EVALUATION OF ACID RESISTANCE IN FOOD-ASSOCIATED BACTERIA

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DECLARATION OF INDEPENDENT WORK

I, Róan Stephanus Slabbert, hereby declare that the dissertation submitted for the degree MAGISTER TECHNOLOGIAE in ENVIRONMENTAL HEALTH, at the Central University of Technology, Free State, is my own original work and has not previously been submitted to any other institutions by myself or any other person in fulfilment of the requirements for the attainment of any qualification.

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SIGNATURE OF STUDENT DATE

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SUMMARY

Although the application of low pH is common practice in food preservation, the emergence of acid tolerance has been reported world-wide amidst a growing concern that preservation with weak acids, such as organic acids may be influenced as a result of food-borne bacteria becoming acid tolerant or acid resistant. The present study was conducted to assess the acid tolerance of a wide range of bacterial species and consequently the sustainable application of organic acids as food preservatives in particularly acidic foodstuffs. Acid tolerance was determined in 19 bacterial strains predominantly associated with food spoilage and food poisoning. After exposure to hydrochloric acid 16% of the isolates were found to be intrinsically tolerant to low pH and included amongst others the enteric bacteria *Escherichia coli* and *Salmonella* spp. The latter organisms are known causative agents in food spoilage and poisoning, and the results highlight the predicaments related to their ability to survive in acidic foodstuffs as well as the human gastric environment. Bacterial strains were further exposed to increasing concentrations of various acidic foodstuffs in order to determine the development of acid tolerance by gradual decrease in pH, as opposed to exposure to acid shock. After induction, the protein profiles of resulting acid tolerant isolates were compared with those of the original un-induced strains. Exposure to acidic foodstuffs resulted in various survival profiles, where not only pH but also the type of acidulant (foodstuff or inorganic acid) were found to be contributing factors in acid tolerance development. Bacterial protein composition after exposure to acidic foodstuffs showed considerable variation which may be indicative of acid tolerance development whereas the mechanisms involved may be the result of multiple modifications in bacterial composition.

After the induction of acid tolerance, susceptibility of induced strains to various organic acids were determined at various pH values. This was done to investigate whether acid tolerance would influence the inhibitory activity of organic acids as antimicrobial agents in acidic food. Decreased susceptibility was not significantly demonstrated with the exception of only selected isolates, the latter including *E. coli* and *S. typhimurium*. Organic acid activity was found to be much more effective at lower pH values and it would be necessary to elucidate

whether this inhibition is the result of a lower pH or more specifically the activity of the organic acids. The effect of exposure to an acidic environment on phenotypic characteristics of Gram-negative bacteria, and more specifically psychrotrophic organisms was evaluated in order to show the combined effect of organic acids and low temperature preservation. The characteristic yellow pigment of various *Chryseobacterium* species was found to be not as apparent after acid exposure while in some cases the colonies were observed as white. In *Pseudomonas aeruginosa* the characteristic green pigment was much more prominent after acid exposure. These morphological alterations may be important factors that should be considered in identification procedures employed in food safety laboratories. Finally, the influence of acidic exposure via acidic foodstuffs and also organic acids on the protein composition and outer membrane protein structure of various bacterial cells was investigated. No specific relationships with the MICs (Minimum Inhibitory Concentrations) of organic acids after induction with the selected acidic foodstuffs could be established, although various differences were found in protein expression. From the results, it may be suggested that the outer membrane of various pathogenic bacteria is involved in acid tolerance development and this supports the reports on the importance of membrane integrity in the protection against low pH. In conclusion, the study endeavoured to add to the body of knowledge with regard to alternative food preservation regimes utilising organic acids, either solely or in combination with selected extrinsic and intrinsic parameters.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABBREVIATION / ACRONYM	DESRIPTION
AR	Acid resistance
ATCC	American Type Culture Collection
ATR	Acid Tolerance Response
A_w	Water Activity
BHI	Brain Heart Infusion
CDC	Centres for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	Colony-Forming Units
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
Dps	DNA binding protein
Eh	Redox Potential
et al.	*et alia* (and others)
FDA	Food and Drug Administration
GRAS	Generally Recognise As Safe
h	Hours (time)
H^+	Protons
HACCP	Hazard Analysis Critical Control Point
IN	Indiana
Inc	Incorporation
kDa	Kilo Daltons
LMG	Laboratory of Microbiology, Ghent
M	Molar Concentration

MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
min.	Minutes (time)
mM	milli-Molar
MW	Molecular Weight
MWM	Molecular Weight Marker
NZ	New Zealand
OM	Outer Membrane
OmpR	Outer membrane protein regulator
pH	Acidity
pH_i	Cytoplasmic pH
pKa	Ionisation constant
RNA	Ribonucleic acid
[Pty] Ltd.	Proprietary Limited
Rpos	Sigma factor σ^s
rRNA	Ribosomal-RNA
RSA	Republic of South Africa
RTE	Ready-to-eat
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
spp.	Species
SSP	Shelf Stable Meat Products
TEMED	Tetramethylethylenediamine
UK	United Kingdom
USA	United States of America
vol	Volume

WHO	World Health Organisation
wt	Weight

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CHAPTER 1

Literature Review

1.1 INTRODUCTION

An estimated three million children under five years of age die in developing countries each year due to diseases caused by food-borne bacteria, while up to 80 million cases of food-borne illnesses are reported in the USA annually. The World Health Organization (WHO) calls it "one of the most widespread health problems and an important cause of reduced economic productivity". Safe food has therefore, become an important public health issue and governments all over the world are enforcing their efforts to improve on this issue (Daniell, 2000).

Food poisoning and food spoilage are the most important reasons why the food and beverage industry is continuously experiencing unnecessary financial expenditures. The United States Ministry of Agriculture published data that demonstrated *Escherichia coli* 0157:H7 and non-0157:H7 shigatoxic *E. coli* to be accountable for more than 2000 reported cases and costing the United States of America more than $ 900 million in the year 2000 alone (Brul *et al*., 2002). This amount was reported to be twice as much for food-related listeriosis. Table 1 illustrates and displays the general percentage of annual food-related illnesses, hospitalisations and deaths as reported during 1999 (Mead *et al*., 1999).

Table 1.1: Food-borne illness, hospitalisation and deaths in the United States (Mead *et al*., 1999).

Health issues	Illness	Hospitalised	Deaths
Total illness / year	173 000 000	774 000	6800
Food-borne illness / year	76 000 000	325 000	5000
Food-borne illness caused by known pathogens, *Salmonella*, *Listeria*, *Toxoplasma* (related)	14 000 000	60 000	1800, 1500

1.2 FOOD POISONING: A SYNOPSIS

Food may be contaminated due to the presence of bacteria, viruses, environmental toxins, or poisons from some seafood and mushrooms that act as toxins. Food poisoning symptoms include gastrointestinal discomfort, such as nausea, abdominal pain, diarrhoea and/or vomiting. Every year millions of people of all ages suffer from outbreaks of vomiting and diarrhoea associated with food poisoning. (Cerexhe and Asthon, 2000). The Centres for Disease Control and Prevention (CDC) (1999), reported millions of cases of food poisoning outbreaks occurring annually in the United States of America. Many cases are mild and of short duration and as a result are never diagnosed.

The human gastric fluid plays an important role in first-line defence against enteric pathogens present in food by killing or inactivating these organisms before they can enter the intestinal tract (Clarke, 1999). However, food

poisoning is still a common infectious disease. It is, therefore, necessary to determine if these pathogens, ingested together with food, are acid-tolerant or if infection occurs before they reach the stomach. Various foodstuffs, especially processed food, sauces and juices have a low pH and bacteria have been reported to survive in such products. Acid substances, such as organic acids are common food preservatives, which also lower the pH of processed foods, and concern has been expressed that decontamination with organic acids could result in the emergence of acid tolerant food-borne pathogens that may overcome the protective barrier of the gastric stomach (Bjornsdottir *et al*., 2006).

The incessant documented outbreaks of food poisoning cases in Europe and the rest of the world, despite new technologies in food preservation, causes substantial public distress (Ogata *et al*., 2009). In addition to this, there are persistent consumer demands for high-quality foods that are more expedient, more natural and less preserved, for example; foods containing less acid, salt and sugar as well as foods that contain less amounts of added preservatives (Russell and Gould, 1991; Lund and Notermans, 1992; Gould, 1995a; Koutsoumanis *et al*., 2008). The majority of these demands lead to a common decrease of fundamental food preservation and exploitation of new packaging (Koutsoumanis *et al*., 2008; Nychas *et al*., 2008). In addition, numerous food poisoning pathogens present in especially food of animal origin, survive food preservatives as well as other preservation methods. A fair assumption could be, therefore, that achieving a considerable decrease in food poisoning cases, prospectively, could be problematic without a better

perceptive of the physiology of the most significant food-borne pathogens (Knochel and Gould, 1995).

Although most bacteria are killed in the stomach due to the acidic environment, their toxins may remain intact and pass through to the intestine. Typical examples of such toxin producing food-borne bacteria are *Staphylococcus aureus* and *Bacillus cereus* (Clarke, 1999; Caspers *et al*., 2011). However, some bacteria may also survive the stomach acid and multiply in the intestine to cause gastro-intestinal infections. Acid-tolerant bacteria such as *Salmonella* spp., *Yersinia enterocolitica* and *Escherichia coli* are notorious for surviving acidic environments. Consumption of foodstuff such as milk may add to this problem by diluting and even neutralising the stomach acid to provide a more acceptable or even optimal environment. Less acid-tolerant bacteria can then survive the stomach environment and enter the intestine where they attach themselves to the lining of the intestine and cause infection (Clarke, 1999). Some bacteria, such as *E. coli* 0157:H7 and *Shigella* spp. can survive acidic conditions between pH 2 and pH 2.5 for several hours, which would provide more than enough time to exit the stomach and enter the intestine (Clarke, 1999).

The stomach and stomach contents are not the only defence mechanisms of the human body that bacteria have to overcome in order to survive. Other defence mechanisms include intestinal micro-flora and antibodies. The process of survival in the stomach, acid resistance and the defeat of various other defence mechanisms, therefore, are complex and food poisoning remains a common problem world-wide (Clarke, 1999).

Some produce, such as fruit juices, have been excluded from food safety concerns due to the presence of naturally occurring organic acids, which cause a decrease in product pH (Parish *et al*., 1997). However, in recent years outbreaks of salmonellosis have often been connected with unpasteurised juices where causative agents such as *Salmonella anatum* (Krause *et al*., 2002), *S*. *hartford* (Centres for Disease Control and Prevention, 1995), *S*. *meunchen* (Centres for Disease Control and Prevention, 1999) and *S. typhimurium* (Centres for Disease Control and Prevention, 1975) were identified in orange juice and apple cider. The Food and Drug Administration (FDA), therefore, was forced to issue Hazard Analysis Critical Control Point (HACCP) regulations for the production of fruit juices due to recorded outbreaks associated with unpasteurised juice. These regulations require a five-log reduction of relevant microorganisms (FDA, 2001).

1.3 FOOD SPOILAGE

A variety of microorganisms may contaminate food during harvesting, processing and handling operations. However, the type of food as well as environmental factors may influence the presence of specific microorganisms or the type of (bio)chemical reactions that will occur during food spoilage. A diversity of reactions can cause food to become contaminated. Some of these reactions are mainly physical or chemical, while others are due to the action of microorganisms. Inherent food properties such as endogenous

enzymes, substrates, sensitivity for light, oxygen and the development of cross-contamination during harvesting, slaughter, food processing and temperature negligence are the principal factors linked with food spoilage. Primary quality alterations in fresh food may be due to bacterial growth and metabolism ensuing potential pH-changes and the development of toxic compounds, gas, slime and off-odours (Koutsoumanis et al., 2008). Quality changes can also occur in fat-containing food where lipids and pigments become oxidised resulting in objectionable flavours and the formation of compounds with undesirable biological effects or even discolouration (Nychas et al., 2008). Microbial activity due to a variety of microorganisms present in food and beverages could result in food spoilage. Product features, processing techniques and storing conditions influence the kind of microbial flora colonising a meticulous food or beverage (Mossel *et al*., 1995). Although food spoilage is a massive universal economic problem, the mechanisms and interactions causing food spoilage are still poorly understood (Huis in't Veld, 1996).

The presence of substantial damage, such as observable growth of microorganisms, slime production or damage caused by insects, would make the assumption or decision of food being contaminated relatively apparent. However, the mechanisms of spoilage due to biochemical or microbial activities that cause changes in consistency or the development of off-flavours is often complex and difficult to identify and sometimes depend on the subjective judgement of the consumer (Nychas et al., 2008; Mossel *et al*., 1995).

Over the years, considerable attention has been given to the microbiology of food spoilage and the identification and portrayal of microflora developing on different types of foods during storage (Mossel *et al*., 1995). Interaction or grouping of microbial and (bio)chemical factors renders food spoilage to be multifaceted. However, the main focus areas are, therefore, the relationship between microbial constitution and the existence of microbial metabolites, associated with the assessment and the potential prediction of microbial spoilage (Borch and Agerhem, 1992; Drosinos and Board, 1994).

Propagation of microorganisms in foodstuffs is often affected by intrinsic, extrinsic and implicit parameters as well as modes of processing and preservation (Nychas et al., 2008). It is important to understand that any of these parameters could influence the effects of the others and that the overall effect due to a grouping of parameters is much higher than the alleged outcome of each of the individual parameters. Structural, chemical and physical characteristics of the foodstuffs themselves are all forming part of the intrinsic parameters, which comprise water activity, acidity, redox potential, natural antimicrobial substances and obtainable nutrients. On the other hand, extrinsic parameters are factors existing in the environment where food is stored and include temperature, humidity and atmospheric composition.

Implicit parameters are mutual influences, which may be synergistic or antagonistic, resulting from the influence of the parameters mentioned above and as such are the cause of the emergence of a microorganism with either a synergistic or antagonistic influence on other microorganisms' activities

present in the food (Mossel *et al*., 1995). For example, the creation of, or accessibility to, necessary nutrients due to the growth of a specific group of microorganisms could lead to the growth of other organisms which otherwise would be incapable to grow. Similarly, alterations in pH, redox potential and water activity may enable groups of organisms less tolerant to these inhibitory factors to develop and cause secondary spoilage. However, competition for essential nutrients, alterations in pH levels or redox potential or the production of antimicrobial substance, such as bacteriocins, may negatively affect the growth or survival of other groups of organisms. The latter process is known as an antagonistic process (Stiles and Hastings 1991; Kim, 1993; Abee *et al*., 1996). Processing and preservation are also known to alter the characteristics of food produce due to physical or chemical treatments. These changes may then influence the type of microbiota associated with the product.

Homeostasis of microorganisms is another important phenomenon, which requires attention in the food industry especially in food preservation (Gould, 1988). When a microorganism experiences a disturbance in its homeostasis, such as a disturbance caused in the internal equilibrium by preservative factors, the microorganism can either be unable to grow or multiply and remain in the lag-phase, or it can die before homeostasis is fully restored. For example, if bacterial cells find themselves in an acidic environment they will actively expel protons (H^+) against the pressure caused by a passive proton influx. Other important homeostatic mechanisms are also participating in the protection of the bacterial cell. One of these mechanisms regulates the

internal osmotic pressure of cells by maintaining a positive turgor. This is done by maintaining the osmolarity of the cell's cytoplasm higher than that of the surrounding environment by osmoprotective compounds such as praline and betaine (Gould, 1988; Leistner and Gorris, 1995).

Current knowledge on the response mechanisms of microorganisms to ever changing environments is derived from results from laboratory experiments, which focus on pure cultures. However, bacterial cells possess a range of mechanisms, which enable the cells to rapidly adapt to a particular environment. This adaptation to various antagonistic surroundings enables bacterial cells to colonise and multiply on a variety of substrates (Huis in't Veld, 1996). The finding that the survival of microorganisms is not restricted to specific temperature, pH and water activity ranges, but that microorganisms can adapt to endure and proliferate when exposed to values outside these ranges, is considered to be one of the most successful breakthroughs of research over the last few years (Huis in't Veld, 1996).

1.3.1 Bacteria in food spoilage

Foodstuffs that are high in protein concentration such as meat, fish, poultry, shellfish, milk and some dairy products are most likely to become spoiled. These attributes permit growth of a variety of microorganisms and include high levels of nutrition, pH properties (neutral or slightly acidic) and also high moisture content. Microbial spoilage of these types of food has been reported to follow similar patterns (Zhang et al., 2009).

The food industry, and indirectly the consumer, annually loses millions because of microbial food spoilage. Not only is there an increase in expenditure by the food industry, but this also implicates vast losses in a precious resource (Roller, 1999). Food losses due to spoilage often originate on the farm and persist throughout handling, storing, and vending until used in the home and in food preparation – a process often referred to as “farm-to-fork”. By making use of distinguished technologies such as gamma irradiation, it is theoretically achievable to produce food that is totally free of contamination. However, such methods are contradicting the existing consumer requirements that food should be less preserved and processed. Consequently, over and above the liberation and production of safe and “fresh” food, the challenge remains to produce first-class products that are trouble-free to prepare, easily accessible and less preserved with synthesized food preservatives (Gálvez et al,. 2007).

1.3.2 Modes of action of food preservatives and microbial physiology

The majority of conventional processes for food preservation has been designed and used without a good perception of the mechanism of activity of the preservative (Roller, 1999). Notwithstanding the shift from using high concentrations of single antimicrobial compounds to using more than one preservative simultaneously at decreased concentrations, the need exists to reconsider the essentials (Gálvez et al., 2007). It is reasonable to assume that with only a comprehensive understanding of the physiology of microorganisms present in food, it is possible to develop and introduce

plausible preservation systems in successful food preservation (Russell and Gould, 1992; Knochel and Gould, 1995).

1.3.3 Survival and resistance to food-associated stresses

Acidity, low water activity, modified atmospheres and high or low temperatures are some of the foremost stresses that food-borne microorganisms are exposed to in food systems. Although spoilage and pathogenic organisms may be exposed to the same types of stresses, there is a tendency that spoilage organisms have the ability to be more adaptable to unfavourable environmental conditions. This attribute often provides these organisms with the ability to develop tolerance to sanitising agents, cleaning agents and also to antimicrobial food preservatives (Roller, 1999).

Regulatory constraints and expenses involved with testing that are currently mandatory for all novel food additives may render the introduction of new food antimicrobials slow, notwithstanding the technological applications of genetic engineering. Development of more refined preservation methods/procedures and novel produce would therefore, require detailed studies on stress responses of microorganisms and evaluation of existing antimicrobials and preservation procedures.

1.4 FOOD PRESERVATION

1.4.1 Basic aspects

Food preservation entails the exposure of microorganisms to an unfavourable environment by restricting the organism's growth or by inactivating or killing the organism. Survival of organisms being targeted is dependent on their viable reactions to the unfavourable surroundings. More studies and investigations are required regarding these reactions, although progress has been made, taking into account the type of stress reaction, metabolic depletion and homeostasis. The establishment of a new concept of "multitarget" preservation for a moderate, yet more successful preservation has also contributed to the progress made towards hurdle technology (Leistner, 1995a, b).

Heat treatments and addition of food preservatives are the most popular preservation methods used by the food industry. However, increasing resistance of pathogens to preservation and the decrease in the concentration of antimicrobial substances currently allowed, create concern and also increase the challenge to produce food that is safe and of high standard (Brul and Coote, 1999; Piper, *et al*., 2001). During food preservation, the key importance remains the limitation and circumvention of opportunities for the development of antimicrobial resistance.

Factors determining and influencing successful preservation of food, referred to as hurdles, are taken into consideration during preservation to provide microbial free/stable food, while preserving dietary class. These are applied to all foods, including conventional foods with intrinsic hurdles as well as new produce for which hurdles are skilfully developed and purposefully used (Leistner, 1995a).

1.4.2 Hurdle technology

Hurdle technology has been formulated and developed to collectively and intentionally introduce different hurdles to certain food groups to enhance their microbial status, sensory, dietary as well as trade and industry attributes (Gálvez *et al*., 2007). Hurdle technology, therefore, is used to enhance the total standard of food by the combination of different hurdles. In recent years much has been done to obtain better insight into the effects of hurdle technology and to expand their uses (Leistner and Gorris, 1994).

In developed countries, hurdle technology is mainly applied to food groups, which are moderately heated or fermented. These include food that requires less preservation, such as ready-to-eat food (RTE) (Leistner, 2000a). Hurdle technology is also applied in supplying food with less risk of becoming spoiled, healthier food (for example food with less fat and reduced salt content) (Leistner, 1997), as well as food that requires less packaging (Leistner, 2000b). In food groups that require refrigeration, low temperatures serve as the main and the sole hurdle. If these food groups are subjected to higher temperatures during product circulation, the hurdle can collapse and put the

product at risk of becoming spoiled and chances of food poisoning become more evident. As a result, additional hurdles for food groups requiring refrigeration should be integrated into the hurdle technology approach. These are called 'invisible technology' (Leistner, 1999).

1.4.3 Hurdles in foods

Temperature (high or low), acidity (pH), redox potential (Eh), water activity (a_w), preservatives as well as competitive microorganisms, are all significant hurdles applied in the food preservation process. More than 60 possible hurdles that increase the quality and stability of food have already been reported and the list of potential hurdles is still growing (Leistner, 1999). Maillard reaction products are examples of some hurdles that play a role in both the standard and safety of food as they are known to enhance the quality and flavour of food, while simultaneously acting as preservatives (Leistner, 2000b).

Depending on its potency a hurdle could either have a constructive or destructive consequence on a product. For example, the cooling down of products to an unfavourable low temperature may be harmful to some food products, especially those of plant origin. This damaging effect is called "chilling injury". However, cooling down of the same product to a modest temperature could be advantageous to the produce shelf life. Another example may be found in fermented sausages where the pH should be adequately low to protect the sausage against bacterial contaminants, but not so low that it will spoil the taste of the product. Modification of a specific

hurdle in a food should be an option if a hurdle is harmful to the product. The capability to change a hurdle's intensity implies that all hurdles in a food system can be maintained in a most favourable scope of reference to deliver food of the highest safety and class (Leistner, 1994a).

Depending on a specific food product, there is a specific set of hurdles, which varies in standard and strength. These hurdles are essential in controlling the 'normal' organisms present in the food from the beginning of preservation throughout storage until consumed by the consumer. This is achieved if the normal microorganisms are prevented from overcoming these hurdles. Leistner (1978) first introduced the hurdle effect, which is considered to be of great significance for the successful preservation of food with high and moderate moisture content (Leistner and Rödel, 1976; Leistner *et al*., 1981).

The use of hurdle technology in developing countries is mainly applied for foodstuffs that require no refrigeration and where the application is of supreme significance for food to maintain their safety, microbial stability and flavour properties. The use of this technology has made remarkable progress, particularly in Latin America, concerning new fruit products that are less processed and high in moisture content. However, deliberate use of hurdle technology has enjoyed much attention in the development of meat products in China and for diary products in India (Leistner, 2000b). Developing countries share the global interest to do away with foodstuffs of moderate moisture content, because it sometimes contains too much sugar or salt and presents a less attractive look and consistency than food with high moisture

content. Such aspiration may be attained by the use of hurdle technology (Leistner, 2000c).

1.4.4 The role of homeostasis

Homeostasis within an organism is defined as the inclination of microorganisms to maintain a constant internal environment. Retaining a distinct internal pH is a requirement for and characteristic of all living cells including microorganisms, as well as human cells (Häussinger, 1988). An understanding of homeostasis in higher organisms should be applied to microorganisms responsible for food spoilage or food poisoning. The maintenance of homeostasis in microorganisms is an important occurrence and warrants much consideration during food preservation. If the homeostasis of microorganisms becomes disrupted by the application of hurdle technology (preservation factors) the organism may not be able to grow and multiply and may even die before there was a re-establishment of homeostasis. As a result, successful preservation of food is accomplished when homeostasis in microorganisms in food is disrupted. Food may also interfere with the homeostasis in microorganisms, although limited information is available on this phenomenon (Gould, 1988 and 1995b).

1.4.5 Metabolic exhaustion

Depletion or exhaustion of the metabolism of microorganisms that can lead to “auto sterilisation” is another preservation method that was first recorded when liver sausage (moderately heated) (95°C core temperature) was inoculated with *Clostridium sporogenes* and subjected to different water

activities by the addition of fat and salt. The food was stored at 37°C and some of the results revealed that clostridial spores that survived the heat treatment, had disappeared during storage of the sausage (Leistner and Karan-Djurdjic´, 1970). *Clostridium* and *Bacillus* spores also exhibited this behaviour when shelf stable meat products (SSP) were stored at ambient temperatures (Leistner, 1994b). It could be reasoned that only the bacterial spores that survived the heat treatments were capable of proliferating in these foodstuffs when exposed to less harsh environments (Leistner, 1992). Food that requires no refrigeration particularly showed a reduction in the number of spores during storage.

It may be possible that during exposure to unfavourable environments, microorganisms deplete their energy supply and also their restoring mechanisms in order to attain normal homeostasis, which may result in complete metabolic exhaustion and consequently auto sterilisation of food (Leistner, 1995b). Hurdle-technology and microbial stable foods can therefore, develop into safer food during storage time, due to the phenomenon of auto sterilisation. For example, *Salmonella* cells that have endured the maturation of fermented sausages will rapidly become extinct if food is stored at higher temperatures, while storage in a refrigerator might cause food poisoning (Leistner, 1995a).

1.5 ACID ADAPTATION

The number of food-borne illnesses is escalating annually, regardless of the increase of knowledge gained concerning microorganisms present in food (Hill *et al.*, 1995). Survival of microorganisms is not restricted to certain values of different environmental factors, such as temperature, pH and water activity. Microorganisms are, however, capable of adapting, surviving and proliferating when subjected to values outside the known ranges. This is not only a significant research theme for the food industry but also for the medical profession, given that organic acids are often used as food preservatives and that the human stomach containing acids acting as a defence against pathogens.

Some foodstuffs naturally contain organic acids, but organic acids may also be added intentionally for preservation purposes and can be found a by-product due to the fermentation processes facilitated by microorganisms. The type and characteristics of a particular organic acid and the ultimate pH of the end product will influence the degree of preservation of food. Various microorganisms are unable to grow at pH levels less than 4.5 and will die if exposed to even lower levels due to massive disturbance of the pH homeostasis. The reason why some microorganisms are able to survive exposure to acidic environments is their ability to control their cytoplasmic pH (pH_i). This practice is effectuated by the movement of positive ions (cations) across the bacterial cell membrane. However, when exposed to severe acidic conditions the ability of microorganisms to attain an internal or cytoplasmic pH

close to pH 7 can be evaded to cause cell death. Some bacterial cells that have been subjected to moderate pH levels or moderate acidic environments showed an ability to develop a tolerance to withstand or survive pH levels that, as a rule, would lead to cell death. This occurrence is called the acid tolerance response (ATR) (Hill *et al*., 1995).

Modifications regarding the physiology of microorganisms exhibiting this response are not yet fully comprehended, but it is becoming progressively evident that this response could entail significant importance for food-borne pathogens' survival in acidic foodstuffs. The low pH of both the stomach- and phagosome content is part of the human body's defence against microorganisms. However, the expression of the ATR response could result in increased virulence and increased invasive properties of food-borne pathogens. Much attention has been paid to this phenomenon especially in *Escherichia coli* (Goodson and Rowbury, 1989) *Salmonella* (Foster, 1993), *Listeria monocytogenes* (Kroll and Patchett, 1992) and *Aeromonas hydrophila* (Karem *et al*., 1994).

1.5.1 The physiological basis of pH homeostasis

Before considering the adaptation and tolerance response it is practical to review the physiological ways by which bacterial cells manage to maintain its cytoplasmic pH (pH_i), which entails maintaining the pH homeostasis (Booth, 1985). The majority of food-borne pathogens are classified as neutrophils with optimum pH levels between pH 6 and pH 7. In various foodstuffs, the main source of acid stress that food-borne pathogens may be subjected to is

lipid-permeable weak acids. Although the bacterial cell membrane is considered to be impermeable to protons (H^+), the un-dissociated weak acids can cross the membrane effortlessly and once inside the cytoplasm they can dissociate to release protons (H^+). If a difference between pHi and the external pH of 2 units or more (i.e. pHi 7.0, external pH 5.0), the weak acid concentration inside the cell can be more than 100 fold higher than the external acid concentration. To illustrate this, if an external acetate concentration is 1mM and the bacterial cell is preserving its cytoplasmic pH at 7, the internal concentration supposedly would be in the range of 100mM. Consequently, 100mM of H^+ would be liberated into the cytoplasm of the bacterial cell (Hill *et al*., 1995).

In reality the liberation of protons to such an extent would exceed the cell's buffering capability and the pH of the cytoplasm would decrease to a level that could inhibit growth or even cause cell death. Substantial or significant fluctuations in the pH_i, due to changes in the external surroundings, are prevented by the low permeability of the membrane to protons. As a result, protons from the surrounding environment are not able to cross the cell membrane to cause a decrease in the cytoplasmic pH. The presence of proteins, cytoplasmic glutamate and polyamines equips the bacterial cell with buffering abilities and is another reason for protection against a major disturbance in the cytoplasmic pH. At the limits of the pH range, the cell's ability to buffer the cytoplasm is functioning optimally. However, conformation is lacking whether bacteria have the ability to modify its cytoplasmic character to amplify the buffering effect. It has been reported that some mutant strains

of *Salmonella typhimurium* that have survived pH exposure, can exchange some of its glutamate pool for citrate and isocitrate (p*K*a 6.4). The optimised buffering of the cytoplasm at neutral conditions is considered to be a significant feature of the mutants (Foster and Hall, 1991).

Resistance is classified as the ability of a microorganism to survive and grow under conditions that would normally kill or inactivate it. If an organism is not susceptible to a specific concentration of an antimicrobial substance applied in a food system or in a medical treatment, it is considered to be resistant. Resistance generally entails increased tolerance developed against antimicrobials as a result of genetic modification and a known biochemical basis. Resistance of microorganisms to antibiotics has been thoroughly studied and is well understood. However, resistance to disinfectants, antiseptics and substances used in food preservation has not been clearly described. The development of microbial resistance, particularly in biofilm forming organisms, has serious repercussions on the environment as well as trade and industry (Hoyle and Costerton, 1991; Breyers, 1993). Attachment mechanisms and problems caused by biofilm forming organisms have been widely investigated, especially in the food and dairy industries (Carpentier and Cerf, 1993; Criado *et al.*, 1994; Zottola, 1994).

Survival in extreme acidic environments, such as the stomach, or moderately acidic environments such as containing organic acids, is an important property of food-borne pathogens (Record *et al*., 1996; Diez-Gonzalez and Russel, 1999; Barua *et al*., 2002). Acid resistance is also essential for enteric bacteria

to colonise in hosts and to form mutual relationships with their hosts (Castanie-Cornet *et al*., 1999; Barua *et al*., 2002). The terms 'acid resistance' and 'acid tolerance' have been used to describe bacterial growth at moderate pH levels and survival after acid shock at low pH levels (Russel, 1991; Benjamin and Datta, 1995; Diez-Gonzalez and Russel, 1999). The lack of specificity for accurately describing these two terms sometimes creates confusion. For example, *Salmonella* is able to grow at lower pH levels than *E. coli* and *Shigella*, but is much more sensitive to acid shock. To eliminate confusion, some science papers use the term 'extreme acid-resistance' to describe the bacterial survival after acid shock (Lin *et al*., 1995; Diez-Gonzalez and Russel, 1999). Other papers classify acid resistance and acid tolerance under the same definition – 'the survival of stationary-phase cells at extremely low pH levels' (Leyer *et al*., 1995).

Various studies have been done on acid tolerance and survival of bacteria, especially on *E. coli,* in food implicated in outbreaks (Leyer *et al*., 1995) and also the ability of organisms to survive acidic conditions in the environment as well as the passage through the stomach to the intestine. A number of acid survival systems have been identified and referred to as the acid tolerance response (ATR), acid resistance (AR) and acid habituation (Goodson and Rowbury, 1989; Foster and Hall, 1990; Small *et al*., 1994; Lin *et al*., 1995).
It is, however, not always possible to determine if there is a difference between the systems described. However, the question arises if it is not just different ways of measuring the same system (Lin *et al*, 1995).

One factor known to be implicated in acid survival is the sigma factor σ^S (RpoS) associated with stationary-phase bacterial cells. This factor is a product of the *rpo*S locus and facilitates the binding of RNA polymerase to DNA. Studies with this sigma factor confirm the difficulty of evaluating acid survival systems measured by different approaches. Although the RpoS sigma factor is normally associated with stationary phase cells, it has been found that this factor can be induced in exponentially growing cells if a stress is imposed (Hengge-Aronis, 1996; Diez-Gonzalez and Russel, 1999). Examples of such stresses are nutrient deficiency and acidic conditions.

Two kinds of acid tolerance responses for stationary-phase cells of *Salmonella* identified are an acid inducible response, which is σ^S-independent and a σ^S-dependent response, which requires no acid induction (Lin *et al*., 1995). The acid inducible response is associated with an increase in the regulator OmpR responsible for the expression of acid-shock proteins. The sigma factor is induced automatically as stress tolerant system when the cells enter stationary-phase. These mechanisms are essential for the organisms to survive in extreme environments (Bearson *et al*., 1996). Other mechanisms that have been proposed to be involved in the development of acid-resistance, are (1) a change in the permeability of the bacterial cell, (2) production of enzymes to repair DNA- damage (RpoS) (Audia *et al*., 2001), or (3) a DNA- binding protein (Dps), reported to be involved in DNA protection or DNA-repair after acid damage (Choi *et al*., 2000).

1.6 BACTERIA INVOLVED IN FOOD POISONING

More than 250 food-borne diseases have been described and infections can be caused by a variety of bacteria, viruses, or fungi. Some organisms also produce harmful toxins or chemicals that may contaminate the food, for example, poisonous mushrooms (Cerexhe and Ashton, 2000). The most common bacteria involved in classic food poisoning cases are *Salmonella*, spp*. Staphylococcus aureus, Campylobacter, Escherichia coli, Shigella* spp.*, Clostridium botulinum* and *Clostridium perfringens.*

1.7 RATIONALE

There is a definite demand in the South African food safety sector for research to determine the current situation with regard to acid-tolerance and acid resistance in food-associated bacteria, particularly the food-borne pathogens. This is warranted by the increasing number of traditional and foreign foodstuffs introduced in the country, because of contemporary consumer demands. A concern has also arisen that the application of organic acids may be implicated in the emergence of acid-tolerance and acid resistance. The aim of this study was to highlight and address specific aspects associated with bacterial exposure to an acidic environment, specifically in relation to food preservation, food spoilage and food poisoning.

1.8 OBJECTIVES

The objectives of the study were:

- To determine the extent of acid tolerance in a wide range of food-associated bacterial pathogens.
- To explore and monitor variations in acid tolerance development of bacterial pathogens when exposed to acidic foodstuffs and hydrochloric acid.
- To investigate concerns about the application of organic acids in acidic foodstuffs and the effectiveness on resulting acid tolerant food-borne bacteria.
- To examine morphological and phenotypic alterations developing in bacterial cells after exposure to reduced pH.
- To determine the influence of acidic foodstuffs and organic acids on total bacterial protein and outer membrane composition.

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Chapter 2

The Prevalence of Acid Tolerance in a Wide Range of Food-Associated Bacteria

2.1 INTRODUCTION

In an ever changing environment, prokaryotes have the well respected and highly valued ability to detect, counter and often adapt to multi-stressful conditions. Cross-protection of stressed food-borne pathogenic bacteria against exposure to otherwise lethal environmental stresses enhances the potential for survival and growth (Foster and Hall, 1990; Leyer and Johnson, 1993; Gill *et al*., 1995; Leyer *et al*., 1995; Ryu and Beuchat, 1998). Survival of some bacteria for extended periods under adverse conditions such as high acidic levels or high temperatures has often been reported (Smith, 1987; Small *et al*., 1994; Lin *et al*., 1996). Extreme acidic environments, one of the stresses encountered by many microorganisms, especially food-borne pathogens, evokes an adaptive stress response that provides the organisms with the necessary capability to endure exposure to such conditions (Lin *et al*., 1996).

An important characteristic of microbial pathogens associated with oral-faecal routes of transmission is not only to survive exposure to extremely acidic environments but also to withstand or combat moderately acidic conditions caused by, for example, the presence of weak acids in food preservation. This characteristic is important for food-borne pathogens to guarantee survival in specific foods containing weak organic acids and also to survive the extreme acidic environment encountered by the organism in the gastrointestinal tract (Eklund, 1983; Kwon and Ricke, 1998). Organic acids are added to food as flavourants but also to prevent the growth of

contaminating organisms. Weak acids are present in a variety of foods due to the fermentation process by the organisms themselves but can also be added as acidulant, because acidulation is frequently used in fermented and acidic foodstuffs (Table 2.1). During food-processing procedures, weak organic acids are also introduced into food as microbial inhibitors to control contaminating pathogens and to achieve well preserved food products (Cutter and Siragusa, 1994; Humphrey *et al*., 1988; Van Netten *et al*., 1994a; Van Netten *et al*., 1994b). However, the ability of organisms to counter or resist these environments may allow pathogenic food-borne organisms to survive acidic foods, animal feeds and preservation processes until the organism is ingested by the consumer (Brackett *et al*., 1994; Conner and Kotrola, 1995; Leyer *et al*., 1995).

Pathogens that successfully survive the weak acidic environments encountered in the foodstuffs must also endure a variety of acid exposures through the gastrointestinal tract after being ingested. The human gastric content has been recognised for a long time as one of the most important first line defences against pathogens, as the main bactericidal barricade is acid dependent (Hewetson, 1904; Giannella *et al*., 1972; Peterson *et al*., 1989). On exiting the stomach and entering into the small intestine, organisms will encounter a less acidic environment (pH 4-6). Although less acidic, the small intestine also provides an environment containing weak acids or fermentation end products resulting from fermentation processes by normal intestinal flora. The presence of weak acids in the small intestine could actually increase acid stress to such levels that are lethal for enteric bacteria. However, pathogens

such as *E. coli* and *Salmonella typhimurium* have been reported to survive the various acidic conditions (Salmond *et al*., 1984) of the gastrointestinal acidity and eventually cause disease (Gilbert and Roberts, 1986).

Table 2.1: List of common food and foodstuffs with pH ≤ 5.

FOODSTUFFS	**Approximate pH**
Apples	3.3 - 3.9
Apricots	3.3 - 4.8
Bananas	4.5 - 5.2
Beer	4.0 - 5.0
Buttermilk	4.4 - 4.8
Cheese	4.8 - 6.4
Chilli sauce	2.8 - 3.7
Cider	2.9 - 3.3
Fruit cocktail	3.6 - 4.0
Grapefruit	3.0 - 3.7
Mustard	3.5 - 6.0
Pickles, sour	3.0 - 3.4
Sauerkraut	3.4 - 3.6
Sherry	3.4
Soft drinks	2.0 - 4.0
Soya sauce	4.4 - 5.4
Tomatoes	4.3 - 4.9
Tomato juice	4.1 - 4.6
Tomato puree	4.3 - 4.5
Vegetable juice	3.9 - 4.3
Wines	2.8 - 3.8

Foodstuffs with pH ≤ 4.5 is regarded as acidic.

With the general conception being that bacteria cannot survive under strong acidic conditions such as the human stomach, the question arises: "Why is food poisoning such a common infectious disease?" Food-borne pathogens cause millions of illnesses and thousands of deaths world-wide every year (Banati, 2003).

There is a growing need in the food safety arena to elucidate current concerns with regard to acid tolerance and acid resistance and its role in survival and growth of food-associated bacteria. It is also becoming imperative to investigate the complex adaptations of these organisms to acidic environments and to help ease "one of the most widespread health problems and an important cause of reduced economic productivity" as referred to by The World Health Organization (WHO) (Daniell, 2000). The aim of this study was, therefore, to investigate the extent of acid tolerance and acid resistance in a wide range of food-associated bacteria by exposure to a synthetic acidic environment.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial isolates

Potential pathogenic and spoilage food-associated bacteria were selected for this study and comprised of various strains of *Bacillus cereus* (1), *Chryseobacterium* spp. (9) *Escherichia coli* (2), *Klebsiella pneumoniae* (1), *Proteus vulgaris* (1), *Pseudomonas aeruginosa* (1), *Salmonella enterica* sv. *Enteritidis* (1), *Salmonella enterica* sv. Typhimurium (1), *Staphylococcus aureus* (1), and *Yersinia enterocolitica* (1). The selection included various species of *Chryseobacterium,* which is known to be phsychrotrophic.

2.2.2 Screening isolates for acid tolerance

The method described by Jordan *et al*. (1999) was adapted in the determination of the prevalence of acid tolerance development. Isolates were cultivated in Mueller-Hinton (MH) broth (Biolab Diagnostics [Pty] Ltd., Auckland, NZ) (pH 7) for 48 h at 35˚C and consequently acid challenged through the reduction of the medium to pH 4.5 with 3M HCl. Viable cell counts were determined prior to acid challenge and at consecutive intervals of 12, 24, 36 and 48 h after pH adjustment. Serial dilutions were performed in 0.1% peptone, 10 µl spread-plated onto MH agar (Biolab Diagnostics [Pty] Ltd.) and incubated for 24 h at 35°C (Jordan *et al*., 1999). All analyses were performed at least in triplicate.

Bacterial culture in broth

3M HCl added to culture
pH lowered to pH5

Every 12 hours
Plate counts

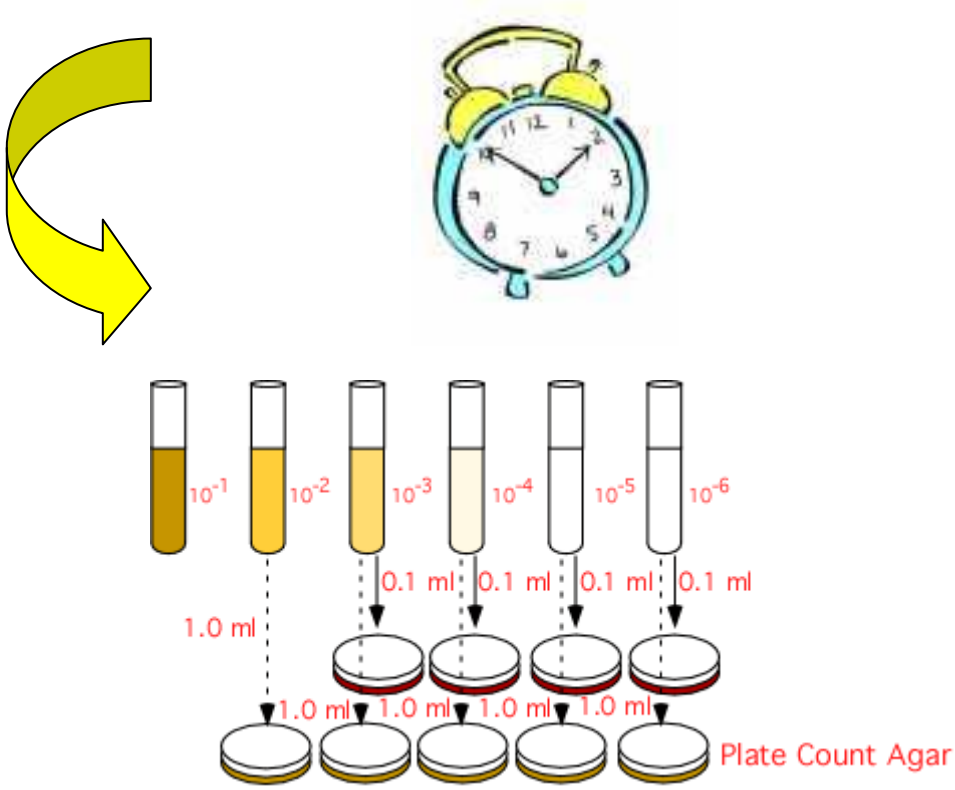


Figure 2.1: Diagrammatic representation of acid challenge of bacterial strains.

2.3 RESULTS AND DISCUSSION

Acquired acid tolerance development was observed in both *S. enterica* sv. Enteritidis and *E. coli* ATCC 25922 after 36h of acid exposure. This is underlined by the increase in total counts after an initial decrease (Figures 2.2 and 2.3). In Figures 2.4 and 2.5 growth is illustrated at each of the time intervals. In *S. enterica* sv. Typhimurium intrinsic acid tolerance was also noted whereas none of the other organisms showed notable acquisition of acid resistance after exposure to pH 4.5 (Figure 2.2). For a summary of the viability of bacterial strains after acid challenge, refer to Table A1 (Appendix).

Organisms that survived after 48 h, although presenting lower viable counts than recorded at 0 h included *E. coli* O111, *Chryseobacterium balustinum, Chryseobacterium indologenes, Klebsiella pneumoniae, Proteus vulgaris,* and *Pseudomonas aeruginosa* (Figures 2.6-2.10). Another group of strains survived for 36 hours of exposure but no growth was detected after 48 hours of acid exposure. These included *Bacillus cereus, Chryseobacterium gleum, Chryseobacterium vrystaatense* and *Yersinia enterocolitica* (Figures 2.11-2.14). *Chryseobacterium joostei* survived for 24 hours after acid exposure (Figure 2.15), while *Chryseobacterium piscium* survived for only 12 hours (Figure 2.16). The remaining four strains did not show any survival after 12 hours acid exposure. These organisms included three of the psychrotrophic bacteria *Chryseobacterium defluvii, Chryseobacterium indoltheticum, Chryseobacterium scophthalmus* and *Staphylococcus aureus* (Figures 2.17-2.20).

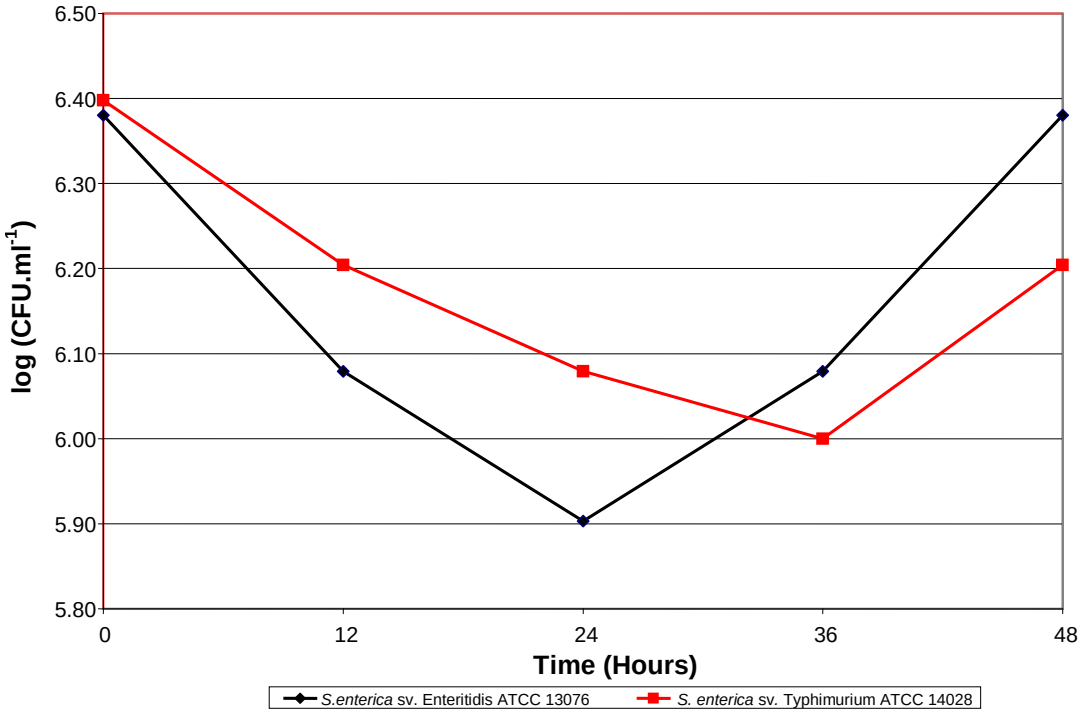


Figure 2.2: Total viable counts for *Salmonella enterica* sv. Enteritidis ATCC 13076 and *Salmonella enterica* sv. Typhimurium ATCC 14028 after acid exposure.

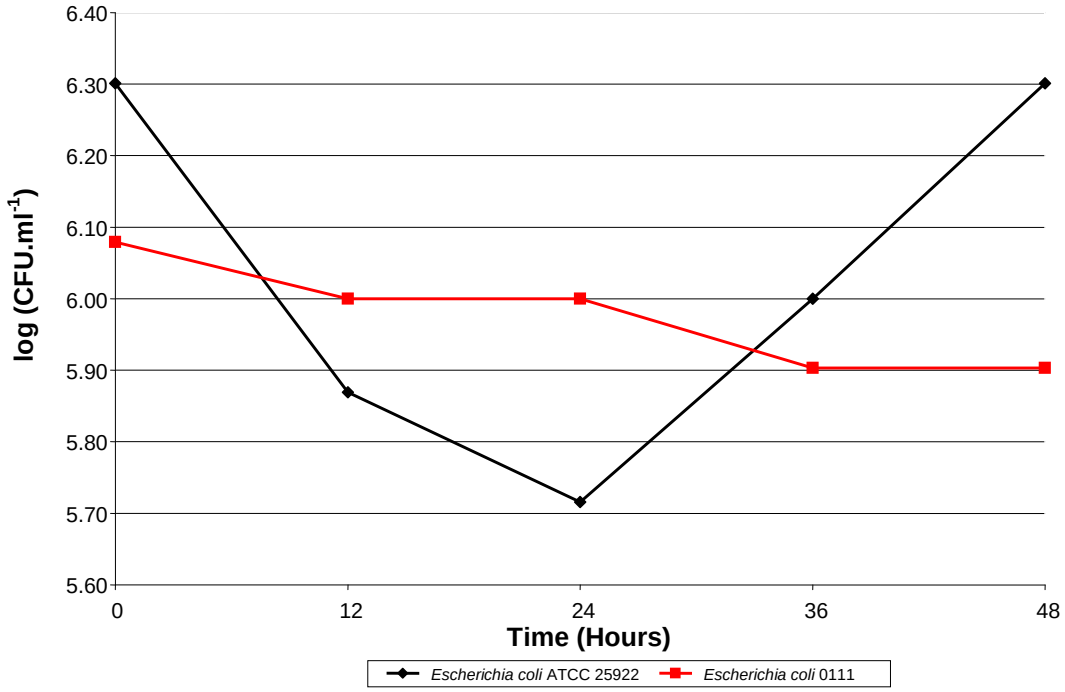
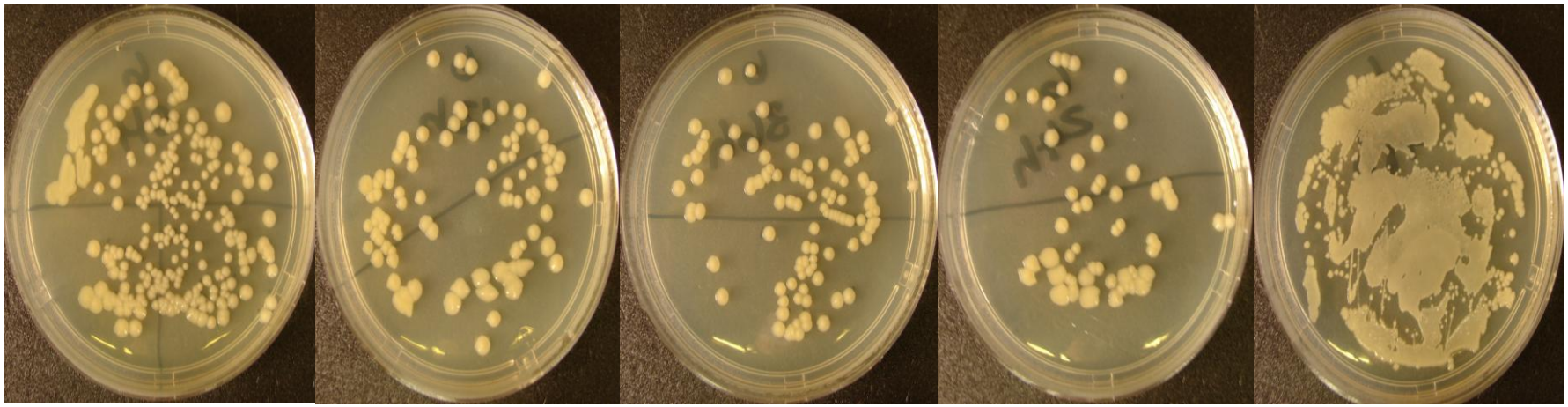
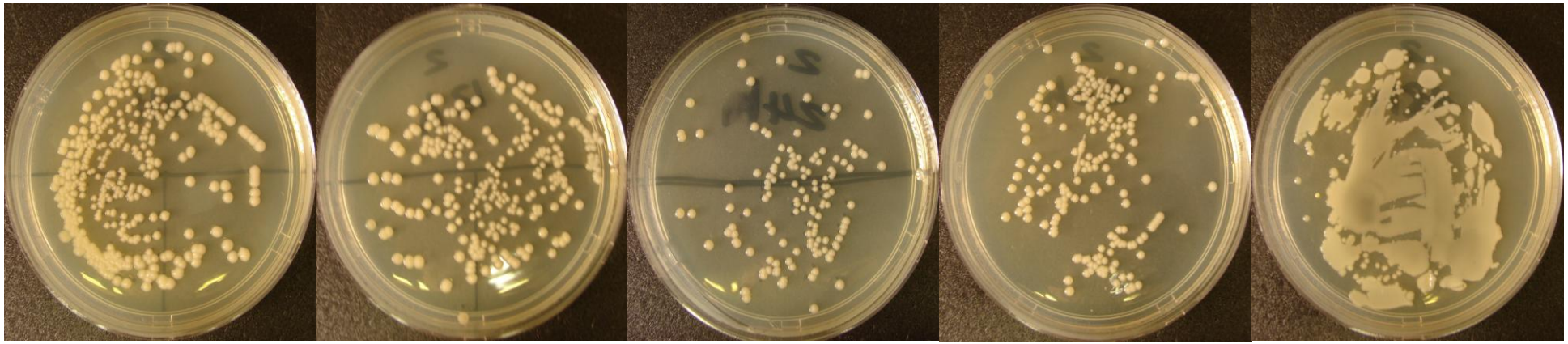


Figure 2.3: Total viable counts for *Escherichia coli* ATCC 25922 and *Escherichia coli* 0111 after acid exposure.



0h **12h** **24h** **36h** **48h**

Figure 2.4: Growth variation of *Escherichia coli* ATCC 25922 after exposure to HCl.



0h **12h** **24h** **36h** **48h**

Figure 2.5: Growth variation of *Salmonella enterica* sv Enteritidis ATCC 13076 after exposure to HCl.

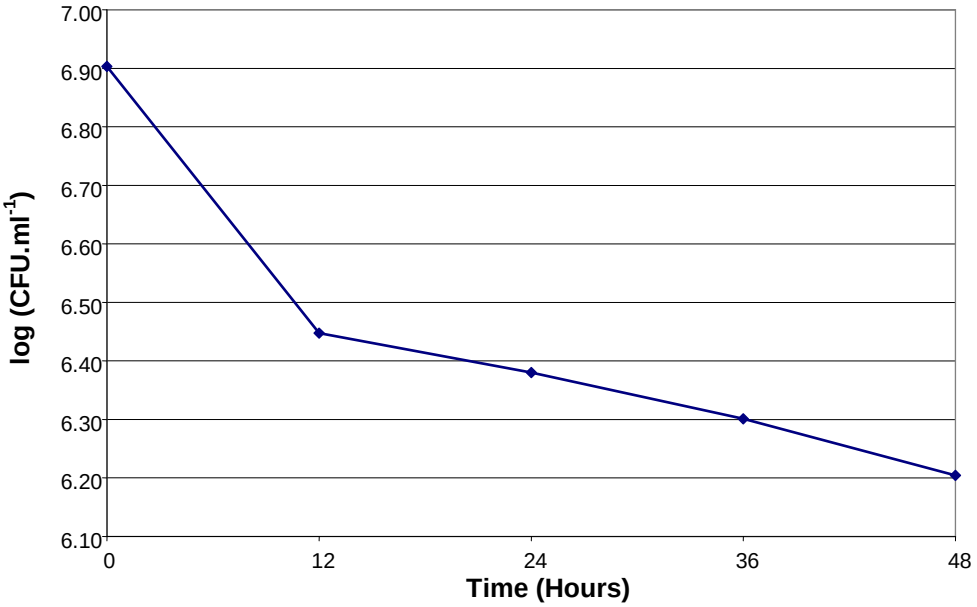


Figure 2.6: Total viable counts for *Chryseobacterium balustinum* LMG 8329 after acid exposure.

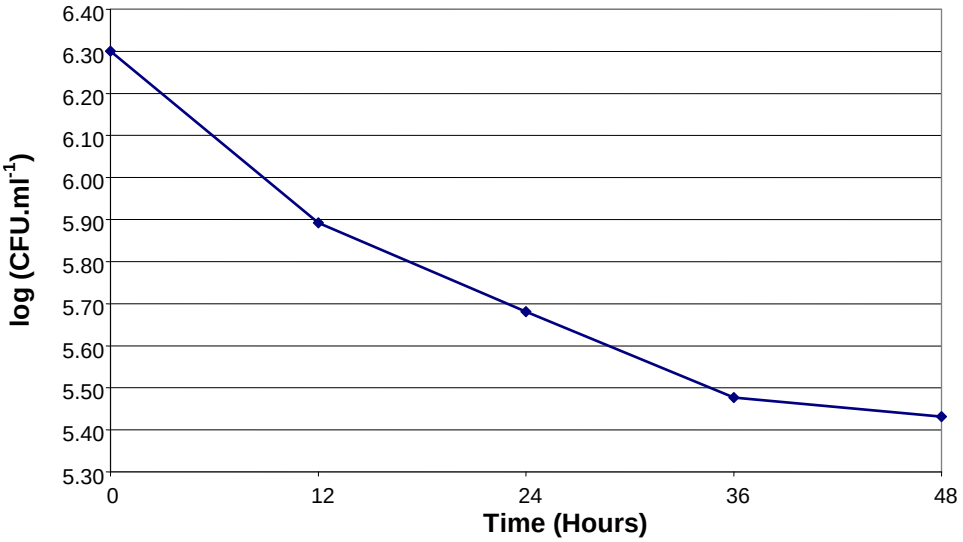


Figure 2.7: Total viable counts for *Chryseobacterium indologenes* LMG 8337 after acid exposure.

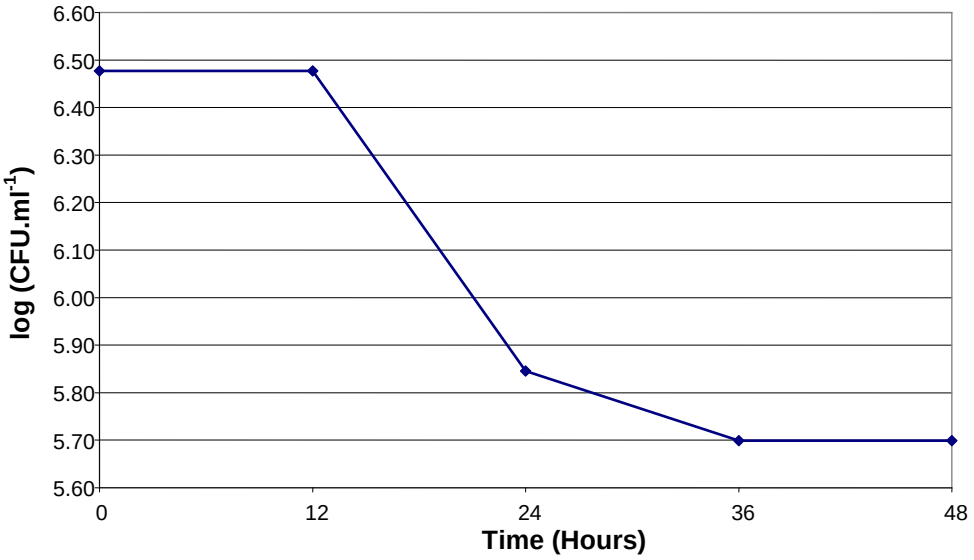


Figure 2.8: Total viable counts for *Klebsiella pneumoniae* ATCC 31488 after acid exposure.

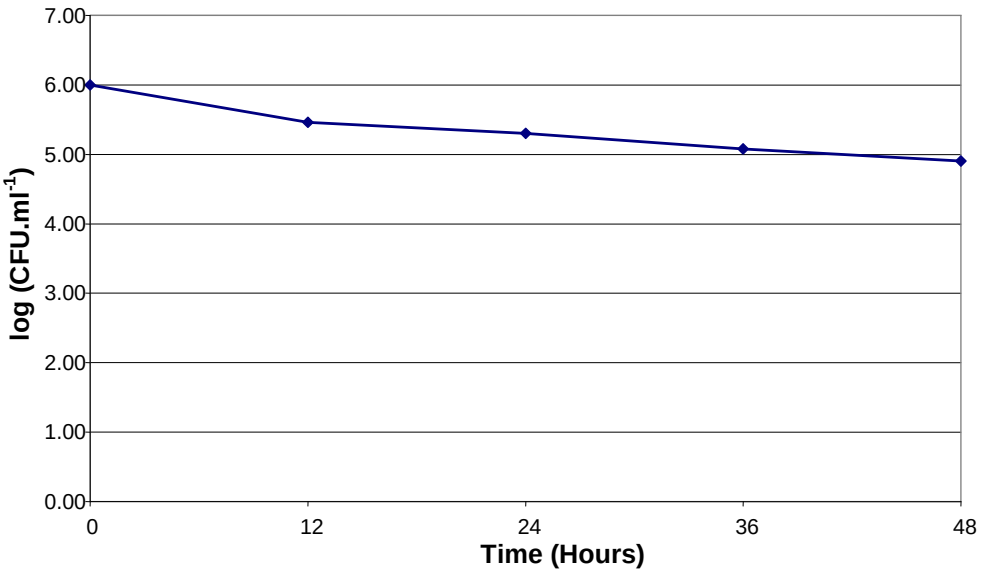


Figure 2.9: Total viable counts for *Proteus vulgaris* ATCC 13315 after acid exposure.

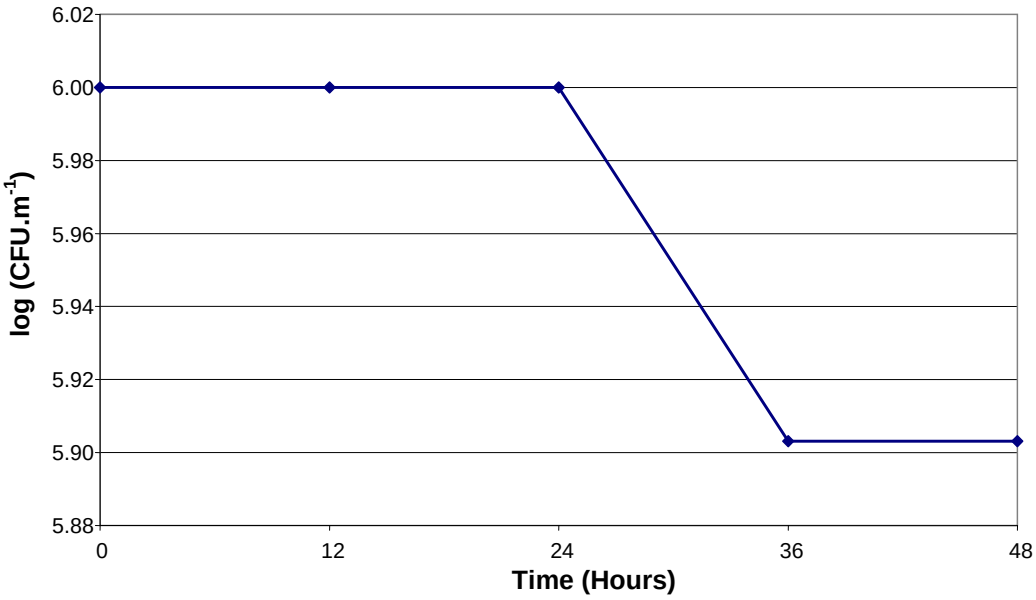


Figure 2.10: Total viable counts for *Pseudomonas aeruginosa* ATCC 27853 after acid exposure.

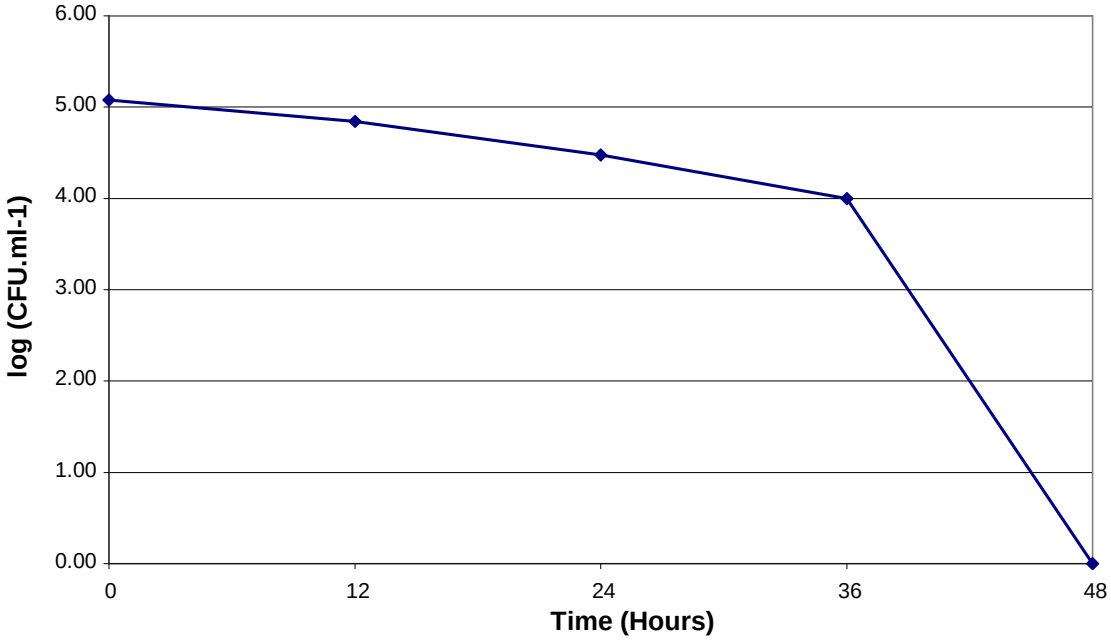


Figure 2.11: Total viable counts for *Bacillus cereus* ATCC 14579 after acid exposure.

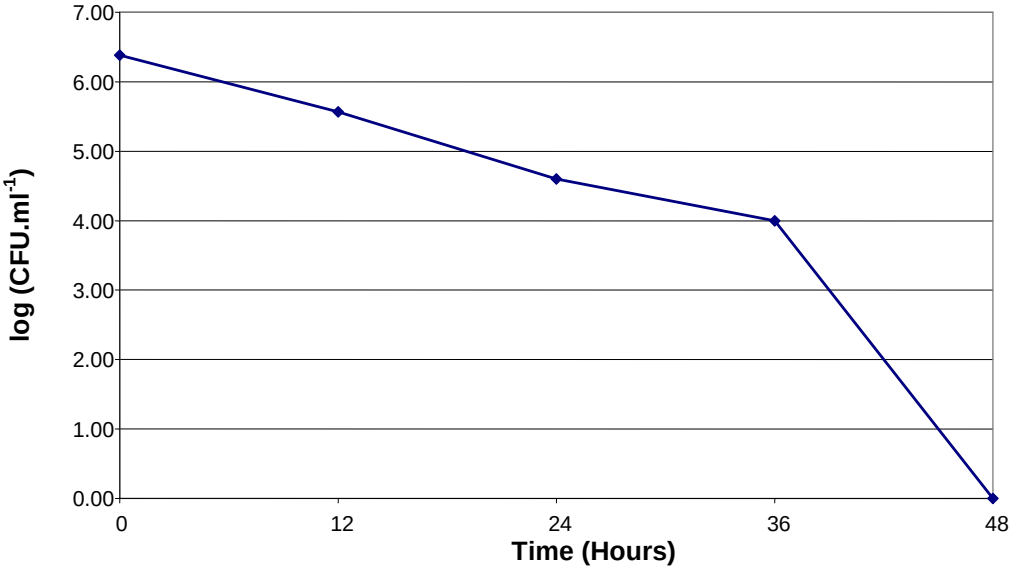


Figure 2.12: Total viable counts for *Chryseobacterium gleum* LMG 8334 after acid exposure.

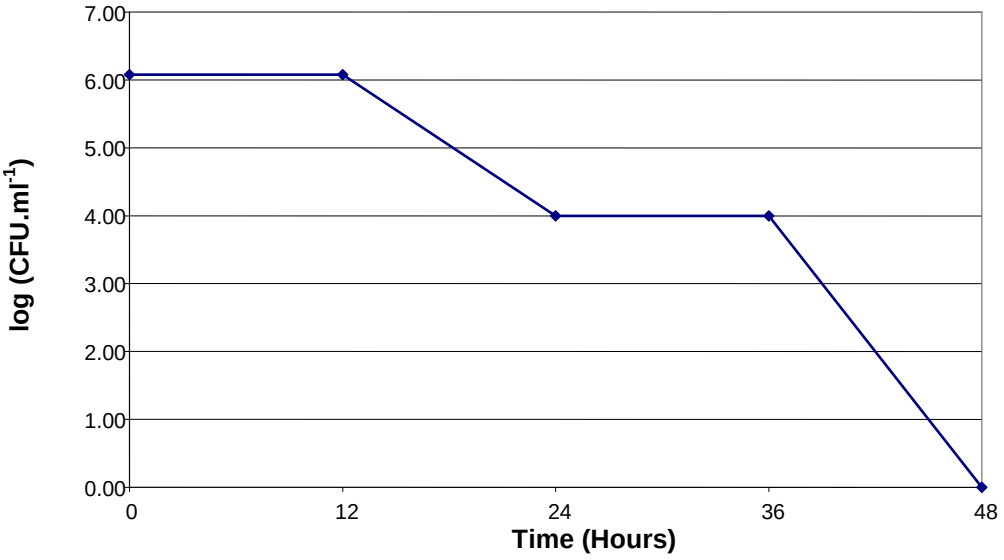


Figure 2.13: Total viable counts for *Chryseobacterium vrystaatense* LMG 22846 after acid exposure.

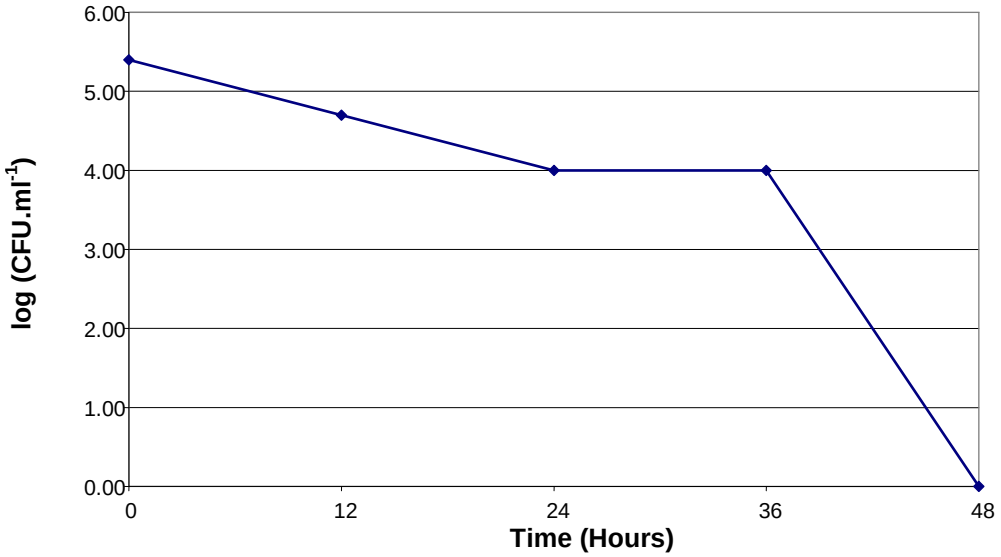


Figure 2.14: Total viable counts for *Yersinia enterocolitica* ATCC 9610 after acid exposure.

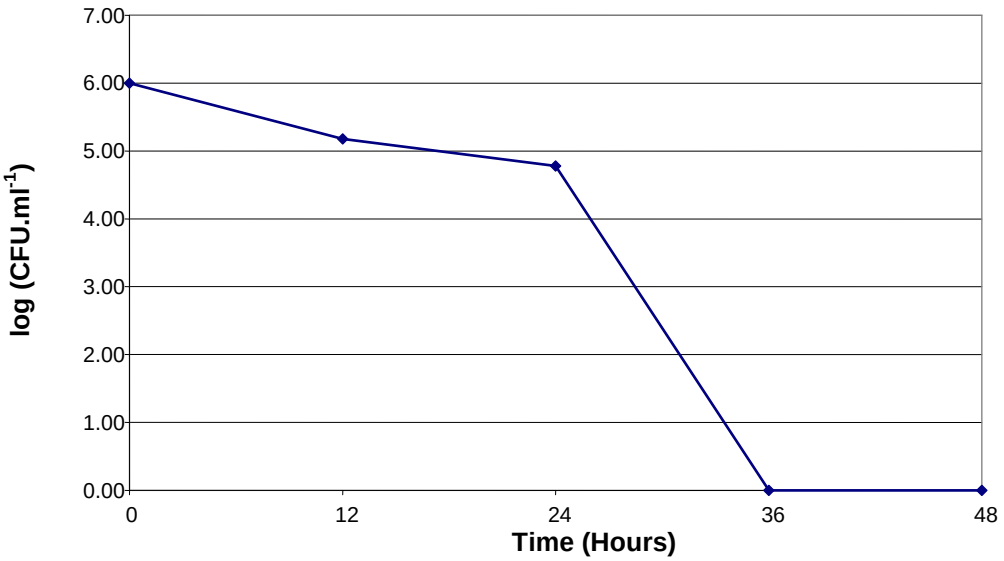


Figure 2.15: Total viable counts for *Chryseobacterium joostei* LMG 18212 after acid exposure.

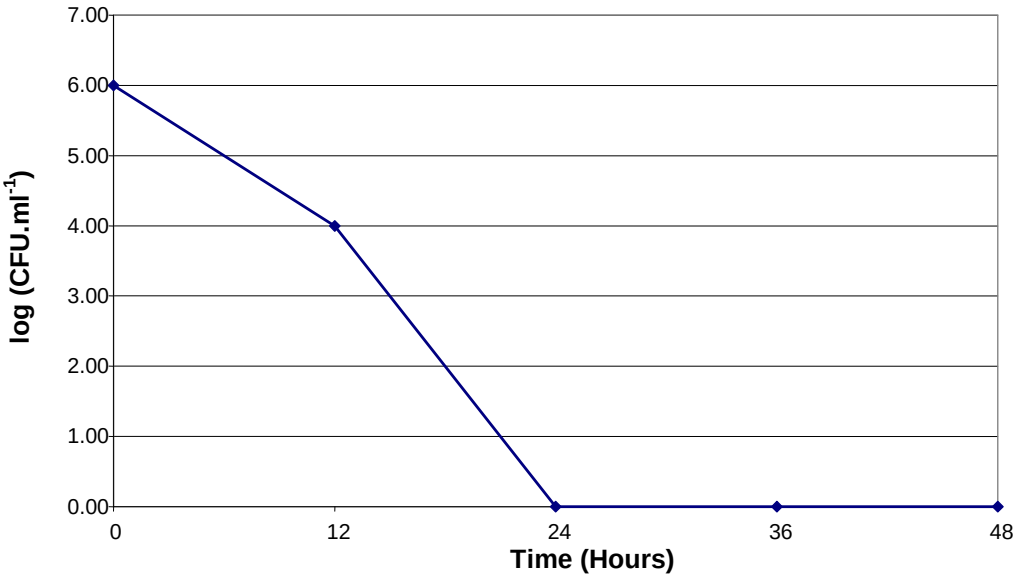


Figure 2.16: Total viable counts for *Chryseobacterium piscium* LMG 23089 after acid exposure.

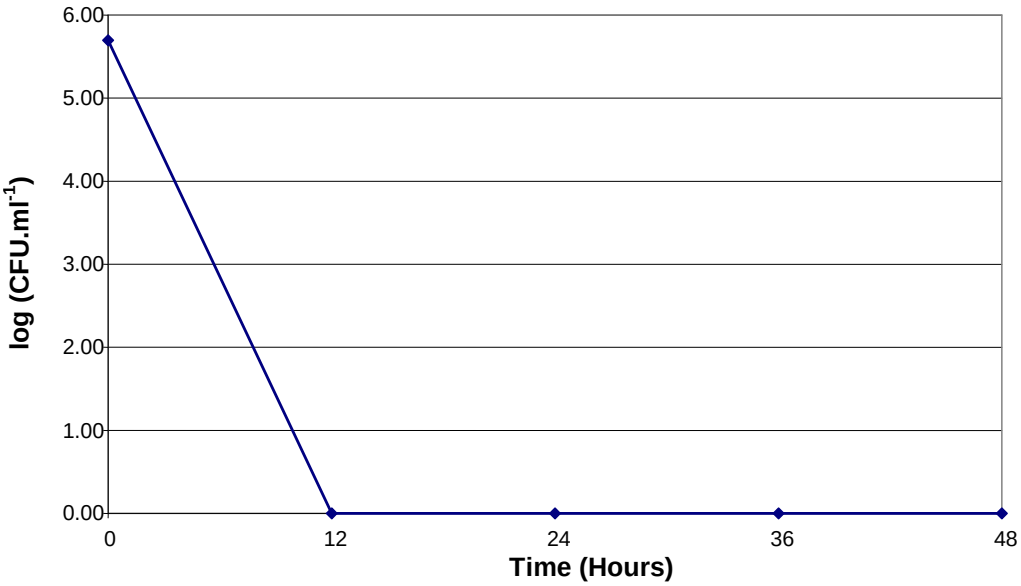


Figure 2.17: Total viable counts for *Chryseobacterium defluvii* LMG 22469 after acid exposure.

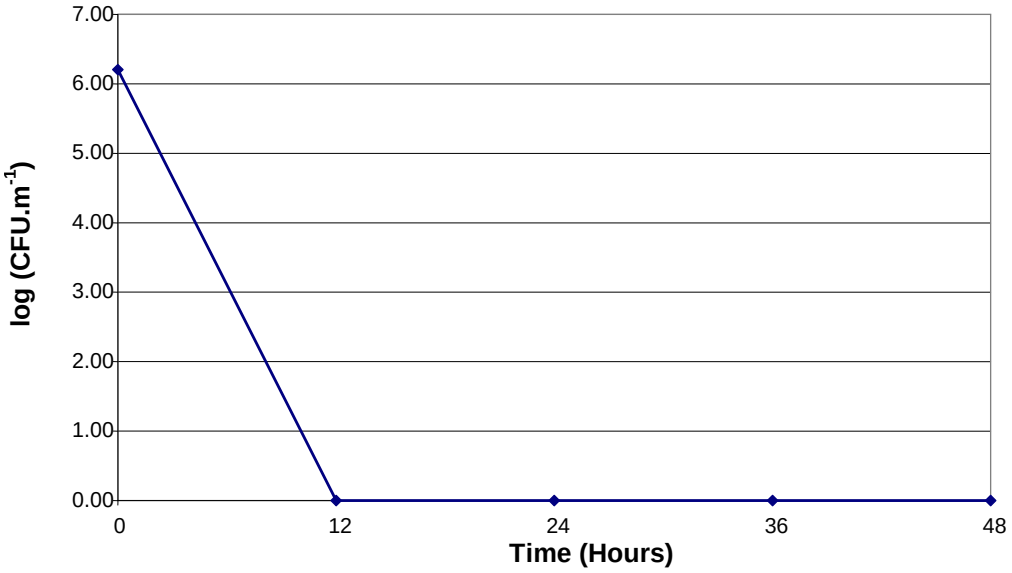


Figure 2.18: Total viable counts for *Chryseobacterium indoltheticum* LMG 4025 after acid exposure.

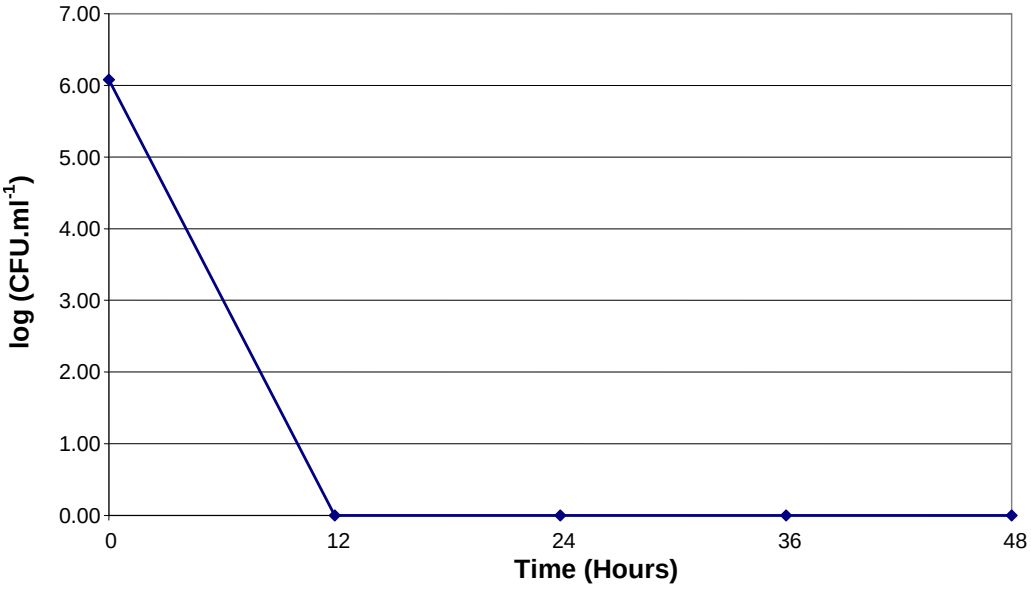


Figure 2.19: Total viable counts for *Chryseobacterium scophthalmus* LMG 13028 after acid exposure.

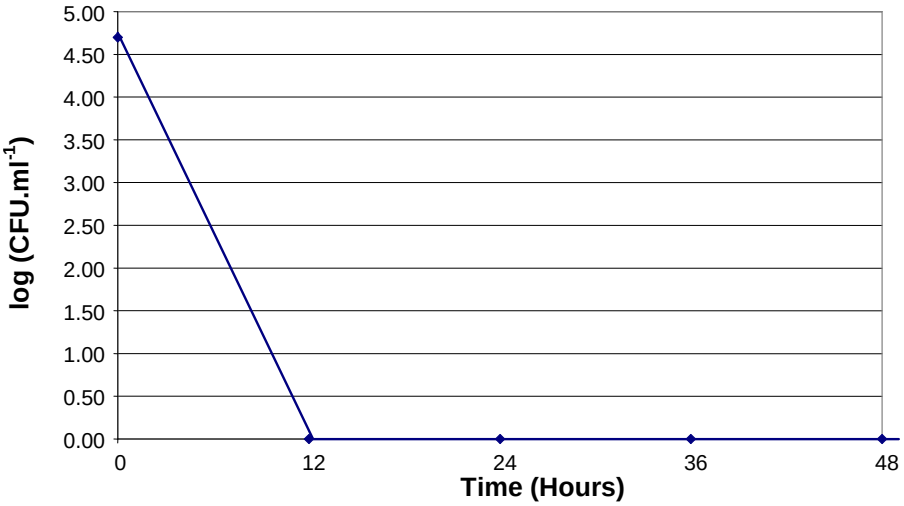


Figure 2.20: Total viable counts for *Staphylococcus aureus* ATCC 25923 after acid exposure.

E. coli and *S. enterica* sv. Enteritidis demonstrated the same trend after acid exposure. This was also observed in *S. enterica* sv. Typhimurium, although this organism showed a lower viable count after 48 h of growth. It has been reported that *S. enterica* sv. Typhimurium is not able to survive an acid challenge at a very low pH (pH 2.5) under conditions similar to those used for *E. coli* (Kieboom and Abee, 2006). Acid adaptation in *S. typhimurium* has also been reported to occur in two-stages, requiring an initial pre-shock exposure to a mild pH in the range of 5.0-6.0, and then followed by acid challenge exposure to a pH below 4.0 (acid shock) (Tosun and Aktug Gonul, 2003).

Growth of *P. aeruginosa* after acid exposure revealed a different trend from the other strains (Figure 2.10), as viable counts only decreased after 24 h, while this also indicated little influence on cell growth. Although *P. aeruginosa* is known to be resistant to many commonly used antibiotics, not much information is available on its acid tolerance (Todar, 2008).

It was evident that among the psychrotrophic bacteria, the survival rate and per implication the acid tolerance were diverse, which could have serious implications on the application of preservation methods at lower or refrigeration temperatures. It is therefore, essential for processors to select high-quality raw materials with low levels of microorganisms, especially psychrotrophs and also to determine the potentially microbiological hazards of ingredients to minimise the risk of contamination in acidic foods (Moberg, 1989).

Food-borne pathogenic bacteria are mainly neutrophils and will grow optimally at pH 6-7. However, these pathogens often encounter acidic environments and have to adapt in order to survive. In food systems such acid-related stresses are often due to the presence of lipid-permeable weak acids (Hill *et al*., 1995). Bacteria in general have the ability to tolerate small changes in environmental parameters and can adapt within minutes, hours or days. However, larger changes away from the optimal required values can cause the induction of more elaborate stress responses (Hill *et al*., 1995). One such response is acid tolerance that can rapidly be induced when enterobacteria are transferred from growing at neutral pH to mildly acidic external pH (Trilla *et al*., 1997). Survival of food-borne pathogens in acidic environments has been shown to be enhanced when a bacterial cell enters the stationary phase and is also enhanced when cells are pre-exposed to moderately acidic environment prior to acid stress (Edelson-Mammel *et al*., 2006). A gradual increase in acidity will allow an induction of acid tolerance or an acid habituation and consequently the survival of organisms to subsequent exposures, which otherwise would be lethal to bacterial cells (Alakomi *et al*., 2000). Such adaption of bacterial cells to a mildly acidic pH before exposure to low pH environments will, therefore, result in the development of cells with increased resistance and longer survival time as opposed to placing cells directly into a low pH environment (Merrell and Camilli, 1999; Cheng *et al*., 2003).

Although acid tolerance was found in only 3/19 (16%) of all the isolates after exposure to HCl, this may be a cause for concern, as these isolates include *E.*

coli and *Salmonella* spp., which are notorious for causing food poisoning in addition to food spoilage. Care should therefore, be taken in the production and processing of acidic foodstuffs.

2.4 CONCLUSIONS

Pathogens associated with transmission via faecal-oral routes are known to survive in extremely acidic environments. These bacteria must be able to withstand acid stress under different conditions and acid tolerance plays an important role in the survival and growth in especially fermented foods (Ricke, 2003). In the current investigation the diversity of acid tolerance among bacterial genera as well as species was obvious. Of concern was the enteric bacteria *Salmonella* spp. and also *E. coli* that demonstrated high levels of acid tolerance, as this would have a serious implication on their survival in acid foodstuffs and consequent resistance to the protective effect of the acidic human gastric environment. Conversely, these organisms may show promise as subjects in future studies into the seat of tolerance and ultimately add to the solutions into addressing the problems associated with tolerance in acidic food.

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CHAPTER 3

Differences in Potential Acid Tolerance During Exposure of Food-Related Bacteria to Acidic Foodstuffs and HCl

3.1 INTRODUCTION

Bacteria have been reported to survive in environments such as low pH foodstuffs, especially processed foods, sauces, salad dressing, yoghurt, tomato ketchup and juices whereas pathogenic organisms are known to adapt and survive at extreme temperatures and osmotic pressures outside the fundamental ranges reported in literature (Cheng *et al*., 2003). One such survival mechanism, the development of acid resistance and tolerance may have serious implications for food safety and concern has been expressed regarding pathogens being able to survive the human GI tract and defence system (Berry and Cutter, 2000). Prolonged exposure to acidic food have been reported to contribute to the induction of an acid tolerance response (ATR) that leads to acid resistance, an important characteristic of organisms such as *E. coli* (Sainz, 2005).

Microorganisms differ with regard to their physiological requirements in surviving organic and inorganic challenges (Ferreira *et al*., 2003). In attempting to assess the ATR response under conditions prevalent in the food industry, the majority of studies have been conducted by utilising acidulates such as hydrochloric acid (Foster, 1991; Kroll and Patchett, 1992; O'Driscoll *et al*., 1996; Greenacre *et al*., 2003). However, hydrochloric acid lowers the pH levels abruptly, whereas lowering the pH in foodstuffs such as yogurt and fermented meats occurs gradually as fermentation proceeds. In attempting to measure the effect of acidic environments on acid tolerance of bacteria and their ability to survive in fermented and acidic foods, cells should rather be

exposed to acidic environments by making use of methods that accurately simulate authentic food systems (Deng *et al*., 1999). There is also some concern that pathogens in a mixed microbial culture, such as a food environment, may react differently to decontamination stresses than their pure cultures in a controlled environment (Samelis *et al*., 2002; Stopforth *et al*., 2003).

A range of different types of foodstuffs has been associated in outbreaks concerning enterohemorrhagic strains of *E. coli* and *E. coli* 0157:H7 with milk, beef and apple cider being a few examples (Besser *et al.*, 1993; Centres for Disease Control and Prevention, 1993; Griffin and Tauxe, 1991; Padhye and Doyle, 1992; Steele *et al.*, 1982; Tarr, 1995). Outbreaks of *E. coli* 0157:H7 have also been reported from fermented hard salami (Centres for Disease Control and Prevention, 1995). Fermentation processes and the acidity of food products such as salami and apple cider contribute to their preservation and studies have shown significant interest in investigating if adaptation to acid could lead to bacterial survival in low pH foodstuffs (Foster and Hall, 1990; Lee *et al*., 1994).

Acid adaptation generally leads to an increase of acid tolerance, while bacterial cells that have acquired acid tolerance due to an acid-shock do not increase tolerance. For example, bacterial cells may develop acid adaptation during the fermentation process of certain foods and not experience an acid shock due to the steady and continuous decrease in pH levels (Ryu and Beuchat, 1999). Earlier studies on acid adaptation have produced data,

which indicate that adaptation to acid can increase enteric bacterial ability to survive in acidic foodstuffs (Leyer and Johnson, 1992; Leyer and Johnson, 1993). It is, therefore, vital for food challenge studies to include adapted cells in their experiments as exponentially growing cells may signify incorrect survival profiles.

The aim of this study was to expose various bacterial strains to acidic foodstuffs as well as HCl in order to demonstrate possible differences in the development of potential acid tolerance. The study should contribute to a better understanding of the response of food-borne bacteria to exposure to acid foods.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial isolates

Bacterial isolates comprised species that have often been implicated in food-borne illnesses resulting from the consumption of acidic foodstuffs. These included bacterial strains *Escherichia coli* ATCC 25922, *Salmonella enterica* sv. Typhimurium ATCC 14028, *Salmonella enterica* sv. Enteritidis ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Yersinia enterocolitica* ATCC 9610 and *Chryseobacterium piscium* LMG 23089.

3.2.2 Induction of acid tolerance

Strains were sub-cultured in Brain-Heart Infusion (BHI) broth (Biolab Diagnostics [Pty] Ltd.) containing respective increasing concentrations of hydrochloric acid as well as a variety of acidic foodstuffs (vinegar, mayonnaise, chopped gherkins and gherkin brine) and incubated at 30˚C for 24 h. These inducing agents were not further sterilised, as it was attempted to simulate a similar acidic environment as in acidic foodstuff and a low pH is commonly used as food preservative. Control broths without growth were included for monitoring pH. The lowest induction pH of each organism and each acidic foodstuff was measured. To distinguish between acid tolerance and acid resistance, viable organisms at lowest pH levels for each induction medium (broth and foodstuffs) were inoculated onto BHI agar (pH 7 and pH 5) and incubated at 30˚C for 48 h. Induced isolates that were not able to grow at pH 5 after induction, were regarded as acid-tolerant. Cultures were harvested and stored at -80˚C.

3.2.3 Protein studies

Protein profiles of induced and control strains were generated by SDS-PAGE. Harvested cells were washed twice in 0.1M phosphate buffer, pH 7, by centrifugation (Eppendorf-Netheler-Hinz, GmbH, Hamburg, Germany) at 10 000 x g for 5 min. Protein concentrations were determined by the Bio-Rad Protein Assay. Equal concentrations of the washed cells were resuspended in 450 µl sample treatment buffer (0.062M Tris-HCl, pH6.8, [Saarchem, Merck Chemicals [PTY] Ltd., Gauteng, RSA], 5 % [vol/vol] 2-mercaptoethanol, [MP Biochemicals Inc, (Solen, Ohio, USA)], 10 % [vol/vol] glycerol [Roche

Diagnostics Corporation, Indianapolis, IN, USA]).) Sodium dodecyl sulphate (SDS) (Saarchem) was added (50 µl of 20% wt/vol) to samples prior to heat suspension at 95 -100˚C for 10 min. Samples were centrifuged at 10 000 x g for 10 min and 10 µl of 0.5% (wt/vol) bromophenol blue (Saarchem) was added to 100 µl of the supernatant. Equal volumes of the samples were loaded onto the stacking gel (0.5 M Tris-HCl [pH 6.8], 10% [wt/wt] SDS, 30% [wt/vol] acrylamide/bisacrylamide [Saarchem], 10 % [wt/vol] ammonium persulphate [Saarchem], TEMED [Saarchem]). A molecular weight marker (AEC-Amersham [Pty] Ltd., Buckinghamshire, UK) was included in each run. Protein separation was performed in a PROTEAN II xi cell (Bio-Rad, USA) attached to a Haake K10 cooling system (Lasec, Bloemfontein, RSA) at a constant current of 16 mA per gel through the stacking gel and 24 mA per gel through the separating gel (1.5 M Tris-HCl [pH 8.8], 10% [wt/wt] SDS, 30% [wt/vol] acrylamide/bisacrylamide, 10% [wt/vol] ammonium persulphate, TEMED). Gels were run in a tris-glycine running buffer (25 mM Tris, 192 mM glycine [Saarchem], 0.1% SDS [pH8.3]) with a running time between 4 to 5 hours. Gels were stained with 0.1% Coommassie brilliant blue (Saarchem) in 40% ethanol (Saarchem) and 10% acetic acid (Saarchem) and gels were destained with 40% methanol (Saarchem) and 10% acetic acid. Protein profiles of induced strains were compared with those of the un-induced strains. All analyses were performed at least in triplicate.

3.3 RESULTS AND DISCUSSION

The majority of isolates were able to grow on acidified media (pH 5) after induction with the various acidic foods and were considered to have acquired acid resistance. Induced strains that were not able to grow on low pH BHI agar (pH 5) after induction were regarded as acid tolerant since a certain amount of tolerance was induced. In Table 3.1 the lowest pH of each acidic foodstuff where each of the induced strains was able to grow, is depicted. It is evident that the lowest pH where all the strains were still viable was found in mayonnaise (pH 5.4) and chopped gherkin (pH 5.3). This may be due to the higher nutritional value of a foodstuff such as mayonnaise, or that the foodstuff provided some protection against acid stress. All induced strains of *E. coli*, *S. enterica* sv. Enteritidis, *S. enterica* sv. Typhimurium, and *Y. enterocolitica* were considered acid resistant, while *P. aeruginosa*, *S. aureus* and *C. piscium* delivered both acid-tolerant and acid resistant strains (Table 3.1).

E. coli is often regarded as acid-resistant and in the current study the organism demonstrated survival at varying pH levels (pH 4.6 - 6.6) for the different acidulants used in the induction process (Figure 3.1). This was also observed with *P. aeruginosa* (pH 4.6 - 6.6, Figure 3.2), *S. enterica* sv. Enteritidis (pH 4.5 - 6.6, Figure 3.3), *S. enterica* sv. Typhimurium (4.5 - 6.6, Figure 3.4), *Y. enterocolitica* (pH 5.0 - 6.6, Figure 3.5), *C. piscium* (pH 4.5 - 6.6, Figure 3.6) and *S. aureus* (pH 5 - 6.7, Figure 3.7).

Table 3.1: The lowest pH where the organisms were able to grow after induction.

Bacterial strains	Lowest exposure pH
***E. coli* ATCC 25922**	
chopped gherkin (solid)	5.3
gherkin brine	6.6
mayonnaise	4.5
vinegar	4.8
HCl	5*
***P. aeruginosa* ATCC 27853**	
chopped gherkin (solid)	5.3*
gherkin brine	6.6
mayonnaise	4.5*
vinegar	4.6
HCl	5*
***S. enterica* sv. Enteritidis ATCC 13076**	
chopped gherkin (solid)	5.3
gherkin brine	6.6
mayonnaise	4.5
vinegar	5
HCl	5*
***S. enterica* sv. Typhimurium ATCC 14028**	
chopped gherkin (solid)	5.3
gherkin brine	6.6
mayonnaise	4.5
vinegar	4.8
HCl	5*
***Y. enterocolitica* ATCC 9610**	
chopped gherkin (solid)	5
gherkin brine	6.6
mayonnaise	5.4
vinegar	5
HCl	5*
***C. piscium* LMG 23089**	
chopped gherkin (solid)	5
gherkin brine	6.6
mayonnaise	5.4*
vinegar	6.7*
HCl	5*
***S. aureus* ATCC 25923**	
chopped gherkin (solid)	5*
gherkin brine	6.6
mayonnaise	4.5*
vinegar	5*
HCl	5*

***** Acid induced isolates not able to grow on low pH agar (pH 5), and regarded as acid-tolerant, whereas the induced isolates that were able to grow at pH 5, were considered acid-resistant.

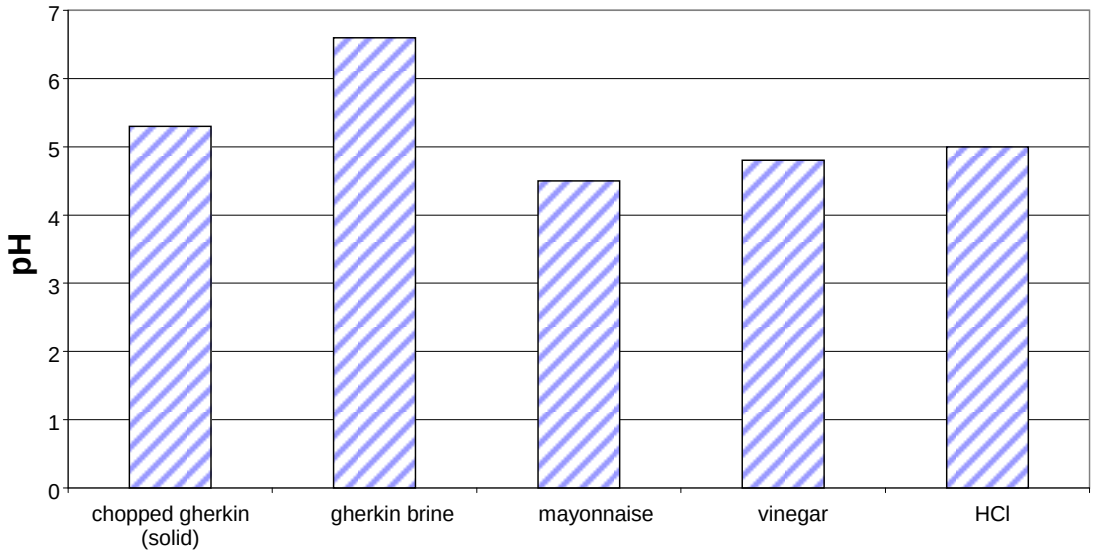

Figure 3.1: The lowest pH for each acidic foodstuffs used during acid tolerance induction in *E. coli* ATCC 25922 (pH range 4.5-6.6).

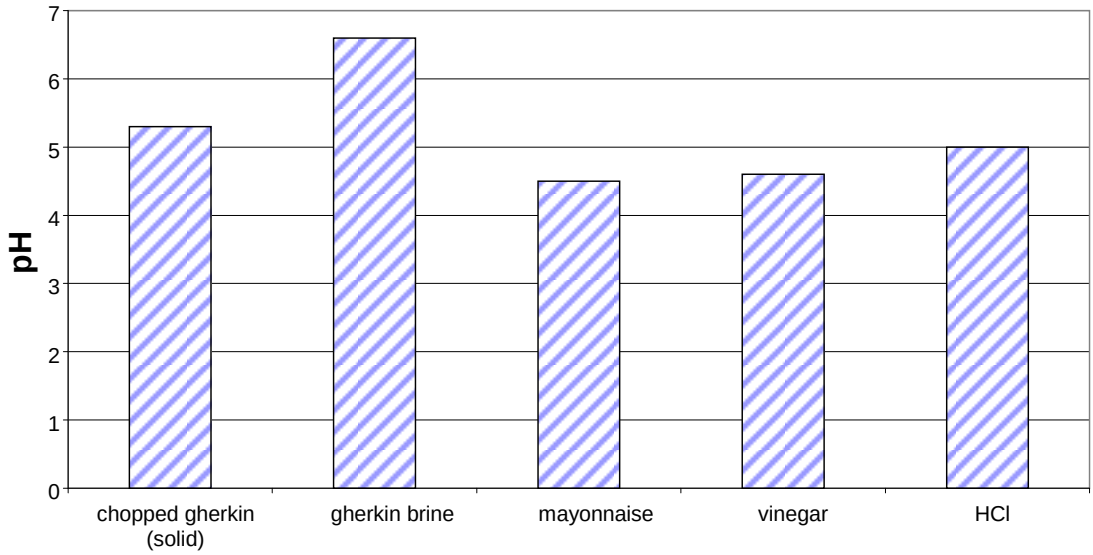

Figure 3.2: The lowest induction pH for each acidic foodstuffs used during acid tolerance induction in *P. aeruginosa* ATCC 27853, (pH range 4.5-6.6).

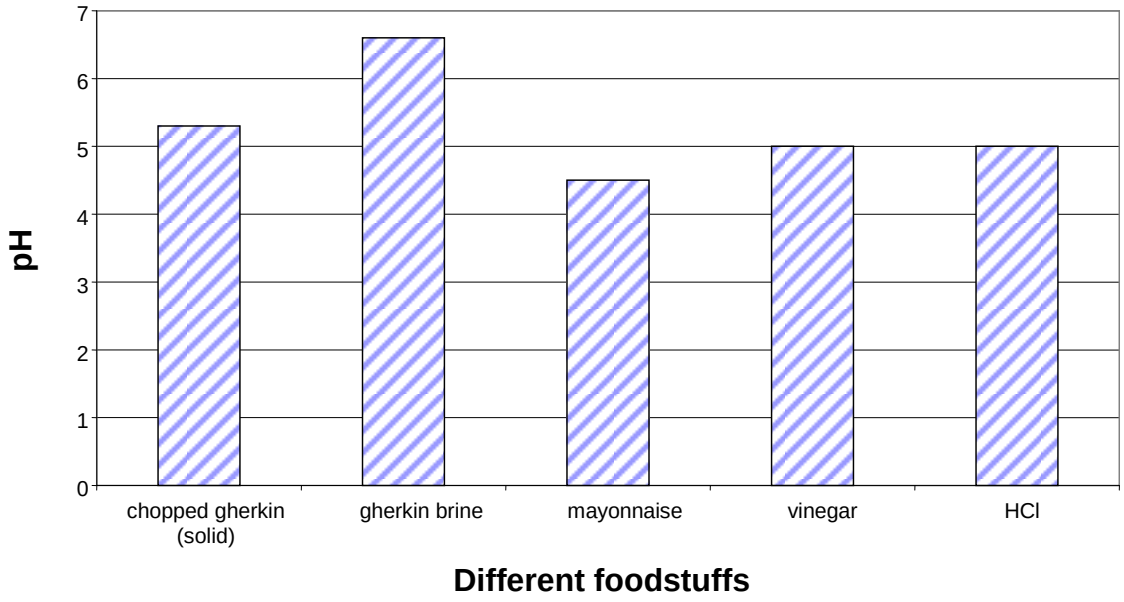

Figure 3.3: The lowest induction pH for each acidic foodstuffs used during acid tolerance induction in *S. enterica* sv. Enteritidis ATCC 13076 (pH range 4.5-6.6).

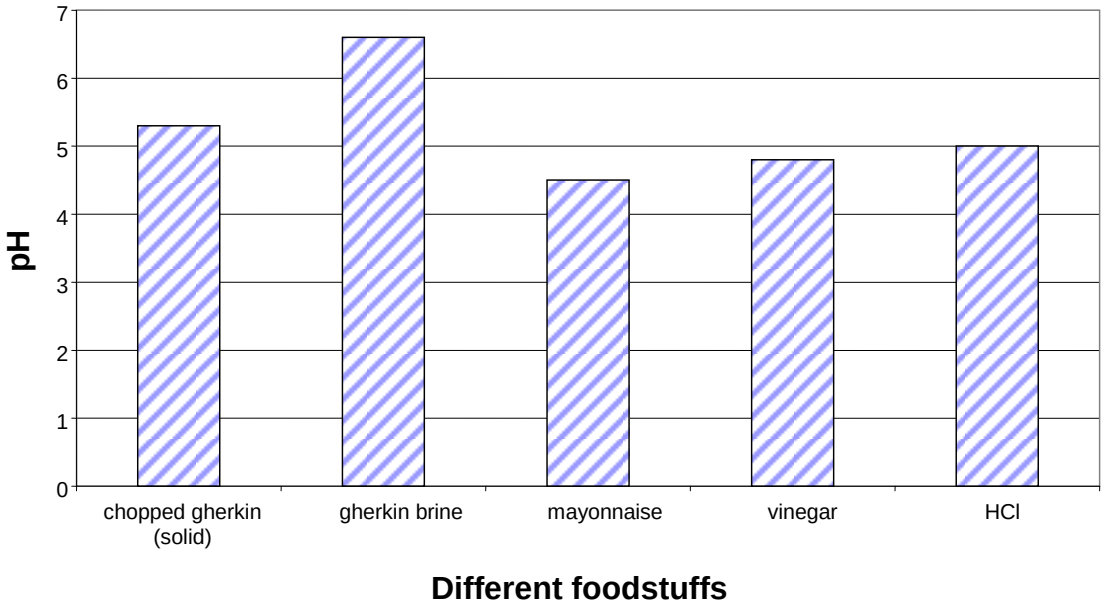

Figure 3.4: The lowest induction pH for each acidic foodstuffs used during acid tolerance induction in *Salmonella enterica* sv. Typhimurium ATCC 14028, (pH range 4.5-6.6).

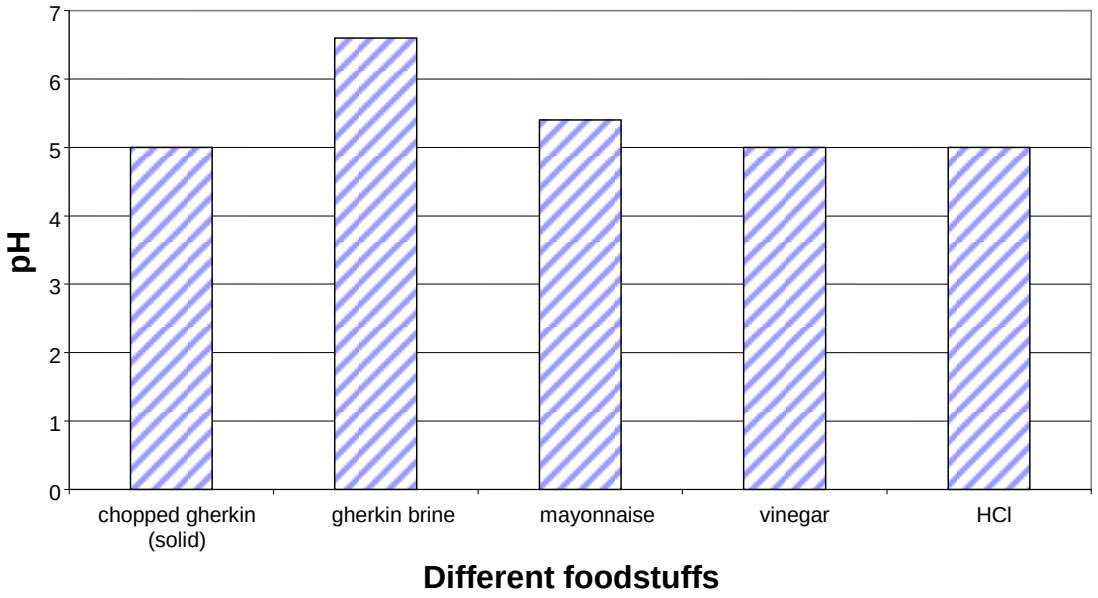

Figure 3.5: The lowest induction pH for each acidic foodstuffs used during acid tolerance induction in *Y. enterocolitica* ATCC 9610 (pH range 5.0-6.6).

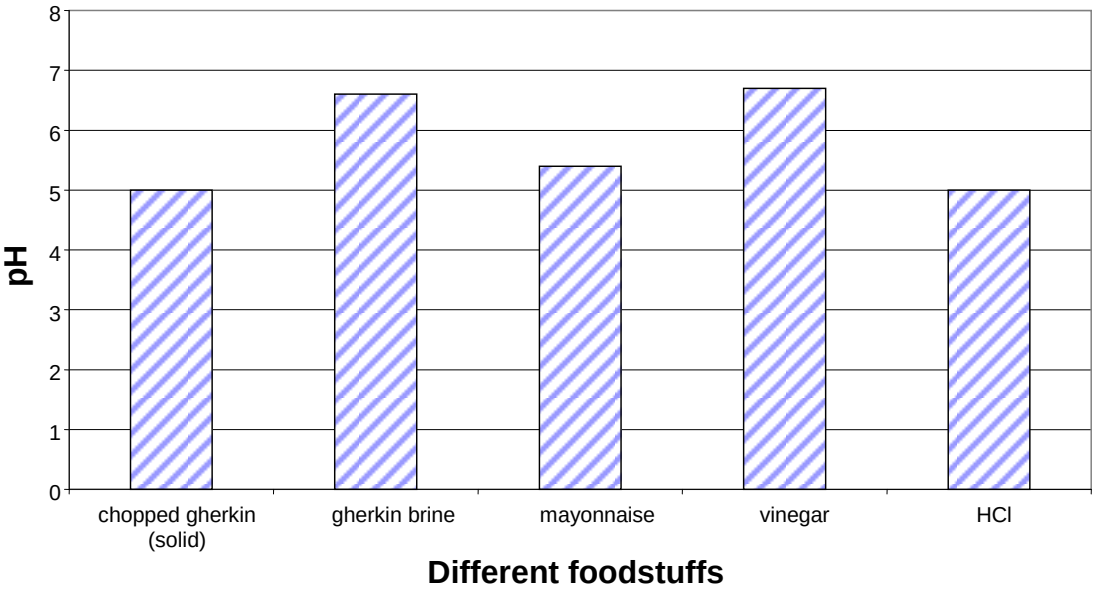

Figure 3.6: The lowest induction pH for each acidic foodstuffs used during acid tolerance induction in *C. piscium* LMG 23089 (pH range 5.0-6.6).

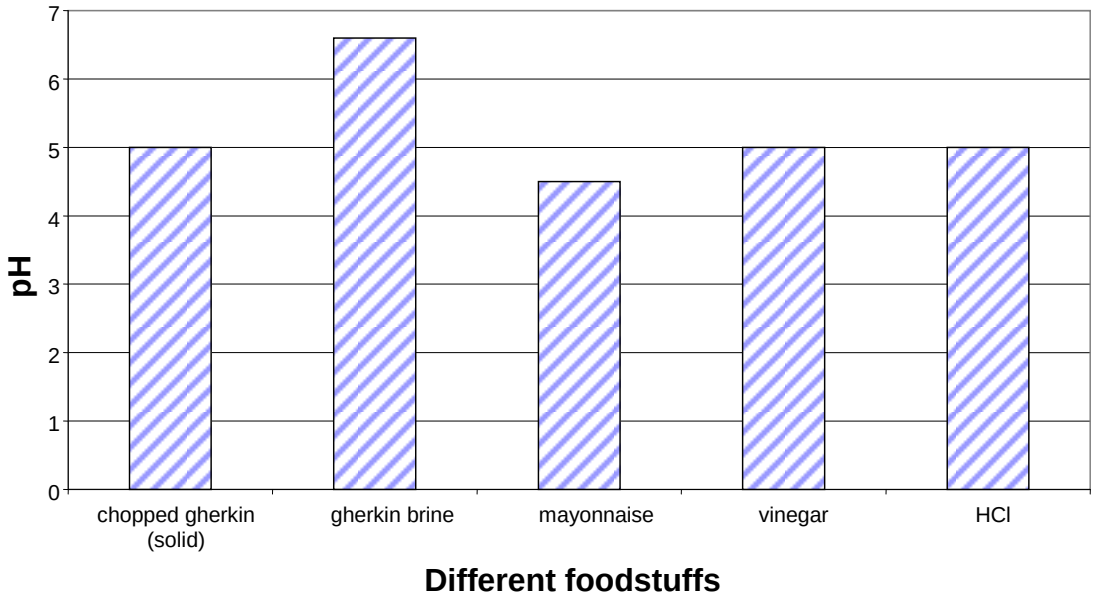

Figure 3.7: The lowest induction pH for each acidic foodstuffs used during acid tolerance induction in *S. aureus* ATCC 25923, (pH range 4.5-6.6).

Tables 3.2 to 3.6 illustrate survival of all the bacterial stains at each induction step and each foodstuff as the pH decreased. With a pH range between 6.4 – 7.3, all bacterial cells survived the first induction step (Table 3.2). Cell death became noticeable during the second induction step with vinegar as induction substance with *C. piscium* being the first organism where no viable cell counts were detected at pH 5.6 (Table 3.3). The remaining foodstuffs caused different survival profiles for all the bacterial cells in each induction step. All the organisms survived the induction process with gherkin brine and the lowest pH induction level where growth was recorded was pH 6.6 (Table 3.6 and Figure 3.8).

Table 3.2: pH values of different foodstuffs at the first induction step where organisms were able to grow.

INOCULATION 1					
	Mayonnaise	Vinegar	Chopped gherkins	Gherkin brine	HCl
	pH 6.8	pH 6.7	pH 7.0	pH 7.3	pH 6.4
S. enterica sv. Enteritidis ATCC 13076	√	√	√	√	√
S. aureus ATCC 25923	√	√	√	√	√
E. coli ATCC 25922	√	√	√	√	√
P. aeruginosa ATCC 27853	√	√	√	√	√
S. enterica sv. Typhimurium ATCC 14028	√	√	√	√	√
Y. enterocolitica ATCC 9610	√	√	√	√	√
C. piscium LMG 23089	√	√	√	√	√

Table 3.3: pH values of different foodstuffs at the second induction step where organisms were able to grow.

INOCULATION 2					
	Mayonnaise	Vinegar	Chopped gherkins	Gherkin brine	HCl
	pH 5.8	pH 5.6	pH 6.5	pH 7.0	pH 6.2
S. enterica sv. Enteritidis ATCC 13076	√	√	√	√	√
S. aureus ATCC 25923	√	√	√	√	√
E. coli ATCC 25922	√	√	√	√	√
P. aeruginosa ATCC 27853	√	√	√	√	√
S. enterica sv. Typhimurium ATCC 14028	√	√	√	√	√
Y. enterocolitica ATCC 9610	√	√	√	√	√
C. piscium LMG 23089	√	X	√	√	√

Table 3.4: pH values of different foodstuffs at the third induction step where organisms were able to grow.

INOCULATION 3					
	Mayonnaise	Vinegar	Chopped gherkins	Gherkin brine	HCl
	pH 5.4	pH 5.0	pH 6.0	pH 6.9	pH 5.8
S. enterica sv. Enteritidis ATCC 13076	√	√	√	√	√
S. aureus ATCC 25923	√	√	√	√	√
E. coli ATCC 25922	√	√	√	√	√
P. aeruginosa ATCC 27853	√	√	√	√	√
S. enterica sv. Typhimurium ATCC 14028	√	√	√	√	√
Y. enterocolitica ATCC 9610	√	√	√	√	√
C. piscium LMG 23089	√	X	√	√	√

Table 3.5: pH values of different foodstuffs at the fourth induction step where organisms were able to grow.

INOCULATION 4					
	Mayonnaise	Vinegar	Chopped gherkins	Gherkin brine	HCl
	pH 4.9	pH 4.8	pH 5.3	pH 6.8	pH 5.5
S. enterica sv. Enteritidis ATCC 13076	√	X	√	√	√
S. aureus ATCC 25923	√	X	√	√	√
E. coli ATCC 25922	√	√	√	√	√
P. aeruginosa ATCC 27853	√	√	√	√	√
S. enterica sv. Typhimurium ATCC 14028	√	√	√	√	√
Y. enterocolitica ATCC 9610	X	X	√	√	√
C. piscium LMG 23089	X	X	√	√	√

Table 3.6: pH values of different foodstuffs at the fifth and last induction step where organisms were able to grow.

INOCULATION 5					
	Mayonnaise	Vinegar	Chopped gherkins	Gherkin brine	HCl
	pH 4.5	pH 4.6	pH 5.0	pH 6.6	pH 5
S. enterica sv. Enteritidis ATCC 13076	√	X	X	√	√
S. aureus ATCC 25923	√	X	√	√	√
E. coli ATCC 25922	√	X	X	√	√
P. aeruginosa ATCC 27853	√	√	X	√	√
S. enterica sv. Typhimurium ATCC 14028	√	X	X	√	√
Y. enterocolitica ATCC 9610	X	X	√	√	√
C. piscium LMG 23089	X	X	√	√	√

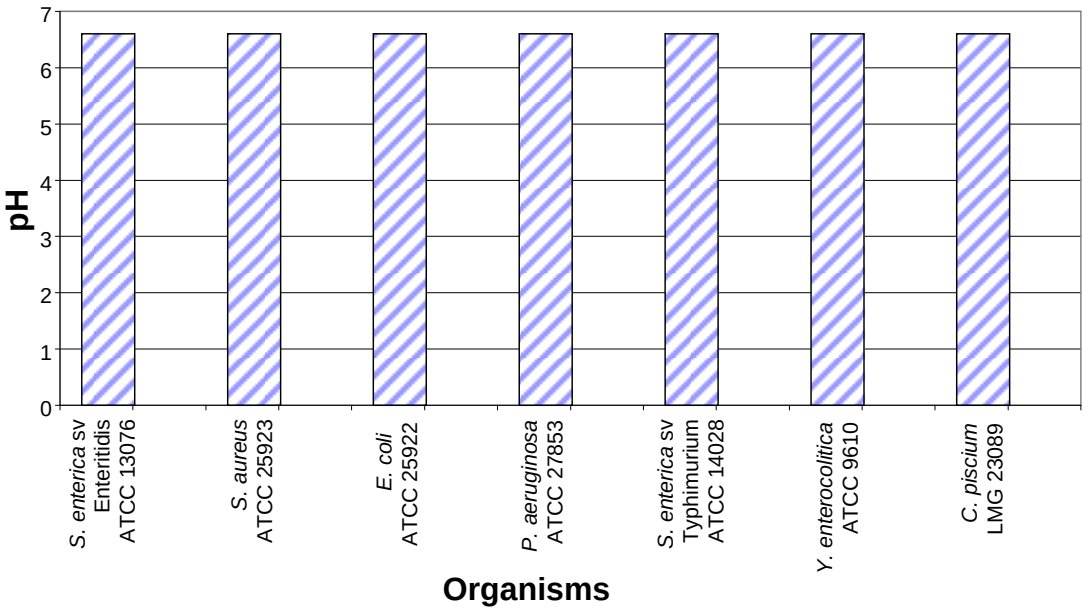

Figure 3.8: The lowest induction pH levels where organisms were able to grow with gherkin brine as acidulant (average pH level, 6.6).

Acidic foodstuff used during the induction supplied varying data on the survival abilities of all isolates. The lowest induction pH recorded at the last induction step with mayonnaise was pH 4.5, but some of the strains, for example *Y. enterocolitica* and *C. piscium* were not able to survive at this pH in mayonnaise. The lowest pH level where growth was detected for these organisms was 5.5 (Figure 3.9). However, *Y. enterocolitica* and *C. piscium* survived the induction with chopped gherkin at a lower pH level of 5 (Figure 3.10).

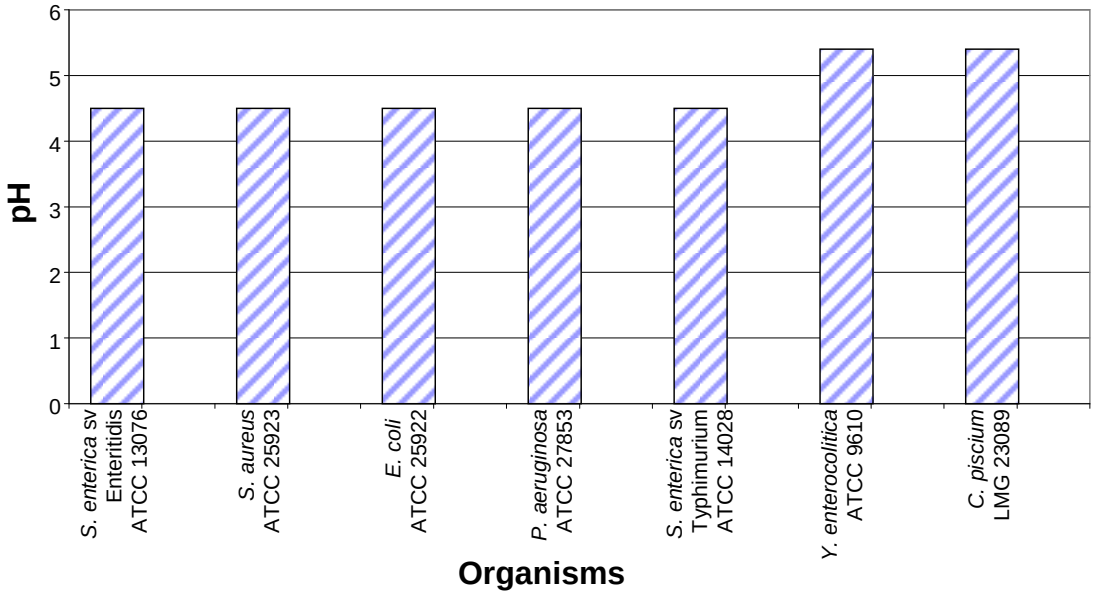

Figure 3.9: The lowest induction pH levels where organisms were able to grow with mayonnaise as acidulant (average pH level: 4.76).

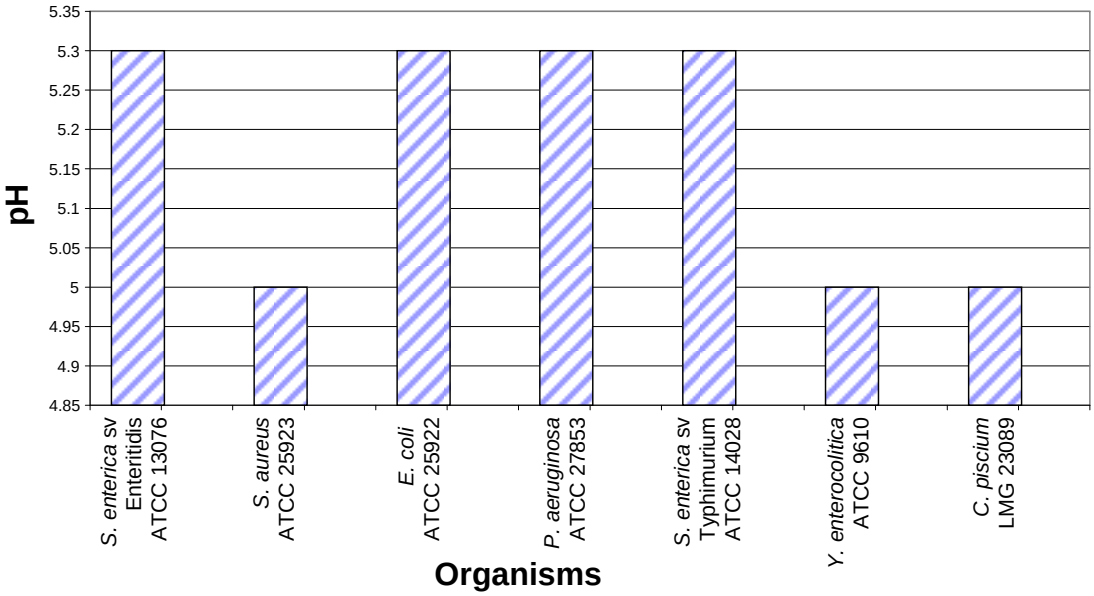

Figure 3.10: The lowest induction pH levels where organisms were able to grow with chopped gherkin as acidulant (average pH level: 5.17).

Vinegar, also known as acetic acid, produced a range of lowest induction pH levels between 4.6 and 6.8 with an average pH of 5.1 for the variety of organisms involved (Table 3.7). Six of the seven organisms produced a lowest induction pH below 5, rendering mayonnaise and vinegar (average induction pH 4.76 and 5.1 respectively) the foodstuffs with the highest ability to induce acid tolerance (Figures 3.9 and 3.11).

When comparing survival profiles after exposure to acidic foodstuffs with those found after exposure to the inorganic acid HCl, it was noted that all the isolates survived the induction process with HCl at induction pH 5, but not all isolates survived induction with chopped gherkins at pH 5 (Table 3.6 and Figure 3.12). It is therefore, imperative that induction studies should not only be conducted by using HCl as described in previous studies (refer to Introduction, page 70) (Foster, 1991; Kroll and Patchett, 1992; O'Driscoll *et al*., 1996; Greenacre *et al*., 2003).

Table 3.7: Average induction pH of each acidulants where organisms were able to grow.

Lowest induction pH levels of acidulants for each organism					
	Mayonnaise	Vinegar	Chopped gherkins	Gherkin brine	HCl
S. enterica sv. Enteritidis ATCC 13076	4.5	5	5.3	6.6	5
S. aureus ATCC 25923	4.5	5	5	6.6	5
E. coli ATCC 25922	4.5	4.8	5.3	6.6	5
P. aeruginosa ATCC 27853	4.5	4.5	5.3	6.6	5
S. enterica sv. Typhimurium ATCC 14028	4.5	4.8	5.3	6.6	5
Y. enterocolitica ATCC 9610	5.4	5	5	6.6	5
C. piscium LMG 23089	5.4	6.6	5	6.6	5
Average induction pH	4.76	5.10	5.17	6.60	5.00

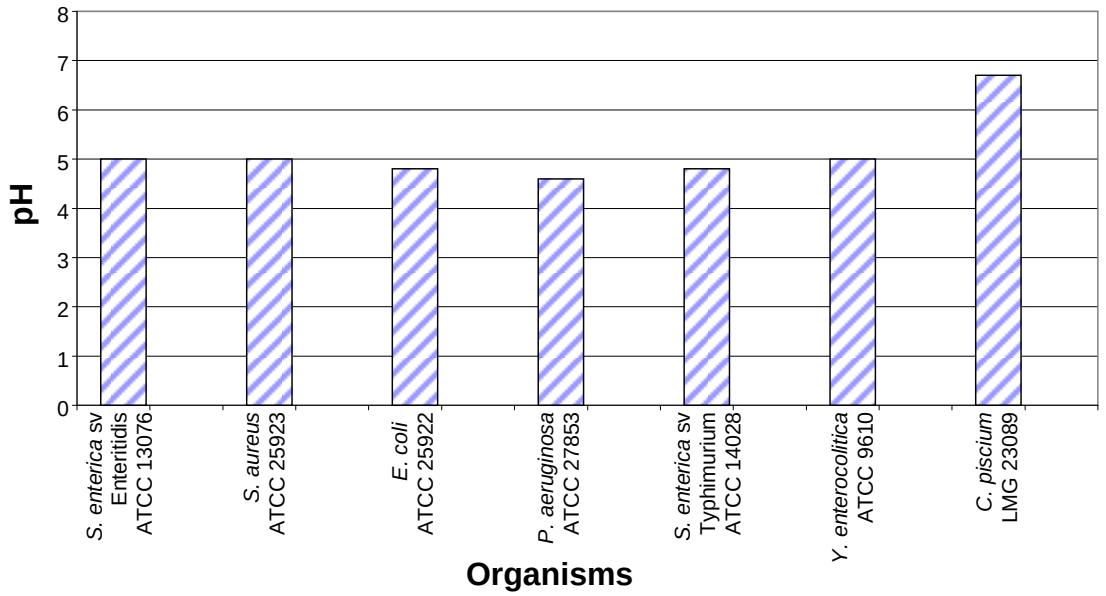

Figure 3.11: The lowest induction pH levels where organisms were able to grow with vinegar as acidulant (average pH level: 5.1).

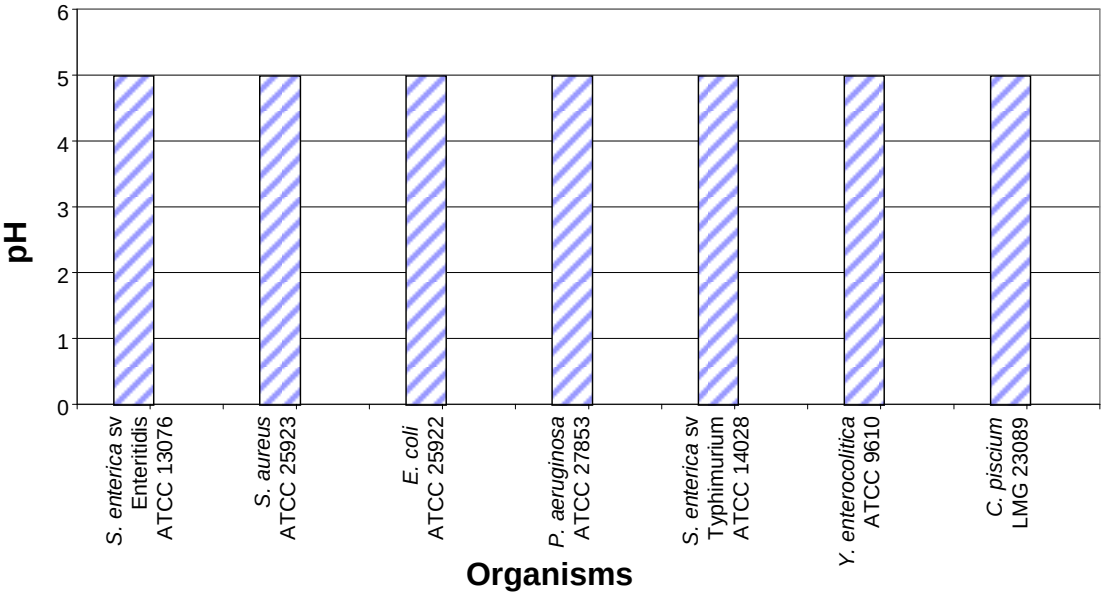

Figure 3.12: The lowest induction pH levels where organisms were able to grow with HCl as acidulant (average pH level: 5).

Protein profiles of induced isolates were compared with the un-induced strains and illustrated in Figures 3.13 to 3.18. After induction with chopped gherkin and vinegar, some protein bands were less visible than in the un-induced strain of *E. coli* ATCC 25922 (approximate sizes 34 and 52 kDa) (Figure 3.13). A similar observation was found in induced strains of *E. coli* after induction with gherkin brine (approximately 30 kDa) (Figure 3.14). However, an additional band at approximately ±49 kDa became visible in the induced strain.

Salmonella enterica sv. Enteritidis showed various additional protein bands (of approximate sizes 20, 23, 25 and 53 kDa) in induced strains after induction with chopped gherkin and gherkin brine in comparison with the wild type but no visible modification of protein profiles was noted in vinegar induced strains (Figure 3.15). An additional protein band at approximately 33 kDa was also visible in *S. enterica* sv. Typhimurium strain induced with chopped gherkin (Figure 3.16), while a band at approximately 59 kDa was not visible in chopped gherkin and vinegar induced strains. A protein band at approximately 37 kDa was visible in the un-induced *S. enterica* sv. Typhimurium but not in the gherkin brine induced strain (Figure 3.17). This was also found in *E. coli* after induction with gherkin brine. However, an additional band at approximately 22 kDa was visible in this induced strain.

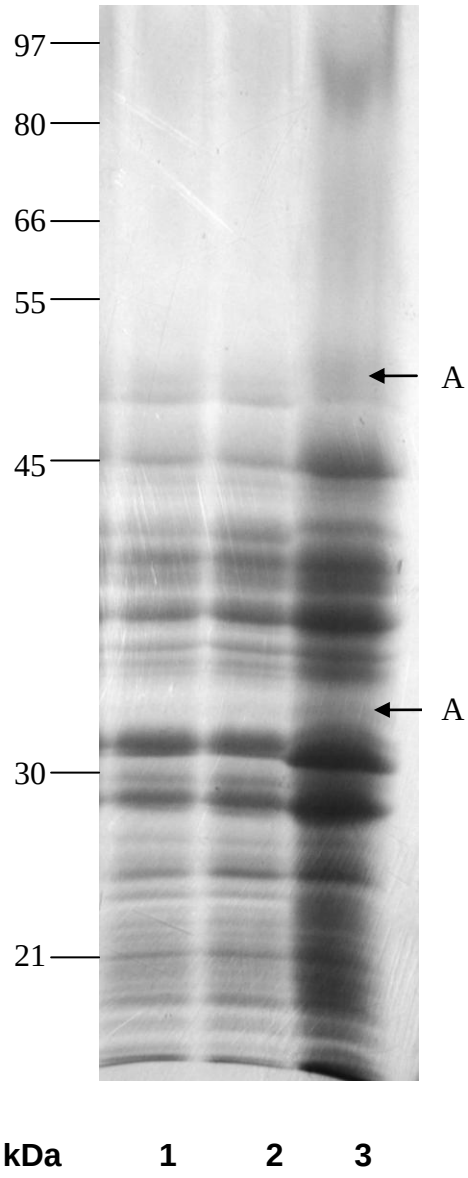

Figure 3.13: Protein profiles of induced strains of *E. coli* ATCC 25922. Lane 1: Chopped gherkin, induced strain; Lane 2: Vinegar, induced strain; Lane 3: Un-induced strain. A = protein band less visible or not at all visible in induced strain(s).

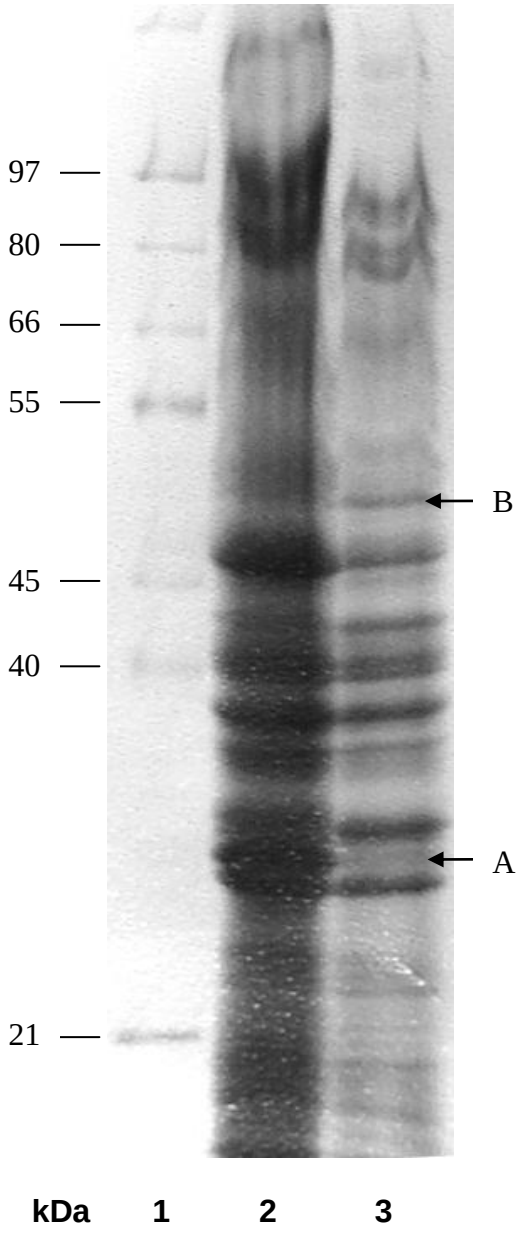

Figure 3.14: Protein profiles of induced strains of *E. coli* ATCC 25922. Lane 1: Molecular Weight Marker; Lane 2: Un-induced strain; Lane 3: gherkin brine induced strain. A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

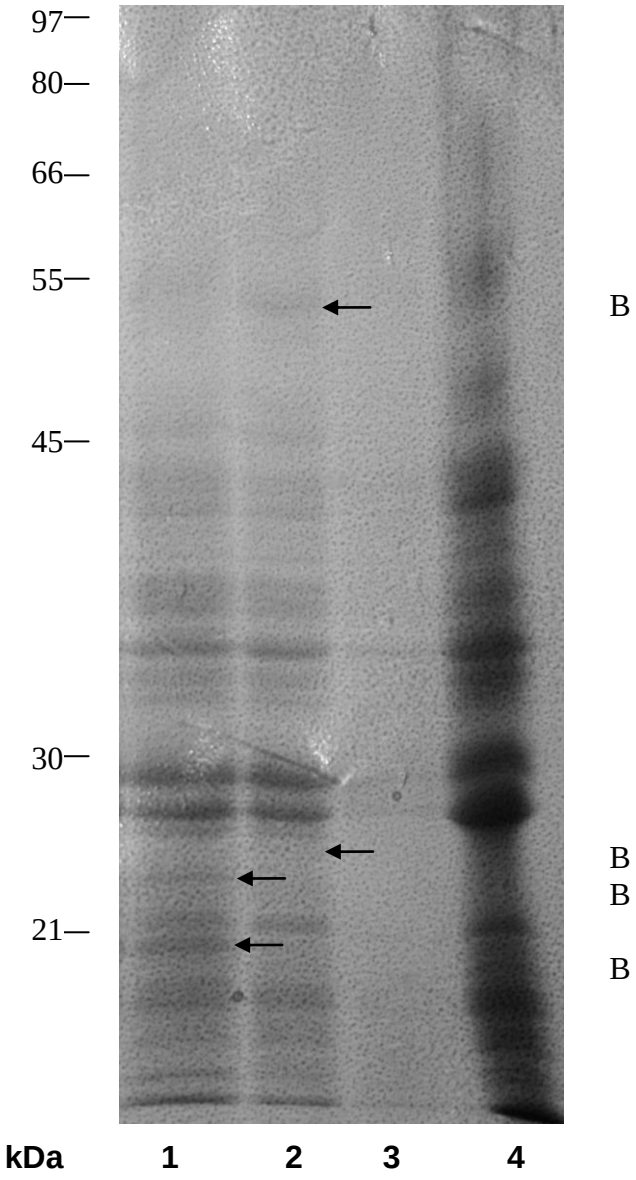

Figure 3.15: Protein profiles of induced strains of *S. enterica* sv. Enteritidis ATCC 13076. Lane 1: Gherkin brine, induced strain; Lane 2: Chopped gherkins, induced strain; Lane 3: Vinegar, induced strain; Lane 4: Un-induced strain. B = additional protein band visible in induced strain(s).

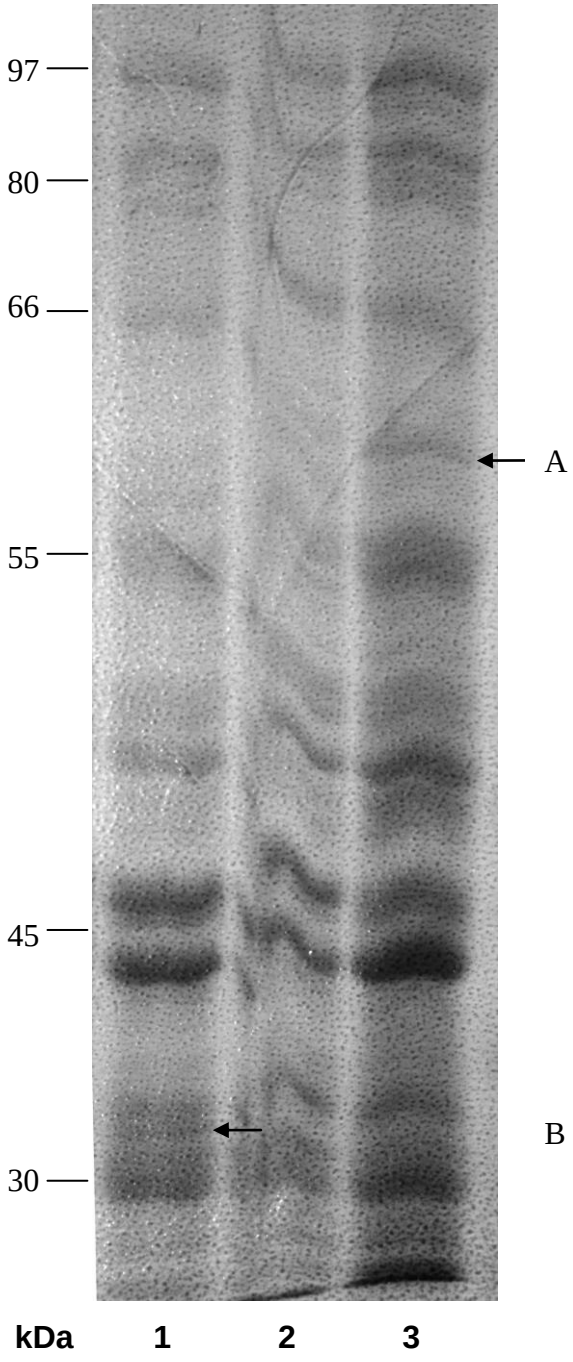

Figure 3.16: Protein profiles of induced strains of *S. enterica* sv. Typhimurium ATCC 14028. Lane 1: Chopped gherkins, induced strain; Lane 2: Vinegar, induced strain. Lane 3: Un-induced strain. A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

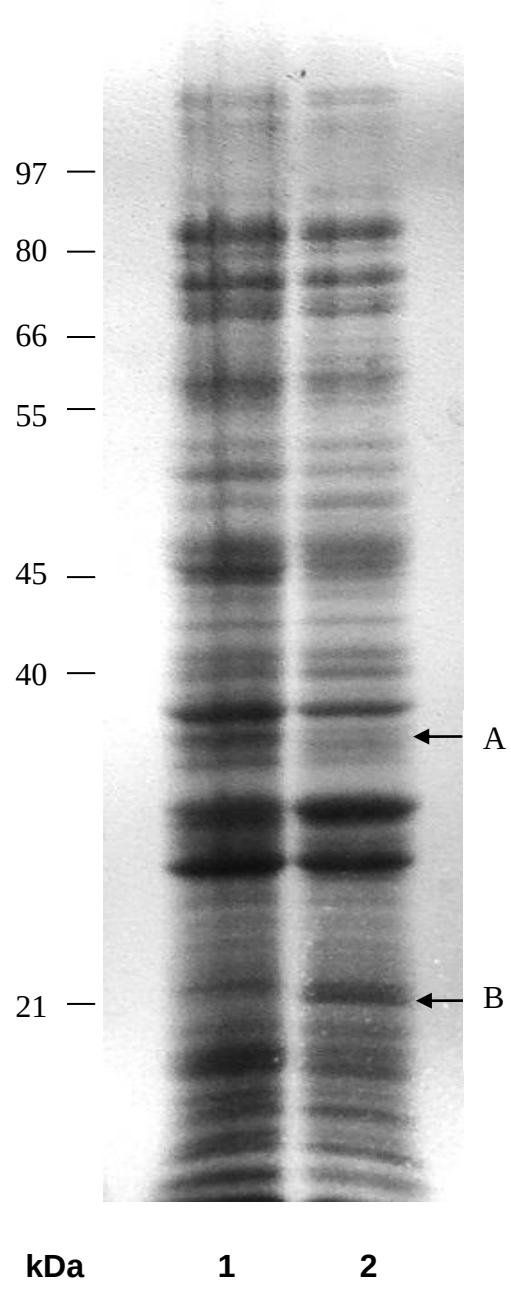

Figure 3.17: Protein profiles of induced strains of *S. enterica* sv. Typhimurium ATCC 14028. Lane 1: Un-induced strain; Lane 2: Gherkin brine, induced strain. A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

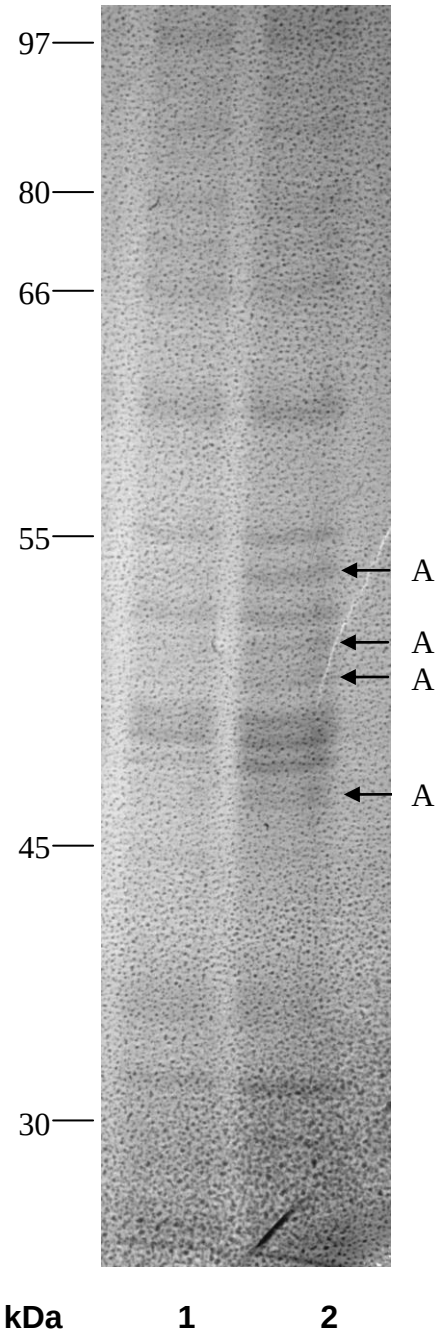

Figure 3.18: Protein profiles of induced strains of *P. aeruginosa* ATCC 27853 Lane 1: Chopped gherkins, induced strain; Lane 2: Un-induced strain. A = protein band less visible or not at all visible in induced strain(s).

Various bands (approximate sizes 47, 49, 50 and 52 kDa) were not visible in the chopped gherkin induced *P. aeruginosa* strain, when compared with the un-induced strain (Figure 3.18). This correlates with a loss of protein bands of *E. coli* and *S. enterica* sv. Typhimurium, when induced with chopped gherkins.

Although acid tolerance is not dependent on pH, it has been reported to be dependent on the growth phase of the cells (Deng *et al*., 1999). However, the ability of *S. typhimurium* to survive at extreme pH (pH 3.0) has been reported to be dependent on the acid used to acidify the growth medium (Álvarez-Ordóñez *et al*., 2009). *S. typhimurium* encounters several low pH environments during its life cycle, and the *cadBA* gene has been implicated in a system responsible for pH homeostasis during exposure to acid stress and has been shown to be composed of a complex cascade of proteins. Protein profile modification found after induction in the current study, may therefore be attributed to a similar cell process.

3.4 CONCLUSIONS

This study demonstrated the development of acid tolerance and acid resistance after exposure to acidic foodstuffs. Such tolerance may have implications in the survival of bacterial pathogens in the human acidic gastric stomach. Moreover, exposure to acidic foodstuffs resulted in various survival profiles, where not only pH value, but also the type of acidulant (foodstuff or inorganic acid) may be contributing factors in acid tolerance development.

Various alterations in bacterial protein composition were noted, indicating that the mechanisms involved in acid tolerance development may entail multiple modifications in bacterial composition and warrants further in-depth investigations.

In the determination of survival and growth characteristics of bacterial pathogens in foods the type of acid that a bacterial cell has been exposed to, as well as exposing conditions and procedures, are important in acid challenge studies. Results from this study may therefore, be useful in predicting survival and growth of an organism in acidic foods, which may provide a better understanding of factors that influence adaptation of food-associated bacteria to acid stress.

3.5 REFERENCES

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CHAPTER 4

Acid tolerance and Associated Organic Acid Susceptibility Amongst Gram-Negative Food-borne Bacteria

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4.1 INTRODUCTION

Preservation of acidic foods often requires a combination of factors where the types of acids play an important role (Hsiao and Siebert, 1999). Since ancient times low pH has been a method of food preservation and is also a natural preservation mechanism in various foodstuffs (Fielding *et al*., 1997). The categorization of food into low and high acid is also a fundamental principle of food safety while preservation treatments are applied accordingly (Nakai and Siebert, 2004). Acid foods and acidified foods are defined in the US Code of Federal Regulations (21 CFR 114) as having a pH of 4.6 or lower (e-CFR, 2010) and such products have for many years been considered safe without further treatments; primarily as a result of the organic acid(s) present in these products (Zagory and Garren, 1999). However, conclusive information is still lacking on the respective inhibitory effects of particular organic acids and pH on pathogens in these products (Breidt *et al*., 2004).

In addition to the ATR being a complex defence system, known to minimize the lethal effects of extreme low pH (pH 3), it has also been reported to defend an organism against the inhibitory activity of weak acids (Baik *et al*., 1996). Although inherent resistance as well as acquired resistance to the organic acids may influence their efficacy as antimicrobial chemicals, environmental stresses may also play an important role (Ricke, 2003). It is, therefore, imperative to determine the two-way role of organic acids in acid tolerance and bacterial inhibition. Although it is not yet clear how food protect bacteria from extreme acidic conditions, this may also be a factor to be

considered in the successful application of acidic food preservatives (Álvarez-Ordóñez *et al*, 2009). The objectives of the study were, therefore, to investigate the effect of acid tolerance induction on organic acid susceptibility amongst some common Gram negative food-borne pathogens in specific foodstuffs. Ultimately the research was aimed at shedding light on concerns that the use of organic acids as food preservatives may contribute to the emergence of acid tolerant pathogens with the ability to survive the protective barrier of the gastric environment.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial isolates

Bacterial isolates comprised species that have often been implicated in food-borne illnesses resulting from the consumption of acidic foodstuffs. These included standard bacterial strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* sv. Typhimurium ATCC 14028 and *Salmonella enterica* sv. Enteritidis ATCC 13076.

4.2.2 Induction of acid tolerance

Strains were sub-cultured in Brain-Heart Infusion (BHI) broth (Biolab Diagnostics [Pty] Ltd.) containing respective increasing concentrations of hydrochloric acid as well as a variety of acidic foodstuffs (vinegar, mayonnaise, chopped gherkins and gherkin brine) and incubated at 30˚C for 24 h. Control broths were included for monitoring pH. The lowest induction

pH of each organism and each acidic foodstuff was measured. To distinguish between acid tolerance and acid resistance, viable organisms at lowest pH levels for each induction medium (broth and foodstuffs) were inoculated onto BHI agar (pH 7 and pH 5) and incubated at 30˚C for 48 h. Induced isolates that were not able to grow at pH 5 after induction, were regarded as acid-tolerant. Cultures were harvested and stored at -80˚C.

4.2.3 Susceptibility testing

Acetic acid, benzoic acid [sodium salt], lactic acid, malic acid, propionic acid and sorbic acid [potassium salt] were obtained from MP Biomedicals, Inc. (Solon, Ohio, USA) and the minimum inhibitory concentrations (MICs) of the six organic acids for both the parent strains and induced strains were determined with an agar-dilution method, at various pH levels ranging from pH 5 to pH 7.5, as described by the Clinical and Laboratory Standards Institute (CLSI, 2006). Cell suspensions were inoculated onto the surface of Mueller-Hinton (MH) agar containing doubling organic acid concentrations (ranging from 0.25-256 mM), using a multipoint inoculator (MultipointElite, Mast Laboratories, Merseyside, UK) to deliver 1 x 10^5 CFU per spot. After 24h incubation at 35°C the MIC was recorded as the lowest concentration of organic acid where no growth was detected. All analyses were performed at least in triplicate.

4.3 RESULTS AND DISCUSSION

Acid tolerance of the four bacterial strains included in the study is illustrated in Figure 4.1 (data obtained from Chapter 2). This information is included to clarify the possible correlation between the development of acid tolerance and the reduced antimicrobial activity of organic acids. Exposure to acidic foodstuffs in addition to hydrochloric acid resulted in diverse susceptibility patterns to the organic acids (Tables 4.1-4.4).

Pseudomonas aeruginosa revealed a wide variety of changes to organic acid susceptibility and is the only strain that showed changes in MIC profiles at all test pHs, although after exposure to different acidic foodstuffs, especially after exposure to vinegar and gherkin brine. Although this organism displayed lower acid tolerance (Figure 4.1) decreased susceptibility to potassium sorbate, sodium benzoate, acetic acid and lactic acid was observed after exposure to gherkin brine.

Contrary to these findings *E. coli, S. enterica* sv. Typhimurium and *S. enterica* sv. Enteritidis, strains that more rapidly acquired acid tolerance after acidic exposure (Figure 4.1), only showed significant changes in susceptibility to the organic acids at lower pH values (pH 5-6) (Tables 4.1-4.4).

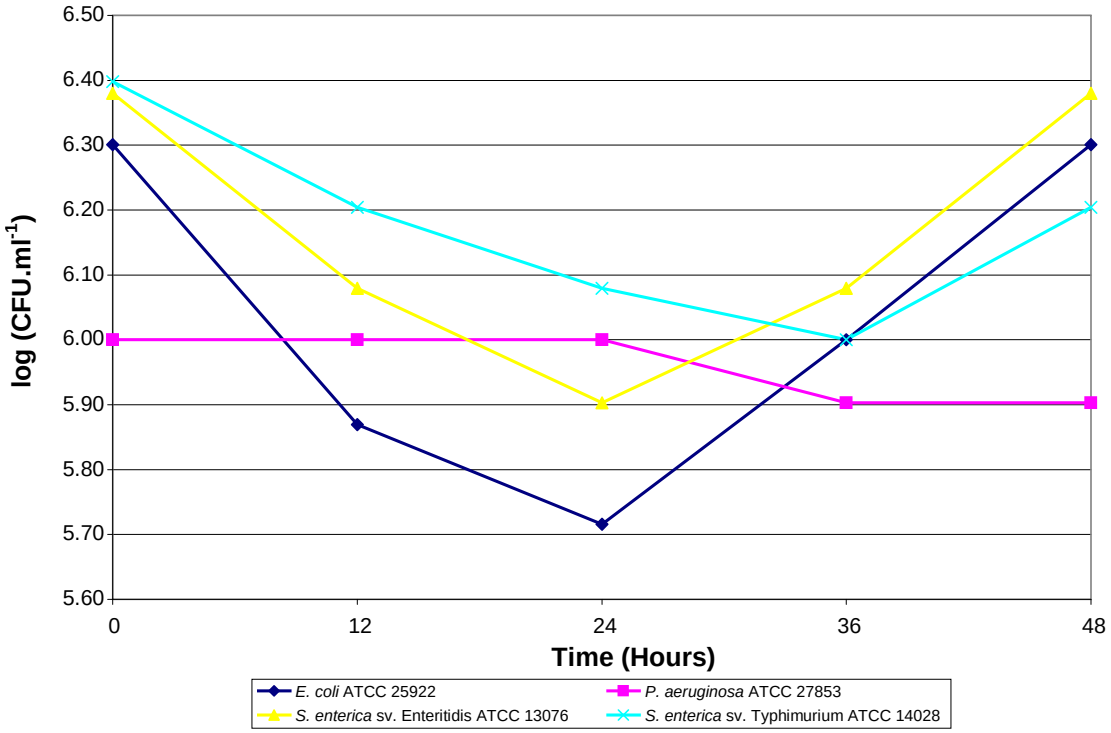

Figure 4.1: Acid tolerance in bacterial strains after exposure to HCl

Table 4.1: MICs of six organic acids for *E. coli* ATCC 25922 at various pH levels to acidic foodstuffs and HCl. SD = standard deviation.

		MIC (mM)					
		pH					
		5.0	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**
Potassium sorbate	Un-induced	8	32	64	128	256	256
	Gherkin brine	16	32	64	128	256	256
	Chopped gherkins	16	32	64	128	256	256
	Mayonnaise	16	32	64	128	256	256
	Vinegar	16	32	64	128	256	256
	HCl	16	32	64	128	256	256
	SD	**3.27**	**0**	**0**	**0**	**0**	**0**
Sodium benzoate	Un-induced	4	8	32	64	128	256
	Gherkin brine	4	8	32	64	128	256
	Chopped gherkins	4	8	32	64	128	256
	Mayonnaise	4	8	32	64	128	256
	Vinegar	4	8	32	64	128	256
	HCl	4	8	32	64	128	256
	SD	**0**	**0**	**0**	**0**	**0**	**0**
Acetic acid	Un-induced	8	8	16	16	32	32
	Gherkin brine	8	8	16	16	16	32
	Chopped gherkins	4	8	16	16	16	32
	Mayonnaise	4	8	16	16	32	32
	Vinegar	4	8	16	16	32	32
	HCl	8	8	16	16	32	32
	SD	**2.19**	**0**	**0**	**0**	**8.26**	**0**
Lactic acid	Un-induced	64	64	64	128	256	>256
	Gherkin brine	64	64	64	128	256	>256
	Chopped gherkins	64	64	64	128	256	>256
	Mayonnaise	64	64	64	128	256	>256
	Vinegar	64	64	64	128	256	>256
	HCl	64	64	64	128	256	>256
	SD	**0**	**0**	**0**	**0**	**0**	**-**
Malic acid	Un-induced	16	16	32	32	32	32
	Gherkin brine	16	32	32	32	32	32
	Chopped gherkins	16	32	32	32	32	32
	Mayonnaise	16	32	32	32	32	64
	Vinegar	16	16	32	32	32	32
	HCl	16	16	32	32	32	32
	SD	**0**	**8.76**	**0**	**0**	**0**	**13.06**
Propionic acid	Un-induced	8	8	16	16	32	32
	Gherkin brine	4	8	16	16	32	32
	Chopped gherkins	8	8	16	16	32	32
	Mayonnaise	4	8	16	16	32	32
	Vinegar	4	8	16	16	32	32
	HCl	8	8	16	16	32	32
	SD	**2.19**	**0**	**0**	**0**	**0**	**0**

Table 4.2: Minimum inhibitory concentrations of six organic acids for *S. enterica* sv. Enteritidis ATCC 13076 at various pH levels to acidic foodstuffs and HCl. SD = standard deviation.

		MIC (mM)					
		pH					
		5.0	5.5	6.0	6.5	7.0	7.5
Potassium sorbate	Un-induced	8	16	64	128	>256	>256
	Gherkin brine	8	32	64	128	>256	>256
	Chopped gherkins	8	16	64	128	256	>256
	Mayonnaise	8	16	64	128	256	>256
	Vinegar	8	16	64	128	>256	>256
	HCl	8	16	64	64	>256	>256
	SD	**0**	**6.53**	**0**	**26.13**	**0**	**-**
Sodium benzoate	Un-induced	4	8	32	64	128	>256
	Gherkin brine	4	8	32	64	128	>256
	Chopped gherkins	4	8	32	64	128	>256
	Mayonnaise	4	8	32	64	128	>256
	Vinegar	4	8	32	64	128	>256
	HCl	4	8	32	64	128	>256
	SD	**0**	**0**	**0**	**0**	**0**	**-**
Acetic acid	Un-induced	4	8	8	16	32	32
	Gherkin brine	4	8	16	16	32	32
	Chopped gherkins	4	8	16	16	16	32
	Mayonnaise	8	8	16	16	16	32
	Vinegar	8	8	16	16	32	32
	HCl	8	8	16	16	32	32
	SD	**2.19**	**0**	**3.27**	**0**	**8.26**	**0**
Lactic acid	Un-induced	64	64	64	128	128	>256
	Gherkin brine	64	64	64	128	128	>256
	Chopped gherkins	64	64	128	256	256	>256
	Mayonnaise	64	64	128	256	256	>256
	Vinegar	64	64	64	128	256	>256
	HCl	64	64	128	128	256	>256
	SD	**0**	**0**	**35.05**	**0**		**-**
Malic acid	Un-induced	32	32	64	32	64	64
	Gherkin brine	32	32	64	64	64	64
	Chopped gherkins	32	32	64	64	64	64
	Mayonnaise	32	32	64	64	64	64
	Vinegar	32	32	64	64	64	64
	HCl	32	32	64	32	64	64
	SD	**0**	**0**	**0**	**16.52**	**0**	**0**
Propionic acid	Un-induced	8	8	8	16	32	16
	Gherkin brine	4	8	16	16	32	16
	Chopped gherkins	4	8	16	16	32	16
	Mayonnaise	8	8	16	16	32	16
	Vinegar	8	8	16	16	32	16
	HCl	8	8	16	16	32	16
	SD	**2.07**	**0**	**3.27**	**0**	**0**	**0**

Table 4.3: Minimum inhibitory concentrations of six organic acids for *S. enterica* sv. Typhimurium ATCC 14028 at various pH levels to acidic foodstuffs and HCl. SD = standard deviation.

		MIC (mM)					
		pH					
		5.0	5.5	6.0	6.5	7.0	7.5
Potassium sorbate	Un-induced	16	64	64	128	>256	>256
	Gherkin brine	16	64	128	128	>256	>256
	Chopped gherkins	16	64	128	128	>256	>256
	Mayonnaise	16	64	64	128	256	256
	Vinegar	16	64	128	256	>256	>256
	HCl	16	64	128	128	256	256
	SD	**0**	**0**	**33.05**	**52.26**	**0**	**0**
Sodium benzoate	Un-induced	8	16	32	64	128	256
	Gherkin brine	8	16	32	64	128	256
	Chopped gherkins	8	16	32	64	128	256
	Mayonnaise	8	16	32	64	128	256
	Vinegar	8	16	32	64	128	256
	HCl	8	16	32	64	128	256
	SD	**0**	**0**	**0**	**0**	**0**	**0**
Acetic acid	Un-induced	4	8	16	16	32	32
	Gherkin brine	8	8	16	16	32	32
	Chopped gherkins	8	8	16	16	32	32
	Mayonnaise	8	8	16	16	32	32
	Vinegar	8	8	16	16	16	32
	HCl	8	8	16	16	16	32
	SD	**1.63**	**0**	**0**	**0**	**8.26**	**0**
Lactic acid	Un-induced	64	64	64	128	256	>256
	Gherkin brine	64	64	128	256	>256	>256
	Chopped gherkins	64	64	128	256	>256	>256
	Mayonnaise	64	64	128	256	>256	>256
	Vinegar	64	64	128	>256	>256	>256
	HCl	64	64	128	256	>256	>256
	SD	**0**	**0**	**26.13**	**-**	**-**	**-**
Malic acid	Un-induced	32	32	64	32	64	64
	Gherkin brine	32	32	64	64	64	64
	Chopped gherkins	32	32	64	32	64	64
	Mayonnaise	32	32	64	64	64	64
	Vinegar	32	32	64	64	64	64
	HCl	32	32	64	64	64	128
	SD	**0**	**0**		**16.52**	**0**	**26.13**
Propionic acid	Un-induced	2	4	8	16	16	16
	Gherkin brine	2	4	8	16	16	16
	Chopped gherkins	2	4	8	16	16	16
	Mayonnaise	4	4	8	16	16	32
	Vinegar	2	4	8	16	16	16
	HCl	2	4	8	16	16	16
	SD	**0.82**	**0**	**0**	**0**	**0**	**6.53**

Table 4.4: Minimum inhibitory concentrations of six organic acids for *Pseudomonas aeruginosa* ATCC 27853 at various pH levels to acidic foodstuffs and HCl. SD = standard deviation.

		MIC (mM)					
		pH					
		5.0	5.5	6.0	6.5	7.0	7.5
Potassium sorbate	Un-induced	16	32	128	256	>256	>256
	Gherkin brine	32	128	256	>256	>256	>256
	Chopped gherkins	64	128	>256	>256	>256	>256
	Mayonnaise	16	64	256	>256	>256	>256
	Vinegar	8	32	64	128	256	256
	HCl	32	128	256	>256	>256	>256
	SD	**20.08**	**48.18**	**-**	**-**	**-**	**-**
Sodium benzoate	Un-induced	8	16	64	128	256	256
	Gherkin brine	16	32	128	256	256	>256
	Chopped gherkins	16	64	128	256	256	>256
	Mayonnaise	8	32	128	256	256	256
	Vinegar	4	8	32	64	128	256
	HCl	8	64	128	256	256	>256
	SD	**4.9**	**23.6**	**42.53**	**85.07**	**52.26**	**-**
Acetic acid	Un-induced	4	4	8	16	16	32
	Gherkin brine	8	8	16	16	32	32
	Chopped gherkins	2	4	8	16	16	32
	Mayonnaise	2	4	8	16	16	32
	Vinegar	8	8	16	16	32	32
	HCl	2	4	8	16	16	32
	SD	**2.94**	**2.07**	**4.13**	**0**	**8.26**	**0**
Lactic acid	Un-induced	16	64	64	64	256	>256
	Gherkin brine	64	64	64	128	256	>256
	Chopped gherkins	16	64	64	64	256	>256
	Mayonnaise	16	32	64	64	256	>256
	Vinegar	64	64	64	128	128	>256
	HCl	16	32	64	64	256	>256
	SD	**24.79**	**16.52**	**0**	**33.05**		**-**
Malic acid	Un-induced	8	16	8	16	32	32
	Gherkin brine	8	16	8	16	32	32
	Chopped gherkins	8	8	8	16	32	32
	Mayonnaise	8	16	8	16	32	32
	Vinegar	16	16	32	32	32	32
	HCl	8	16	8	16	32	32
	SD	**3.27**	**3.27**	**9.8**	**6.53**	**0**	**0**
Propionic acid	Un-induced	2	4	8	16	16	32
	Gherkin brine	4	8	16	16	32	32
	Chopped gherkins	2	4	8	16	16	16
	Mayonnaise	2	4	8	16	16	16
	Vinegar	8	8	16	16	32	32
	HCl	2	4	8	16	16	16
	SD	**2.42**	**2.07**	**4.13**	**0**	**8.26**	**8.76**

E. coli showed differences only at pH 5 and 5.5 and more specifically to potassium sorbate (decreased susceptibility after exposure to all foodstuffs and HCl) (16 mM vs 8 mM) at pH 5 and propionic acid (increased susceptibility after exposure to gherkin brine, mayonnaise and vinegar) (4 mM vs 8 mM) at pH 5 and to malic acid (decreased susceptibility after exposure to gherkin brine, chopped gherkin and mayonnaise) (32 mM vs 16 mM) at pH 5.5. At pH 5 *S. enterica* sv. Enteritidis showed increased susceptibility to propionic acid after exposure to gherkin brine and chopped gherkins (4 mM vs 8 mM), and decreased susceptibility to acetic acid after exposure to mayonnaise, vinegar and HCl (8 mM vs 4 mM). *S. enterica* sv. Typhimurium showed changes in susceptibility (decrease) after exposure to all the foodstuffs and HCl only to acetic acid at pH 5 (8 mM vs 4 mM) and to lactic acid (128 mM vs 64 mM) at pH 6.

It has been reported that although acetic acid and sorbic acid has the same pK_a value, a 10-fold higher concentration may be necessary to produce the same effect as sorbic acid in inhibiting microorganisms (Papadimitriou *et al*., 2007). However, in the current study acetic acid evidently demonstrated much higher activity than potassium sorbate, the salt derivative of sorbic acid (Tables 4.1-4.4). Moreover, acetic acid has been found to be more inhibitory than lactic acid, due to its higher pK_a value and it has also been demonstrated that acetic acid can significantly decrease the survival time for the *E. coli* strains tested at a given pH when compared with effects attributed to pH alone (Røssland *et al*., 2005; Breidt *et al*., 2004).

Lactic acid is known for its wide industrial applications, being classified as GRAS by the FDA. This acid is, therefore, often used in foods as acidulant, flavouring agent, pH buffering agent, or preservative (Valli *et al*., 2006). In a previous study done by Buchanan *et al.* (2004) with a pH range between 4.0 and 5.5 it was found that among five acidulants (lactic, acetic, citric, malic and hydrochloric acids) lactic acid had the greatest consistent activity against enterohemorrhagic *E. coli,* while HCl had the least. However, in the current study, lactic acid did not live up to its expectation as it was least effective in inhibiting any of the four organisms (Tables 4.1-4.4). In the same study by Buchanan *et al*. (2004) cultures were found to be least affected by acetic acid and most affected by malic acid. This was also not applicable to the current study, as acetic acid and propionic acid had the highest activity against all the organisms, followed by malic acid and sodium benzoate (Tables 4.1-4.4).

Although sorbic acid and its salts have several advantages as food preservatives and are also considered harmless, sodium benzoate has been reported to demonstrate higher inhibitory activity against *E. coli* O157:H7 populations in apple cider than potassium sorbate (Comes and Beelman, 2002; González-Fandoz and Dominguez, 2007). This was also found in the current study, especially at pH 5 and 5.5 (Table 4.1). In food preservation organic acids are often applied in their sodium, potassium or calcium form since this application is often more practical as these salts are much more readily soluble in water (Gauthier, 2005). In this study the salts sodium benzoate and potassium sorbate were included as opposed to benzoic acid and sorbic acid.

Although the mechanism by which organic acids inhibit growth has not yet been fully elucidated, it is known that when concentrated within cells they reduce the internal pH below normal physiological range tolerated by the cell and growth is inhibited as a result of destabilisation of proteins (Kasemets *et al*., 2006). In acidic food the low pH increases the proportion of un-dissociated acid present (Adams and Nicolaides, 1997). These weak acids enter cells in their uncharged, un-dissociated, protonated form, after which they are deprotonated internally, thereby lowering the intracellular pH (Price-Carter *et al*., 2005). This acidification of cell cytoplasm has for many years been assumed to be the primary bactericidal action of organic acids. Another explanation is the accumulation of acid anion in the interior of bacterial cells, because of an internal pH that is higher than the external environment (Breidt *et al*., 2004).

The results from the organic acid susceptibility testing demonstrated varying responses from the four different bacterial strains when exposed to low pH environments and acidic foodstuffs and no specific relationship was noted between the type of foodstuff and the effectiveness of a specific organic acid. It was however, evident that decreased susceptibility occurred in each organism to at least one organic acid tested and after exposure to at least one of the acidic foodstuffs (Tables 4.1-4.4).

Organic acids are generally considered more effective against food-borne pathogens than hydrochloric acid. This assumption originates from the fact that antimicrobial activity is associated with the anion portion of the acid

molecule and may also vary among different organic acids (Buchanan *et al*., 2004). The total inhibitory action of organic acids is, however dependent on the combined effects from un-dissociated molecules as well as dissociated ions. These multifunctional effects are dependent on time and temperature of exposure, microbial strains, composition of assay medium as well as the kind of organic acid, its concentration and pK_a, and of course the pH of the environment (Taniguchi *et al*., 1998). Some organic acids may also enter the cell more easily than others and alter the pH_i of the cell more readily (Greenacre *et al*., 2003). It is important to remember that the concentration of un-dissociated form of organic acid and pH are interdependent variables, linked by the Henderson-Hasselbalch equation (Breidt *et al*., 2004):

$$[H^+] = K_a \frac{[HA]}{[A^-]} \qquad \text{or} \qquad pH = pKa + \log\left(\frac{[HA]}{[A^-]}\right)$$

Where: HA = acid

A^- = conjugate base

The Henderson-Hasselbalch equation (or buffer equation) is used to describe the derivation of pH as a measure of acidity in biological and chemical systems, by employing the acid dissociation constant (pK_a).

Acid tolerance development has on various occasions been demonstrated to protect *Salmonella* against the lethal effects of organic acids and to subsequently increase their survival in fermented foods (Ricke, 2003). In an

investigation by Baik *et al*. (1996) it was attempted to induce resistance to weak acids by these acids themselves in a sub-lethal concentration of organic acid and to determine if acid shock is required to induce resistance to organic acids. It was established that none of these growth conditions resulted in an increase in resistance to the organic acids at low pH and that acid shock adaptation was indeed required for development of such resistance (Baik *et al*., 1996).

In the current study acid adaptation did not appear to cause significant decreased susceptibility to the organic acids, except for *S. enterica* sv. Typhimurium at pH 5 against acetic acid (Table 4.3) and *E. coli* at pH 5 against potassium sorbate (Table 4.1). The process of acid adaptation of microorganisms is complex and many physiological changes take place, including stress proteins being expressed and also damage to cell membranes (Leyer and Johnson, 1993). The degree of acid tolerance is dependent on the nature of the physiological changes as well as the intensity of the stress factors. In some cases the effects of cellular damage might, however, exceed the shielding effect of acid-shock proteins or other protective metabolic changes induced by low pH, and stressed cells could die if exposed to more harsh environments (Deng *et al*., 1999). It would be worth investigating possible alterations in the cell membrane proteins, specifically after acid induction.

On the other end of the scale organic acids have been observed to enhance survivability of acid sensitive pathogens by induction of an acid tolerance

response and that this tolerance may be linked to increased virulence. Such a situation may have serious implications regarding the use of organic acids, although this may only be applicable to situations where reduced acid levels have induced resistance in exposed organisms (Ricke, 2003). In addition to the preservative function organic acids may also significantly affect the flavour and quality of food (Yang and Choong, 2001) and US FDA regulations for acidified foods currently do not take into account the amount or type of organic acid that is needed to lower the pH (Bjornsdottir *et al*., 2006). It may be necessary to investigate the implication of such a two-way resistance development in food-borne pathogens.

4.4 CONCLUSIONS

Although acid-adapted cells are known to be more resistant under various stress conditions, in the current study this was not obvious in their susceptibility to the organic acids. However, it remains imperative that organic acids be carefully applied and pH of foodstuffs monitored for effective food preservation and safety control. It is also essential to consider the extent to which bacteria can withstand stress when attempting to provide effective barriers. The extent of tolerance that Gram-negative pathogenic and spoilage bacteria can develop and the underlying control of stress responses are ongoing areas of investigation and have not yet been elucidated. It is also necessary to determine the extent of the influence of acid tolerance on the sustainability of organic acids as food preservatives in acid foodstuffs. The

increased inhibitory activity of organic acids at lower pH values was evident in this study and this should be further investigated in acid susceptible strains to determine if this inhibition is the result of a lower pH or more specifically the activity of the organic acids.

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CHAPTER 5

The Effect of Acid Exposure on Selected Phenotypic Characteristics of Psychrotrophic Food-borne Bacteria

5.1 INTRODUCTION

Bacteria have developed variety of strategies for protection against acid stress (Barua *et al*., 2002). For example, enhanced extreme acid resistance have been reported for non-pathogenic *E. coli* as well as extreme acid resistant *E. coli* 0157:H7 after exposure to butyric acid and propionic acid (Guilfoyle and Hershfield, 1996), while benzoate may be less effective in endorsing survival of *E. coli* O157:H7 than acetate (Diez-Gonzalez and Russell, 1999).

Flavobacteria and pseudomonads are conventionally acknowledged as food spoilage bacteria (Forsythe, 2000). Flavobacteria is the name generally used in literature when describing yellow pigmented rods (Hendrie *et al*., 1969). *Salmonella* and *Campylobacter* are two pathogens normally allied with poultry, but numerous other bacteria known to cause food spoilage are also found on poultry carcasses. These comprise the alleged flavobacteria accountable for causing food spoilage and that could originate from either the poultry meat product itself or from processing in the slaughterhouse (Hang'ombe *et al*., 1999). Some studies support this when the occurrence of members of the genus *Pseudomonas* and flavobacteria on chicken carcasses was reported to be 17 and 16 % respectively (Mai and Conner, 2001).

The first identification of the *Flavobacterium* genus was done in 1923. This included the unsporulating Gram-negative rods associated with the production of yellow pigments (Holmes *et al*., 1984; Jooste and Hugo, 1999). Some members of the *Flavobacterium* have also for years been classified as

pathogens causing a variety of infections including nosocomial infections, bacteraemia as well as meningitis (Siegman-Igra *et al*., 1987).

The existence and effects of food-borne pathogens on food systems as well as in the human body are common food safety issues globally (Odumeru *et al.*, 1999). Detection and identification of bacterial pathogens present in food samples require rapid and accurate methods, which is not just vital for food quality assurance but also for monitoring and tracing of outbreaks of pathogens in the food chain (Odumeru *et al*., 1999). Food microbiology and clinical laboratories make extensively use of automated microbial identification systems, which provide many advantages over conventional methods. One such conventional method, which has been used over decades, is the rapid presumptive identification method used on pigments from certain bacterial strains that yields characteristic colour reactions when treated with various acids and bases (Jones and Watkins, 1973). Important to note is that the exposure of these pigments to acids and bases occurs after the pigments have already been produced and not during the growing phase of bacterial cells. Reports from studies such as Christakis *et al.* (2005), focusing on isolating and identifying pathogens, confirmed that yellow-pigmented colonies producing the flexirubin type of pigment changes to a red colour if exposed to an alkaline solution (such as 10% KOH). Jones and Watkins (1973) also reported that yellow pigmented surface growths produced colour changes only on exposure to strong bases and acids and that colour changes are not produced while growing cells were exposed to weak bases and acids.

Organic acids, also known to be weak acids, are used as preservatives in the food industry. The antimicrobial effect of organic acids as well as their salts and the preservative effect of these compounds during the food storage enjoyed much attention during the last couple of years, especially in the meat industries such as pork, beef and poultry (Bogaert and Naidu, 2000). The US Food and Drug Administration regard these preservatives as GRAS (Generally Recognized as Safe) and these agents are used in a variety of food systems. However, concern has been expressed that decontamination and preservation with organic acids and their salts could render bacterial pathogens more acid tolerant, which could play an important role in the virulence of the pathogen (Bjornsdottir *et al*., 2006). Cells that have undergone acid-adaptation or acid-shock have been reported to differ in their resistance to thermal stress (Ryu and Beuchat, 1999). It would be interesting, therefore, to investigate the influence of acid exposure on the bacterial cell.

The aim of this study was to investigate the effect of acids on psychrotrophic bacteria in order to understand the implication of preservation with organic acids under refrigerating conditions. Objectives therefore, were to investigate possible morphological changes associated with the bacterial cell after exposure to low pH and to compare to resulting structural changes in the cell.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial isolates

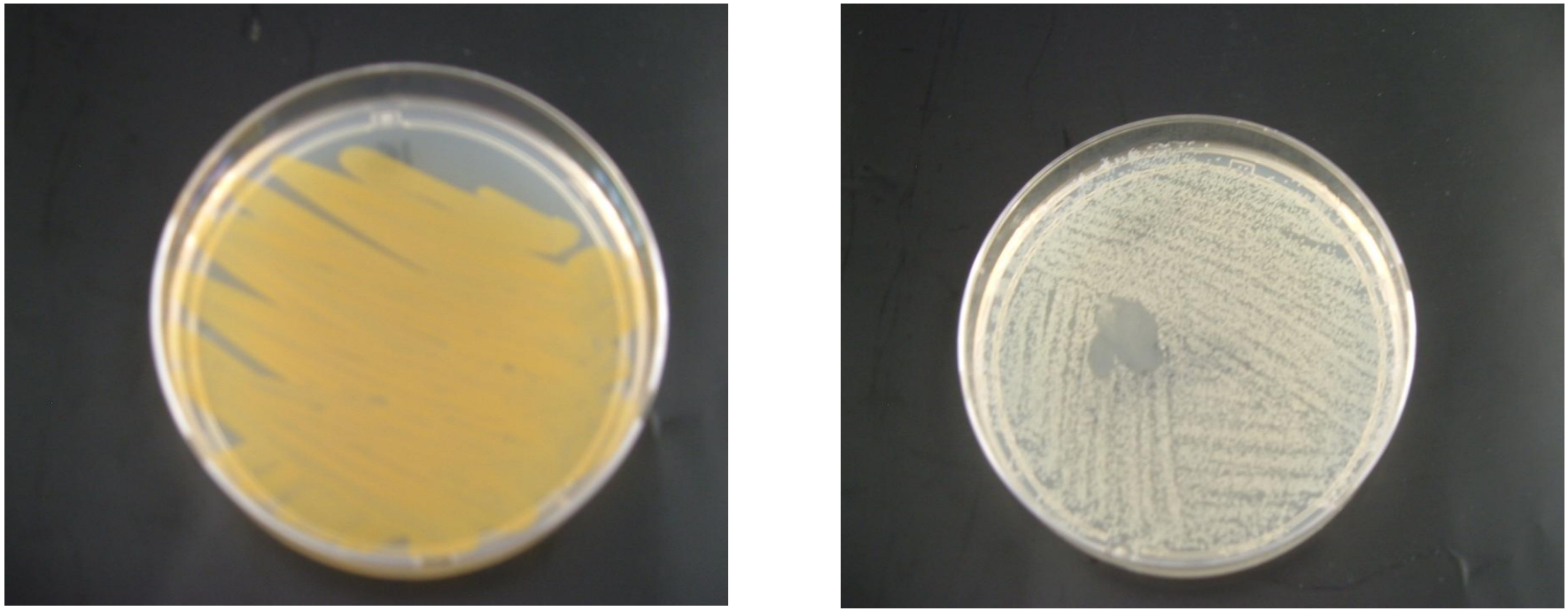
The same isolates that were screened for acid tolerance in Chapter 2 were included in this investigation. All the *Chryseobacterium* spp., in particular, were selected for observation of possible morphological changes in colony formation. *Chryseobacterium* was previously classified under the genus *Flavobacterium*.

5.2.2 Protein studies

Protein profiles of the unexposed and exposed cells were generated by SDS-PAGE and the procedure followed was similar to that described in Chapter 3. Protein profiles were compared and any alterations in protein band composition were recorded. All analyses were performed at least in triplicate.

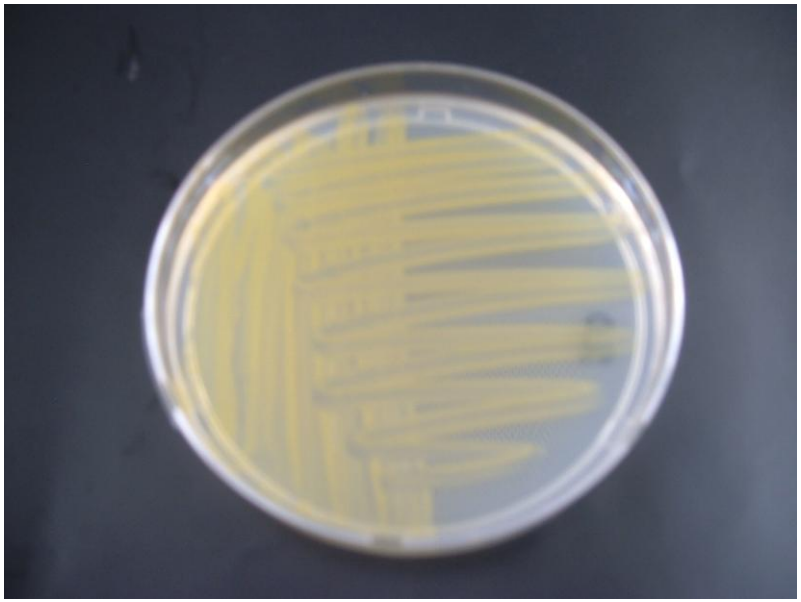
5.3 RESULTS AND DISCUSSION

After acid exposure a colour change from the characteristic bright yellow pigment to white was observed in the bacterial colonies of *Chryseobacterium defluvii* LMG 22469, *Chryseobacterium gleum* LMG 8334, *Chryseobacterium indoltheticum* LMG 4025, *Chryseobacterium joostei* LMG 18212, *Chryseobacterium piscium* LMG 23089, *Chryseobacterium vrystaatense* LMG 22846 and *Chryseobacterium scophthalmus* LMG13028. Some of these colour changes are pictured in Figures 5.1-5.3.

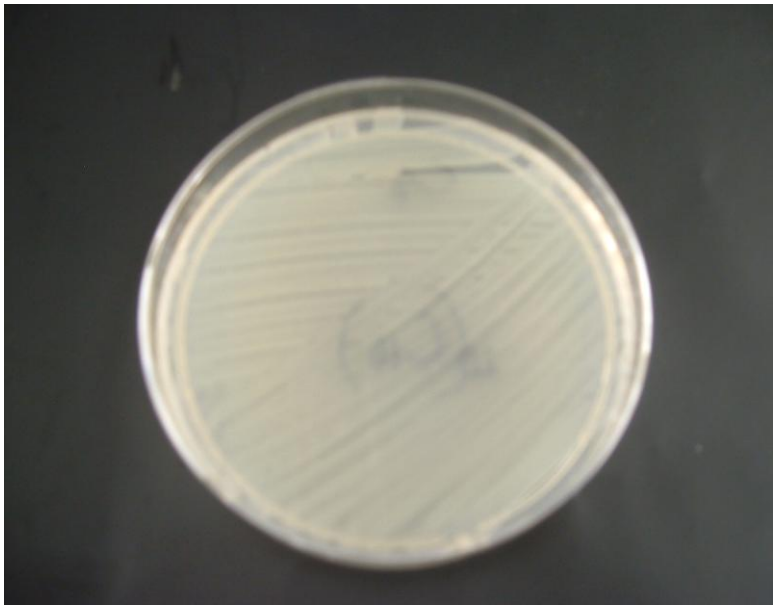


BEFORE ACID EXPOSURE *AFTER 24H ACID EXPOSURE*

Figure 5.1: Colour changes in colonial growth of *Chryseobacterium gleum* LMG 8334 colonies after 24 hours of acid exposure to 3M HCl.



BEFORE ACID EXPOSURE



AFTER 12H ACID EXPOSURE

Figure 5.2: Colour changes in colonial growth of *Chryseobacterium piscium* LMG 23089 colonies after 12 hours of acid exposure to 3M HCl.

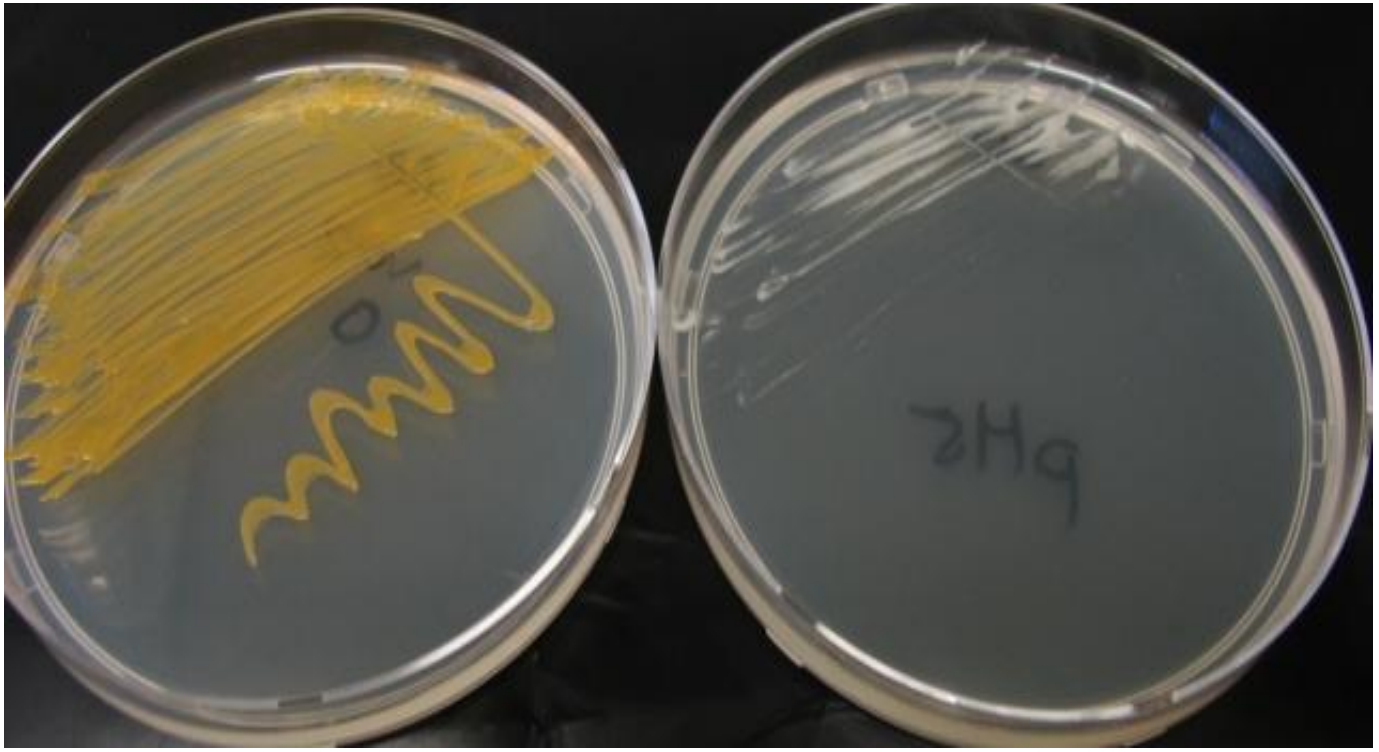


Figure 5.3: *Chryseobacterium defluvii* LMG 22469 exposed to 3M HCl

No noticeable colour changes were observed in *Chryseobacterium balustinum* LMG 8329 and *Chryseobacterium indologenes* LMG 8337 as these colonies remained yellow. *Chryseobacterium* spp. that displayed no colour changes during the acid exposure also showed higher total viable counts than in those that displayed colour changes (Table 5.1). For a summarised version of the graphs illustrating total viable counts after acid challenge (data from Chapter 2), refer to Appendix A (Figures A1-A3).

Table 5.1: Total viable counts of *Chryseobacterium* spp. and *Pseudomonas aeruginosa*, after screening for acid tolerance, at 0, 12, 24, 36 and 48 hours (Data from Chapter 2).

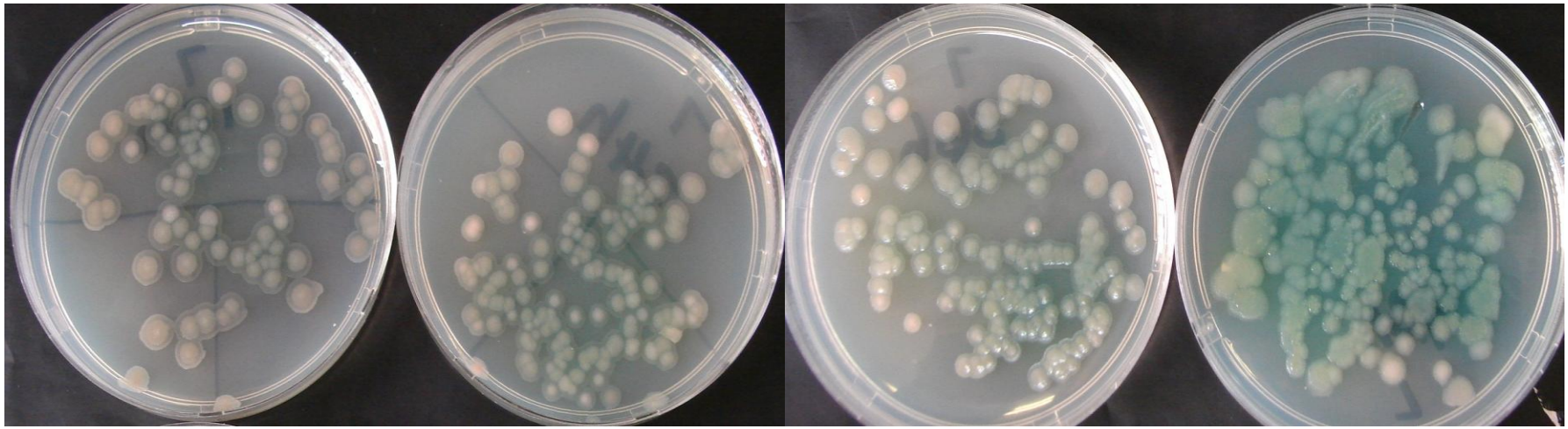
Bacterial Isolate	Total Viable Counts (CFU.ml^{-1})				
	0 h	**12 h**	**24 h**	**36 h**	**48 h**
**C. gleum* LMG 8334	2.4×10^6	3.7×10^5	4×10^4	1×10^4	0
**C. vrystaatense* LMG 22846	1.2×10^6	1.2×10^6	1×10^4	1×10^4	0
**C. joostei* LMG 18212	1×10^6	1.5×10^5	6×10^4	0	0
**C. piscium* LMG 23089	1×10^6	1×10^4	0	0	0
**C. indoltheticum* LMG 4025	1.6×10^6	0	0	0	0
**C. scophthalmus* LMG 13028	1.2×10^6	0	0	0	0
**C. defluvii* LMG 22469	5×10^5	0	0	0	0
C. indologenes LMG 8337	2×10^6	7.8×10^5	4.8×10^5	3×10^5	2.7×10^5
C. balustinum LMG 8329	8×10^6	2.8×10^6	2.4×10^6	2×10^6	1.6×10^6
P. aeruginosa ATCC 27853	3×10^6	3×10^6	7×10^5	5×10^5	5×10^5

* *Chryseobacterium* strains where a colour change (from yellow to white) was observed in colony growth.

The colonies of *Pseudomonas aeruginosa* demonstrated a brighter green colour after acid exposure (Figure 5.4). Although this observation may be significant, various factors may also be implicated in such a colour reaction. However, the total viable counts of the organisms remained unchanged for 24 hours after acid challenge, with a slight decrease between 24 and 36 hours (Table 5.1). From 36 hours to 48 hours after acid exposure, the total viable counts remained constant.

In Figures 5.5 and 5.6 protein profiles of isolates before acid challenge are compared with the resulting acid-tolerant strains. In *C. gleum* LMG 8334 and *C. piscium* LMG 23089 additional bands were visible in the acid exposed strains (at approximately 60 kDa), while this additional band was less visible in *C. indoltheticum* LMG 4025. These additional protein bands may have been produced in response to acid-stress. All these isolates also demonstrated a colour change from yellow to white.

In *C. balustinum* LMG 8329, where no colour change was observed, the protein bands remained similar in both the unexposed and the exposed cells (Figure 5.5). However, in *C. indologens* LMG 8337, where the yellow colour was also unaltered in the exposed cells, a protein band (at approximately 60 kDa) was less visible or lost in the exposed cells. In *C. defluvii* LMG 22469, which exhibited a colour change (Figure 5.3), a protein band was also less visible or not produced (at approximately 67 kDa). The protein bands that were not so prominent after acid exposure may be an indication of a suppression of protein production in response to acid exposure.



12 h **24 h** **36 h** **48 h**

Figure 5.4: Colour changes in colonial growth of *Pseudomonas aeruginosa* ATCC 27853 colonies after acid exposure to 3M HCl.

It is evident that the colour changes in some of the strains were the result of a bacterial response to acid exposure. This may also indicate a defence mechanism of the bacteria against acid shock, since the isolates that did not show any colour change, appeared to be more acid tolerant. The colour change that was observed during culturing (after acid exposure) differs from the colour change from yellow to red in colonies producing flexirubin type of pigment (as described in the Introduction, page 125), as this change in colour is observed as a result of growth in an acidic environment and not as a result of the surface addition of an alkaline solution.

In a study on the phylogeny of *Chryseomonas* and *Pseudomonas* done in 1997, the 16S rRNA sequences of the two genera were compared. Similarities were found, which indicated a strong relationship between the two genera (Anzai *et al*., 1997). Both genera are also known for their characteristic pigmentation. *P. aeruginosa* produces two types of soluble pigments pyoverdin and pyocyanin. Pyoverdin is a fluorescent blue-green pigment, and pyocyanin is a blue pigment (Todar, 2008). The latter has been reported as a virulence determinant of this organism. *P. aeruginosa* is also naturally resistant to various antibiotics, but not much is known about its reaction to acidic stresses. However, this organism is a natural inhabitant of soil, water and vegetation, in association with the actinomycetes and also fungi, and as a result, has developed resistance to a wide range of environmental factors and natural antibiotics. These factors are likely to have an impact on the structure and also pigmentation of the cell.

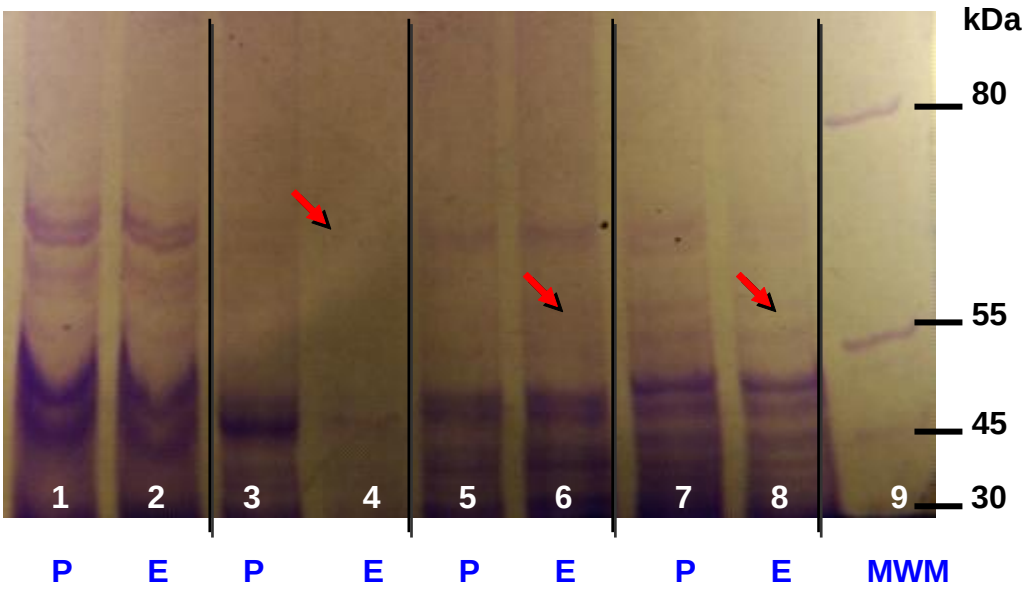


Figure 5.5: Protein profiles of *Chryseobacterium* spp. before and after exposure to 3M HCl respectively (P = parent strain and E = exposed strain). Arrows indicate alterations in protein bands detected.

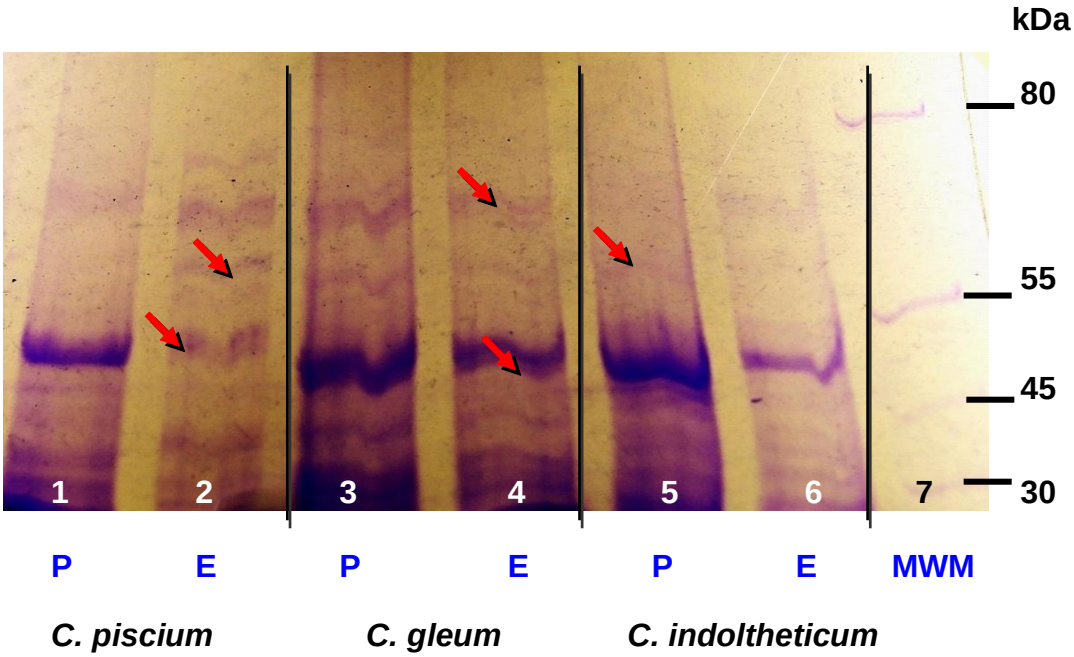


Figure 5.6: Protein profiles of *Chryseobacterium* spp. before and after exposure to 3M HCl respectively (P = parent strain and E = exposed strain). Arrows indicate alterations in protein bands detected.

5.4 CONCLUSIONS

Bacterial cells that have been exposed to environmental challenges, such as acid exposure, are known to produce various responses. In this study some of these responses were highlighted and include various colour changes as well as alterations in protein structure. The observation that the expression of a bright yellow pigment appeared to be suppressed in some *Chryseobacterium* spp. after acid exposure may be an important factor that should be considered in identification procedures employed in food safety laboratories. In addition, concurrent protein modifications resulting in response to acid exposure may play a pivotal role in possible acid tolerance development of food-associated pathogenic bacteria.

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CHAPTER 6

Changes in Protein Profiles of Food-borne Bacteria during Exposure to Low pH and Weak Acid Preservatives

6.1 INTRODUCTION

Bacterial survival against organic as well as inorganic challenges varies in relation to various physiological requirements (Ferreira *et al.,* 2003). Intrinsic differences among bacteria also cause organisms to respond differently to antimicrobial substances and include unique composition of cell envelope or proteins. However, bacterial adaptation, genetic exchange or induction could also be responsible for the differences found among organisms and preservatives or any other antimicrobial substance must possess the necessary attributes to cross the cell membrane. One of the major reasons for development of resistance to antimicrobials may therefore, be due to the adaptation of the cell membrane, and in order to ensure antibacterial inactivation, high concentrations of the antimicrobial agent have to be achieved at the target site (Cloete, 2003).

In Gram-negative bacteria, for example, protection against unfavourable conditions or environments is mainly attributed to the presence of an outer membrane (OM). Implanted proteins present in the outer membranes execute various important functions in bacterial cells, which include the translocation of solutes and proteins and also signal transduction (Beis *et al.*, 2006). Investigations into the biochemical mechanisms involved in the development of acid resistance have found several alterations in the outer membrane structures of the bacterial cell (Leyer *et al.*, 1993).

Various organic acids exert their antimicrobial functions by disrupting the outer membrane and to cause oxidative stress (Hazan *et al*., 2004). This is achieved by the un-dissociated as well as dissociated forms of these weak acids (Alakomi *et al*., 2000). After dissociation within the cell, the anionic part of organic acids cannot leave the cell, but accumulates inside the cytoplasm to cause disruption of metabolic functions. These disruptions can also lead to an increase in osmotic pressure which will ultimately cause cell death (Gauthier, 2005). Changes in the fluidity status of bacterial membranes and the dispelling of proton gradients, due to the disruption of membrane structures, also occur when bacterial cells are exposed to alcohol, ethanol in particular. A similar mechanism of inhibition is noted with sorbic acid and sorbic alcohol, which could eventually produce ethanol tolerance among preservative-resistant bacteria and yeasts (Stratford and Anslow, 1998). Benzoic acid as well as sorbic acid function as membrane perturbing agents (Hazan *et al*., 2004).

The aim of this study was to investigate the influence of acidic exposure from acidic foodstuffs and also organic acids on the protein composition as well as the outer membrane protein structure of a bacterial cell.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial isolates

Isolates comprised eight bacterial strains: *Escherichia coli* ATCC 25922 *Escherichia coli* 0111, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* sv. Typhimurium ATCC 14028, *Salmonella enterica* sv. Enteritidis ATCC 13076, *Chryseobacterium balustinum* LMG 8329, *Weeksella virosa* LMG 12995 and *Bacillus cereus* LMG 6923. Only five of these strains were selected for the extraction of total proteins after induction with organic acids (*E. coli* ATCC 25922, *E. coli* 0111, *C. balustinum* LMG 8329, *W. virosa* LMG 12995 and *B. cereus* LMG 6923).

6.2.2 Induction of acid tolerance

Bacterial strains were sub-cultured in Brain-Heart Infusion (BHI) (Biolab Diagnostics [Pty] Ltd., Auckland, NZ) broth containing increasing concentrations of two organic acids (acetic and citric acid) and a variety of acidic foodstuffs including, vinegar, mayonnaise, chopped gherkins and gherkin brine (refer to Chapter 4). Cultures were incubated at 30˚C for 24 h. Control broths without any organism growth were included for monitoring the pH. Viable organisms at the lowest pH levels for each induction were inoculated onto BHI agar (pH 5) and incubated at 30˚C for 48 h. Acid-tolerant cells were harvested and stored at -80˚C.

6.2.3 Susceptibility determination

Minimum inhibitory concentrations (MICs) of two organic acids (acetic- and citric acid) were determined before and after induction similar to the method described in Chapter 4 (CLSI, 2006). The organic acids used for induction were selected on the grounds of their popularity in application as preservatives in acidic foodstuffs. MIC results for selected strains induced with acidic foodstuffs were obtained from Chapter 4.

6.2.4 Total proteins

Protein extraction was performed and protein profiles prepared as described in Chapter 3.

6.2.5 Outer Membrane preparation

Outer membranes were prepared as described by Livermore and Williams (1996). Bacterial cells were harvested from overnight cultures on MH (Mueller Hinton) agar plates (pH 7 for parent strains or pH 5 for induced strains). Cells were harvested by centrifugation at 5000 x g at 4°C (Eppendorf-Netheler-Hinz, GmbH, Hamburg, Germany) and washed with phosphate buffer (0.01M, pH 7) containing 140 mmol/liter β–mercaptoethanol (MP Biochemicals Inc, [Solen, Ohio, USA]) and resuspended in the same buffer. Cells were disrupted by three 30-second bursts of sonication (Misonix, Inc., NY) at maximum power, with intermediate cooling on ice. Residual cells and debris were removed by centrifugation at 5000 x g for 10 minutes at 4°C. Membranes were harvested by ultracentrifugation (ProteomeLab™ XL-A/XL-I, [Beckman Coulter]) at 100 000 x g for 30 minutes at 4°C, washed and

resuspended in 0.1 M phosphate buffer (pH7). Outer membrane protein profiles were generated by SDS-PAGE (as described in Chapter 3). Molecular weight marker (Kaleidoscope Prestained Standards) included in this run, was obtained from Bio-Rad (South Africa). After protein separation outer membrane profiles were captured with a GelDoc XR (Bio-Rad) and molecular weight determined by Quantity One® 1-D Analysis Software (Bio-Rad). All analyses were performed at least in triplicate.

6.3 RESULTS AND DISCUSSION

Induction of *E. coli* 0111 with acetic acid resulted in an increase in minimum inhibitory concentration (MIC) of acetic acid from 8 mM to 32 mM (Table 6.1). In Figure 6.1 protein bands of *E. coli* 0111 before and after induction are compared and differences were visible in protein bands with approximate sizes 30.5, 32 and 54 kDa. The latter band was visible in the original un-induced strain, but not after induction.

A decrease in organic acid susceptibility after induction with acetic and citric acid was also noticed in *C. balustinum* LMG 8329, *E. coli* ATCC 25922, *Bacillus cereus* LMG 6923 and *Weeksella virosa* LMG 12995 (Table 6.1). MICs of acetic acid for *C. balustinum* increased from 8 mM to 32 mM, while the MICs of citric acid for *Bacillus cereus* and *Weeksella virosa* similarly increased from 8 mM to 32 mM (Table 6.1). A smaller increase was found in *E. coli* ATCC 25922 for acetic acid (16 to 32 mM).

Table 6.1: Comparison of minimum inhibitory concentrations of acetic acid and citric acid after respective induction.

Organism	Inducing agent	Acetic acid MIC (mM)	
		Before induction	**After induction**
E. coli 0111	acetic acid	8	32
E. coli ATCC 25922	acetic acid	16	32
C. balustinum LMG 8329	acetic acid	8	32
		Citric acid MIC (mM)	
E. coli ATCC 25922	citric acid	16	32
W. virosa LMG 12995	citric acid	4	32
B. cereus LMG 6923	citric acid	8	32

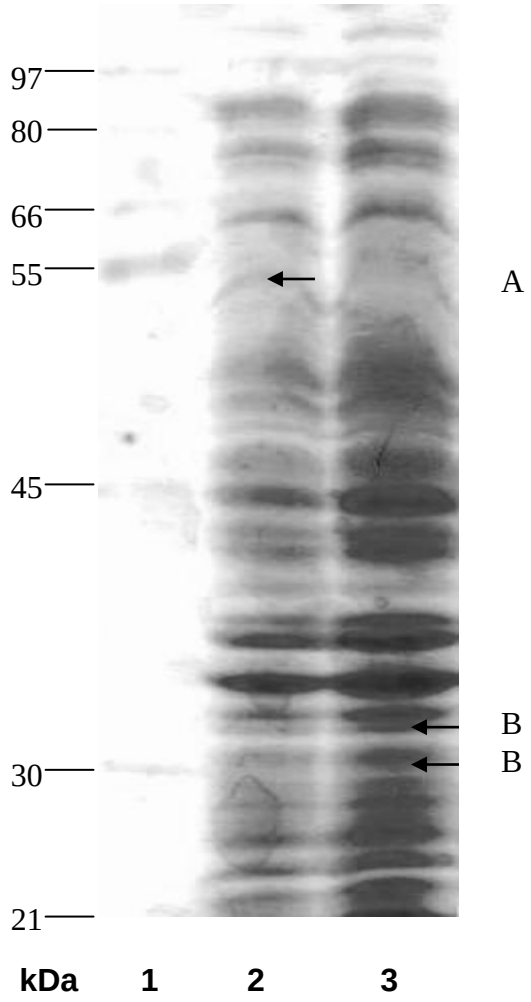

Figure 6.1: Protein profiles after acetic acid induction of *E. coli* 0111. (Lane 1: Molecular weight marker; Lane 2: Un-induced strain [acetic acid MIC 8 mM]; Lane 3: Induced strain [acetic acid MIC 32 mM]). A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

Protein profiles of *C. balustinum* after induction with acetic acid are illustrated in Figure 6.2. Two additional protein bands were visible in the induced strain at approximately 32 and 56 kDa, while a loss of two protein bands was observed at approximately 38 and 74 kDa.

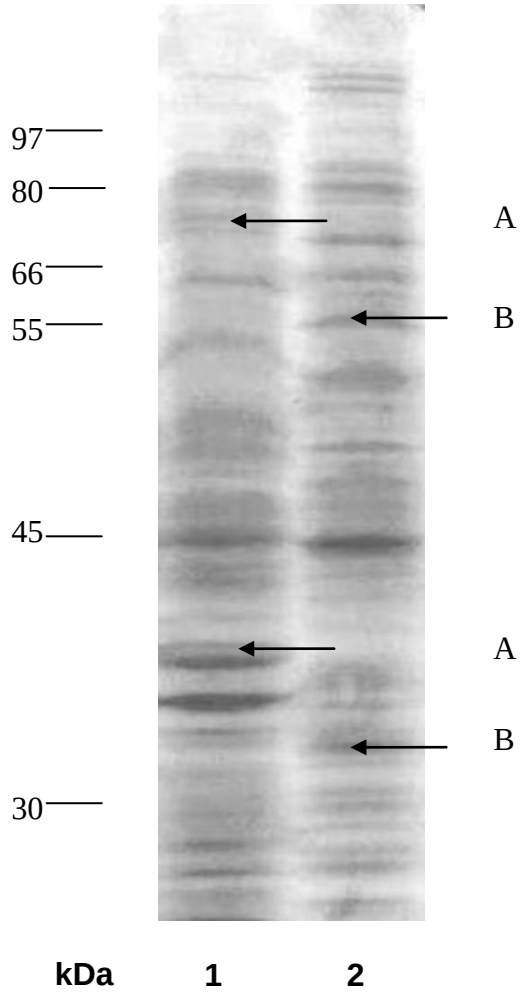

Figure 6.2: Protein profiles after acetic acid induction of *Chryseobacterium balustinum* LMG 8329. (Lane 1: Un-induced strain [MIC 8 mM]; Lane 2: Induced strain [MIC 32 mM]). A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

After induction of *E. coli* 25922 with acetic acid as well as citric acid additional protein bands were visible at approximately 53 and 54 kDa, while a protein band of approximately 74 kDa could not be seen in both induced strains (Figure 6.3).

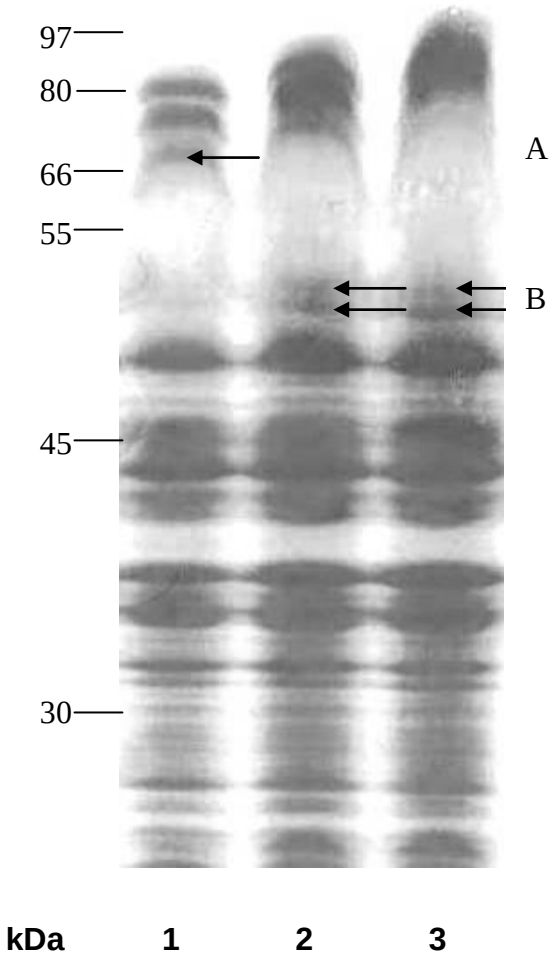

Figure 6.3: Protein profiles after organic acid induction of *E. coli* 25922. (Lane 1: Un-induced strain [acetic acid and citric acid MIC 16 mM]; Lane 2: Acetic acid induction [acetic acid MIC 32 mM]; Lane 3: citric acid induction [citric acid MIC 32 mM]). A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

Induction with citric acid also produced vast different protein profiles in *B. cereus* (Figure 6.4). Additional protein bands were visible in the induced strain at approximately 28 and 97 kDa, while five bands were not as visible as in the un-induced strain (approximate sizes 30, 33, 40, 47 and 70 kDa).

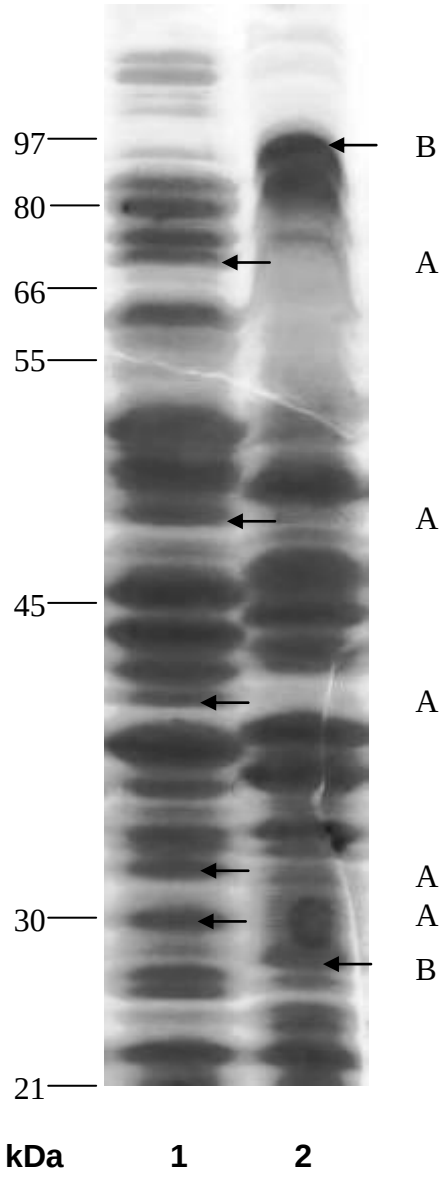

Figure 6.4: Protein profiles after citric acid induction of *Bacillus cereus* LMG 6923. (Lane 1: Un-induced strain [citric acid MIC 8 mM]; Lane 2: citric acid induction [citric acid MIC 32 mM]). A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

In *W. virosa*, after induction with citric acid, bands of approximate sizes 36, 38, 45 and 51 kDa were more visible in the induced strain, while bands of approximate sizes 33 and 54 kDa were less visible in the induced strain (Figure 6.5).

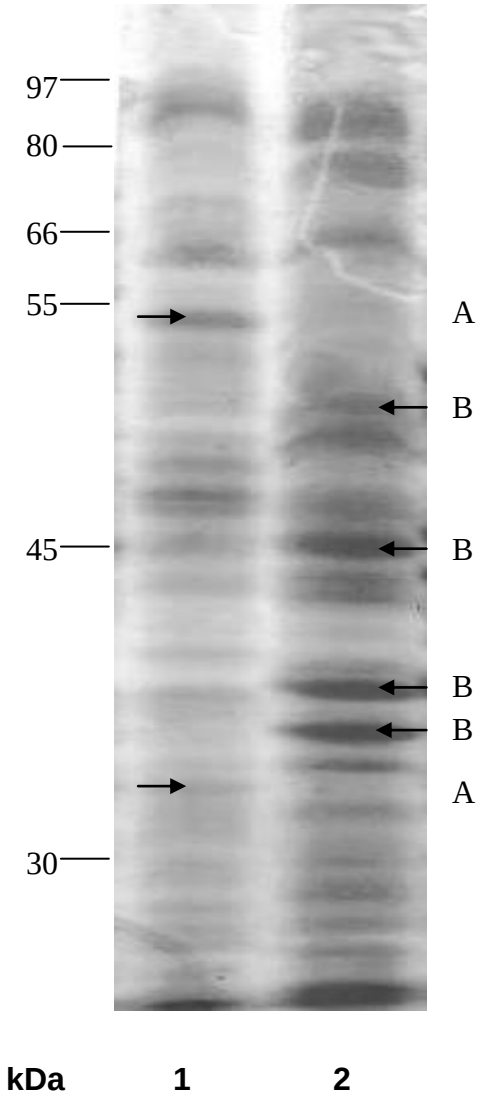

Figure 6.5: Protein profiles after citric acid induction for *Weeksella virosa* LMG 12995. (Lane 1: Un-induced strain [citric acid MIC 4 mM]; Lane 2: citric acid induction [citric acid MIC 32 mM]). A = protein band less visible or not at all visible in induced strain(s), B = additional protein band visible in induced strain(s).

It was evident from the protein profiles that various alterations occurred after induction with acetic acid as well as citric acid. Although no specific pattern was obvious, some similarities were found. In *E. coli* O111 and *C. balustinum* LMG 8329 both strains induced with acetic acid appeared to have produced an additional protein of approximate size 32 kDa (Figures 6.1 and 6.2). On the other hand, in *E. coli* ATCC 25922 and *C. balustinum* LMG 8329 both strains that were also induced with acetic acid indicated a loss of a protein of approximate size 74 kDa (Figures 6.2 and 6.3). Furthermore, after induction with citric acid, the induced strains of *B. cereus* LMG 6923 and *W. virosa* LMG 12995 also appeared to have not produced a protein of approximate size 33 kDa, which could be seen in both un-induced strains (Figures 6.4 and 6.5). After induction of *E. coli* O111 and *W. virosa* LMG 12995 with acetic acid and citric acid respectively, a protein of approximate size 54 kDa was not visible as in the original un-induced strains (Figures 6.1 and 6.5).

Outer membrane protein gels did not produce clear results on SDS-PAGE (Gels shown in Appendix A, Figures A4 and A5). However, it was obvious from the resulting outer membrane profiles (Tables 6.2, 6.3, 6.4 and 6.5) that, although no specific correlation with the MICs of organic acids after induction with the selected acidic foodstuffs (Chapter 4) could be shown; various differences in protein expression were recorded. Of interest was the outer membrane protein profile of *P. aeruginosa* ATCC 27853, where some correlation was observed in strains after exposure to gherkin brine and hydrochloric acid (Figure A5).

Table 6.2: Outer membrane protein profiles of *Escherichia coli* ATCC 25922 after induction with various acidic foodstuffs and hydrochloric acid.

Induced strains of *E.coli* ATCC 25922*						Visible protein bands (kDa)
NI	**GB**	**CG**	**MA**	**HCl**	**AA**	
					+	36.95
		+				37.52
	+					37.84
			+	+		38.2
					+	38.3
		+				39.06
	+					40.48
+				+		40.8
			+		+	40.9
		+				41.36
	+					42.06

* NI = not induced, GB = gherkin brine, CG = chopped gherkin, MA = mayonnaise, HCl = hydrochloric acid, AA = acetic acid.

Table 6.3: Outer membrane protein profiles of *Salmonella enterica* sv Enteritidis ATCC 13076 after induction with various acidic foodstuffs and hydrochloric acid.

Induced strains of *Salmonella enterica* sv Enteritidis ATCC 13076*						Visible protein bands (kDa)
NI	**GB**	**CG**	**MA**	**HCl**	**AA**	
				+		16.89
		+				18.24
	+					31.96
				+		34.23
			+			38.42
		+				39.33
				+		40.28
		+				40.83
				+		50.61
		+				50.92
				+		63.04
			+			64.50
+						74.8
		+				93.0
				+		97.26

* NI = not induced, GB = gherkin brine, CG = chopped gherkin, MA = mayonnaise, HCl = hydrochloric acid, AA = acetic acid.

Table 6.4: Outer membrane protein profiles of *Pseudomonas aeruginosa* ATCC 27853 after induction with various acidic foodstuffs and hydrochloric acid.

Induced strains of *Pseudomonas aeruginosa* ATCC 27853*						Visible protein bands (kDa)
NI	**GB**	**CG**	**MA**	**HCl**	**AA**	
		+				7.38
	+			+		7.43
					+	7.65
		+				35.53
	+					35.68
					+	35.8
					+	37.17
	+					37.81
		+				38.0
		+				46.11
	+			+		46.43
				+		46.5
						49.05
	+					84.37
		+		+		88.70
						89.44
		+				97.84
	+			+		101.76
						111.06
	+			+		155.34
+		+				157.83
						178.31

* NI = not induced, GB = gherkin brine, CG = chopped gherkin, MA = mayonnaise, HCl = hydrochloric acid, AA = acetic acid.

Table 6.5: Outer membrane protein profiles of *Salmonella enterica* sv Typhimurium ATCC 14028 after induction with various acidic foodstuffs and hydrochloric acid.

Induced strains of *Salmonella enterica* sv Typhimurium ATCC 14028*						Visible protein bands (kDa)
NI	**GB**	**CG**	**MA**	**HCl**	**AA**	
				+		7.12
					+	7.83
				+		39.32
		+				46.8
				+		46.95
		+				58.18
				+		58.89
				+		61.13
		+				63.44
		+				72.69
				+		85.73
				+		92.72

* NI = not induced, GB = gherkin brine, CG = chopped gherkin, MA = mayonnaise, HCl = hydrochloric acid, AA = acetic acid.

6.4 CONCLUSIONS

Numerous studies have been conducted on the mechanisms involved in the development of acid tolerance in Gram-negative bacteria, especially *E. coli* and other enterobacteria, but few of these studies have identified specific mechanisms (Jordan *et al.,* 1999). However, it is known that the outer membrane of Gram-negative bacteria plays an important role in the development of acid tolerance and resistance to the organic acids (Theron and Lues, 2010). The results found in the current study confirm such involvement of the outer membrane of various pathogenic bacteria and support the essential role of membrane integrity in the protection against low pH. The study and the results found should provide a valuable foundation on which further in-depth studies can be constructed.

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CHAPTER 7

Conclusions

7.1 CONCLUDING REMARKS

Acid tolerance and acid resistance may have serious implications in the survival of bacterial pathogens in the human acidic gastric stomach. The study was done in response to a need in the South African food safety research to determine the current situation with regard to acid-tolerance and acid resistance in food-associated bacteria. The focus was on the prevalence of acid tolerance in known bacterial strains, the development of acid tolerance because of exposure to acidic food and acids and the response of bacterial cells on acidic stress.

The extent of acid tolerance was investigated in a wide range of food-associated bacteria. An obvious diversity in acid tolerance was found, demonstrating the complexity of controlling the quality of foodstuffs even in an acidic environment. Of specific concern were the enteric bacteria *E. coli* and *Salmonella* spp., which demonstrated intrinsic acid tolerance, as this would have a serious implication on their survival in acid foodstuffs and consequent resistance to the protective effect of the acidic human gastric environment. There may be a serious need to apply hurdle technology in preservation of acidic foodstuff, as a low pH may not be enough to combat bacterial pathogens.

In addition to this, bacterial strains were exposed to increasing concentrations of acidic foodstuffs and hydrochloric acid to monitor variations in the development of acid tolerance. The survival patterns of the various bacterial

strains were diverse and it was obvious that low pH as well as the type of acidulant are factors to be considered in acid tolerance development. The bacterial protein compositions recorded after acid exposure also indicated that growth in acidic food could result in acid tolerance as a result of protein modifications. This chapter made an important contribution to a better understanding of factors that influence adaptation of food-associated bacteria to acid stress.

The investigation was further focused on the influence of acid tolerance development on the susceptibility of food-borne pathogens to various organic acids. No specific relationship was evident between the type of foodstuff and the effectiveness of a specific organic acid. Acid adaptation, therefore, did not appear to result in significant resistance development to the organic acids, except for some decreases in susceptibility in *S. enterica* sv. Typhimurium. It should, however, be concluded that the application of organic acids in effective food preservation and safety control should be executed with caution, specifically in acidic foodstuffs. Organic acid activity was also much more effective at lower pH values, which may have an influence on the specific activity of organic acids in acid susceptible strains.

Morphological changes in the bacterial cell were also investigated after exposure to lower pH environments. Colour changes resulting after acid exposure may be of importance in the response of bacteria to such environmental stresses such as acid shock. These modifications may even

be a factor in potential difficulties encountered in identification of pathogens, specifically psychrotrophic bacteria and would require further in-depth studies.

Finally the influence of acidic foodstuffs and organic acids on protein composition and outer membrane protein composition of a bacterial cell was investigated. These investigations revealed alterations in protein profiles occurring after induction with organic acids, also showing some similarities. These results confirm the involvement of various outer membrane proteins of Gram-negative pathogenic bacteria after exposure to acidic foodstuffs as well as weak acid preservatives and should provide a valuable foundation on which to construct further essential studies.

In Chapter 2 the extent of acid tolerance was investigated in a wide range of food-associated bacteria. *E. coli* and *Salmonella* demonstrated intrinsic acid tolerance, while in *P. aeruginosa* cell counts did show a decline, but acid exposure did not seem to have a serious effect on cell growth. Among the psychrotrophic bacteria, acid tolerance development was evident and may cause problems in preservation of foodstuffs kept at lower or refrigeration temperatures. The diversity of acid tolerance among a wide range of bacterial genera was obvious, highlighting concern regarding enteric bacteria *Salmonella* spp. and *E. coli* that demonstrated high levels of acid tolerance, as this would have a serious implication on their survival in acid foodstuffs and consequent resistance to the protective effect of the acidic human gastric environment.

In Chapter 3 bacterial strains were exposed to increasing concentrations of acidic foodstuffs and hydrochloric acid to monitor variations in the development of acid tolerance. It is known that acid adaptation in bacteria is complex and many physiological changes take place, such as damaged cell membranes and expression of stress proteins. In this study, various survival patterns could be distinguished and it was found that low pH as well as the type of acidulant is factors to be considered in acid tolerance development. Alterations in bacterial protein composition were recorded after growing in acidic food and indicate involvement in acid tolerance development. This chapter made an important contribution to a better understanding of factors that influence adaptation of food-associated bacteria to acid stress.

In Chapter 4 the investigation was focused on the influence of acid tolerance development on the susceptibility of food-borne pathogens to various organic acids. This aim was to address concerns about the application of organic acids in acidic foodstuffs and the effectiveness on resulting acid tolerant food-borne bacteria. Diverse susceptibility patterns to the organic acids were evident after exposure, although no specific relationship was found between the type of foodstuff and the effectiveness of a specific organic acid.

Acid adaptation did not appear to result in significant resistance development to the organic acids, except for some decreases in susceptibility in *S. enterica* sv. Typhimurium. Although acid adapted cells have been reported to be more resistant under various stress conditions, this was not significantly obvious in the current study, specifically with regard to the organic acids. However, it is

important to be mindful of potential resistance in the application of organic acids in effective food preservation and safety control. Organic acid activity was also much more effective at lower pH values. In acid susceptible strains this may have an influence on the specific activity of organic acids.

In Chapter 5 morphological changes in the bacterial cell were investigated after exposure to a low pH. A definite colour change from the characteristic bright yellow to white was observed in various *Chryseobacterium* spp. These strains were also less tolerant to acid exposure than the strains showing no change in colour. Another colour change was recorded after acid exposure in *Pseudomonas aeruginosa*, demonstrating a brighter green colour.

Colour changes resulting after acid exposure may play a role in the defence mechanism of the bacteria against acid shock, as the isolates not showing any colour change, appeared to more acid tolerant. Various responses to acid exposure were highlighted in this chapter and include a range of colour changes and alterations in protein structure. Such modifications may play an important role in bacterial identification and acid tolerance development in food-borne pathogens.

In Chapter 6 the influence of acidic foodstuffs and organic acids on protein composition and outer membrane protein composition of a bacterial cell was investigated. Alterations in protein profiles occurred after induction with organic acids, and some similarities could be demonstrated. Outer membrane protein gels did not produce clear results on SDS-PAGE, but

various differences in protein expression were recorded and a correlation was observed between strains after exposure to gherkin brine as well as hydrochloric acid.

The outer membrane of Gram-negative bacteria plays an important role in the development of acid tolerance and resistance to the organic acids. Results found in the current study confirm involvement of the outer membrane of various pathogenic bacteria and should provide a valuable foundation on which to construct further in-depth studies.

7.2 FUTURE RESEARCH

The study has highlighted various aspects that would be worth investigating in future studies. These include:

- Possible alterations in the cell membrane proteins, specifically after acid induction.
- Enhancement of survivability of acid sensitive pathogens after exposure to organic acids.
- Development of increased virulence as a result of acid tolerance development.
- The relationship between the amount of organic acid used in food preservation and the resulting pH reduction, which could also have an effect on preservative action.

- Determining the extent of the influence of acid tolerance on the sustainability of organic acids as food preservatives in acidic foodstuffs.
- Investigating whether a reduction in pH by organic acid is the cause of inhibition, or actually the specific action of the organic acids.
- The extent of tolerance that pathogenic and spoilage bacteria can develop, and the underlying control of stress responses.

APPENDICES

Table A1: Total viable counts of bacterial strains after screening of acid tolerance at 0, 12, 24, 36 and 48 hours.

Bacterial Isolate	Total Viable Counts (CFU.ml^{-1})				
	0 h	**12 h**	**24 h**	**36 h**	**48 h**
Bacillus cereus ATCC 14579	1.2×10^5	7×10^4	3×10^4	1×10^4	0
Chryseobacterium balustinum LMG 8329	8×10^6	2.8×10^6	2.4×10^6	2×10^6	1.6×10^6
Chryseobacterium defluvii LMG 22469	5×10^5	0	0	0	0
Chryseobacterium gleum LMG 8334	2.4×10^6	3.7×10^5	4×10^4	1×10^4	0
Chryseobacterium indologenes LMG 8337	2×10^6	7.8×10^5	4.8×10^5	3×10^5	2.7×10^5
Chryseobacterium indoltheticum LMG 4025	1.6×10^6	0	0	0	0
Chryseobacterium joostei LMG 18212	1×10^6	1.5×10^5	6×10^4	0	0
Chryseobacterium piscium LMG 23089	1×10^6	1×10^4	0	0	0
Chryseobacterium vrystaatense LMG 22846	1.2×10^6	1.2×10^6	1×10^4	1×10^4	0
Chryseobacterium scophthalmus LMG 13028	1.2×10^6	0	0	0	0
Escherichia coli ATCC 25922	2×10^6	7.4×10^5	5.2×10^5	1×10^6	$>2 \times 10^6$
Escherichia coli 0111	1.2×10^6	1×10^6	1×10^6	8×10^5	8×10^5
Klebsiella pneumoniae ATCC 31488	3×10^6	3×10^6	7×10^5	5×10^5	5×10^5
Proteus vulgaris ATCC 13315	1×10^6	2.9×10^5	2×10^5	1.2×10^5	8×10^4
Pseudomonas aeruginosa ATCC 27853	1×10^6	1×10^6	1×10^6	8×10^5	8×10^5
Salmonella enterica sv. Enteritidis ATCC 13076	2.4×10^6	1.2×10^6	8×10^5	1.2×10^6	$>2.4 \times 10^6$
Salmonella enterica sv. Typhimurium ATCC 14028	2.5×10^6	1.6×10^6	1.2×10^6	1×10^6	1.6×10^6
Staphylococcus aureus ATCC 25923	5×10^4	0	0	0	0
Yersinia enterocolitica ATCC 9610	2.5×10^5	5×10^4	1×10^4	1×10^4	0

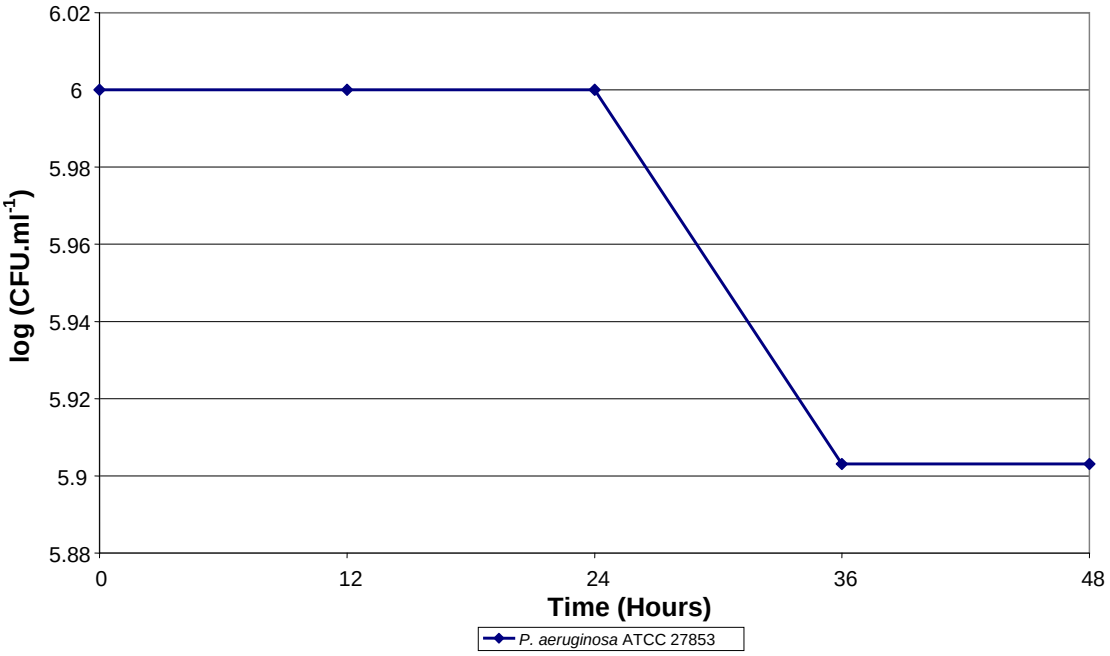

Figure A1: Total viable counts for *Pseudomonas aeruginosa* ATCC 27853 after exposure to acid challenge.

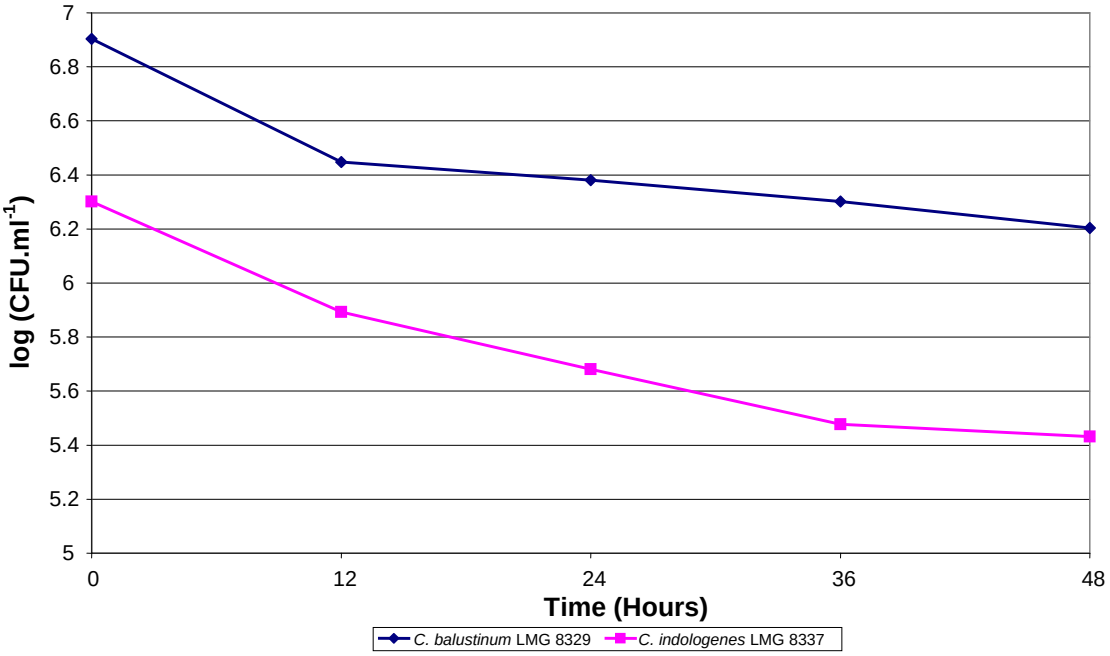

Figure A2: Total viable counts for *Chryseobacterium balustinum* LMG 8329 and *Chryseobacterium indologenes* LMG 8337 after exposure to acid challenge.

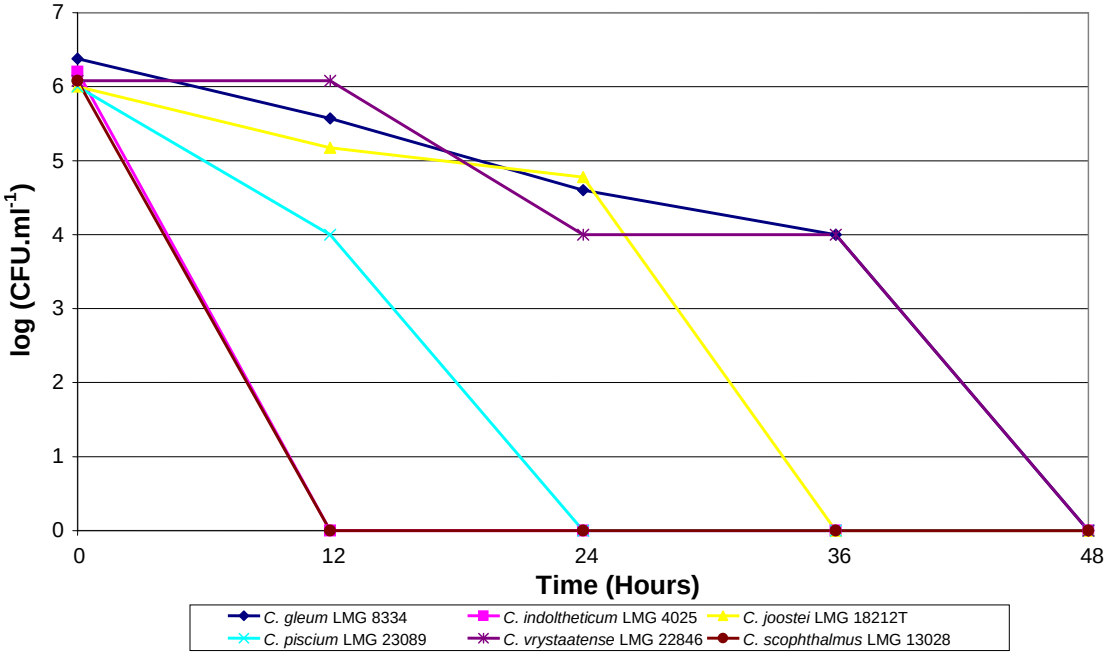

Figure A3: Total viable counts for *C. gleum* LMG 8334, *C. indoltheticum* LMG 4025, *C. joostei* LMG 18212T, *C. piscium* LMG 23089, *C. vrystaatense* LMG 22845 and *C. scophthalmus* LMG 13028 exposed to acid challenge.

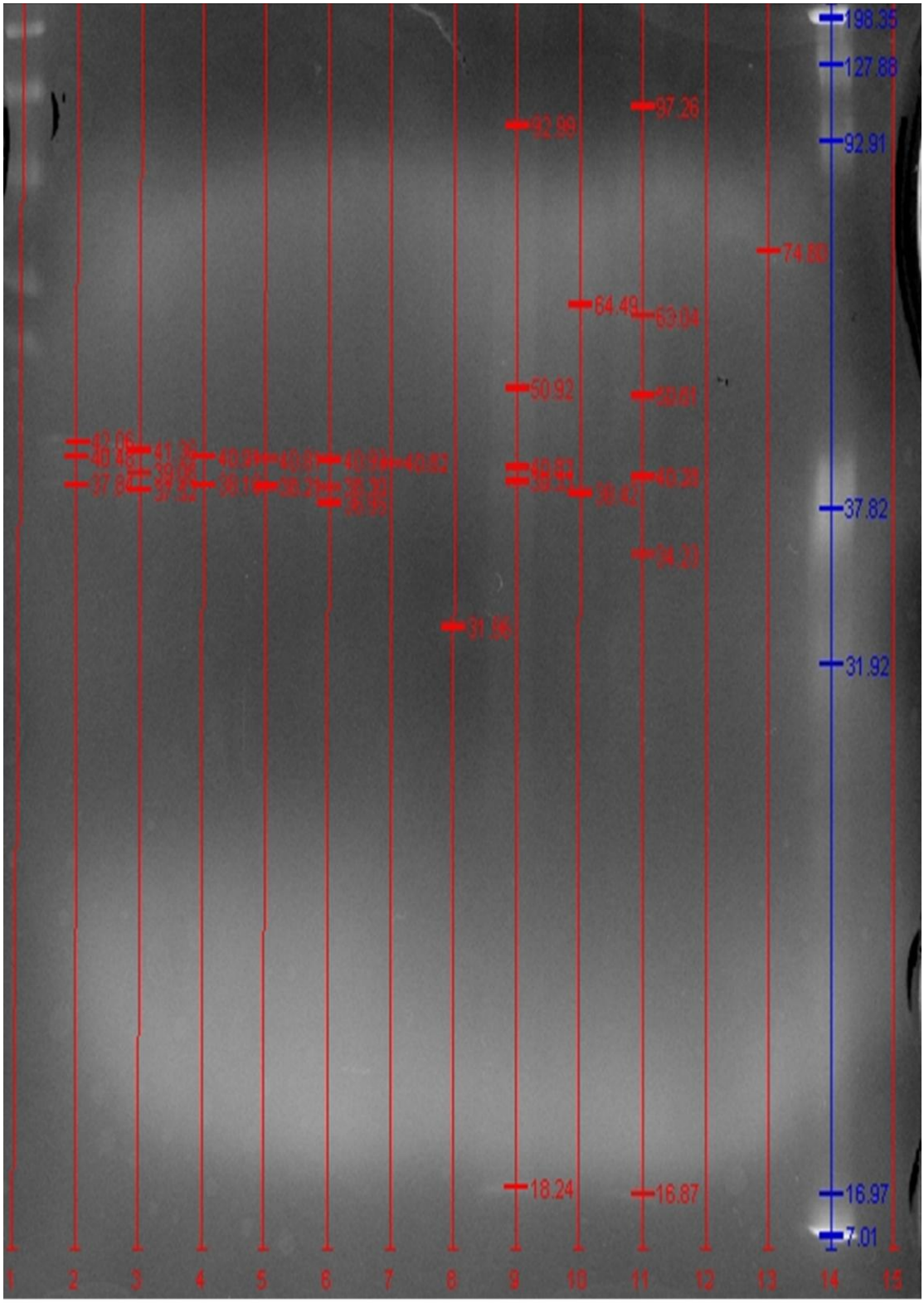

Figure A4: Outer membrane profiles after acid induction of *E. coli* 25922 and *Salmonella enterica* sv. Enteritidis ATCC 13076 (Legend on p.173).

Table A2: Legend for Figure A4.

Lane number	Organisms	Acidulant used for induction
1	Molecular weight marker	N/A
2	*E. coli* ATCC 25922	Gherkin brine
3	*E. coli* ATCC 25922	Chopped gherkin
4	*E. coli* ATCC 25922	Mayonnaise
5	*E. coli* ATCC 25922	Hydrochloric acid
6	*E. coli* ATCC 25922	Acetic acid
7	*E. coli* ATCC 25922	Un-induced strain
8	*S. enterica* sv. Enteritidis ATCC 13076	Gherkin brine
9	*S. enterica* sv. Enteritidis ATCC 13076	Chopped gherkin
10	*S. enterica* sv. Enteritidis ATCC 13076	Mayonnaise
11	*S. enterica* sv. Enteritidis ATCC 13076	Hydrochloric acid
12	*S. enterica* sv. Enteritidis ATCC 13076	Acetic acid
13	*S. enterica* sv. Enteritidis ATCC 13076	Un-induced strain
14	Molecular weight marker	N/A
15	Open lane	N/A

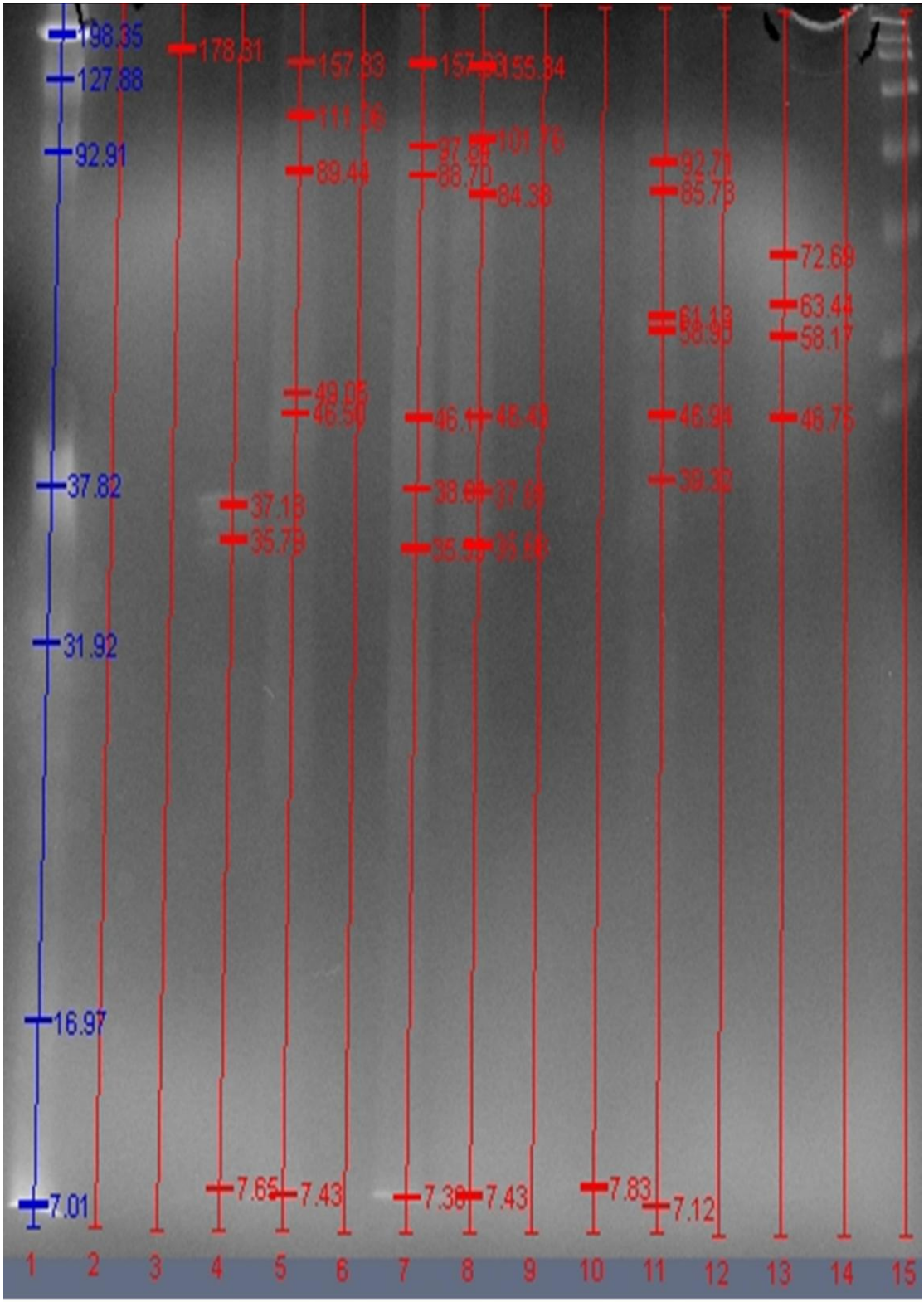

Figure A5: Outer membrane profiles after acid induction of *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella enterica* sv. Typhimurium ATCC 14028 (Legend on p. 175).

Table A3: Legend for Figure A5.

Lane number	Organisms	Acidulant used for induction
1	Molecular weight marker	N/A
2	Open lane	N/A
3	*P. aeruginosa* ATCC 27853	Un-induced strain
4	*P. aeruginosa* ATCC 27853	Acetic acid
5	*P. aeruginosa* ATCC 27853	Hydrochloric acid
6	*P. aeruginosa* ATCC 27853	Mayonnaise
7	*P. aeruginosa* ATCC 27853	Chopped gherkin
8	*P. aeruginosa* ATCC 27853	Gherkin brine
9	*S. enterica* sv. Typhimurium ATCC 14028	Un-induced strain
10	*S. enterica* sv. Typhimurium ATCC 14028	Acetic acid
11	*S. enterica* sv. Typhimurium ATCC 14028	Hydrochloric acid
12	*S. enterica* sv. Typhimurium ATCC 14028	Mayonnaise
13	*S. enterica* sv. Typhimurium ATCC 14028	Chopped gherkin
14	*S. enterica* sv. Typhimurium ATCC 14028	Gherkin brine
15	Molecular weight marker	N/A