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Effects of orange juice pH on survival, urease activity and DNA profiles of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* stored at 4 degree C

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Abstract

The objective of this study was to determine the survival, growth rate and possible cellular adaptation mechanisms of *Y. pseudotuberculosis* and *Y. enterocolitica* in orange juice under different pH conditions. *Yersinia* was inoculated in orange juice with adjusted pH levels of 3.9, 4.0, and 7.0 and stored at 4 C for 3, 24, 72 and 168 hours (h). The inter- and intra-species variation is significant to the pH and time of incubation variables ($p < 0.05$). At 3.9 pH the CFU (colony forming units) count decreased significantly.

At pH 3.9 and 4.0, *Y. enterocolitica* and *Y. pseudotuberculosis* survived for at least 30 days and 15 days, respectively. *Yersinia* that survived under low pH in orange juice revealed enhanced urease activity within 12 h of incubation. The attachment gene (*ail*) could not be detected by PCR in *Y. enterocolitica* from undiluted sample incubated for 24 h or longer. Moreover, the *FesI*-restriction profile was altered when *Y. pseudotuberculosis* was stored at pH 4.0 orange juice for 7 days. These results indicate that *Yersinia* could survive and grow at low pH and the survival mechanisms could also enable the bacteria to survive the stomach pH barrier to cause enteric infection.

Practical Applications

The threat of contamination in the nation's food supplies has become an alarming issue. *Y. pseudotuberculosis* and *Y. enterocolitica* are known for their ability to grow at low temperatures and contaminate food supplies. *Yersinia* spp were isolated and reported in different food matrices; however the risk of contamination was not sufficiently addressed in orange juice. The focus and application of our study was to investigate the ability of *Y. pseudotuberculosis* and *Y. enterocolitica* to survive and grow in orange juice at low pH and incubated at standard storage temperature (4 C). There is a Knowledge gap to explain what contribute to the survival and propagation of *Yersinia* in orange juice. Because of their similar evolutionary origin, the results extracted about *Y. pseudotuberculosis* will provide valuable information for *Y. pestis*. Survival in acidic environments is important for successful infection of gastrointestinal pathogens. Therefore, these findings could explain the mechanisms that facilitate *Yersinia* to tolerate the low pH in the stomach and establish infection.

Introduction

Currently, contamination of food, either non-intentional or in the form of a covert biological attack, is an alarming global issue. *Y. pseudotuberculosis* and *Y. enterocolitica* are psychrophilic bacteria that can proliferate in food matrices from a very low level of contamination to clinically significant levels when stored at 4 C. The enteropathogenic *Yersiniae* are fecal-oral pathogens, which are well known to cause a range of invasive gastrointestinal diseases and for their ability to grow at low temperatures (Sommers and Niemira, 2007; Souza and Santos, 2009). Occasional dissemination of these organisms into the blood, liver, and spleen gives rise to enteric fever. Pathogenicity or virulence in these species is a complex interplay between ecology, geographic distribution, biochemical and antigenic properties, and chromosomal and plasmid-encoded genes (Zink et al., 1982). *Y. pseudotuberculosis* and *Y. enterocolitica* can survive in most natural environments or in food matrices by means of highly adaptable metabolic pathways that are typical of free-living enterobacteria (Rosso et al., 2008).

A number of reports associated with *Yersinia* spp in food matrices have been registered (Rimhanen-Finne et al., 2009; Stenstad et al., 2007). Human yersiniosis is reported and attributed to contaminated pork (Fredriksson-Ahomaa et al., 2001b), chocolate and milk (Otero et al., 2010, Black et al., 1978), egg shells (Musgrove et al. 2004, Favier et al. 2008). mountain stream and well water contamination in Japan (Inoue et al., 1988), contaminated iceberg lettuce in Finland (Nuorti et al., 2004), and tofu consumption, as well as blood transfusions (Morimoto et al., 1990). Although, there is no abundant data regarding *Yersinia* related illness due to consumption of orange juice, food borne illness has been reported from drinking juices such as apple juice (Deliganis and. 1998). The Center for Disease Control and prevention (CDC) has reported 21 juice associated outbreaks between 1995 and 2005 (Vojdani et al., 2008). Ten of the outbreaks were apple juice or ciders related and eight were linked to orange juice (Vojdani et al., 2008).

Y. pseudotuberculosis and *Y. enterocolitica* must overcome the acidic stress in the stomach for successful enteric colonization, but how these pathogens survive in acidic conditions remains largely unknown (Hu et al., 2009). These enterogenic bacteria have a unique ability to maintain their structural integrity over a wide pH range and to survive and propagate under extreme environmental conditions. An association between environmental change and gene regulation has long been established (Young et al., 1996). *Yersinia* have biphasic life cycles during which gene expression adapts them to a spectrum of environments, including niches that become progressively altered through the combined effects of bacterial colonization and host response (Straley and Perry, 1995). *Yersinia* genome contains genes associated with environmental stress modulations. For example, gene *rpoS* encodes an alternative sigma factor (Badger and Miller, 1995), which is critical for the ability of *Y. enterocolitica* to survive diverse environmental insults such as high temperature, hydrogen peroxide, osmolarity, and low pH (Badger and Miller, 1995).

PCR assays and other molecular methods have been developed as efficient tools for identifying pathogenic *Y. enterocolitica*, targeting chromosomal genes such as *ail* (attachment invasion locus, which mediates cell invasion) (Miller et al., 1990), *inv* (invasive gene, which mediates cell invasion) (Rasmussen et al., 1994), *ystA* (which is responsible for the production of a heat-stable enterotoxin in virulent *Y. enterocolitica*) (Delor et al., 1990). Molecular methods have also been used for typing and in epidemiological studies of *Y. enterocolitica* and *Y. pseudotuberculosis*, including restriction analysis of both plasmids and chromosomes, randomly amplified polymorphic DNA analysis, ribotyping and pulsed-field gel electrophoresis (Niskanen et al., 2003)

Acid adaptation prolonged the survival of some pathogens in various food systems, and may have a significant implication in food safety (van Netten et al., 1997). Enzymatic activity such as urease has been well documented and is considered an important factor in acid tolerance (Stoof et al., 2008). Urease has been implicated in playing a role in the pathogenesis of many bacteria such as *Helicobacter pylori*, *Proteus mirabilis*, and *Brucella abortus* (Jones et al., 1990; Marshall et al., 1990; Sangari et al., 2007). *Y. enterocolitica* invariably produces urease which has been reported to enable *biovar 1B* and *biovar 4* strains to survive in the acidic environment of the stomach (De Koning-Ward and Robins-Browne, 1995; de Koning-Ward et al., 1995; Gripenberg-Lerche et al., 2000).

Currently, there is limited information about the survival and growth of *Yersinia* species in orange juice and about the cellular mechanisms this species may use to adapt to an acidic environment. The objective of this study was to examine the growth and genetic alterations of enteropathogenic *Yersinia* caused by the stress of pH and time of exposure. Results from this study will contribute to the fundamental knowledge base that is imperative to characterize the fate of *Yersinia* in contaminated orange juice. Furthermore, the study also investigated the urease activity and genomic profile alterations in *Yersinia* inoculated into orange juice at different pHs and incubated at a standard storage temperature (4 C).

Materials and Methods

1.1. Bacteria strains

Yersinia enterocolitica strain 27729 and *Yersinia pseudotuberculosis* strain 29838 were purchased from the American Type Cultural Collection (ATCC, Manassas, VA). *Y. pseudotuberculosis* (strain NR-804) and *Y. enterocolitica* (NR-214) were generously provided from Biodefense & Emerging Infections Research Resources Repository (BEI Resources; Manassas, VA). *Yersinia* were cultured in CIN (Cefsulodin-Irgasan-Novobiocin; Difco, Sparks, MD) agar, a differential and selective medium used for *Yersinia* species. A known bacterial concentration of all the strains was made to 1.0 OD at 600 nm Abs (NanoDrop, Wilmington, DE) corresponding to an average of $\sim 10^{12}$ cells/ml. A 5 μ l aliquot of this bacterial suspension was used to inoculate 1 ml orange juice samples.

1.2. Orange juice inoculation

Commercially available undiluted (UD) orange juice with a pH of 3.9 (Dole; total fat 0, cholesterol 0, Na 25 mg, K 450 mg, sugar 23 gm and protein < 1 mg) was adjusted to two additional pH conditions; pH 4.0 (diluted with double distilled water to 1:4) and pH 7.0 adjusted with 1N NaOH. Orange juice under these three pH conditions was inoculated with strains of *Y. pseudotuberculosis* and *Y. enterocolitica* and incubated for 3 h, 24 h, 72 h (3 days) and 168 h (7 days) at 4 C temperature, which mimics the actual storage temperature.

1.3. Enumeration of *Yersinia*

Bacterial counts were completed by serial dilution and plating procedures. A tenfold serial dilution was performed aseptically after each treatment and incubation period. 10 μ l of the inoculated orange juice was mixed with 90 μ l (10^{-1}) of peptone water followed by serial dilutions up to 10^{-8} . A direct drop plating of 10 μ l on CIN agar plates was used to enumerate the number of colony forming units per ml (CFU/ml). The number of surviving cells (CFU/ml) was then compared among the different time points and the pH treatments. T_0 was considered as the time of inoculation. Plates inoculated with a sample dilution that yielded between 30 and 300 colonies were used for counting and the viable counting method was carried out in triplicate.

1.4. Urease Activity

The urease activity of *Yersinia* strains was measured *in vitro* as described previously (Cussac et al., 1992). Briefly, *Yersinia* species from each treatment group was inoculated on TSA (Trypticase Soy Agar) slant media containing 29 mg/ml of urea (Remel, Lenexa, KS) supplement and incubated for 48 h. Urease activity was visually monitored and recorded after each hour for 12 h and at 24 and 48 h after inoculation.

1.5. DNA extraction and Real-Time PCR

DNA extraction was performed using PrepMan (Applied Biosystems, Carlsbad, CA) at T₀ and after each incubation period and quantified using the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). About 10 ng of the extracted DNA was used as a template for the amplification of four target genes (*ypi*, *inv*, *ail*, and *yst*) involved in the pathogenicity of *Yersinia*. A SYBR green real time PCR assay (Stratagene, La Jolla, CA) was used to amplify these genes.

Primers specific for the above four genes (Table 1) were designed using Primer Designer software (Primer 3 software, versions 2.01 and 3.0, Scientific & Educational Software, Durham, N.C.) and according to previous publications (Straley and Perry, 1995). The reaction volume was fixed at 25 µl with: 2X SYBR Green (400 uM of each dNTP, 50 µM Taq polymerase, 6 mM MgCl₂); 10 µM of each primer forward and reverse; and 1 µl of DNA template. All the PCR assays were performed in duplicates. The PCR parameters consisted of an initial denaturation step at 95 C for 10 min followed by 30 cycles of denaturation at 95 C for 15 sec, annealing at 60 C for 15 sec, and elongation at 72 C for 30 sec in the Stratagene Mx3000P^R real time PCR instrument (Stratagene, La Jolla, CA).

1.6. Pulsed-field gel electrophoresis

Plugs for restriction enzyme analysis were prepared according to the standard protocol for molecular sub-typing of *Y. pestis* (PulseNet, CDC, http://www.cdc.gov/pulsenet/protocols/yersinia_Apr2006.pdf). Briefly, cells were re-suspended in 200 µl of cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0) and mixed gently with 10 µl of proteinase K (20 mg/ml stock). One millimeter-thick plugs were prepared with a 1:1 mixture of cell suspension and 1% SeaKem Gold: 1% SDS agarose in TE Buffer (10 mM Tris: 1 mM EDTA, pH 8.0) using Plug Molds (Bio-Rad, Hercules, CA). Based on genomic analysis of sequenced strains *Y. pseudotuberculosis* IP 31758 (CP000720.1) and *Y. enterocolitica* subsp. *enterocolitica* 8081 (AM286415.1), four rarely cutting enzymes *FseI*, *NotI*, *SpeI*, and *XbaI* were selected.

Prior to restriction enzyme digestion, all plugs were equilibrated for 30 min in restriction buffer. For digests using *FseI* and *NotI*, gels were run at 5 V/cm for 18 h with the pulse ramp time varied from 2 – 30 sec and 1 – 25 sec, respectively. For restriction enzyme *XbaI* 1.5% gel concentrations were run with pulse ramp time from 0.1 – 7s, for 16 h at 6V/cm. Finally, for fragments generated by restriction enzyme *SpeI*, gels were run with pulse ramp time from 1 – 16 sec for 19 h with 5 V/cm voltage gradient using 1.4% gel concentration. Electrophoresis was conducted in 0.5 X TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.2) at 14 C with angle of 120° in a two-state mode.

The digested fragments in plugs were separated on 1.2 % pulsed-field certified agarose (Bio-Rad, Hercules, CA) gels. The CHEF-DR III (Bio-Rad, Hercules, CA) was used for fragment separation and the running conditions were adjusted according to the restriction enzyme used. *Insilco* simulation (Bikandi et al., 2004) and Vector-NTI 11 (Invitrogen, Carlsbad, CA) software were used to verify the restriction profiles. A lambda ladder and low-range

PFGE marker (New England Biolabs, Ipswich, MA) were used as molecular weight markers.

The gels were then stained with 1 µg/ml ethidium bromide for 15 min, destained with distilled water for 15 min, and the DNA bands were visualized by UV transilluminator and documented.

1.7. Data analysis

All the experiments were performed in triplicates. Colony forming unit (CFU) count was expressed as $\log_{10} N - \log_{10} t_0$, where t_0 is the initial count in orange juice and N was the count after each treatment. The mean and the standard deviation were calculated for the log reduction/increase of each *Yersinia* strain for each of the pH and time of exposure variables. SigmaPlot and Systat Software were used to prepare the graphs and the statistical analysis. Analysis of variance (ANOVA) was used to determine the effects of pH, time of exposure, and the intra-and inter-species differences

Results

The primary focus of this study was to investigate the survival and growth of *Y. pseudotuberculosis* and *Y. enterocolitica* in orange juice. Furthermore, the study assessed changes in urease activity and genomic profile stability of the enteropathogenic *Yersinia* cultured in orange juice with different pH settings. Three pH conditions were: undiluted (UD), pH 3.9; 1:4 diluted in distilled water, pH 4.0; and NaOH titrated, pH 7.0. The inoculated orange juice was stored for 3 h, 24 h, 72 h and 168 h at 4 C. The pH of undiluted orange juice was only slightly less than that of the 1:4 (orange juice: water) dilution with distilled water.

All tested *Yersinia* strains survived in orange juice under the three-pH conditions (Fig. 1 and 2). Based on our results, there was a rapid reduction, ranging in average from 0.2 – 2 log CFU/ml, observed between T_0 and 3 h of incubation at 4 C (Fig. 1 and 2). During the initial 3 h of incubation, the maximum average population reduction (i.e., ~2 log CFU/ml) was observed for *Y. enterocolitica* strain 27729 incubated in the undiluted orange juice (Fig. 1B). The minimum average reduction of viable cells population (i.e., ~0.2 log CFU/ml) was observed in *Y. enterocolitica* strain NR-214 (Fig. 1A) incubated in the NaOH titrated orange juice (pH 7.0). The growth reduction for the two *Y. pseudotuberculosis* strains exposed to the UD orange juice was 1.7 log CFU/ml and 0.7 log CFU/ml for strains 29838 and NR-804, respectively (Fig. 2A and 2B). In the 1:4 diluted orange juice, both *Y. enterocolitica* strains showed an average of 0.8 log CFU/ml reduction (Fig. 1A and 1B); whereas, for *Y. pseudotuberculosis* strains, the average viable bacteria population reductions were 1.33 and 0.55 log CFU/ml for strains 29838 and NR-804, respectively (Fig. 2A and 2B). The average number of viable cells continued to decrease for up to 24 h of incubation for all the strains in the UD and 1:4 diluted orange juice. The bacterial population increase/reduction ratio was calculated by subtracting the average number of live bacteria after a specific incubation time from that of the initial bacteria count at T_0 .

The results suggest that the period between 24 h and 72 h corresponds to the growth recovery phase for almost all the strains under the tested pH conditions. The adaptive and recovery phase continued until at least 168 h of incubation for two *Y. enterocolitica* of the strains tested. The first strain was *Y. enterocolitica* 27729, where the organism recovered and reached an average of 7.3 log CFU/ml at 4 C in the NaOH titrated orange juice (Fig. 1B). This value is slightly lower than the average number of viable cells at T_0 which was 7.96 log CFU/ml. Compared to the values of the 72 h incubation period, strain 27729 showed a minimal increase of 0.25 and 0.28 log CFU/ml in the UD and 1:4 diluted orange

samples, respectively. The second strain that exhibited low pH tolerance and re-growth was *Y. pseudotuberculosis* strain NR-804. *Y. pseudotuberculosis* strain NR-804 reached a mean log CFU/ml of 7.0 in the 1:4 diluted orange juice, which was 0.8 log CFU/ml higher than the value on the third day of incubation (Fig. 2B).

In undiluted orange juice (pH 3.9), the average number of viable cells for *Y. pseudotuberculosis* and *Y. enterocolitica* strains declined between the T_0 and 24 h incubation periods. Between the 24 h and 72 h incubation period, the number of viable cells increased for 27729, 29838 and NR-804 but continued to decline for *Y. enterocolitica* (NR-214). For the 72 h to 168 h incubation period, the growth rates for *Y. enterocolitica* strains (NR-214 and 27729) continued to increase while those for *Y. pseudotuberculosis* (29838 and NR-804) declined (Fig. 1A, 1B, 2A and 2B).

Between the T_0 and 24 h incubation period, there was a decrease in the average log CFU/ml of all strains of *Yersinia* under the pH 4.0 incubation conditions. This decline continued until the 72 h incubation period for 27729 with the three remaining strains demonstrating an increase during this same period. The average log CFU/ml increased during the 72 h to 168 h incubation period for NR-804 and 27729 and decreased for NR-214 and 29838. In NaOH titrated orange juice (pH 7.0) *Y. pseudotuberculosis* and *Y. enterocolitica* strains manifested overall better recovery and higher growth rate than at lower pHs. At this pH, the growth rate of all strains declined between T_0 and 3 h of incubation and either leveled off or increased between 3 h and 24 h. This increased growth rate continued for up to 168 h of incubation in all strains except for *Y. enterocolitica* (NR-214), which showed a slight decline in growth rate (Fig. 1A).

The number of CFU/ml of these *Yersinia* species from the undiluted orange juice sample between T_0 and 168 h of incubation period revealed the ability of these species to tolerate low pH conditions and survive in the orange juice. This phenomenon was further supported by a parallel experiment conducted to see the survival of these species over an extended period of time for the shelf life of most of the commercially available orange juices. Both organisms survived the extended incubation of lower pH in orange juice for 15–30 days. The statistical analysis was conducted to interpret the inter- and intra-species differences in the CFU counts. Statistical comparison of all the species denoted that there was a significant variation both from the pH and incubation periods and their interaction. The statistical value of CFU counts among *Y. enterocolitica* indicated that difference in the incubation periods were predominantly significant. Analysis of the incubation period and its interaction with the pH were predominantly significant in *Y. pseudotuberculosis* ($p < 0.05$).

A comparison of urease enzyme activity was performed between T_0 and after the extended incubation in orange juice and revealed an increased urease activity at later incubation periods. It was found that both *Yersinia* species were positive for urease after 2 h of incubation when the bacteria were exposed to orange juice, as compared to the non-treated bacteria, which gave positive urease results between 24–48 h of incubation.

Additionally, *ypi*, *inv*, *ail*, and *yst* genes were detected by real time PCR. Cycle threshold value in real time PCR array was used to evaluate the genes. The gene amplification change was explained by the absence of cycle threshold (Ct) value in the real time PCR assay (Fig 3). *Y. enterocolitica* attachment gene (*ail*) was detectable after 3 h incubation in all the samples. However, this gene was no longer detectable in *Y. enterocolitica* incubated in undiluted orange juice at 24 h and later.

Genomic profiles of *Yersinia* were assessed using PFGE technique from all the experimental groups before and after treatments. Alterations in the genomic profiles were observed only in *Y. pseudotuberculosis* strain 29838 analyzed by restriction enzyme FseI.

Interestingly, the change in the genomic profile was observed in bacteria recovered from the 1:4 diluted orange juice sample (pH 4.0), stored for 168 h. The *FseI*-restriction profile was uniquely altered by the generation of a ~210 Kbp fragment (Fig. 4, arrow head 1) and the absence of a ~110 Kbp fragment (Fig. 4, arrow head 2). There was no other detectable genetic profile variation digested by *NotI*, *SpeI*, and *XbaI*-pulsotypes.

Discussion

Bacteria in general exhibit a rapid molecular response with coordinated gene expression when they are exposed to conditions threatening their survival, including variations in pH, sudden elevated temperature, oxidative damage, nutrient limitation and starvation, and chemical stress (Morimoto et al., 1990). In our study, we identified the survival, recovery and growth performance of *Y. pseudotuberculosis* and *Y. enterocolitica* in orange juice under different pH conditions. The results suggested that there is a variation between strains of the same *Yersinia* species inoculated and incubated under all the three pH conditions and stored for 72 h to 168 h. The survival, recovery and growth of the enteropathogenic *Yersinia* in orange juice showed that the acidity level in orange juice and the low incubation temperature seem inadequate to completely inhibit the growth of the organism.

An earlier study has demonstrated that ascorbic acid and citric acid from orange juices have less inhibitory effect on the bacteria as compared to acetic and lactic acids (Adams et al., 1991). Culturing two pathogenic and one environmental serotype of *Y. enterocolitica* under both 4C and 25C temperatures showed the pH of acetic acid to have maximum growth inhibitory effects (Adams et al., 1991). Parallel to our findings, Ellison et al. (2004) demonstrated that stressful conditions such as high concentration of salts and low pH decrease the expression of an invasion gene at 25 C (Ellison et al., 2004). Although no detailed study has been conducted to justify the change in the genomic profile due to adaptation, it was shown that *ompR* was involved in the adaptation of this bacterium to multiple stresses as well as in the survival and replication in macrophages (Brzostek et al., 2007).

Microarray analysis has provided insights into species-specific gene functions and the inter- and intra-species differences between the high, low, and nonpathogenic *Yersinia* species. Moreover, wider investigations looking at the patterns of gene loss and gain in the *Yersinia* have highlighted common themes in the genome evolution of other human enteropathogens (Nicholas R. et al. 2006). Two genetic (*inv*, *ail*) loci that confer invasiveness of *Y. enterocolitica* and *Y. pseudotuberculosis* are necessary for virulence have been identified on the bacterial chromosome (Isberg and Falkow., 1985). Pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* were identified by PCR targeting the chromosomal genes *ail* and *inv*, respectively (Martinez et al 2009). Thoerner et al., (2003) reported that virulence plasmid *pYV* could easily be lost depending on the culture conditions. Therefore, differentiation of pathogenic strains should not rely solely on the expression or the detection of the virulence plasmid but also on the detection of chromosomal virulence factors.

In our result, the amplification of the attachment gene (*ail*) was not detectable in *Y. enterocolitica* incubated in undiluted orange juice at 24 h and later (Fig 3). It has been reported that because of the unstable nature of *pYV* in *Y. enterocolitica* when virulent strains are cultivated *in vitro*, the *pYV* can be spontaneously lost during cell division, resulting in a mixed population of virulent and avirulent clones (Bhaduri, 2001; Kwaga and Iverson, 1991; Robins-Browne, 2001; Straley, 1991). Loss could be also facilitated by culturing at 37 C, repeated transfer of cultures, extended storage at 4 C or 20 C, and during laboratory manipulation (Bhaduri, 2001; Robins-Browne, 2001; Weagant et al., 2007). Recently Hu et al., (2011) indicated that the expression level of eight proteins involved in carbohydrate

metabolism of *Yersinia pseudotuberculosis* were up- or down-regulated over twofold at pH 4.5 compared with pH 7.0.

Yuk and Schneider (2006) described the moderate acid adaptation of intestinal bacteria such as *Salmonella* in fruit juices that render the organism to be more resistant to gastric acid and increase the risk of illness. It has been additionally shown that acid adaptation in juice can provide a cross-protection against thermal and osmotic stresses rendering the organism more pathogenic (Souza and Santos, 2009; Yuk and Schneider, 2006). Our study indicated that the ability of *Yersinia* species to survive and grow in orange juice under commonly practiced storage conditions, possibly by genomic modification, could play a role in the survival and adaptation of the organism in extreme environmental conditions. Although there was a drastic reduction of the load of the organisms in the UD and 1:4 diluted orange juices, it appeared that the pH in orange juice and the low incubation temperature seemed to be ineffective in the total inhibition of both *Y. pseudotuberculosis* and *Y. enterocolitica*. It has been previously documented that acid resistance in orange juice may render the bacteria to be more resistant to the acidic environment of the gastrointestinal tract or provide cross protection to other environmental stresses (Chen et al., 2009).

There is a stoichiometric match between amino acid oxidation and urea formation (Jackson, 1994). Due to the limited energy supply in orange juice, bacteria may be imposed to utilize all the available nutrient supply including the available protein, which may result in urea production. The presence of urea in the vicinity of the bacteria activates the enzyme urease. As a consequence urea is exclusively hydrolyzed by bacterial urease, consequently helping the organisms to tolerate and survive the extreme pH conditions (pH 3.9 and 4.0). It has been suggested that the resistance of *Y. pseudotuberculosis* and *Y. enterocolitica* to acid depends also on the bacterial growth phase and the concentration of urea in the medium, being maximal during the stationary phase, with a minimal presence of 0.3 mM urea (de Koning-Ward et al., 1995).

Under natural conditions, the presence of urease is assumed to be important for survival in acidic environments where urea is available, such as in the stomach, or potentially, in an acidified phagosome of a macrophage, in polymorphonuclear leukocytes or other host cells (de Koning-Ward et al., 1995; Sebbane et al., 2002). Previous experiments indicated that the activity of urease promotes the survival of *Y. enterocolitica* in extreme acidity when urea is available (Lavermicocca et al., 2008). Hu et al. (2009) and others also reported that enhanced urease expression has a key role in the survival of *Y. pseudotuberculosis* and in addition the same research demonstrated the importance of *OmpR* gene in the regulation of urease at acidic pH of 4.5 or lower (Chen et al., 2009; Hu et al., 2009).

To date, there is no documentation of *Yersinia* contamination of orange juice. Nonetheless, the findings reported here suggest that there is a potential food safety risk from orange juice if it is accidentally or intentionally contaminated by *Yersinia* species. In conclusion, *Yersinia* has the potential to survive in orange juice (low pH) at standard storage temperature. This characteristics of the bacterium makes it a potential orange juice contaminant, which could pose a health risk, even when the juice is stored at 4 C. Mechanisms of adaptation of *Yersinia* to survive and grow in orange juice may include urease production and genomic alteration.

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Figure 1. Survival of *Y. enterocolitica* strain NR-214 (A) and strain 27729 (B) in orange juice exposed to three pH conditions UD (pH 3.9), 1:4 diluted (pH 4.0), NaOH adjusted (pH 7.0), during storage for 3, 24, 72 and 168 hours period at 4 C.

(A) *Y. pseudotuberculosis* strain 29838 (B) *Y. pseudotuberculosis* strain NR-804

Figure 2.

Survival and growth of *Y. pseudotuberculosis* strain 29838 (**A**) and strain NR-804 (**B**) in orange juice exposed to three pH conditions. UD (pH 3.9), 1:4 diluted (pH 4.0), NaOH adjusted (pH 7.0); stored for 3 hrs, 24 hrs, 3 days and 7 days at 4 C.

Figure 3.

A real time PCR amplification of attachment gene *ail* from *Y. enterocolitica* strain 27729 after the 3 hrs, 24 hrs, 72 h (3 days) and 168 h (7 days) of incubation at 4 C in the undiluted orange juice (UD) = pH 3.9, orange juice: distilled water diluted (1:4) = pH 4.0, and NaOH neutralized= pH 7.0 orange juice sample. No amplification of this gene was obtained in the undiluted orange juice sample incubated for 72 h and 168 h at 4 C, where as the band prominently amplified at 3 h and faintly expressed at 24 h. The electrophoresis data was in agreement with the real time data plot showing the absence of amplification in undiluted orange juice sample.

Figure 4.

PFGE profile of *Y. pseudotuberculosis* strain 29838 obtained by restriction enzyme *FseI* digestion (Arrow head 1= presence of an additional restriction segment; Arrow head 2 = missing restriction segment). Undiluted orange juice (UD) = pH 3.9, orange juice: distilled water diluted (1:4) = pH 4.0, and NaOH neutralized= pH 7.0. LL: lambda ladder; YP: *Yersinia pseudotuberculosis* not challenged in orange juice.

Table 1

Primers used for the amplification of selected genes and amplicon size

Target gene	Sequence (5'-3')	Amplicon length (bp)
<i>ail</i>	TAAATGTGTACGCTGCGAG GACGTCCTTACTTGCACTG	351
<i>ysjA</i>	ATCGACACCAATAACCGCTGAG CCAAATCACTACTGACTTCGGCT	79
<i>inv</i>	CGGTACGGGCTCAAAGTTAAATCTG CCGTTCTCCAATGTAGGTATCC	183
<i>ypi</i>	CCCAAAATCGGTGGGATATACG GTTTCAAAATCCGTGCTGGT	231