2014

Antibacterial activity of organic acids and an aqueous lime-peel extract against selected foodborne pathogens

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Antibacterial activity of organic acids and an aqueous lime-peel extract against selected foodborne pathogens

By

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A thesis submitted to the University of Plymouth in

partial fulfilment for the degree of

MASTER OF PHILOSOPHY

School of Biomedical and Biological Sciences

Faculty of Science and Technology

March 2014

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Abstract

Food of animal origin contaminated with food-borne pathogens still concerns the public and food experts due to its harm toward public health. Using organic acids is one of the most important interventions for controlling the microbiological safety and quality of food and has been widely used. An increase of bacterial acid resistant species gave the motivation to find an alternative way to control foodborne pathogens in meat. Limited experimental studies have investigated the antibacterial activity of organic acids and an aqueous lime-peel extract combination on chicken meat against foodborne pathogens. The inhibitory effects of citric, propionic, acetic and lactic acid in combination with an aqueous lime-peel extract (*Citrus aurantifolia*) against foodborne pathogens and on the organoleptic properties of chicken meat were examined.

The minimum inhibitory concentration (MIC) of an aqueous lime-peel extract was investigated using spectrophotometer (TECAN) at 30°C. The MIC of an aqueous lime-peel extract with lactic, citric, acetic and propionic acids in nutrient broth individually against *Salmonella* Typhimurium DT104 were 2 % w/v, 8 mmol/l, 5.5 mmol/l, 5 mmol/l and 5mmol/l respectively, while against *E.coli* K12 were 1.5 % w/v, 7 mmol/l, 4 mmol/l, 4 mmol/l and 4mmol/l respectively at 30°C. MIC of lactic acid combined with citric or acetic or propionic acids against *Salmonella* Typhimurium DT104 were 8 mmol/l - 4.5 mmol/l, 7 mmol/l - 4.5 mmol/l and 7.5 mmol/l - 4 mmol/l respectively. While, against *E.coli K12* the MIC of lactic acid combined with citric or acetic or propionic acids were 7 mmol/l - 3 mmol/l, and 6.5 mmol/l - 3.5 mmol/l 6 mmol/l - 3.5 mmol/l respectively.

Immersion treatment (10 minutes) of lactic acid combined with acetic acid (208 mmol/l - 96 mmol/l) was used against *Salmonella* on chicken meat showed a negligible effect at 30°C. A combination of an aqueous lime-peel extract and acetic acid (41.6 % w/v – 1.12 mmol/l) including 1% NaCl on chicken meat was able to significantly inhibit the growth of *Salmonella* after 9 hours. Applying a sensory evaluation experiment revealed that treating raw chicken meat with lime added a citrusy flavour and made chicken more palatable after cooking. In spite of the induction of virulence and acid tolerance genes of salmonellae which were grow in nutrient broth (pH 2.25) after been treated with an aqueous lime-peel extract and acetic acid combination, salmonellae did not survive more than 2.5 hours at pH 2.5. This study shows that combining both organic acids and an aqueous lime-peel extract on chicken meat can inhibit the bacterial growth of *Salmonella* and make it more palatable than an untreated sample (control).

Table of Contents

List of Tables

List of Figures

List of abbreviations

µg	Microgram
µl	Microliter
ANOVA	Analysis of variance
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
nal^r	Nalidixic acid resistance
EDTA	Ethylenediaminetetraacetic acid
HPLC	High Performance Liquid Chromatography
mm	Millimetre
OD	Optical density
TE	Tris- EDTA
%	Percentage
FSA	Food Standards Agency
GLM	General linear model
HACCP	Hazard Analyses and Critical Control Point
MIC	Minimum inhibitory concentration
P	Statistical probability
PCR	Polymerase chain reaction
SE	Standard error
XLD	Xylose lysine deoxycholate
WHO	World Health Organization
PHE	Public Health England
LPS	Lipopolysaccharides
ERS	Economic Research Service
FDA	Food and Drug Administration
ATR	Acid tolerance response
LA	Lactic acid
AC	Acetic acid

CA	Citric acid
PR	Propionic acid
NPC	Natural plant compounds

Dedication

I dedicate this dissertation to my family (small family), who have supported me throughout the process, my lovely wife Khamael Al-Faris and my adorable child Sama Al-Khanaq.

I also dedicate my dissertation work to my family (big family), a special feeling of gratitude to my parents, Naji Al-Khanaq (God bless his soul) and Nidal Al-Saadi whose words of encouragement have pushed me all the time through. My sisters May, Noor, Luma, Huda and my brother Mohammed Ali Al-Khanaq who have stood by my side.

Acknowledgement

First, thanks God for everything and I wish to thank my supervisors who were generous with their expertise and precious time. A special thanks to Dr. Jane Beal, my director of study for her countless hours of reflecting, reading, encouraging, and most of all patience throughout the entire process. I would also like to thank my second supervisor Dr. Victor Kuri for his invaluable advice and support.

I would like to acknowledge and thank the technicians of the third and eighth floor Davy building, Professors David Coslett and Mick Fuller, Dr Michele Kiernan, Dr Mohammed Al-Issawi, Matthew Emery, Sarah Jamieson, and my friends Peter Philips and Beverly Stout for helping me to achieve this. Appreciation also goes out to Liz Preston who helped me in the Nutrition lab to do the sensory evaluation and all administrators of the Faculty of Science and Technology and international student advice office (ISAS) at Plymouth University. I apologize if I have missed anyone!

Above all, I really appreciate all the efforts that have been made by my wife Khamael, who has given me the love and encouragement to finish my thesis. I ask Almighty Allah to help her to fulfil her thesis and get the PhD. Finally, I could never ever forget my daughter Sama who has given a meaning to my life. Thanks all!

Author's Declaration

At no time during the registration for the degree of Master of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of Ministry of the Higher Education and scientific research/ Iraq.

Relevant scientific seminars and conferences attended at which work was presented are as follows:

Al-Khanaq H, Kuri V. Beal J. (2011). Minimum inhibitory concentrations of organic acids and their antibacterial activity on the growth of foodborne pathogens (Poster). In: Book of abstracts of the Society for Applied Microbiology, Summer Conference 4-7 July 2011 Dublin, Ireland.

Al-Khanaq H, Kuri V. Beal J. (2012). Inhibition of *Salmonella* Typhimurium DT104 on marinated chicken by combination of organic acids and an aqueous extract of lime (Poster). In: Book of abstracts of the Society for Applied Microbiology, Summer Conference 4-7 July 2011 Edinburgh, UK.

Al-Khanaq H, Kuri V. Beal J. (2012). The effect of acidic food additives on the gene expression of *Salmonella* Typhimurium DT104 at 30°C (Poster No. 514A). In: Book of abstracts of the ICSB (The 13th International conference on System Biology), 19-23 August 2012 Toronto, Canada.

Al-Khanaq H, Kuri V. Beal J. (2013). Using food additives and their effect on gene expression of *Salmonella* Typhimurium on chicken meat (Poster). In: Book of abstracts of the Early Career Researchers in Food Sector conference on 14th November 2012 Edinburgh, UK. Available on-line https://connect.innovateuk.org/c/document_library/get_file?folderId=10031450&name=DLFE-112600.pdf

Al-Khanaq H and Beal J. (2013). Inhibition of microbial food contamination on raw chicken meat by using combination of natural food additives (Poster). In Book of abstracts of Microbial Spoilers Congress in Food 2013 on 1st -3rd July 2013, Quimper, France.

Al-Khanaq H and Beal J. (2013). Inhibition of acid resistant *Salmonella* on raw chicken meat using a combination of natural food additives (Oral). In Book of abstracts of BioMicroWorld 2013 conference on 2nd -4th October 2013, Madrid, Spain. Available on- line http://www.formatex.info/biomicroworld2013/acceptedabstracts.php

Al-Khanaq H and Beal J. (2013). The impact of using acidic food additives on gene expressions of acid tolerant strains of *Salmonella* Typhimurium at 30°C (Oral). In Book of abstracts of BioMicroWorld 2013 conference on 2nd -4th October 2013, Madrid, Spain. Available on- line http://www.formatex.info/biomicroworld2013/acceptedabstracts.php

Word count of main body of the thesis (including references and appendixes): 34,060

Signed

Date: 24/03/2014

Chapter 1

General introduction and literature review

Chapter 1

1.1 General Introduction

Poultry consumption is expected to rise as a response to the world population increase. The current estimated poultry meat consumption in the world will grow at an annual rate of 3% (an increase of 60 million tonne/year)(Sarris, Food & Nations, 2003). By 2020, world production of poultry meat will approach 122.5 million metric tons per year (Best, 2012; WHO, 2007). In addition, there is an increase in the public demand for poultry products in the world (Clare A. Narrod, 2008; Guerrero-Legarreta et al., 2010; Narrod, Pray & Tiongco, 2008), which is due to consumer awareness of the influence of the dietary fat on health and fitness (Edelstein, 2010; Pearson, Dutson & Dutson, 1997). This diet, in combination with our modern lifestyle and large portion of meat is blamed to be the reason for the increase in obesity and associated diseases (high blood pressure, coronary heart disease, stroke, gallbladder disease).

Poultry meat is considered a favourable host for bacteria especially *Salmonella, E.coli* and *Campylobacter,* which occur naturally in the chicken gut and are very easily spread by both broiler and food processing techniques (Guerrero-Legarreta *et al.*, 2010). Raw chicken meat is one of the main sources of foodborne pathogens for several reasons such as: the suitability for microbial multiplication, high level of water activity, and the glucose (Forsythe, 2011). As a result, it becomes essential to find a suitable decontaminant which is able to inhibit the bacterial growth on chicken meat keep the food safe.

Throughout the world, food production standards have been introduced such as Hazard Analysis Critical Control Point (HACCP) aimed at producing safe food products by applying several precautions to minimize the risk of contamination due to physical, chemical, and microbiological hazards as reported by Food Standards Agency.

Despite that, the number of people infected with foodborne pathogens like *Salmonella* and *E.coli* continues to increase in undeveloped countries (Goodkind, 2012). Foodborne disease still concerns many governments due to its social and economic harm that costs $152 million annually in developed countries like the USA (Buzby, 2004; Pew Health Group, 2013); Also, yet 76 million illnesses and around 3000 estimated deaths number due to food-borne pathogen infections still occur each year in the USA.

In the EU, foodborne disease costs nearly £1.5 billion annually and around one million people have reported suffering from an infection of foodborne pathogens (FSA, 2011). In the Middle East and especially in Iraq, there is a lack of information about the economic cost of foodborne disease. However, in one set of experiments, British soldiers who suffered from foodborne disease in Iraq, remarkably suffering more food- borne health problems than battlefield injuries (Bailey et al., 2005).

In the industrial sector, two techniques have been in use in the decontamination of chicken meat: physical and chemical methods (Toldr, 2010). In terms of the physical method, dipping and spraying with hot water, and ultra violet light has been used widely (Coote et al., 2001). Organic acids, trisodium phosphate, and ozone have been considered as antibacterial treatments and showed a respectable antibacterial activity against foodborne pathogens (Toldr, 2010). In the UK and EU, using chemical decontamination of carcasses with organic acids is not permitted under

regulation (EC) No. 853/ 2004 (EFSA, 2011). Contradicting this, chemicals such as organic acids as antibacterial agents in meat is considered a safe decontaminant in the USA.

Using high concentrations of organic acid treatments affects the organoleptic properties of food products (taste, colour, and texture), making the product unsuitable for human consumption. Hence, researchers and food experts have investigated alternative natural decontamination techniques. The decontamination tactic not only needs to be effective, but also must be acceptable, inexpensive, safe to be in the food, and not harmful to our environment.

For centuries, herbs have been in use for several reasons, such as their ability to keep food safe, adding natural flavour, and their beneficial role in medicine (Sisson, 2011). Therefore, many studies have focused on using herbs in food (Ismail et al., 2001). In addition, most plants are considered as safe natural food additives, such as *Citrus aurantifolia* as it gives the food flavour and acidity (Al-Jazairy, 2012). Organic acids are intensively used to inhibit and reduce the microbial population in food products and their antimicrobial performance may due to the level of acidity (Bjornsdottir, 2006). However, the information about the antibacterial activity of herbal extracts and organic acids combined on raw chicken meat is still limited (de Oliveira et al., 2010).

The aim of this project was to investigate the antibacterial activity of an aqueous lime-peel extract and organic acid combination against selected foodborne pathogens on raw chicken meat for worldwide use, especially in Iraq, instead of water when poultry carcasses are washed.

The objectives were to:

- Determine the minimum inhibitory concentration (MIC) of organic acids (lactic, citric, acetic and propionic acids) alone and in combination with nutrient broth and then on raw chicken meat at 30°C (room temperature in Iraq) against foodborne pathogens.
- Determine the MIC of an aqueous extract of *Citrus aurantifolia* against *Salmonella* Typhimurium DT104 and *Escherichia coli* K12 alone and in combination with organic acids in nutrient broth and then evaluate the antibacterial activity of the concentration below the MICs against *Salmonella* Typhimurium in raw chicken meat at 30°C.
- Assess the effect of a lime and organic acids combination treatment on the sensory evaluation properties (aroma, flavour, colour, etc.) of cooked chicken breast meat.

1.2 Literature review

1.2.1 Foodborne disease

Many countries (developed and undeveloped) are still struggling with foodborne disease, which leads to sickness and economic losses (James, 2006). *Salmonella, Campylobacter* and *E.coli* cause gastroenteritis, typhoid, paratyphoid, bacteraemia and septicaemia throughout the world (Fleisher & Ludwig, 2010). For infections from *Escherichia coli* or *Salmonella,* the symptoms are similar, such as abdominal cramps, vomiting, diarrhoea and fever (Taylor, 2003).

Foster (2004a) has reported that foodborne illness in the USA infected 3.3–6.5 million people each year and there are five million fatalities each year in third world countries (Gould & Russell, 2003). According to several studies, the economic cost of salmonellosis in the USA was estimated between '$64-$114.5' million each year (Bryan & Doyle, 1995; Castillo, Martinez & Apodaca, 2008; Heres et al., 2004). In 2011, the total cost of foodborne disease in the USA economy was $77.7 billion (CDC, 2013a). According to WHO, the global incidence of foodborne illness is difficult to be estimated, but in 2005 alone, 1.8 million people died from diarrhoea (WHO, 2007). In the UK, the incidence of food-borne disease due to *Salmonella* Typhimurium was more than 400 cases in 2010 (HPA, 2011c).

The increasing number of food-borne disease cases from consumption of infected meat, especially acid resistant and antibiotic resistant bacteria, has attracted the attention and amplified the concern of food experts and food hygiene agents (MRA, 2009). Therefore, food safety societies, food experts, and governments have started educating people to be aware of the danger of foodborne diseases.

As a result, the number of infected people was slightly reduced until 2004, when the number of infected people started to increase again (HPA, 2011b). The number of reported cases in 2011 has increased due to a 10% rise in the number of *Salmonella* cases (Goodkind, 2012). The rate of poultry consumption has exceeded the production rate remarkably over the last ten years in many world countries such as China, Japan, Russia and the USA (Table 1) (FDA, 2005). By 2020, the production of poultry meat will increase by 36.04% (Best, 2012).

Table 1: Basic statistics on the number of production and consumption of poultry sectors in six countries (thousand tone) from FDA (2005)

Countries	Production	Consumption
Brazil	4597	3969
China	10978	11762
EU	8375	7588
Japan	1190	1697
Russia	640	1516
USA	15131	12623
Other Countries	12148	11925

Faeces, spillage of gut contents (in the abattoir), and contaminated equipment lead to cross-contamination in slaughter houses and factories (Bassett, 2012). The positive number of contaminated raw chicken meat differs in carcass parts (Table 2). Chicken breast meat and wings have a high incident number of *Salmonella*; this might be due to a high fat level which is considered as typical for bacterial growth. Hence, the contamination of raw chicken meat by *Salmonella* and *Campylobacter* is unavoidable (WHO, 2009).

Table 2: Incidence of *Salmonella* in retail raw chicken products in the UK adapted from (Bell & Kyriakides, 2007)

Part of slaughter	Number of samples positive for *Salmonella* / *total number*	%
Whole birds	19/64	29
Breasts	28/91	30
Quarters	12/75	16
Drumsticks	5/29	17
Thighs	1/28	3
Wings	3/10	30
Mixed portions	6/24	25

In fact, foodborne disease cases are complicated and many points should be taken into account to prevent the contamination of food products, such as: (1) The size of meat pieces and their density, (2) cleaning status of equipment's and packaging manner, (3) a sufficient cooking time (Bell & Kyriakides, 2002). Hence, many researchers have investigated several new strategies by trying to minimise the microbial load on chicken carcasses. For instance, dipping the carcasses with chilled water or hot water, a combination of acid and salt, and using chlorinated water (Dubal et al., 2004).

1.2.2 Foodborne pathogens

Salmonella is considered a member of Enterobacteriaceae and a Gram-negative bacterium, facultative anaerobic, non-lactose fermenter and an external rod shape (Wray & Wray, 2000). *Salmonella* has several species and subspecies. *Salmonella* enterica subsp. enterica is the most pathogenic subspecies according to food safety experts and the high number of human outbreaks (Table 3) (Tindall et al., 2005). In terms of growth conditions, the range of temperature is from 8°C to 45°C, *Salmonella* can grow in a pH range of 4 to 9, and the typical water activity level for *Salmonella* is above 0.94 (Bell & Kyriakides, 2001).

Additionally, most species of *Salmonella* can tolerate and survive within a salt environment of 20 % and survive up to seven years in cold temperatures (from -23 to -18 °C)(Bell & Kyriakides, 2001).

Table 3: *Salmonella* species and subspecies adapted from (Palmer, Torgerson & Brown, 2011)

***Salmonella* enterica subsp.**		
I	*Enterica*	1539
II	*Salamae*	
IIIa	*Arizonae*	96
IIIb	*Diarizonae*	334
IV	*Houtenae*	71
VI	*Indica*	14
	S.bongori	22
Total		2579

Salmonella enters the human body by contaminated water and food. Once it reaches the intestine of the human body, *Salmonella* starts to colonize, penetrate the intestinal cells and can easily remain in the intestine for many weeks (Boyer, Wright & Manns, 2011). In order to be inside the intestine cells, *Salmonella* uses several structural components, for example, fimbriae, which are responsible for the adhesion of bacteria to external host surface (Wooldridge, 2009). In addition, fimbriae are considered as one of main virulence factors in *Salmonella* (Pichpol, 2009; Quinn & Markey, 2003).

The infective dose for causing salmonellosis still varies due to several reasons such as the age, defence system and general health of the host (Wray & Wray, 2000). However, 10^5–10^6 CFU ml^{-1} of *Salmonella* per gram of food definitely led to foodborne disease (Pichpol, 2009).

The food prepares the environment and protects *Salmonella* through the acidic environment in the stomach. For example, the high fat content in food has the ability to buffer the stomach environment. Then a low number of *Salmonella* can easily increase and severely infect intestinal cells (Pichpol, 2009). Furthermore, *Salmonella* can easily survive in the presence of antibiotics such as chloramphenicol, ampicillin and tetracycline (Mayers et al., 2009) as it is multidrug resistant among the most commonly isolated serovars from various sources (human and animal) (Zhao et al., 2003). It is challenging to control *Salmonella* due to its ability to survive within various stressed environments (Russell, 2012). Therefore, there is an urgent necessity from scientific, public health and governments to control and regulate salmonellae species (Wray & Wray, 2000). Economic losses due to *Escherichia coli* have been estimated at 5 million / year according to USDA (United State Department of Agriculture) and ERS (The Economic Research Service in the USA) (ERS, 2011).

Escherichia coli is one of the Gram-negative bacteria, non-spore forming, rod-shaped, and facultative anaerobic, which can be motile by flagella or not motile (Ray & Bhunia, 2008). *E.coli* belongs to the *Enterobacteriaceae* as foodborne pathogens and is considered as a part of human and animal (Acton, 2013b). Pathogenic species of *E.coli* produce a toxin called Shiga like *E.coli* O157:H7, causing a gastrointestinal illness. Similar to salmonellosis symptoms; diarrhoea, vomiting, abdominal cramps are the main symptoms of gastrointestinal illness, which appear after ingestion of contaminated food (Forsythe, 2010).

Campylobacter is a genus belonging to Campylobacteraceae and described as a pathogenic genes of food borne pathogens (Ketley & Konkel, 2005). The general characteristics of *Campylobacter* are a Gram-negative bacteria, spiral in shape, 0.2-0.8 µm width and 0.5-5µm length, and most species of *Campylobacter* are motile (de los Santos & Arkansas, 2008). *Campylobacter* is one of the most common causes of diarrhoeal illness in the USA (Bope & Kellerman, 2012). *Campylobacter jejuni* grows typically at 37°C to 42°C also it seems to be well adapted to live in birds, who carry it without becoming affected (CDC, 2013b).

In the UK, there are 50,000 reported gastroenteritis cases caused by *Campylobacter* each year but many more cases go unreported because most people recover naturally after a few days. Nonetheless, disease caused by *Campylobacter* costs the UK economy an estimated £500 million (Titball, 2011). In the USA, *Campylobacter* causes an estimated 845,000 cases of food-borne illness and 76 deaths each year (Swayne et al., 2013).

Salmonellosis and campylobacteriosis are among the most frequently reported foodborne diseases worldwide and commercial chicken meat has been identified as one of the most important food vehicles for these organisms (Organization, 2009). Although specific data on the burden of foodborne disease associated with *Salmonella* and *Campylobacter* in poultry is limited, the fresh and processed poultry account for ~29% of all *Salmonella* infections in humans (Braden, 2006). Furthermore, the presence of these organisms in poultry is also affecting trade, and recently the detection of *Salmonella* in poultry products led to rejection of large consignments of raw poultry meat (Organization, 2009).

Out of the total 314 samples (144 of raw red meat and meat products, 128 of raw poultry meat and poultry products, and 42 of processed meat products) collected from various retail, 61 (19.43%) were tested positive for *Salmonella* (Acton, 2013a). In addition, the contamination problem of *Salmonella* was recently highlighted in a 2010 salmonellosis outbreak caused by *S. enterica* serovar Enteritidis that was traced back to contaminated eggs from Iowa (Kuehn, 2010).

1.2.3 Decontamination of poultry meat with organic acids and herbal extracts

There are many ways to decontaminate poultry meat, some of them physical (heat, cold and salt), and others are chemical (organic acids and inorganic acids). One of the best routes to inhibit the bacterial growth in food is using organic acids. The positive alteration of adding herbs into food may enhance the meat taste, kill microorganisms and eliminate the risk of cancer components like heterocyclic amines (HCAs). The positive health benefits of polyphenols (natural extract contents) in reducing several HCAs such as 2-amino-3,8- dimethyl-imidazo [4,5-f] quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenyl-imidazo[4,5- b] pyridine (PhIP) in meat has been reported (Smith, Ameri & Gadgil, 2008).

Organic acids have several types of acids like lactic, citric, propionic and acetic acids. In addition, treating food with herbs might inhibit the bacterial growth and keep food safe and healthy as many food safety specialists have confirmed (Peterson, Organics & Freeman, 2006). Organic acids are considered safe antimicrobials and food preservatives (Bell & Kyriakides, 2007; Nakai & Siebert, 2004; Xiong et al., 1998).

For many years, organic acids have been used as food additives, preservatives and shelf life enhancers (Cherrington et al., 1991). The United States Food and Drug Administration (FDA) has stated that the concentration of 1.5 % to 2.5 % of organic acids such as citric acid (CA) and lactic acid (LA) are recognized as safe and are also suitable to inhibit microorganisms in poultry products (FDA, 2011; MIS, 2006).

Lactic acid has been used extensively due to high sensory qualities and the antimicrobial performance; nowadays lactic acid is used widely to rinse carcasses (beef, poultry and pork) (Davidson, 2005). Lactic acid is considered one of the common food additives, which naturally occurs in food due to fermentation (Benninga, 1990; Theron & Lues, 2009). According to the FDA, lactic acid has been classified as a safe food preserver, flavour enhancer, pH buffering agent and organoleptic properties enhancer (flavour and aroma) (FDA, 2011; Theron & Lues, 2009). One of the best routes for elimination of foodborne pathogens in meat is to treat it with some decontaminants before packaging, like lactic acid in different concentrations (FSA, 2009). The antibacterial activity mechanism of organic acids starts when the undissociated acid form entering the cytoplasm start to dissociate; hence, the internal pH will be lower than normal level and that disrupts many cellular functions within the cell (Abou-Taleb & Kawai, 2008; Anang et al., 2007; Lück & Jager, 1997).

The antibacterial effect of organic acids is due to the dissociation form and the hydrogen ions' donation abilities in an aqueous environment (Uljas & Ingham, 1998). Many studies have investigated the antibacterial activity of organic acids against a wide range of bacteria. Nevertheless, the mechanisms of bacterial inhibition are still not clear. However, three proposed suggestions have been accepted, which are: cytoplasmic acidification, acid anions, and intracellular anions accumulation and the disruption of the bacterial plasma membrane (Yildirim et al., 2010).

The undissociated form of organic acids is responsible for letting acid molecules invading the cytoplasmic membrane into the bacterial cell (Keklikci et al., 2010). Acid molecules decreased the pH level immediately after inflowing into the bacterial cytoplasm (Hensel, 2004).

The main cellular functions of the bacterial cell, which will be affected by using organic acids, are: enzyme secretion, proton transportation, and energy compounds, depletion, which due to the transportation of protons and that acidify the external cell environment. It is an efficient bacterial way to confront this type of harsh circumstances by adjusting the cytoplasmic pH level and keep it in the normal level by removing protons outside of the cytoplasm (Lambert & Stratford, 1999).

In addition, when the bacteria cell is exposed to organic acids, many toxic anions invade the cytoplasm leading to anions accumulation inside the bacteria. The accumulation of anions within the cytoplasm will increase the osmolarity significantly (Bacon et al., 2003). As a result, the bacterial cell will be ruptured because of the huge amount of osmotic pressure. The third mechanism of acid inhibition is called disruption of the cell membrane, which has been reported within lactic and Gram-negative bacteria (Yildirim *et al.*, 2010).

Lactic acid is a slightly lipid soluble and its ability to diffuse across the cell membrane happens slowly, and that might affect its ability as an antibacterial compared with other types of organic acids (Gravesen et al., 2004). For instance, concentrations of lactic acid from 1 to 2 % are recommended in food for good flavour and inhibitory action while lactic acid below 0.5% might have an inhibitory effect against Gram negative bacteria (Dickson & Siragusa, 1994; Ray, 2004). Moreover, using high concentrations, like 10 % lactic acid, has been avoided due to detectable changes in food products such as odour, colour and texture (Kim & Marshall, 1999).

However, dipping treatment with 1.5 % and 2.0 % lactic acid for 10, 20 and 30 minutes caused a significant reduction in the total number of *Salmonella* in chicken breast at 4°C (Table 4) (Anang *et al.*, 2007). *Campylobacter jejuni* has the ability to survive for extended periods in different stressful environments. However, a 1 minute dipping treatment of 2.5 % lactic acid solution was able to reduce the viable cell of *Campylobacter* on the chicken meat and skin by 0.8 and 1.7 log CFU g^{-1} respectively (Riedel et al., 2009).

Table 4: *Salmonella enteritidis* on chicken breast meat dipped in different concentrations of lactic acid for 10, 20, and 30 minute adapted from Anang *et al.* (2007)

Lactic acid concentration % (mM)	Dipping duration (minutes)	Log CFU /g *Salmonella*
0.5 (55mM)	10	0.77
	20	0.77
	30	0.92
1.0 (111mM)	10	0.79
	20	0.98
	30	0.96
1.5 (166mM)	10	0.81
	20	0.89
	30	1.07
2.0 (222mM)	10	0.91
	20	1.22
	30	1.77

Increasing organic acid concentration increases the antibacterial effectiveness, but with unacceptable changes in the organoleptic properties (colour, texture, and smell), especially with organic acid concentrations above 4% (Theron & Lues, 2009).

Therefore, a number of researchers have tried to use and combine organic acids at low concentrations to reduce the impact on food properties and increase the bacterial inhibition (del et al., 2007; Nazer et al., 2005).

As a result, all treatments of organic acid combinations have reduced microbial populations significantly as compared with the control (untreated) samples. Acetic acid is a component of household vinegar, which has 5 % acetic acid (Kerth & Braden, 2007). Some factors have been reported to be responsible for the antibacterial activity of acetic acid such as: the acidity and the polyphenolic compounds like tannins in 'vinegar' (Theron & Lues, 2009).

Regarding the dissociation ability through the bacterial cell membrane, acetic acid is more effective than lactic acid at lower pH (Davidson, 2005). Particularly, organic acids have different dissociation levels depending on the acidity and dissociation constant the lower the pKa value, the stronger the acid (Charalampopoulos & Rastall, 2009). Therefore, pKa value is different between organic acids (Table 5). The pKa value leads to high proportion of undissociated acids, and this might be the reason why acetic acid is effective against wide range of foodborne pathogens (Saltmarsh et al., 2013).

The pKa may explain the antibacterial activity of organic acids; as the pH increases, fewer acid molecules are dissociated and the antibacterial activity shrinks (Schmidl & Labuza, 2000). According to Loretz et al (2010), the range of effective and acceptable concentrations of acetic acid in food against a wide range of bacteria is 0.9 to 2.0 %. Moreover, acetic acid is considered as bacteriostatic at 0.2% (Kim, Shin & Hwang, 2001; Ray, 2004). All organic acids show the strongest bactericidal effect on *Campylobacter* at pH 4.0. In contrast, at pH 5.0 and 5.5, the bactericidal activity of organic acids was low (Chaveerach et al., 2002).

Table 5: pKa values of regulatory approved organic acids from (Armstrong & Kellee Hollyman, 2011; Davidson, 2005 ; Theron & Lues, 2009).

Organic acids	pKa
Acetic acid	4.75
Lactic acid	3.79
Propionic acid	4.87
Citric acid	3.08

In addition, at low pH levels and high temperature, acetic acid was more effective to inhibit foodborne pathogens than lactic and citric acids (Davidson, 2005). Regarding the surface tension theory, acetic acid is more effective than citric acid (Permprasert & Devahastin, 2005; Theron & Lues, 2009).

According to Greenacre et al (2003), acetic acid is a more effective antimicrobial than lactic acid, mainly due to non-lipid soluble properties and the slow diffusion through the bacterial cell membrane. Over all, both dipping and spraying treatments have shown a significant reduction in microorganisms, especially *Salmonella* (Adams & Moss, 2002; Beal et al., 2004; Birk et al., 2010a). For instance, *Salmonella* has been significantly reduced by 1.3 $\log_{10}$ CFU ml^{-1} with spray treatment and 2.3 $\log_{10}$ CFU ml^{-1} for dipping treatment using a concentration of 2.5% lactic and acetic acids (Laury et al., 2009).

The reduction of *Escherichia coli* cells on chicken meat surfaces dipped into 1, 2 and 3% lactic acid was 0.5, 1.8 and 2.1 $\log_{10}$ CFU cm^2 respectively, and lactic acid was more effective against *Escherichia coli* than acetic acid (Bin Jasass, 2008). The antibacterial activity of acetic and lactic acid against *Campylobacter jejuni* was tested in suspension at 4°C.

As a result, 20 minutes of treatment with 1 % lactic acid did not significantly reduce *C. jejuni* populations; also substituting 0.5 % lactic acid for 0.5 % acetic acid was not effective with a reduction of *C. jejuni* (Zhao & Doyle, 2006).

Citric acid is one of the commonly used food acidulants. It is a natural consitutent of many fruit types called ‘citrus fruit’ and widely used in canned food as a preserver (Davidson & Branen, 1993; Davidson, Sofos & Branen, 2005; Naidu, 2000). In the USA, citric acid has been used for many years to inhibit foodborne pathogens and prevent contamination of food products (FDA, 2011).

Nowadays, as an alternative to using citric acid, citrus fruit (lime, lemon etc.) has been used due to a high level of citric acid (Theron & Lues, 2009; Valero et al., 2000). In addition, citrus fruits have various levels of organic acid and vitamins such as vitamin C. Therefore, citrus fruits are a good supplier of organic acids and vitamins (Wu et al., 1995).

In general, the range of an effective concentration of organic acids (citric, lactic and acetic acids) is between 1.5 - 2.5 % in the decontamination of meat (CFIA, 2011), which is able to reduce the negative aspects of using organic acid in food (colour change, acidic smell etc.) (Ray, 2004). In one set of experiments, *C. jejuni* strains were exposed to tartaric, acetic, lactic, malic, and citric acids in broth and on chicken meat at 4°C. As a result, an organic acids concentration of 0.5 % was able to reduce the bacterial populations of *Campylobacter* in broth (chicken juice and brain heart infusion broth) by 4 to 6 log units after 24 hours of treatment (Birk et al., 2010b).

Propionic acid is one of the legislated food preservatives (FSA, 2012) and as a food additive and an antimicrobial reagent in the USA (FDA, 2010). Propionic acid is a traditional food preservative due to its inhibitory effect against yeast, molds, and bacteria (Suhr & Nielsen, 2004).

Propionic acid exists naturally in many food types by natural processing as result of fermenting food (Table 6). Treatments of propionic and acetic acids have been reported to have bactericidal effects on *Campylobacter* (Ricke et al., 2012).

Table 6: Natural occurrence of citric and propionic acids from Theron and Lues (2009)

Organic acids	Source found	Natural function
Citric acid	Citrus fruits	Essential for citric acid cycle in respiration of plants and animal cells
Propionic acid	In foods by natural processing (fermentation)	fermented foods

On the other hand, the value of using herbal extracts as antibacterial and food preservers has been documented as herbs have various level of antibacterial activity, which give them the ability to inhibit the bacteria (Table 7).

Usually, many herbs are stored in dried form, aiming to extend the shelf life and preservation due to concentrated contents (Shababy, 2010).

Table 7: Antimicrobial effectiveness of spices and herbs adapted from Snyder (1997)

Spices and Herbs	Inhibitory effect
Cinnamon, cloves, mustard	Strong
Allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage, thyme	Medium
Black pepper, red pepper, ginger	Weak

In European countries, a wide range of herbal essential oils are accepted as flavourings and considered as safe food additives such as carvacrol, cinnamaldehyde, citral, limonene and menthol (Falcone, 2005; Tajkarimi, Ibrahim & Cliver, 2010). Some herbs are considered as a safe treatment either in food or in medicine in the UK referring to legislation 2004/24/EC (MHRA, 2010).

In the USA, as regards to Federal legislation No. 21 CFR, the essential oils for cinnamon, lemon and clove are described as safe to be in food (Turgis et al., 2009).

Many researchers investigated the antibacterial properties of herbs in many countries like China, India, and Indonesia (Tajkarimi, Ibrahim & Cliver, 2010; Zaika, 1988). These kinds of plants are prepared in different ways, such as fermenting in the sun, air-drying, roasting, and grinding techniques (CDC, 2012). In the Middle East and Asian countries, many herbs are still in use as a food flavour enhancer such as clove, cinnamon, mustard and garlic, besides using them as remedies. Additionally, there are 1340 plants that contain antimicrobial compounds and more than 30,000 compounds have been utilized in factories (Tajkarimi, Ibrahim & Cliver, 2010). Some points must be taken into account when using herbs as antibacterials, such as using concentrations of herbal extracts to protect the sensory properties of the product at levels appropriate for decontamination. Fisher and Phillips (2006) have investigated the minimum inhibitory concentration for some types of herbs and spices against Gram positive and Gram negative bacteria. The minimum inhibitory concentration of bergamot, orange, lemon, citral and linalool against both of *Escherichia coli* O157 and *Staphylococcus aureus* were (0.5, 1, 1, 0, 0.25 % v/v) and (1, 0, >4, 0.06 and 0.125 % v/v) respectively.

The inhibitory effects of herbs and spices were reported against a wide range of bacteria (Table 8) (Snyder, 1997). Tajkarimi et al (2010) has reported the antimicrobial activity of many types of plants against *Escherichia coli* and *Salmonella* Typhimurium (Table 9).

Herbal extracts have several natural plant compounds, which are most effective against *Campylobacter jejuni* such as flavonoids, terpines and tannins, by inhibiting the bacterial growth at 62.5-125 µg mL (Dholvitayakhun, Cushnie & Trachoo, 2011). In general, plants are known to have an enormous variety of antibiotics, which are classified as 'phytoalexins' that included: terpenoids, glycosteroids, flavonoids and polyphenols (Anja, Sonja Smole & Qijing, 2012).

Phytoalexin have been screened for potential anti-*Campylobacter* effects (Anja, Sonja Smole & Qijing, 2012). In addition, Citrus limon and turmeric (*Curcuma longa*) has been reported to contain high amounts of polyphenols, and that might be the reason for the high level of antibacterial against a wide range of bacteria (Murali et al., 2012). Marination of chicken meat in different food ingredients can be used to reduce populations of *Campylobacter jejuni*. For instance, vinegar, lemon juice, pomegranate syrup, and soy sauce were reported to reduce the total counts of *C. jejuni* by at least 0.8 log units on meat medallions at 4°C (Birk *et al.*, 2010b).

Table 8: Inhibitory effects of spices and herbs against wide of microorganism adapted from Snyder (1997)

Spice / Herb	Microorganisms
Garlic	*Salmonella* Typhimurium*, Escherichia coli, Staphylococcus aureus, Bacillus cereus, Bacillus subtilis,* Mycotoxigenic *Aspergillus, Candida albicans*
Onion	*Aspergillus flavis, Aspergillus parasiticus*
Cinnamon	Mycotoxigenic *Aspergillus, Aspergillus parasiticus*
Cloves	Mycotoxigenic *Aspergillus*
Mustard	Mycotoxigenic *Aspergillus*
Allspice	Mycotoxigenic *Aspergillus*
Oregano	Mycotoxigenic *Aspergillus, Salmonella* spp*., Vibrio parahaemolyticus*
Rosemary	*Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus*
Sage	*Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus*
Thyme	*Vibrio parahaemolyticus*

Table 9: Inhibitory activities of plant origin antimicrobials against pathogenic bacteria (*E.coli* and *Salmonella*) adapted from Tajkarimi, Ibrahim and Cliver (2010).

Organism	Inhibitors
Escherichia coli	Cinnamon, oregano oil, pure essential oils, leaf volatile oil, eugenic, bark volatile oil, bark oleoresin, carvacrole, oregano oil, Citra, lemongrass oil, cinnamaldehyde, cinnamon oil ,thyme, clove, mustard, bersama engleriana, cabbage juice, Aristo lochia, ground yellow mustard, brassica oleracea juice, dried garlic powder, commercial garlic products, garlic oil, onion, marjoram, basil essential oil, forsythia suspense and rosemary and clove oil with 75% ethanol, cassia bark derived substances, bay, ion extracts, crude extract of lycoris chine sis(bulbs).
Salmonella Spp.	Teas, leaf volatile oil, leaf oleoresin, eugenol, bark volatile oil, bark oleoresin, oregano, cinnamon, lemongrass, thyme, Citra, carvacrole, cinnamaldehyde, Citral, methanol extract of Aspilia mussambicensis, bersama engleriana, brassica oleracea juice, dried garlic powder, commercial garlic products, garlic oil marjoram, basil essential oil, cassia bark-derived substances ,lemongrass ,bay, methanol and acetone extracts of 14 plants belonging to different families and thymol.

Various levels of antibacterial activity have been achieved by using the herbal extracts depending on the level of plant compounds such as phenol, alcohols, aldehydes, ketones, ethers, and hydrocarbons (Patra, 2012). Initially, natural plant compounds (NPC) such as glycosides, saponins, alkaloids, and organic acids are in use in plants' protection systems against the microbial infection (Ceylan & Fung, 2004b).

In addition, some NPC such as limonene, linalool, and cineole have stated to be effective in the elimination of tough species of foodborne pathogens such as, *Campylobacter jejuni*, *Escherichia coli* and *S.aureus* (Chen et al., 2008; Sandasi, Leonard & Viljoen, 2008). Therefore, in recent years, treatments of spice and herbal extracts have become widely used and recommended for controlling of food spoilage and foodborne disease (AL-Branen, 1993). The severity of the antibacterial effect of herbal extract depends on several factors, like the pH level, the temperature, the total concentration of extract and the level of NPC compounds (Burt, 2004; Ceylan & Fung, 2004b; Gutierrez, Barry-Ryan & Bourke, 2008; Tajkarimi, Ibrahim & Cliver, 2010).

Despite having a high level of antibacterial activity, some herbal extracts are not used because of the pungent odour of their chemical contents, such as thiosulfates in garlic and glucosinolates in cabbage (Almajano et al., 2008; Graumann & Holley, 2008). When concerns about using chemicals to disinfect and preserve the food are elevated, treatment of herbs will increase. Therefore, some studies have tried to investigate the beneficial aspects of using lime extracts in meat (Hyldgaard, Mygind & Meyer, 2012; Tserennadmid et al., 2010). In Iraq as one of the Middle East countries, many herbs such as *Citrus aurantifolia,* cardamom and *Stachys monnieri* are still in use in various life aspects especially in the kitchen.

For instance, on chicken, citrus fruits are likely be used to give the citrusy flavour as well as to preserve the food (Magazine, 2011). *Citrus aurantifolia* (Figure 1) is one of citrus species usually picked green but the mature fruit is yellow. In Egypt and Sudan they are called ‘limûn baladi’, ‘baladi’ in Morocco, ‘limun Basra’ in Iraq, and in the USA called ‘key limes’(Katzer, 2000a).

Figure 1: *Citrus aurantifolia* (dried on the sun) adapted from Katzer (2000a)

Citrus aurantifolia belongs to Rutaceae originating from Asian countries (Indonesia, Iran and Iraq) (Table 10). Due to the unique citrusy flavor, citrus fruit is consider to be good be added to fish and meat or cold, hot drinks and aperitifs (Katzer, 2000b). In addition, citrus fruits, lemons and limes were analyzed to find the chemical contents (Table 11). Citrus fruits are in use in medicine (traditional medicine) as painkillers and anti-flu protection. Recently, the use of citrus species has attracted food experts and researchers (Aibinu et al., 2007; Chanthaphon & Chanthachum, 2008).

Table 10: Classification of *Citrus aurantifolia* from Woodford (2005)

Taxonomy	
Kingdom	Plantae
Division	Spermatophyta
Sub Division	Angiospermae
Class	Dicotyledonae
Order	Rutales
Family	Rutaceae
Genus	Citrus
Species	*Citrus aurantifolia*

In order to find the reason that makes citrus very effective as an antibacterial, some studies have investigated the content of citrus (Table 11). The main contents of lime essential oil are citral, limonene, β-pinene and fenchone (Katzer, 2000a). Limonene, linalool, and cineole were reported to be the reasons for making citrus fruits very effective in the elimination of foodborne pathogens (Chen *et al.*, 2008; Sandasi, Leonard & Viljoen, 2008; Tajkarimi, Ibrahim & Cliver, 2010).

Table 11: The nutrition analysis of lemons and limes from WHFoods (2011)

lemons and limes (61.00 grams – 0.25 cup)	
Nutrient	**Amount**
Lutein + zeaxanthin	5.49 mg
Choline	3.11 mg
Organic acid	2.85 mg
Citric acid	2.85 mg
Potassium	75.64 mg
Magnesium	3.66 mg
Calcium	4.27 mg
Carbohydrates	5.26 g
Calories	15.25 unit
Water	55.35 g
Vitamin A (IU)	12.20 IU
Vitamin C	28.06 mg
Folate	7.93 mcg

Regardless of the environmental conditions (pH and temperature etc.) and the enrichment levels (protein and carbohydrates etc.), the level of antimicrobial activity between herbal species is different (Adams & Moss, 2002; Gutierrez, Barry-Ryan & Bourke, 2008). As a result, several points need to be established such as the extension of food shelf life, reducing economic losses, and minimizing the cost of using many food additives (Pliego, 2007).

It has been confirmed that the antibacterial activity of phenolic compounds is due to their effect against the phospholipid bilayer of cytoplasmic membrane in bacteria (Campo et al., 2003). Added to this, the permeability of the cytoplasmic membrane has been increased, the intracellular defence barriers have been depressed, and the bacterial enzyme routes in bacteria have been disrupted (de Souza et al., 2005).

It is important to realize that herbal extracts have been suggested as an alternative antibacterial agent and food flavor enhancer, according to the high level of antibacterial activity of herbal extract, medical benefits (anticancer, anti-flu and anti-allergic activities), unique citrusy flavor, and low price as compared to other preservatives.

Marinating is defined as the addition of a liquid which includes functional ingredients, spices, herbs and flavorings to food before the cooking (Hui, 2012). Marinating meat has been in use for a long time, and recently there has been a large increase in the marination of poultry carcasses, parts, and deboned meat (Guerrero-Legarreta *et al.*, 2010). The marinating of meat is important to improve tenderness and juiciness of meat, and for adding flavor, but the marination time before the cooking varies (Hui, 2012; Tuntivanich & University, 2008). Despite the safety of using refrigerator temperature to marinate the meat (McCracken, 2011), some people who live in hot weather countries that struggle with the lack of electricity usually use room temperature (30°C). In this study therefore, using 30°C was used for marination when the antibacterial activity of organic acids and lime-peel extract applied in nutrient broth or on raw chicken meat.

1.2.4 Acid tolerance response (ATR)

All pathogenic bacteria show different strategies to evade the human body defence system and to cause infection. The human defence and immune system includes the acidity of the stomach, the physical barriers in epithelial tissue, and the immune defence of microphages; *Salmonella* and *Escherichia coli* as gastrointestinal pathogens use several tactics which facilitate their arrival at the site of infection and avoid the human defence and immune system (Gahan & Hill, 1999). Each type of bacteria has protective schemes that allow the bacteria to sense the stress and persist in adverse environments (Paul, 2012).

The Acid Tolerance Response (ATR) is one of these protections, which has been intensively investigated due to the effect on bacterial behaviour (Gawande & Bhagwat, 2002; Greenacre *et al.*, 2003; Hall & Foster, 1996a). ATR is a complex bacterial phenomenon usually including changes in protein levels (Gahan & Hill, 1999). More than 11 kinds of acid tolerance proteins have been identified when bacteria encounter various conditions like chemicals, acidic rain and acidic agricultural operations (Table 12). As an enteric pathogen, *Salmonella* Typhimurium can easily survive within the intestinal environment and host's tissue (Garcia-del Portillo, Foster & Finlay, 1993; Miller, 1991; WilmesRiesenberg et al., 1996).

In order to avoid the human immune system, *Salmonella* has applied several strategies, e.g. prompting the virulence factors to invade the cells and reduce the maturation of phagosome (Gahan & Hill, 1999). Many studies have investigated the strategy of *Salmonella* to survive and become acid tolerant in an acidic environment (Foster & Hall, 1990; WilmesRiesenberg *et al.*, 1996). In the beginning, the log-phase ATR appears in growing cells at low pH (pH 4.5). Then, during stationary-phase (pH ≥ 5.8) stationary-phase ATR is induced (Lee, Slonczewski & Foster, 1994;

WilmesRiesenberg *et al.*, 1996). A third ATR is prompted due to general stress resistance (Lee, Slonczewski & Foster, 1994). It is confirmed that each bacterial specie required a specific period in acid to be adaptive and increased their acid tolerance ability and that has raised food safety concern in contaminated food with ATR bacterial species (Samelis, Ikeda & Sofos, 2003). Theoretically, long periods of acid treatment at low pH might motivate the mutation in the acid tolerance and virulence factors in the bacteria (Archer, 1996). According to Abshire and Neidhardt (1993), in both aerobic and anaerobic conditions, many genes are induced such as stress genes (*rpoS* and *phoPQ* regulons), chaperones, and universal stress proteins (Table 12).

Table 12: Stress regulators and their relationship to virulence in *Salmonella* enterica adapted from Hermans (2007)

Proteins	Functions
ArcAB	Anaerobiosis/aerobiosis
ClpP	Heat shock protease
DnaK/DnaJ	Heat shock chaperone
Fnr	Anaerobiosis/aerobiosis
Fur	Acid pH, oxidative and nitrosative stress
GroEL/ES	Heat shock chaperone
HtrA	Heat shock protease
LuxS	Quorum sensing
OmpR/EnvZ	Osmotic shock and acid response
OxyR	Oxidative and nitrosative stress
PhoPQ	Acid pH and bile salts
RelA/SpoT	Stringent response
RpoE	Extracytoplasmic shock
RpoH	Heat shock
RpoS	Acid pH resistance
SdiA	Quorum sensing
SoxRS	Oxidative and nitrosative stress

ATR of *Salmonella* regulates by *rpoS* (as alternative sigma factor ó^{S}) and two sensory regulators called *PhoP/Q* and *Fur* (ferric uptake regulator) (Requena, 2012). When *Salmonella* is exposed to acid, several genes will be induced that code for transcriptional regulators *ropS*, *phoP* and *Fur* (Hermans, 2007).

Most of ATR regulators are induced when H^{+} attack and invade the bacterial cell, while *PhoP* is inducing organic acid molecules surrounded the outside of the cell membrane (Table 13).

Table 13: Different stresses expressed by *Salmonella* when colonizing a susceptible host. Experiencing one form of stress always makes *Salmonella* of increased resistance to the stress likely to be encountered during the next step of infection, e.g., acid stress increases *Salmonella* resistance to bile adapted from (Hermans, 2007; Rychlik & Barrow, 2005).

Environment	Stress factor	Regulons induced	Results
out of host 1	cold, low nutrients	*rpoS, csp*	general stress resistance
Stomach	extreme acid pH	*rpoS, Fur, ompR*	PhoP induced, bile resistance induced
		phoP	RpoS induced, short chain fatty acids (SCFA) resistance induced
duodenum	Bile	*phoP*	membrane modifications, invasion suppressed
Ileum	Decreased O2 supply	*fnr, arcA*	switch from aerobiosis to anaerobiosis
	SCFA	*rpoS*	acid-induced cross-resistance to SCFA, SCFA induced cationic antimicrobial peptides (CAMP) resistance
	competitive flora, quorum sensing	*sdiA, luxS*	Virulence regulation, acid stress
epithelium	CAMP	*phoP*	LPS modifications, resistance to macrophage CAMPs
out of host 2	cold, low nutrients, aerobiosis	*rpoS, csp, fnr, arcA, oxyR, soxRS*	

ASP may either repair or protect the cellular gene molecules. For instance, *rpoS* induced by *dps*, which is important for avoiding the DNA degradation (Halsey et al., 2004; Hermans, 2007).

The $ó^S$ is considered as an effective factor in the regulation of both stationary and exponential phases of proteins which are responsible for stress adaptation and virulence (Figure 2). Additionally, *Mig5* was found on virulence plasmid and reported to associate with macrophage-inducible putative carbonic anhydrase (Weir et al., 2008).

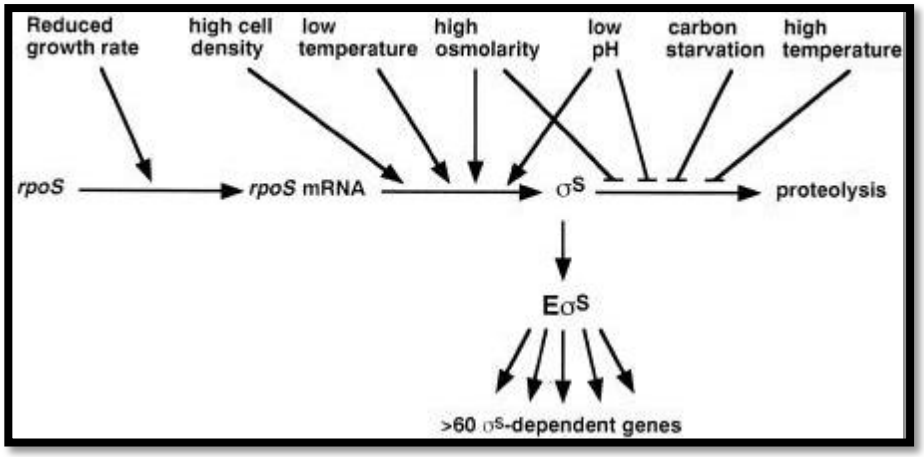

Figure 2: Various levels of $Ó^S$ regulation are differentially affected by various stress conditions. An increase of the cellular $Ó^S$ level can be obtained either by stimulating $Ó^S$ synthesis at the levels of *rpoS* transcription or translation or by inhibiting $Ó^S$ proteolysis adopted from (Hermans, 2007)

In addition, *Fur* play an important role in regulation of ATR in *Salmonella* Typhimurium (Hall & Foster, 1996b). When *Fur* is disrupted, both ATR and virulence will be effected (WilmesRiesenberg *et al.*, 1996). Hence, the regulatory systems (*rpoS*, *PhoP/Q* and *Fur*) have an impact in ATR genes of *Salmonella* and their virulence factors (Gahan & Hill, 1999; Hermans, 2007). In contrast, some studies were reported that a mutation within *Fur* has depressed the adaptation of *Salmonella* to survive within the acidic environment (Foster, 1991; Foster & Hall, 1992).

In terms of organic acids stress, *rpoS* and *Fur* are playing an important role in adapting the bacterial cell against low pH level (Requena, 2012). However, acid tolerant species of *Salmonella* Typhimurium treated with food additives (acidic food additives) might extend the ability to survive within the human intestinal acidity and amplify the illness. Unfortunately, there is a lack of evidence about the effect of food additives such as the combination of organic acids and herbal extract on ATR genes.

1.3 Aims

Dipping or spraying poultry products with high concentrations of organic acids has been used safely and effectively to eliminate foodborne pathogens in poultry meat (*Salmonella* Typhimurium and *Escherichia coli*). Nevertheless, organic acids have many negative effects such as unacceptable sensory properties of meat like acidic smell, colour changes. Hence, the aims of this research are to identify and evaluate the antibacterial activity of organic acids for the concentration below the minimum inhibitory concentration alone or in combination with an aqueous extract of citrus (*Citrus aurantifolia*). Thereafter, the results will be applied on raw chicken breast meat to reduce the microbial population of *Salmonella* Typhimurium DT104 and *Salmonella* Typhimurium 1344nal^{r} at room temperature in Iraq (30°C), as most marinating takes place at this temperature due to lack of electricity. The effect of food additives on the gene expression of *Salmonella* will then be tested to see if that exposure has increased the acid tolerance and virulence of two acid tolerant species of *Salmonella,* making them survive within the human intestine and if so, for how long.

The main objectives of this study are:

1. Identify the minimum inhibitory concentration of acetic, lactic, citric and propionic acids alone against selected foodborne pathogens (*Salmonella* and *E.coli*) in nutrient broth at 30°C, which is average room temperature in Iraq.
2. Evaluate the efficiency of lactic acid combined with acetic, citric and propionic acids below the minimum inhibitory concentration to foodborne pathogens in nutrient broth and then apply the results on raw chicken breast meat at 30°C.
3. Investigate the antibacterial activity of some herbal extracts and find the minimum inhibitory concentration in nutrient broth against selected foodborne pathogens at 30°C.
4. Combine both organic acids and an aqueous lime-peel extract in concentrations below the minimum inhibitory concentration in nutrient broth and then in raw chicken breast meat at 30°C.
5. Apply sensory evaluation experiments to test several food organoleptic properties such as the aroma, flavour, texture, and acidity in order to evaluate the acceptability of treated cooked chicken meat (treated with combination of organic acids and an aqueous lime-peel extract).
6. Assess the effect of treatment (combination of organic acids and an aqueous lime-peel extract) on the induction of acid tolerance and virulence genes of *Salmonella* Typhimurium in nutrient broth at 30°C by Quantitative Polymerase Chain Reaction (Q-PCR), and determine how that affects the ability of *Salmonella* to survive within an acidic environment (human gastrointestinal).

Chapter 2: Materials and Methods

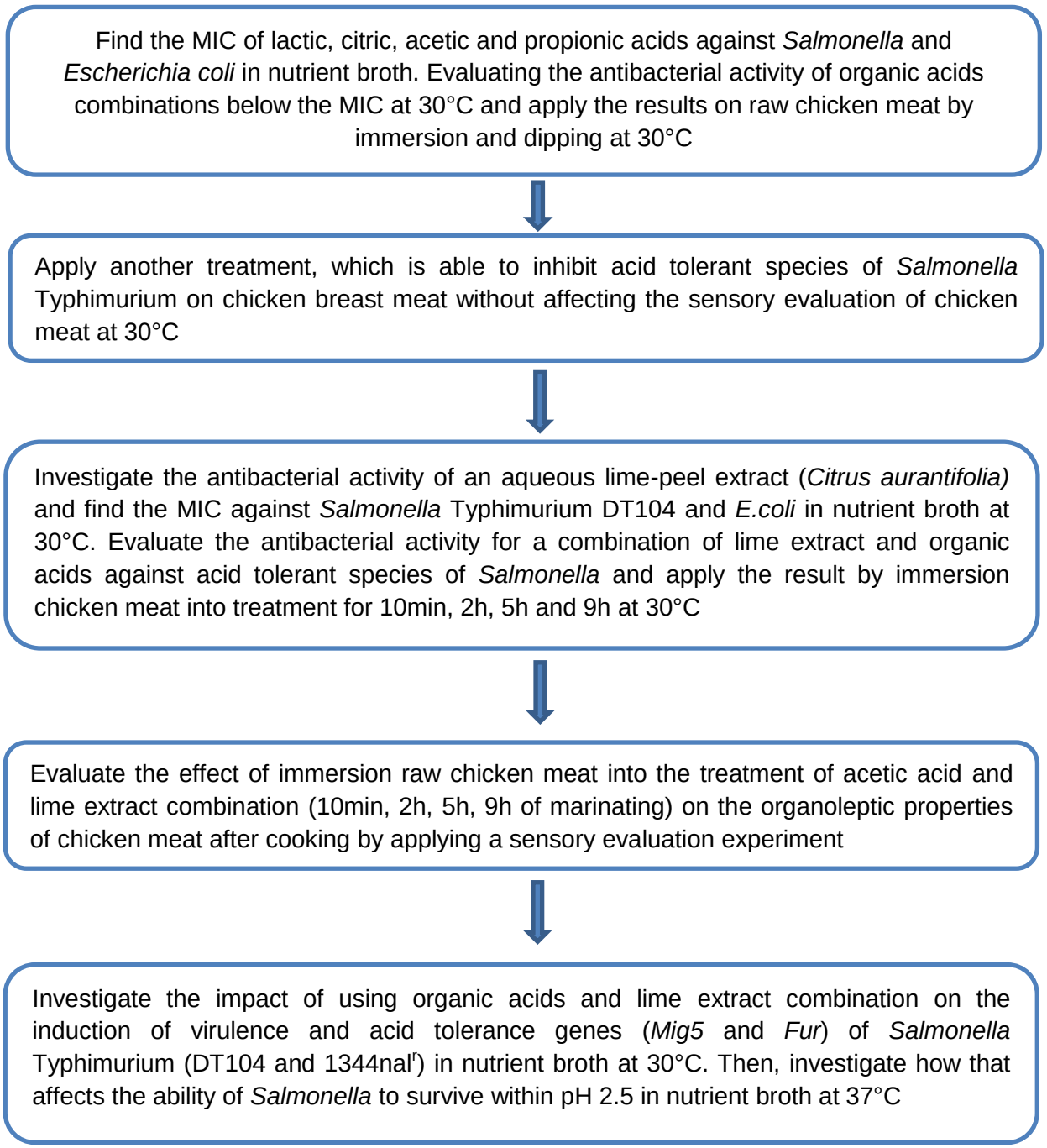

Figure 3: A schematic outline of experiments

2.1 Preparation of bacterial cultures, media and inoculum

- Culture media

Nutrient broth (NB-CM0001), Nutrient agar, Xylose lysine Desoxycolate agar (XLD-CM0469) and coliform selective agar (Oxoid Ltd., Basingstoke, Hampshire, UK) were prepared according to the manufacturer's instructions, then sterilized at 121ºC for 15 minutes (autoclaving).

- Bacterial cultures:

Escherichia coli K12, *Salmonella* Typhimurium DT104, and *Salmonella* Typhimurium 1344nal^{r} were obtained from the School of Biomedical and Biological Sciences - University of Plymouth / stock culture (-18°C), purchased from Public Health England, Salisbury, UK. Cultures were grown by plating on nutrient agar plates, incubated for 24 hours at 37°C, and then stored in the fridge for maximum period of seven days. After this time, fresh cultures were prepared from the frozen stocks which were kept in -18C.

- Preparation of inoculum

Pure cultures of salmonellae (DT104 and 1344nal^{r}) and *Escherichia coli* K12 were used to inoculate 10 ml of fresh nutrient broth and incubated aerobically for 18 to 20 hours at 37ºC (LEEC incubator, Cowlick Industrial Estate, Nottingham, UK).

2.2 Preparation of reagents

- Chemicals

Stock solutions (400 mmol) of the following acids (Fisher Scientific, UK) were prepared. Working concentrations were prepared by diluting with sterilized distilled water.

- Propionic acid (29.63 g/L)
- Citric acid (84.04 g/L)
- Lactic acid (36 g/L)

- Acetic acid (24.02g/L)

- Phosphate buffered saline (PBS) pH7

One tablet of saline (BR0053) was dissolved up to 100 ml of distilled water and autoclaved at 121ºC for 15 minutes.

2.3 Preparation and characterisation of lime extract

2.3.1 Preparation of an aqueous lime-peel extract

The sun- dried fruit of *Citrus aurantifolia* was purchased from a botanical shop in Baghdad, Iraq. Lime seeds were removed and the peel was ground for 5 seconds using a grinder and left in a desiccator at 20ºC for 24 hours. An aqueous lime-peel extract was prepared by adding 10 g of ground lime peel up to 100 ml of distilled water. Then, the mixture was left for an hour with stirring at room temperature (22±1°C), followed by filtration through filter paper (Whatman 1) (Nasar-Abbas & Halkman, 2004).

2.3.2 Phenolic compound test

- Calibration curve preparation (Gallic acid)

The standard of Gallic acid was prepared by dissolving 0.05 g Gallic acid up to 100 ml of distilled water and followed by serial dilution. A volume of 25 µl of an aqueous lime-peel extract was added. Folin-Ciocaleu reagent 625 µl was prepared in serial dilution with distilled water (1:10). Samples were treated with 500 µl of Na_2Co_3 (7.5 %) and the tubes were vortexed for 5 seconds, all samples were then incubated for 5 minutes at 50°C.

Samples were transferred to spectrophotometer to measure the optical density at 760 nm. All samples and Gallic acid reagent have been prepared in triplicate. The data was exported to Excel and compared with the calibration curve (Shirsat, 2012).

2.3.3 Determination of citric acid level in an aqueous lime-peel extract

High-performance liquid chromatography (HPLC) was used to determine the level of citric acid in lime extract. To neutralise the sample, di-potassium carbonate (1 M) was mixed with a double volume of the sample in triplicate. The sample volume was then mixed with 20 µl of 7 % H_2SO_4 converting the acid equilibrium to protonated type.

The sample was vortexed for 10 seconds and centrifuged at 1300 rpm for 10 minutes (RC5C centrifuge, Sovall instrument, USA). Finally, 20 µl of the supernatant was taken to be analysed by HPLC for presence of citric acid (Niven, Beal & Brooks, 2004). Ion exclusion chromatography (IEC) was used to separate organic acids. IEC consisted of a Gynkotek pump (Dionex Crop., Sunnyvale, USA), Gynkotek GINA 50 autosampler and a Shode x RI-71 refractive indicator (Showa Denko K.K., Tokyo, Japan). To separate citric acid from the lime extract, a separation column MetaCarb 87H 300 mm $_X$ 7.8 mm (MetaChem Technologies Inc., Torrance, USA) was used. The data was transferred to the computer in order to be analysed by Chromeleon ^{TM} software (Dionex Crop., Sunnyvale, USA).

2.3.4 Phytochemical screening (qualitative analysis)

An aqueous lime-peel extract was tested for the presence or absence of the phytochemical compounds (amino acids, tannins, phlobatanins and saponin) at room temperature (22±1°C) (Ashok kumar et al., 2011) as follows:

- **Amino acids test**: an aqueous lime-peel extract (1 ml) was treated with Ninhydrin reagent (1-3 drops). Appearance of a purple colour shows the presence of amino acids.

- **Tannins test:** drops of 0.1 % ferric chloride were added to investigate the presence of tannins into 1 ml of the extract at room temperature. A positive result was revealed when the extract become blue-black; that indicated that the lime extract has tannins.

- **Phlobatanins test:** Hydrochloric acid 1 % was mixed with 2 ml of an aqueous lime-peel extract, then, boiled for 2-3 minutes; the redness precipitation was an indicator for the presence of phlobatanins.

- **Test for saponin:** 1 gram of dried lime peel was weighed and mixed with 10 ml of distilled water in a conical flask and boiled for 5 minutes. Then, the sample was filtered and 2.5 ml was mixed with 10 ml of sterilized water in test tubes. The mixture was in a vortex for 30 seconds and left for 30 minutes at room temperature. The formation of a honeycomb was observed, indicating the presence of saponins in the lime extract.

2.4 Determination of MIC of organic acids in nutrient broth

Growth curves of *E.coli* and *Salmonella* were obtained by OD using a plate reader (Tecan Infinite®200 PRO Readers, UK).

Optical density (OD) measured the bacterial growth in nutrient broth with different concentrations of lactic, citric, propionic, and acetic acids at 30°C. The range of tested concentrations for lactic, citric, propionic and acetic acids were 3 to 9 mmol/l, 2 to 6 mmol/l, 2 to 7 mmol/l and 2 to 6 mmol/l respectively. In addition, concentrations below the minimum inhibitory concentration of organic acids (acetic, citric and propionic acids) in combination with lactic acid were investigated at the same temperature (all experimental steps are shown in the Figure 3). The wells of microtitre plate (48 wells) were filled with 600 µl of double strength nutrient broth and mixed with 600 µl of double strength of organic acids alone or in combinations.

For instance, 600 µl of 6 % acid were mixed with 600 µl of double strength nutrient broth to get 3 % final acid concentration. Then, the inoculation of media was accomplished by adding 0.1 ml of ~10^6-10^5 CFU ml^{-1} (the required dose to cause illness) of bacterial serial dilution (*E.coli* and *Salmonella*) (Figure 4).

The plate reader was pre-programmed as a kinetic cycle for 24 hours period and measured every 2 hours with a shaking duration 5 seconds performed before each measurement at 600 nm. The incubation temperature for the experiment was set at 30ºC. pH- values for combinations of organic acids and nutrient broth in the wells were measured by using a standardized pH meter (pH meter, Hanna instrument, Lisbon, Portugal). Growth curves for *E.coli* K12 and *Salmonella* Typhimurium DT104 were obtained after transferring the data to the Excel program.

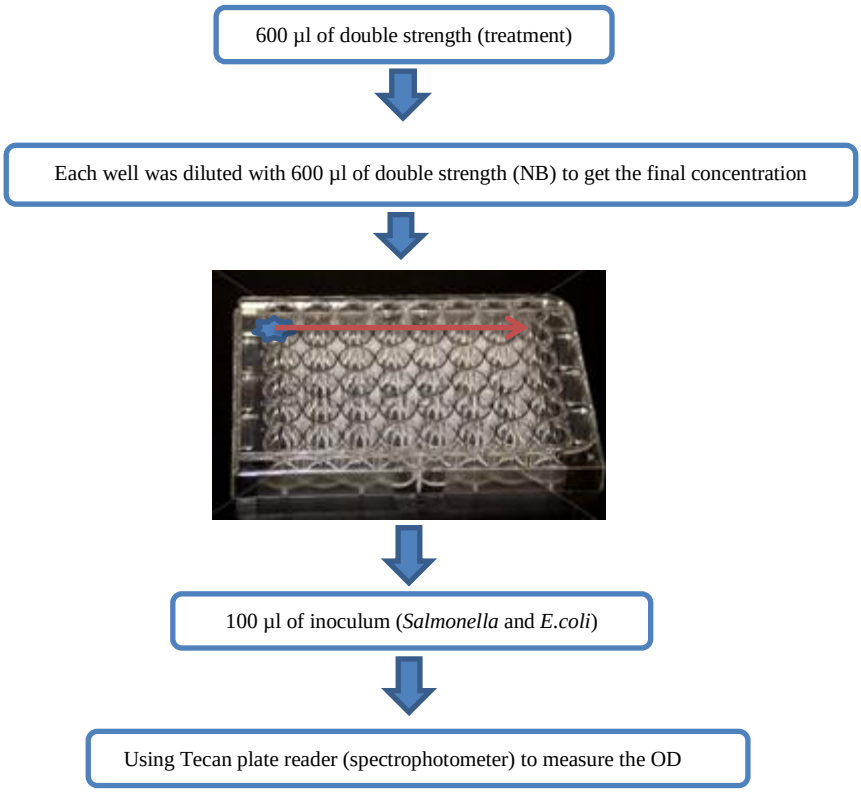

Figure 4: Steps of experiment investigating the MIC of organic acids

For both *Salmonella* and *Escherichia coli*, ΔOD values were calculated from the growth curves data from spectrophotometer (Tecan Infinite® 200 PRO Readers, UK). Two points in the bacteria growth curve were taken. The first one was taken when the exponential phase has started. The end of the log phase (maximum optical density reading, exponential phase) was considered as the second point (Figure 5).

The ΔOD value equation is:

ΔOD value = L (last reading) – F (first reading in Exponential phase)/100

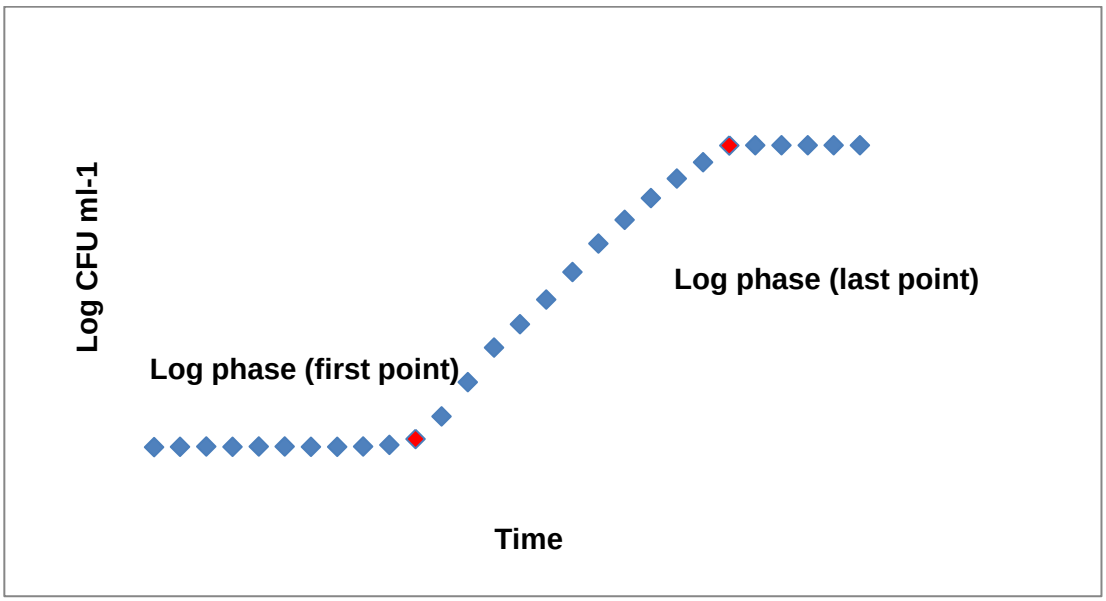

Figure 5: Bacterial growth curve and the measurement of ΔOD value

2.5 Preparation of chicken breast meat

The antibacterial activity of organic acids and an aqueous lime-peel extract (alone or in combination) to reduce the number of *Salmonella* on raw chicken meat at 30°C, was determined. Raw chicken breast meat was purchased fresh from Sainsbury's, UK and stored at -18±1ºC. The chicken meat was left overnight to thaw in a fridge (4±1 ºC) and then, chopped aseptically into cubes and kept in the freezer (-18±1 ºC) until used.

2.6 Chicken meat inoculation:

The chicken breast cubes were inoculated with *Salmonella* Typhimurium DT104 and *Salmonella* Typhimurium 1344nal^{r} by immersion into a suspension of *Salmonella* (~6-7 $\log_{10}$ CFU ml^{-1}) for 10 minutes at room temperature (22±1 °C).

After the inoculation, three chicken pieces were separately dried at room temperature for 1-2 minutes using sterilized filter papers. To ensure that each piece of immersed chicken meat was covered with an equal number of *Salmonella,* two groups of six pieces of raw chicken meat were inoculated with *Salmonella* for 10 minutes and washed with PBS. One ml of washing suspension was serially diluted (10 fold) and 0.1 ml was plated out on XLD agar (in triplicate) and incubated for 24 hours at 37±1ºC (LEEC incubator, Cowlick Industrial Estate, Nottingham, UK). The calculation of the CFU number per gram on raw chicken meat for *Salmonella* was made by counting the number of typical colonies (30-300 colonies), which were obtained by plating the sample on XLD agar (xylose-lysine-deoxycholate- selective media) and confirmed by biochemical such as the SIM (Sulfur – indole- motility media) and urea reaction (Taormina, 2012).

2.7 Decontamination of chicken meat by combination of lime and organic acids

Chicken pieces were immersed into a volume of 50 ml of organic acid and aqueous lime-peel extract combinations for 10 minutes at room temperature (22±1°C) gently to avoid washing off the cells of *Salmonella* by the treatment. The final concentrations of an aqueous lime-peel extract (% w/v) with acetic acid combination were (5.2/0.28, 10.4/0.28, 20.8/0.56 and 41.6/1.12) or with propionic acid were (5.2/0.35, 10.4/0.35, 20.8/0.65 and 41.6/1.3) respectively. These combinations were chosen based on OD readings in previous experiments using spectrophotometer (Tecan-infinity 200, UK).

The chicken pieces were kept immersed into the prior treatment for different times (10 minutes, 2, 5, 9 hours) at 30±1°C separately. The control was the inoculated chicken breast meat without any treatment.

1 % NaCl was used as a treatment with lime and organic acid combinations on raw chicken meat. To determine the number of viable cells of *Salmonella,* treated chicken meat samples were taken out of the immersion treatment after each time (10 minutes, 2, 5, 9 and 24 hours) and washed with PBS, using hand shaking to avoid smashing of chicken pieces in case of using the homogeniser. *Salmonella* was enumerated by plating 0.1 ml of serial dilution on XLD agar plates and incubated for 24 hours at 37±1°C (Lu & Wu, 2012). Then, the calculation of the CFU number per gram on raw chicken meat for *Salmonella* was made by accounting the number of typical colonies, which obtained on XLD agar and was confirmed by biochemical tests.

2.8 Microbiological analysis

In order to make sure that raw chicken meat was not contaminated with *Salmonella*, a pilot study was applied. Chicken pieces (6) were washed with PBS in a stomacher bag and homogenised for 2 minutes by hand. Then, 1 ml of the homogenised suspension was taken for the serial dilution and plating on XLD agar (in triplicate) and incubated for 24hours at 37±1°C.

Chicken pieces as small as 5 g (minimises the losses of the experiment) were mixed with 95 ml of PBS in stomacher bags and homogenised by hand for 2 minutes. One ml volume of mixture was diluted in serial dilution of 9 ml of PBS and 0.1 ml was plated out on XLD agar according to National Standard Methods (Downes, Ito & Association, 2001). Cells of *Salmonella* and *E.coli* were enumerated on XLD and coliform selective agar plates respectively after being incubated for 24 hours at

37±1ºC (HPA, 2011a). The number of viable cells of bacteria was converted to $\log_{10}$ CFU g^{-1}.

- Ethical approval for sensory evaluation

The Human Ethics faculty of Science / Plymouth University approved a sensory evaluation experiment, which involved volunteers tasting chicken meat with lime extract. All the participants were from Plymouth University (students or staff) who were invited by email and provided with a consent form. Each participant had the right to withdraw at any time without any excuse.

2.9 Sensory evaluation of cooked chicken meat with lime

Halal cubed chicken breast meat (10 kg) was purchased from Mullaco (West Yorkshire, UK) and kept at -80°C (Williams Refrigeration and Air-Conditioning, installation by general refrigeration, Limited) then thawed overnight at 4±1°C before the day of the experiment.

After that, chicken meats were divided into 5 groups (30 pieces each group): one was cooked as a control, while the rest were immersed into a treatment of an aqueous lime-peel extract and acetic acid (41.6-1.12 %) for 10 minutes, 2, 5, 9 hours including 1 % NaCl (Figure 6). In terms of cooking, cubes of chicken meat were cooked using a gas oven (Zanussi Combi Wave-Italia) at 200°C for 7±1 minutes to reach an internal temperature of 85°C in the centre of the chicken cubes.

Figure 6: Chicken breast cubes were prepared for cooking after marinating

Then, chicken meat cubes were kept at room temperature (22±1°C) to be served to participants in order to evaluate the acceptability of cooked chicken meat regarding colour, flavour, appearance, texture, overall acceptance, and aroma. The oven temperature and the central temperature of the chicken meat cubes were checked constantly using thermo probes, which were connected to a temperature data logger (Comark Electronics, Ltd., Littlehampton, UK)(Figure 7).

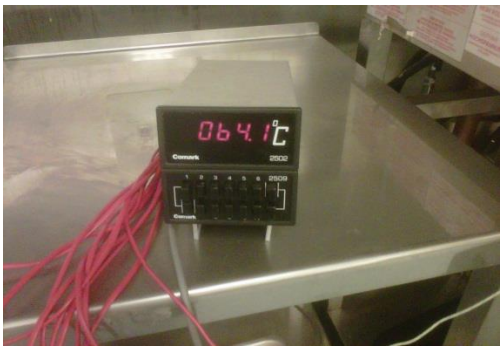

Figure 7: A Comark -data logger was used to measure the temperature

This evaluation took place only once in the sensory laboratory/ food and nutrition lab at Plymouth University (Figure 8).

Figure 8: One of the assessors during the sensory evaluation in food and nutrition lab

Each assessor evaluated the control first and treatment samples later as samples were given codes and placed separately on a small white plate (Figure 9).

For cleansing the palate after each sample, water was provided to assessors to drink at room temperature. Participants were asked to evaluate the acceptability by ordering the samples for each of the following attributes: Aroma, colour, appearance, flavour, acidity, texture, and overall acceptance of the samples (Appendix- A).

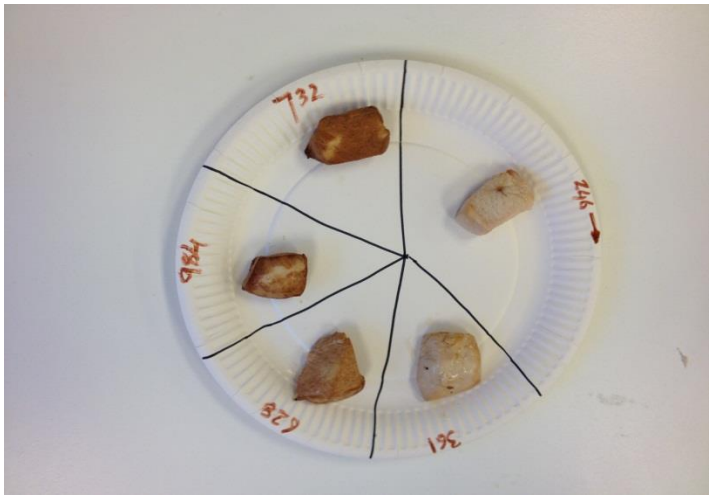

Figure 9: Cooked chicken meat cubes presented on a white plate during the sensory evaluation

The total number of the panel was 30 persons, who volunteered to evaluate the coded samples. According to the hedonic scale scheme, the participants evaluated the samples on 9 points, with number one as very unacceptable, and number nine as very acceptable, regarding their qualitative opinion of the product.

2.10 Statistical Analysis:

MINITAB version 16 (Minitab Ltd, Coventry, UK), one-way analysis of variance (ANOVA), and general linear model (GLM) were used to compare the different data for treatment groups, followed by one of the appropriate multiple comparison tests (Tukey); $P < 0.05$ is considered significant data.

2.11 Acid tolerance gene expression

After approving the antibacterial activity of acid and lime combination against species of *Salmonella* Typhimurium, the effect of using this combination (organic acid and lime) on the induction of acid tolerant and virulence genes of *Salmonella* Typhimurium was investigated. *Salmonella* Typhimurium DT104 and *Salmonella* Typhimurium 1344nal^{r} were prepared overnight (20 hours incubation) by inoculating 10 ml of nutrient broth with a *Salmonella* colony at 37±1ºC.

Cells of *Salmonella* Typhimurium were then washed with PBS and centrifuged (8000 rpm) twice for 5 minutes at room temperature. As a result, ~6-7 $\log_{10}$ CFU ml^{-1} of *Salmonella* were prepared in a container ready to be treated in the next step. The treatment was a combination of an aqueous lime-peel extract (5.2 %) and acetic acid (0.28 %), including 1% NaCl in nutrient broth at 30±1°C. Then, *Salmonella* was immersed into the treatment for 10 minutes, 1.5 or 2.5 hours at 30±1ºC and washed with PBS to remove acidic stress and the residue of lime. Cells were centrifuged (8000 rpm) for 5 minutes and the pellets of *Salmonella* were then stored at **-**80±1ºC. In terms of control samples, no treatment was used and both *Salmonella* species were grown in the same circumstances of treated samples and kept at -80±1ºC.

2.11.1 Optimal culture conditions for Quantitative PCR

- Culture media

Fresh nutrient broth was used as the minimal media to reduce the variation as prepared in chapter 2.1

- Harvesting time

Salmonellae were harvested within the stationary phase after 10 minutes, 1.5 and 2.5 hours of growing in nutrient broth with combination of an aqueous lime-peel extract (5.2 %) and acetic acid (0.28 %) with pH 4.69 including 1% NaCl at 30±1°C.

- **Determining the correct amount of bacteria**

The amount of starting total RNA from bacterial lysates is considered a critical factor. The maximum cell numbers that were applied to cultures of bacteria grown in nutrient broth was 5 x 10^8 to give RNA yield 25 µg.

In order to avoid the inefficient lysis and small yield of RNA, using up to that amount of cells is recommended using RNeasy Mini Kit spin column. However, it is not recommended to rely on OD values due to the difference between bacterial species.

Therefore, viable cells of salmonellae were calibrated with OD readings at 600 nm by plating out the cells on XLD agar. The *Salmonella* culture of 10^9 $\log_{10}$ CFU ml^{-1} was diluted 1:4 with PBS to get optical density readings of 0.25 to give up to 7.5x10^8 per mini spin column. RNAprotect® Reagent was calculated depending on the original amount of sample which were added directly and mixed gently in order to keep the RNA stable. RNAprotect® Reagent (100 µl) was mixed gently with 500 µl of sample and vortex for 5 seconds. Then, samples were incubated for 5 minutes at room temperature and centrifuged for 10 minutes at 5000x g. Supernatant was decanted and the residual was removed by gently dabbing on a paper towel for 20 seconds. Here, salmonellae pellets become ready to be stored up to a month at -80±1ºC.

2.11.2 Enzymatic Lysis and Proteinase K Digestion of Salmonellae (Protocol)

- Preparation of buffers (TE and RLT buffers)

TE buffer was prepared by mixing 30 mM of Tris-Cl and 1mM of EDTA (Ethylenediamine tetra acetic acid) containing 15mg\ml of lysozyme (Guerrero-Legarreta *et al.*, 2010). 1 ml of RLT buffer solution was added to 10 µl of ß-mercaptoethanol (Fisher Scientific, UK) in the hood (Bigneat, USA). Once mixed with ß-mercaptoethanol, RLT buffer is stable for 30 days and stored at room temperature. QIAGEN Proteinase K of 20 µl was added to 200 µl of TE buffer and mixed with the

pellets gently. Contents were mixed by vortexing the sample for 10 seconds and then incubating at 22±1ºC for 10 minutes. During the incubation, sample tubes were shaken frequently at 22±1ºC using Stuart Shaking Incubator S1500 (Gene flow, UK). RLT buffer 700 µl was added to the samples and vortexing strongly for 5 seconds and only the supernatant was used for the next step. In order to ensure the highest amount of RNA, pure ethanol (~100%) 500 µl (Sigma Ltd., UK) was mixed with the supernatant by pipetting to get the final volume of 1.4 ml and this mixture is called Lysate.

Lysate 700 µl were transferred to a RNeasy mini column (2 ml) and centrifuged for 15 seconds at ≥ 8000 rpm. Due to the volume of Lysate, samples were centrifuged twice through the same spin column successively. RW1 solution is an astringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids etc..(Edelstein, 2010). Therefore, 700 µl of RW1 solution was added and centrifuged for 15 seconds at 8000 rpm to wash the column membrane. Then solution flows were discarded and the columns were kept for the next step. Columns were placed in new 2 ml-collection tubes and 500 µl of RPE buffer solution was added to the column to remove traces of salts, which are still on the column due to buffers used earlier in the protocol (Long, Pickering & Prober, 2012). Then, samples were centrifuged for 15 seconds at 8000 rpm to wash the spin column twice. Flows were discarded and collection tubes were kept for the next step. To wash the spin column and ensured that no ethanol still carried over, another 500µl of RPE buffer solution was added to the columns and centrifuged for 2 minutes at 8000 rpm. Carefully, RNeasy mini spin columns were removed from collection tubes to keep the sample free of Ethanol.

Finally, columns were placed into new collection tubes, mixed gently with 40 µl of RNase-free water directly and centrifuged for 1 minute at 8000 rpm to elute the RNA (QIAGEN, 2005).

- DNase Protocol

In order to purify the extracted mRNA from any DNA either contamination or residue, DNase I (Sigma: AMP-D I) kit was used. DNase I (1 µl) and 1 µl of DNase buffer were mixed gently with each 10 µl of sample. Then, the samples were incubated for 15 minutes at room temperature. One µl of stop solution (50 mM EDTA) was added to stop the digestion of DNA by binding both calcium and magnesium ions leading to inhibit the DNase I. The next step was heating the samples by incubation at 70°C for 10 minutes to denature DNase I. Directly; samples were placed into ice for immediate use or kept in -80±1ºC for long time storage.

- Measurement of RNA

After extraction and purification of samples, Nanodrop (NanoVue Plus™) was used to measure the total concentration of RNA in each sample. According to (Warburg & Christian, 1942), RNA molecules were measured with spectrophotometric absorbance of 260 \ 280nm.

- Converting the RNA to cDNA protocol

First, control and treated samples were normalized in order to have the same amount of RNA in each sample. A High Capacity RNA-to-cDNA™ Kit (A&B applied Bio system, USA) was used. The High Capacity RNA-to-cDNA™ Kit is designed for optimum performance with various gene expression solutions. The kit of two tubes was provided from A&B Applied Bio-system; these solutions were working together earning an accurate, specific and high performance reverse transcription.

The High Capacity RNA-to-cDNA™ Kit reaction tubes contain 2x RT Buffer and 20x Enzyme mix, including Nuclease-free water, which was stored according to the manufacturer's instructions.

A volume of 2 µl RNA was used in total reaction of 20 µl and left to thaw on the ice. Reactions volumes were calculated as 10 µl of 2 X RT buffers, 1 µl of 20X Enzyme mix and up to 9 µl of RNA sample to get the final volume of 20 µl per reaction. The aliquots solution of RT was mixed in tubes and centrifuged to spin down the contents for 5 seconds. Reverse transcription of samples was started by incubation for 60 minutes at 37ºC followed by heating up to 95ºC for five minutes. Then, samples were held at 4ºC and samples became ready for use in real time PCR or stored in the freezer (-20±ºC).

- Primers preparation

According to Weir *et al.* (2008), some genes were responsible for making *Salmonella* Typhimurium DT104 acid tolerant and survived within the acidic environment. Two primers, which were *Mig5* and *Fur* which were selected due to their ability to make *Salmonella* survived in the acidic environment; the housekeeper gene was 16S rRNA, and all primers were purchased from eurofins (eurofins Ltd., UK).

The sequence of *Mig5* forward primer was (AAACATGATTACCTGGCACAGA) and the reverse sequence for *Mig5* was (CTACGCGGGAATTAAACGTC). The sequence for forward *Fur* primer was (TTAAAGAAGGCTGGCCTGAA) and for the reverse primer sequence was (ACGGTATACGGTTGCCAGAC). In addition, 16S rRNA was used as a housekeeper gene. The sequence of 16SrRNA was (CAGAAGAAGCACCGGCTAAC) for the forward primer while the sequence was (AATGCAGTTCCCAGGTTGAG) for the reverse primer. All primers were diluted with Nuclease-free water (1:10 dilution) and stored at -80±1ºC.

- Run cDNA by StepOne QPCR machine

The StepOnePlus™ Real-Time PCR machine (Applied Bio systems) was used to run the cDNA and analysed the gene expression of target genes. SYBER Green Master Mix was added to samples to bind all double-stranded DNA.

The following steps were followed successively:

Master Mix per primer was prepared as following for each sample to get a total reaction volume of 20 µl (10 µl SYBR Green Master Mix, 1 µl F primer (10 µM), 1 µl R primer (10 µM), 3 µl of Nuclease-free water and 5 µl (1 µl cDNA Sample template + 4 µl of Nuclease-free water)). The mix was then vortexed for 3 seconds and centrifuged for 5 seconds to collect components at the bottom of tubes. The cDNA of each sample was optimized by diluting the template of cDNA as 1:10 dilution with Nuclease-free water. A volume of 10 µl of diluted cDNA template was used per well. After preparing the sufficient amount of reactions, the template of PCR was prepared in triplicate for each sample in each well, the plate then covered with film securely and spine for 15 minutes to bring the mixture to the bottom of the wells and eliminated the air bubbles in the plate at room temperature.

Applied Biosystems StepOnePlus™ Real-Time PCR System and StepOnePlus™ software was used to run the samples and identify the target of genes expression. In order to set the machine (StepOnePlus™ RT PCR), many points were applied such as enter experimental name, select 96-well setting, select for standard curve, select SYBR® Green reagent, select standard (2 hours), plate set up and select number of targets (primers name), determine targets to selected wells, select passive reference, select “enable veriflex block” due to using a range of annealing temperatures in the plate, run method, holding stage at temperature of 94˚C for 5 minutes.

In terms of the cycling stage, a 40 cycle's option was selected with 3 steps which were temp 94˚C for 15 seconds, select annealing temp, unselect fluorescence for 1 minute and temperature of 72˚C, select fluorescence on for 1 minute. The melt curve was set as three steps which were temp 94˚C for 15 seconds, temp 60˚C for 1 minutes and temp 94˚C for 15 seconds.

Chapter 3: Minimum inhibitory concentrations of organic acids alone or in combination against *Escherichia coli* K12 and *Salmonella* Typhimurium DT104 at 30°C

Minimum inhibitory concentrations of organic acids alone or in combination against *Escherichia coli* K12 and *Salmonella* Typhimurium DT104 at 30°C

3.1 Introduction

Foodborne pathogens can be reduced by using various types of preservative (Bhat, Alias & Paliyath, 2012). Organic acids are described as a natural and attractive way to keep food safe against a wide range of bacteria (Nazer *et al.*, 2005; Shimizu, Takahata & Kato, 1995; Sirsat, Muthaiyan & Ricke, 2009; Theron & Lues, 2009). In addition, organic acids are used due to several factors such as low toxicity value, low price and availability. Although organic acids are used worldwide to disinfect meat and poultry carcases, this disinfectant is unauthorised in the EU because of the toxicity effect of undissociated acid molecules which might remain in our bodies (Van Boxtel, Santoso & Edwards, 2008).

There is no doubt that the lethal effect of organic acids on microorganisms is largely due to the ability of organic acids to increase the concentration of hydrogen ions in the bacterial cell (Davidson, 2005). A direct relation between the concentration of organic acids and their antibacterial activity has been reported. For instance, 4% of organic acids were more effective than a range of concentrations between 1 to 3% against *Salmonella* Typhimurium, but high levels of organic acids affected the organoleptic properties of meat (Theron & Lues, 2009). This treatment therefore became unacceptable for commercial use. Salmonellae were reported to be more active and resistant when attached to the skin of poultry, thereby increasing their ability to resist the fatal effect of organic acids (Magazine, 2011).

The high content of lipid and the pH neutral environment of chicken meat are the main factors which protect *Salmonella*. Therefore, any treatment should consider eliminating the number of foodborne pathogens. One of these treatments consists of using the synergistic effect of a combination of organic acids to inhibit the bacteria and enhance the ability of each acid alone (Dufour, Simmonds & Bremer, 2003). Historically, a combination of organic acids has been employed in food preservation (Woodford, 2005) and several studies have therefore been conducted on the beneficial aspects of combining organic acids against foodborne pathogens (Dolezalova et al., 2010; Woodford, 2005).

Matsuda et al (2012) demonstrated the various antibacterial activities of organic acids against different kinds of microorganisms. However, the antibacterial activity of organic acid combinations depends on several factors, such as the type of microorganism, the pH level, the concentration of acids and water activity (Hsiao & Siebert, 1999). Most environmental stresses, such as thermal and acidic stresses, which are not strong enough to kill the bacteria, might convert the normal species of bacteria into being resistant (Knipe & Rust, 2009). Both external and internal factors are responsible for the tolerance mechanisms of foodborne pathogens. The external factor is the concentration of hydrogen ions outside and inside of the bacterial cytoplasmic membrane (Naidu, 2000). The bacterial cell defences against acid stress are revealed as changes in the chemistry of the bacterial cell membrane, and also in the stability of internal pH homeostasis (Richard and Foster, 2004). The reduction of pH level might affect both the activity of organic acids and dissociation capacity through the bacterial cell membrane (Leyer & Johnson, 1993; Ricke, 2003).

The internal factor, which is responsible for adapting the bacteria within the acidic environment, is the up-regulation of several genes such as *Mig5* & *Fur* during the exposure to organic acids (Weir *et al.*, 2008). The antibacterial activity of organic acids was investigated by Khan and Katamay (1969), who found that *Salmonella* was reduced using 3% propionic, lactic, citric and acetic acids by 25.2, 19.6, 19.4 and 24.6 mm respectively (zone inhibition method). The greatest bacterial reduction (*Listeria Monocytogenes*) was gained by using acetic acid rather than lactic and citric acids (Ita & Hutkins, 1991). In contrast, however, Cheng Yu et al. (2003) concluded that the antibacterial activity of lactic acid was stronger than acetic acid against *Escherichia coli* 0157:H7.

Concentrations of 10% and 5% lactic acid were able to reduce the number of viable cells of *Salmonella* and *Listeria innocua* significantly by 4.59 $\log_{10}$ immediately after treatment (Lecompte et al., 2009). Increasing the concentration of organic acids from 5 % to 10%, (dipping treatment) significantly affected the bacterial growth. However, increasing the concentration of organic acids has negatively affected the organoleptic properties of raw chicken meat, especially the colour, and that makes the product unacceptable to many consumers (FDA, 2001). The antibacterial activity of organic acids is various, and depends on the pKa value of each acid (Bjornsdottir, Breidt & McFeeters, 2006).

The pKa value of acids is defined as *'A number that indicates how strong or weak a particular acid is, the strong acid have negatively or slightly positive pKa values, and the larger the pKa value, the weaker the acid'* (Suggs, 2002). Therefore, providing a foodborne pathogen decontaminant has therefore become essential in order to inhibit the bacterial population and enhance the safety of consuming poultry meat.

The objectives of this study were to evaluate the antibacterial activity of organic acid combinations below the MIC against *Escherichia coli* K12 and *Salmonella* Typhimurium DT104.

3.2 Materials and Methods

The challenges of *E.coli* and *Salmonella* with organic acids (lactic, citric, acetic and propionic acids) and combinations of lactic with citric or acetic or propionic acids in nutrient broth have been investigated as in section 2.2 and section 2.4

Chicken breast cubes were inoculated with *Salmonella* Typhimurium DT104 and *Salmonella* Typhimurium 1344nal^{r} by immersion into a suspension of *Salmonella* (~6-7 $\log_{10}$ CFU ml^{-1}) for 10 minutes at room temperature (22±1°C) as in section 2.7.

In order to determine the MIC for organic acids against *Escherichia coli* K12 and *Salmonella* Typhimurium DT104, different ranges have been investigated to be used in nutrient broth at 30°C. For example, the antibacterial activity of lactic acid concentration 3 to 9 mmol/L was investigated against *Salmonella* Typhimurium. Due to its palatable flavour in food, lactic acid was combined with acetic or citric or propionic acids in concentrations below the MIC using a Tecan plate reader at 30°C. Two points within the bacterial growth curve were manually determined from data by measuring the changing of OD (ΔOD) for each bacterium by subtracting the first optical density reading (when the exponential phase was started) from the maximum optical reading in log phase.

3.3 Results

The minimum inhibitory concentrations of lactic, citric, acetic and propionic acids against *Escherichia coli* K12 were 7, 4, 4 and 4 mmol/L respectively (Table14). The MICs of the same organic acids were 8, 5.5, 5 and 5 mmol/L against *Salmonella* Typhimurium DT104 respectively (Table 15). In terms of citric, acetic and propionic acids combinations with lactic acid below the MIC and their effect against both *Salmonella* Typhimurium DT104 and *Escherichia coli* K12, small differences have been shown in the strength of acid concentrations. The MIC of lactic and citric acids against *Escherichia coli* K12 was 7 mmol/l and 4 mmol/l respectively (Table 14); while for the combination of lactic and citric acids (below the MIC), the MIC was 7 and 3 mmol/l respectively against *Escherichia coli* (Table 16). The same scenario was used against *Salmonella* Typhimurium DT104 (Table 17).

Table 14: Minimum inhibitory concentrations of organic acids alone and their pH values against *Escherichia coli* at 30°C

	Lactic acid	Citric acid	Acetic acid	Propionic acid
MIC mmol/L	7	4	4	4
pH values	4.26	4.27	4.19	4.21

Table 15: Minimum inhibitory concentrations of organic acids alone and their pH values against *Salmonella* Typhimurium DT104 at 30°C

	Lactic acid	Citric acid	Acetic acid	Propionic acid
MIC mmol/L	8	5.5	5	5
pH values	4.14	4.12	4.09	4.10

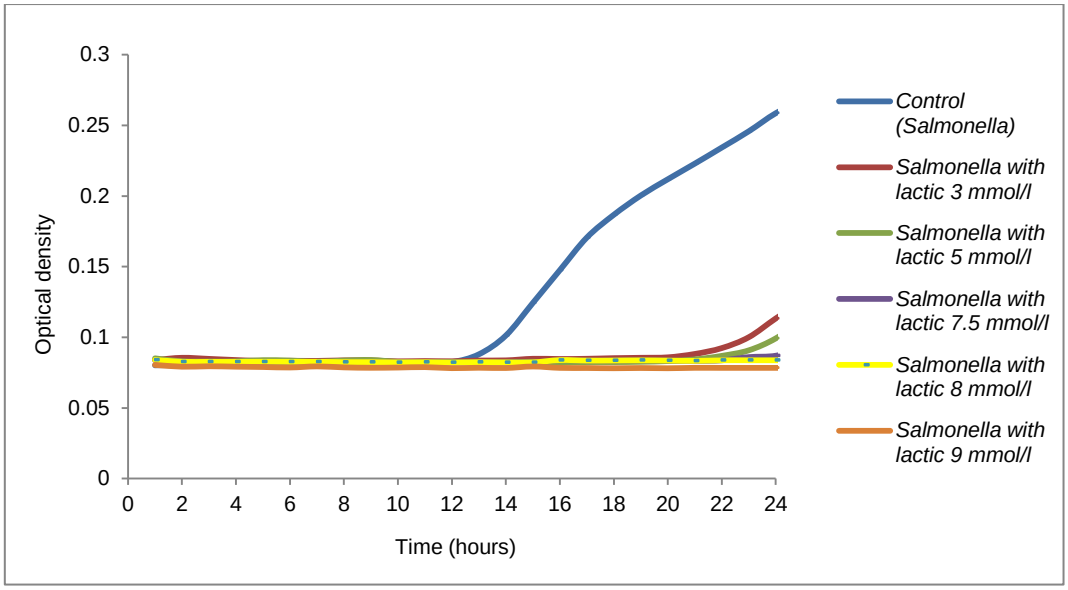

Figure 10: The antibacterial activity of a range of lactic acid concentrations (3 to 9 mmol/L) against *Salmonella* Typhimurium DT104 in nutrient broth at 30°C

Table 16: Minimum inhibitory concentrations of lactic acid and organic acid combination and their pH values against *Escherichia coli* at 30°C

	Organic acids combinations		
	Lactic and citric acids	**Lactic and propionic acids**	**Lactic and acetic Acids**
MIC mmol/L	LA 7 – CA 3	LA 6 – PR 3.5	LA 6.5- AC 3.5
pH values	4.22	4.28	4.18

Table 17: Minimum inhibitory concentrations of organic acid combinations and their pH values against *Salmonella* Typhimurium DT104 at 30°C

	Organic acids combinations		
	Lactic and citric acids	**Lactic and propionic acids**	**Lactic and acetic acids**
MIC mmol/L	LA 8 – CA 4.5	LA 7– PR 4.5	LA 7.5 – AC 4
pH values	4.18	4.20	4.16

When organic acids (the concentrations below the MIC) were combined, the combination of LA (5.5) & AC (2) was significantly stronger then LA (5.5) & PR (2) and LA (5.5) & CA (2) against *E.coli* K12 (Table 18). The combination of LA (6.5) & AC (3) was significantly the strongest against *Salmonella* Typhimurium DT104. In addition, combining acetic acid with lactic acid was able to increase the antibacterial activity for each separately against *E.coli* by increasing the lag phase time up to 20hrs and ΔOD 76.7%. The synergistic effect of organic acids (CA, PR and AC with LA) against *Salmonella* Typhimurium DT104 was noticed when either citric acid or acetic acid was combined with lactic acid. The ΔOD of the following combinations LA (6.5) & CA (3), LA (6.5) & PR (3) and LA (6.5) & AC (3) against *Salmonella* Typhimurium DT104 were 52.5 %, 60.6 % and 76.3 % respectively. The combination of acetic and lactic acids was more effective than either propionic or citric with lactic acid against both *Salmonella* Typhimurium DT104 and *E.coli* K12 at 30˚C.

Table 18: The antibacterial activity of lactic acid and organic acid combination in concentrations below the MIC against *Escherichia coli* K12 and *Salmonella* Typhimurium DT104 in nutrient broth at 30ºC

Acid concentration (mmol/L) A	Time of lag phase (hours)	ΔOD of *E.coli* K12 for 24 hours %	pH values
CA 2	15	48.6 [J]	4.59
LA 5.5	19	65.9 [c]	4.43
LA (5.5) & CA (2)	16	31.6 [L]	4.55
PR 2	16	49.4 [J]	4.49
LA 5.5	19	65.9 [c]	4.43
LA (5.5) & PR (2)	18	55.5 [e]	4.44
AC 2	15	51.6 [n]	4.42
LA 5.5	19	65.9 [c]	4.43
LA (5.5) & AC (2)	20	76.7 [a]	4.40
Acid concentration (mmol/L) B	**Time of lag phase (hours)**	**ΔOD of *Salmonella* for 24 hours %**	**pH values**
CA 3	15	46.6 [k]	4.48
LA 6.5	18	68.8 [b]	4.38
LA (6.5) & CA (3)	16	52.5 [g]	4.46
PR 3	15	50.6 [I]	4.31
LA 6.5	18	68.8 [b]	4.38
LA (6.5) & PR (3)	16	60.6 [d]	4.39
AC 3	16	53.3 [f]	4.33
LA 6.5	18	68.7 [b]	4.38
LA (6.5) & AC (3)	19	76.3 [a]	4.32

(A) against *E.coli*, (B) against *Salmonella* Typhimurium DT104. Means with the same superscript are not significantly different (*P>0.05*)

The immersion treatment of lactic and acetic acids combination (LA 13 - AC 6) for 10 minutes was not able to reduce the number of *Salmonella*. Nevertheless, when the concentration of the combination (LA 13- AC 6) was doubled as LA 208 and AC 96 mmol/l, the reduction of the bacterial population was 1.8 $\log_{10}$ CFU g^{-1} (Table 19). However, after keeping chicken pieces for a slightly longer time (4 hours), the total viable cells of *Salmonella* were increased. For example, the number of *Salmonella* in the lactic acid 13 mmol/l and acetic acid 6 mmol/l combinations was 6.34 $\log_{10}$ CFU g^{-1}, while after 4 hours of storage it was 6.43 $\log_{10}$ CFU g^{-1} and increased significantly after 24 hours of treatment to 8.43 $\log_{10}$ CFU g^{-1}. The effect of 10 minutes immersion treatment with lactic and acetic acid combinations (LA 13 - AC 6) had no significant difference against the growth of *Salmonella* as compared with the control, while most of the treatments were significantly effective against *Salmonella* (Table 19).

Table 19: The number of viable cells of *Salmonella* Typhimurium DT104 after immersion treatment of lactic and acetic acids combination on chicken meat at 30ºC

Treatment mmol/L	Time of storage (hours)		
	***Salmonella* Typhimurium Log_{10} CFU/g (n=3)**		
	10min.	**4 hrs.**	**24 hrs.**
Control	6.36 ^{gh}	7.94 ^{e}	8.90 ^{a}
Combination LA 13 - AC 6	6.34 ^{n}	6.43 ^{g}	8.43 ^{b}
Combination LA 26 - AC 12	5.77 ^{J}	6.24 ^{I}	8.33 ^{c}
Combination LA 104 - AC 48	5.55 ^{k}	5.37 ^{I}	8.22 ^{d}
Combination LA 208- AC 96	4.54 ^{m}	4.02 ^{n}	7.08 ^{f}

Means with the same superscript are not significantly different (*P>0.05*)

Combining two types of organic acids might increase the internal stress (number of dissociated acid molecules) and also increase the lethal effect of the combination, compared with organic acid alone, against *Salmonella* within the pH range of 2.38 to 3.95 (Table 20).

Table 20: PH values of various combinations of lactic and acetic acids

Acids concentrations mmol/L	pH values
Combination LA 13 - AC 6	3.95
Combination LA 26 - AC 12	3.82
Combination LA 104 - AC 48	3.52
Combination LA 208- AC 96	2.38

In this study, the acetic and lactic acid combination was able to reduce the bacterial population, and as the acidity became higher, the reduction was bigger. As a result, elongating the time of the immersion treatment was effective (Table 21). Moreover, *Salmonella* was undetectable (limit of detection =200) after 5 hours of treatment (immersion into the lactic and acetic acid combination), and that treatment was able to reduce the number of *Salmonella* by 0.84, 1.42 and 3.85 $\log_{10}$ CFU g^{-1} after 10 minutes, 2 hours and 4 hours respectively at 30±1ºC.

Table 21: The total viable cell of *Salmonella* Typhimurium DT104 on chicken meat after immersion treatment (lactic and acetic acids combination) at 30ºC

Treatments mmol/L	Number of *Salmonella* after several times of immersion (Log_{10} CFU/g) (n=3)					
	10 min	2hrs	4hrs	5hrs	6hrs	24hrs
Control	6.34	6.74	7.95	8.43	8.58	8.90
Combination LA 208 - AC 96	5.50	5.32	4.1	ND	ND	ND

ND= below the limit of detection. Means with the same superscript are not significantly different (*P>0.05*)

3.4 Discussion

Salmonella is one of the acid resistant *Enterobacteriaceae* species, while *Escherichia coli* K12 is classified as a non-acid resistant species (Winfield & Groisman, 2004): this may explain why the *Salmonella* was more durable in its behaviour than *E. coli* and may be the reason for the difference between MICs for both microorganisms.

In general, pH values of MICs of organic acids alone were in ranges of 4 to 5, which indicated that the reason for reduction of the number of viable bacterial cells might be the effect of acidity (González-Fandos & Dominguez, 2006). *Escherichia coli* K12 was the most affected organism compared with *Salmonella* Typhimurium DT104. The combination of lactic and acetic acids (LA 6.5 and AC 3 mmol/l) showed a synergistic effect against the growth of *Salmonella,* leading to 76.3 % of OD reduction and 19 hours delay of the lag time and this was in accordance with Narendranath, Thomas and Ingledew (2001) experiment applied in minimal media against yeast (Saccharomyces cerevisiae) at 30°C. Their results showed that the length of the lag phase of the growth curve increased exponentially with increasing concentrations of acetic or lactic acid, and that the inhibitory effect of lactic with acetic is highly synergistic (Narendranath, Thomas & Ingledew, 2001).

The current study found that the ΔOD due to combinations of lactic acid with acetic, propionic and citric acids against *Salmonella* Typhimurium DT104 were 76.3%, 60.6%, and 52.5% respectively, while, against *Escherichia coli,* the ΔOD due to lactic and acetic acids combination (LA 5.5 and AC 2 mmol/l) was 76.7% and the log time has started after 20 hours of treatment.

However, the ΔOD of the combinations of lactic with acetic, propionic and citric acids against *Escherichia coli* were 76.7%, 55.5 % and 31.6 % respectively (Table 18).

The immersion treatment of lactic and acetic acids in combination (LA 13 - AC 6 mmol/l) after 10 minutes was not able to reduce the number of *Salmonella.* Nevertheless, when the concentration of the combination was doubled as LA 208 and AC 96 mmol/l (LA 1.87%- AC 0.57%), the reduction of the bacterial population was 1.8 $\log_{10}$ CFU g^{-1} possibly owing to its greater effective acidity (Table 19). This result agreed with the experiment outcomes of Smulders and Greer (1998), which were that acid dipping of 1 to 3 % organic acids on meat tissues was able to produce a 2 log reduction in the number foodborne pathogens.

In this study, after keeping chicken pieces for a longer time (4 hours), the total viable number of *Salmonella* increased. This result could be because the effect of acid molecules on chicken meat tissue was to damage cells and release water, leading to a buffering of the environment. As a result, the pH level was higher than 6, which was quite typical for bacterial growth. Therefore, *Salmonella* started to grow and the number of *Salmonella* increased. The effect of acid after 10 minutes of immersion has a slight effect on the bacterial growth but, when the storage time was extended, the tissue started to release the water into the surface and buffered the environment, creating a favourable environment for bacterial growth and increasing the bacterial number (Table 19).

The immersion treatment into organic acid was able to inhibit the bacterial growth of *Salmonella* on chicken meat. The same results were achieved by Okolocha & Ellerbroek (2005), when chicken meat was dipped into 1% of lactic acid. This was more effective than spraying with the same concentration, which resulted in a reduction of 0.6 $\log_{10}$ CFU ml^{-1}.

Another explanation was reported: *Salmonella* after the exposure to acid, especially at low pH levels, becomes more resistant over time and harder to kill within a high resource of carbohydrate (chicken meat) (Kwon & Ricke, 1998). The antibacterial activity of organic acids has been explained as the effect of undissociated acid molecules and their reaction against the bacterial metabolism and obstruction of the cellular functions (Weir *et al.*, 2008). Therefore, combining two types of organic acids might increase the internal stress (number of dissociated acid molecules) and increase the lethal effect of the combination, as compared with the internal stress of acid alone, against *Salmonella* within a pH range of 2.38 to 3.95 (Theron & Lues, 2010).

3.5 Conclusion

The results in this chapter showed that lactic and acetic acids in combination resulted in a high level of antibacterial activity as compared with a propionic and citric acids combination in concentrations below MIC, against both *Salmonella* Typhimurium DT104 and *Escherichia coli* K12. Acetic acid was more effective than the others because of its capability to dissociate within the cytoplasm, and that leading to a lowering of the pH level and an increase in the anions number; as a result all turgor pressure inside the cell will be great, which is compatible with the findings of Roe et al (1998). The antibacterial activity of organic acids has been explained as the effect of undissociated acid molecules and their reaction against the bacterial metabolism and obstruction of the cellular function (Weir *et al.*, 2008).

Therefore, combining two types of organic acid might increase the internal stress (number of dissociated acid molecules) and increase the lethal effect of the combination compared with acid alone against *Salmonella* within the range of pH 2.38 to 3.95 (Table 20). *Salmonella* was not detectable in chicken breast meat after 5 hours treatment, but did have an effect on the organoleptic properties of raw chicken meat (colour and acidic smell). Therefore, this research strongly recommended finding an alternative natural treatment that is able to inhibit the *Salmonella* and enhance the organoleptic properties of raw chicken meat at 30°C.

Chapter 4:

Inhibition of selected foodborne pathogens on chicken breast meat by immersion into organic acids and an aqueous lime-peel extract at 30°C

Inhibition of selected foodborne pathogens on chicken breast meat by immersion into organic acids and an aqueous lime-peel extract at 30°C

4.1 Introduction:

In the USA and many Middle East countries, several types of organic acids, e.g. citric, acetic and propionic acids, have been approved as treatments to reduce the amount of viable bacteria on meat (USDA, 2011b). Organic acids have shown an ability to inhibit foodborne pathogens and are described as bacteriostatic (Helander & Mattila-Sandholm, 2000). In the UK and the EU, these treatments are not legislated as bacterial decontaminators in meat.

The benefit of using organic acids in reduction of the bacterial population has been reported widely except against acid tolerant species such as *Salmonella* Typhimurium DT104, which survived easily in acidic conditions throughout processing of food and human digestion (Sampathkumar & Food Security Assessment Unit for Somalia., 2004). Food preservation methods (heat, sugar and salt) have been used to reduce the potential risk of *Salmonella* in food, but not without affecting the natural food aroma (Olasupo et al., 2003). According to the perception of consumers, natural food products are more favourable, and also better able to maintain human health (Pei et al., 2009; Watson & Preedy, 2008).

Organic acids are used in the range of 1-3 % without affecting the sensory properties of meat products (Theron & Lues, 2010). Acetic, citric and propionic acids are commonly preferred for domestic use. In food industries, organic acids are considered as safe and efficient antimicrobial agents for spraying and immersion

treatment to reduce the population of foodborne pathogens on poultry meat (Van Immerseel et al., 2006). In addition, organic acids are most effective at high temperatures (Quinn et al., 2011) and especially at concentrations of 2 to 4% (Sirsat, Muthaiyan & Ricke, 2009).

On the other hand, many studies have focused on the antimicrobial activity of herbal extracts as an alternative decontamination treatment in food, emphasising cost and safety factors (de Souza *et al.*, 2005; Fazeli et al., 2007; Olasupo *et al.*, 2003). Citrus fruits are used in several areas of life such as medicine, preservation and drinks (Magazine, 2011; Vasudeva & Sharma, 2012). The antibacterial activity of some herbal compounds is still not clear, and for that reason these compounds have been investigated widely (Ceylan & Fung, 2004a; Karatzas et al., 2000). According to Sharma & Tripathi (2008), many species of citrus contain aromatic compounds such as dl-limonene, β-myrcene, α-pinene and sabinene. These aromatic compounds are responsible for the antibacterial, antioxidant and anti-inflammatory properties of citrus fruits (Fisher & Phillips, 2008a; Zia ur, 2006). It has been confirmed that the antibacterial activity of natural herbal compounds (phenolic and aromatic compounds) affects the structure of the phospholipid bilayer of cytoplasmic membrane in bacteria, increases the permeability of cytoplasmic membrane, depresses the intracellular defence barriers and disrupts the bacterial enzyme routes (de Souza *et al.*, 2005).

In food preservation, one of the main goals is extending the shelf life of food by eliminating and preventing the growth of foodborne pathogens (Aibinu *et al.*, 2007). Many preservative methods, including salt, vinegar and sugar, are used in food(Hyldgaard, Mygind & Meyer, 2012). Recently, the activity of preservation due to combining several preservatives has been investigated, with the aim of reducing the severity of each method individually (Requena, 2012).

Food factories and food experts are still seeking new substances as alternatives for food preservation, to reduce both the economic cost and the risk of cross contamination. Herbal extracts have been the subject of particular interest as a natural food additive and there is a demand from the public and retail sectors to use natural ingredients due to their flavours (Peter, 2004). There remains nonetheless a shortage of studies and researches about the antibacterial activity of organic acids and herbal extracts used in combination against acid tolerant species of *Salmonella*. The present study is concerned with demonstrating the antibacterial activity of concentrations below the minimum inhibitory concentration of organic acids in combination with an aqueous lime-peel extract on raw chicken meat against *Salmonella* Typhimurium (DT104 and 1344nal^{r}) at 30°C.

4.2 Methods

An extract of *Citrus aurantifolia* was prepared by grinding the dried peel of lime and mixing it with distilled water at room temperature (22±1°C) see chapter 2.3.1. In addition, organic acids (acetic and propionic acids) and NaCl were prepared as in chapter 2.2

Phenolic and phytochemical compounds of an aqueous lime-peel extract were tested and calibrated with Gallic acid, as detailed in sections 2.3.2 and 2.3.4.

Chicken meat pieces were inoculated with *Salmonella* Typhimurium (DT104 and 1344nal^{r}) by immersion into a suspension of *Salmonella,* as fully explained in chapter 2.7.

After the inoculation, chicken meat was treated with combinations of lime and acetic acid as 5.2 % - 0.28 %, 10.4 % - 0.28 %, and 20.8 %- 0.56 % and 41.6 % - 1.12 % w/v respectively. The treatments of lime with propionic acid were (5.2 %- 0.35 %, 10.4 %- 0.35 %, 20.8 % -0.65 % and 41.6 % -1.3 %) w/v respectively.

The inoculated pieces of chicken meat were immersed into organic acids (acetic and propionic acids) and an aqueous lime-peel extract combination for 10 minutes, 2, 5, 9 and 24 hours. The choice of these combinations was based on a previous experiment, as detailed in chapter 3.

To evaluate the effect of using the combination of an aqueous lime-peel extract and acetic acid on chicken meat, a sensory evaluation experiment was applied. Chicken breast meat was treated by immersion into the combination of an aqueous lime-peel extract and acetic acid (LI 41.6 % - AC 1.12 % w/v) for 10 min, 2hrs, 5hrs and 9hrs, as fully detailed in chapter 2.9

4.2.1 Statistical analysis

Analysis of variance of log reduction was measured using MINITAB program (version 16). To compare the means, Tukey's test was performed in the same program, using general linear anova and- one way anova, and the significant result was at $P<0.05$ (chapter 2.10).

4.3 Results

Both combinations of propionic / acetic acids with an aqueous lime-peel extract were able to increase the lag time of both organisms (*Salmonella* Typhimurium DT104 and *Escherichia coli* K12) by more than their individual effect (Table 22).

Both propionic acid and an aqueous lime-peel extract alone delayed the lag phase of *Escherichia coli* K12 for 16 hours, while 18 hours delay occurred as a result of their combination. In addition, the lag phase of *E.coli* was elongated for 15 hours by acetic acid alone, and 19 hours due to a combination of acetic acid and an aqueous lime-peel extract at 30°C. Against *Salmonella* Typhimurium DT104, propionic acid and an aqueous lime-peel extract separately delayed the lag time up to 15 hours while, when the two were combined, the lag phase was extended to 16 hours.

In addition, acetic acid and an aqueous lime-peel extract combination was significantly able to delay the lag phase up to 17 hours; acetic acid was more effective than propionic acid and an aqueous lime-peel extract alone by 16 hours delay. Additionally, the reduction of the bacterial growth by propionic acid, an aqueous lime-peel extract, and their combination against *E.coli* K12 for 24 hours were 49.4 %, 35.7 % and 53.8 % respectively at 30°C. The ΔOD of acetic acid, an aqueous lime-peel extract and the combination, were 51.6 %, 35.7 % and 61.4 % against *E.coli* respectively at 30°C (Table 22). The ΔOD for propionic acid, an aqueous lime-peel extract, and their combination against *Salmonella* Typhimurium DT104 were 50.6 %, 38.9 %, and 55.7 % respectively at 30°C. The ΔOD for acetic acid, an aqueous lime-peel extract, and their combination were 53.3 %, 38.9 %, and 58.2 % respectively at 30°C (Table 22).

Table 22: The antibacterial activity of organic acids and an aqueous lime-peel extract alone and their combinations below the MIC against *Salmonella* Typhimurium DT104 and *Escherichia coli* K12 in nutrient broth at 30±1ºC (n=3)

Acids and lime combinations % (A)	Time of lag phase (hours)	ΔOD of *E.coli* K12 for 24 hours %	pH Values
PR 0.014	16	49.4 [f]	4.49
LI 0.25	16	35.7 [h]	4.82
LI 0.250 and PR 0.014	18	53.8 [d]	4.55
AC 0.012	15	51.6 [e]	4.42
LI 0.250	16	35.7 [h]	4.82
LI 0.25 and AC 0.012	19	61.4 [a]	4.47
Acids and lime combinations % (B)	**Time of lag phase (hours)**	**ΔOD of *Salmonella* Typhimurium for 24 hours %**	**pH Values**
PR 0.022	15	50.6 [e]	4.31
LI 0.325	15	38.9 [g]	4.60
LI 0.35 and PR 0.022	16	55.7 [c]	4.42
AC 0.018	16	53.3 [d]	4.33
LI 0.325	15	38.9 [g]	4.60
LI 0.325 and AC 0.018	17	58.2 [b]	4.39

(A)Against *E.coli* K12 (B) against *Salmonella* Typhimurium DT104. Means with the same superscript are not significantly different (p>0.05)

In terms of lime analysis, several tests were applied in order to indicate the presence or absence of phytochemical compounds such as amino acids, tannins, phlobatanins, and saponin. As a result, all tests showed positive reactions (presence of phytochemical compounds). In addition, the level of citric acid in lime (indigenous citric acid) was determined by using high performance liquid chromatography: the concentration of citric acid in lime-peel extract was 230 mM (4.41 %). Moreover, the level of phenolic compounds in an aqueous lime-peel extract was 2.4 %.

The concentration of acetic and propionic acids with lime combinations, which were obtained by spectrophotometer below the MIC, were doubled, keeping the proportions of each (lime and organic acids) equally on chicken meat until the effective treatment was obtained and *Salmonella* was not detectable. For instance, a treatment of acetic acid and lime combination (LI 0.325 % and AC 0.018 %), which showed a remarkable antibacterial performance in nutrient broth, was doubled several times on raw chicken meat until there was no *Salmonella,* and that treatment was LI 41.6 % with AC 1.12 %. As a result, the lowest concentration of lime and acetic acid combination (5.2 % - 0.28 %) achieved half a log of *Salmonella* at pH 4.69 after 10 minutes of immersion into the treatment at 30±1°C (Table 23).

Table 23: The total viable cell count of *Salmonella* Typhimurium DT104 after immersion treatment (lime and acetic and propionic acids combinations) on chicken breast meat at 30±1°C (n=3)

Time (hours)	Lime - acetic acid (LI-AC) %				Lime - propionic (LI-PR) %				Control
	(5.2-0.28)	(10.4-0.28)	(20.8-0.56)	(41.6-1.12)	(5.2-0.35)	(10.4-0.35)	(20.8-0.65)	(41.6-1.3)	
	pH 4.69	*pH 4.45*	*pH 3.90*	*pH 2.67*	*pH 4.71*	*pH 4.49*	*pH 3.98*	*pH 2.88*	
0:10	5.85 ^{e}	5.79 ^{ef}	5.59 ^{ghij}	5.26 ^{m}	5.78 ^{efg}	5.6 ^{ghij}	5.53 ^{ijkl}	5.35 ^{lm}	6.34
2:00	5.63 ^{fghi}	5.57 ^{hijk}	5.42 ^{jklm}	4.56 ^{no}	5.5 ^{ijkl}	5.43 ^{jklm}	5.37 ^{klm}	4.68 ^{n}	6.74
5:00	6.26 ^{cd}	5.88 ^{e}	5.78 ^{efg}	4.4 ^{o}	6.16 ^{d}	6.14 ^{d}	5.74 ^{efgh}	3.6 ^{p}	8.43
9:00	6.41 ^{c}	6.4 ^{c}	6.24 ^{cd}	2 ^{r}	6.3 ^{cd}	6.28 ^{cd}	6.19 ^{d}	3.15 ^{q}	8.69
24:00	7.51 ^{a}	7.43 ^{a}	6.97 ^{b}	2 ^{r}	7.45 ^{a}	7.37 ^{a}	6.88 ^{b}	2 ^{r}	8.91

Means with the same superscript are not significantly different (*P>0.05)*

However, after 24 hours of immersion into the same treatment, the total log reduction of *Salmonella* Typhimurium DT104 was 1.4 $\log_{10}$ CFU g-1. Increasing the lime concentration up to 10.4 % and keeping the same concentration of acetic acid (0.28 %), showed no significant difference in most of the immersion times. The concentration of lime and acetic acid (20.8% – 0.56%) reduced the number of *Salmonella* after 10 minutes, 2, 5, 9 and 24 hours by 0.75, 1.32, 2.65, 2.45 and 1.94

$\log_{10}$ CFU g^{-1} respectively at 30±1°C. *Salmonella* Typhimurium DT104 was not detectable after 9 hours of immersion into the combination of lime-peel extract and acetic acid (41.6 % - 1.12 %) by total reduction of 8.69 $\log_{10}$ CFU g^{-1} in pH of 2.67 at 30±1°C. Treating the inoculated chicken meat with the combination of lime with propionic acid (5.2 % – 0.35 %) and lime with acetic acid combination (5.2 % – 0.28 %) for 10 minutes, resulted in no significant difference in the total bacterial reduction. The effective treatment of propionic acid and lime combination reduced the total viable number of *Salmonella* Typhimurium DT104 after 10 minutes, 2, 5, 9 and 24 hours by 0.99, 2.06, 4.83, 5.54, and 8.91 $\log_{10}$ CFU g^{-1} respectively.

The pH values of immersion treatments were in range of 2.62 - 3.51 (Table 24). The antibacterial activity of acetic acid and lime was significantly different from propionic and lime combination after 9 hours of immersion, when no *Salmonella* was detected on chicken meat at 30±1°C.

Table 24: pH- values for immersion treatments of propionic, acetic acids and an aqueous lime-peel extract alone in various concentrations

Treatments %	pH values
Propionic acid 1.3	2.62
Acetic acid 1.12	2.66
Lime 41.6	2.81
Acetic acid 0.56	2.85
Propionic acid 0.65	2.79
Lime 20.8	3.12
Acetic acid 0.28	3.31
Propionic acid 0.35	3.24
Lime 10.4	3.39
Lime 5.2	3.51

Comparing the antibacterial activity of each treatment alone, acetic acid (1.12 %) and an aqueous lime-peel extract (41.6 %) showed no significant difference until 6 hours of treatment, and then the lime was more effective than acetic acid by reducing the total amount of *Salmonella* Typhimurium DT104 by 4.27 and 3.46 1.4 log_{10} CFU g^{-1} respectively (Table 23). Moreover, adding 1% NaCl to acetic acid and lime combination has reduced the population of *Salmonella* Typhimurium DT104 on chicken meat by 0.3 log_{10} CFU g^{-1}, but no significant difference was noticed after 10 minutes of immersion (Figure 11).

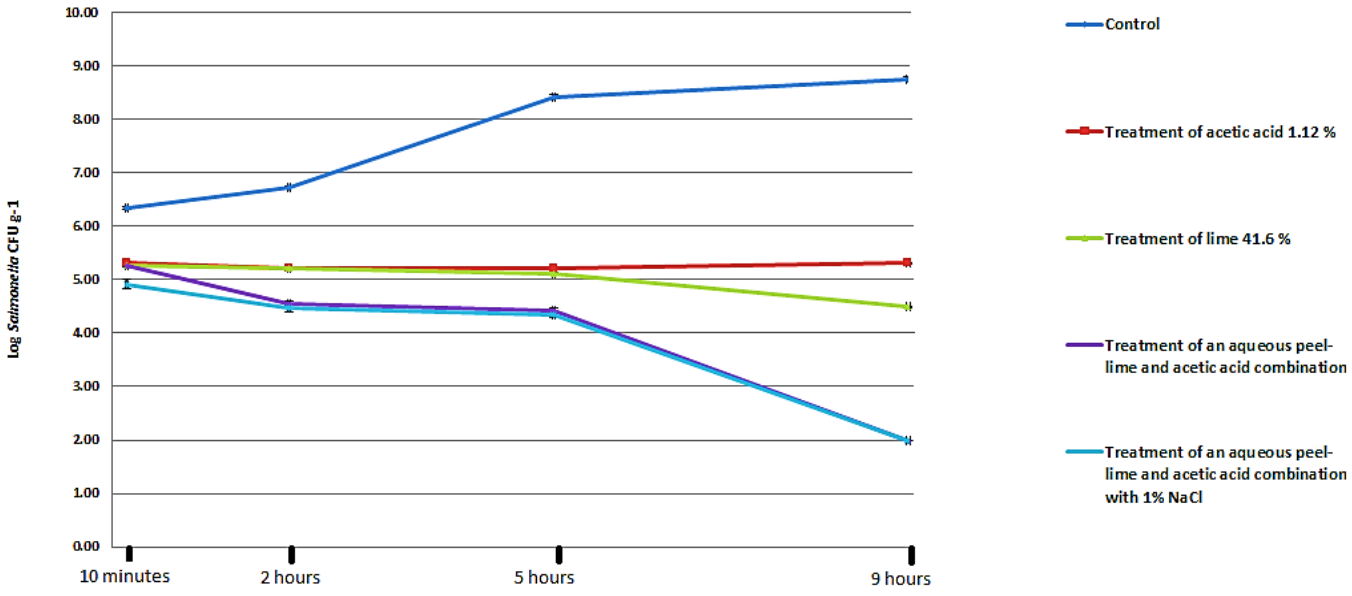

Figure 11: The antibacterial activity of acetic acid and an aqueous lime-peel extract alone or in combination with and without 1% NaCl against *Salmonella* Typhimurium DT104 on chicken meat at 30±1°C (n=3)

The antibacterial activity of acetic acid and lime combination (41.6 % – 1.12 %), including 1% NaCl, was investigated against *Salmonella* Typhimurium 1344nal^{r} on chicken meat at 30±1°C (Figure 12). As a result, no significant difference was noticed by comparison with *Salmonella* Typhimurium 1344nal^{r} and no *Salmonella* was detected after 9 hours of immersion.

However, after immersion into lime and acetic acid combination (41.6% - 1.12%) including 1% NaCl for 10 minutes, 2 and 5 hours, the number of *Salmonella* Typhimurium 1344nal^{r} was lower than the control by 1.58, 2.48 and 4.64 log_{10} CFU g^{-1} respectively at 30±1°C, while, numbers of *Salmonella* Typhimurium DT104 were 1.43, 2.25, and 4.09 log_{10} CFU g^{-1} respectively at 30±1°C.

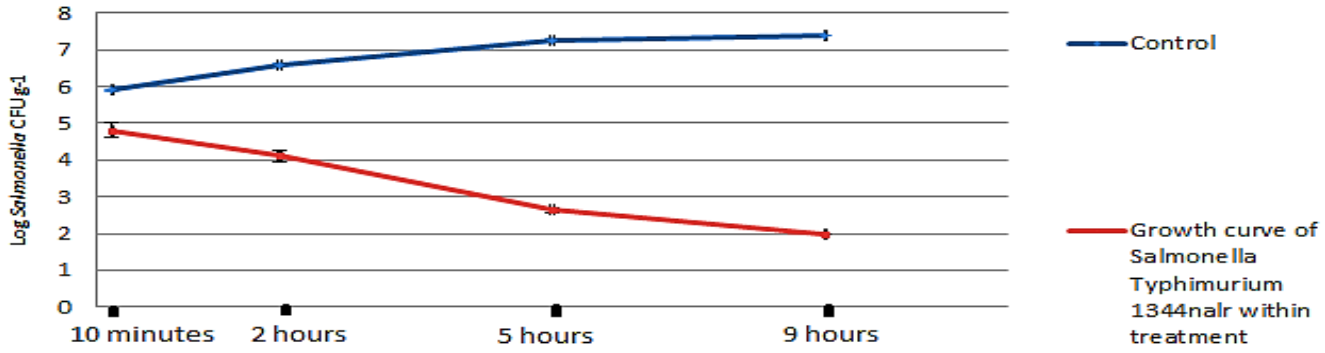

Figure 12: Antibacterial activity of acetic acid and lime combination including 1% NaCl against *Salmonella* Typhimutium 1344nal^{r} on raw chicken breast meat at 30±1°C (n=3)

The treatment of an aqueous lime-peel extract and acetic acid combination (41.6% - 1.12%) was more effective than the treatment of an aqueous lime-peel extract and propionic acid combination (41.6 % – 1.3 %) against *Salmonella* after 9 hours of immersion on raw chicken meat at 30°C. Therefore, and in order to evaluate the effect of an aqueous lime-peel extract and acetic acid combination (41.6 % – 1.12 %) on chicken breast meat, sensory evaluation was assessed. The chicken breast meat cubes were immersed into the treatment (LI 41.6 %, AC 1.12 %), including 1 % NaCl ,for the maximum time of 9 hours.

Thirty participants were invited from Plymouth University (staff and students) to assess 5 pieces of cooked chicken meat in total (4 marinating cooked chicken meat and one piece of cooked chicken without any additives was considered as a control). Chicken meat pieces were marinated for four time-periods, which were 10 minutes(T1), 2 hours (T2), 5 hours (T3) and 9 hours (T4) into the treatment of an aqueous lime-peel extract and acetic acid combination (41.6 %, 1.12 %) including 1% NaCl, as recomended with cooked chicken meat. In general, each participant evaluated 7 types of attributes of chicken meat cubes: aroma, colour, apperance, flavour, acidity, texture, and overall acceptance. In terms of the overall acceptance score, the most preferred samples were T2 and T3 significantly (*P>0.05*).

In addition, no significant difference was observed between the control, T1 and T4 respectively. The scoring of colour as one of the attributes revealed that T1 and T2 were the most liked treatments, with no significant difference between them. In terms of the aroma, no significant difference was noticed between the treatments, while a significant difference occurred comparing all treatments with the control.

Most of the assessors preferred T2 and T1 in termsof the appearance. Regarding the flavour, T1 was the most favoured treatment, with no significant difference shown between other treatments. When assessors evaluated the acidity, T2 was the significantly preferred treatment, as compared to other treatments. In addition, no significant difference was observed between the control, T1 and T3 ,T4 in terms of the texture. In overall acceptance, no significant difference existed between the control, T4 and T1 and the most preferred treatment was T2 (Figure 13).

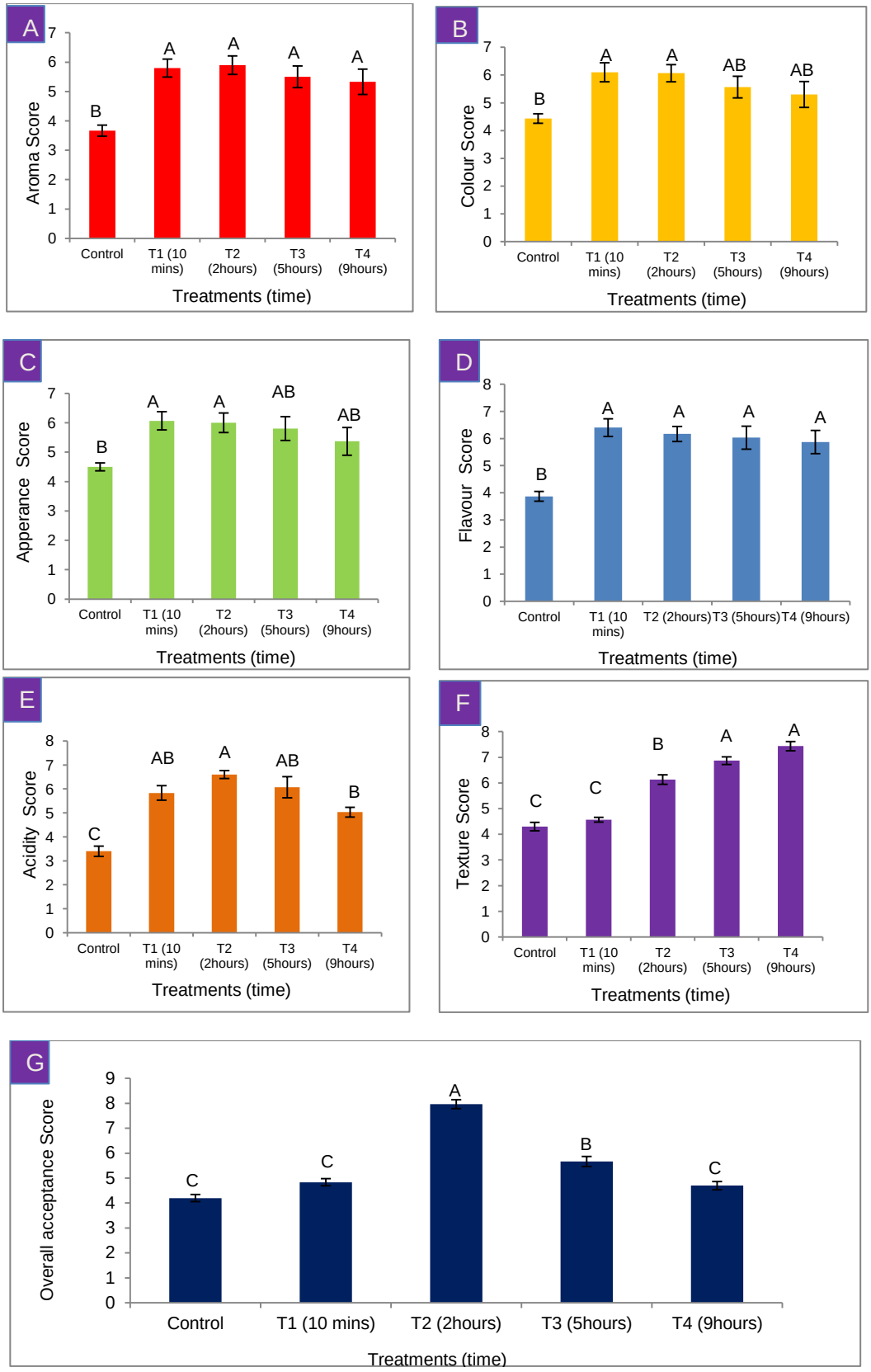

Figure 13: Sensory evolution attributes: aroma (A), colour (B), apperance (C), flavour (D), acidity (E), texture (F), overall acceptance (G) of cooked marinated chicken breast meat

4.4 Discussion

Combining various preservative techniques can be considered as an alternative antimicrobial and flavour enhancer, keeping food in high microbial quality and tasty (Leistner & Gould, 2002; Nazer et al., 2005). This study investigated the antibacterial activity of some combinations of an aqueous lime-peel extract and organic acids (both acetic and propionic acids) for concentrations below the MIC, aiming to inhibit the growth of *Salmonella* Typhimurium DT104 and *Salmonella* Typhimurium 1344nal^{r} on chicken breast meat at 30±1°C.

Organic acids (propionic and acetic acids) were selected according to their capability in inhibiting foodborne pathogens, as several studies have reported (Dickens & Whittemore, 1997; Nazer *et al.*, 2005; Rhee et al., 2003; Theron & Lues, 2009). The antibacterial activity of citrus fruits, especially *Citrus aurantifolia,* has been reported as due to the citric acid level and some phenolic compounds (Bhat, Alias & Paliyath, 2012). The combination of lime with acetic acid was more effective than propionic acid and lime after 9 hours of immersion: this may be due to the additional toxic effect of acetic acid on the bacterial cells (Jensen et al., 2003). The Intracellular pH and undissociated acid anions are reported to be responsible for making acetic acid more effective than other types of organic acids as an antibacterial (Jensen *et al.*, 2003). Treatment of acetic acid and lime extract combination for 10 minutes immersion was able to achieve nearly half a log reduction of *Salmonella* Typhimurium DT104, which was due not only to the environmental acidity (pH= 4.69), but also to the level of phenolic compounds (2.4 %) and the concentration of indigenous citric acid (230 Mm).

The antibacterial activity of phenolic compounds comes from its ability to act as a non-ionic surface-active agent, which is able to disrupt the lipid-protein interface (Leszczynski, 2012). The antibacterial activity of phenolics at low concentrations has an effect on bacterial enzyme activities, while at high concentrations, phenolics cause protein denaturalization (Hemming, 2011). A great number of antibacterial agents has been reported in herbal extract due to the presence of several natural plant compounds such as alkaloids, glycosides, tannins, steroids, saponins, phlobatanins, and flavonoids (Arekemase, 2011). Vinoth, Manivasagaperumal and Balamurugan (2012) have reported *Salmonella*, *E.coli* and *S.aureus* to be sensitive to aqueous extract of herbal leaf extract (*Moringa olifera*) due to the presence of flavonoids, tannins, steroids and glycosides. As a result, the presence of phytochemicals indicates possible preventive properties against food borne pathogens.

When an aqueous lime-peel extract concentration was doubled, the level of water was also increased, diluting the environment's acidity and reducing the antibacterial activity of acetic and propionic acids. Therefore, most of the 10 minute treatments showed no significant difference, except the treatment of lime and acetic acid combination (41.6 % –1.12 %) with pH of 2.67. Presumably, combining a high level of acidity and phenolic compounds worked together synergistically to achieve that high reduction of *Salmonella* (Ohlsson & Bengtsson, 2002). The antibacterial activity of both lime and acetic acid alone has shown no significant difference with up to 6 hours of immersion. Lime alone achieved 4.27 $\log_{10}$ CFU g^{-1} log reduction, while 3.46 $\log_{10}$ CFU g^{-1} of *Salmonella* was the result of using acetic acid alone.

The exposure to acid for such a long time has an effect on the meat tissue structure, causing severe damage in the external cells, releasing water and diluting the environment, while the occurrence of phenolic compound and the indigenous citric acid in lime were able to inhibit the *Salmonella* after 6 hours of treatment.

The above data may be the reason why the food industries and nutrition experts are looking to this sort of treatment as a means of 'hitting two birds with one stone.' Diluting the salt during the time of immersion was the reason why 1 % NaCl was ineffective (against growth of *Salmonella*) after ten minutes of treatment, with negligible reduction in the total viable cell of *Salmonella.* The addition of salt nonetheless successively maintained the chicken texture and kept the chicken meat tasty (Skipp, 2009). *Salmonella* Typhimurium 1344nal^{r} was reported to be responsible for infecting humans with Salmonellosis (Sarris, Food & Nations, 2003). In addition, *Salmonella* Typhimurium 1344nal^{r} is described as a tough strain of *Salmonella* due to its ability to adapt and its resistance to nalidixic acid (Narrod, Pray & Tiongco, 2008).

In the results of this chapter, no significant difference in log reduction was noticed between the two strains of *Salmonella* and both strains were not detected after 9 hours of immersion into the treatment (acetic acid and lime combination (41.6%-1.12%)). However, immersion contaminated chicken breast meat into an acetic acid and lime combination including 1 % NaCl for 10 minutes, 2 and 5 hours reduced the number of *Salmonella* by 1.58, 2.48 and 4.64 $\log_{10}$ CFU g^{-1} respectively at 30±1°C. Several natural plant compounds have been reported to have an antibacterial activity. For instance, in lime extract, plant volatiles, alkaloids and flavonoids have been reported. Plant volatiles have been recognized as safe and as a flavour source (Utama et al., 2002).

Moreover, the natural antibacterial activity of citrus fruit extract comes from many complex compound structures, such as alkaloids, flavonoids, tanins, coumarins and phenolic compounds (de Souza *et al.*, 2005).

Organic acids are indigenous in citrus fruits and several mechanisms have impacted the antibacterial activity of organic acids, such as metabolic antagonism and the inhibition of many cellular functions like cell wall synthesis, cytoplasmic membrane function, protein synthesis and nucleic acid synthesis (Levic´, 2008). Hence, using an aqueous herbal extract has the benefit of minimizing the acidic smell and colour changes which might exist due to using high level of organic acids in poultry products (de Souza et al., 2005). Moreover, using this type of acidic herbal extract as a chicken marinade has given the products savoury flavour, tenderization, and makes them juicier (USDA, 2011a). According to Foster (2004b), *Salmonella* Typhimurium has the ability to resist the acidic environment, especially in the stomach, and to then invade the intestinal cells. Therefore, applying an aqueous lime-peel extract and acetic acid combination (5.2 % – 0.28 %), or even increasing the concentration of lime (10.4 % – 0.28 %), made no significant difference in the log reduction of *Salmonella* within the pH range of 4.45 to 4.69 after 9 and 24 hours of immersion at 30±1°C.

Acetic acid is one of the common options among organic acids, which is used currently in decontamination of meat and food products (Rhee *et al.*, 2003; Stivarius et al., 2002). However, this is usually accompanied by some faults, including the effect of high concentrations on the organoleptic properties, such as a colour changes, taste and acidic smell (Stivarius *et al.*, 2002). As an antibacterial agent, acetic acid has the ability to reduce the total viability of the bacterial cell and to elongate the lag phase of foodborne pathogens' growth (Toldrá, 2008).

As a result of the sensory evaluation experiment, in terms of the aroma, all treatments were more palatable than the control due to the concentrated level of aromatic compounds in citrus fruits, as González-Mas et al. (2011) have reported. One of the bad effects of using organic acids is the colour change in meat (Nollet et al., 2012).

Evaluation of the colour has nonetheless demonstrated that T1 and T2 were more acceptable than other treatments. In addition, the same results were revealed when assessors evaluated the appearance of cooked chicken meat. Interestingly, all treatments were significantly more palatable than the control, which might be due to the citrusy flavour, the effect of indigenous level of organic acids and the aromatic compounds. T2 has given the highest acidity score, as the participants preferred moderate acidity with chicken meat. This treatment is widely used in the Middle East, especially in Iraq, where it is mixed with different kinds of vegetable soups, enhancing the aroma and acting as an appetizer. Not surprisingly, the effect of the acidic treatment on the texture of chicken meat was obvious, due to the organic acid's ability to degrade the proteins, and thus to affect the connective tissue (Kahraman et al., 2012). Ten minutes was insufficient to make any significant difference as compared with the control. Therefore, no significant difference has been reported in the acidity score. In overall acceptance, T2 was the most favourable sample, followed by T3 significantly.

4.5 Conclusion

The minimum inhibitory concentration of the combination of organic acids and an aqueous lime-peel extract which is required to inhibit the growth of *Salmonella* on raw chicken breast meat at 30°C was investigated. Additionally, this combination (organic acids with an aqueous lime-peel extract) was able to minimize the effect of organic acids on the organoleptic properties of food and to reduce the acidic smell due to the level of water.

The results of the sensory evaluation of marinated cooked chicken meat has demonstrated that all treatments were more palatable than the control, possibly indicating a good means of avoiding the cross contamination of chicken meat and reducing existing contamination as well as adding the citrusy flavour which causes chicken meat to be more appetizing. Thus, success was achieved in creating a marinade with an acceptable taste that reduced the total count of *Salmonella*.

Chapter 5: The effect of lime and acetic acid combination on gene expressions of acid tolerant species of *Salmonella* Typhimurium growing in nutrient broth

The effect of lime and acetic acid combination on gene expressions of acid tolerant species of *Salmonella* Typhimurium growing in nutrient broth

5.1 Introduction

The rise in the number of medical reports which refer to *Salmonella* Typhimurium has increased concern in the community (Health, 2004). Even within stressed environments (osmosis, cold and acid stresses), *Salmonella* behaves strangely and survives for a long time (Xu, Lee & Ahn, 2008; Zhou et al., 2011). When antimicrobials are used, *Salmonella* is able to stay viable for a long time, with a high level of pathogenicity (van Duijkeren & Houwers, 2000).

Antimicrobials have been responsible for prolonging the time of faecal shedding of *Salmonella*, inducing virulence factors in *Salmonella* and prohibiting the endogenous microflora (Weir *et al.*, 2008). Acidic food additives are reported to act as natural antimicrobials due to the level of acid and phenolic compounds (K. Ashok kumar, 2011; Taiwo, Oyekanmi & Opaleye, 2007). The antibacterial activities of organic acids, such as lactic, acetic and citric acids, have been studied widely (Hirshfield, Terzulli & O'Byrne, 2003; Jensen *et al.*, 2003). As an alternative natural and cheap antimicrobial, herbal extract and organic acids have been combined as an treatment to inhibit *Salmonella* (Levic´, 2008). There is a clear correlation between using antimicrobials and their effect on the persistence and virulence of *Salmonella* (Weir et al., 2008).

Acid tolerance response (ATR) is an explanation for how *Salmonella* survives when exposed to harsh environments such as acidic environments (low or moderate levels) and how it subsequently adapts by synthesis of proteins and induction of several genes (Ericson, 2011). *Fur* (ferric uptake regulator) and *Mig5* (macrophage-inducible gene coding for putative carbonic anhydrase) have been reported to be responsible for ATR in *Salmonella* (Weir et al., 2008). *Mig5* is one of the genes originating from the *Salmonella* serovar specific virulence plasmid, allowing for the evolution of highly virulent and antibiotic resistant clones of *S. enterica* (Rychlik, Gregorova & Hradecka, 2006). In addition, *Mig5* gene is important for bacterial colonization inside the host (Chiu, Su & Chu, 2004). However, there are few studies investigating the effect of combining organic acids and herbal extract on genes expression of foodborne pathogens.

Food experts and agencies are trying to eliminate the side effects of using chemical preservatives (Callahan, 2011; Sizer et al., 2006), and the combination of an aqueous lime-peel extract and acetic acid could be an effective multi-factor antimicrobial against bacteria (*Salmonella* Typhimurium DT104 – multi acid tolerant species), enhancing the organoleptic properties of the product by reducing the effect of acidity. This study aimed to ascertain whether exposure of *Salmonella* Typhimurium to a sub- lethal acidic environment for a maximum period of 2.5 hours of immersion treatment (an aqueous lime-peel extract and acetic acid combination) subsequently induced the acid tolerance and virulence genes *Fur* and *Mig5,* as several researchers have reported (Bearson, Wilson & Foster, 1998; Kwon & Ricke, 1998); and if so, whether the *Salmonella* would have the capacity to survive within the nutrient broth and stomach environment acidity (pH 2.5).

5.2 Materials and methods

Salmonella Typhimurium DT104 and *Salmonella* Typhimurium 1344nal^{r} were treated with the combination of an aqueous lime-peel extract (5.2) and acetic acid (0.28 %) with pH 4.69 including 1 % NaCl, as prepared in chapter 2.3.

The viability of *Salmonella* after the treatment has been checked by growing treated cells in nutrient broth at pH 2.5 for several times (10 minutes, 30 minutes, 1 hour, 1.5 hour, 2 hours and 2.5 hours) at 37±1°C. After each time, pure cells were washed with PBS and spread on nutrient agar and XLD (selective media) and kept overnight at 37±1°C to investigate the absence and presence of salmonellae. The control was pure cells of *Salmonella* growing in nutrient broth at pH 2.5 at 37±1°C.

As per manufacturer's instructions, fresh minimal media (nutrient broth) was used to grow the bacteria. In addition, the stationary phase is considered as the time to harvest the bacteria in order to get the RNA. All samples (cDNA) were run by step one Q-PCR machine using SYBER Green Master Mix SYBR® Green JumpStart™ Taq ReadyMix™ from SIGMA- ALDRICH, UK. All steps, which were followed, have been described in chapter 2.10.2.

In addition, Relative Quantitation (RQs) data were imported using 2 (Delta Delta C(T)) method (Livak & Schmittgen, 2001) directly from Step One Plus real time machine and organized as figures using the Excel program.

5.3 Results

The ability of *Salmonella* to survive within a harsh acidic environment was increased, making *Salmonella* more adaptive (Figure 14, 15). Both genes (*Fur* and *Mig5*) were up - regulated in all times, except the gene *Fur* after 10 minutes treatment (Figure 14).

It appears that 10 minutes of immersion was not sufficient to up-regulate this gene at 30±1°C. The duration of exposure to the treatment of an aqueous lime-peel extract (5.2) and acetic acid (0.28 %) combination was selected randomly, taking into account the level of RNA.

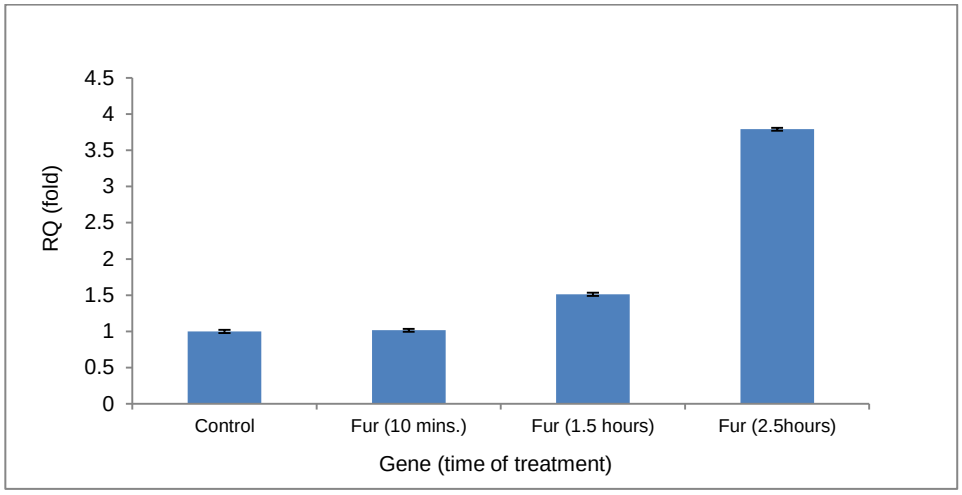

Figure 14: Relative Quantitation *of Fur* gene expression of *Salmonella* Typhimurium DT104 during times of immersion into a treatment of lime and acetic acid combination at 30±1°C(n=3)

Relative Quantitations (RQs) of *Fur* gene expression of *Salmonella* Typhimurium DT104 during 10 minutes, 1.5 and 2.5 hours were 1.01, 1.51 and 3.79 fold respectively at 30±1°C. In this study, *Mig5* gene was increased significantly after growing the *Salmonella* within the acidic environment by 1.26, 1.87 and 5.8 fold after 10 minutes, 1.5 and 2.5 hours respectively at 30±1°C (Figure 15).

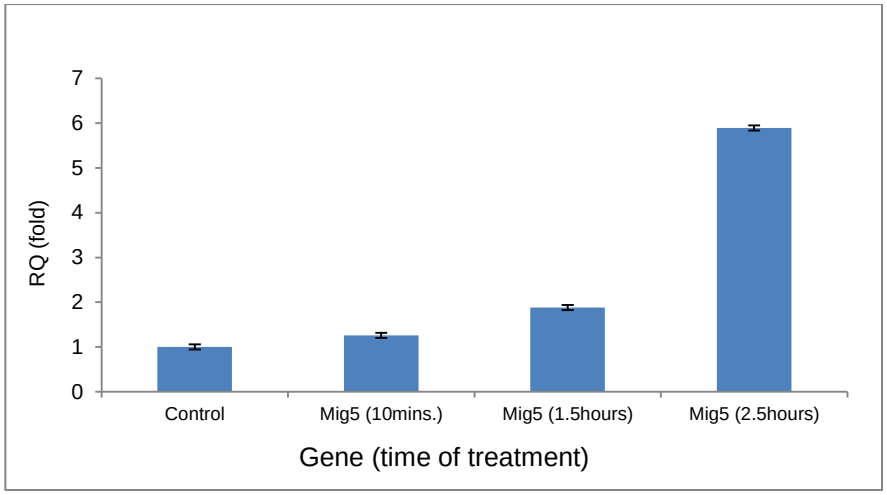

Figure 15: Relative Quantitation of *Mig5* gene expression of *Salmonella* Typhimurium DT104 during times of immersion into a treatment of lime and acetic acid combination at 30±1°C (n=3)

After 10 minutes and 1.5 hours of treatment, Relative Quantitations (RQs) of *Fur* expression in *Salmonella* Typhimurium 1344nal^{r} were 1.5 and 2.9 fold respectively at 30±1°C. The inductions of *Mig5* gene after the same time were 2.8 and 7.8 fold respectively at 30±1°C (Figure 16, 17).

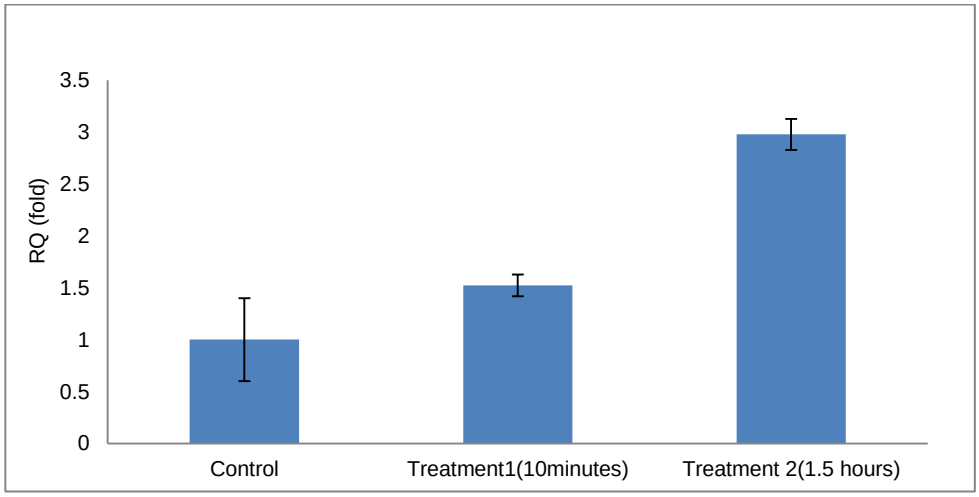

Figure 16: Relative Quantitation of *Fur* expression in *Salmonella* Typhimurium 1344nal^{r} after two times of immersion into a treatment of lime and acetic acid combination at 30±1°C (n=3)

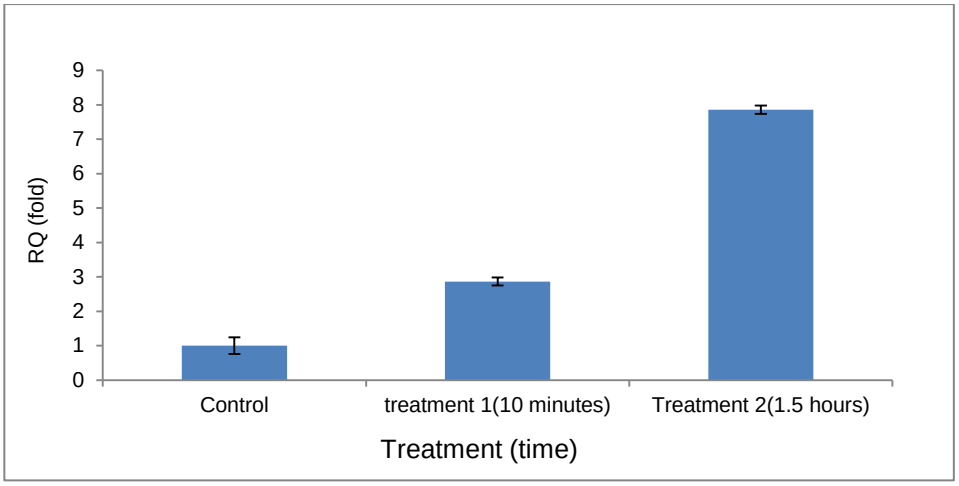

Figure 17: Relative Quantitation of *Mig5* expression for *Salmonella* Typhimurium 1344nal^{r} after two times of immersion into a treatment of lime and acetic acid combination at 30±1°C (n=3)

Increasing the time of immersion made a significant difference regarding the induction of *Fur* gene. The expressions of *Fur* and *Mig5* genes in *Salmonella* Typhimurium 1344nal^{r} were significantly greater than in *Salmonella* Typhimurium DT104. Later, survival cells of *Salmonella* at pH 2.5 in nutrient broth were assessed. As a result, *Salmonella* Typhimurium 1344nal^{r} managed to survive within the acidic environment (pH 2.5) for 2.5 hours, an extra half an hour compared with the other species (*Salmonella* Typhimurium DT104, which survived for 2 hours). In addition, the RQ level of *Mig5* gene was increased significantly in *Salmonella* 1344nal^{r} by 7.8 fold compared with 1.8 fold in *Salmonella* DT104.

In terms of absence and presence of *Salmonella* after the treatment and growing within stomach acidity (pH 2.5), *Salmonella* DT104 was able to survive up to 1.5 hours and 2 hours for *Salmonella* 1344nal^{r}, whilst the control (pure cells of *Salmonella*) was not able to survive after 1 hour of growing in nutrient broth (pH 2.5) at 37±1°C. However, exposure to this treatment increased the ability of *Salmonella* to survive for an extra hour and one and a half hours within the acidic treatment, compared with one hour of survival for control *Salmonella* Typhimurium (DT104 and 1344nal^{r}) respectively.

5.4 Discussion

The results of this chapter accorded with the outcomes of several researchers, who concluded that within a sub- lethal acidic environment (pH= 4.5), to which the sample was exposed, the bacteria might become adapted (Kwon & Ricke, 1998; Seputiene et al., 2006). In this study, it was confirmed that short-term exposure to a sub- lethal concentration of the acidic food additive induced the transcription of acid tolerance and virulence genes (*Fur and Mig5*) of *Salmonella* Typhimurium.

One of the main adaptive tolerance mechanisms of bacteria is acid tolerance response in a wide range of Gram positive and negative bacteria, as many studies have reported (CDC, 2013a; Palmer, Torgerson & Brown, 2011). *Mig5* gene is reported to be expressed by *Salmonella* when ingested by macrophages and to be responsible for making *Salmonella* more resistant within harsh environments (Valdivia & Falkow, 1997).

The *Fur* gene has been reported to be associated with acid tolerance of *Salmonella* Typhimurium DT104, as well as its virulence within stressful environments (Garcia-del Portillo, Foster & Finlay, 1993; Tsolis et al., 1995; Weir *et al.*, 2008). The *Fur* gene is an essential regulator in the bacterial cell; it is controlled by intracellular iron levels that help the bacteria to survive within stressful environments (general stressful, iron limitation and acidic environments) (Weir et al., 2008). In other words, the *Fur* gene is a global transcriptional regulator which is able to regulate the expression of many operons by using ferrous iron as a repressor, as has also been identified in many other bacterial species (Smith, Ameri & Gadgil, 2008).

In addition, the *Fur* gene is also involved in aerobic respiration, chemotaxis, synthesis of amino acids and DNA precursors, sugar metabolism, protecting the bacterial cell from oxidative damage, and protecting the genes encoding bacterial toxins (Maiyo et al., 2010; Smith, Ameri & Gadgil, 2008; Suggs, 2002). *Mig5* and *Fur* genes were up-regulated mostly in all times, except the gene *Fur* after 10 minutes treatment (Figure 20). It appears that 10 minutes of immersion was not sufficient to up-regulate this gene at 30±1°C.

According to (Chiu, Su & Chu, 2004; Weir *et al.*, 2008), *Mig5* gene is associated with virulence of *Salmonella* Typhimurium DT104, as is confirmed in this study. Furthermore, the positive correlation between acid tolerance and virulence genes of *Salmonella* Typhimurium was clearly indicated, as many studies have stated (Requena, 2012; Tsolis *et al.*, 1995; Weir *et al.*, 2008). Induction of acid tolerance and virulence genes of *Salmonella* Typhimurium due to the use of food additives is a concern for food experts and food industries. Several factors were reported to be responsible for making *Salmonella* survived such as cell-cell interactions, temperature and predation (García et al., 2010). Current food preservation methods may not be entirely sufficient to prevent *Salmonella* transmission and the elevation of the adaptive ability of *Salmonella* Typhimurium to grow normally in the intestine of the host (Requena, 2012). Treatment with organic acid and an aqueous lime-peel extract combination was applied against *Salmonella* Typhimurium 1344nal^{r}, which is considered as an antibiotic resistant and survives when exposed to nalidixic acid. The expressions of *Fur* and *Mig5* genes of *Salmonella* Typhimurium 1344nal^{r} were significantly greater than of *Salmonella* Typhimurium DT104.

Therefore, *Salmonella* Typhimurium 1344nal^{r} managed to survive within the acidic environment (pH 2.5) for 2.5 hours, half an hour more than the other species (*Salmonella* Typhimurium DT104 which survived for 2 hours). This might be due to the level of *Fur* expression, which was nearly doubled compared with *Salmonella* DT104 at 30±1°C. Therefore, such a treatment should use with precautious as it might convert *Salmonella* hard to kill inside the human body.

The RQ level of *Mig5* induction was increased significantly in *Salmonella* 1344nal^{r} and this may be the reason for *Salmonella* 1344nal^{r} surviving up to 2.5 hours. According to Hall & Foster (1996a), the *Fur* gene also has an impact on acid tolerance, especially in *Salmonella* Typhimurium. As a result, *Salmonella* is able to adapt and survive within the intestinal tract of the host. There was a positive correlation between the increased time of exposure to acid and further stimulation of the genes, as Jaykus et al. (2009) and Hall & Foster (1996b) have also demonstrated.

5.5 Conclusion

Despite their effective antibacterial activity, the treatment of organic acids and an aqueous lime-peel extract combination increased the ability of salmonellae to survive and increased the induction of both *Fur* and *Mig5* genes, which were enhanced significantly by increasing the time of immersion. However, treated salmonellae were not able to survive within the stomach environment (pH 2.5) and that might be due to their antibacterial activity and their ability to disturb the bacterial cell structure. To reduce the ability of *Salmonella* to survive within an acidic environment, combining another type of herb is suggested for further studies in the future. This study has demonstrated that both *Fur* and *Mig5* genes have a role in the acid tolerance response of *Salmonella*. In addition, there is an obvious relationship between the virulence and acid tolerance response of *Salmonella* within the acidic environment at 30±1°C.

Chapter 6: General discussion

General discussion

Iraq as one of the Middle East countries is still struggles with food borne pathogen incidents in meat. In most local slaughterhouses, washing chicken carcasses after slaughter with tap water in one big bowl is the start of contamination of all chicken carcasses. One contaminated chicken maybe the reason for infecting the whole contents of a washing bowl. During the transfer process (from the storage to markets), the temperature increases due to poor temperature control which allows microorganisms to multiply in chicken meat. Hence, the consumer gets the product with a high dose of microorganisms, leading to food borne disease, particularly if the chicken meat was not completely cooked (like a barbecued chicken meat) and that affects the public health and economy.

Marination of organic acids (acetic, citric and lactic acids) is a common preservative method (Mead, 2004). Lactic acid was combined with organic acids (propionic, citric and acetic acids) for several reasons such as its flavour, the ability to reduce the bacterial population and its popularity to use in decontamination of the meat (Sun, 2012).

In this study, combining organic acids might disrupt the outer membrane of Gram negative bacteria and acting synergistically (Alakomi et al., 2000). The synergistic effect of organic acids (CA, PR and AC with LA) against *Salmonella* Typhimurium DT104 was noticed when either citric acid or acetic acid was combined with lactic acid. Combining acetic acid with lactic acid was able to increase the antibacterial activity for each separately against *E.coli* by increasing the lag phase time up to 20 hours and ΔOD was 76.7 %.

When organic acids (the concentrations below the MIC) were combined, the combination of LA (5.5) & AC (2) was significantly strongest then LA (5.5) & PR (2) and LA (5.5) & CA (2) against *E.coli* K12 (Table 18). The combination of LA (6.5) & AC (3) was significantly strongest against *Salmonella* Typhimurium DT104. The ΔOD of the following combinations LA (6.5) & CA (3), LA (6.5) & PR (3) and LA (6.5) & AC (3) against *Salmonella* Typhimurium DT104 were 52.5%, 60.6% and 76.3% respectively.

No *Salmonella* was detected in this study on chicken breast meat after 5 hours of immersion into a treatment of organic acids combination but with an obvious effect on the sensory properties of chicken meat (colour and acidic smell) as many researchers have previously reported (Theron & Lues, 2010; Xiong, Ho & Shahidi, 1999). When the treatment of organic acid combinations was applied on the chicken meat, immersion treatment (10 minutes) of lactic acid combined with acetic acid (208 mmol/l - 96 mmol/l) against *Salmonella* on chicken meat showed a negligible antibacterial effect accompanied with a negative effect on sensory attributes of the chicken meat. Using a high concentration of organic acids was able to inhibit the microbial loads on meat but not without affecting the sensory properties as reported by Theron and Lues (2010). Therefore, the treatment of both organic acid and herbal water extract (lime-peel water extract) was suggested on chicken meat (as an alternative of using organic acids alone or in combination) to increase the antibacterial activity and reduce the effect on the sensory properties of chicken meat as some researchers have suggested (Leistner & Gould, 2002; Nazer *et al.*, 2005).

A combination of an aqueous lime-peel extract and acetic acid (41.6 w/v % – 1.12 mmol/l) including 1% NaCl on chicken meat was used to inhibit the growth of *Salmonella*.

In order to find out whether this treatment has affected the sensory properties of chicken meat or not, a sensory evaluation experiment of cooked chicken meat was applied, evaluating the product after the treatment measured consumer preference of chicken meat. As a result, significant differences were noticed in aroma, flavour, and texture and most of treatments were scored higher (liking score) than the control. In terms of the effect of lime-peel extract and acetic acid combination on the sensory of chicken meat, ten minutes treatment was not able to reduce the bacterial population but when the concentration of combination doubled, a significant reduction was demonstrated. In other words, increasing the number of hydrogen ions will increase their pressure on the bacterial growth as reported by Sumbali (2009). In addition, using herbal extract is considered an effective way to inhibit the bacteria on meat due to their indigenous compounds like phytochemicals and phenols (Patra, 2012). *Citrus aurantifolia* is still in use in different life aspects such as anti- inflammation, antibacterial, antifungal and as a flavour enhancer in food (Aibinu *et al.*, 2007; Chutia et al., 2009; Fisher & Phillips, 2008b; Reviewes, 2011). Therefore, citrus fruits are selected, especially those dried on the sun. Dried citrus fruit has concentrated contents after the evaporation of the water through the drying out. Combining organic acids and herbal or spice extracts has increased the antibacterial activity level of each separately asJuneja, Dwivedi and Yan (2012) have investigated.

Moreover, when organic acids and lime-peel extract were combined in concentration below the minimum inhibitory concentration, a synergistic effect was observed against bacteria and positive effect on the sensory properties of meat and that agreed with Kim (2013), who reported that combining lactic acid with acetic acid has worked together against bacteria synergistically more than each acid separately.

In addition, lime-peel extract was more effective against foodborne pathogens regarding two main elements (Bhat, Alias & Paliyath, 2012) as this study confirmed, the indigenous level of citric acid (230 mM) and phenolic compounds (2.4 %). Jensen et al., 2003 has illustrated that acetic acid has an additional toxic effect on the bacterial cells compared with propionic acid, and that might be the reason for making the combination of acetic acid and lime more effective than the propionic acid and lime-peel extract combination. Combining the high level of acidity and phenolic compounds they have worked together synergistically to achieve the high reduction of *Salmonella* as illustrated when the same pH level was applied. Therefore, immersion chicken breast meat into a treatment of an aqueous lime-peel extract and acetic acid (41.6 % –1.12%) was able to inhibit almost 9 $\log_{10}$ CFU g^{-1} after 9 hours at 30±1°C. In terms of sensory attributes of chicken meat, overall acceptance score-T2 (2 hours immersion treatment) was the more palatable between the treatments significantly as two hours of immersion was sufficient to give the meat a moderate level of flavour and acidity. Despite that, T2 was not able to reduce the number of *Salmonella* to the detection limit. Although in theory, T2 was not effective to inhibit the *Salmonella* but in reality the dose of *Salmonella* was high and not expected on chicken meat.

Most participants selected T2 (2 hours immersion) as the most acceptable treatment; this might be because this type of additive (acidic food) is not usually in our daily food menu. However, all treatments were acceptable to be used in food without affecting the sensory evaluation badly. Hence, organic acids with an aqueous lime-peel extract treatment was able to minimize the effect of organic acids on the organoleptic properties of chicken meat, inhibit the high dose of *Salmonella* after 9 hours of immersion into the treatment and adding citrusy flavour makes chicken meat more

palatable. In spite of the recommended temperature for marinating meat is fridge temperature (Calvert & DeVere, 2010) but to avoid the use of electricity, most housewives tend to use room temperature (In Iraq, the temperature can reach 30±1°C in a room).

On the other hand, contaminated chicken meat with *Campylobacter* and multi-resistant *Salmonella* Typhimurium is still a threat to public health worldwide, and elevates the importance of finding more information about the existence and survival of foodborne pathogens associated with chicken meat particularly in acidic environments. In order to find if exposure of *Salmonella* Typhimurium to a sub lethal acidic environment (lime-peel extract and acetic acid combination- pH 4.69) for maximum period of 2.5 hours of immersion has induced or not the acid tolerance and virulence genes *Mig5* and *Fur,* a molecular experiment was applied.

As a result, there was a positive correlation between the increased time of exposure to acid and further stimulation of the genes, as both Jaykus *et al.* (2009) and Hall & Foster (1996b) have demonstrated. Add to this, applying this treatment was able to induce these genes significantly. However, treated cells of *Salmonella* Typhimurium 1344nal^{r} and DT104 has showed remarkable ability to survive within the acidic environment pH 2.5 (stomach acidity) for 2.5 hours and 2 hours respectively as compared to an hour to the control. It seems that *Salmonella* becomes more adaptive after being treated with such treatment. Despite that, the acidity of the stomach was able to inhibit *Salmonella* after almost half the time of gastric emptying (4 to 6 hrs) at 37°C (Gropper & Smith, 2012). Hence, using such a treatment is increasing the like hood of infection with *Salmonella*. Therefore, such a treatment should be used on chicken meat with a precaution, bearing in mind that the infected dose that we used was high.

Moreover, adding another treatment with the recent combination (lime and acetic acid combination) may reduce the total inhibition time of *Salmonella* (9hours immersion) without affecting the sensory evaluation properties of chicken meat like using another herb or spice or even another preservative (combine three types of organic acids with the lime-peel extract). Further studies should be applied to investigate more about the ability of foodborne pathogens to survive within stressful environments by up-regulated other genes using microarray techniques.

7. References

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Chapter 8: APPENDIX

Appendix- A

Sensory evaluation of cooked chicken meat

You will be given 5 samples of chicken meat.

You are asked to taste one sample at a time in the order presented to you, scoring each product for acceptability by ticking the appropriate box for each attribute, following the acceptability scale; *i.e.* from like extremely to dislike extremely. Each sample has a three-figure code. Please ensure your results are placed under the correct code.

Ingredients: (Chicken meat, vinegar, dried limes and salt)
Allergy advice: If you are allergic or dislike the ingredients, you may not participate in this exercise

Consent information:

Please feel free to leave your name (although you are not obliged to do so), and any comments on the space provided or on the reverse of the sheet.

The objectives of this research have been explained to me.
I don't have known allergies to the ingredients of the products
I understand that I am free to withdraw from the research at any stage, and ask for my data to be destroyed if I wish.
I understand that data will be anonymous, unless I expressly state otherwise.
Photos may be taken, however no faces will be shown. Please state on the form if you do not wish to be photographed.
I understand that the Principal Investigator of this work will have attempted, as far as possible, to avoid any risks, and that risk assessment has been carried out
Under these circumstances, I agree to participate in the research.

Thank you for your participation in this panel.

UNIVERSITY OF PLYMOUTH

FACULTY OF SCIENCE AND TECHNOLOGY

RESEARCH INFORMATION SHEET

Name of Principal Investigator

Haider Naji Al-Khanaq

Title of Research

Evaluation of sensory properties of cooked chicken breast meat

Aim of research

Evaluate sensory evaluation properties of marinated and cooked breast chicken meat.

Description of procedure

Participants will be given four samples of chicken breast meat (cooked) samples as well as the control. You will be given a questionnaire to tell us your preference for every sample by scoring them, on flavour, colour and acidity.

Description of risks

Allergy advice http://foodallergy.org
Benefits of proposed research

A novel meat product of acceptable sensory quality and safety is being studied, particularly the use natural antibacterial components (herbal extract)

Right to withdraw

The participant has the ability to withdraw from the panel at any time, without penalty or having to give a reason during the experiment
If you are dissatisfied with the way the research is conducted, please contact the principal investigator in the first instance: telephone number Haider.alkhanaq@plymouth.ac.uk , telephone number +44 07971059773. If you feel the problem has not been resolved please contact the secretary to the Faculty of Science and Technology Human Ethics Committee: Mrs Paula Simson 01752 584503.

Evaluation of sensory properties of cooked chicken meat (assessing form)

Panellist code/initials… Product code: ……....

Please evaluate and indicate your opinion about each attribute by marking (√) a suitable box

Attributes	Liking scale Strongly dislike ← → Strongly like								
	1	2	3	4	5	6	7	8	9
Aroma									
Colour									
Appearance									
Flavour									
Acidity									
Texture									
Overall acceptance									

Additional comments:

Appendix- B

Conferences

1. Marine Biology conference, University of Plymouth 20/12/2010.
2. Plymouth PG conference, Plymouth –UK, PG society 29/06/2011.
3. SFAM conference July 2011 Dublin, Ireland.
4. First Annual Meeting for Ecotoxicology Research and Innovation Centre, University of Plymouth on 4th April 2011
5. SFAM conference on April 2012 in Edinburgh, UK.
6. Postgraduate Society Short Conference, Plymouth –UK, PG society 14/3/2012
7. Plymouth PG conference, Plymouth –UK, PG society 26/06/2012.
8. The 13th International conference on System Biology, 19-23/8/2012 Toronto, Canada.
9. Plymouth PG conference, Plymouth –UK, PG society 21/11/2012.
10. Early Career Researchers in Food Sector conference on 14th November 2012 in Edinburgh, UK.
11. Spoilers in Food 2013, Quimper, France 1st to 3rd July 2013
12. BioMicro World 2013, Madrid, Spain 2nd to 4th October 2013

Courses attended

1. English language summer school, academic writing (April to September 2009), University of Plymouth.
2. Postgraduate Research Skills and Methods in Biology (2010), University of Plymouth.
3. General Teaching Associates (GTA) Course (03/03/2011), University of Plymouth.
4. Postgraduate Certificate in Academic Practice (PGCAP) 2012, University of Plymouth.
5. Training session about the PCR technique (23/11/2011), Exeter.
6. Clinical Trials and Regulatory Affairs Course (10/01/2012), Plymouth University.

7. Application and Principles in Electron Microscope (Bio 5102) (2012), full attendance (Course work report) Plymouth University.
8. Microbial life Biol 2409 (10/10/2010), partial attendance, Plymouth University.
9. Award in Food Safety in Catering (Level 2), 09/11/2011, UK.
10. Laboratory based teaching methods and practice 2012, full attendance, Plymouth University.
11. Practical Molecular workshop- Postgraduate Society, (16–19/07/12), full attendance, Plymouth University.
12. The IEEE Workshop for writing scientific skills (13/06/ 2013), full attendance, Plymouth University.

Taught sessions attended

1. Overview to searching information resources (11/5/2010)
2. Gen Stat training session (25/10/2010)
3. Take to the trees (16/4/2010)
4. SPSS (02/11/2010)
5. Preparing Effective Poster Presentations (10/5/2010)
6. Research Owning and Using (06/5/2010)
7. Excel conditional formatting charts (16/12/2010)
8. Presenting to an Audience (10/11/2010)
9. PowerPoint Creating a presentation (15/11/2010)
10. Developing Professional Writing Skills (16/11/2011)
11. Word structuring your thesis (17/3/2011)
12. SPSS (27/3/2011)
13. PowerPoint 2007 creating a presentation (22/2/2011)
14. Master document (19/04/2012)
15. Developing Professional Writing Skills (28/3/2012).
16. Alpha laboratories pipetting techniques workshop (03/05/2012)
17. Getting the Most from Conferences (26/6/2012)
18. Cryogenic gases Safety Awareness workshop (25/06/2012)
19. Safe handling gases workshop (26/06/2012)
20. EndNote Clinic (18/02/2012)
21. Professional writing skills (28/03/2012)

22. Research employability event, Wembley London (19/04/2012)
23. Preparing to submit on PEARL, including copyright and open access (24/10/2013)

Professional memberships

1. Student full membership in SFAM (Society for Applied Microbiology) in the UK from 2011, 2012, 2013 and 2014
2. Membership in Associate of the British Higher Education Teaching Academy from 2013
3. Student full membership in SCI (Society of chemical industry) in the UK (2011-2012)
4. Student full membership in SGM (Social of general Microbiology) in the UK (2011 and 2012)
5. Student full membership in SAM (Society of applied Microbiology) in the USA (2012)
6. Student full membership in ASM (American Society of Microbiology) in the USA (2012, 2013 and 2014)

Awards

- Travel grant for Early Career Researchers in Food Sector conference on 14th November 2012 in Edinburgh, UK (£150).
- The cost of attending Food Micro 2014 Conference to be held in Nantes, France on 01 – 04 September 2014, covered by Society of applied Microbiology (*SfAM*) in the UK (£300).

Publications:

- Al-Khanaq H, Kuri V. Beal J. (2014). Inhibition of acid resistant *Salmonella* Typhimurium on raw chicken meat using a combination of natural food additives. In: Industrial, medical and environmental applications of microorganisms: current status and trends. Proceedings of the V International Conference on Environmental, Industrial and Applied Microbiology - BioMicroWorld2013 (Madrid, Spain, 2-4 October 2013). [ISBN E-book: 978-90-8686-795-0].

Inhibition of acid resistant *Salmonella* Typhimurium on raw chicken meat using a combination of natural food additives

H. Al-Khanaq, V. Kuri and J. Beal

School of Bio-medical and Biological Sciences, University of Plymouth, United Kingdom

Corresponding author: e-mail Hayder_alkhanaq@yahoo.com, Phone: + 44 7771059773

Keywords: *Salmonella*, food additives and chicken meat

Organic acids at low concentrations may be used as an antimicrobial treatment for meat without adversely affecting the sensory properties. Such treatments may be enhanced by adding a further hurdle in the form of antimicrobial herbal treatments. In this study, chicken meat was treated with combinations of acetic or propionic acids and an aqueous extract of lime- peel (*Citrus aurantifolia*) for different durations (10min, 2h, 5h, 9h and 24h). The combination of lime- peel extract with acetic acid was more effective than with propionic acid. *Salmonella* Typhimurium was reduced from $\log_{10}$ 5.26 to non-detectable levels after 9h treatment with lime- peel extract and acetic acid, whereas an equivalent reduction was only achieved after 24h treatment with lime- peel extract and propionic acid. A sensory evaluation of chicken meat treated with lime- peel extract and acetic acid showed that treating for 2h with lime- peel and acetic acid gave the highest overall acceptance score. This study showed that using a combination of food additives was effective in reducing *Salmonella* on raw chicken meat with no adverse effects on the sensory properties.

1. Introduction:

In the USA and many Middle East countries, several types of organic acids e.g. citric, acetic and propionic acids have been approved as treatments to reduce the amount of viable bacteria on meat [1]. Organic acids have the ability to inhibit foodborne pathogens and are described as bacteriostatic [2]. In the UK and the EU, these treatments are not permitted as bacterial decontaminators in meat. The benefit of using organic acids in reducing the bacterial population has been reported widely [3]. Organic acids are used in the range of 1-3% without affecting the sensory properties of meat products [4], however, this may be insufficient to prevent the growth of acid tolerant organisms such as salmonellae. Herbal extracts may also be used as antimicrobial treatments in food [5-7]. The antibacterial activity of some natural herbal compounds such as phenolic and aromatic compounds affects the structure of phospholipid bilayer of cytoplasmic membrane and increases the permeability of cytoplasmic membrane in bacteria, depresses the intracellular defence barriers and disrupts the bacterial enzyme activity. Thus, combining an herbal treatment with an organic acid may produce an effective antimicrobial treatment. In addition, there is considerable interest in herbal extracts as natural food additives from the public and retail sectors due to their perceived health giving properties.[8]. The aim of this study was to determine the effect of a combination of organic acids and herbal extract against acid tolerant species of *Salmonella* Typhimurium DT104 on chicken meat, and to assess the sensory properties of such treatments.

This is to certify that
Haider Naji Kazim Al-Khanaq

has achieved the status of
Associate Fellow of
The Higher Education Academy

Recognition reference:
PR055768
18/01/2013

Professor Craig Mahoney
Chief Executive
The Higher Education Academy

Professor Sir Robert Burgess
Chairman of the Board of Directors
The Higher Education Academy Board

Chartered Institute of Environmental Health

Level 2 Award in Food Safety in Catering

1 credit

Haider Naji K Al-Khanaq

has successfully completed a programme of training and an assessment which concluded the course

Course Director

Chief Executive Graham Jukes
Chartered Institute of Environmental Health

Examination Date *09 November 2010*

CIEH recommends you refresh your training by *09 November 2013*

Centre number *FF23168*

Certificate number *8119704*

Issue Number 1

Qualification accreditation number – 500/5671/4
Accredited only for England, Wales and Northern Ireland

University of Plymouth

Certificate of Professional Development

Learning and Teaching for General Teaching Associates

A course run by Educational Development:
see www.gtacourse.net for further details

This is to certify that

Haider Alkhanaq

has attended the General Teaching Associates course, which included taught sessions and online activities as detailed below

Sessions Attended

Theories of Learning and Teaching
Presentation Skills
Learning in Groups
Evaluating Teaching
Assessment
Feedback

Online Activities

Dealing with Difficult Situations
Issues in Assessment

Signed: .....

Date of issue: 21st March 2011

Jennie Winter – Educational Developer and GTA Lead

Inhibition of *Salmonella* Typhimurium DT104 in marinated chicken by a combination of organic acids and an aqueous extract of lime

Haider Al-Khanaq, Jane Beal, Victor Kuri
School of Biomedical and Biological Sciences, Plymouth University, Plymouth, UK

Introduction

*Poultry products are described as the main source of food borne diseases such as salmonellosis. In many Middle East countries, organic acids and herbs are approved as treatments to reduce the total amount of viable bacteria on poultry carcasses.To reduce the *Salmonella* load in chicken meat, alternative preservative methods such as herb extracts have potential benefits as they may also add flavour. The use of high concentrations of organic acids (≤3.5 %) negatively affect the organoleptic properties of poultry products.

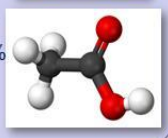
*The aim of this study was to determine the effect of the combination of organic acids and lime extract on the survival of *Salmonella* on chicken meat.

Materials and Methods

*Skinless chicken breasts were inoculated with *Salmonella* Typhimurium DT104 by immersing in broth containing 10^6 ~10^7 CFU ml^{-1} at room temperature.

*Contaminated chicken pieces were then treated by immersing into various concentrations of combinations of organic acids (propionic and acetic acids) with an aqueous extract of dried lime (*Citrus aurantifolia*) at 30°C.

*The combinations were:- lime (Li) : acetic acid (AC) ; 5.2%:0.28% ; 10.4%:0.28%, 20.8%-0.56% and 41.6 % 1.12% while Li : propionic acid (Prop); 5.2%-0.35%, 10.4%-0.35%, 20.8%-0.65% and 41.6%-1.3%.



* The level of citric acid in the aqueous lime extract was 17.7% measured by HPLC.

Results

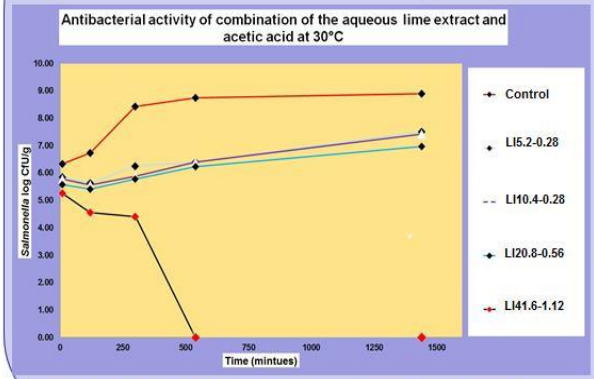


Figure 1: Antibacterial activity of combination of acetic acid and an aqueous lime extract

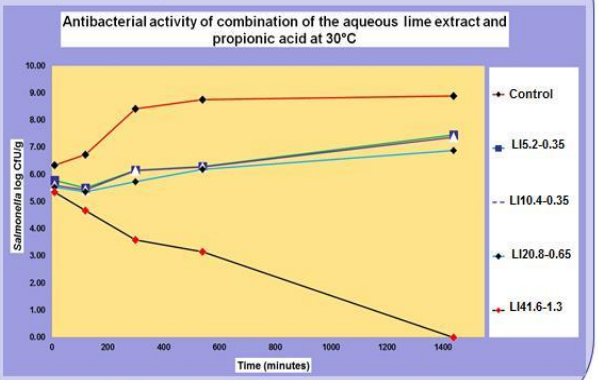


Figure 2: Antibacterial activity of combination of Propionic acid and an aqueous lime extract

Conclusion

*Using a combination of lime extract and organic acids reduced the number of *Salmonella* at concentrations of 41.6%Li:1.12%AC and 41.6%Li:1.3%Prop with none detectable after 9 h and 24 h respectively. Lower concentrations of lime and acids reduced the growth rate of *Salmonella* compared with the control but the organism still grew. Acetic acid was more effective than propionic acid. Such treatments may be used to control the growth of *Salmonella* in chicken meat at ~30° C.

Acknowledgements

The authors gratefully acknowledge:

*The financial support of the Iraqi Government Ministry of Higher Education and Scientific Research.

*Plymouth University, technicians staff.

*Hayder_alkhanaq@yahoo.com
*Haider.alkhanaq@plymouth.ac.uk

References

1- HPA (2011) 'Annual report and Account 2010/2011'. 2-FSA (2011) 'Foodborne disease strategy 2010-2015'. Food Standard Agency.

The effect of acidic food additives on the expression of acid tolerance genes in multi- resistant *Salmonella* Typhimurium DT104

Haider Al-Khanaq, Victor Kuri, Jane Beal
School of Biomedical and Biological Sciences, Plymouth University, Plymouth, UK

Introduction

The occurrence of resistance *Salmonella* such as *S.* Typhimurium DT104 in food products of animal origin considers as a serious threat to human health. Therefore, it is necessary to understand how the environment and processing conditions could affect the bacterial acid tolerance response, in order to estimate the level of the lethality and the efficient way to control (1). Few studies have investigated the adaptation of *Salmonella* into acidic conditions (2). However, the modification in cytoplasmic membrane (3) and gene mutations considered as adaptive bacterial responses to the environmental stress and that called the Acid Tolerance Response (ATR). The invocation of the ATR response may enhance several cellular functions e.g. the ability of *Salmonella* to survive through the acid barrier of the stomach, invade the intestinal epithelium and survive components of the immune system (1).

Therefore , this study aimed to whether exposing *Salmonella* to acids and lime treatment influenced the acid tolerance genes *fur* and *mig5* using quantitative-PCR techniques.

Materials and Methods

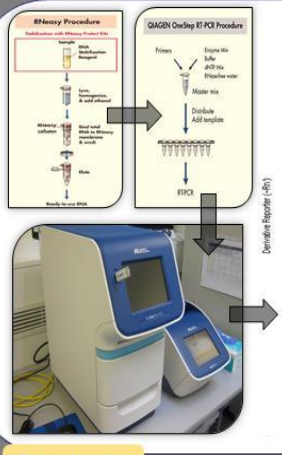
1- *Salmonella* was exposed to a combination of acetic acid with lime 0.28 % - 5.2 % in nutrient broth for 30 minutes at 30°C, control consisted of *Salmonella* in nutrient broth.

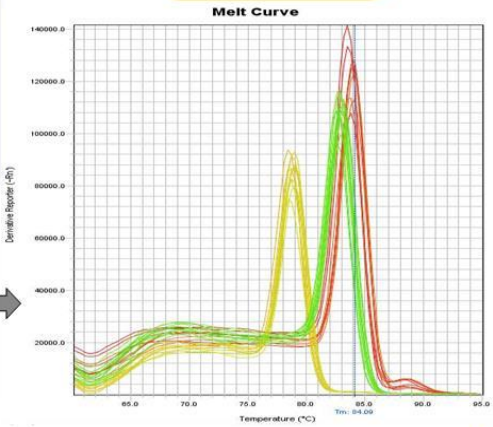
2- *Salmonella* were washed (PBS) and centrifuged twice for 5 minutes.

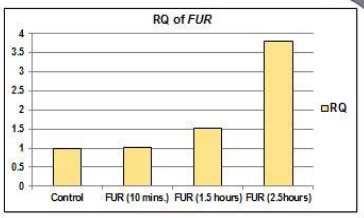
3- Pure cells of *Salmonella* were extracted and converted to RNA using RNeasy protect Bacteria Mini Kit (Qiagen). The resulting RNA was treated with RNase- free DNase and converted to cDNA by using high capacity RNA to cDNA kit (Applied Bio System) then, running by step one plus QPCR machine from Applied Bio System.

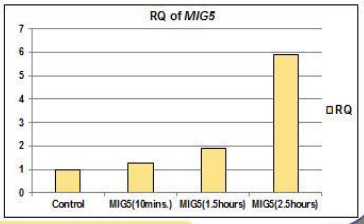
4- Primers for *fur, mig5* and 16S rRNA (housekeeper gene) were used for real time reverse transcription .

Results









Conclusion

* Relative transcripts level (RQs) with the endogenous control of *fur* samples for 10mintues, 1.30 hour and 2.30 hours were 1.014, 1.511 and 3.791 respectively. While, during the same time, RQs of *mig5* were 1.261, 1.878 and 5.888 respectively.

*Exposure *Salmonella* to a specific acidic treatment has up regulated of both *fur* and *mig5*. Despite of that, treated *Salmonella* did not survive after 2.5 hours in pH=2.25 at 37°C while the natural resistance of control did not exceed an hour.

Acknowledgements

The authors gratefully acknowledge:

* The financial support of the Iraqi Government Ministry of Higher Education and Scientific Research.

* Plymouth University, technicians staff.

*Haider.alkhanaq@plymouth.ac.uk

References

1- Effects of Acid Adaptation of *Escherichia coli* O157:H7 on Efficacy of Acetic Acid Spray Washes To Decontaminate Beef Carcass Tissue. *Applied and Environmental Microbiology*, 66,1493-1498.

2- The Acid Tolerance Response of *Salmonella* spp.: An adaptive strategy to survive in stressful environments prevailing in foods and the host. *Food Research International*.

3- Effects of exposure to poultry chemical decontaminants on the membrane fluidity of *Listeria monocytogenes* and *Salmonella* enterica strains. *International Journal of Food Microbiology*, 137, 130-136.