Journal of Microbiology (2017) Vol. 55, No. 2, pp.
DOI 10.1007/s12275-017-6300-3

eISSN 1976-3794
pISSN 1225-8873

Candida krusei isolated from fruit juices ultrafiltration membranes promotes colonization of *Escherichia coli* O157:H7 and *Salmonella enterica* on stainless steel surfaces

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(Received Jun 23, 2016 / Revised Aug 31, 2016 / Accepted Oct 13, 2016)

To clarify the interactions between a common food spoilage yeast and two pathogenic bacteria involved in outbreaks associated with fruit juices, the present paper studies the effect of the interplay of *C. krusei*, collected from UF membranes, with *Escherichia coli* O157:H7 and *Salmonella enterica* in the overall process of adhesion and colonization of abiotic surfaces. Two different cases were tested: a) co-adhesion by pathogenic bacteria and yeasts, and b) incorporation of bacteria to pre-adhered *C. krusei* cells. Cultures were made on stainless steel at 25°C using apple juice as culture medium. After 24 h of co-adhesion with *C. krusei*, both *E. coli* O157:H7 and *S. enterica* increased their counts 1.05 and 1.11 log CFU cm^2, respectively. Similar increases were obtained when incorporating bacteria to pre-adhered cells of *Candida*. Nevertheless *C. krusei* counts decreased in both experimental conditions, in a) 0.40 log CFU cm^2 and 0.55 log CFU cm^2 when exposed to *E. coli* O157:H7 and *S. enterica* and in b) 0.18 and 0.68 log CFU cm^2, respectively. This suggests that *C. krusei*, *E. coli* O157:H7 and *S. enterica* have a complex relationship involving physical and chemical interactions on food contact surfaces. This study supports the possibility that pathogen interactions with members of spoilage microbiota, such as *C. krusei,* might play an important role for the survival and dissemination of *E. coli* O157:H7 and *Salmonella enterica* in food-processing environments. Based on the data obtained from the present study, much more attention should be given to prevent the contamination of these pathogens in acidic drinks.

Keywords: *Candida krusei*, *Escherichia coli* O157:H7, *Salmonella enterica*, apple juice, stainless steel

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Introduction

Acid fruit juices below pH 4.6 were once deemed a minor health threat due to the high acidity, and when juice spoilage did occur, it was usually a reflection of the indigenous microbiota: yeast, mould, and lactic acid bacteria. However, the Food and Drug Administration in the final ruling stated in the Federal Register (FR, 2001), identified *E. coli* O157:H7, *S. enterica*, and *Listeria monocytogenes* as the bacterial pathogens pertinent to juice safety. Several *S. enterica* and *E. coli* O157:H7 outbreaks have been attributed to consumption of apple and citrus juices (Cheng *et al*., 2003; Alonzo, 2013). Moreover, it has been reported that 80% of all microbial diseases, including foodborne illnesses are caused by microorganisms in biofilms (Fux *et al*., 2005). The success of biofilm formation depens on the first microorganisms which could be attached to a surface on their own are sometimes able to anchor themselves to the matrix or directly to earlier colonists. Biofilm formation is a stepwise and dynamical process consisting of a series of steps (Srey *et al*., 2013); interactions encountered at the stage of microbial adhesion determine the initial community structure of the developing biofilm (Giaouris *et al*., 2015) thus leading to a big concern for food safety.

Bacteria and fungi are found together in a myriad of environments, where adherent species interact through diverse mechanisms (Shirtliff *et al*., 2009). However, relatively little is known about the behavior of communities of mixed microorganisms, particularly mixed fungal-bacterial communities. Most of the work addressed in the literature investigating fungal-bacterial attachment focuses only on clinically important fungal-bacterial interactions. As far as we know, a few studies focus on interactions between yeast isolated from food industry premises and food pathogens. One of the few works involving inter-kingdom interactions to the detriment of the food sector was made by our group (Tarifa *et al*., 2015). Research in this area should be of great interest, since the coexistence of multiple microbial species is frequently observed in commercial food processing plants (Uhlich *et al*., 2010).

The effects of yeast spoilage leads to substantial financial losses, both directly through loss of the food product and indirectly through recall of the affected product, loss of consumer confidence and support, as well as potential compensation and legal costs (Fleet, 2007; O'Brien *et al*., 2007). In food processing lines, yeasts belonging to *Saccharomyces*, *Candida*, and *Rhodotorula* have been isolated from biofilms on conveyor tracks, and on can and bottle warmers in the

packaging department of a beverage industry (Salo and Wirtanen, 2005).

Candida krusei possesses a range of properties enabling it to tolerate the conditions encountered in foods resulting in their subsequent spoilage. A tolerance for low pH environments and preservative concentrations has facilitated the spoilage of acid preserved food, where the fermentation of available sugars including, glucose and sucrose can result in excessive CO_2 formation leading to bloating and packaging's rupturing (Casey and Dobson, 2003). In addition, *C. krusei* spoilage is often characterized as a surface-growing film-forming yeast on foods, with a range of proteolytic and lipolytic activities making the yeast problematic to fruit concentrates, alcoholic beverages and a range of dairy products (Deak and Beuchat, 1996; Pitt and Hocking, 1997).

Brugnoni *et al.* (2007, 2011) found that *C. krusei* isolated from a large-scale apple juice processing plant presents the fastest colonization and the highest covered surface on stainless steel compared with other yeasts. Although *C. krusei* represents less than 3% of the total population in apple juice, it seems to be one of the most frequently isolated yeast species in fruit concentrates (Deak and Beuchat, 1996; Brugnoni *et al.*, 2012a): this might be due to its great potential for biofilm formation. Early data (Sancho *et al.*, 2000; Brugnoni *et al.*, 2014) established that *C. krusei* outnumber in mixed biofilms subjected to varying hydrodynamic conditions commonly found in apple juice facilities. This strain can therefore be regarded as the dominant one in such environments and here it is assumed to mimic the microbial population that a foodborne pathogenic strain might encounter in food industry premises. As described in bacterial biofilms (Liu *et al.*, 2014), it could be speculated that the presence of yeast strains on food surface equipment could potentially promote colonization by pathogenic bacteria. In the case of foodborne pathogenic bacteria like *E. coli* O157:H7 and *Salmonella*, their extremely low infectious dose combined with their ability to associate in multispecies communities brings to an extra challenge from the food safety standpoint.

When testing situations related to food industry, it is important to use procedures that are relevant to the environment in which the microorganisms will be exposed, and so in the present study the following factors were taken in mind to approximate to a real model: 1) yeast strain isolated from a plant after cleaning and disinfection; 2) apple juice as culture medium; 3) incubation of cultures at 25°C which is usual in apple juice processing plants; 4) stainless steel AISI 304 used as food contact material.

To study the interactions between *E. coli* O157:H7, *S. enterica*, and *C. krusei*, two different cases were tested: a) simultaneous (co-adhesion) surface colonization by pathogenic bacteria and yeasts (condition A), and b) incorporation of bacteria to pre-adhered *C. krusei* cells (condition B). The incorporation of yeasts to pre-adhered bacteria biofilms is not contemplated, assuming that this one is the least probable case on the basis of the microbial population frequencies present in fruit juice processing plant.

Materials and Methods

Microorganisms and culture conditions

For the present study two bacteria strains were used, *Escherichia coli* O157:H7 (EDL 933) and *Salmonella enterica* serotype Enteritidis, isolated from poultry in our laboratory, and one yeast strain, *Candida krusei*, previously isolated from ultrafiltration (UF) membranes, as described in Tarifa *et al.* (2013).

Stock culture of *C. krusei* stored at -70°C in 20% (v/v) glycerol was suspended in yeast extract-glucose-chloramphenicol (YGC) broth at 25 ± 1°C for 48 h on an orbital shaker at 50 rpm (Vicking M23, Vicking s.r.l.), harvested by centrifugation at 1,200 × *g* for 5 min (Labofuge 200, Kendro). The supernatant was discarded and the cell pellet was washed twice with phosphate-buffered saline (PBS: 0.15 mol/L NaCl, 0.05 mol/L KH_2PO_4, 0.05 mol/L K_2HPO_4, pH 7.2) and subsequently resuspended in sterile clarified 12° Brix apple juice, to achieve a population of ca. 6.0 log CFU/ml.

For bacterial assays, stock cultures of the strains were cultivated in Triptic Soy Broth (TSB, Difco) at 37 ± 1°C for 24 h, harvested by centrifugation at 5,000 × *g* for 10 min (Labofuge 200, Kendro) and cell pellets were set up as previously described to achieve a population of ca. 8.0 log CFU/ml.

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). The approximate composition of the clarified apple juice can be seen in Lozano (2006).

Preparation of acid-adapted cells of foodborne pathogenic bacteria

In apple juice processing plants, to attach on surfaces and proliferate, microbial pathogens must survive in the food system, in this work apple juice. In turn, 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 food process environment. Therefore, for bacterial assays, stock cultures of *E. coli* O157:H7 and *S. enterica* were acid-adapted in apple juice in accordance with Tarifa *et al.* (2015) to mimic a worst-case scenario, for instance, the contamination of apple juice with acid-adapted cells during manufacture and post-processing. Briefly, *E. coli* O157:H7 and *S. enterica* were pre-adapted by inoculating them in apple juice pH 4.5 for 4 h at 25°C. Then these acid-adapted cells were harvested by centrifugation at 5,000 × *g* for 10 min and suspended in apple juice for further testing. In all cases mentioned bacteria suspensions were prepared at a concentration of ca. 8.0 log CFU/ml.

Co-aggregation assays

In the co-aggregation test, suspensions of pathogenic bacteria and *C. krusei* were prepared as described in 2.1, for each strain equal volumes (2.5 ml) of adjusted suspensions were mixed and incubated at room temperature (21 ± 1°C) without agitation.

The absorbance of the mixture and each strain suspension alone was monitored at different time intervals (5, 10, 15, 30, 60, 90, and 120 min): 0.2 ml of the surface constant liquid layer was collected without disturbing the suspension and the optical density at 600 nm (OD_{600}) was measured in a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation). Co-aggregation (%) was calculated according to the equation described by Malik *et al.* (2003) with some modifications: $[(A_{bacteria} + A_{yeast}) - (A_{mixture}) / (A_{bacteria} + A_{yeast})] \times 100$, where $A_{bacteria} + A_{yeast}$ represent A_{600} nm of the mixed suspensions at time 0 min and $A_{mixture}$ represents A_{600} nm of the mixed suspensions at different times.

Autoaggregation assays

So as to determine de effect of each species sedimentation in the co-aggregation phenomena, autoaggregation of *C. krusei*, *E. coli* O157:H7, and *S. enterica* was measured as described by Collado *et al.* (2008) using the autoaggregation percentage (A%). Suspensions were made in 12° Brix apple juice as stated in 2.1. Suspensions were incubated at room temperature (21 ± 1°C) without agitation and monitored at different time intervals (0, 5, 10, 15, 30, 60, 90, and 120 min) in the same way, as stated in the previous section. The percentages of autoaggregation were expressed as: $A\%=(A_0 - A_t)/A_0 \times 100$ where A_0 represents the OD_{600} at 0 min and A_t represents the OD_{600} at 0, 5, 10, 15, 30, 60, 90, and 120 min.

Attachment assays

Abiotic substratum : The abiotic substrate used for attachment assays were stainless steel coupons (SSC) (25 × 15 × 1 mm) type AISI-304, material widely used for the manufacture of food processing equipment. Before the experiments, the coupons were degreased and sterilized according the protocol described in Brugnoni *et al.* (2007). The experiments were carried out in sterile glass Petri dishes divided in six sections by glass pieces. The divisions were made by fusing the Petri dish base and the glass division to avoid overlapping of the coupons during the experiment (Brugnoni *et al.*, 2007).

Attachment procedures : To determine the effect of interspecies relations in the attachment process different conditions (A and B) were tested.

The first step previous to each culturing conditions was to assess the behavior of each species alone so as to have a reference point. To do so adjusted suspensions of *C. krusei, E. coli* O157:H7 and *S. enterica* were prepared as described in 2.1 and attachment tests were done for each species alone. Each suspension was put in contact with SSCs for 24 h at 25 °C.

Co-adhesion yeast/bacteria (*condition A*): To see the net effect of the interaction between *C. krusei* and each pathogenic bacteria strain, equal volumes of the standardized suspensions (section 2.1) were mixed immediately before used. The adjusted suspensions of *C. krusei*/*E. coli* O157:H7 and *C. krusei*/*S. enterica* were put in contact with the SSC and incubated at 25°C for 24 h.

Pre-adhered C. krusei cells (*condition B*): The following condition tested was the scenario where pathogenic bacteria encounter an already colonized surface, in this case by yeasts (the scenario of pre-colonized surface by bacteria is the least possible scenario). To do so, an adjusted suspension of *C. krusei* (prepared as stated in 2.1) was put in contact with SSCs for 8 h at 25°C, time after which they were removed from the divisions and rinsed by immersing it for 2 min in 5 ml of PBS with agitation (50 rpm) in order to remove the loosely attached cells. Afterwards, these SSCs were carefully placed in a Petri dish containing an adjusted suspension of acid adapted *E. coli* O157:H7 and *S. enterica* (ca. 8.0 log CFU/ml) and incubated at 25°C for 16 h (completing 24 h trial).

In each situation (A and B) adjusted suspensions were poured into each Petri dish division containing a sterilized SSC and incubated under static conditions. After each, the coupons were carefully removed from the divisions using sterile forceps and thereafter rinsed by immersing it for 2 min in 5 ml of PBS with agitation (50 rpm) in order to remove the loosely attached cells.

Supernatant assay

To see the potential presence of inhibiting molecules with antimicrobial activity, cell-free supernatants were used. To obtain extracellular filtrates, each species was grown as stated in 2.1 and the supernatants were collected by centrifugation at 5,000 × *g* for 10 min. The resulting supernatants were sterilized by microfiltration (pore size 0.45 µm) (Metricel®Grid, GelmanSciences) and immediately used. This modified medium contains all molecules released from the cells during growth.

To examine the antimicrobial effect on the adhesion capacity, bacterial supernatants were tested against adjusted suspensions of *C. krusei*, and vice versa yeast supernatant was tested against each bacteria suspension. In all cases cell-free supernatants were mixed with the corresponding adjusted suspension of *E. coli* O157:H7, *S. enterica*, and *C. krusei* in a ratio 1:1. These suspensions were put in contact with the SSC and incubated at 25°C for 24 h.

Quantification of single and mixed cultures

To determine the final composition of the resulting surface attached cultures in each assay, the number was estimated by placing the SSCs into a test tube with glass beads and vortexed to full speed for 3 min (in order to remove the adherent microorganisms) (Lindsay and von Holy, 1997). In each incubation period samples were serially diluted with PBS and determined by pour plate technique by plating bacteria 1 ml on Tryptic Soy Agar (TSA, Difco) and for yeast 1 ml in YGC agar (Merck) by triplicate. Samples were incubated at 37°C and 25°C, respectively, for 24–48 h. The results were expressed as CFU/cm^2.

Scanning electron microscopy (SEM)

SEM was used to determine the adhesion patterns on SSCs. The coupons were fixed with glutaraldehyde (2.5%) in phosphate buffer (0.1 mol/L, pH 7.2); washed three times with the same buffer and dehydrated by critical point drying (E3000, Polaron Instruments). 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) at 7.0 kV acceleration voltage.

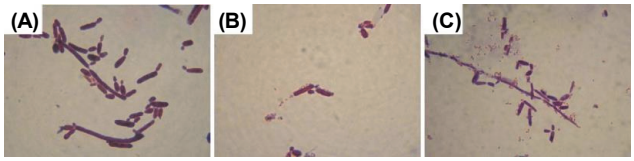

Fig. 1. Optical microscopy (Gram staining) of 120 min cultures of: (A) Pure cultures of *Candida krusei*, (B) Co-cultures of *Candida krusei* and *Escherichia coli* O157:H7, (C) Co-cultures of *Candida krusei* and *Salmonella enterica*. Magnification 100x.

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 means and standard deviations (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

Aggregation activity

Defining co-aggregation as more than 20% decrease in the OD_{600} relative to the initial OD_{600} (Furukawa *et al.*, 2011), co-aggregations between yeast cells and both pathogenic bacteria occurred within 5 min with values of 83 and 66% for *E. coli* O157:H7 and *S. enterica*, respectively. As seen in Table 1, the greater percentages of co-aggregation were seen for *C. krusei* and *E. coli* O157:H7 with no significant differences ($P > 0.05$) along the time intervals assayed; whereas for *C. krusei* and *S. enterica* co-aggregation raised from 66 to 76% ($P < 0.01$) from 5 to 120 min.

After 120 min trial a sample from the bottom of each testing tube was taken, Gram stained, and watched at optical microscope (Primo Star-Zeiss).

Figure 1A to C show the morphological changes from pure cultures of *C. krusei* (Fig. 1A) to co-culture with *E. coli* O157:H7 (Fig. 1B) and *S. enterica* (Fig. 1C). To evaluate if the co-aggregation coefficients were mainly due to the auto-aggregation and sedimentation of yeast cells, the A% (auto-aggregation percentage) of each species involved was calculated and compared to the co-aggregation values. The percentages of auto-aggregation can be seen in Table 1; in all time intervals the differences between A% and co-aggregation % are statically significant.

Mono and dual attachment assays

In mono species adhesion assays, the number of attached cells of *E. coli* O157:H7 was of 4.60 ± 0.14 CFU/cm^2 whereas for *S. enterica* was of 4.42 ± 0.02 CFU/cm^2. As can be seen in Fig. 2A and B, *S. enterica* covers a greater area of the SSC than *E. coli* within 24 h exposure, showing in the case of *S. enterica* small aggregates. Moreover, *C. krusei* demonstrated a major colonization of SS in short periods of time being the number of cells attached of 6.03 ± 0.001 CFU/cm^2. In Fig. 2C it can be seen yeast cells gathered together forming chains that resulted in an arrangement of micro-colonies covering the majority of the surface.

After 24 h incubation the co-adhesion (condition A) resulted in an increase of bacteria counts (Fig. 3A and B) with changes of 23% for *E. coli* O157:H7 (1.05 CFU/ml) and 25% for *S. enterica* (1.11 CFU/ml). Through visualization by SEM dual species interactions showed that both bacteria strains adhered closely to yeast cells (Fig. 4A and B), preferring the adhesion to blastospores rather than to the SS surfaces after 24 h exposure. In the co-adhesion bacteria/yeast (condition

Table 1. Aggregation activity as Mean percentages (± SD) of: [(a)] Co-aggregation between *Candida krusei* and *Escherichia coli* O157:H7 and *Salmonella enterica* [(b)] Auto-aggregation of *Candida krusei*, *E. coli* O157:H7, and *S. enterica*. Significant differences between co-aggregation and auto-aggregation are expressed through the following nomenclature * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Time (min)	Co-aggregation[a]		Auto-aggregation[b]		
	Candida krusei				
	Escherichia coli O157:H7	*S. enterica*	*E. coli* O157:H7	*S. enterica*	*Candida krusei*
5	83 ± 0.97***	66 ± 1.15***	4 ± 0.2	1 ± 0.4	5 ± 5.3
10	83 ± 0.86***	67 ± 1.02***	3 ± 0.4	5 ± 4.5	6 ± 4.1
15	83 ± 0.86***	67 ± 1.02***	5 ± 0.2	5 ± 3.7	8 ± 2.3
30	83 ± 1.05***	67 ± 1.02***	5 ± 0.0	6 ± 4.5	14 ± 0.8
60	83 ± 0.64***	67 ± 1.13***	7 ± 0.1	6 ± 5.1	48 ± 0.6
90	83 ± 0.83***	69 ± 1.47*	8 ± 0.4	49 ± 4.7	60 ± 4.2
120	84 ± 0.60***	76 ± 1.90*	7 ± 0.6	56 ± 3.3	69 ± 2.3

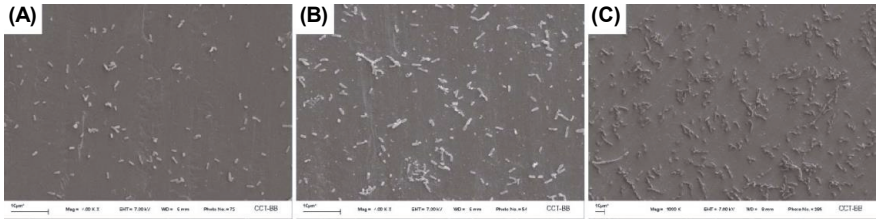

Fig. 2. SEM observation of attached cells on stainless steel AISI 304 incubated at 25°C in 12° Brix apple juice for 24 h: (A) *Escherichia coli* O157:H7, (B) *Salmonella enterica*, (C) *Candida krusei*.

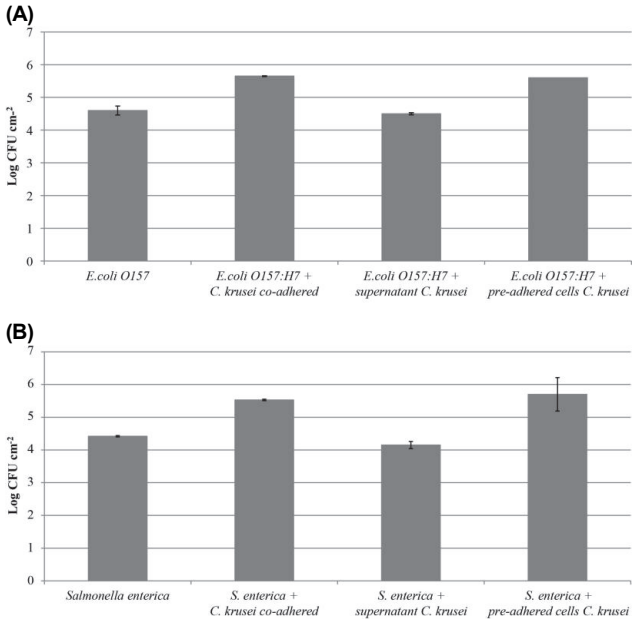

Fig. 3. Populations (log CFU/cm^2 ± SD) of attached cells on SSCs of (A) *Escherichia coli* O157:H7, (B) *Salmonella enterica*. Attachment conditions: mono cultures, co-adhesion with *Candida krusei*, in contact with pre-adhered cells of *C. krusei* and yeast supernatant in a ratio 1:1. The incubation conditions were 24 h at 25°C.

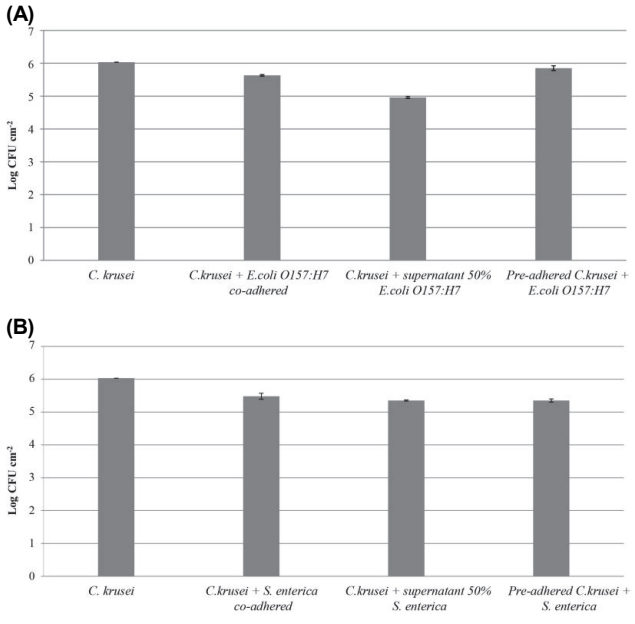

Fig. 5. Populations (log CFU/cm^2 ± SD) of attached cells of *Candida krusei* on SSCs of mono cultures and co-cultures with (A) *Escherichia coli* O157:H7, (B) *Salmonella enterica*; co-adhesion, in contact with pre-adhered cells of *C. krusei* and bacteria supernatant in a ratio 1:1. The incubation conditions were 24 h at 25°C.

A), after 24 h exposure *C. krusei* counts decreased 0.4 ($P < 0.001$) log units when faced to *E. coli* O157:H7 (Fig. 5A) whereas with *S. enterica* the drop was of 0.55 log units (Fig. 5B) ($P < 0.01$). These changes represent a 7 and 9%, respectively, of the counts reached in mono cultures of *Candida* in the same conditions of incubation.

As was stated in previous works (Brugnoni *et al.*, 2007, 2012a, 2012b; Tarifa *et al.*, 2013, 2015) yeasts represent the indigenous microbiota of apple juice processing industries, having a high adherence rate. So, following this assay the potential scenario that bacteria could encounter is an established layer of yeast cells (*pre-adhered cells*, condition B). As already mentioned to evaluate the outcome of pathogenic bacteria facing an already established layer of yeast, *C. krusei* was incubated alone for 8 h and put in contact with the corresponding suspensions of *E. coli* O157:H7/*S. enterica* and cultivated for 16 h. Changes in the adhesion capability of bacteria and yeast species were statistically significant ($P < 0.05$) compared to the performance of each one alone. *E. coli* counts increased in 1.0 log units which represents a 22% rise, whereas *S. enterica* counts increased in 1.28 log units representing a 29% (Fig. 3A and B). In the case of *C. krusei*, the results were opposite with a decrease in the counts of 3 and 11% when exposed to *E. coli* and *S. enterica*, respectively, representing 0.18 and 0.68 log units (Fig. 5A and B).

Supernatant assay: To assess the effect of cell-free cultures on the adhesion capacity, supernatants were made, showing a negative effect on the viability of *C. krusei* while bacteria performance was not affected when faced to yeast supernatant (Fig. 3A and B). As shown in Fig. 5A and B "50% supernatant" treatment of *E. coli*'s and *Salmonella*'s drove to a significant ($P < 0.001$) net reduction in *Candida* cell adhesion of 1.07 and 0.68 log units, respectively. On the other hand, *Candida*'s supernatant showed no significant ($P > 0.05$) effect in the adhesion capability of *E. coli* and *S. enterica* to SS at 24 h exposure.

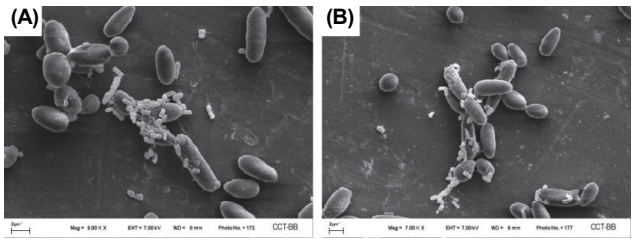

Fig. 4. SEM observation of *Candida krusei* and *Salmonella enterica* in co-adhesion incubated in 12° Brix apple juice at 25°C. Magnification (A) 7000 × (B) 9000 ×.

Discussion

The goal of the present study was to determine how interactions between bacteria and yeast alter their ability to attach and colonize abiotic surfaces.

In an attempt to clarify the interactions between a common food spoilage yeast and two pathogenic bacteria involved in outbreaks associated with fruit juices, the present paper studies the effect of the interplay of *C. krusei*, collected from UF membranes, with *E. coli* O157:H7 and *S. enterica* in the overall process of adhesion and colonization of abiotic surfaces.

The ability of *Candida krusei* to tolerate a diverse range of environmental conditions (low pH, low aw, preservatives, and oxygen tension) together with a number of metabolic activities (carbohydrate metabolism, protein, and polysaccharide hydrolysis) and a great capacity to adhere and form biofilms in food processing facilities under a wide range of conditions (Brugnoni *et al*., 2007; Srey *et al*., 2013; Tarifa *et al*., 2013) warrants this investigations.

There has been a resurgence of interest in interactions between microorganisms, such as those mediated through cell-cell signaling and coaggregation, in order to decipher which are the factors that contribute to competition or cooperation between them. Conceivably, the ability to coaggregate could aid one bacterial species to kill/inactivate another or to protect itself and others from predation. However, it is equally possible that coaggregation can serve as a method for one microorganism to prey upon another, and thus to confer benefits for one species (but not the other coaggregating partner) (Katharios-Lanwermeyer *et al*., 2014).

In this study, when testing situations related to fruit juice processing industry, we found that *S. enterica* and *E. coli* O157:H7 are able to produce various cell surface structures that may result in the efficient co-aggregation of its own cells with each other, as well as with cells of *C. krusei*, facilitating thereby the formation of multi-species communities on food contact surfaces, supporting the colonization and expansion of these foodborne pathogens. Jenkinson and Douglas (2002) postulated that co-aggregation, together with co-adhesion, represent significant colonization factors for micro-organisms because they enable development, stabilization and maintenance of complex communities. Observation by optical microscopy (Fig. 1) revealed extensive physical interactions between *E. coli* O157:H7 and *S. enterica* and *C. krusei* in coaggregation assays. These interactions between cell surface structures were clearly observed by SEM for *Candida krusei* and *S. enterica* (Fig. 4B) and for *Candida tropicalis* and *E. coli* O157:H7 in a previous work (Tarifa *et al*., 2015). These structures are also known to be involved in biofilm development, mostly by resulting in co-aggregation of cells (of the same and/or different species) (Giaouris *et al*., 2015).

In recent years, there has gradually been a shift in focus towards examining the complexity and interactions in multi-species communities, as inter-species interactions can shape its development, structure and function (Yang *et al*., 2011; Elias and Banin, 2012; Rendueles and Ghigo, 2012; Burmølle *et al*., 2014). Under this scenario bacteria would be in disadvantage in terms of competition for space but as described in bacterial biofilms (Liu *et al*., 2014), it could be assumed that the presence of yeast strains strong adhering on food industry premises could potentially promote colonization by pathogenic bacteria. As *C. krusei* has survived typical cleaning and disinfection procedures, it was assumed that it 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. Results of the present study demonstrated that bacterial pathogens such as *E. coli* O157:H7 and *S. enterica*, and spoilage yeast such as *C. krusei*, can be entrapped in multi-species sessile communities formed on inadequately cleaned and disinfected food processing surfaces. The study of these mixed communities revealed a complex relationship between the species that is characterized by antagonistic effects on one another. In both tested conditions (A and B), bacteria showed a proper adhesion to SSC and interaction with yeast. This interaction showed antagonistic results with a positive net effect for bacteria in contrast to the outcome for *C. krusei*. Interestingly, bacteria cells preferred the adhesion to yeast cells rather than to the SS surfaces, leading to think the possible ability to encounter an eligible niche. This finding is a matter of concern particularly for fruit juice processing industries using modern processing equipment as it represents a hazard state. With mechanical and process automation, the surfaces are in repeated contact with raw juice, thus increasing the opportunities for *S. enterica* and *E. coli* O157:H7 to transfer and attach leading to biofilm formation.

To the best for our knowledge, there is no information on interactions between *C. krusei* isolated from food processing equipment and *E. coli* O157:H7 and *S. enterica*, both known to attach to abiotic surfaces such as those of industrial facilities (Giaouris *et al*., 2015). Most biofilm studies are on mixed cultures of bacteria, but two studies have also demonstrated interactions between *Salmonella* spp. and fungi. Tampakakis *et al*. (2009) showed that *S.* Typhimurium was able to secrete a heat stable substance that inhibited filamentation and biofilm formation of *Candida albicans* on silicone pads. In another study *S*. Typhimurium was reported to rapidly attach on the hyphae of the fungus, *Aspergillus niger*. Interactions between cellulose produced by S. Typhimurium and chitin of *A. niger* was required for the production of the mixed biofilms (Brandl *et al*., 2011).

For *E. coli* O157:H7, Uhlich *et al.* (2010) noted cell-to-cell dependent interactions promoting retention of bacteria in biofilms. Other authors found that strains isolated from water and food processing environments have shown to stimulate co-adhesion and biofilm formation of *E. coli*. For instance, Castonguay *et al*. (2006) showed that biofilm formation of non- adherent *E. coli* from drinking water reservoirs was stimulated, in dual and other multi-culture biofilms, with biofilm proficient bacteria from the same environment. The mechanism of biofilm stimulation required direct cell-to-cell contact. Marouani-Gadri *et al*. (2009) found that all but one of 20 randomly collected bacterial isolates obtained after being cleaned and disinfected from a beef processing plant increased the counts of adhered *E. coli* O157:H7 in dual-cultures. Carter *et al.* (2012) examined the interaction of *E. coli* O157:H7 with spinach leaf indigenous microorganisms during co-colonization and establishment of a mixed biofilm on a stainless steel surface. Although the detailed mechanisms are not known, it is likely that the early colonizer provides surface structures or surfactants promoting co- adhesion of *E. coli*. Liu *et al.* (2014) observed that strong biofilm forming plant-associated bacteria promoted the incorporation of *E. coli* O157:H7 in biofilms at 30°C.

On the other hand, *C. krusei* showed a negative response after interact with both foodborne pathogens. A significant reduction in the counts of *C. krusei* was noted with *E. coli* O157:H7 and *S. enterica* in both (A and B) conditions. In a previous study, Tarifa *et al*. (2015) faced *C. tropicalis* with *E. coli* and *Salmonella* sp. and observed a beneficial outcome

for bacteria in detriment of yeast development. Nair and Samaranayake (1996) also demonstrated the suppression of *C. krusei* adhesion in the presence of a high concentration of *E. coli* (10^5 and 10^6 cells/ml). A significant quantitative reduction in CFU counts of *C. krusei* was noted by Bandara *et al.* (2009) on incubation with *E. coli* ATCC 25922 in comparison with their mono species biofilm counterparts.

There are only a few studies on bacterial biofilm supernatant and its effect on modulation of *Candida* biofilms in a mixed species environment (Tampakakis *et al.*, 2009; Holcombe *et al.*, 2010). Hence, our study focused on identifying the effects of *E. coli* 0157:H7 and *S. enterica* extracellular products on *C. krusei* attachment and colonization at a cellular level, but also the potential implication of *Candida* supernatant in the performance of both bacteria.

We found that *Candida krusei* showed a reduction in the adhesion on SSC surface when exposed to *E. coli* O157:H7's and *S. enterica*'s supernatant. The negative effect of O157:H7's and *Salmonella*'s supernatants in the adhesion of *Candida krusei* to SSC surface clearly shows a negative interaction through the presence of molecules or changes in the media affecting significantly yeast adhesion, which not necessarily implies the presence of bacterial cells for a lethal effect. Of the same way, Bandara *et al.* (2013) noted that an active compound secreted by *E. coli* affected the size and shape of *C. krusei* at initial colonization phase (90 min to 24 h). Similarly, they noted a growth retardation of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* at the initial colonization stage in the presence of *E. coli* supernatant, but most significantly at maturation stage after 24 h. Park *et al.* (2014) stated that inhibiting action of bacteria could be induced by a bacterial metabolite that synthesizes during the phase of multiplication and growth, and/or by the differential gene expression response of co-cultures.

These negative effects were not seen in the case of bacteria cultures faced with *Candida*'s supernatant, in which the performance of both species was not affected; suggesting that in the conditions assayed the profit of bacteria is ought to cell-cell interaction rather than a secreted molecule.

To date, the exact mechanisms underlying fungal-bacterial interactions are not fully understood. Also, within typical multi-species biofilms, there are many thousands of potential cell-cell interactions and within real food environments; the possible presence of many other microbial species clearly adds additional complexity. Is evident that eukaryotes and prokaryotes possess diverse signaling mechanisms to detect and respond to each other.

The above findings suggest that the interplay of primary microbiota with pathogens may enhance or reduce its capacity to adhere and colonize contact surfaces of juice facilities, thereby contributing to cross-contamination during juice processing.

This is of significant relevance since it highlights that the control of environmental microorganisms promoting adherence of foodborne bacteria can be an important measure to prevent establishment of pathogenic *E. coli* and *Salmonella enterica* in food processing environments.

Undoubtedly, future studies on multi-species biofilms formed under food relevant conditions will shed light on this fascinating research area.

Acknowledgements

The authors would like to thank Dr Al Cunningham, Professor Emeritus of Civil Engineering at Montana State University (MSU), for her useful comments and suggestions on the language during preparation of this manuscript.

We thank the Consejo Nacional de Investigaciones Científicasy Técnicas de la República Argentina (PIP 112-201101-00438) and Agencia de Promoción Científicay Tecnológica, Ministerio de Educación Ciencia y Tecnología of Argentina (PICT 2011, N° 0697) for funding this research.

Conflict of Interest

The authors have no conflicts of interest to declare.

References

Alonzo, A.G. 2013. Influences of simultaneous physicochemical stresses on injury and subsequent heat and acid resistances of *Salmonella* Enteritidis in apple juice. *Food Control* **31**, 28–34.

Bandara, H.M.H.N., Cheung, B.P., Watt, R.M., Jin, L.J. and Samaranayake, L.P. 2013. Secretory products of *Escherichia coli* biofilm modulate *Candida* biofilm formation and hyphal development. *J. Investig. Clin. Dent.* **4**, 186–199.

Bandara, H.M.H.N., Yau, J.Y., Watt, R.M., Jin, L.J., and Samaranayake, L.P. 2009. *Escherichia coli* and its lipopolysaccharide modulate *in vitro Candida* biofilm formation. *J Med Microbiol.* **58**, 1623–1631.

Brandl, M.T., Carter, M.Q., Parker, C.T., Chapman, M.R., Huynh, S., and Zhou, Y. 2011. *Salmonella* biofilm formation on *Aspergillus niger* involves cellulose-chitin interactions. *PLoS ONE* **6**, e25553.

Brugnoni, L.I., Cubitto, M.A., and Lozano, J.E. 2012a. *Candida krusei* development on turbulent flow regimes: Biofilm formation and efficiency of cleaning and disinfection program. *J. Food Eng.* **111**, 546–552.

Brugnoni, L.I., Lozano, J.E., and Cubitto, M.A. 2007. Potential of yeast isolated from an apple juice to adhere to stainless steel surfaces in the apple juice processing industry. *Food Res. Int.* **40**, 332–340.

Brugnoni, L.I., Lozano, J.E., and Cubitto, M.A. 2011. Biofilm formation under laminar flow conditions of yeast isolated from an apple juice processing plant. *J. Food Process Eng.* **34**, 49–66.

Brugnoni, L.I., Lozano, J.E., and Cubitto, M.A. 2012b. Efficacy of sodium hypochlorite and quaternary ammonium compounds on yeasts isolated from apple juice. *J. Food Process Eng.* **35**, 104–119.

Brugnoni, L.I., Tarifa, M.C., Lozano, J.E. and Genovese, D. 2014. *In situ* rheology of yeast biofilms. *Biofouling* **30**, 1269–1279.

Burmølle, M., Ren, D., Bjarnsholt, T., and Sorensen, S.J. 2014. Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* **22**, 84–91.

Carter, M.Q., Xue, K., Brandl, M.T., Liu, F., Wu, L., Louie, J.W., Mandrell, R.E. and Zhou, J. 2012. Functional metagenomics of *Escherichia coli* O157:H7 interactions with spinach indigenous microorganisms during biofilm formation. *PLoS ONE* **7**, e44186.

Casey, G.D. and Dobson, A.D.W. 2003. Molecular detection of *Candida krusei* contamination in fruit juice using the citrate synthase gene cs1 and a potential role for this gene in the adaptive response to acetic acid. *J. Appl. Microbiol.* **95**, 431–442.

Castonguay, M.H., van der Schaaf, S., Koester, W., Krooneman, J., van der Meer, W., Harmsen, H., and Landini, P. 2006. Biofilm formation by *Escherichia coli* is stimulated by synergistic interactions

and co-adhesion mechanisms with adherence-proficient bacteria. *Res. Microbiol.* **157**, 471–478.

Cheng, H.Y., Yu, R.C., and Chou, C.C. 2003. Increased acid tolerance of *Escherichia coli* O157:H7 as affected by acid adaptation time and conditions of acid challenge. *Food Res. Int*. **36**, 49–56.

Collado, M.C., Meriluoto, J., and Salminen, S. 2008. Adhesion and aggregation properties of probiotic and pathogen strains. *Eur. Food Res. Tech.* **226**, 1065–1073.

Deak, T. and Beuchat, L.R. 1996. Yeasts in specific types of foods, pp. 61–95. *In* Deak, T. and Beuchat, L.R. (eds.), Handbook of Food Spoilage Yeasts. CRC Press, New York, USA.

Elias, S. and Banin, E. 2012. Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol. Rev.* **36**, 990–1004.

Federal Register (FR). 2001. Hazard analysis and critical control points (HACCP); procedures for the safe and sanitary processing and importing of juice. U.S. Food and Drug Administration. Final rule. Federal Register 66, 6138–6202.

Fleet, G.H. 2007. Wine, pp. 863–890. *In* Doyle, M.P. and Beuchat, L.R. (eds.), Food Microbiology: Fundamentals and Frontiers. American Society for Microbiology, Washington, D.C., USA.

Furukawa, S., Nojima, N., Yoshida, K., Hirayama, S., Ogihara, H. and Morigana, Y. 2011. The importance of inter-species cell-cell co-aggregation between *Lactobacillus plantarum* ML11-11 and *Saccharomyces cerevisiae* BY4741 in mixed-species biofilm formation. *Biosci. Biotechnol. Biochem.* **75**, 1430–1434.

Fux, C.A., Costerton, J.W., Stewart, P.S., and Stoodley, P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol.* **13**, 34–40.

Giaouris, E., Heir, E., Desvaux, M., Hébraud, M., Møretrø, T., Langsrud, S., Doulgeraki, A., Nychas, G.J., Kačániová, M., Czaczyk, K., *et al.* 2015. Intra-and inter-species interactions within biofilms of important foodborne bacterial pathogens. *Front. Microbiol.* **6,** doi: 10.3389/fmicb.2015.00841.

Holcombe, L.J., McAlester, G., Munro, C.A., Enjalbert, B., Brown, A.J, Gow, N.A., Ding, C., Butler, G., O'Gara, F. and Morrisey, J.P. 2010. *Pseudomonas aeruginosa* secreted factors impair biofilm development in *Candida albicans*. *Microbiology* **156**, 1476–1486.

Jenkinson, H.F. and Douglas, L.J. 2002. *Candida* interactions with bacterial biofilms, pp. 357–373. *In* Brogden, K.A. and Guthmiller, J.M. (eds.), *Polymicrobial Infections and Disease*. American Society for Microbiology, Washington, D.C., USA.

Katharios-Lanwermeyer, S., Xi, C., Jakubovics, N.S. and Rickard, A.H. 2014. Mini-review: microbial coaggregation: ubiquity and implications for biofilm development. *Biofouling* **30**, 1235–1251.

Lindsay, D. and von Holy, A. 1997. Evaluation of dislodging methods for laboratory-grown bacteria biofilms. *Food Microbiol.* **14**, 383–390.

Liu, N.T., Nou, X., Lefcourt, A.M., Shelton, D.R., and Lo, Y.M. 2014. Dual-species biofilm formation by *Escherichia coli* O157:H7 and environmental bacteria isolated from fresh-cut processing facilities. *Int. J. Food Microbiol.* **171**, 15–20.

Lozano, V. 1990. Preparación de muestras biológicas para microscopía de barrido [Biological samples preparation for scanning electron mircoscopy], pp. 133–156. *In* Lozano, V. and Morales, A. (eds.), Introducción a la microscopía electrónica [Introduction to Electron Microscopy]. CRIBABB-CONICET. Bahía Blanca, Argentina.

Lozano, J.E. 2006. Fruit manufacturing: scientific basis, engineering properties and deteriorative reactions of technological importance, pp. 133–149. *In* Barbosa-Cánovas, G.V. (ed.), Food engineering series. Springer Verlag, New York, USA.

Malik, A., Sakamoto, M., Ono, T., and Kakii, K. 2003. Coaggregation between *Acinetobacter johnsonii* S35 and *Microbacterium esteraromaticum* strains isolated from sewage activated sludge. *J. Biosci. Bioeng*. **96**, 10–15.

Marouani-Gadri, N., Augier, G., and Carpentier, B. 2009. Characterization of bacterial strains isolated from a beef-processing plant following cleaning and disinfection – Influence of isolated strains on biofilm formation by Sakai and EDL933 *E. coli* O157:H7. *Int. J. Food Microbiol.* **133**, 62–67.

Nair, R.G. and Samaranayake, L.P. 1996. The effect of oral commensal bacteria on candidal adhesion to human buccal epithelial cells *in vitro*. *J. Med. Microbiol.* **45**, 179–185.

O'Brien, S.S., Lindsay, D., and von Holy, A. 2007. Biofilms associated with the final stages of Baker's compressed yeast production. *B. Food J.* **109**, 20–30.

Park, S.J., Han, K.H., Park, J.Y., Choi, S.J., and Lee, K.H. 2014. Influence of bacterial presence on biofilm formation of *Candida albicans*. *Yonsei Med. J.* **55**, 449–458.

Pitt, J.I. and Hocking, A.D. 1997. Yeasts, pp. 447–448. *In* Fungi and Food Spoilage Pitt, J.I. and Hocking, A.D. (eds), Blackie Academic and Professional Press. London, UK.

Rendueles, O. and Ghigo, J.M. 2012. Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiol. Rev.* **36**, 972–989.

Salo, S. and Wirtanen, G. 2005. Disinfectant efficacy of food borne spoilage yeast strains. *Trans. I. Chem. E. Part C* **83**, 288–296.

Sancho, T., Giménez-Jurado, G., Malfeito-Ferreira, M., and Loureiro, V. 2000. Zymological indicators: a new concept applied to the detection of potential spoilage yeast species associated with fruit pulps and concentrates. *Food Microbiol.* **17**, 613–624.

Shirtliff, M.E., Peters, B.M., and Jabra-Rizk, M.A. 2009. Cross-kingdom interactions: *Candida albicans* and bacteria. *FEMS Microbiol. Lett.* **299**, 1–8.

Srey, S., Jahid, I.K., and Ha, S.D. 2013. Biofilm formation in food industries: a food safety concern. *Food Control* **31**, 572–585.

Tampakakis, E., Peleg, A.Y., and Mylonakis, E. 2009. Interaction of *Candida albicans* with an intestinal pathogen, *Salmonella enterica* serovar Typhimurium. *Eukaryot. Cell* **8**, 732–737.

Tarifa, M.C., Brugnoni, L.I., and Lozano, J. 2013. Role of hydrophobicity in adhesion of wild yeast isolated from ultrafiltration membranes of an apple juice processing plant. *Biofouling* **29**, 841–853.

Tarifa, M.C., Lozano, J.E., and Brugnoni, L.I. 2015. Dual-species relations between *Candida tropicalis* isolated from apple juice ultrafiltration membranes, with *Escherichia coli* O157:H7 and *Salmonella* sp. *J. Appl. Microbiol.* **118**, 431–442.

Uhlich, G.A., Rogers, D.P., and Mosier, D.A. 2010. *Escherichia coli* serotype O157:H7 retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. *Foodborne Pathog. Dis.* **7**, 935–943.

Yang, L., Liu, Y., Wu, H., Hoiby, N., Molin, S., and Song, Z.J. 2011. Current understanding of multi-species biofilms. *Int. J. Oral Sci.* **3**, 74–81.