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ORIGINAL ARTICLE

Dual-species relations between *Candida tropicalis* isolated from apple juice ultrafiltration membranes, with *Escherichia coli* O157:H7 and *Salmonella* sp.

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Keywords
apple juice, *Candida tropicalis*, co-cultures, *Escherichia coli* O157:H7, *Salmonella* sp., stainless steel.

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Abstract

Aims: The objective of this study was to determine the interactions between common spoilage yeast, *Candida tropicalis*, isolated from ultrafiltration membranes, and *Escherichia coli* O157:H7 and *Salmonella* sp. on stainless steel surfaces.

Methods and Results: Single and dual-species attachment assays were performed on stainless steel at 25°C using apple juice as culture medium. The growth of *Salmonella* sp. rose when it was co-cultivated with *C. tropicalis* in dual biofilms at 16 and 24 h; the same effect was observed for *E. coli* O157:H7 at 24 h. The colonization of *C. tropicalis* on stainless steel surfaces was reduced when it was co-cultivated with both pathogenic bacteria, reducing *C. tropicalis* population by at least 1·0 log unit. Visualization by SEM demonstrated that *E. coli* O157:H7 and *Salmonella* sp. adhere closely to hyphal elements using anchorage structures to attach to the surface and other cells.

Conclusions: These results suggest a route for potential increased survival of pathogens in juice processing environments. These support the notion that the species involved interact in mixed yeast–bacteria communities favouring the development of bacteria over yeast.

Significance and Impact of the Study: This study support the plausibility that pathogen interactions with strong biofilm forming members of spoilage microbiota, such as *C. tropicalis*, might play an important role for the survival and dissemination of *E. coli* O157:H7 and *Salmonella* sp. in food-processing environments.

Introduction

Contamination of food by spoilage and pathogenic microorganisms costs the food industry millions of dollars annually; much of this contamination may be attributed to the presence of biofilms in the processing plants (Brooks and Flint 2008).

In food industry, the presence of undesirable biofilms causes serious problems such as impeding the flow of heat across the surface, increase in the fluid frictional resistance at the surface and increase in the corrosion rate at the surface leading to energy and product losses. In addition, the biofilms, including spoilage and pathogenic microflora also offer considerable problems of cross-contamination and postprocessing contamination (Kumar and Anand 1998). The risk becomes even more serious, as it has been observed that the antimicrobial resistance of biofilm cells is significantly increased compared to planktonic ones (Bridier *et al.* 2011; Brugnoni *et al.* 2012a).

As the recognition of biofilms as microbial phenomenon, most investigations have been focused on bacterial biofilms, with little reference to the involvement of yeasts in either single-species phenomena, or as part of mixed-species communities (El-Azizi *et al.* 2004), even less in the food sector.

Yeasts predominate in spoilage of acid food products as they have the ability to grow at low pH, high sugar concentration and low water activity conditions and resist inactivation by heat processing which enables them to survive or grow in fruit or fruit products (Stratford *et al.* 2000). The Food and Drug Administration in the final ruling stated in the Federal Register (FR 2001), identified *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* as the bacterial pathogens pertinent to juice safety. Several *Salm. enterica* and *E. coli* O157:H7 outbreaks have however been attributed to consumption of apple juice and citrus juices (Cheng *et al.* 2003; Alonzo 2013).

As described in bacterial biofilms (Liu *et al.* 2014), it could be assumed that the presence of yeast strains strong biofilm producers on food industry premises could potentially promote colonization by pathogenic bacteria, which many are poor biofilm formers. These food-borne bacterial pathogens, even in small numbers, would take advantage of this protective mechanism by interacting with yeasts, which is of great concern from the food safety standpoint.

In food-processing lines, yeasts belonging to *Saccharomyces*, *Candida* and *Rhodotorula* have been isolated from biofilms on conveyor tracks and can and bottle warmers in packaging departments of a beverage industry (Salo and Wirtanen 2005). Yeasts are able to attach and develop biofilms on ultrafiltration (UF) membranes used in beverage clarification (Tarifa *et al.* 2013) and in pre- and post-UF processing equipment (Brugnoni *et al.* 2007). As yeast in such sites cannot be easily removed by cleaning (Brugnoni *et al.* 2012a; Tarifa *et al.* 2013) and as attached yeast are highly resistant to biocidal agents (Brugnoni *et al.* 2012b), even low initial numbers of surviving yeasts may grow to form biofilm in these sites.

Most of the previous studies on interactions between yeast and bacteria (Nair and Samaranayake 1996a,b; Peters *et al.* 2010; Thein *et al.* 2006) focused only *Candida albicans* and bacteria of human health concern.

As far as we know, there is no information on interactions between yeast isolated from food industry premises and food pathogens. Research in this area should be of great interest, because the coexistence of multiple microbial species is frequently observed in commercial food-processing plants (Uhlich *et al.* 2010).

The present paper studies the effect of *Candida tropicalis,* isolated from an UF membrane used in apple juice clarification, on the colonization of stainless steel by *E. coli* O157:H7 and *Salmonella* sp.

It is not possible to perfectly reproduce the field conditions in experimental studies, but at least four factors were set to model as closely as possible the juice plant situation. Those were as follows: (i) strain isolated from a plant after cleaning and disinfection for co-culturing with *E. coli* 0157:H7 and *Salmonella* sp.; (ii) apple juice as culture medium; (iii) incubation of cultures at 25°C which is usual in apple juice processing plants; (iv) stainless steel (SS) AISI 304 used as food-contact material.

Materials and methods

Micro-organisms and preparation of inocula

For the present study, two bacteria strains, *Escherichia coli* O157:H7 (EDL 933) and *Salmonella* sp. (isolated from poultry in our laboratory), and one yeast strains, *C. tropicalis,* were used. The yeast strain was isolated from the surface of a polyvinylidene fluoride UF membrane used in a large-scale apple juice processing industry located in Argentina as described in Tarifa *et al.* (2013). For the correct ID of the yeast strain, a phenotypic identification was performed through the analysis of typical morphological features (Kurtzman and Fell 1998), along with a biochemical and physiological characterization (fermentation of seven carbohydrates, assimilation of two nitrogen and nineteen carbon sources, urea hydrolysis and growth at 37°C). Genotypic identification was also performed, through the analysis of the single gene sequence of the domains D1/D2 region of 26S rDNA and from the internal transcribed space (ITS) region, as described by Taverna *et al.* (2013). The DNA extraction was performed using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA, USA) and preserved at −20°C until use. For the purification of the PCR products, a PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) was used and sequenced in the reference laboratory Instituto Nacional de Enfermedades Infecciosas 'Dr. Carlos G. Malbrán' (Buenos Aires, Argentina). Once obtained, the sequences were edited using BioEdit 7.0.0 and the similarity was obtained using blastn tool of the National Center for Biotechnology Information.

Stock culture of the strain was suspended in 20% (v/v) glycerol in yeast extract–glucose–chloramphenicol (YGC) broth: 0·5% w/v yeast extract (Merck KGaA, Darmstadt, Germany), 2% w/v glucose (Merck KGaA, Darmstadt, Germany) and 0·01% w/v chloramphenicol (Fluka Chemie AG, Buchs, switzerland) and stored at −70°C.

For bacteria assays, stock cultures of the strains were cultivated in Triptic Soy Broth (TSB, Difco, Detroit, MI) at 37 ± 1°C for 24 h and harvested by centrifugation at 5000 ***g*** for 10 min (Labofuge 200, Kendro, Germany). The supernatant was discarded and cell pellets were washed twice with phosphate-buffered saline (PBS: 0·15 mol l^{-1} NaCl, 0·05 mol l^{-1} KH_2PO_4, 0·05 mol l^{-1} K_2HPO_4, pH 7·2). In all cases mentioned from now on, the suspensions were prepared until reaching a population of ca. 8·0 log colony forming units (CFU) ml^{-1}.

For experiments, a loop of frozen cells of *C. tropicalis* was suspended in YGC broth at 25 ± 1°C and 50 rev min^{-1} on an orbital shaker (Vicking M23, Vicking s.r.l., Argentina) until reaching the stationary phase (48 h), harvested by centrifugation at 1200 ***g*** for 5 min (Labofuge 200, Kendro, Germany) and subsequently resuspended in sterile clarified 12 °Brix apple juice, to achieve a population of ca. 6·0 log CFU ml^{-1}.

The 12 °Brix clarified apple juice used in the successive assays was prepared from 72 °Brix concentrated apple juice obtained from a large-scale apple juice processing industry located in Argentina and sterilized by microfiltration (pore size 0·45 μm) (Metricel®Grid, Gelman-Sciences, Ann Arbor, MI, USA). The approximate composition of the clarified apple juice can be seen in Lozano (2006).

Bacterial survival studies in apple juice

To adhere and form a biofilm, microbial cells must be able to survive in the food system under study; in this work apple juice.

To study the survival capability of *E. coli* O157:H7 and *Salmonella* sp. in apple juice, the cell suspensions prepared as stated in 2·1 were used as inocula. Samples were then incubated at 25 ± 1°C for 4, 18 and 24 h. Moreover, a precondition in apple juice was carried out to mimic a worst-case scenarios, for instance, the contamination of apple juice with acid-adapted cells during manufacturing process, postprocessing or during transportation.

To do so, experiments were performed in apple juice inoculated with a suspension of *E. coli* O157:H7 and *Salmonella* sp. for 4 h at 25°C. Then, these acid-adapted (AA) cells were harvested by centrifugation at 5000 ***g*** for 10 min and suspended in apple juice. Bacterial survival was determinate at 25°C and cell viability at 0, 4, 18 and 24 h.

For the enumeration of viable populations of *E. coli* O157:H7 and *Salmonella* sp. in each incubation period, samples were serially diluted with PBS and determined by plating 1 ml on Tryptic Soy Agar (TSA, Difco) by triplicate and incubated at 37°C for 24–48 h.

Mono and dual attachment assays

SS-coupons (25 × 15 × 1 mm, type AISI-304) were the abiotic substrates used for biofilm formation because this material is the most commonly used for the manufacture of food-processing equipment (Wijman *et al.* 2007). Before the experiments, the chips were soaked for 15 min with 2% of a detergent solution (Extran MA 02 neutral, Merck KGaA, Darmstadt, Germany) at 50°C and rinsed five times for 5 min each with hot tap water followed by five rinses with distilled water. Finally, the chips were autoclaved for 15 min at 120°C. For each micro-organism, the experiments were carried out in sterile glass Petri dishes divided in six sections by glass pieces (Brugnoni *et al.* 2007). The divisions were made by fusing the Petri dish base and the glass division to avoid overlapping of the coupons during the experiment.

For bacteria, experiments were performed with acid-adapted cells in apple juice for 4 h at 25°C. Then, these acid-adapted cells were harvested by centrifugation at 5000 ***g*** for 10 min and suspended in apple juice to achieve a population of ca. 8·0 log CFU ml^{-1}. For yeast cells, the suspensions were prepared as stated in 2·1.

For mono-species colonization assays, suspensions of each species were poured into each Petri dish division containing a sterilized SS coupon, followed by incubation at 25°C for 2, 8, 16 and 24 h, under static conditions. After each time, the coupons were carefully removed from the division using sterile forceps and thereafter rinsed by immersing it for 2 min in 5 ml of PBS with shaking (50 rev min^{-1}), to remove the loosely attached cells. Coupons for each micro-organism were later used for scanning electron microscopy (SEM), epifluorescence microscopy (EM) and viable counts. Triplicate tests were performed under identical conditions in two independent trials.

For dual-species colonization, the following combinations were assayed: *C. tropicalis*/*E. coli* O157:H7 and *C. tropicalis*/*Salmonella* sp., and equal volumes of the standardized suspensions of each pair were mixed immediately before use. The mixed suspensions of bacteria and yeast were prepared as described above, and attachment test was made as explained in the previous paragraph.

SEM and EM

SEM was used to determine the adhesion patterns on SS surfaces. The coupons were fixed with glutaraldehyde (2·5%) in phosphate buffer (0·1 mol l^{-1}, pH 7·2); washed three times with the same buffer and dehydrated by critical point drying (E3000, Polaron Instruments, Hatfield, PA, USA). Samples were gold coated (300 Å) in a Pelco Model 3 Sputter Coater 91000 metal evaporator (Lozano 1990) and viewed with a Scanning Electronic Microscope (LEO EVO 40, Cambridge, UK) at 7·0 kV acceleration voltage.

For EM, the chips were stained with fluorescein diacetate (FDA). The principle behind a test using FDA is that only live cells will convert FDA to fluorescein. FDA specifically stains cells possessing esterase activities and intact cell membranes. This fluorescent probe is widely used as an indicator of cell viability (Ki-Bong and Hideaki 2002).

A standard stock solution of 2 mg ml^{-1} (0·2% w/v) FDA, ($C_{24}H_{16}0_7$, Sigma–Aldrich Chemical Co., St. Louis,

MO) was prepared in acetone (Dorwil, Industria Argentina) and stored to −18°C. The coupons from each experimental condition were stained with sterile FDA acetonic solution in 0·1 mol l^{-1} phosphate buffer (0·04% v/v), pH 7·5. After 90 min shaking at 25 ± 1°C in darkness, the coupons were rinsed twice with sterile distilled water. Chips were then allowed to air-dry and observed with an epifluorescence microscope (Olympus BX 51, NY, USA) using a 100× oil-immersion objective, blue excitation U-MWB2. The total number of bacterial cells per field and attached bacteria per hyphae were counted. Per cent attachment was calculated by dividing the number of attached bacteria by the total number of bacteria. A total of 10 random fields per coupon were analysed.

Quantification of cells

To analyse the proportion of bacteria and yeast in each assay, the number was estimated by placing SS-coupons into a test tube with glass beads and vortexed to full speed for 3 min (to remove the adherent micro-organisms) (Lindsay and von Holy 1997). In each incubation period, samples were serially diluted with PBS and determined by plating 1 ml on TSA (Difco) and YGC agar (Merck, Germany) by triplicate and incubated at 37 and 25°C, respectively, for 24–48 h. The results were expressed as CFU cm^{-2}.

Statistical analysis

Counts were converted to decimal logarithmic values (log CFU cm^{-2}) to nearly match the assumption of a normal distribution.

In all analyses, triplicate tests were performed under identical conditions in two independent trials and the results expressed as mean and standard deviation (mean ± SD). When appropriate, Student's *t*-test was used for comparison of means. Confidence level equal or higher than 95% was considered statistically significant.

Results

Survival studies in apple juice

As shown in Fig. 1, the viable population of *Escherichia coli* O157:H7 in apple juice regardless of acid adaptation, declined when incubated at 25°C for 24 h. The population of the acid-adapted (AA) cells of *E. coli* O157:H7 decreased 2·90 log CFU ml^{-1} at 18 h and 2·72 log CFU ml^{-1} at 24 h, whereas not adapted (NA) ones decreased 4·7 log CFU ml^{-1} at 18 h and 4·6 log CFU ml^{-1} at 24 h. Comparing the results between AA and NA cells, the differences in the reductions were significant ($P < 0·05$) along the experience. Nonetheless, acid tolerance of *Salmonella* sp. was not dependent on prior exposure to a low pH. No significant differences ($P > 0·05$) were found when AA and NA cells were incubated for 24 h in apple juice. Apple juice inoculated with *Salmonella* sp. contained ca. 8·0 and 8·4 log CFU ml^{-1} of the AA and NA, respectively, after 24 h of incubation at 25°C. Unlike *E. coli* 0157:H7, *Salmonella* sp. not only survived but also grew quickly in apple juice between 4 and 18 h of incubation.

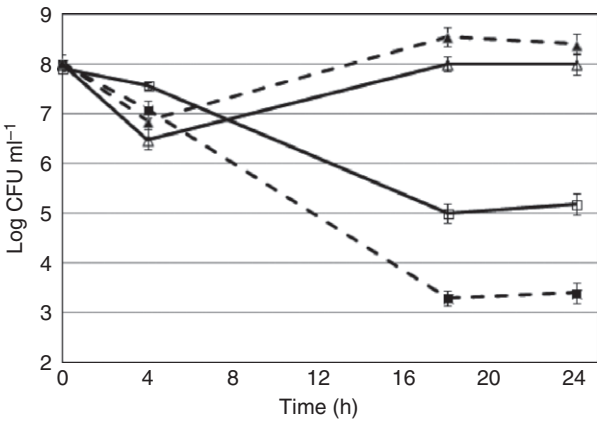

Figure 1 Survival studies of bacteria in 12 °Brix apple juice (pH 4·3 ± 0·2) during 24 h at 25°C (□) acid-adapted *Escherichia coli* 0157:H7, (■) nonadapted *E. coli* O157:H7, (△) acid-adapted *Salmonella* sp. and (▲) nonadapted *Salmonella* sp. Results are means ± standard deviations ($n = 3$).

Mono-species attachment assays

The number of attached cells of *E. coli* O157:H7 and *Salmonella* sp. on the SS surfaces along the time intervals assayed (2, 8, 16 and 24 h) ranged between 4·98–6·59 log CFU cm^{-2} and 6·00–6·69 log CFU cm^{-2}, respectively, as shown in Table 1. When comparing mono-species attachment and in co-culture, significant differences were observed in the adhesion of *Salmonella* sp. and *E. coli* O157:H7 at 2 and 8 h ($P < 0·05$).

As expected, mono-specie cultures of *C. tropicalis* on stainless steel surfaces demonstrated a profuse growth

Table 1 Attached cells of *Candida tropicalis*, *Escherichia coli* O157:H7 and *Salmonella* sp. on the stainless steel surfaces grown in 12 °Bx apple juice for 2, 8, 16, 24 h. Data are expressed as mean log CFU cm^{-2} (±SD)

Time (h)	*Candida tropicalis*	*Escherichia coli* O157:H7	*Salmonella* sp.
2	5·66 ± 0·035	4·98 ± 0·125	6·00 ± 0·088
8	6·26 ± 0·081	6·54 ± 0·155	6·22 ± 0·088
16	6·58 ± 0·032	6·64 ± 0·190	6·51 ± 0·022
24	6·74 ± 0·015	6·59 ± 0·002	6·69 ± 0·073

and dense colonization of the substrate (Fig. 2a,d) with a number of cells ranging from 5·66 to 6·74 log CFU cm^{-2} (Table 1). It was observed that following the adhesion of *C. tropicalis* blastospores (yeast form) to SS surface at 2 h, the adhered cells multiplied continuously and blastospores became hyphae which formed three-dimensional structures. The three-dimensional structures of biofilms are generally comprised of yeast cells, pseudohyphae and hyphae. The mature biofilm enables *Candida* yeast to fix onto extracellular surface, and the hyphae form a cross-sectional structure with structural frames.

In addition SEM images of the mono-species cultures of S*almonella* sp. (Fig. 2b,e) and *E. coli* O157:H7 (Fig. 2c, f) show the presence of single cells distributed all over the SS surface or forming small clusters of cells already after 16 and 24 h incubation.

As seen in Fig. 3, the cells of *Salmonella* sp. (a and c) and *E. coli* O157:H7 (b and d) attached to SS and other counterparts using flagella or fibril-like structures, which are surface structures projecting away from the cell wall. As seen in these images, *Salmonella* sp. showed a higher development of anchorage structures compared to what happened with *E. coli* O157:H7.

Dual-species attachment assays

Once the survival of *E. coli* O157:H7 and *Salmonella* sp. in apple juice was established, the interactions in co-culture between them and *C. tropicalis* were studied.

The growth of *Salmonella* sp. rose when it was co-cultivated with *C. tropicalis* at 16 and 24 h (Table 2) and the same effect was observed for *E. coli* O157:H7 at 24 h (Table 2), indicating a global positive interaction in what respects bacterial strains.

On the other hand, it was observed that the colonization of *C. tropicalis* on SS surfaces was reduced when it was co-cultivated with both pathogenic bacteria strains as can be seen in Fig. 4a–h, suggesting that *Salmonella* sp. and *E. coli* O157:H7 have a strong negative effect on the formation of *C. tropicalis* biofilms (Table 2). SEM observations of dual co-cultures shows that bacteria attach to *C. tropicalis* hyphae and insert between *C. tropicalis* cells (Fig. 5a–e).

A similar behaviour to the one seen in mono-species cultures was registered by bacteria strains in the presence of *C. tropicalis* as what respects anchorage structures. *Salmonella* sp. showed a higher development of fibril structures since 2 h of attachment (Fig. 5a–d) and *E. coli* O157:H7 at 24 h (Fig. 5e).

In dual co-cultures with *C. tropicalis*, per cent counts demonstrated that *Salmonella* sp. and *E. coli* 157:H7 (Fig. 5f) have a high hyphal association through the experience (between 58–80% and 30–74%, respectively).

Discussion

Acidification is one of the important measures commonly employed to control growth and survival of spoilage and

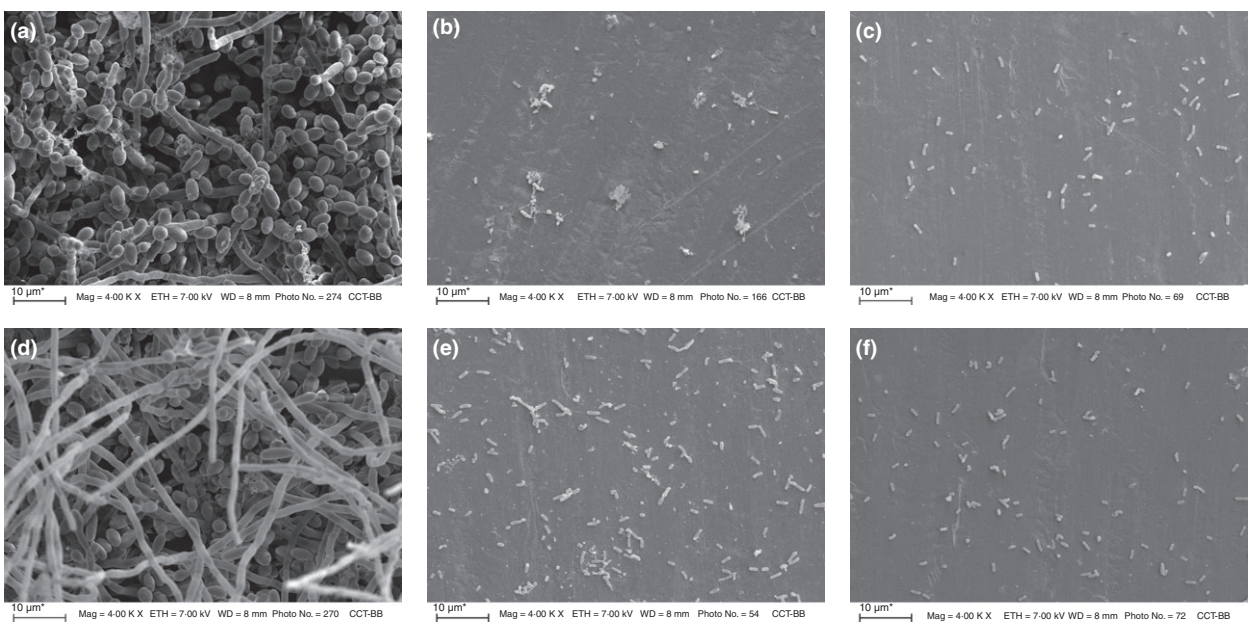

Figure 2 SEM images of mono-species attachment on stainless steel AISI 304 incubated in 12 °Brix apple juice at 25°C. First row: 16 h exposure, second row: 24 h exposure. (a and d) *Candida tropicalis,* (b and e) *Salmonella* sp. and (c and f) *Escherichia coli* O157:H7.

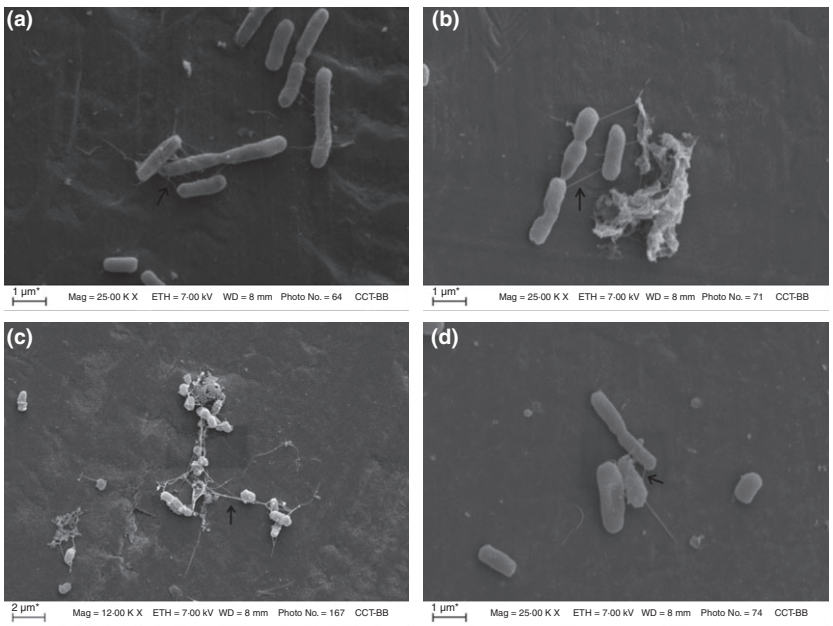

Figure 3 SEM images of anchorage structures used by *Salmonella* sp. and *Escherichia coli* O157:H7 for cell–cell and/or cell-surface adhesion on stainless steel AISI 304 and incubated in 12 °Brix apple juice at 25°C. First row: 16 h exposure, second row: 24 h exposure. (a and c) *Salmonella* sp. and (b and d) *E. coli* O157:H7.

Table 2 Attached cells of co-cultures of *Candida tropicalis* with *Escherichia coli* O157:H7 and *Salmonella* sp. on stainless steel surfaces, grown in 12 °Bx apple juice for 2, 8, 16, 24 h. Data are expressed as mean log cm^{-2} (±SD)

	Candida tropicalis			
Time (h)	*E. coli* O157:H7	*Salmonella* sp.	*E. coli* O157:H7	*Salmonella* sp.
2	3·80 ± 0·312***	4·00 ± 0·707***	5·00 ± 0·494	6·07 ± 0·068
8	4·34 ± 0·102***	4·52 ± 0·213***	6·78 ± 0·345	6·29 ± 0·168
16	5·63 ± 0·299*	5·72 ± 0·152*	6·81 ± 0·061	6·99 ± 0·034***
24	5·60 ± 0·295**	5·92 ± 0·082**	7·01 ± 0·046**	7·10 ± 0·022***

Significant differences between mono species and in co-culture are expressed through the following nomenclature $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

pathogenic micro-organisms in food (Brown and Booth 1991). However, various acidic foods such as apple cider (Besser *et al.* 1993), mayonnaise (Weagant *et al.* 1994) and yoghurt (Morgan *et al.* 1993) have been implicated in the outbreaks of food-borne disease caused by *Escherichia coli* O157:H7 and *Salmonella*.

Results of the present study further demonstrated that acid adaptation increased the acid tolerance of *E. coli* O157:H7 in acid fruit juices. Acid adaptation response is a phenomenon by which micro-organisms show an increased resistance to environmental stress after the exposure to a moderate acid environment. It was also reported that acid adaptation prolonged the survival of some pathogens in various food systems, having feasible important implications in food safety (Leyer and Johnson 1997; Tsai and Ingham 1997). Acid tolerance is probably an important component of virulence for *E. coli* O157:H7, and it allows a small number of cells to cause illness by their being protected in the gastric tract. Hence, the tolerance of *E. coli* O157:H7, which has a low infectious dose, to acidic food compounds leads a serious scenario for this bacterium as a food-borne pathogen (Mao *et al.* 2001). On the other hand, survival of acid-adapted *Salmonella* sp. was less than its nonadapted counterpart in apple juice. Growth and survival of *Salmonella* within fruits and vegetables of various acidities has been demonstrated by a number of investigators (Asplund and Nurmi 1991; Golden *et al.* 1993; Zhuang *et al.* 1995; Parish 1997). The mechanism of acid resistance is not precisely known for *Salmonella*, but similar to *E. coli*, exposure to acid results in the production of numerous acid shock proteins (Foster 1991; Foster and Hall 1991).

In this study, the experiences were carry out with apple juice obtained from a producer/exporter company to model the main intrinsic factors of apple juice, represented by low pH and high sugar concentrations that these micro-organisms face when exposed to this media. In fruit juice processing plants, so as to attach on surfaces

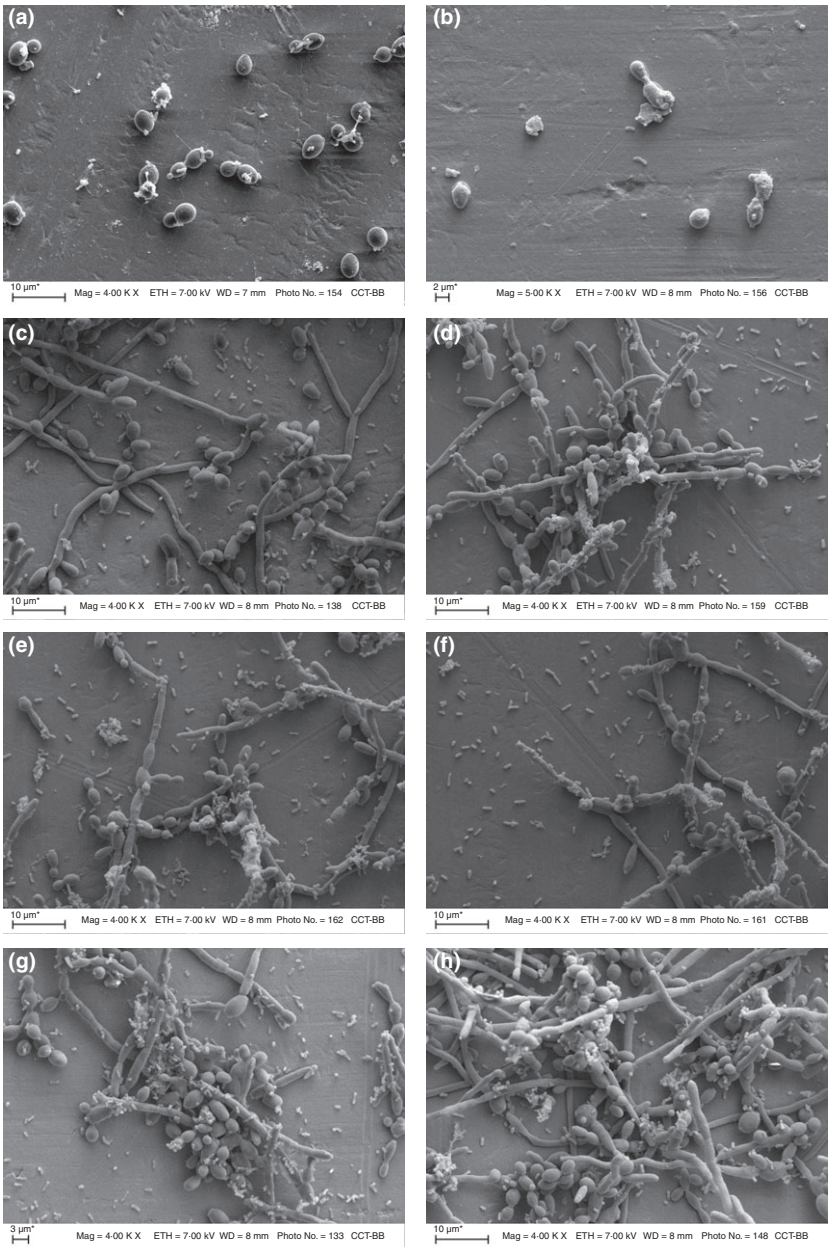

Figure 4 SEM images of mixed fungal–bacterial communities on stainless steel AISI 304 incubated in 12 °Brix apple juice at 25°C. Left panel: *Candida tropicalis*/*Salmonella* sp. and right panel: *C. tropicalis*/*Escherichia coli* O157:H7; (a, b) 2 h, (c, d) 8 h, (e, f) 16 h and (g, h) 24 h.

and proliferate, microbial pathogens must survive in this acidic liquid food. Using 12 °Brix apple juice pH~ 4·3 in adhesion assays would be useful for obtaining more realistic information compared with standard laboratory growth media. This finding is a matter of concern particularly for fruit juice processing industries using modern processing equipments, as represents a hazard state. With mechanical and process automation, the surfaces are in repeated contact with raw juice, thus increasing the opportunities for *Salmonella* and *E. coli* O157:H7 to transfer and attach leading to biofilm formation. Due to this, we believe that it is important to study and consider the use of stressed or adapted cells, as the use of healthy exponentially growing cultures may inaccurately represent their survival state in the natural environment.

Although recent decades have witnessed a surge in the area of biofilm research, relatively little is known about the behaviour of communities of mixed micro-organisms, particularly mixed fungal–bacterial communities.

Candida tropicalis, as mentioned before, was isolated from UF membrane samples of a large-scale apple juice processing plant. As described in this work, *C. tropicalis* in monoculture can adhere and colonize the stainless steel surface and consequently form a biofilm. The

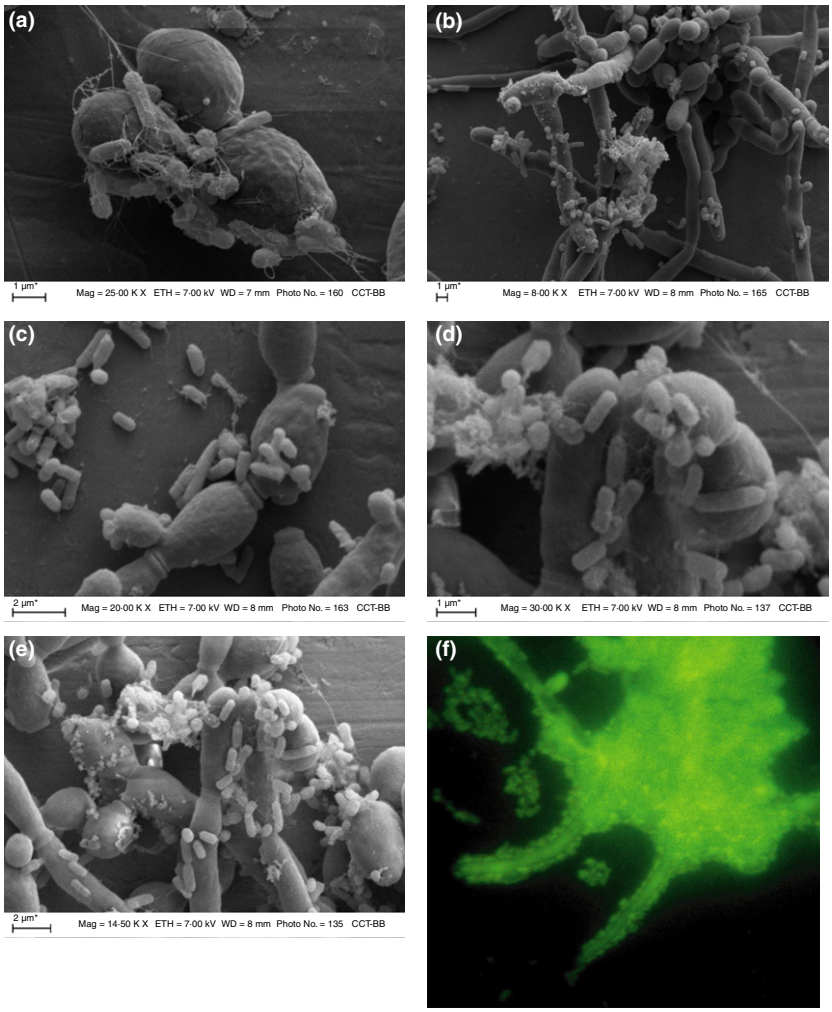

Figure 5 SEM images shows the anchorage structures used by the bacteria strains for cell–cell and/or cell-surface adhesion. (a, b, c, d) *Candida tropicalis*/*Salmonella* sp. at 2, 8, 16 and 24 h, respectively; (e) *C. tropicalis*/*Escherichia coli* O157:H7at 24 h. (f) epifluorescence microscopy image of the interaction between *E. coli* O157:H7 and hyphae of *C. tropicalis* stained with fluorescein diacetate. Magnification 200×.

dimorphism of *C. tropicalis* serves as a major factor that influenced the formation of the biofilm. The ability to switch the morphology between yeast and hyphal form it has been seen in other strains with known development of hyphae. This feature could be crucial for the ability to adhere and colonize surfaces (Calderone 2002; Saville *et al.* 2003). In *C. albicans*, De Bernardis *et al.* (1998) found that for yeast–hyphae morphogenesis, the pH of the host niche around 4·5, acts as an environmental signal that regulates gene expression. Previous works established that expression of *PHR1* and *PHR2* (genes encode a function required for *C. albicans* morphogenesis) *in vitro* is regulated in response to the acidic pH of the growth environment, independent of temperature, nutritional factors or morphology (Saporito-Irwin *et al.* 1995; Mühlschlegel and Fonzi 1997).

At this stage, the molecular mechanisms that regulate phenotypic switching and biofilm development in *C. tropicalis* remain unknown. Regardless of the gap of information in this matter, in our study, *C. tropicalis* formed a mature biofilm in an acidic environment with high sugar content at 25°C.

In our study, *Salmonella* sp. and *E. coli* O157:H7 grown in apple juice showed to easily establish on SS surfaces in short times relevant to the fruit juice industry, particularly overnight or during idle hours. Several studies have shown that extracellular appendages such as flagella, pili or curli present in bacteria may play an important role in the initial stages of adhesion, assisting the cells in the attachment to abiotic surfaces, including food-contact ones (Austin *et al.* 1998; O'Toole and Kolter 1998; Pawar *et al.* 2005; Van Houdt and Michiels 2010).

As *C. tropicalis* has survived typical cleaning and disinfection procedures, it was assumed that was firmly attached to the UF membrane. Therefore, they inevitably encounter and interact with many other microbial species, and these interactions might concern the survival, colonization and pathogenesis of these organisms involved. Co-aggregation and co-adhesion reactions of

micro-organisms are significant colonization factors because they enable development, stabilization and maintenance of complex communities (Jenkinson and Douglas 2002). Co-aggregation may help increase the mass of the species (yeast/bacteria) allowing them to form clumps of higher sedimentation velocity, or it may expose adhesion receptors found in the cell wall of the yeast (Peters *et al.* 2012).

As mentioned above, *C. tropicalis* forms a real biofilm in monoculture assays. In co-culture assays with *E. coli* O157:H7 and *Salmonella*, it was seen that bacteria encounter an eligible niche in the yeast hyphae, suggesting some interspecies interaction. This co-adhesion with bacteria is thus a complex multimodal interaction by which mixed-species colonization would be promoted.

It has been reported (Handley *et al.* 1999; Jenkinson and Douglas 2002) that for oral streptococci, oral actinomyces and other plaque bacteria, many of the co-aggregation adhesins are carried on fimbriae or fibrils. The distancing of co-aggregation adhesins away from the cell surface on fibrils and fimbriae will help partner organisms make effective contact with each other. The thin fibrils and fimbriae act to penetrate the electrostatic barrier that operates between cells in close contact (10–20 nm apart) (Busscher *et al.* 1992), thus acting as probes to locate the appropriate receptor on the partner organism successfully. These interactions cells-fimbriae were clearly observed by SEM (Fig. 3). In addition, some chemical interaction might occur between the two species that induces phenotypic changes in *E. coli* 0157:H7 and *Salmonella* sp. allowing them to attach more effectively.

Most of the work addressed in the literature investigating fungal–bacterial attachment focused only on clinically important fungal–bacterial interactions. As far as we know to date, there is no information on the effect of direct cell interaction in co-cultures between non-*Candida albicans* species and food-borne pathogens. Due of this, the comparative discussion of the obtained results can only be performed with reference of yeast and bacteria of human health concern.

A number of bacterial species including *S. pyogenes* and *Acinetobacter baumannii* have been reported to have preference in binding to *C. albicans* hyphae (Cunningham 2000; Peleg *et al.* 2008; Bamford *et al.* 2009). Another tight association between bacterial cells and fungal hyphae has been observed between *C. albicans* and S*taphylococcus aureus,* which is assumed to promote the invasion ability of *S. aureus* through epithelial layers (Thein *et al.* 2006; Peters *et al.* 2010) in a nonlethal interaction. Peters *et al.* (2010), to assess the potential for hyphal–bacterial interactions of various bacterial species on *Candida albicans* hyphae, demonstrated that *Staphylococcus aureus* had the highest hyphal association (56%), followed by *Streptococcus pyogenes* and *S. epidermidis* (25%). *Pseudomonas aeruginosa*, a known hyphae binder, had a hyphal association of 17%, while *E. coli* and *Bacillus subtilis*, demonstrated the lowest hyphal binding (5·7 and 2·5%, respectively).

Another observation performed in this study was that the co-culture of yeast with bacteria clearly demonstrated the antagonistic effect of *E. coli* O157:H7 and *Salmonella* sp. on *C. tropicalis* biofilm formation.

Many of the previously identified *C. albicans*–bacteria interactions result in fungal and/or bacterial killing during co-culture (Hogan *et al.* 2004; Brand *et al.* 2008; Kim and Mylonakis 2011). As seen in Fig. 5f, cells of *E. coli* O157:H7 interact closely with *C. tropicalis* hyphae. The same was observed in co-cultures with *Salmonella* sp. As FDA staining is widely used to indicate cell viability (Hassan *et al.* 2002; Rocha-Valadez *et al.* 2005; Lecault *et al.* 2007), we can conclude that the negative effect of *E. coli* O157:H7 and *Salmonella* sp. on *C. tropicalis* biofilm formation does not occur through the death of the hyphae.

In one of the earliest studies, Nair and Samaranayake (1996a,b) reported that *E. coli* significantly suppressed the adhesion of *C. albicans* and *Candida krusei* to human oral epithelial cells (Nair and Samaranayake 1996b). More recently, the same group (Thein *et al.* 2006) confirmed that *E. coli* and *Ps. aeruginosa* can significantly compromise *C. albicans* biofilm formation in a dose-dependent manner. Bandara *et al.* (2009) noted that *E. coli* ATCC 25922 suppressed the adhesion of others relatively common *Candida* species: *C. krusei*, *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis*. The authors indicate that *E. coli* and *Candida* species in a mixed-species environment mutually modulate biofilm development, both quantitatively and qualitatively, and that *E. coli* LPS appears to be a key component in mediating these outcomes. In a more recent study, Bandara *et al.* (2010) demonstrated that *Salmonella* LPS significantly suppressed the metabolic activity of *Candida* spp. in general.

Although the exact mechanisms underlying fungal–bacterial interactions are not fully understood, recent researches indicate that a range of molecules secreted by both species interacting play a role in such complex interactions. Bacterial and fungal quorum sensing molecules appear to be predominantly involved in these multi-species interactions (McAlester *et al.* 2008; De Sordi and Muhlschlegel 2009).

Evidently, bacterial pathogens, such as *E. coli* 0157:H7 and *Salmonella* sp., and spoilage yeast such as *C. tropicalis*, can be entrapped in multi-species sessile communities formed on inadequately cleaned and disinfected food-processing surfaces. Undoubtedly, further work is necessary to clarify the molecular basis of these bacterial–fungal interactions.

Findings in this study support the plausibility that pathogen interactions with strong biofilm forming members of spoilage microbiota, such as *C. tropicalis*, might play an important role for the survival and dissemination of *E. coli* O157:H7 and *Salmonella* sp. in food-processing environments.

Based on the data obtained from the present study, much attention should be given to prevent the contamination of these pathogens in acidic drinks. Effective measures to reduce or eliminate vegetative pathogens in fruit or vegetable products, especially those with a low infective dose, are thus required.

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Conflict of interest

The authors have no conflicts of interest to declare.

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