# Comparison of three patterns of feed supplementation with live *Saccharomyces cerevisiae* yeast on postweaning diarrhea, health status, and blood metabolic profile of susceptible weaning pigs orally challenged with *Escherichia coli* F4ac[1]

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**ABSTRACT:** The development of effective feeding strategies to reduce the detrimental effect of enterotoxigenic *Escherichia coli* F4ac (ETEC) plays a crucial role in reducing the occurrence of therapeutic intervention with antibiotics in livestock. The ability of *Saccharomyces cerevisiae* CNCM I-4407 (SCC), supplied in different patterns to counteract ETEC infection in weaned pigs, was evaluated. Fifty pigs weaned at 24 d were then divided into 5 groups: control (CO), CO + colistin (AB), CO + $5 \times 10^{10}$ cfu of SCC/ kg feed, from d 0 to 21 (PR), CO + $5 \times 10^{10}$ cfu of SCC/ kg feed from d 7 to 11 (CM), and CO + 1 shot of $2 \times 10^{11}$ cfu of SCC when the first diarrhea appeared (CU). On d 7 postweaning, all the pigs were orally challenged with $10^8$ cfu of ETEC. Blood samples were taken from the pigs (d 7, 8, 12, and 21) while the fecal excretion of ETEC was assessed on d 7 and 10. Fecal consistency was scored from 12 h before infection to 144 h postinfection (p.i.). On d 21, the pigs were sacrificed. The in vitro adhesion test on the intestinal villi confirmed individual susceptibility to ETEC, excluding the presence of resistant pigs. Growth performance did not differ between the treatments. Mortality was reduced in the AB group ($P < 0.01$) and, marginally, in the PR group ($P = 0.089$) when compared to the CO group. The CO group had a higher fecal score than AB in the period of observation (from $P = 0.01$ to $P < 0.001$). Yeast administration reduced the fecal score when compared to the CO group 12 and 48 h p.i. ($P = 0.04$). Total IgA never differed among the treatments, but the ETEC-specific IgA concentration was lower in the AB group than in CO ($P = 0.04$) at d 12. Four days p.i., the pigs fed live yeast had reduced ETEC excretion compared with the CO pigs ($P = 0.05$). Blood concentrations of dodecenoyl-L-carnitine ($P < 0.01$), glutaryl-L-carnitine/hydroxyhex¬anoyl-L-carnitine, phosphatidylcholine diacyl and phosphatidylcholine diacyl ($P = 0.01$ and $P < 0.01$, respectively), and α-amino adipic acid ($P < 0.01$) were reduced in the AB group compared to the CO group; PR + CM reduced the concentration of sphingomyelin-ceramide ($P = 0.02$) and increased the concentration of decadienyl-L-carnitine (C10:2; $P = 0.02$) vs. CO. The CM group had an increased concentration of C10:2 ($P < 0.01$) compared to the PR group. In conclusion, the administration of live yeast, even in concomitance with ETEC infections, reduces pig illness and mortality. The strain of SCC tested did not show a therapeutic effect.

**Key words:** blood metabolic profile, *Escherichia coli* F4ac, health status, pig, *Saccharomyces cerevisiae* CNCM I-4407

## INTRODUCTION

In 2006, the European Union banned the use of antibiotics as growth promoters; there is diffuse agreement that a strong restriction of the use of therapeutics

in livestock feed may reduce the risk of spreading bacterial antibiotic resistance. This implies significant changes in animal feeding. Developing new feeding strategies is particularly important in reducing postweaning digestive disorders, which are a relevant cause of illness in pigs fostered by intensive feeding practices (Heo et al., 2013). The most important etiological agent is enterotoxigenic *Escherichia coli* F4ac (**ETEC**; Nagy and Fekete, 2005) and the response to feeding strategies may vary due to the existence of different phenotypes for ETEC adhesion on the intestinal villi of pigs (Sellwood et al., 1975).

The concept of probiosis originated approximately a century ago, but its use in animal production is still valid in reducing the detrimental effects of pathogen infection (Armstrong et al., 2014). *Saccharomyces* spp. is the yeast most studied for counteracting intestinal disorders in young mammals (Farthing et al., 2013; Shan et al., 2013). The administration of *Saccharomyces cerevisiae* modulates the activation of inflammation in mice infected with *Salmonella enterica* serovar Typhimurium (Martins et al., 2011). Moreover, in the pig model, *S. cerevisiae* yields positive effects in controlling ETEC infection, reducing the severity of diarrhea in weaned piglets (Trckova et al., 2014).

For the first time, the effectiveness of *S. cerevisiae* CNCM I-4407 (**SCC**) dosed in different patterns was compared to counteract the detrimental effect of ETEC on the health status of weaned pigs orally challenged with this pathogen. Moreover, considering that exposure to postweaning stress and challenge with pathogenic *E. coli* affect several metabolites (Sugiharto et al., 2014), the blood metabolic profile of the pigs was evaluated to determine the interaction among the yeast, ETEC, and the host.

## MATERIALS AND METHODS

The procedures complied with Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, Italy.

### General Experimental Design

Fifty piglets were obtained from a commercial piggery where ETEC infections had been reported; this indicated the presence in the herd of pigs susceptible to ETEC. During the suckling period, no creep feed was supplied. At $24 \pm 2$ d of age (d 0), the pigs were weaned and moved to the experimental farm, divided into 5 groups balanced for litter and BW, and were housed in pens with a mesh floor. The pigs were kept at a controlled temperature (30°C at the beginning and 25°C at the end of the experiment, with a 1°C decrease every 3 d). Infrared lamps

**Table 1.** Ingredients and calculated composition of the basal diet (percentage as-fed basis)

| Ingredient | % | Calculated composition[1] | % or otherwise |
|---|---|---|---|
| Wheat shorts | 20 | CP | 18.13 |
| Corn | 17 | Crude fat | 6.01 |
| Barley | 15 | Total lysine | 1.28 |
| Barley, extruded | 15 | Total threonine | 0.87 |
| Soybean meal, 50 | 13.4 | Total methionine | 0.50 |
| Whey, dehydrated, skimmed | 6 | Total methionine and cysteine | 0.81 |
| Potato, protein concentrate | 4 | Total tryptophan | 0.28 |
| Vegetable oil | 4 | DE, growing pig, kcal/kg | 3,355 |
| Beet pulp, dehydrated | 2 | NE, growing pig kcal/kg | 2,424 |
| Calcium carbonate | 1.38 | | |
| Monosodium phosphate hydrated | 0.6 | | |
| L-Lysine HCl | 0.4 | | |
| Sodium chloride | 0.3 | | |
| DL-Methionine | 0.2 | | |
| L-Threonine | 0.15 | | |
| L-Tryptophan | 0.07 | | |
| Vitamin and trace mineral mixture[2] | 0.5 | | |

[1]Values were estimated by the EvaPig database (Noblet et al., 2008).

[2]Provided per kilogram of diet: vitamin A, 9,000 IU; vitamin $D_3$, 1,500 IU; vitamin $K_3$, 2 mg; vitamin E, 50 mg; vitamin $B_1$, 2 mg; vitamin $B_2$, 4 mg; vitamin $B_6$, 4 mg; vitamin $B_{12}$, 0.04 mg; niacin, 55 mg; biotin, 0.15 mg; d-pantothenic acid, 30 mg; folacin, 2 mg; choline chloride, 400 mg; iron as $FeSO_4$, 150 mg; zinc as $ZnSO_4$, 110 mg; copper as $CuSO_4$, 25 mg; manganese as $MnSO_4$, 70 mg; iodine as KI, 1 mg; selenium as $Na_2SeO_4$, 0.3 mg.

were located above the piglets for the first 7 d. The piglets had free access to feed and water throughout the experimental period; feed was supplied in a dry feeder. On d 7 postweaning, all the pigs were orally dosed with 1.5 mL of suspension containing $10^8$ cfu ETEC O149/mL. The bacteria solution was prepared as described by Bosi et al. (2004). The product tested was a lyophilized live yeast strain (Actisaf; Lesaffre Feed Additives, Marcq-en-Baroeul, France) of SCC mixed in the diet formula.

The piglets were assigned to 1 of 5 diets: control (**CO**; typical weaning diet—Table 1), CO + 1 g colistin/kg of feed (**AB**), CO + $5 \times 10^{10}$ cfu SCC/kg of feed, from d 0 to d 21 (preventive dose [**PR**]), CO + $5 \times 10^{10}$ cfu SCC/kg of feed from d 7 (day of infection with ETEC) to d 11 (competitive dose [**CM**]), and CO + 1 shot of $2 \times 10^{11}$ cfu SCC/kg of feed when the first diarrhea appeared (curative dose [**CU**]). Colistin treatment was used as a positive control because it is active against the ETEC strain used for the challenge. Colistin has strong properties against gram-negative bacteria, and it is frequently used for this purpose in other trials involving an ETEC challenge (Torrallardona et al., 2003; Bosi et al., 2004). The pigs were individually penned in cages, except for the first 2 d when they were kept in groups of 2 having the same dietary treatment for the purpose of improving their adaptation and feed intake.

## Experimental Procedure

Starting on d 0, each group received its experimental diet. The pigs were sacrificed at the end of the trial (d 21). At slaughter, the animals were deeply anesthetized with sodium thiopental (10 mg/kg BW) and sacrificed via an intracardiac injection of Tanax (0.5 mL/kg BW).

## Experimental Controls

The pigs were weighed individually at the start of the trial, on d 7 (prechallenge), on d 14, and at sacrifice (d 21). The feed intake of each pig was recorded individually.

Blood was sampled on d 7 (prechallenge), d 8, d 12, and on d 21 (day of sacrifice) by venipuncture of the vena cava and centrifuged at $3,000 \times g$ for 10 min at 4°C; the serum was then removed. The serum samples collected on d 7, 12, and 21 were inactivated at 56°C for 30 min and stored at −20°C until analysis. On the other hand, the serum collected on d 8 was stored at −80°C after centrifugation. Individual fecal samples were obtained on d 7 (prechallenge) and d 10 for the ETEC plate counts following the protocol described by Bosi et al. (2004). The severity of the diarrhea was evaluated daily in each subject by 5 point fecal scores (1 to 5: 1 = hard, 5 = watery feces) and by the same operator from 12 h before to 144 h after infection.

On d 21, the piglets were sacrificed to collect a sample from the distal jejunum to determine the phenotype for adhesion of the ETEC to the intestinal villi, as described in Trevisi et al. (2009).

## Total IgA and Escherichia coli F4ac-Specific IgA Titers

Total IgA determination was performed by ELISA, using Pig Immunoglobulin Reference Serum (Bethyl laboratories, Montgomery, TX) as the specific antibody for the standard curve, Goat anti-Pig IgA-HRP conjugate (Bethyl Laboratories) as a secondary antibody, and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Roche Diagnostics, San Francisco, CA) for chromogenic detection. The concentration was expressed as micrograms per milliliter. The ETEC-specific IgA quantification was performed by ELISA according to Van den Broeck et al. (1999), using F4 fimbriae isolated from ETEC cultures as reported by Bosi et al. (2004). Briefly, the F4 antigen was added at a concentration of 50 mg/mL in ELISA-diluted buffer to coated wells with F4 fimbrial adhesin Mab (CVL, Addlestone, UK). Pooled serum obtained from 5 subjects, all ETEC-challenged and positive for the ETEC adhesion test was used as a calibrant. The concentration values of specific IgA were expressed as arbitrary units per gram of total IgA.

## Metabolic Profile of Blood Serum

A targeted metabolic technique, designed to quantify the concentration of 188 endogenous metabolites from 5 different compound classes taken from 10 µL of plasma, was performed using the AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Innsbruck, Austria). Sample analyses were performed on the API 4000 QTrap LC/MS/MS System (Applied Biosystems, Foster City, CA). Measurements were performed on the same plate and analyzed by MetIQ software packages, which are an integral part of the AbsoluteIDQ Kit.

## Statistical Analysis

Performance data were analyzed by ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) with a completely randomized design, 2 blocks (time), sows within block, and 5 dietary treatments. Degrees of freedom for the dietary treatments were used to test the following orthogonal contrasts: CO vs. yeast (PR, CM, CU), PR vs. (CM and CU), CM vs. CU, and CO vs. AB. However, for prechallenge observations, the CM and CU groups received the same diet and, thus, the contrasts were PR vs. (CM + CU + CO), PR vs. AB, and AB vs. CO.

$P < 0.05$ was statistically significant and $0.05 < P < 0.10$ was considered a trend.

For mortality data, Fisher's exact test was performed comparing CO with each of the other dietary treatments.

The metabolomic data were analyzed using linear mixed models (Pinheiro and Bates, 2009), taking the concentration of a given metabolite as a dependent variable and including a random effect for litter. Body weight at d 7 and fecal score were considered to be possible confounding factors, the latter taken after centering with respect to the diet-specific mean fecal score. To establish which of these factors should be included in the model for each metabolite, a backward elimination procedure, based on bootstrap testing (Davison and Hinkley, 1997) was performed on the corresponding linear mixed model. The analysis was focused on diets AB, CO, CM, and PR, and examined the following contrasts: AB vs. CO, CM + PR vs. CO, and CM vs. PR. For each null hypothesis, a leave-one-out (**LOO**) procedure was implemented (Hastie et al., 2009) to account for the possible presence of influential observations (Cook and Weisberg, 1982). The applied procedure consisted of testing the given null hypothesis on 38 different datasets, each one obtained after excluding 1 animal at a time; finally, the rejection of the null hypothesis was deemed to be "most stable" when it occurred on each one of the 38 different LOO datasets.

**Table 2.** Effect of dietary supplementation with *Saccharomyces cerevisiae* CNCM I-4407 on different patterns regarding the mortality of weaned pigs challenged with ETEC

|  | Diet[1] | | | | | *P*, Fisher's exact test | | | |
|  | CO | AB | PR | CM | CU | CO vs. AB | CO vs. PR | CO vs. CM | CO vs. CU |
|---|---|---|---|---|---|---|---|---|---|
| Alive | 3 | 9 | 7 | 6 | 4 | | | | |
| Dead | 7 | 1 | 3 | 4 | 5 | <0.01 | 0.089 | 0.181 | 0.430 |

[1]CO, no live yeast + F4 challenge; AB, antibiotic + F4 challenge; PR, preventive administration pattern of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 0 to 21) + F4 challenge; CM, competitive administration of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 7 to 11) + F4 challenge; CU, curative administration of live yeast (1 shot of $2 \times 10^{11}$ cfu when the first diarrhea appears) + F4 challenge.

# RESULTS

## Growth Performances

No difference in growth performance was observed among the experimental groups. The ADG was 72.0, 71.0, 63.4, 86.1, and 95.2 g (SEM = 17.4) from d 0 to 7 (before the challenge), and 197, 188, 210, 204, and 202 g (SEM = 73.2), from d 7 to 21, for groups CO, AB, PR, CM, and CU, respectively.

## Severity of Diarrhea and Mortality

The in vitro tests confirmed the presence of specific receptors for ETEC on the intestinal villi of all pigs.

Table 2 lists the number of pig deaths during the trial for each group. Mortality in the CO group was significantly higher than in the AB group ($P < 0.01$) and a trend of reduction was seen also for PR ($P = 0.089$). Figure 1 shows the time course of pig survival during the trial. Twenty-four hours after infection (d 8), the first pig died in the CO group; in the PR and CM groups, the first pig died on d 10. In the AB group, only 1 pig died on d 11. Finally, in the CU group, even if the pigs started to die on d 10 as in the other yeast-treated groups, the survival curve decreased faster than in the PR and CM groups.

## Fecal Scores

Table 3 shows the effect of the dietary supplementation with live yeast on the fecal scores of weaned pigs challenged with ETEC at different times and doses. Before the challenge, the maximum fecal score was 2.4, indicating that no diarrhea occurred and no differences emerged among the groups. From 12 to 144 h after infection, the CO group showed a higher fecal score than the AB group (from $P = 0.01$ to $P < 0.001$). Conversely, the administration of yeast significantly reduced the fe-
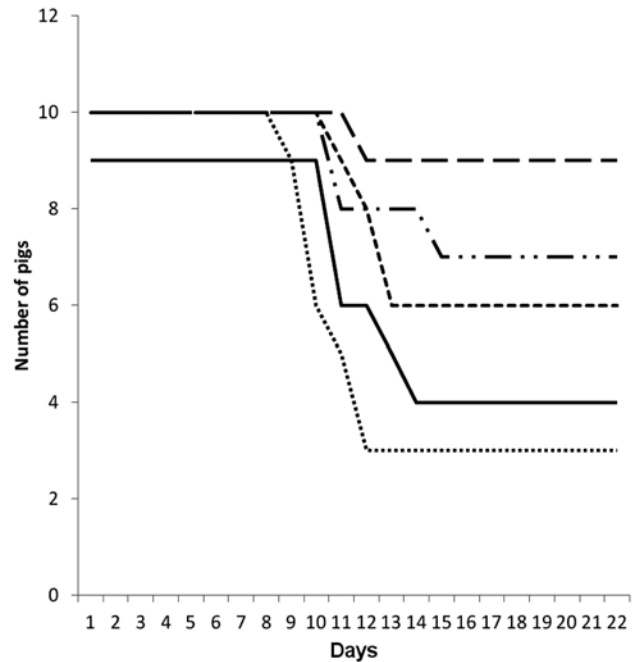


**Figure 1.** Effect of dietary supplementation with *Saccharomyces cerevisiae* CNCM I-4407 at different times and doses on the survival of weaned pigs challenged with enterotoxigenic *Escherichia coli* F4ac (ETEC; ···· CO: no live yeast + F4 challenge; —— AB: antibiotic + F4 challenge; —··—·· PR: preventive administration pattern of live yeast [$5 \times 10^{10}$ cfu/kg of feed from d 0 to 21] + F4 challenge; – – – CM: competitive administration of live yeast [$5 \times 10^{10}$ cfu/kg of feed from d 7 to 11] + F4 challenge; —— CU: curative administration of live yeast [1 shot of $2 \times 10^{11}$ cfu when the first diarrhea appears] + F4 challenge).

cal score as compared with the CO diet 12 and 48 h after infection ($P = 0.04$ and $P = 0.04$, respectively), and a tendency to reduce this parameter against the same groups was seen 24 h postchallenge ($P = 0.08$). Moreover, during the entire period of observation, no significant differences were observed among the groups supplied with live yeast, even if 96 h after the infection, the PR group tended to reduce the fecal score as compared with the CM and CU groups ($P = 0.08$).

## Immune Response and *Escherichia coli* F4ac Shedding in Feces

Table 4 shows the data related to the IgA concentration in the blood serum and to the fecal excretion of ETEC. The total IgA never differed among the experimental groups at any of the time points considered. Moreover, before the challenge, no difference was observed in ETEC-specific IgA concentration among the experimental groups. At d 12, the ETEC-specific IgA concentration was lower in the AB group than in the CO group ($P = 0.04$), and the administration of live yeast tended to reduce the specific IgA concentration as compared with the CO group ($P = 0.10$).

On d 7 (before the challenge), no pigs were found to be positive for fecal excretion of ETEC while, 4

**Table 3.** Effect of dietary supplementation with *Saccharomyces cerevisiae* CNCM I-4407 with different patterns on the fecal score of weaned pigs challenged with enterotoxigenic Escherichia coli F4ac (ETEC)

| Hours | Diet[1] | | | | | SEM | AB vs. CO | Yeast vs. CO | PR vs. CM | PR vs. CM + CU | CM vs. CU |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CO | AB | PR | CM | CU | | | | | | |
| −12[2] | 2.4 | 2.0 | 2.1 | 1.9 | 1.7 | 0.2 | 0.89 | 0.77[3] | | | |
| 12 | 2.9 | 2.2 | 2.5 | 2.3 | 2.3 | 0.2 | 0.02 | 0.04[4] | 0.51 | | |
| 24 | 3.7 | 2.4 | 3.0 | 3.2 | 2.8 | 0.3 | 0.001 | 0.08[4] | 0.57 | | |
| 48 | 4.2 | 2.4 | 3.4 | 3.7 | 3.2 | 0.3 | <0.001 | 0.04 | | 0.93 | 0.38 |
| 72 | 4.1 | 2.6 | 3.4 | 3.9 | 3.5 | 0.4 | <0.05 | 0.41 | | 0.67 | 0.42 |
| 96 | 4.0 | 2.5 | 2.9 | 3.9 | 3.5 | 0.4 | 0.01 | 0.38 | | 0.08 | 0.60 |
| 120 | 3.3 | 1.9 | 3.5 | 3.9 | 3.7 | 0.3 | 0.01 | 0.42 | | 0.58 | 0.81 |
| 144 | 3.0 | 1.9 | 3.2 | 3.3 | 3.1 | 0.3 | 0.02 | 0.73 | | 0.93 | 0.77 |

[1] CO, no live yeast + F4 challenge; AB, antibiotic + F4 challenge; PR, preventive administration pattern of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 0 to 21) + F4 challenge; CM, competitive administration of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 7 to 11) + F4 challenge; CU, curative administration of live yeast (1 shot of $2 \times 10^{11}$ cfu when the first diarrhea appears) + F4 challenge.

[2] Contrast before the challenge.

[3] CM and CU were combined with CO because the pigs had not yet been given yeast.

[4] Yeast includes PR and CM only while CU was not considered in the contrast.

d after infection, the subjects fed with live yeast excreted less ETEC as compared with the CO group ($P = 0.05$). No other significant differences among the groups were observed.

### Blood Metabolic Profile

The differences between the most stable metabolites (i.e., the metabolites for which a given null hypothesis was rejected in all 38 LOO datasets) in the blood serum 24 h after infection with ETEC in weaned pigs are shown in Table 5. Compared with the antibiotic-treated pigs, in the CO group, there were increased concentrations of dodecenoyl-L-carnitine (**C12:1**; $P < 0.01$), glutaryl-L-carnitine/hydroxyhexanoyl-L-carnitine (**C5DC [C6-OH]**; $P = 0.02$), phosphatidylcholine diacyl C 40:1 and phosphatidylcholine diacyl C 40:6 (**PC_aa_C40:1** and **PC_aa_C40:6**; C 40 stands for total carbon numbers of the couples of acyls and :1 and :6 for total double bond numbers; $P = 0.01$ and $P < 0.01$, respectively). Moreover, the concentration of the α-amino adipic acid (**α-AAA**) was also higher in the CO group than in the AB group ($P < 0.01$), but this difference was affected by the fecal score factor.

In CM + PR vs. CO, the fecal score was responsible for the decreasing concentration of

**Table 4.** Effect of dietary supplementation with *Saccharomyces cerevisiae* CNCM I-4407 with different patterns on total and specific immunoglobulins against enterotoxigenic Escherichia coli F4ac (ETEC) and on the fecal excretion of ETEC of weaned pigs challenged with this strain

| | Diet[1] | | | | | SME | PR vs. CM + CU + CO[2] | PR vs. AB | AB vs. CO | Yeast vs. CO[3] | PR vs. CM + CU[3] | CM vs. CU[3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CO | AB | PR | CM | CU | | | | | | | |
| Total IgA (mg/L) | | | | | | | | | | | | |
| d 7[4] | 400 | 391 | 439 | 344 | 371 | 51 | 0.25 | 0.70 | 0.90 | | | |
| d 12 | 801 | 717 | 666 | 1045 | 711 | 102 | | | | 0.61 | 0.96 | 0.28 | 0.11 |
| d 21 | 1,090 | 1,312 | 1,203 | 1,298 | 1,269 | 227 | | | | 0.53 | 0.60 | 0.76 | 0.93 |
| Specific IgA against ETEC (UI) | | | | | | | | | | | | |
| d 7[4] | 0.5 | 0.21 | 0.18 | 0.14 | 0.33 | 0.12 | 0.13 | 0.56 | 0.12 | | | |
| d 12 | 88.3 | 13.2 | 11.7 | 26.3 | 66.1 | 21.4 | | | | 0.04 | 0.10 | 0.16 | 0.26 |
| d 21 | 182 | 45 | 340 | 228 | 210 | 114 | | | | 0.23 | 0.97 | 0.93 | 0.98 |
| ETEC fecal counts (log10 cfu/g) | | | | | | | | | | | | |
| d 10[5] | 8.9 | 8.4 | 7.3 | 8.2 | 7.7 | 0.5 | | | | 0.52 | 0.05 | 0.45 | 0.54 |

[1] CO, no live yeast + F4 challenge; AB, antibiotic + F4 challenge; PR, preventive administration pattern of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 0 to 21) + F4 challenge; CM, competitive administration of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 7 to 11) + F4 challenge; CU, curative administration of live yeast (1 shot of $2 \times 10^{11}$ cfu when the first diarrhea appears) + F4 challenge.

[2] CM and CU were combined with CO because the yeast had not yet been given to the pigs.

[3] Contrast after the challenge.

[4] Contrast before the challenge.

[5] Four days postchallenge.

**Table 5.** Effect of *Saccharomyces cerevisiae* CNCM I-4407 on blood metabolic profile metabolites 24 h after infection with enterotoxigenic Escherichia coli F4ac (ETEC) in weaned pigs

| Diet[1]/metabolites | *P*-value | Direction |
|---|---|---|
| AB vs. CO | | |
| C12:1[2] | <0.01 | CO |
| C5DC (C6-OH)[3] | 0.02 | CO |
| PC_aa_C40:1[4] | 0.01 | CO |
| PC_aa_C40:6[5] | <0.01 | CO |
| alpha-AAA[6,7] | <0.01 | CO |
| CM + PR vs. CO | | |
| SM_C18:0[8,7] | 0.02 | CM + PR ↓ |
| C10:2[9] | 0.02 | CM + PR |
| CM vs. PR | | |
| C10:2 | <0.01 | CM |

[1]CO, no live yeast + F4 challenge; AB, antibiotic + F4 challenge; PR, preventive administration pattern of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 0 to 21) + F4 challenge; CM, competitive administration of live yeast ($5 \times 10^{10}$ cfu/kg of feed from d 7 to 11) + F4 challenge.

[2]Dodecenoyl-L-carnitine.

[3]Glutaryl-L-carnitine/hydroxyhexanoyl-L-carnitine.

[4]Phosphatidylcholine diacyl C 40:1.

[5]Phosphatidylcholine diacyl C 40:6.

[6]Alpha-amino adipic acid.

[7]Affected by the confounding factor "fecal score."

[8]Sphingomyelin-ceramide.

[9]Decadienyl-L-carnitine.

sphingomyelin-ceramide (**SM_C18:0**; *P* = 0.02) in the yeast-treated pigs. On the other hand, the yeast treatments increased the concentration of decadienyl-L-carnitine (**C10:2**; *P* = 0.02). However, when compared with the PR group, the CM group exhibited an increased concentration of C10:2 (*P* < 0.01).

## DISCUSSION

This study evaluated the protective effect of 3 different patterns of SCC supplementation in the feed of sensitive ETEC-challenged piglets: the preventive, the competitive, and the curative; a group treated with the antibiotic colistin, frequently used against gram-negative enterobacteria, was also included as a positive reference.

Due to the experimental design, the absence of differences for growth parameters is not surprising. An experiment on a larger scale is necessary to evaluate the growth performance differences in susceptible challenged pigs fed live yeast.

However, in experimental challenge trials with ETEC, health parameters provided relevant indications regarding the entire effect of testing feeding practices; of these, mortality was an important parameter to be evaluated (Fairbrother et al., 2005). Moreover, a proper evaluation of the sensitivity of the animals used in

the trials is a prerequisite for avoiding false-negative responses. In the present study, specific receptors for ETEC on the intestinal villi were present in all the piglets, strengthening the relevancy of the experimental results. Furthermore, the ETEC strain used to infect the piglets was proven to be sensitive to the antibiotic used here as a positive control. The low mortality rate of the pigs, the low concentration of specific IgA against ETEC in the blood serum and the lowest diarrhea score compared with the CO group confirm the effectiveness of the antibiotic. Only 1 pig in the AB group died as a result of diarrhea immediately after weaning as a consequence of the reduction in feed intake and the subsequent reduction in antibiotic ingestion. Between the 3 feeding strategies studied in the trial supplying SCC in the feed, the preventive method was the classic method of supplying probiotics to livestock feed to protect animals against the risk of pathogenic infection. In the literature, there is evidence of the preventive effect of *S. cerevisiae* spp. supplied in weaned pigs challenged with lipopolysaccharide (**LPS**) from *E. coli* (Collier et al., 2011) to reduce the inflammatory response and mortality in pigs. Moreover, a protective effect of *S. cerevisiae* on porcine epithelial cell lines reducing the increased expression of genes related to inflammation on ETEC stimulation was observed (Badia et al., 2012). Furthermore, a continuous supply of SCC to the sows from late gestation and to the piglets, before and after weaning, reduced the severity and duration of diarrhea on ETEC challenge (Trckova et al., 2014).

In the present trial, 70% of untreated piglets died after infection with ETEC while SCC halved pig mortality when administered in a preventive way. Similarly, Collier et al. (2011) reported that *S. cerevisiae* var. *boulardii* reduced the mortality of LPS-challenged pigs by 20%. Furthermore, an examination of the time course of pig survival reveals that, when yeast is supplied after weaning, a reduction in diarrhea severity is associated with delayed mortality. From a practical point of view, this fact implies a delay in the appearance of pig cachexia and more time for eventual therapeutic intervention. The protective effect in the PR group could also be ascribed to the ability of SCC to modulate the immune response in the gut mucosa, as reported by in vitro tests (Zanello et al., 2011a,b).

Currently, precision feeding is a new targeted technique for modern livestock production to reduce the environmental footprint and improve growth efficiency; feed additives should also be utilized in a similar manner, to be supplied ideally only when it is necessary. For this reason, the competitive and curative uses of a probiotic product in piglet feeding were tested. To our knowledge, this is the first trial aimed at studying pigs exposed to an ETEC challenge and the ability of SCC to compete

with the pathogen. Furthermore, focusing on the potential therapeutic properties of SCC when diarrhea was already present was really challenging and innovative. The SCC used in the diet of the present trials was lyophilized. The pig survival curve of the CM group, which shows an effect comparable to that of the PR group, may be explained by the sudden activation of the yeasts in the gastrointestinal tract (**GIT**). There is evidence of the capability of *S. cerevisiae* to produce ethanol along the intestinal tract, fermenting the sugar derived from the digestive process or provided by the diet (Etienne-Mesmin et al., 2011). The ethanol concentration in the gut was not quantified in this study. However, on the basis of the data of Bode et al. (1984), SCC should be able to produce ethanol in the stomach by means of the fermentation of the sugar provided by the milk-derived product supplied with the feed formula. This, in turn, could have reduced the quantity of viable ETEC available to adhere to the intestinal receptors and/or the gut sensitivity to the bacterial toxins, as demonstrated in macrophages in vitro or in the liver of mice challenged with *E. coli* lipopolysaccharide (Nishiyama et al., 2002). Moreover, the continuous supply of live yeast for an additional 4 d in the CM group may have been responsible for containing the inflammation of the intestinal mucosa, thereby reducing the consequences of the ETEC challenge (Zanello et al., 2011a).

Other studies in the scientific literature targeted to human gut health and therapy against diarrhea suggest a curative approach using probiotics. In clinical trials on children, *Lactobacillus rhamnosus* GG seems to shorten the duration of acute diarrhea (Shornikova et al., 1997; Guandalini et al., 2000). On the other hand, *Saccharomyces* spp. are considered to be broad-spectrum probiotics because they are not commonly found on or adherent to the mucosa of the GIT in mammals (Blehaut et al., 1989). Thus, an interspecific effect is conceivable, as suggested by the positive results obtained with the same yeast strain in human and animal models (McFarland, 2010; Kurugöl and Koturoğlu, 2005). Our therapeutic dose of SCC was 1 shot, 4 times more concentrated than the dose used in the PR and CM groups, but the resulting health data did not show any reduction in the detrimental effects of ETEC infection. This suggests that when ETEC has already exerted its pathogenicity adhering to the mucosa and producing its toxins yeast is not capable of interfering with the pathogenic mechanisms of ETEC. This finding partially disagrees with the meta-analyses of Szajewska et al. (2007), which indicated a moderate clinical benefit of *S. cerevisiae* boulardii therapy in infants and children with acute gastroenteritis, with a shortened duration of diarrhea; nevertheless, the same authors indicated some methodological limitations in the study. We observed only a slight delay in the time course of mortality in comparison to untreated animals; the number of dead piglets

did not differ between the CU and CO groups. As a confirmation of the general effect of SCC against ETEC, there is a global lowering effect of the yeast treatments on the specific IgA against ETEC, even if the greatest effect was attributable to the PR group. This fact could indirectly indicate the ability of the yeast to reduce the antigenic presence in the gut, reducing the antigen exposure and the specific immune response.

In the present study, the blood plasma metabolic profile was considered to support the clinical evidence and to reveal the metabolic effects resulting from the interaction among ETEC, yeast, and the host. In pigs, abrupt modifications in the microbial population in the GIT can occur after weaning with a negative impact on the mucosal homeostasis and, consequently, on the blood metabolic profile (Wikoff et al., 2009; Campbell et al., 2013). In this study, a sudden impact of ETEC infection was observed on some bioactive metabolites involved in cell signals and in the activation of immune pathways. In the CO group, 2 phosphatidylcholine diacyls (C40:1, and C40:6) and 2-aminoadipic acid were upregulated. Phosphatidylcholine is by far the most abundant phospholipid component in plasma and is largely found in diacylated form (Floegel etal., 2013). Lipopolysaccharide, a bioactive component of the cell wall of gram-negative bacteria, stimulates phosphatidylcholine breakdown in macrophages (Grove et al., 1990). T cells, by means of acyltransferases and phospholipases, manipulate phospholipid composition on stimulation (Robichaud et al., 2013). No specific reference to the 2 diacyl compounds which were affected herein is reported; however, due to the time proximity to the ETEC challenge, it can be hypothesized that this was related to the metabolic action of ETEC on inflammatory or immune cells and that this action was reduced by the antibiotic. Alpha-AAA is a product of lysine degradation in tissues after oxidant stress (Sell et al., 2007) and the higher blood values in the CO group may agree with the clinical observations and indirectly indicate that ETEC infection stimulated the inflammatory pathways with additional oxidative stress. Moreover, in all the experimental groups except for the AB group, the carnitine metabolism was affected by an increase in the concentration of medium-chain acylcarnitine compounds in the blood plasma. This finding agrees with the results of Bene et al. (2006) regarding the increase in the level of decadienyl-L-carnitine in patients affected by an acute inflammation of the hindgut. Moreover, increases in the acylcarnitine compounds in the CO and CM groups, supported by evidence of their involvement in the activation of the proinflammatory signaling pathways (Rutkowsky et al., 2014) indicated the low protection rate against ETEC in these groups. Conversely, ceramide, a sphingolipid involved in the regulation of cell growth, survival, immune cell trafficking, and

epithelial integrity, which plays a key role in inflammation (Maceyka and Spiegel, 2014), was downregulated in the PR and CM groups as compared with animals fed the CO diet. These findings, particularly in the CM group, indicate regulation between pro- and anti-inflammatory signals, which helps to explain the survival curve when pigs are fed SCC in a competitive way.

In summary, our results demonstrated the effectiveness of SCC in delaying cachexia in ETEC-susceptible piglets, providing a window for therapeutic intervention. Moreover, preliminary evidence was provided regarding new perspectives for the use of live yeast in livestock to reduce the use of antibiotics. Unfortunately, our evidence suggested that this yeast strain alone is not completely capable of exerting a therapeutic action when ETEC has already adhered to its specific receptors; however, it is effective as a preventive treatment.

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