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Experimental Studies on the Potential for Acid Tolerance, Growth and Survival of *Salmonella enterica* Serovar *Typhimurium* and *Escherichia coli* O157: H7 in Orange Juices

[1]*Youssuf Abdul-Malik Al tayib and* [2]*Munir Mustafa Al-Bashan*

[1]Department of Chemistry, College of Science, Taif University, KSA
[2]Department of Biologyy, College of Science, Taif University, KSA

Abstract: Outbreaks of diarrhea, haemolytic uraemic syndrome and food poisoning have been associated with the consumption of orange ciders and juices. The organisms implicated in these outbreaks have been *Escherichia coli* O157:H7 serotype and Salmonella enterica serovar typhimurium, indicating the resistance of the serotypes of these organisms to acidic pH. Experimental studies on the potential for acid tolerance, growth and survival of Salmonella enterica serovar typhimurium and *Escherichia coli* O157:H7 in both natural (unpasteurized, untreated) and pasteurized, treated orange juices and ciders. The effects of benzoic acid, as a preservative, on the growth of the mentioned bacteria subjected to examination, in typticase soy broth and orange juices and ciders were also examined. It was evident that *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium organisms grew well in trypticase soy broth at pH level ranging from 3.0 to 9.0. The growth of both microorganisms subjected to study were inhibited by adding 0.05% of benzoic acid. Similarly, *E. coli* 0157:H7 serotype and Salmonella enterica serovar typhimurium strains grew well in both natural (unpasteurized) as well as in pasteurized orange juices and ciders and the growth was inhibited by adding 0.1% of benzoic acid. The possible sources of contamination of natural orange juices and ciders with *E. coli* 0157:H7 serotype and Salmonella enterica serovar typhimurium are discussed. The study declared the efficacy of acids, their salts and derivates in controlling microbial growth in food in general. The results provide evidence that internalization, survival and growth of human bacterial pathogens may occur within oranges. There is a need to establish the likelihood and levels of technologies needed to assure the microbiological safety of juices.

Key words: Acid tolerance · growth · survival · *Salmonella* · *Escherichia coli* · orange juices

INTRODUCTION

Fresh fruit and vegetable juices are recognized as an emerging cause of foodborne illness [1]. A contributing factor is that these products are raw agricultural commodities, which may become contaminated by animal or human waste and consumed without a processing step that will kill or remove associated pathogens. While a single piece of contaminated produce may infect a single person, contaminated produce that is co-mingled, juiced and served may infect many individuals [2].

Contamination, infiltration and survival of microorganisms in fruits and vegetables have been documented, although most reported data concern plant pathogens and spoilage organisms. One potential source of entry of microorganisms into fruits and vegetable is by environmental exposure with uptake occurring through either specific morphological structure in plant and/or through breaks in tissues that occur as a result of punctures, wounds, cuts and splits [2].

Escherichia is a genus of gram-negative, facultatively anaerobic, rod-shaped bacteria of the tribe Escherichieae, family Enterobactriaceae, 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 [3]. *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 [4].

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[5].

Corresponding Author: Dr. Youssuf Abdul-Malik Al Tayib, Dept. of Chemistry, College of Science, Taif University, KSA

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 [6].

Outbreaks of strain O157:H7 have been associated with a range of foods, including ground beef, raw milk and contaminated water [7].

This microbe is a persistent problem in cattle [8] and outbreaks of disease involving fresh produce and fruit juices, have been reported with increasing frequency [9]. The first outbreak of this disease by consuming apple cider was in the fall (autumn) of 1991 [10].

Recent outbreaks of strain 0157:H7 involving fresh apple juice indicate the resistance of the bacterium to acidic pH and have raised doubts about the safety of unpasteurized 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 [3].

There is strong evidence that Salmonella enterica serovar typhimurium has established reservoirs in wild-living birds and hedgehogs in Norway [11]. Wild-living birds and hedgehogs may function as effective spreaders of Salmonella bacteria to humans and to different animal species through contamination of the environment [11, 12]. In Norway, sporadic indigenous cases and national outbreak of human Salmonellosis, caused by serovar typhimurium, have been related to infections in small passerines [12].

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 [13].

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 environmental 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) [14, 15].

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 [16].

It is therefore important to gain further knowledge of epidemiology of Salmonella bacteria in a range of foods, involving fruits ciders and juices, in particular of the endemically distributed serovar typhimurium.

The objectives of the current study were to determine the potential for human bacterial pathogens to internalized within oranges and to determine the potential for acid tolerance, growth and survival of Salmonella enterica serovar typhimurium and *Escherichia coli* O157:H7 within natural orange juices.

MATERIALS AND METHODS

Microorganisms: *Escherichia coli* O157:H7 and Salmonella enterica serovar typhimurium 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 tools during an outbreak of haemorrhagic colitis in Taif, K. S. A. and from raw food implicated in a haemorrhagic colitis outbreaks.

The Salmonella enterica serovar typhimurium strains were originally isolated from contaminated food during an outbreaks of Salmonellosis and food poisoning. Permanent cultures were maintained at-70°C.

Inoculum: Working cultures were maintained on Brain Heart Infusion (BHI) and on Typticas Soy Agar (TSA) slants, [BHI and TSA; pH 7.3, Difco Laboratories, Detroit, Michigan, U.S.A.] and stored at 4°C. Wherever needed, these were activated by transferring loop inocula into 7 ml BHI broth at pH 7.2 (Difco Laboratories, Detroit, Michigan, USA), grown overnight at 37°C and then refrigerated. This broth was used to inoculate overnight cultures and was not kept for longer than 1-week before a new culture was generated from the slant culture. Overnight cultures were started by inoculating into tryptic soy broth containing 1% dextrose (TSB + 1% G) medium using a 0.1 ml of the culture broth and were incubated at 37°C without shaking for at least 18 h.

Oranges: Egyptian oranges (42, 7.3-cm average diameter) purchased from a local produce market were used for all experiments. Oranges were examined and those with visual defects were not used.

Media: Xylose Lysine Deoxychocolate agar was from BBL. All other media were from Difco. Brilliant Blue FCF dye was from Warner-Jenkinson.

All bacteriological procedures were performed according to Forbes *et al.* [3], Koodie and Dhople [17] and Walderhaug *et al.* [2].

Infiltration Studies: Oranges were selected and placed in a 37°C incubator overnight to equilibrate. Concurrently, a 10-ml culture of *E. coli* O157:H7 and Salmonella enterica serovar typhimurium were inoculated separately in each orange and allowed to grow overnight at 37°C. The next morning 8-mg of Brilliant Blue FCF was added to the bacterial culture and 0.1 ml of the dye-culture solution containing approximately 7-log cfu *E. coli* O157:H7 and *Salmonella enterica* were also inoculated solely onto the stem scar of the warm oranges, after removal of residual stem material. Oranges were then transferred to a 4°C incubator and allowed to equilibrate for 3-hours. During this time the solution appeared to be internalized by the oranges and the internal temperature was observed to decrease from 35°C to 11°C. Oranges were then juiced using the following protocol: Inoculated stem scar areas were submerged in a 80°C water bath for one minute to sanitize the contaminated surfaces [18]. Afterwards oranges were halved with a sterile knife through the midline of the orange lateral to the stem scar and juiced in a juicer. The juicers were cleaned and rinsed after each orange was juiced. Juice was collected in sterile 50-ml centrifuge tubes and the volume was recorded. Duplicate samples were plated on BHI agar and Sorbitol MacConkey plates using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda MD). Plates were incubated either at 37°C overnight or at room temperature (21°C) for 48-hours and counted on a laser colony scanner Model 500 A (Spiral Biotech). *E. coli* O157:H7 and Salmonella enterica serovar typhimurium colonies detection and identification were carried out according to Forbes *et al.* [3].

Survival studies 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 16X100 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 studies in orange juices: Orange juices were purchased from tow sources, one from 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 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 orange juices was adjusted using hydrochloric acid and sodium hydroxide.

Each kind of juice was distributed in 7 ml volumes into 16X100 mm screw cap tubes, inoculated with the respective strains of *E. coli* or Salmonella enterica and incubated at 37°C. OD reading 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 orange juice to achieve the final concentration of acid at 0.025, 0.05 and 0.1% (w/v).

The TSB or orange juices samples were inoculated with either strains of *E. coli* O157:H7 or with Salmonella enterica serovar typhymurium. 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 [19] procedure. The least significant difference test was used to determine whether significant differences ($p \leq 0.05$) existed between the two types of organisms (*E. coli* O157:H7 and Salmonella enterica serovar typhimurium.) subjected to examination in this investigation.

RESULTS

Results obtained were recorded in (Table 1-5). Each experiment was repeated three times and triplicate tubes were used for each variable.

The present investigations were carried out using TSB adjusted to a pH ranging from 1.0 to 12.0 to define the survival of *E. coli* O157:H7 and Salmonella enterica serovar typhimurium at extreme pH values (Table 1).

Table 1: Survival of *E. coli* O157:H7 serotype and *Salmonella enterica* serovar *typhimurium* in TSB at extreme pH levels

TSB (pH)	Growth (OD* at 540 nm) of *E. coli* O157:H7 serotype	Growth (OD* at 540 nm) of *Salmonella enterica* serovar *typhimurium*	Blank
1.0	0.964	0.895	0.791
3.0	0.714	0.673	0.630
5.0	0.744	0.727	0.703
7.0	0.74	1.77	0.602
9.0	1.9	1.86	0.709
12.0	0.0	0.0	0.801

* OD, read as absorbance

Table 2: Survival of *E. coli* O157:H7 Serotype and Salmonella enterica serovar typhimurium in TSB containing benzoic acid as a preservative

		Growth (OD* at 540 nm) of *E. coli* O157:H7 serotype and *Salmonella enterica* serovar *Typhimurium* in presence of:		
TSB (pH)	Concentration of BA** (%)	Benzoic acid: control	*E. coli* O157:H7 serotype	Salmonella enterica serovar Typhimurium
3.0	0.025	0.0	1.692	1.489
	0.05	0.0	1.704	1.396
	0.1	0.0	0.0	0.0
5.0	0.025	0.2460	1.803	1.591
	0.05	0.0	1.814	0.501
	0.1	0.0	0.0	0.0
7.0	0.025	0.9599	1.970	1.624
	0.05	0.0	1.894	1.597
	0.1	0.0	0.0	0.0

* OD, Optical Density at 540 nm, giving an absorbance reading,** BA, benzoic acid, Note: The blank at all concentrations of BA was (0.600)

Table 3: Survival of *E. coli* O157:H7 serotype and *Salmonella enterica* serovar *typhimurium* in natural inoculated orange juice

Orange juice (pH)	Growth (OD* at 540 nm) of *E. coli* serotype	Growth (OD* at 540 nm) of *Salmonella enterica* serovar *typhimurium*
1.0	0.994	0.997
3.0	0.859	0.865
5.0	0.891	0.897
7.0	0.87	0.90
9.0	1.75	1.82
12.0	0.91	0.95

* OD, read as absorbance Note: The blank for natural orange juice was 1.63.

Table 4: Survival of *E. coli* O157:H7 Serotype and *Salmonella enterica* serovar *typhimurium* in natural and pasteurized orange juice

	Growth (OD* at 540 nm) of *E. coli* O157:H7 serotype in:		Growth (OD* at 540 nm) of *Salmonella enterica* serovar *typhimurium* in:	
Orange juice (pH)	Natural orange juice	Pasteurized orange juice	Natural orange juice	Pasteurized orange juice
1.0	0.879	0.342	0.964	0.299
3.0	0.689	0.273	0.800	0.272
5.0	0.710	0.298	0.811	0.337
7.0	0.72	0.304	0.81	0.36
9.0	1.51	0.64	1.66	0.72
12.0	0.78	0.316	0.86	0.38

* OD, read as absorbance, Note: The blanks for natural orange juice and orange pasteurized juice were 1.55 and 0.69 consecutively

Table 5: Survival of *E. coli* O157:H7 serotype and *Salmonella enterica* serovar *typhimurium* and *Escherichia coli* O157:H7 in orange juice supplemented with benzoic acid

Orange juice (pH)	Concentration of BA*	Growth (OD* at 540 nm) of *E. coli* O157:H7 serotype	Growth (OD* at 540 nm) of Salmonella enterica serovar typhimurium
Natural	0.0	1.7555	1.8228
	0.025	1.3370	1.4130
	0.050	0.0	0.0
	0.10	0.0	0.0
Pasteurized	0.0	1.6022	1.7794
	0.025	1.4199	1.6184
	0.050	0.0	0.0
	0.10	0.0	0.0

*BA, benzoic acid,** OD, read as absorbance

The *E. coli* O157:H7 serotype and Salmonella enterica serovar strains were resistant to low pH.

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 enterica serovar typhimurium 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.

The survival of *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium in natural inoculated orange juices was observed distinctly. The growth of the two organisms at pH values of 1.0 to 12.0 was increased and variant at all measured values of pH, but at pH 9.0 the growth of these organisms were increasingly. Both types of organisms revealed growth at pH 12.0 (Table 3).

The pasteurized orange juices and the supernatant from natural orange juices were examined for any indigenous micro-organisms.

Aliquots of 7 ml from each were dispensed in 16 X100 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 orange juices. The pH of both the natural and pasteurized orange juices was adjusted to 3.0, 5.0 and 7.0 (The original pH level for both the juices were 3.6).

The results on the survival of each *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium in natural and pasteurized orange juices are recorded in Table 4. The growth of these organisms was higher and significantly in natural orange juices than pasteurized orange juices.

The present study demonstrated that *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium strains exhibited normal growth at all six pH levels for orange juices.

In order to investigate the effects of benzoic acid as preservative in orange juices, the compound was added to the orange juices, at their natural pH of 3.6, at final concentrations ranging from 0.025 to 0.1% (w/v) (Table 5).

In this investigation the pH of orange juices was changed and the inherent pH of orange juices was 3.6. The results again stated that *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium strains grew well in 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 enterica serovar typhimurium strains in orange juices.

In order to establish the relationship between the number of bacteria present in orange juices to occurrence of visible growth, aliquots of natural 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 enterica serovar typhimurium and incubated at an ambient room temperature of 22-23°C. The OD reading were taken daily. With either 50 or 100 CFU pre ml of the juice, the first visible sign of growth occurred on the third day, but with CFU per ml, it took 6 days for the visible growth to occur.

DISCUSSION

Key findings of the current study were the demonstration that infiltration of human pathogens into oranges can occur and that survival and growth can occur under conditions. The demonstration in this study that a human pathogen can be internalized by oranges at an uptake frequency of 3% and at a level of 0.001 to 0.0001 of the challenge levels is an agreement with the earlier dye-uptake studies [20]. Oranges having visually or non-visually obvious peel defects may allow for higher infiltration frequencies and levels. These findings are consistent with pathogen uptake studies using other fruit and vegetable commodities, including apples and tomatoes [21].

Unpasteurized apple cider and juice have been associated with outbreaks of *E. coli* O157:H7 infection, cryptosporidiosis and Salmonellosis [22, 23].

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 [10] and apples can become contaminated by resting on ground contaminated with manure. In an outbreak of *E. coli* O157:H7 infections in 1991 [10], 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 [24]. 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 [25, 26]. Outbreaks associated with highly acidic foods are of particular concern because acidic conditions are normally considered sufficient not only to inhibit the growth of some bacteria but also to kill most foodborne pathogens. Hence, the tolerance of *E. coli* O157:H7, which has a low infections dose, to acidic foods compounds the serious nature of this bacterium as a foodborne pathogen [27]. As it is, 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 contant [5]. 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 [28].

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* [17].

In relation to Salmonella, Bearson *et al.* [29] 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 [30, 31]. The results presented in this investigation seem to confirm this view.

The survival of the *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium was distinct at both pH 3.0 and 5.0. The result revealed that the growth of *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium in TSB at pH 3.0 and 5.0 was similar to that at pH 7.0. The same was true in 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 orange juices.

Furthermore, *E. coli* O157:H7 serotype and Salmonella enterica serovar typhimurium microorganisms exhibited growth even at pH 3.0. Both the natural (unpasteurized) and pasteurized orange juices were free of indigenous flora due centrifugation and pasteurization of the respective juices.

Earlier, Salmonella had been identified in one outbreak involving the consumption of apple juice [32] and recently by the consumption of orange juice [33].

However, such acid tolerance has been shown to be the adaptation of Salmonella to an acidic environment [34] 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 [17]. The results of our study indicated that at 0.05% concentration, benzoic acid inhibit the growth of booth *E. coli* O157:H7 serotype and

Salmonella enterica serovar typhimurium strains in TSB as well as in orange juices. Similar results were reported earlier by Zhao *et al.* [35] and Zhao *et al.* [36].

Unpasteurized fresh 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 cause by *E. coli* O157:H7, Salmonellosis and cryptosporidiosis [22, 26]. This has raised doubts about the safety of unpasteurized juices.

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 [37]. 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 safetly of apple cider [17].

The current results support research on other commodities that demonstrate the survival of *E. coli* O157:H7 and Salmonella spp. in low pH environments [21, 38].

While the present study has provide evidence for the potential for human bacterial pathogens to enter, survive and grow within intact oranges, their natural occurrence on or within oranges is unknown. Further research is required to better characterize the factors that lead to contamination and infiltration of human pathogens within oranges. Most critically however, there is a need for data to establish the likelihood of these events occurring during cultivation, harvesting, transport, storage, or processing. In addition, data are needed to establish the natural levels of human pathogens on or within oranges. This information would provide a rationale basis for designing intervention technologies that are needed to assure the microbiological safety of juices.

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