3.06 ± 0.42 **(a)**	0.2695 ±0.1527	0.9849 ± 0.0138
	Rosemary	16.53 ± 0.87 **(d)**	0.2279 ± 0.0617	0.9803 ± 0.0102
	Spearmint	15.01 ± 0.24 **(cd)**	0.2035 ± 0.0774	0.9826 ± 0.0125
	Thyme	6.13 ± 1.06 **(b)**	0.1201 ± 0.0642	0.9971 ± 0.0028

Different lowercase letters within the same column of a single strain indicate statistical differences among different plant extracts for significance level p=0.05 according to Tukey's HSD

Table 4.5: Order of antimicrobial potential of the hydro-distilled aqueous extracts based on their 4D estimates

***Salmonella* Typhimurium strains**	**Order of antimicrobial potential**
S. Typhimurium 4/74	Oregano > Thyme > Calendula > C. oregano > Spearmint = Rosemary > Basil > Laurel > Corn silk
S. Typhimurium FS8	Oregano > Thyme > Calendula > C. oregano > Spearmint ≥ Rosemary = Basil > Laurel > Corn silk
S. Typhimurium	Oregano > Thyme = Calendula > C. oregano ≥ Spearmint = Rosemary > Basil >Laurel > Corn silk

Overall, the aqueous extracts effectively reduced the levels or the growth rate of three *S.* Typhimurium strains, in a plant-, temperature- and strain-dependent manner.

Antimicrobial activity of hydro-distilled oregano extract and industrial oregano hydrolate against S. Typhimurium *FS8 in pork meat*

Hydro-distilled oregano extract and industrial oregano hydrolate exhibiting the strongest *in vitro* antibacterial performance were examined for their efficacy in improving the safety of pork meat stored at 4 °C. The individual or combined effect of marination and edible coatings supplemented with or without low concentrations of OEO was assessed for the application of the extracts on the meat surface. Given that the *in vitro* inactivation profiles of the three strains did not significantly differ during incubation at 4 °C, FS8 strain (minced pork isolate) was chosen.

Effect of marination

Depending on the treatment, application of 3 h marination resulted in 0.6-2.4 log reductions ($P < 0.05$) of the initial bacterial population (Figure 4.5). When samples were marinated in water and water + OEO, a 1.2 and 1.6 log reduction ($P < 0.05$), respectively, was observed compared to the inoculated untreated samples immediately after the application of the treatment. No further reduction ($P < 0.05$) of *Salmonella* populations was observed when oregano, oregano + OEO or industrial hydrolate were

applied as marination solutions. On the contrary, industrial oregano hydrolate + OEO had the highest antimicrobial activity ($P < 0.05$), lowering the initial bacterial population by 2.4 log CFU/g compared to the inoculated untreated samples and by ~ 1 log CFU/g compared to the samples marinated in water + OEO (Figure 4.5). Significant differences were not observed between extracts supplemented with and those without OEO ($P > 0.05$). Furthermore, there was not any residual antimicrobial activity after storage at 4 °C for 4 days, regardless of the marination solution ($P < 0.05$) (Figure 4.5). Therefore, marination of pork meat samples resulted in a rapid decrease of *Salmonella* populations, especially when industrial + OEO was used in the marinades.

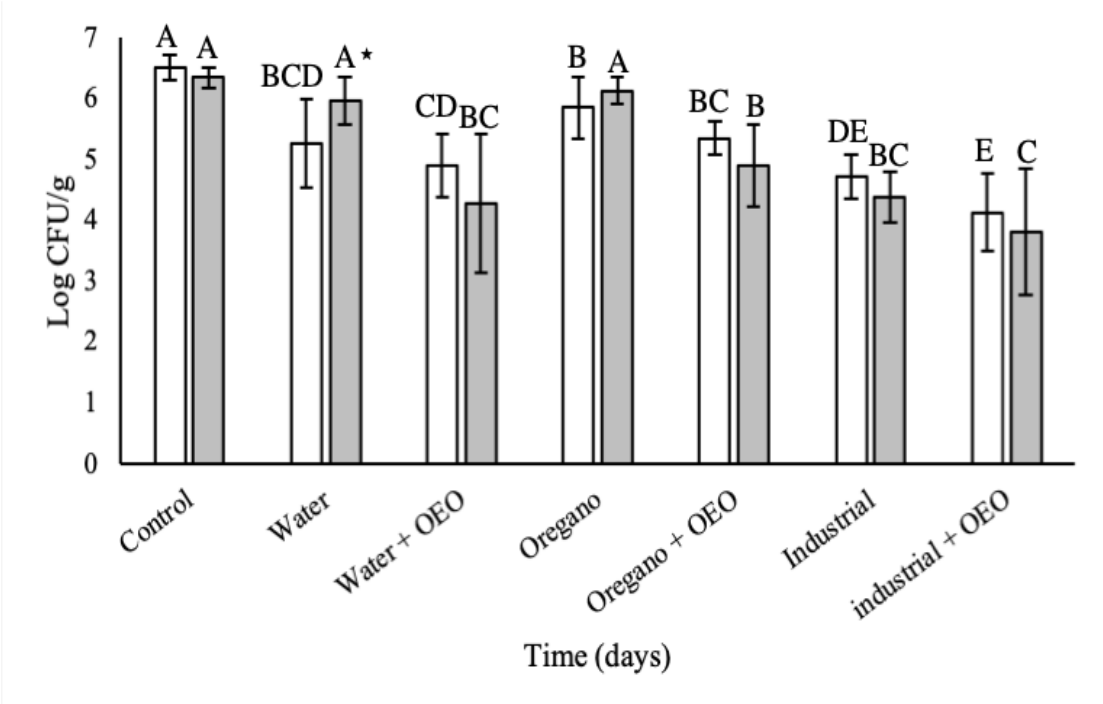

Figure 4.5: Effect of 3-hour marination on the survival of *S.* Typhmurium FS8 on pork meat. Hydro-distilled oregano extract and industrial oregano hydrolate supplemented with or without 0.2% oregano essential oil (OEO) were used as marination solutions. Water and inoculated untreated pork meat samples were used as controls. Samples were stored at 4^{o}C. Sampling was performed immediately after treatment (white bars) and after 4 days of storage (grey bars). Bars represent an average (± standard deviation) of six replicates.

Different capital letters indicate statistical differences among different treatments of the same storage time for a significant level p=0.05 according to Tukey's HSD

Star indicate statistical differences between identically treated samples of different storage time for a significant level p=0.05 according to Tukey's HSD

Effect of edible coatings

The effect of sodium alginate edible coatings prepared of water, 50% oregano, centrifuged oregano, 50% centrifuged oregano, industrial oregano and 50% industrial oregano supplemented with or without 0.5% OEO on pathogen survival was studied. Application of coatings with no added OEO resulted in approximately 1.0 log CFU/g reduction ($P < 0.05$) during storage for all extract-treated samples (Table 4.6). At the end of storage period, no differences ($P>0.05$) were found among different extract-coated samples, though lower survivors were enumerated compared to uncoated and water-coated meat samples ($P < 0.05$) (Table 4.6).

Supplementation of edible coatings with OEO was initially performed using a concentration of 0.2% (data not shown). Since no effect was found, a higher concentration of 0.5% was tested. Incorporation of 0.5% OEO increased the antibacterial effect of the coatings compared to those with no added OEO: all treated samples had 1.0-1.5 log ($P < 0.05$) lower bacterial levels compared to the inoculated untreated samples immediately after treatment, regardless of the film-forming solution (Table 4.7). In addition, a gradual reduction of up to 1.3 log CFU/g ($P < 0.05$) was observed at the end of storage period (5^{th} day of storage) for most of the samples tested, apart from those coated in centrifuged oregano + OEO ($P >0.05$). Nonetheless, coatings prepared of industrial oregano hydrolate + OEO were the most potent in decreasing the levels of *Salmonella*, resulting in a total reduction of 2.6 log CFU/g on day 5 compared to inoculated uncoated samples and ~ 1 log CFU/g compared to samples coated with water + OEO (Table 4.7). Therefore, addition of low levels of OEO enhanced the antimicrobial activity of industrial oregano hydrolate incorporated through edible coatings.

Table 4.6: Effect of edible coatings prepared of water, hydro-distilled oregano extract and industrial oregano hydrolate on the survival of *S.* Typhimurium FS8 inoculated in pork meat at 4 ºC. Inoculated uncoated pork meat samples were used as controls. Each value represents an average (± standard deviation) of six replicates

	Extracts						
Time (days)	**Control**	**Water**	**50% Oregano**	**Centrifuged oregano**	**50% Centrifuged oregano**	**Industrial oregano**	**50% Industrial oregano**
0	6.5 ± 0.2 **A, a**	6.1 ± 0.2 **B, a**	6.2 ± 0.1 **AB, a**	6.2 ± 0.2 **AB, a**	6.3 ± 0.2 **AB, a**	6.1 ± 0.3 **B, a**	6.0 ± 0.2 **B, a**
1	6.3 ± 0.3 **A, abc**	6.2 ± 0.2 **AB, a**	6.1 ± 0.2 **AB, a**	6.0 ± 0.3 **AB, a**	6.3 ± 0.3 **A, a**	5.9 ± 0.2 **B, a**	5.9 ± 0.4 **AB, a**
2	6.4 ± 0.3 **A, ab**	6.1 ± 0.3 **ABC, a**	6.2 ± 0.1 **AB, a**	6.0 ± 0.3 **BC, a**	6.3 ± 0.2 **AB, a**	5.7 ± 0.2 **C, ab**	6.1 ± 0.2 **ABC, a**
3	6.0 ± 0.2 **A, c**	5.7 ± 0.4 **AB, a**	5.5 ± 0.2 **ABC, b**	5.3 ± 0.4 **BC, b**	5.1 ± 0.3 **C, b**	5.3 ± 0.3 **BC, b**	5.3 ± 0.3 **BC, bc**
4	6.1 ± 0.4 **A, bc**	5.9 ± 0.4 **AB, a**	5.5 ± 0.6 **AB, b**	5.3 ± 0.3 **AB, b**	5.5 ± 0.1 **AB, b**	5.6 ± 0.8 **AB, ab**	5.0 ± 0.2 **B, c**
5	6.3 ± 0.2 **A, abc**	6.1 ± 0.5 **AB, a**	5.4 ± 0.2 **C, b**	5.3 ± 0.3 **C, b**	5.2 ± 0.4 **C, b**	5.3 ± 0.6 **C, b**	5.7 ± 0.3 **BC, ab**

Different capital letters within the same row indicate statistical differences at a significance level p=0.05 according to Tukey's HSD

Different lowercase letters within the same column indicate statistical differences at a significance level p=0.05 according to Tukey's HSD

Table 4.7: Effect of edible coatings prepared of water, hydro-distilled oregano extract and industrial oregano hydrolate supplemented with 0.5 % oregano essential oil (OEO) on the survival of *S.* Typhimurium FS8 inoculated in pork meat at 4 ºC. Inoculated uncoated pork meat samples were used as controls. Each value represents an average (± standard deviation) of six replicates

	Extracts						
Time (days)	**Control**	**Water + EO**	**50% Oregano + OEO**	**Centrifuged oregano + OEO**	**50% Centrifuged oregano + OEO**	**Industrial oregano + OEO**	**50% Industrial oregano + OEO**
0	6.5 ± 0.2 **A, a**	5.4 ± 0.2 **B, a**	5.4 ± 0.2 **B, a**	4.8 ± 0.9 **C, a**	5.5 ± 0.4 **B, a**	5.2 ± 0.3 **BC, a**	5.2 ± 0.3 **BC, a**
1	6.3 ± 0.3 **A, abc**	5.2 ± 0.2 **B, abc**	4.8 ± 0.5 **B, bc**	5.2 ± 0.3 **B, a**	5.2 ± 0.4 **B, ab**	4.7 ± 0.3 **B, ab**	4.9 ± 0.2 **B, ab**
2	6.4 ± 0.3 **A, ab**	5.3 ± 0.5 **B, ab**	5.1 ± 0.3 **BC, ab**	4.8 ± 0.3 **BC, a**	5.1 ± 0.2 **BC, ab**	4.7 ± 0.3 **C, ab**	5.0 ± 0.4 **BC, ab**
3	6.0 ± 0.2 **A, c**	4.6 ± 0.3 **BC, c**	4.5 ± 0.3 **BC, c**	4.6 ± 0.7 **BC, a**	4.8 ± 0.1 **B, b**	4.1 ± 0.5 **C, bc**	4.7 ± 0.4 **B, abc**
4	6.1 ± 0.4 **A, bc**	5.1 ± 0.4 **B, abc**	4.3 ± 0.2 **B, c**	4.7 ± 0.4 **B, a**	5.0 ± 0.2 **B, ab**	4.7 ± 0.7 **B, ab**	4.5 ± 0.2 **B, bc**
5	6.3 ± 0.2 **A, abc**	4.8 ± 0.5 **BC, bc**	4.8 ± 0.1 **BC, bc**	4.6 ± 0.5 **BC, a**	5.1 ± 0.2 **B, ab**	3.9 ± 0.4 **D, b**	4.3 ± 0.5 **CD, b**

Different capital letters within the same row indicate statistical differences at a significance level p=0.05 according to Tukey's HSD

Different lowercase letters within the same column indicate statistical differences at a significance level p=0.05) according to Tukey's HSD

Combined effect of marination and edible coatings

The combined effect of antimicrobial marination and edible coatings on the survival of *S.* Typhimurium FS8 on pork meat was evaluated (Table 4.6). Edible coatings enhanced the antimicrobial effect of marination for some of the treatments tested, resulting in log reductions during storage. Combination of marination in hydro-distilled oregano extract and water + OEO coatings or hydro-distilled oregano + OEO coatings enhanced the performance of the extract, leading to a reduction of 0.9 and 1.4 log units ($P < 0.05$) during storage, respectively. The total reduction was 1.4 and 2.0 log CFU/g, respectively, compared to the inoculated untreated pork meat samples. (Table 4.8).

In all experiments, *Salmonella* levels remained stable/unchanged on inoculated untreated samples, whereas no *Salmonella* population was detected in none of the uninoculated untreated meat samples.

Table 4.8: Combined effect of marinades and edible coatings prepared of water, hydro-distilled oregano extract and industrial oregano hydrolate supplemented with or without 0.2 % OEO in the inactivation of *S.* Typhimurium FS8 in pork meat at 4ºC. Combinations resulting in reductions during storage are indicated in bold. Each value represents an average (± standard deviation) of six replicates

Marination	Coatings	Days		
		0	2	4
No	No	6.5 ± 0.2 **a**	6.2 ± 0.6 **a**	6.3 ± 0.2 **a**
Water	Water	5.3 ±0.7 **A, b**	5.5 ± 0.2 **A, abc**	5.1 ± 0.5 **A, bc**
	Water + OEO	5.3 ± 0.7 **A, b**	4.5 ± 0.3 **A, d**	4.9 ± 0.5 **A, c**
	50% Oregano	5.3 ±0.7 **A, b**	5.5 ± 0.4 **A, ab**	5.8 ± 0.4 **A, ab**
	50% Oregano + OEO	**5.3 ± 0.7 A, b**	**4.9 ± 0.8 AB, bcd**	**4.4 ± 0.4 B, c**
	Industrial	5.3 ±0.7 **A, b**	4.6 ± 0.3 **A, cd**	4.9± 9.5 **A, c**
	Industrial + OEO	5.3 ± 0.7 **A, b**	4.6 ± 0.5 **A, d**	5.0 ± 0.6 **A, c**
Water + OEO	**Water**	**4.9 ± 0.6 A, b**	**4.3 ± 0.2 B, b**	**4.2± 0.6 B, bc**
	Water + OEO	**4.9 ± 0.6 A, b**	**4.0 ± 0.3 B, b**	**3.7 ± 0.5 B, c**
	50% Oregano	**4.9 ± 0.6 A, b**	**4.2 ± 0.1 B, b**	**4.7 ± 0.3 AB, b**
	50% Oregano + OEO	**4.9 ± 0.6 A, b**	**3.7 ± 1.0 B, b**	**3.9 ± 0.3 B, c**
	Industrial	**4.9 ± 0.6 A, b**	**4.2 ± 0.7 AB, b**	**3.9 ± 0.3 B, c**
	Industrial + OEO	**4.9 ± 0.6 A, b**	**3.5 ± 1.0 B, b**	**4.1 ± 0.5 AB, bc**
Oregano	Water	5.9 ± 0.5 **A, b**	6.1 ± 0.8 **A, a**	5.5 ± 0.7 **A, ab**
	Water + OEO	**5.9 ± 0.5 A, b**	**5.5 ± 0.3 AB, a**	**5.1 ± 0.6 B, bc**
	50% Oregano	5.9 ± 0.5 **A, b**	5.7 ± 0.6 **A, a**	5.8 ± 0.2 **A, ab**
	50% Oregano + OEO	**5.9 ± 0.5 A, b**	**5.5 ± 0.7 A, a**	**4.5 ± 1.0 B, c**
	Industrial	5.9 ± 0.5 **A, b**	5.5 ± 0.4 **A, a**	5.4 ± 0.3 **A, abc**
	Industrial + OEO	5.9 ± 0.5 **A, b**	5.2 ± 0.5 **A, a**	5.2 ± 0.5 **A, bc**
Oregano + OEO	Water	5.4 ± 0.3 **A, b**	5.4 ± 0.7 **A, ab**	4.8 ± 0.4 **A, b**

	Water + OEO	**5.4 ± 0.3 A, b**	**4.8 ± 0.5 B, bc**	**4.7 ± 0.7 B, bc**
	50% Oregano	**5.4 ± 0.3 A, b**	**4.0 ± 0.7 B, c**	**4.5 ± 0.8 B, bc**
	50% Oregano + OEO	**5.4 ± 0.3 A, b**	**4.3 ± 1.0 B, bc**	**4.0 ± 0.6 B, c**
	Industrial	**5.4 ± 0.3 A, b**	**4.6 ± 0.2 B, bc**	**4.4 ± 0.1 B, bc**
	Industrial + OEO	**5.4 ± 0.3 A, b**	**4.8 ± 0.5 B, bc**	**4.4 ± 0.4 B, bc**
Industrial	Water	4.7 ± 0.4 **A, b**	4.1 ± 0.8 **A, b**	3.8 ± 0.9 **A, b**
	Water + OEO	**4.7 ± 0.4 A, b**	**4.0 ± 0.3 AB, b**	**3.8 ± 1.0 B, b**
	50% Oregano	4.7 ± 0.4 **A, b**	4.4 ± 0.4 **A, b**	4.4 ± 0.4 **A, b**
	50% Oregano + OEO	4.7 ± 0.4 **A, b**	4.4 ± 0.2 **A, b**	4.4 ± 0.3 **A, b**
	Industrial	4.7 ± 0.4 **A, b**	4.5 ± 0.2 **A, b**	4.4 ± 0.2 **A, b**
	Industrial + OEO	4.7 ± 0.4 **A, b**	4.1 ± 0.8 **AB, b**	3.9 ± 0.3 **B, b**
Industrial + OEO	Water	4.1 ± 0.6 **A, b**	4.2 ± 0.4 **A, b**	4.0 ± 0.8 **A, b**
	Water + OEO	4.1 ± 0.6 **A, b**	4.1 ± 0.4 **A, b**	3.8 ± 0.7 **A, b**
	50% Oregano	4.1 ± 0.6 **A, b**	4.4 ± 0.7 **A, b**	4.5 ± 0.4 **A, b**
	50% Oregano + OEO	**4.1 ± 0.6 AB, b**	**4.4 ± 0.2 A, b**	**3.3 ± 0.7 B, b**
	Industrial	4.1 ± 0.6 **A, b**	3.9 ± 0.6 **A, b**	4.4 ± 0.5 **A, b**
	Industrial + OEO	4.1 ± 0.6 **A, b**	3.9 ± 0.3 **A, b**	4.2 ± 0.4 **A, b**

Different capital letters within the same row indicate statistical differences at a significance level p=0.05 according to Tukey's HSD

Different lowercase letters within the same column indicate statistical differences at a significance level p=0.05 according to Tukey's HSD among samples marinated in the same solution but covered with different coatings

Changes in the pH during storage

Slight/no alterations were recorded at pH values of most treated samples. On the contrary, pH of untreated samples increased from 5.8 to approximately 6.4-6.5 during storage (Figures 4.6-4.8).

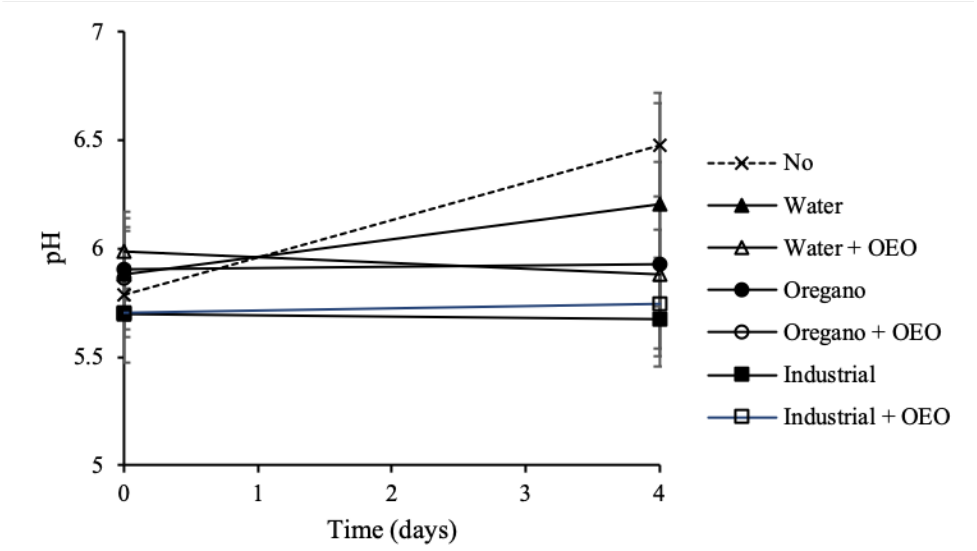

Figure 4.6: pH changes of inoculated pork meat samples during storage at 4ºC following3-h marination to water, hydrodistilled oregano extract or industrial oregano hydrolate supplemented with or without 0.2% OEO. Each data point is an average (± standard deviation) of six replicates.

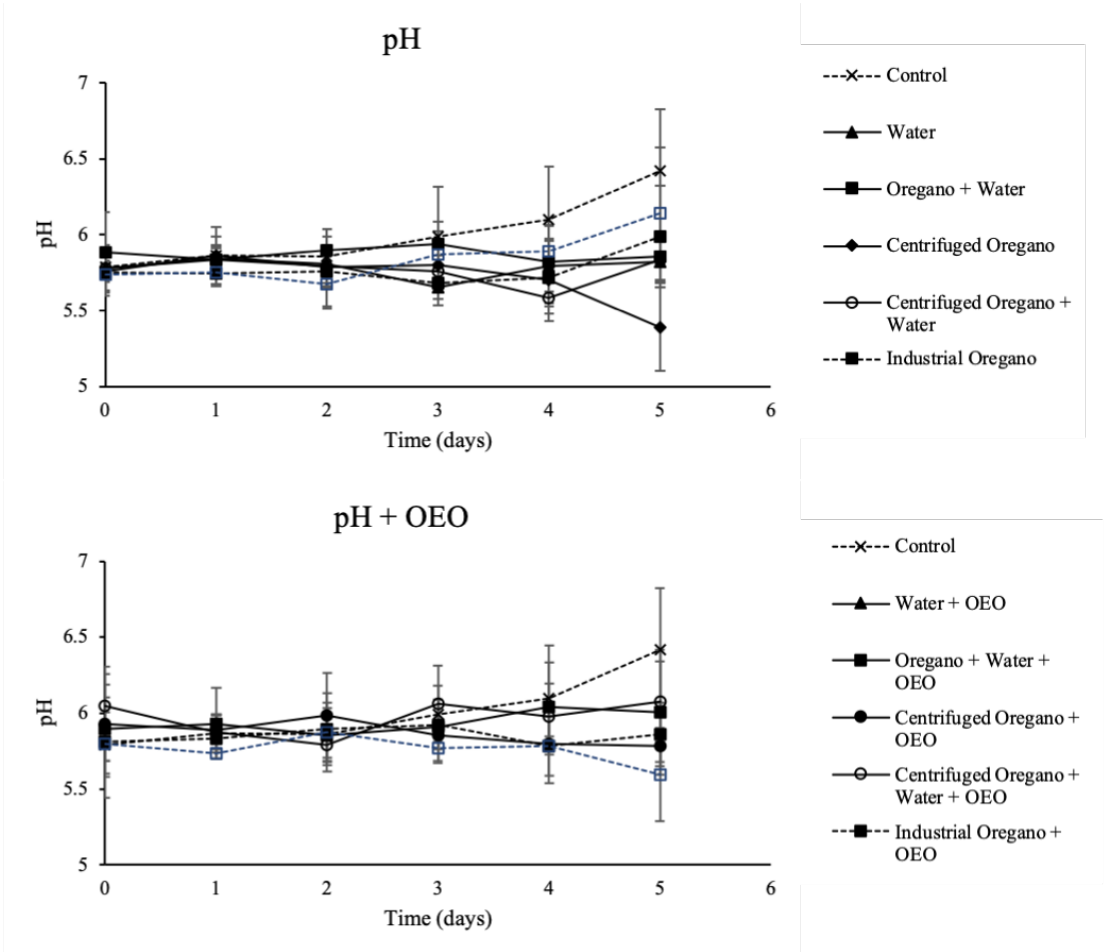

Figure 4.7: pH changes of inoculated pork meat samples coated with water, hydro-distilled oregano extract or oregano hydrolate supplemented with or without 0.5% OEO during storage at 4ºC. Each data point is an average (± standard deviation) of six replicates.

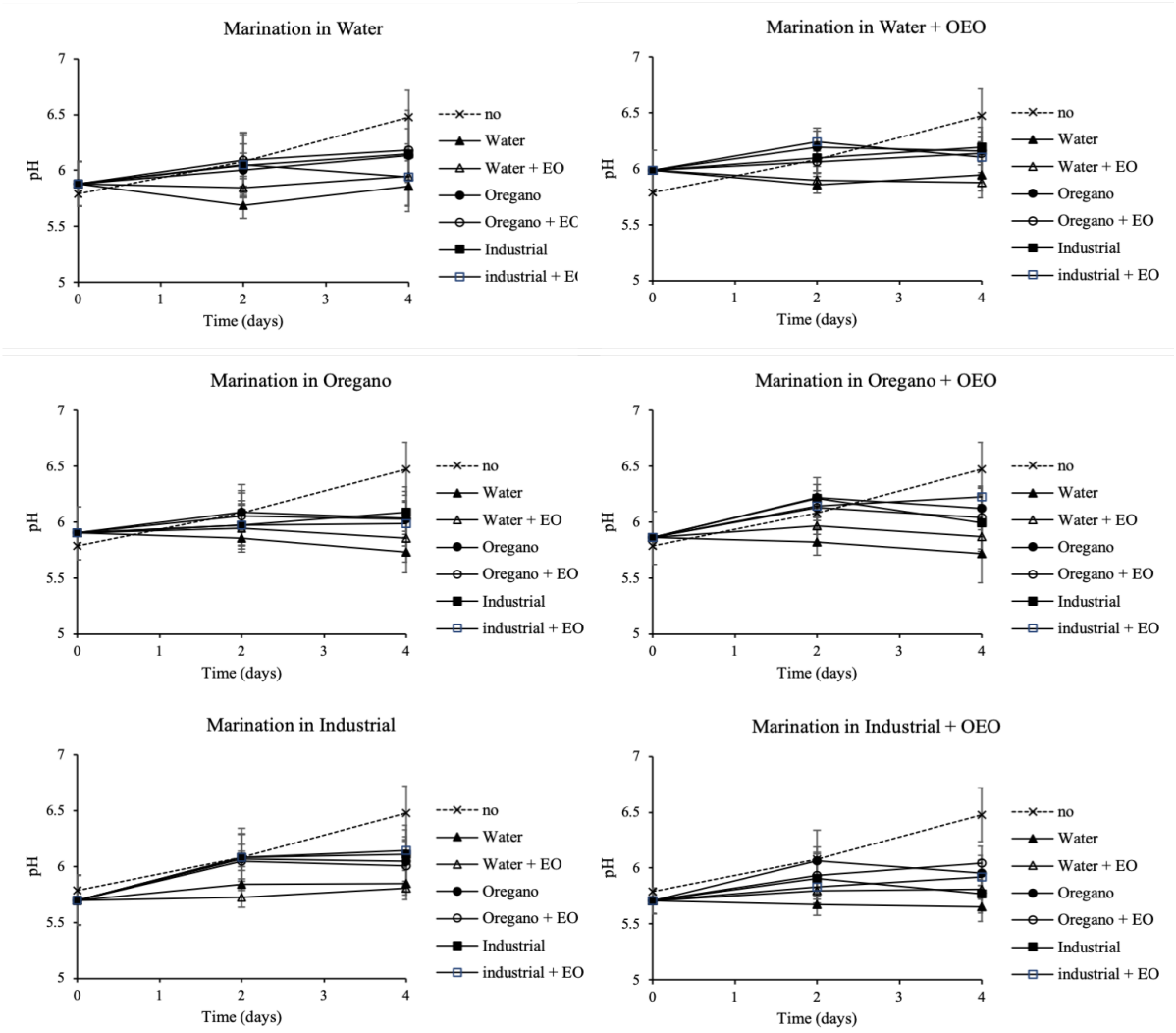

Figure 4.8: pH changes of inoculated pork meat samples marinated in water, hydro-distilled oregano extract or oregano hydrolate supplemented with or without 0.2% OEO and coated with water, hydro-distilled oregano extract or oregano hydrolate supplemented with or without 0.2% OEO at 4ºC. Each diagram represents samples marinated in different marinating solution, whereas lines within each diagram samples coated with a different forming material. Each data point is a mean of 6 replicates (± standard deviation).

Discussion

Plant derived extracts and compounds have been widely investigated as alternatives to chemical preservatives. Their *in vitro* antimicrobial activity has been tested against a wide variety of foodborne bacteria, such as *Salmonella* spp., *Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* (Bouarab-Chibane et al., 2018; Côté et al., 2011; Gonelimali et al., 2018; Higginbotham et al., 2014; Y. Liu et al., 2017; Miceli et al., 2014; Mostafa et al., 2018). The bactericidal effect of these compounds has been attributed to the presence of polyphenolic fraction (Chun et al., 2005; Poimenidou et al., 2016; Shan et al., 2007). In fact, it has been shown that antibacterial activity is closely related to the phenolic content (Shan et al., 2007). Agro-industrial by-products comprise potential sources of phenolic compounds with a reported antimicrobial effect (Hayrapetyan et al., 2012; Poimenidou et al., 2016; Shahamirian et al., 2019). By-products of essential oil distillation are inexpensive and were found effective in delivering an important bactericidal effect when applied as antimicrobial interventions in lettuce, even though their commercial or industrial utilization has not been established (Poimenidou et al., 2016). In addition, these extracts have no (hydro-distilled) or reduced (industrial oregano hydrolate) odor compared to essentials oils, and, thus, can be used without compromising the organoleptical quality of the food products. Therefore, in the present study, the effect of eleven rich-phenolic aqueous extracts collected as by-products of essential oil purification procedure was tested against three *S.* Typhimurium strains (4/74, FS8 and FS115).

Most of the extracts tested (apart from garlic at 4^{o}C) inhibited or reduced *Salmonella* strains when *in vitro* examined. However, the susceptibility of the strains was mainly affected by incubation temperature and plant extract. At 37^{o}C, incubation in the extracts caused either inactivation or growth inhibition of the strains. On the other hand, the combination of low temperature and antimicrobial compounds during incubation at 4^{o}C resulted in inactivation of all pathogens for most of the extracts tested. In addition, a marked variability in the antimicrobial potential of the extracts was observed at both incubation temperatures. This may be attributed to variations of the phytocompounds present in the extracts (Gonelimali et al., 2018; Moreno et al., 2006; Mostafa et al., 2018) as well as variations in their volatile nature (industrial hydrolate *vs* hydro-distilled extracts) (Gonelimali et al., 2018; Mostafa et al., 2018). For instance, a unique composition was observed for each one of the hydro-distilled extracts, with

rosmarinic acid being the most abundant component in half of the extracts tested. The antimicrobial activity of pure phenolic compounds, such as chloragenic acid, caffeic acid, ellagic acid and quercetin has been documented (Fattouch et al., 2007; Rodríguez Vaquero and Manca De Nadra, 2008). However, no direct correlation can be established between the total observed antimicrobial activity and the individual components present in the tested extracts, since their combination may produce a synergistic, additive or even antagonistic effect (Rodriguez Vaquero et al., 2010). In line with the above results, Seeram et al. (2005) reported that pomegranate juice had a superior bioactivity against human tumor cell lines compared to its purified polyphenols, indicating synergistic or additive effects among the phytochemicals present in the juice. Regarding industrial oregano hydrolate having the highest antimicrobial potency, it was composed of the volatile compounds thymol and carvacrol, the main components of oregano essential oil (De Souza et al., 2016). The superior antimicrobial effect of essential oils compared to their corresponding water extracts has also been reported elsewhere (Iturriaga et al., 2012; Tepe et al., 2004).

A strain-dependent effect was observed when *Salmonella* strains were incubated in the presence of laurel extract at 37 °C. Miceli et al., (2014) also reported that the susceptibility of several strains of *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and *Enterobacter* spp. to two different plant water extracts was strain-specific. Food preservation methods or stresses widely occurring in the food chain can stimulate diverse strain-specific phenotypic responses. For instance, variations in the thermal (Lianou and Koutsoumanis, 2013b) or acid (Jørgensen et al., 2000; Lianou and Koutsoumanis, 2013b) resistance has been reported among *Salmonella* strains. According to Melo et al. (2013), three strains of *Listeria monocytogenes* adapted to a cheese stimulated medium used different proteomic repertoires in order to survive gastric stress, leading to a unique proteomic profile for each strain.

The *in situ* efficacy of both hydro-distilled oregano extract and industrial oregano hydrolate having the most prominent *in vitro* antimicrobial potency was further evaluated in pork meat under refrigeration (4 °C). Marination, edible coatings or their combinations supplemented with or without low levels of OEO were used as antimicrobial interventions for distributing the extracts onto the food surface. In all cases, both extracts exhibited reduced antimicrobial activity against *S.* Typhimurium FS8 compared to the *in vitro* assays, regardless of the method of application. It is

generally accepted that the antimicrobial effect of plant extracts may be markedly moderated in food substrates (Higginbotham et al., 2014; Kim et al., 2004; Miceli et al., 2014; Piskernik et al., 2011). Food with a complex composition such as meat and poultry products can limit effective application of natural antimicrobials due to their inherent characteristics, such as their nonhomogeneous substrate, their neutral pH and their high concentrations of lipids and proteins (Davidson et al., 2013). Indeed, in complex matrices, lipids and proteins can strongly interact with bioactive phenolic compounds, lowering their antimicrobial efficacy (Weiss et al., 2015). Del Campo et al. (2000), demonstrated that the presence of serum albumin in TSB and dairy cream in TSB and zucchini broth reduced the inhibitory effect of a rosemary extract. Similarly, Bouarab-Chibane et al. (2018) reported that the antimicrobial activity of some phenolics against *Staphylococcus aureus* were abolished in the presence of bovine meat proteins, probably due to limitations in the active free quantity of these antimicrobials. Furthermore, the lower water content of food substrates compared to the laboratory media could also hinder the transfer of antimicrobial molecules into the active site within the microbial cell (Cabral et al., 2013). Regarding edible coatings, interactions of the forming biopolymer with the bioactive compounds may also affect the free residual concentration of the bioactive compounds. Gómez-Estaca et al. (2009) demonstrated reduced free phenol content in tuna-skin gelatin films as a result of polyphenol-protein interactions. Interactions of alginates with polyphenols has also been reported (Plazinski and Plazinska, 2011). Finally, the level of the initial bacterial concentration has been found to affect the antimicrobial profile of the extracts, with low levels of *Camylobacter jejuni* cell populations being more susceptible compared to high inocula levels (Piskernik et al., 2011).

Despite the reduced antimicrobial potency of phenolics in food substrates, some studies have demonstrated the effectiveness of these substances in reducing pathogens in foods, with regard to factors such as food matrix, storage temperature and type and concentration of plant extract (Chen et al., 2013; Hayrapetyan et al., 2012; Kim et al., 2001; Poimenidou et al., 2016; Stojković et al., 2013; Vodnar, 2012). In the current study, industrial oregano hydrolate supplemented with low concentrations of OEO effectively reduced the levels of S. Typhimurium FS8 in pork meat when incorporated either in marination or inside edible coatings. Nonetheless, the type of the applied treatment affected its antimicrobial performance. Marination proved a rapid and effective intervention, even though no residual activity was observed during storage.

Direct application of phytochemicals into the food system results in rapid diffusion of the antimicrobial agents into the mass of the food (Aloui and Khwaldia, 2016). Application of edible coatings prepared of industrial oregano hydrolate + OEO, on the other hand, apart from a rapid reduction immediately after implementation, had also a gradual reduction during storage. Edible coatings are reported to allow a more gradual and controlled diffusion of bioactive compounds into the food system (Bouarab-Chibane et al., 2019; Gyawali and Ibrahim, 2014), maintaining, therefore, their concentrations on the surface of the foods at appropriated levels over time (Bouarab-Chibane et al., 2019). Application of a multi-hurdle approach combining different interventions and antimicrobials enhanced the performance of hydro-distilled oregano extract. This result is in line with other studies, demonstrating that the combination of natural antimicrobials or their use together with other preservation technologies may enhance their effectiveness (Careaga et al., 2003; Piskernik et al., 2011; Poimenidou et al., 2016). The use of essential oils in food products required for sufficient antimicrobial activity usually exceed their organoleptically acceptable levels (Ribeiro-Santos et al., 2017), due to negative effects in the flavor and aroma. Therefore, combinations of plant extracts could minimize the required dose (Gutierrez et al., 2008), leading to acceptable sensory properties of treated products

In conclusion, aqueous by-products of essential oil production with reduced odor effectively controlled *S.* Typhimurium in a temperature-, plant- and strain-dependent manner when *in vitro* examined. Utilization of by-products of oregano essential oil purification procedure exhibited prominent bactericidal potency against FS8 strain inoculated on pork meat at 4 ^{o}C, even though the type of intervention applied (marination, edible coatings or their combination) determined the *in situ* effectiveness of each extract (hydro-distilled *vs* industrial hydrolate). In any case, the application of these natural phytochemicals could provide an alternative and cost-effective way of improving the safety of pork meat without compromising the sensory properties of the food product. Future work could focus on investigating the strain-dependent effect of laurel at 37°C by assembling the proteomic profiles of the strains. In addition, the ability of natural extracts to improve food safety or extend shelf-life could be tested in a variety of food products.

Supplementary data

S4.1 Table: *In vitro* antimicrobial effect of hydro-distilled aqueous plant extracts on *S.* Typhimurium strains strains at 37 ºC

Strain	Plant Extract	Time (h)				
		0	3	6	9	24
4/74	Basil	5.5 ± 0.2 **(bc)**	5.5 ± 0.3 **(d)**	5.3 ± 0.2 **(de)**	5.2 ± 0.3 **(f)**	1.7 ± 1.1 **(g)**
	Calendula	5.8 ± 0.2 **(a)**	5.3 ± 0.2 **(d)**	3.7 ± 0.1 **(h)**	3.3 ± 0.1 **(h)**	1.0 ± 0.0 **(h)**
	Centrifuged oregano	5.7 ± 0.3 **(ab)**	5.4 ± 0.3 **(d)**	4.8 ± 0.3 **(fg)**	4.0 ± 0.9 **(g)**	1.1 ± 0.2 **(h)**
	Corn silk	5.8 ± 0.1 **(ab)**	5.7 ± 0.1 **(cd)**	6.3 ± 0.3 **(c)**	7.4 ± 0.2 **(c)**	7.6 ± 0.2 **(c)**
	Garlic	5.8 ± 0.1 **(a)**	7.1 ± 0.1 **(b)**	8.3 ± 0.1 **(b)**	8.5± 0.1 **(b)**	8.4 ± 0.1 **(b)**
	Laurel	5.6 ± 0.2 **(ab)**	5.6 ± 0.2 **(cd)**	5.5 ± 0.1 **(de)**	5.4 ± 0.1 **(f)**	3.4 ± 0.5 **(f)**
	Oregano	5.9 ± 0.2 **(a)**	4.6 ± 0.5 **(e)**	1.4 ± 0.5 **(i)**	1.0 ± 0.2 **(i)**	1.0 ± 0.0 **(h)**
	Rosemary	5.3 ± 0.1 **(c)**	5.3 ± 0.2 **(d)**	5.2 ± 0.1 **(ef)**	5.3 ± 0.2 **(f)**	6.9 ±0.1 **(d)**
	Spearmint	5.6 ± 0.2 **(ab)**	5.5 ± 0.1 **(cd)**	5.7 ± 0.1 **(d)**	6.8 ± 0.1 **(d)**	7.3 ± 0.2 **(cd)**
	Thyme	5.8 ± 0.1 **(a)**	5.6 ± 0.4 **(cd)**	4.4 ± 0.9 **(g)**	4.0 ± 0.5 **(g)**	1.0± 0.1 **(h)**
	TSB	5.7 ± 0.2 **(ab)**	7.8 ± 0.2 **(a)**	9.2 ± 0.4 **(a)**	9.5 ± 0.2 **(a)**	9.5 ± 0.3 **(a)**
	TSB pH 5.5	5.8 ± 0.1 **(ab)**	6.8 ± 0.1 **(b)**	8.2 ± 0.2 **(b)**	8.7 ± 0.2 **(b)**	9.2 ± 0.2 **(a)**
	Water	5.7 ± 0.2 **(ab)**	5.9 ± 0.1 **(c)**	5.6 ± 0.2 **(d)**	5.9 ± 0.1 **(e)**	5.7 ± 0.2 **(e)**
FS8	Basil	5.3 ± 0.3 **(cd)**	5.3 ± 0.2 **(fg)**	5.3 ± 0.2 **(ef)**	5.4 ± 0.1 **(d)**	1.5 ± 1.0 **(g)**
	Calendula	5.8 ± 0.1 **(ab)**	5.4 ± 0.1 **(efg)**	3.9 ± 0.1 **(h)**	3.6 ± 0.1 **(f)**	1.0 ± 0.0 **(g)**
	Centrifuged oregano	5.7 ± 0.3 **(ab)**	5.4 ± 0.4 **(efg)**	4.8 ± 0.4 **(g)**	4.3 ± 0.9 **(e)**	1.4 ± 0.4 **(g)**
	Corn silk	5.7 ± 0.1 **(ab)**	5.7 ± 0.2 **(de)**	6.7 ± 0.2 **(d)**	7.4 ± 0.1 **(c)**	7.7 ± 0.1 **(c)**
	Garlic	5.9 ± 0.1 **(a)**	6.3 ± 0.3 **(c)**	7.7 ± 0.8 **(c)**	8.4 ± 0.3 **(b)**	8.4 ± 0.1 **(b)**
	Laurel	5.6 ± 0.2 **(bc)**	5.6 ± 0.1 **(def)**	5.5 ± 0.2 **(ef)**	5.6 ± 0.1 **(d)**	6.8 ± 0.3 **(e)**
	Oregano	5.9 ± 0.2 **(ab)**	4.7 ± 0.4 **(h)**	2.1 ± 0.5 **(i)**	1.0 ± 0.0 **(g)**	1.0 ± 0.0 **(g)**
	Rosemary	5.2 ±0.1 **(d)**	5.1 ± 0.1 **(g)**	5.1 ± 0.1 **(fg)**	5.4 ± 0.2 **(d)**	7.0 ± 0.1 **(de)**
	Spearmint	5.7 ± 0.1 **(ab)**	5.6 ± 0.2 **(def)**	6.5 ± 0.4 **(d)**	7.4 ± 0.2 **(c)**	7.3 ± 0.2 **(cd)**
	Thyme	5.7 ± 0.2 **(ab)**	5.6 ± 0.2 **(def)**	4.6 ± 0.3 **(g)**	4.4 ± 0.4 **(e)**	1.0 ± 0.1 **(g)**
	TSB	5.7 ± 0.3 **(ab)**	7.9 ± 0.2 **(a)**	9.0 ± 0.5 **(a)**	9.3 ± 0.4 **(a)**	9.5 ± 0.3 **(a)**
	TSB pH 5.5	5.8 ± 0.1 **(ab)**	6.9 ± 0.1 **(b)**	8.2 ± 0.3 **(b)**	8.7 ± 0.1 **(b)**	9.2 ± 0.0 **(a)**
	Water	5.7 ± 0.2 **(ab)**	5.8 ± 0.1 **(d)**	5.6 ± 0.2 **(e)**	5.6 ± 0.3 **(d)**	5.6 ± 0.3 **(f)**
FS115	Basil	5.5 ± 0.3 **(cd)**	5.5 ± 0.3 **(de)**	5.5 ± 0.2 **(e)**	5.5 ± 0.1 **(ef)**	2.1 ± 1.3 **(g)**
	Calendula	5.9 ± 0.1 **(a)**	5.4 ± 0.1 **(e)**	4.0 ± 0.2 **(h)**	3.2 ± 0.1 **(h)**	1.1 ± 0.1 **(h)**
	Centrifuged oregano	5.7 ± 0.2 **(abc)**	5.5 ± 0.3 **(de)**	5.0 ± 0.3 **(fg)**	4.2 ± 0.9 **(g)**	1.0 ± 0.1 **(h)**

Corn silk	5.7 ± 0.1 **(abc)**	5.7 ± 0.1 **(cd)**	7.1 ± 0.2 **(c)**	7.5 ± 0.1 **(d)**	7.7 ± 0.1 **(bc)**
Garlic	5.9 ± 0.1 **(a)**	7.2 ± 0.1 **(b)**	8.2 ± 0.3 **(b)**	8.1 ± 0.4 **(c)**	8.2 ± 0.2 **(b)**
Laurel	5.6 ± 0.2 **(bcd)**	5.6 ± 0.2 **(cde)**	5.5 ± 0.2 **(de)**	5.5 ± 0.2 **(ef)**	4.1 ± 1.0 **(f)**
Oregano	5.9 ± 0.2 **(a)**	5.0 ±0.4 **(f)**	1.6 ± 0.7 **(i)**	1.2 ± 0.6 **(i)**	1.0 ± 0.0 **(h)**
Rosemary	5.3 ± 0.1 **(d)**	5.4 ± 0.1 **(e)**	5.3 ± 0.1 **(ef)**	5.2 ± 0.1 **(f)**	6.5 ± 0.1 **(d)**
Spearmint	5.8 ± 0.1 **(ab)**	5.6 ± 0.1 **(cde)**	5.9 ± 0.2 **(d)**	7.1 ± 0.2 **(d)**	7.1 ± 0.2 **(cd)**
Thyme	5.8 ± 0.1 **(ab)**	5.6 ± 0.2 **(de)**	4.8 ± 0.2 **(g)**	4.5 ± 0.3 **(g)**	1.0 ± 0.0 **(h)**
TSB	5.7 ± 0.2 **(ab)**	7.8 ± 0.2 **(a)**	9.1 ± 0.4 **(a)**	9.5 ± 0.2 **(a)**	9.5± 0.3 **(a)**
TSB pH 5.5	5.8 ± 0.1 **(ab)**	6.9 ± 0.2 **(b)**	8.5 ± 0.1 **(b)**	8.7 ± 0.1 **(b)**	9.2 ± 0.1 **(a)**
Water	5.7 ± 0.2 **(abc)**	5.9 ± 0.1 **(c)**	5.7 ± 0.2 **(de)**	5.8 ± 0.1 **(e)**	5.6 ± 0.3 **(e)**

Different lowercase letters within the same column of a single strain indicate statistical differences different plant extracts of the same time interval for significance level p=0.05 according to Tukey's HSD

S4.2 Table: Effect of 3-hour marination treatment in the inactivation of *S.* Typhmurium FS8 inoculated in pork meat and stored at 4ºC. Untreated pork meat samples inoculated with the pathogen were used as controls

Time (days)	Extracts						
	Control	Water	Water + OEO	Oregano	Oregano + OEO	Industrial	Industrial + OEO
0	6.5 ± 0.2 **A, a**	5.3 ± 0.7 **BCD, a**	4.9 ± 0.5 **CD, a**	5.9 ± 0.5 **B, a**	5.4 ± 0.3 **BC, a**	4.7 ± 0.4 **DE, a**	4.1 ± 0.6 **E, a**
4	6.3 ± 0.2 **A, a**	6.0 ± 0.4 **A, b**	4.3 ± 0 1.1 **BC, a**	6.1 ± 0.2 **A, a**	4..9 ± 0.7 **B, a**	4.4 ± 0.4 **BC, a**	3.8 ± 1.0 **C, a**

Different capital letters within the same row indicate statistical differences at a significance level p=0.05 according to Tukey's HSD

Different small letters within the column indicate statistical differences at a significance level p=0.05 according to t-test

CHAPTER 5

General conclusions and future perspectives

This thesis was conducted in order to investigate the responses of *Salmonella* cunder various stressful environments. The effects of acid adaptation (chapters 2 & 3) and natural antimicrobials (chapter 4) were examined taking into consideration strain-dependent variations.

So far, different protocols (e.g. glucose supplementation method or short- or long-time pre-exposure to different acid conditions) have been used to unravel the role of acid adaptation on the physiology of *Salmonella*. Despite the available number of studies on the impact of acid adaptation on *Salmonella* spp. acid resistance, the impact of undissociated acetic acid, the drastic form of organic acids which can enter the cell and dissociate intracellularly, has received little attention. During this PhD thesis, 25 different combinations of pH and undissociated acetic acid concentrations were screened for their ability to increase the acid resistance of *Salmonella* spp.. The results shed light on the effect of undissociated acetic acid on the acid adaptive responses of the pathogen. Therefore, the present thesis updated our prior knowledge about adaptive acid response of *Salmonella*, by providing additional insights on the combined role of adaptation medium, the pH and temperature of acid exposure and intrinsic food factors. These factors may act collectively to determine the acid adaptive potential of the pathogen and, thus, the extent of acid stress that can be endured by *Salmonella*.

In accordance with the existing literature, this thesis also recorded extensive strain-dependent variations in the innate and acid adaptive potential of six *Salmonella* spp. strains. In extent to what has been practiced in previous studies, where the strain-dependent acid adaptive responses were examined in relation to a single acid adaptive treatment (e.g. using the glucose supplementation method or long-time pre-exposure to a given pH value), in the current PhD thesis, three different pH/undissociated acetic acid combinations were used as acid adaptive treatments and examined for their ability to promote the survival of the tested strains. In addition, it was shown that the transcriptional activation of lysine/cadaverine and arginine/agmatine decarboxylase systems was variously affected by the levels of undissociated acetic acid. Nevertheless, previous studies have shown that the contribution of these systems to the induced acid tolerance is facilitated by other factors as well (Kieboom and Abee, 2006; Viala et al., 2011), with the presence of the cognate amino acid in the challenge substrate being an absolutely necessary requirement for their function (Álvarez-Ordóñez et al., 2010b; de Jonge et al., 2003; Kieboom and Abee, 2006). Tarama salad used as challenge substrate in the current thesis is considered a poor protein source, something that can explain why

the transcriptional activation of these systems did not always correlate with the observed phenotypes of the pathogen.

Finally, in the last chapter of this PhD thesis, the *in vitro* and *in situ* antimicrobial activity of rich-phenolic natural antimicrobials collected as by-products of essential oil purification procedure against *S.* Typhimurium was demonstrated. So far, the majority of the scientific research regarding the effect of natural antimicrobials has been focused on essential oils. Despite the perceived inhibitory effect of some of these naturally occurring substances, their practical application is often restricted due to their strong flavor and aroma. Therefore, seeking for new natural antimicrobials other than essential oils that can improve food safety without compromising the sensory properties of the final products is essential for the evolution of food industry towards "green solutions". Nevertheless, the *in situ* antimicrobial effectiveness of these substances must not be taken for granted, due to their milder antimicrobial nature and their interactions with food compounds, such as lipids and proteins. The observed antimicrobial activity of aqueous oregano by-products tested in this PhD thesis enables their utilization in the food industry when applied in the context of a multi-hurdle approach, providing, therefore, an alternative, eco-friendly and cost-effective solution for improving food safety in a way consistent with market's requirements.

The last decades have seen massive changes in consumer eating habits. Consumers are not only searching for healthy food products, but also for products that, apart from healthy, are convenient to prepare and use (Nychas et al., 2008); yet, at the same time food must be profitable for the suppliers. These, often, contradictory demands have created new challenges for the Food Industry, which has to ensure the safety of their final products beyond the commercial requirements of nutrition and convenience. Eliminating microbial food hazards is a not easy task, since the ability of foodborne pathogens to survive hurdles widely employed in the food chain can be highly affected by several factors, such as the history of the cell, the innate characteristics of the strain as well as the type and intensity of the hurdle applied. Dependent on the strain, acid adaptation can enhance the resistance of *Salmonella* under subsequent lethal factors of the same or multiple stress (cross protection), such as during heat processing (Álvarez-Ordóñez et al., 2010a, 2009b; Berk et al., 2005; Lianou and Koutsoumanis, 2013b; Malheiros et al., 2009; this thesis). This raises considerable concerns regarding the safety of the food products, especially when taking into consideration the emergence of resistance or multi-drug resistant pathogens over the

last decades, hard to control with conventional antibiotics (Frieri et al., 2017). For instance, acid adapted clinically isolates of *S.* Typhimurium DT104, a virulent and multi drug-resistant strain, demonstrated also higher acid-resistance compared to *S.* Typhimurium DT104 food isolates (Berk et al., 2005). In addition, food matrix can have a protective effect towards bacterial cells, as also shown in this thesis (chapter 2). *In vitro* studies have demonstrated that combinations of organic acid and NaCl, both present in several acid food products, such as mayonnaise or deli-type based salads, can increase pathogen resistance due to an increase in intracellular pH (Lee and Kang, 2016). The ability of *Salmonella* to adapt to sublethal acid conditions should not be underestimated or overlooked by the Food Industry that needs to apply all necessary prerequisites to ensure the safety of their products, starting with good sanitation practices. With GHP established as a strategy indispensable for food safety, plant-derived natural antimicrobials can be further exploited as an alternative, economical and eco-friendly way for the improvement of food safety.

Future work could focus on using more strains in order to ensure the accuracy of the obtained results and to better evaluate the effect of *Salmonella* inter-strain variations in the exposure assessment studies. In addition, examining the proteomic profiles of *Salmonella* strains could explain the reasons why equal combinations of undissociated acetic acid during adaptation resulted in different phenotypes and possibly unravel the mechanisms that led to strain variations following inoculation in laurel at 37ºC. Furthermore, future studies could also include assessment of the virulence potential of acid adapted cells in response to different levels of undissociated acetic acid and low temperature adaptation, in order to mimic real conditions that prevail in the food industry. Finally, assessment of natural antimicrobials against acid adapted *Salmonella* cells could also provide valuable information in terms of simulating the worst-case scenario of stress tolerant cells in several food matrices.

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URL links

- https://www.who.int/activities/estimating-the-burden-of-foodborne-diseases
- https://www.cdc.gov/training/SIC_CaseStudy/Infection_Salmonella_ptversion.pdf
- https://cit.kuleuven.be/biotec/software/GinaFit

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List of publications

Publications leading to this thesis

- *Gavriil, A., Thanasoulia, A. and Skandamis, P. N.* (2020) '**Sublethal concentrations of undissociated acetic acid may not always stimulate acid resistance in *Salmonella enterica* sub. *enterica* serovar Enteritidis Phage Type 4: Implications of challenge substrate associated factors'**, *PLoS ONE*, 15(7), p. e0234999.

- *Alkmini Gavriil, Spiros Paramithiotis, Asimina Skordaki, Eleni Tsiripov, Adamantia Papaioannou, Panagiotis N. Skandamis* (2021). '**Prior exposure to different combinations of pH and undissociated acetic acid can affect the induced resistance of *Salmonella* spp. strains in mayonnaise stored under refrigeration and the regulation of acid-resistance related genes**', *Food Microbiology*, 95, 103680

- *Gavriil Alkmini, Zilelidou Evangelia, Papadopoulos Angelis-Evangelos, Siderakou Danae, Kasiotis M. Konstantinos, Haroutounian A. Serkos, Gardeli Chryssavgi, Giannenas Ilias, Skandamis N. Panagiotis*. '**Evaluation of antimicrobial activities of plant aqueous extracts against *Salmonella* Typhimurium and their application to improve safety of pork meat**'