Effect of Citrus Pulp on the Viability of *Saccharomyces boulardii* in the Presence of Enteric Pathogens †

J. G. Wilson¹, T. C. McLaurin¹, J. A. Carroll², S. Shields-Menard¹, T. B. Schmidt³, T. R. Callaway⁴, and J. R. Donaldson¹

¹Department of Biological Sciences, Mississippi State University, Mississippi State, MS
²Livestock Issues Research Unit, U. S. Department of Agriculture, Agriculture Research Service, Lubbock, TX
³Animal Science Department, University of Nebraska, Lincoln, NE
⁴Food and Feed Safety Research Unit, U. S. Department of Agriculture, Agriculture Research Service, College Station, TX

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ABSTRACT

*Saccharomyces cerevisiae boulardii* is frequently used as a dietary supplement to promote intestinal health and reduce the impact of growth of enteric pathogens in livestock, including cattle and swine. Citrus by-products are also fed as dietary supplements that have the additional benefit of inhibiting the growth of enteric pathogens. Previous research identified that supplementation of *Saccharomyces boulardii* to feed containing citrus pulp significantly reduced the average daily gain of weanling pigs challenged with *Salmonella enterica*, suggesting citrus pulp reduces the effectiveness of *Saccharomyces boulardii*. To investigate this possibility, an *in vitro* analysis was conducted on the activity of *Saccharomyces boulardii* in swine fecal microbial media supplemented with citrus pulp. citrus pulp inclusion reduced (P < 0.01) populations of *Saccharomyces boulardii* within 48 h post-exposure, suggesting that this product may exhibit antifungal properties. Co-incubation of *Salmonella* with *Saccharomyces boulardii* reduced populations of both microbes; inclusion of citrus pulp did not lead to a further reduction of yeast populations in the co-culture. The cell lysate from *Saccharomyces boulardii* was also found to provide a carbon source that was utilizable by *Escherichia coli*, but not *Salmonella*. Together, these results suggest that citrus pulp reduces the viability of *Saccharomyces boulardii* and that the subsequent effects of this interaction on enterics are varied. Though further research is needed to determine how citrus pulp influences the activity of *Saccharomyces boulardii in vivo*, these data strongly suggest caution should be exercised in providing citrus pulp to livestock being fed diets supplemented with live yeast probiotics.

**Keywords:** probiotics, citrus pulp, *Salmonella Typhi*, *Escherichia coli*, *E. coli O157:H7*, *Saccharomyces boulardii*, enterics, swine, feed supplement, antifungal

INTRODUCTION

The microbial communities associated with the gastrointestinal (GI) tract of animals can be altered in response to changes in environment, food consumption or exercise (Chaucheyras-Durand and Durand, 2010). This can cause severe distress, which is problematic particularly in livestock. Abrupt changes in the gastrointestinal microbial community due to lifestyle or environmental changes can lead to acidosis, increased colonization of pathogens, and other harmful effects. In order to reduce these deleterious effects, probiotics have often been administered to livestock (Chaucheyras-Durand and Durand 2010; Siragusa and Ricke, 2012). Probiotics are microorganisms that provide a benefit to the host by improving health and growth. The mechanisms by which probiotics function are varied and debated, but are primarily attributed to a competitive ability to prevent pathogens from having access to colonization sites within the host and also to prevent pathogens from acquiring nutrients (Boirivant and Strober, 2007; Rolfe, 2000). These changes alter the GI population and influence immune parameters and responses, which ultimately improve growth efficiency (Isolauri et al., 2001; Vanbelle et al., 1990).

Saccharomyces cerevisiae subtype boulardii is a probiotic yeast that has been extensively studied in relation to preventing or alleviating intestinal distress (Rolfe, 2000). Along with pathogen inhibitory effects, evidence suggests Saccharomyces cerevisiae helps to stabilize the rumen microbial community, which may decrease the risk of acidosis in ruminants (Chaucheyras-Durand et al., 2005, Newbold et al., 1996; Nisbet and Martin, 1991). Furthermore, weanling pigs provided a diet supplemented with Saccharomyces boulardii had an improved average daily weight gain (ADG) and reduced mortality associated with endotoxemia (Collier et al., 2011).

Citrus pulp is a by-product produced from citrus processing and is used as a low cost alternative carbohydrate source in livestock diets, particularly in citrus producing regions of the United States and South America (Ariza et al., 2001, Bampidis and Robinson 2006). Previous studies have reported that citrus by-product feeds also act as antimicrobial agents against the enteric pathogens Escherichia coli O157:H7 and Salmonella enterica (Callaway et al., 2008, Fett and Cooke 2003). This antimicrobial activity is likely attributed to the essential oils associated with these citrus products, including, but not limited to citrullene, linool, and limonene (Nannapaneni et al., 2008).

Citrus products have been reported to promote the growth of Bacillus subtilis (Sen et al., 2011), which indicates that supplementation of diets with citrus by-products may promote growth of certain microorganisms within the GI tract. However, depending upon the source, citrus by-products can also have inhibitory effects on the probiotic Bifidobacterium bifidum (Sendra et al., 2008). Carroll and colleagues have reported that weanling pigs provided a diet supplemented with both Saccharomyces boulardii and citrus by-products experienced a decline in ADG post-exposure to Salmonella (unpublished results), suggesting an undesirable interaction occurred between the yeast and pathogen in the gut. The aim of the current study was to analyze the interaction between Saccharomyces boulardii and enteric bacteria to determine if the viability of Saccharomyces boulardii is altered in the presence of citrus pulp using an in vitro swine fecal microbial fermentation system.

MATERIALS AND METHODS

Microbial strains and growth conditions

Escherichia coli O157:H7 (ATCC 43895) and Salmonella enterica ssp. Typhi (ATCC 6539) were routinely cultured in the general culture medium tryptic soy broth (TSB) at 37°C. E. coli and S. Typhi were transformed with the plasmid pXEN-13 to allow for selection onto TSB supplemented with 100 μg/ml ampicillin (TSB amp) as previously described by our group (Free et al., 2012). Saccharomyces cerevisiae ssp. boulardii was obtained from a commercial supplier (Saccharomyces cerevisiae I-1077, Lallemand Animal Nutrition). Saccharomyces boulardii was routinely cultured in yeast peptone dextrose media (YPD, Sigma-Aldrich) at 37°C.
Survival in fecal growth medium

Fecal samples were collected from pigs at the Leveck Animal Research Center at Mississippi State University (Mississippi State, MS). Fecal medium was prepared essentially as previously described (Free et al., 2012; Russell and Martin, 1984). Briefly, 33.3g of fresh feces were vortex-mixed in 33.3mL of sterile water prior to addition to 1L of base medium. The fecal medium was incubated overnight at 39°C in a shaker incubator. The following day 5.0 g of ground citrus pulp (Texas Citrus Exchange, Mission, TX) was added to 100 mL of fecal growth medium.

Bacterial cultures were grown overnight at 39°C in 5 mL TSB amp. Cultures were then diluted 1:100 and allowed to grow for 4 h to reach log phase (Optical density, OD$_{600}$ approximately 0.4), at which time cultures were centrifuged for 5 min at 10,000 x g to remove antibiotics. Resulting cell pellets were re-suspended in an equal volume of freshly prepared swine fecal fluid media supplemented with either 0% or 5% citrus by-products. Cultures were subsequently incubated at 39°C for 48 h. For enumeration of yeast, aliquots were diluted in 1X phosphate buffered saline (1X PBS) and plated on YPD agar supplemented with 100 U/mL of penicillin and 100 μg/mL streptomycin and 0.25 μg/mL fungizone (Invitrogen). Cultivation trials confirmed that this medium did not inhibit the growth of *Saccharomyces boulardii*.

Survival in *Saccharomyces boulardii* lysate

Cultures of *E. coli*, *S. Typhi*, and *Saccharomyces boulardii* were grown overnight at 37°C as described in the previous section. Cells were then pelleted, washed with 1X PBS, and resuspended in mineral salts media (MSM) lacking a carbon source (Alvarez et al., 1996). *Saccharomyces boulardii* cultures were lysed via sonication (Fisher Scientific Sonic Dismembrator Model 120; setting 3, 30 sec pulse; Pittsburgh, PA) and filtered through a 0.22 μm syringe filter. *Salmonella* and *E. coli* were diluted 1:100 in 0.2mL of fresh MSM supplemented with either 20% filtrate from *Saccharomyces boulardii* or 2% glucose. Growth was monitored by OD$_{600}$ readings over a 24 h period with a Biotek Synergy HT microplate reader (Biotek, Winooski, VT). A minimum of three independent replicates was performed.

**Statistical analysis**

The fold change ($\log_{10} N_{treated}$ CFU/mL / $\log_{10} N_{original}$ CFU/mL) and log10 CFU/mL of yeast and bacterial populations were analyzed as means across each treatment. Data were analyzed by analysis of variance (ANOVA) using the Glimmix procedures of SAS (version 9.2, 2013, Institute, Inc, Cary, NC), with significance declared at P < 0.05.

**RESULTS AND DISCUSSION**

Citrus pulp reduces the viability of *Saccharomyces boulardii* in vitro

*Saccharomyces boulardii* was grown in swine fecal microbial fluid and viability was assessed over a
48 h growth period. Viability decreased by 0.85 log$_{10}$ CFU/mL (from 7.95 to 7.10 log$_{10}$ CFU/mL) after 48 h in this cultivation medium (P = 0.005; Table 1). In the presence of 5% citrus pulp, viability was reduced by 1.65 log$_{10}$ after 48 h (7.97 to 6.31 log$_{10}$ CFU/mL, P < 0.0001). This was an approximate 10% reduction beyond what was attributed to the medium alone. This suggests that citrus pulp can directly impact yeast viability.

To further analyze this interaction with citrus pulp, *Saccharomyces boulardii* was incubated in YPD broth in the presence (or absence) of 5% citrus pulp and the integrity of the cell walls were assessed by scanning electron microscopy (Figure 1). Alterations in the cell wall morphology were evident in yeast treated with citrus pulp, indicating that citrus pulp introduces damage into the cell wall of *Saccharomyces boulardii*. Together, these data suggest that citrus by-products may exhibit slight fungicidal activity, or that the mechanism by which the products were processed confers this activity to the product. The essential oils from citrus products are known to

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**Table 1. Fold change of *Saccharomyces cerevisiae boulardii* (SCB) populations (Log$_{10}$ CFU/mL) cultured with citrus pulp (CP), *Salmonella typhi* and/or *Escherichia coli* O157:H7**

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
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<tbody>
<tr>
<td>SCB</td>
<td>0.99</td>
<td>0.96</td>
<td>0.89</td>
</tr>
<tr>
<td>+CP</td>
<td>0.95</td>
<td>0.89</td>
<td>0.79</td>
</tr>
<tr>
<td>+Salmonella</td>
<td>0.99</td>
<td>0.86</td>
<td>0.80</td>
</tr>
<tr>
<td>+Salmonella +CP</td>
<td>0.99</td>
<td>0.84</td>
<td>0.83</td>
</tr>
<tr>
<td>+E. coli</td>
<td>1.14</td>
<td>1.09</td>
<td>0.96</td>
</tr>
<tr>
<td>+E. coli +CP</td>
<td>1.11</td>
<td>0.99</td>
<td>0.84</td>
</tr>
</tbody>
</table>

*a,b,c* Means within a column sharing a common superscript are not different. Significance declared at P < 0.05. 

*x,y,z* Means within a row sharing a common superscript are not different. Significance declared at P < 0.05.

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**Figure 1.** Citrus pulp introduces alterations into the cell surface of S. boulardii. S. boulardii was cultured in the absence (A) or presence (B) of citrus pulp for 24 h and samples were subsequently analyzed by scanning electron microscopy. Scale bars represent 1μm.
exhibit antifungal, as well as antibacterial activities (Caccioni et al., 1998; Cvetnia and Vladimir-Knee-via, 2004). A study using Saccharomyces cerevisiae reported that while not all of the oils from citrus products eradicated Saccharomyces cerevisiae, all seemed to have an inhibitory effect (Belletti et al., 2004). This suggests that the presence of any citrus essential oils may directly alter the overall effectiveness of live yeast probiotics.

**Interactions between Saccharomyces boulardii and Salmonella**

To investigate the possibility that the reduced viability of Saccharomyces boulardii in the presence of citrus pulp would alter the competitive activity of this probiotic against Salmonella, Saccharomyces boulardii and S. Typhi were cultured concurrently in a swine fecal microbial fermentation system and viability for both microbes was assessed over a 48 h period. The addition of S. Typhi reduced the viability of Saccharomyces boulardii by 14% within 24 h (8.23 to 7.05 log\(_{10}\) CFU/mL, P = 0.005) and by 20% within 48 h (8.23 to 6.55 log\(_{10}\) CFU/mL, P = 0.005; Table 1). Co-cultivation of S. Typhi and Saccharomyces boulardii reduced populations of S. Typhi by 8% within 24 h (7.03 to 6.43 log\(_{10}\) CFU/mL, P = 0.003) and by 17% within 48 h (7.03 to 5.86 log10 CFU/mL, P < 0.0001; Table 2). Since in a co-culture condition the reductions in populations of Saccharomyces boulardii were more severe than those of S. Typhi within 24 h (P = 0.04), it is possible that S. Typhi utilizes nutrients in the fecal fluid media first or may be more efficient at utilization of nutrients in a mixed culture. This data warrants further investigation.

The addition of citrus pulp decreased populations of Salmonella as expected based on a previous study (Callaway et al., 2008). Within 48 h post exposure, populations of S. Typhi were reduced by 16% in the presence of citrus pulp (6.80 to 5.73 log\(_{10}\) CFU/mL, P < 0.0001). Populations also decreased by 17% within 48 h of cultivation in the presence of Saccharomyces boulardii (7.04 to 5.87 log\(_{10}\) CFU/mL, P < 0.0001). However, in the presence of both citrus pulp and Saccharomyces boulardii, populations of S. typhi were reduced by 12% within 24 h (P = 0.0585) and by 21% within 48 h (P < 0.0001; Table 2).

These data indicate that a combination of citrus pulp and Saccharomyces boulardii might lead to an enhanced lysis of S. Typhi. Though this is a promising result, it does not necessarily correlate with a beneficial synergy in vivo. A previous study found that the combination of Saccharomyces boulardii and citrus pulp reduced the ADG of weanling pigs following Salmonella infections (Carroll et al., unpublished results). Therefore, an alternative interpretation of these data could suggest that the enhanced lysis of S. Typhi from the combination of

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
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<th>48 h</th>
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<tbody>
<tr>
<td><em>Salmonella</em></td>
<td>0.94</td>
<td>0.92</td>
<td>0.88 (^a)</td>
</tr>
<tr>
<td>+CP</td>
<td>1.01 (^x)</td>
<td>0.94 (^y)</td>
<td>0.84 (^a, b, z)</td>
</tr>
<tr>
<td>+SCB</td>
<td>0.99 (^x)</td>
<td>0.91 (^y)</td>
<td>0.83 (^a, b, z)</td>
</tr>
<tr>
<td>+SCB+CP</td>
<td>0.94 (^x)</td>
<td>0.88 (^x)</td>
<td>0.79 (^b, y)</td>
</tr>
</tbody>
</table>

\(^a,b\) Means within a column sharing a common superscript are not different. Significance declared at P < 0.05.

\(^x,y,z\) Means within a row sharing a common superscript are not different. Significance declared at P < 0.05.
Saccharomyces boulardii and citrus pulp may lead to an increase in cytotoxin release. This must be taken into account when analyzing potential antimicrobial compounds in vivo and warrants further investigation.

**Interactions between Saccharomyces boulardii and E. coli O157:H7**

Since enhanced reductions of S. Typhi populations were observed in the presence of citrus pulp and Saccharomyces boulardii, Escherichia coli O157:H7 was also examined to determine whether this effect would extend to other gram-negative bacteria. Populations of E. coli O157:H7 remained stable in the fecal growth medium during the 48 h (P = 0.7; Table 3). Reductions in E. coli populations in the presence of citrus pulp were only observed after 48 h (7.78 to 6.01 log_{10} CFU/mL reduction, P < 0.001). Saccharomyces boulardii did not reduce populations of E. coli, but the combination of Saccharomyces boulardii and citrus pulp reduced populations of E. coli by 22% within 48 h (8.43 to 6.60 log_{10} CFU/mL reduction, P = 0.0076; Table 3). These results indicate that the presence of Saccharomyces boulardii does not affect the viability of E. coli O157:H7 and that even in a mixed culture the effects are due to the presence of citrus pulp-related factors.

**Cell lysate of Saccharomyces boulardii as a potential carbon source for other microorganisms**

Variations in the growth analysis of Saccharomyces boulardii populations may be due to the reduced viability of Saccharomyces boulardii in the presence of citrus pulp. This reduction in viability could have potentially two effects on the other microorganisms in the system: 1) removes competition for nutrients, or 2) provides an additional source of nutrients that can be utilized by other microorganisms in the system. To determine whether it was possible that lysed Saccharomyces boulardii could provide an additional source of nutrients to enteric bacteria, Saccharomyces boulardii cells were lysed and the filter-sterilized lysate was analyzed as a potential carbon source. Cultures of E. coli O157:H7 or S. Typhi were grown in MSM supplemented with either glucose or Saccharomyces boulardii lysate. MSM without the addition of a carbon source did not support growth of either E. coli or S. Typhi; the addition of glucose to this medium did allow for growth of both microorganisms (data not shown). Surprisingly, E. coli O157:H7, but not S. Typhi, utilized the lysate from Saccharomyces boulardii as a carbon source (Figure 2). Though the growth was minimal, this could potentially allow for sustainability of the population as Saccharomyces boulardii are reduced by citrus pulp.

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Table 3. Fold change of Escherichia coli O157:H7 populations (Log_{10} CFU/mL) cultured with citrus pulp (CP) and/or Saccharomyces cerevisiae boulardii (SCB).

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<th></th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>1.02</td>
<td>0.99</td>
<td>0.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+CP</td>
<td>1.03&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.98&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.77&lt;sup&gt;b,y&lt;/sup&gt;</td>
</tr>
<tr>
<td>+SCB</td>
<td>1.00</td>
<td>0.99</td>
<td>0.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+SCB+CP</td>
<td>0.93&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;x,y&lt;/sup&gt;</td>
<td>0.78&lt;sup&gt;b,y&lt;/sup&gt;</td>
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</table>

<sup>a,b</sup> Means within a column sharing a common superscript are not different. Significance declared at P < 0.05.

<sup>x,y</sup> Means within a row sharing a common superscript are not different. Significance declared at P < 0.05.
In co-cultures with *Saccharomyces boulardii* and *E. coli* O157:H7, citrus pulp may affect *E. coli* initially. This is evident by an increase in populations of *Saccharomyces boulardii* and a decrease in *E. coli* populations (Tables 1 and 3). However, as exposure increased, the populations of *Saccharomyces boulardii* decreased (through lysis with citrus pulp by-products). The populations of *E. coli* did not decrease to the same level as cultures in the presence of citrus pulp alone. The co-culture data, along with the ability of *E. coli* O157:H7 to utilize *Saccharomyces boulardii* lysate as a carbon source, suggests that extended exposure to citrus pulp would decrease populations of *Saccharomyces boulardii*, which may potentially lead to a stabilization of populations of *E. coli* O157:H7.

**CONCLUSIONS**

These findings suggest that caution must be extended when providing live yeast in combination with citrus by-products as the antimicrobial factors of the supplements may result in undesirable growth of enteric pathogen populations. Further research is needed to determine how this relationship alters the gastrointestinal microbiome in vivo.

**ACKNOWLEDGEMENTS**

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