INVESTIGATING THE DEVELOPMENT OF ACID TOLERANCE IN FOOD-BORNE PATHOGENS *ESCHERICHIA COLI, SALMONELLA* SPP., AND *PSEUDOMONAS AERUGINOSA* AND THE IMPLICATION ON THE SUSCEPTIBILITY TO ORGANIC ACIDS

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ABSTRACT

Various foodstuffs have a very low pH and bacteria have been reported to survive such products. Acid substances, such as organic acids are common food preservatives. These substances also lower the pH of processed foods. Decontamination with organic acids which could result in the emergence of acid tolerant food-borne pathogens is causing concern. The objectives of the study were to determine the development of acid tolerance in important food-borne pathogenic bacteria, to investigate evolving changes in the phenotypic characteristics as a result this acid tolerance, and to explore the possibility of repercussions in successful food preservation with organic acids. Bacterial strains were screened for acid-tolerance, by determining viable counts immediately before acid challenge and at various times after challenge. Strains were exposed to increasing concentrations of hydrochloric acid, acidic foodstuff and two organic acids (acetic and citric acid). Protein profiles were generated by SDS-PAGE examined for possible modification(s) as a result of acid tolerance development. Susceptibility to seven organic acids levels were scrutinised to evaluate the probability of a correlation between altered antimicrobial activity and acid tolerance. *Salmonella enterica* sv. Enteritidis ATCC 13076 and *Escherichia coli* ATCC 25922 indicated rapid development of acid tolerance, after 36h of acid exposure. In *Salmonella enterica* sv. Typhimurium ATCC 14028, *E. coli* 0111 and *Pseudomonas aeruginosa* ATCC 27853 intermediate intrinsic acid tolerance was obvious. On comparing susceptibility of these pathogens to the organic acids, it was demonstrated that pH played a significant role in inhibitory activity, as it is known that these compounds exhibit optimum antimicrobial activity at a lower pH ($pH \leq 5$). Further investigations will be conducted to elucidate the two-way role of pH in foodstuff and the addition of an organic acid, in determining if microorganisms are losing their susceptibility for the preservative as a result of sub-optimal pH levels, or if they become acid-tolerant after surviving exposure to organic acids.

Keywords: Food-borne pathogens

1. INTRODUCTION

The human gastric fluid plays an important role in first-line defense against enteric pathogens present in food by killing or inactivating these organisms

before they enter the intestinal tract. Yet, the question arises: "Why is food poisoning such a common infectious disease?". It is 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 very low pH and bacteria have been reported to survive in such products. Acid substances, such as organic acids are common food preservatives, which also lowers 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.

Food-borne pathogenic bacteria reveal stress responses, which improve their continued existence in undesirable environmental conditions. A universally encountered stress in foods is an acidic environment, where survival could lead to induction of an acid tolerance response (ATR). The ATR is defined as the resistance of organisms to reduced optimum pH levels when they have either been grown at moderately low pH or previously been exposed to sub-optimal pH levels (Dilworth and Glenn, 1999). Faber and Pagotto (1992) reported that bacterial cells, tolerant to a certain stress may reveal elevated adaptation abilities to other types of stresses. Cross-resistance enhances bacteria's prospective survival and growth (Foster and Hall, 1990) and some studies have shown evidence of increased levels in bacterial virulence (Gahan and Hill, 1999; O'Driscoll et al. 1996). The development of tolerance to low pH levels and the existence of cross-protection could favour and enhance survival of bacteria in acidic foodstuff and in acidic environments such as the human stomach, in so doing escalating the probability of food poisoning (Gorden and Small, 1993). After acid adaptation, increased resistance to the inactivation cells at lower pH was reported in some studies of *S. enterica* serovar Typhimurium. Cross-protection against various environmental stresses was also documented in these studies (Leyer and Johnson, 1993). The acid tolerance response, consequently, plays an important role in both prognostic modeling and risk assessment approaches to microbiological food safety (Greenacre et al. 2003).

The consumption of undercooked ground beef or raw milk, (Doyle et al. 1997) has mainly been the reason for outbreaks of food poisoning caused by *E. coli* O157:H7, but the occurrences of infections have also been associated with acidic foods such as apple cider (Besser et al. 1993) and salami (Centers for Disease control and prevention, 1995). Greenacre et al. (2003) reported that by using either acetic or lactic acid for the exposure of *S. enterica* serovar Typhimurium to moderately pH levels at 20°C, an ATR is notable where the adaptation time and pH represented the distinct conditions for ATR expression. The effect of a lowered internal pH (pHi) of bacteria cells lead to an acid death, as proven by previous studies (Foster and Hall, 1991; O'Driscoll et al. 1997), but acid adapted cells were able to maintain their pHi more successfully, thus increasing their survival in an acidic environment.

The process of acid adaptation of microorganisms is complex and many physiological changes take place, including the expression of shielding stress proteins and even damage to cell membranes (Leyer and Johnson, 1993; Beuchat, 1978). The degree of acid tolerance is depended on the nature of the physiological changes and also the intensity of the subsequent stress factors. In some cases the devastating effects of cellular damage might exceed the shielding effect of acid-shock proteins or other protective metabolic changes induced by low pH, thus stresses cells could die if exposed to more harsh environments (Deng et al. 1999).

The majority of studies concerning the development and evaluation of the ATR have been conducted at temperatures of 30 or 37°C and made use of acidulants such as mineral acids (HCl) (Foster, 1991; Kroll and Patchett, 1992; O'Driscoll et al. 1996). As a result, the ATR expressed in response to these conditions is of most relevance to food. Extensive studies on *S. enterica* serovar Typhimurium proved that acid shock results in tolerance to diverse ecological stresses (Foster and Hall, 1990, 1991; Foster, 1995) including environments in fermented foods such as cheese (Leyer and Johnson, 1992). However, these extensive studies used hydrochloric acid to lower pH levels abruptly. The lowering pH in foodstuff like yogurt and fermented meats occurs gradually as fermentation proceeds. To fully comprehend the effect of acidic environments on acid tolerance of bacteria and to fully appreciate their ability to survive in fermented and acidic foods, cells should be exposed to acidic environments by making use of methods that replicate authentic food systems (Deng et al. 1999). Food-borne pathogenic bacteria are generally in contact with weak organic acids in food systems, either as intrinsically produced i.e. fermentation or as food preservatives or sensory enhancements. Organic acids are normally used for these processes, thus the use of organic acids would be more appropriate to reduce pH in media (Deng et al. 1999). In the designing of an acid challenge study organic and inorganic acids were included in the current study as found in acidic foodstuff and the human stomach.

There is a need in the South African food safety research to determine the current situation with regard to acid-tolerance and acid resistance which plays an important role in the survival and growth of food-associated bacteria, particularly the food-borne pathogens. Studies like this could help with the understanding of the complex adaptations of microorganisms and help ease "one of the most widespread health problems and an important cause of reduced economic productivity" as The World Health Organization (WHO) calls it.

Objectives of his study was, therefore, to evaluate the development of acid tolerance in bacterial strains often implicated with food-borne illnesses, to investigate evolving changes in the phenotypic characteristics as a result of this acid tolerance, and explore the possibility of repercussions in successful food preservation with organic acids.

2. MATERIALS AND METHODS

Bacterial isolates

Bacterial isolates tested 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.

Development of acid tolerance

A method described by Jordan et al. (1999), was adapted and modified for determining the development of acid tolerance. Test isolates were grown in Mueller Hinton broth (MH) (pH 7) for 48 hrs at 35°C. Cultures were exposed to an acid challenged by the reduction of the medium to pH 4.5 with 3M HCl. Viable cell counts were determined before acid challenge and at time intervals of 12, 24, 36 and 48 hours after pH adjustment. Serial dilutions were performed in 0.1% peptone and 10 µl of each dilution was spread onto MH agar and incubated for 24 hours at 35°C.

Induction of acid tolerance

Bacterial strains were subcultured in Brain-Heart Infusion (BHI) broth containing increasing concentrations of hydrochloric acid, acidic foodstuff (Vinegar, Mayonnaise, Gherkins and Gherkin juice) and two organic acids (acetic and citric acid) and incubated at 30°C for 24 hours. Control broths were included for pH measurements. Cultures at lowest survival pH for each induction substance were plated on BHI agar (pH7 and pH5) and incubated at 30°C for 48 hours. Acid-tolerance induced cells were harvested and stored at -80°C.

Protein profiles

Harvested cells were washed in 0.1M phosphate buffer, pH7 and total cell proteins prepared. Protein profiles were obtained by SDS-PAGE and profiles of induced strains were compared with those of the parent strains.

MIC determination at various pH levels

Minimum inhibitory concentrations (MICs) of seven organic acids for the parent strains and induced strains were determined at pH levels ranging from pH 5 pH 8 with an agar-dilution method, as described by the Clinical & Laboratory Standard Institute (CLSI, 2006). MIC was recorded as the lowest concentration of organic acid where no growth was detected. The MIC_{50} and MIC_{90} values were used to interpret the MIC results found. These values will indicate the concentration of organic acid that would inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates.

3. RESULTS AND DISCUSSION

It was evident that of the four bacterial isolates, *P. aeruginosa* was the only strain that did not acquire acid-resistance after being exposed to a low pH of 4.5, although the organism did survive after 48 hours (Table 1). *S. enterica* sv. Enteritidis, *S. enterica* sv. Typhimurium and *E. coli* demonstrated a decrease in bacterial count after 12 or 24 hours, but these counts increased again after 36 hours, and after 48h hours exposure, bacterial counts were higher than in the original culture.

Table 1. Degree of acid tolerance in bacterial strains as demonstrated after exposure to 3M HCl

	Total Viable Counts (CFU/ml)				
Bacterial Isolate	**0 h**	**12 h**	**24 h**	**36 h**	**48 h**
Escherichia coli ATCC 25922	2×10^6	7.4×10^5	5.2×10^5	1×10^6	$>2 \times 10^6$
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

Table 2. The pH of the highest concentration of acidic foodstuff where the organism was able to grow.

Bacterial strains	PH of highest induction concentration	Growth on pH 5 agar after induction*
***E. coli* ATCC 25922**		
gherkin (solid)	5.3	+
gherkin (juice)	6.6	+
mayonnaise	4.5	+
vinegar	4.8	+
HCl	5	+
***P. aeruginosa* ATCC 27853**		
gherkin (solid)	5.3	-
gherkin (juice)	6.6	+
mayonnaise	4.5	-
vinegar	4.6	+
HCl	5	+
***S. enterica* sv Enteritidis ATCC 13076**		
gherkin (solid)	5.3	+
gherkin (juice)	6.6	+
mayonnaise	4.5	+
vinegar	5	+
HCl	5	+
***S. enteritidis* sv. Typhimurium ATCC 14028**		
gherkin (solid)	5.3	+
gherkin (juice)	6.6	+
mayonnaise	4.5	+
vinegar	4.8	+
HCl	5	+

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

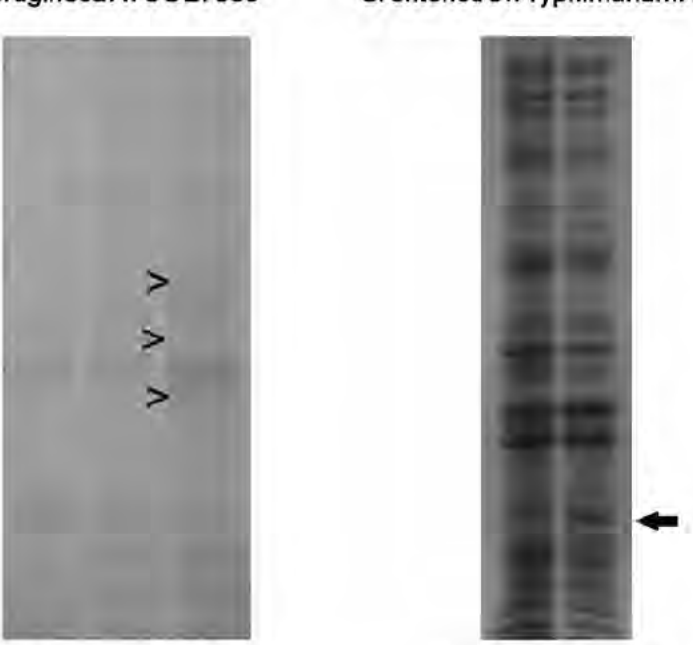
Exposure to the various acidic foodstuffs had different effects on the susceptibility of the bacterial strains after induction (Table 3). It was interesting to find that *P. aeruginosa*, which revealed lower acid tolerance in Table 1, demonstrated decreased susceptibility to potassium sorbate, sodium benzoate, acetic acid and lactic acid after exposure to gherkin juice. Growth in chopped gherkin rendered the organism more susceptible to acetic acid, while growth in mayonnaise and HCl did not significantly decrease the susceptibility of *P. aeruginosa* for any of the organic acids, except against potassium sorbate where susceptibility was decreased after exposure to HCl. In fact, the susceptibility to acetic acid and propionic acid appeared to have increased. Both *S. enterica* sv. Enteritidis and *S. enterica* sv. Typhimurium, that were shown to easily adapt to acidic stress, were found to become less susceptible only to acetic acid after exposure to mayonnaise, vinegar and HCl (both strains) as well as gherkin and

gherkin juice (only *S. enterica* sv. Enteritidis). *E. coli* showed a decrease in susceptibility only to potassium sorbate, but that was the case after growth in all the foodstuffs tested. Protein profiles obtained by SDS-PAGE of *P. aeruginosa* (lower acid tolerance) and *S. enterica* sv. Typhimurium (high acid tolerance) are shown in Figure 1.

Table 3. Comparison of MICs at pH 5 of potassium sorbate, sodium benzoate, acetic acid, lactic acid and propionic acid for test bacterial strains prior to acid tolerance induction and after acid-tolerance induction.

	MIC (mM)				
	Potassium sorbate	**Sodium benzoate**	**Acetic acid**	**Lactic acid**	**Propionic acid**
***E. coli* ATCC 25922**					
Uninduced strain	8	4	8	64	32
Induced - gherkin (solid)	16	4	4	64	32
Induced - gherkin (juice)	16	4	8	64	32
Induced - mayonnaise	16	4	4	64	32
Induced - vinegar	16	4	4	64	32
Induced - HCl	16	4	8	64	32
***P. aeruginosa* ATCC 27853**					
Uninduced strain	16	8	4	16	32
Induced - gherkin (solid)	64	16	2	16	32
Induced - gherkin (juice)	32	16	8	64	32
Induced - mayonnaise	16	8	2	16	16
Induced - vinegar	8	4	8	64	32
Induced - HCl	32	8	2	16	16
***S. enterica* sv Enteritidis ATCC 13076**					
Uninduced strain	8	4	4	64	32
Induced - gherkin (solid)	8	4	4	64	32
Induced - gherkin (juice)	8	4	4	64	32
Induced - mayonnaise	8	4	8	64	32
Induced - vinegar	8	4	8	64	32
Induced - HCl	8	4	8	64	32
***S. enterica* sv. Typhimurium ATCC 14028**					
Uninduced strain	16	8	4	64	16
Induced - gherkin (solid)	16	8	8	64	16
Induced - gherkin (juice)	16	8	8	64	16
Induced - mayonnaise	16	8	8	64	32
Induced - vinegar	16	8	8	64	16
Induced - HCl	16	8	8	64	16

P. aeruginosa ATCC 27853 *S. enterica* sv. Typhimurium ATCC 14028



HCl gherkin control control gherkin juice

Figure 1. Protein profiles of *P. aeruginosa* ATCC 27853 and *S. enterica* sv. Typhimurium ATCC 14028 before and after exposure to chopped gherkin and gherkin juice respectively. Arrows indicate alterations in protein bands detected.

The results have indicated the various responses of only four different bacterial strains when exposed to the low pH of acidic foodstuff. No clear correlation was found between the type of foodstuff or the effectiveness of a specific organic acid. It was however, evident that there was decreased susceptibility for each organism to at least one organic acid tested and after exposure to at least one of the acidic foodstuffs. This altered susceptibility may well be attributed to, amongst others, a change in protein structure. An investigation into the role of outer membrane proteins in acid-tolerance and reduced susceptibility to organic acids is currently underway. It may also be necessary to elucidate the two-way role of pH and the addition of an organic acid in the preservation of foodstuffs, in determining if bacterial isolates are losing susceptibility as a result of the influence of acidic stress, or becoming acid-tolerant as a result of ineffectiveness of organic acids as antimicrobial agents.

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