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Acid Tolerance of *Escherichia coli* O157: H7 Serotype and *Salmonella typhi* (A Group D Serotype) and Their Survival in Apple and Orange Juices

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Abstract: Outbreaks of diarrhoea, haemolytic uraemic syndrome and food poisoning have been associated with the consumption of (apple and orange ciders) and (apple and orange juices). The organisms implicated in these outbreaks have been *Escherichia coli* O157:H7 serotype and *Salmonella typhi* (a group D serotype), indicating the resistance of the serotypes of these organisms to acidic pH. The acid tolerance, the growth, and the survival of these organisms were investigated in both natural (unpasteurized, untreated) and pasteurized, treated apple and orange juices and ciders. The effects of benzoic acid, as a preservative, on the growth of the mentioned bacteria subjected to examination, in trypticase soy broth and apple and orange juices and ciders were also examined. It was evident that *E. coli* O157:H7 serotype and S. typhi (a group D serotype) organisms grew well in trypticase soy broth at pH level ranging from 3.0 to 9.0. The growth of both mentioned serotypes were inhibited by adding 0.05% of benzoic acid. Similarly, E. coli O157:H7 and S. typhi strains grew well in both natural (unpasteurized) as well as in pasteurized apple and orange juices and ciders, and the growth was inhibited by adding 0.1% of benzoic acid. The possible sources of contamination of natural apple and orange juices and ciders with *E. coli* O157:H7 serotype and S. typhi (a group D serotype) are discussed. The study declared the efficacy of acids, their salts and derivatives in controlling microbial growth in food in general.

Key words: *Escherichia coli* · *Salmonella typhi* · Apple and orange juices · Benzoic acid · Survival

INTRODUCTION

Escherichia is a genus of gram-negative, Facultatively anaerobic, rod-shaped bacteria of the tribe Escherichieae, family Enterobacteriaceae, found in the large intestine of warm-blooded animals. The organisms are nonpathogenic or opportunistic pathogens. They are members of the "coliform" group of bacteria, their presence in water supplies being used as an indicator of fecal contamination [1]. *E. coli* O157 : H7 has been noted for its acid adaptive and acid tolerant properties in a number of foods and under a variety of conditions [2].

Escherichia coli strain O157 : H7 has emerged with increasing frequency in the past decade as an important food-borne pathogen causing haemolytic uraemic syndrome and haemolytic colitis in human beings [3]. This enterohaemorrhagic serovar of *E. coli* produces Shiga-like toxins, also known as verotoxins. The clinical manifestations caused by this bacterium include bloody diarrhea severe abdominal cramps, with little or no fever. Since this is an emerging pathogen, clinicians often fail to suspect *E. coli* O157:H7 in the early stages of infection and thus, the stools from such patients are rarely screened for strain O157:H7. It is regarded as the third most frequently isolated pathogen from stools, after Campylobacter and Salmonella.

E. coli O157:H7 was first isolated from a patient in 1975; however, it was not until 1982 that it was recognized as a human pathogen [4]. Outbreaks of strain O157: H7 have been associated with a range of foods, including ground beef, raw milk, and contaminated water [5-7]. This microbe is a persistent problem in cattle [8,9] and outbreaks of disease involving fresh produce, and fruit juices, have been reported with increasing frequency [10]. The first outbreak of this disease by consuming apple cider was in the fall (autumn) of 1991 [11]. Recent outbreaks of strain O157: H7 involving fresh apple juice indicate the resistance of the bacterium to acidic pH and have raised doubts about the safety of unpasteruized fruit juices.

On the other hand, Salmonella is a genus of gram-negative, Facultatively anaerobic bacteria of the family Enterobacteriaceae, made up of nonspore-forming rods, usually motile with peritrichous flagella. *Salmonella typhi*

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is a group D serotype, a strict parasite of humans and the cause of typhoid fever. Strains containing the Vi (virulence) antigen are designated V strains; those that have partially lost Vi antigen, V-W strains; and those that do not contain Vi antigen, W strains. The organism is transmitted by water or food contaminated by human excreta [1].

Tolerance to acidic environments is an important property of free-living and pathogenic enteric bacteria. Salmonella enterica serovar typhimurium possesses two general forms of inducible acid tolerance. One is evident in exponentially growing cells exposed to a sudden acid shock. The other is induced when stationary-phase cells are subjected to a similar shock. These log-phase and stationary-phase acid tolerance responses (ATRs) are distinct in that genes identified as participating in log-phase ATR have little to no effect on the stationary-phase ATR [12].

Salmonella typhimurium encounters a variety of acid stress situations during pathogenesis and in the natural environment. These include the extreme low pH encountered in the stomach and a less acidic intestinal environment containing large amounts of organic weak acids (volatile fatty acids). The acid tolerance response (ATR) is a complex defence system that can minimize the lethal effects of extreme low pH (pH3) [13,14].

Previous studies showed that salmonella serovar typhimurium cells in exponential and stationary growth phase which are subjected to acid challenge in planktonic and surface-associated states, acquired increased acid tolerance upon surface contact with various surfaces, such as fresh-cut apples, agar and polyethersulphone membranes. The alternative sigma transcription factor was not required to acquire surface contact-mediated acid tolerance [15].

A major goal of the present study was to investigate the acid tolerance of *Escherichia coli* O157: H7 strain and Salmonella typhi strain (\a group D serotype) and their survival in apple and orange juices and ciders.

The growth of strain *E. coli* and strain *Salmonella typhi* (a group D serotype) in trypticase soy broth adjusted to pH levels ranging from 1.0 to 12.0 was examined.

The bacterial growth was also assessed in natural (unpasteurized, untreated) and pasteurized, treated apple and orange juices. The effects of benzoic acid, as a preservative, on the growth of strain *E. coli* O157: H7 and strain *Salmonella typhi* (a group D serotype) in trypticase soy broth and apple and orange juices and ciders were also examined.

MATERIALS AND METHODS

Experiments were carried out using laboratory media as well as apple and orange juices, as the growth media, to investigate the growth patterns in the absence and in the presence of chemical preservatives.

Organisms: *Escherichia coli* O157: H7 and strain Salmonella typhi (a group D serotype) were obtained from the Al-Hada Armed Forces Hospital, Department of Laboratory Medicine, Microbiology Section, in Taif governorate, Kingdom of Saudi Arabia. The *E. coli* O157:H7 strains were isolated from human stools during an outbreak of haemorrhagic colitis in Taif, K. S. A, and from raw foods implicated in a haemorrhagic colitis outbreaks. The Salmonella typhi (a group D serotype) strains were originally isolated from contaminated food during an outbreaks of typhoid fever and food poisoning. All the organisms are maintained on trypticase soy agar (TSA; pH 7.3, Difco Laboratories, Detroit, Michigan, U. S. A.) and stored at 4°C. Whenever needed, these were activated by transferring loop inocula into 7 ml trypticase soy broth (TSB) at pH 7.2 (Difco Laboratories, Detroit, Michigan, U. S. A.) and incubating for (18-24) hours. All bacteriological procedures were performed according to [1] and Koodie and Dhople [16].

The *Escherichia coli* strain and Salmonella typhi (a group D serotype) used in this study, were re-serotyped using commercially available antisera supplied by : (Oxoid Limited, Laboratory, Texas, USA).

Survival at Low and High Ph: The TSB was adjusted to the desired pH (1.0 , 3.0 , 5.0 , 7.0, 9.0 and 12.0) with 1N hydrochloric acid or 1N sodium hydroxide, and was dispensed in 7 ml volumes into 16 x 100 mm screw cap tubes. The tubes were sterilized for 20 min at 121°C. The pH of the TSB did not change after autoclaving. The tubes were inoculated with 0.1 ml of (18-24) hours culture of the respective organism (with an absorbance of ± 0.7 at 540 nm) and incubated at 37°C. The optical density (OD) readings were taken (18-24) hours later at 540 nm using a spectronic - 20 spectrophotometer.

Survival in Apple and Orange Juices: Apple and orange juices were purchased from two sources, one form a local grocery store and the other from natural food store. The former was clear liquid which had been pasteurized, while the latter (natural) was cloudy, untreated and unpasteurized. Both were free of any preservatives. The

natural apple and orange juices were first centrifuged for 15 min at 5,000 x g and the clear supernatant was used. Like the TSB, the pH of each kind of apple and orange juices was adjusted using hydrochloric acid and sodium hydroxide. Each kind of juice was distributed in 7 ml volumes into 16 x 100 mm screw cap tubes, inoculated with the respective strains of *E. coli* or *Salmonella typhi* and incubated at 37°C. OD readings were taken (18-24) hours later as described above.

Effect of Preservative: One common preservative was used, benzoic acid. Pasteurized aqueous solutions of that acid was added singly to either TSB medium or to apple juice and orange juice to achieve the final concentration of acid at 0.025% , 0.05% and 0.1% (w/v).

The TSB or apple and orange juices samples were inoculated with either strains of *E. coli* O157: H7 or with *Salmonella typhi* (a group D serotype). The tubes were incubated at 37°C for (18-24) hours before taking OD reading.

Statistical Analysis: All data were analysed using the general liner model of the Statistical Analysis System [17] procedure. The least significant difference test was used to determine whether significant differences (p = 0.05) existed between the two types of organisms [*E. coli* O157:H7 and *Salmonella typhi* (a group D serotype)] subjected to examination in this investigation.

RESULTS

Results are presented in (Tables 1, 2, 3, 4, 5 and 6). Each experiment was repeated three times, and triplicate tubes were used for each variable.

Investigations were undertaken using TSB adjusted to a pH ranging from 1.0 to 12.0 to define the survival of *E. coli* O157: H7 and *Salmonella typhi* (a group D serotype) at extreme pH values (Table 1). The *E. coli* O157:H7 serotype and Salmonella typhi (a group D serotype) strains were resistant to low pH.

Table 1: Survival of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in TSB at extreme pH levels

TSB (pH)	Growth (OD* at 540 nm) of *E. coli* O157:H7	Growth (OD* at 540 nm) of *Salmonella typhi* (a group D serotype)	Blank
1.0	0.963	0.894	0.790
3.0	0.713	0.672	0.628
5.0	0.742	0.726	0.701
7.0	0.720	1.760	0.600
9.0	1.700	1.850	0.707
12.0	0.000	0.000	0.800

* OD, read as absorbance.

Table 2: Survival of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in TSB containing benzoic acid as a preservative

			Growth (OD* at 540 nm) of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in presence of :	
TSB(pH)	Concentration of BA* (%)	Benzoic acid : Control	*E. coli* O157:H7 serotype	*S. typhi* (a group D serotype)
3.0	0.025	0.000	1.690	1.487
	0.050	0.000	1.703	1.394
	0.100	0.000	0.000	0.000
5.0	0.025	0.2452	1.801	1.589
	0.050	0.000	1.812	1.499
	0.100	0.000	0.000	0.000
7.0	0.025	0.959	1.968	1.622
	0.050	0.000	1.892	1.595
	0.100	0.000	0.000	0.000

* OD, optical density at 540 nm, giving an absorbance reading.

** BA, benzoic acid; S. typhi, Salmonella typhi.

Note: The blank at all concentrations of BA was (0.600).

Table 3: Survival of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in natural and pasteurized apple juice

	Growth (OD* at 540 nm) of *E. coli* in :		Growth (OD* at 540 nm) of *Salmonella typhi* in :	
Apple juice (pH)	Natural apple juice	Pasteurized apple juice	Natural apple juice	Pasteurized apple juice
1.0	0.883	0.340	0.963	0.298
3.0	0.699	0.272	0.799	0.271
5.0	0.711	0.297	0.810	0.336
7.0	0.730	0.303	0.800	0.350
9.0	1.520	0.630	1.640	0.700
12.0	0.790	0.315	0.850	0.370

* OD, read as absorbance

Note: The blanks for natural apple juice and pasteurized apple juice were 1.53 and 0.67 consecutively.

Table 4: Survival of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in natural and pasteurized orange juice

	Growth (OD* at 540 nm) of *E. coli* in:		Growth (OD* at 540 nm) of *Salmonella typhi* in:	
Orange juice (pH)	Natural orange juice	Pasteurized orange juice	Natural orange juice	Pasteurized orange juice
1.0	0.6820	0.301	0.9640	1.267
3.0	0.4170	0.116	0.7270	1.064
5.0	0.4211	0.117	0.7332	1.077
7.0	0.5660	0.115	0.7450	1.070
9.0	1.1500	2.190	1.4900	2.120
12.0	0.5750	1.120	0.7550	1.090

* OD, read as absorbance

Note: The blanks for natural orange juice and pasteurized apple juice were 1.06 and 2.25 consecutively.

Table 5: Survival of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in apple juice supplemented with benzoic acid

Apple juice	Concentration of BA*	Growth (OD** at 540 nm) of *E. coli* O157: H7 serotype	Growth (OD** at 540 nm) of *S. typhi* (a group D serotype)
Natural	0.0	1.1520	1.1499
	0.025	1.0063	1.0084
	0.050	0.0	0.0
	0.10	0.0	0.0
Pasteurized	0.0	1.308	1.303
	0.025	1.2189	1.2170
	0.050	0.0	0.0
	0.10	0.0	0.0

* BA, benzoic acid.

** OD, read as absorbance.

Table 6: Survival of *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) in orange juice supplemented with benzoic acid

Orange juice	Concentration of BA*	Growth (OD** at 540 nm) of *E. coli* O157: H7 serotype	Growth (OD** at 540 nm) of *S. typhi* (a group D serotype)
Natural	0.0	1.7542	1.8222
	0.025	1.3367	1.4127
	0.050	0.0	0.0
	0.10	0.0	0.0
Pasteurized	0.0	1.6020	1.7791
	0.025	1.4190	1.6182
	0.050	0.0	0.0
	0.10	0.0	0.0

* BA, benzoic acid.

** OD, read as absorbance

The growth of the two organisms at pH values of 3.0 to 12.0 was similar at all values of pH approximately, but at pH 5.0 the growth of these organisms were significantly. Both types of organisms showed no growth at pH 12.0.

The effects of benzoic acid on the growth of both organisms of *E. coli* O157:H7 serotype and *Salmonella typhi* (a group D serotype) are given in Table 2.

Benzoic acid, when incorporated in TSB at 0.025% concentration exhibited any inhibition of the growth of these organisms. This was true for all pH values tested.

However, when added to TSB at concentrations of 0.05% and above, the growth of both organisms subjected to investigation was completely inhibited.

Both the pasteurized apple and orange juices and the supenatants from natural apple and orange juices were examined for any indigenous mirco-organisms.

Aliquots of 7 ml from each were dispensed in 16 x 100 mm sterile screw cap tubes and incubated at 37° C for 7 days. Similarly, aliquots from each were inoculated on trypticase soy agar, malt extract agar and sabouraud dextrose agar, and the plates were inoculated for 7 days at 37° C. No indigenous organism was present in either of the two kinds of apple and orange juices. The pH of both the natural and pasteurized apple and orange juices was adjusted to 3.0, 5.0 and 7.0 (the original pH levels for both the juices were 3.6).

The results on the survival of each *E. coli* O157:H7 serotype and *Salmonella typhi* (a group D serotype) in these apple and orange juices are presented in tables 3 and 4. Again, *E. coli* O157:H7 serotype and *S. typhi* (a group D serotype) strains exhibited normal growth at all six pH levels for both types of apple and orange juices. In order to study the effects of benzoic acid as preservative in apple and orange juices, the compound was added to both the juices, at their natural pH of 3.6, at final concentrations ranging from 0.025% to 0.1% (w/v) (Tables 5 and 6).

In this experiment the pH of neither apple or orange juices was changed, and the inherent pH of each of the two types of apple and orange juices was 3.6. The data again showed that the *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) strains grew well in both types of apple and orange juices.

The addition of 0.05% and higher concentrations of benzoic acid prevented the growth of the *E. coli* O157: H7 serotype and *Salmonella typhi* (a group D serotype) strains in both types of apple and orange juices. In order to establish the relationship between the number of bacteria present in the apple and orange juices to the occurrence of visible growth, aliquots of natural apple and orange juices were inoculated with 10 , 50 and 100 colony forming units (CFU; viable organisms as determined by inoculating on TSA) of *E. coli* O157:H7 serotype and *Salmonella typhi* (a group D serotype) and incubated at an ambient room temperature of 22-23°C. The OD readings were taken daily. With either 50 or 100 CFU per ml of the juice, the first visible sign of growth occurred on the third day, but with 10 CFU per ml, it took 6 days for the visible growth to occur.

DISCUSSION

Unpasteurized apple cider and juice have been associated with outbreaks of *E. coli* O157:H7 infection, cryptosporidiosis, and salmonellosis [18,19].

Animals are the primary reservoir for the pathogenic organisms associated with these outbreaks. In particular, cattle, deer, and sheep can asymptomatically carry *E. coli* O157:H7 and Cryptosporidium, and many animals, including cattle, chickens, and pigs, can asymptomatically carry Salmonella.

The practice of using drop apples for making apple cider is common [11] and apples can become contaminated by resting on ground contaminated with manure. In an outbreak of *E. coli* O157: H7 infections in 1991 [11] the cider press operator also raised cattle, and cattle grazed in a field adjacent to the mill. The presence of animals near a cider mill can result in manure inadvertently contacting apples, equipment, or workers' hands. In addition, apples or oranges can become contaminated if transported or stored in areas that contain manure, or if rinsed with contaminated water.

Escherichia coli O157:H7 is a serious foodborne pathogen, causing life-threatening maladies including hemorrhagic colitis, hemolytic-uremic syndrome, and thrombotic-thrombocytopenic purpura [6,20]. Although outbreaks of *E. coli* O157:H7 infection are frequently associated with eating undercooked ground beef, a variety of other foods, including dry and acidic foods also have been implicated as vehicles of infection [21,22]. Outbreaks associated with highly acidic foods are of particular concern because acidic conditions are normally considered sufficient not only to inhibit the growth of but also to kill most foodborne pathogens. Hence, the tolerance of *E. coli* O157: H7, which has a low infectious dose, to acidic foods compounds the serious nature of this bacterium as a foodborne pathogen [23]. On the other hand, most of the cases involving *E. coli* O157: H7 strain were associated with the consumption of

undercooked ground meat, unpasteurized milk or person - to - person contact [3]. This condition was of concern because of the acidic pH of the apple cider, which is normally about 3.8-4.0, due to the presence of malic acid and lactic acid [24].

The outbreaks of haemolytic ureamic syndrome by the consumption of unpasteurized apple and orange juices indicated that the resistance to acidic pH might be another characteristic which distinguished the *E. coli* O157: H7 serotype from other *E. coli* [16].

In relation to Salmonella, Bearson [25] stated that the acid tolerance response enables Salmonella typhimurium to survive exposures to potentially lethal acidic environments. The acid stress imposed in a typical assay for acid tolerance (log-phase cells in minimal glucose medium) was shown to comprise both inorganic (i. e., low pH) and organic acid components. *Salmonella* spp. and *E. coli* can adapt and grow at low pH values if sequential acid adaptation is performed [26,27]. The results presented in this investigation seem to confirm this view.

The survival of the *E. coli* O157: H7 serotype and *S. typhi* (a group D serotype) was distinct at both pH 3.0 and 5.0. The result showed that the growth of *E. coli* O157: H7 serotype and S. typhi (a group D serotype) in TSB at pH 3.0 and 5.0 was similar to that at pH 7.0. The same was true in apple and orange juices. No growth was obtained at pH 1.0, while the growth at pH 5.0 was similar to that at pH 7.0., perhaps due to presence of additional nutrient factors in the apple and orange juices.

Furthermore, *E. coli* O157: H7 serotype and *S. typhi* (a group D serotype) microorganisms exhibited growth even at pH 3.0. Both the natural (unpasterurized) and pasteurized apple and orange juices were free of indigenous flora due to centrifugation and pasteurization of the respective juices.

Earlier, Salmonella had been identified in one outbreak involving the consumption of apple cider [28] and recently by the consumption of orange juice [29].

However, such acid tolerance has been shown to be the adaptation of Salmonella to an acidic environment [30] and not a characteristic of strain *E. coli.* O157:H7 as seen in the present study. The preservatives such as sodium benzoate, sorbic acid and benzoic acid, are generally introduced to prevent or delay food spoilage, and are added to food mainly to prevent the growth of mould and yeast, but can also kill bacteria [16]. The results of our study indicated that at 0.05% concentration, benzoic acid inhibit the growth of both *E. coli* O157:H7 and *S. typhi* strains in TSB as well as in apple and orange juices. Similar results were reported earlier by Zhao *et al.* [31].

Unpasteurized fresh apple and orange juices are traditional, commercial and imported products. However, quite a few of these products have been implicated as the vehicle for food-borne diseases, particularly haemolytic uraemic syndrome caused by *E. coli* O157: H7, Salmonellosis and cryptosporidiosis [18,22]. This has raised doubts about the safety of unpasteurized juices.

An important, but as yet ananswered, question is the source(s) of contamination of apple and orange ciders and juices and with *E. coli* O157:H7 strain. Cattle, sheep and deer may serve as reservoirs for this organism [32,33]. Recent studies have shown the potential of fruit flies (Drosophila melanogaster) to transmit *E. coli* O157: H7 strain to apples [34].

A wide variety of acids, their salts and derivatives are used as chemical preservatives. Acids can be added to foods to lower the pH value which can eliminate the growth of certain microbial populations [35]. It is evident that using 0.05% of sodium benzoate or sodium sorbate is an option which the processors have in order to increase substantially the safety of apple cider [16].

Finally, it would be concluded that using acids, their salts and derivatives is efficient and has some usefulness in controlling microbial growth in foods.

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