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Running head: Yeast reduces ETEC effect in challenged pigs

Comparison of three patterns of feed supplementation with live *Saccharomyces cerevisiae* yeast on post-weaning diarrhea, health status and blood metabolic profile of susceptible weaning pigs orally challenged with *Escherichia coli* F4ac^{1,2}

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Abstract

The development of more 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 (*Sc*), supplied in different patterns to counteract ETEC infection in weaned pigs, was evaluated. Fifty pigs were weaned at 24 days and were then divided into five groups: control (CO), CO + colistin (AB), CO + 5×10^{10} CFU of *Sc*/kg feed, from d 0–21 (PR), CO + 5×10^{10} CFU of *Sc*/kg feed from d 7–11 (CM) and CO + one shot of 2×10^{11} CFU of *Sc* when the first diarrhea appeared (CU). On d 7 post-weaning, all the pigs were orally challenged with 10^8 CFU of ETEC. Blood samples were taken from the pigs (d 7, d 8, d 12, d 21) while the fecal excretion of ETEC was assessed on d 7 and d 10. Fecal consistency was scored from 12 h before infection to 144 h post-infection (p.i.). On d 21, the piglets 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 CO group. The CO group had a higher fecal score than AB during the entire period of observation (from $P = 0.01$ to $P < 0.001$). Conversely, yeast administration reduced the fecal score when compared to CO group 12 h and 48 h after infection ($P = 0.04$). Total IgA never differed among the experimental groups, but the ETEC-specific IgA concentration was lower in the AB group than in the CO group ($P = 0.04$) at d 12. Four days p.i., the subjects fed with live yeast had reduced ETEC excretion as compared with the CO group ($P = 0.05$). Blood metabolite concentrations of C12:1 ($P < 0.01$), C5DC (C6-OH) ($P = 0.02$), PC_aa_C40:1 and PC_aa_C40:6 ($P = 0.01$ and $P < 0.01$, respectively) and alpha-AAA ($P < 0.01$) were reduced in the AB group as

compared with the CO group; PR+CM reduced the concentration of SM_C18:0 ($P = 0.02$) and increased the concentration of C10:2 ($P = 0.02$), vs. CO. Furthermore, the CM group had an increased concentration of C10:2 ($P < 0.01$) as compared with the PR group. In conclusion, the administration of live yeast, even in concomitance with ETEC infections, reduces pig illness and mortality. Moreover, the strain of *Sc* tested did not show a therapeutic effect.

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 post-weaning 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 *Escherichia coli* F4 (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 counteract intestinal disorders in young mammals (Farthing et al., 2013; Shan et al., 2013). The administration of *Saccharomyces cerevisiae* (*S. 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 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 post-weaning 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 ± 2 days of age (d 0), the pigs were weaned and moved to the experimental farm, divided into five groups balanced for litter and body weight 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 were located above the piglets for the first 7 days. The piglets had free access to feed and water throughout the experimental period; feed was supplied in a dry feeder. On d 7 post-weaning, all the pigs were orally dosed with 1.5 mL suspension containing 10^8 CFU of 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, France) of *S. cerevisiae* CNCM I-4407 (Sc) mixed in the diet formula.

The piglets were assigned to one of five diets: control (CO, typical weaning diet – Table 1), CO + 1 g colistin/kg of feed (AB), CO + 5×10^{10} colony-forming units (CFU) of Sc/kg of feed, from d 0 to d 21 (PR, preventive dose), CO + 5×10^{10} CFU of Sc/kg of feed from d 7 (day of infection with ETEC) to d 11 (CM, competitive dose) and CO + 1 shot of 2×10^{11} CFU of Sc/kg of feed when the first diarrhea appeared (CU, curative dose). 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 days when they were kept in groups of two 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 body weight) 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 (pre-challenge), on d 14 and at sacrifice (d 21). The feed intake of each pig was recorded individually.

Blood was sampled on d 7 (pre-challenge), d 8, d 12 and on d 21 (day of sacrifice) by venipuncture of the vena cava, centrifuged at $3,000 \times g$ for 10 min at 4°C; the serum was

then removed. The serum samples collected on d 7, d 12 and d 21 were inactivated at 56°C for 30 min and stored at -20°C until analysis. On the other hand, the serum collected at d 8 was stored at -80°C after centrifugation. Individual fecal samples were obtained on d 7 (pre-challenge) 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 five 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 in order 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 carried out 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) (ABTS) (Roche Diagnostics, San Francisco, CA) for chromogenic detection. The concentration was expressed as micrograms per milliliter ($\mu\text{g mL}^{-1}$). The ETEC-specific IgA quantification was carried out 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^{-1} in ELISA-diluted buffer to coated wells with F4 fimbrial adhesin Mab (CVL, Addlestone, UK). Pooled serum obtained from five 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 (AU mg^{-1}) 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 μ L plasma, was performed using the AbsoluteIDQ p180 Kit, (BIOCRATES, Life Science AG, Innsbruck, Austria). Sample analyses were carried out on the API 4000 QTrap LC/MS/MS System (Applied Biosystems, Foster City, CA,). Measurements were carried out 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 general linear model (GLM) procedure of SAS (SAS Inst., Inc., Cary, NC) with a completely randomized design, two blocks (time), sows within block and five 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 pre-challenge 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 were carried out 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. In order 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 carried out on the corresponding linear mixed model. The analysis was focussed 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) in order 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 one 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.

Results

Growth performances

No difference in growth performance was observed among the experimental groups. The average daily gain (ADG) was 72.0, 71.0, 63.4, 86.1 and 95.2 g (SEM = 17.4), from d 0 to d 7 (before the challenge), and 197, 188, 210, 204 and 202 g (SEM = 73.2), from d 7 to d 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 one 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 h 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 fecal score as compared with the CO diet 12 h 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 post-challenge ($P = 0.08$). 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, four days 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, 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 alpha-amino adipic acid (alpha-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 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 three different patterns of *S. cerevisiae* CNCM I-4407 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 experimental design, the absence of differences for growth parameters is not surprising. An experiment on a larger scale is necessary to evaluate 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 one 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 three feeding strategies studied in the trial supplying *S. cerevisiae* CNCM I-4407 in the feed, the preventive method was the classic method of supplying probiotics to livestock feed in order 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) in order 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 upon ETEC stimulation was observed (Badia et al., 2012). Furthermore, a

270 continuous supply of *S. cerevisiae* CNCM I-4407 to the sows from late gestation and to the
271 piglets, before and after weaning, reduced the severity and duration of diarrhea upon ETEC
272 challenge (Trckova et al., 2014).

273 In the present trial, 70% of untreated piglets died after infection with ETEC while *S.*
274 *cerevisiae* CNCM I-4407 halved pig mortality when administered in a preventive way.

275 Similarly, Collier et al. (2011) reported that *S. cerevisiae* var. *boulardii* reduced the
276 mortality of LPS-challenged pigs by 20%. Furthermore, an examination of the time course
277 of pig survival reveals that, when yeast is supplied after weaning, a reduction in diarrhea
278 severity is associated with delayed mortality. From a practical point of view, this fact
279 implies a delay in the appearance of pig cachexia, and more time for eventual therapeutic
280 intervention. The protective effect in the PR group could also be ascribed to the ability of *S.*
281 *cerevisiae* CNCM I-4407 to modulate the immune response in the gut mucosa, as reported
282 by *in vitro* tests (Zanello et al., 2011a,b).

283 Currently, precision feeding is a new targeted technique for modern livestock production in
284 order to reduce the environmental footprint and improve growth efficiency; feed additives
285 should also be utilized in a similar manner, to be supplied ideally only when it is necessary.

286 For this reason, the competitive and curative uses of a probiotic product in piglet feeding
287 were tested. To our knowledge, this is the first trial aimed at studying pigs exposed to an
288 ETEC challenge and the ability of *S. cerevisiae* CNCM I-4407 to compete with the
289 pathogen. Furthermore, focusing on the potential therapeutic properties of *S. cerevisiae*
290 CNCM I-4407 when diarrhea was already present was really challenging and innovative.

291 The *S. cerevisiae* CNCM I-4407 used in the diet of the present trials was lyophilized. The
292 pig survival curve of the CM group, which shows an effect comparable to that of the PR
293 group, may be explained by the sudden activation of the yeasts in the gastrointestinal tract
294 (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), *S. cerevisiae* CNCM I-4407 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 four days 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., 2011b).

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 *S. cerevisiae* CNCM I-4407 was one shot, four 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

320 clinical benefit of *S. cerevisiae* boulardii therapy in infants and children with acute
321 gastroenteritis, with a shortened duration of diarrhea; nevertheless, the same authors
322 indicated some methodological limitations in the study. We observed only a slight delay in
323 the time course of mortality in comparison to untreated animals; the number of dead piglets
324 did not differ between the CU and CO groups. As a confirmation of the **general** effect of *S.*
325 *cerevisiae* CNCM I-4407 against ETEC, there is a global lowering effect of the yeast
326 treatments on the specific IgA against ETEC, even if the **greatest** effect was **attributable** to
327 the PR group. **This fact could indirectly indicate the ability of the yeast to reduce the**
328 **antigenic presence in the gut, reducing the antigen exposure and the specific immune**
329 **response.**

330 **In the present study, the** blood plasma metabolic profile was considered to support the
331 clinical evidence and to reveal the metabolic effects resulting from the interaction among
332 ETEC, yeast and the host. In pigs, abrupt modifications in the microbial population in the
333 GIT can occur after weaning with a negative impact on the mucosal homeostasis and
334 consequently on the blood metabolic profile (Wikoff et al., 2009; Campbell et al., 2013). In
335 this study, a sudden impact of ETEC infection was observed on some bioactive metabolites
336 involved in cell signals and in the activation of immune pathways. **In the CO group, two**
337 **phosphatidylcholine diacyls (C40:1, and C40:6) and 2-Aminoadipic acid were**
338 **upregulated. Phosphatidylcholine is by far the most abundant phospholipid component in**
339 **plasma and is largely found in diacylated form (Flögel et al., 2013). Lipopolysaccharide, a**
340 **bioactive component of the cell wall of gram-negative bacteria, stimulates**
341 **phosphatidylcholine breakdown in macrophages (Grove et al., 1990). T cells, by means of**
342 **acyltransferases, and phospholipases, manipulate phospholipid composition upon**
343 **stimulation (Robichaud et al., 2013). No specific reference to the two diacyl compounds**
344 **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 pro-inflammatory 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 control 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 *S. cerevisiae* CNCM I-4407 in a competitive way.

In summary, our results demonstrated the effectiveness of *S. cerevisiae* CNCM I-4407 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 but it is, however, effective as preventive treatment.

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499

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

| Ingredient | % | Calculated composition ¹ | % or otherwise |
|--|------|-------------------------------------|----------------|
| Wheat shorts | 20 | CP | 18.13 |
| Corn | 17 | Crude fat | 6.01 |
| Barley | 15 | Total Lys | 1.28 |
| Barley, extruded | 15 | Total Thr | 0.87 |
| Soybean meal, 50 | 13.4 | Total Met | 0.50 |
| Whey, dehydrated, skimmed | 6 | Total Met+Cys | 0.81 |
| Potato, protein concentrate | 4 | Total Trp | 0.28 |
| Vegetable oil | 4 | DE, growing pig, kcal/kg | 3355 |
| Beet pulp, dehydrated | 2 | NE, growing pig kcal/kg | 2424 |
| 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 | | |

501 ¹Values were estimated by the EvaPig® database (Noblet et al., 2008); ² Provided per
502 kilogram of diet: vitamin A, 9000 IU; vitamin D₃, 1500 IU; vitamin K₃, 2 mg; vitamin E, 50
503 mg; vitamin B₁, 2 mg; vitamin B₂, 4 mg; vitamin B₆, 4 mg; vitamin B₁₂, 0.04 mg; niacin, 55
504 mg; biotin, 0.15 mg; d-pantothenic acid, 30 mg; folacin, 2 mg; choline chloride, 400 mg; iron
505 as FeSO₄, 150 mg; zinc as ZnSO₄, 110 mg; copper as CuSO₄, 25 mg; manganese as MnSO₄,

506 70 mg; iodine as KI, 1 mg; selenium as Na_2SeO_4 , 0.3 mg.

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

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

509 ¹ **CO:** no live yeast + F4 challenge; **AB:** antibiotic + F4 challenge; **PR:** Preventive
 510 administration pattern of live yeast (5×10^{10} CFU/kg of feed from d 0 to d 21) + F4
 511 challenge; **CM:** Competitive administration of live yeast (5×10^{10} CFU/kg of feed from d 7
 512 to d 11) + F4 challenge; **CU:** Curative administration of live yeast (1 shot of 2×10^{11} CFU
 513 when the first diarrhea appears) + F4 challenge.

515 **Table 3.** Effect of dietary supplementation with *Saccharomyces cerevisiae* CNCM I-4407 with different patterns on the fecal score of weaned
516 pigs challenged with 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 ⁴ | 2.4 | 2.0 | 2.1 | 1.9 | 1.7 | 0.2 | 0.89 | 0.77 ² | - | - | - |
| 12 | 2.9 | 2.2 | 2.5 | 2.3 | 2.3 | 0.2 | 0.02 | 0.04 ³ | 0.51 | - | - |
| 24 | 3.7 | 2.4 | 3.0 | 3.2 | 2.8 | 0.3 | 0.001 | 0.08 ³ | 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 |

517 ¹ **CO:** no live yeast + F4 challenge; **AB:** antibiotic + F4 challenge; **PR:** Preventive administration pattern of live yeast (5×10^{10} CFU/kg of feed
518 from d 0 to d 21) + F4 challenge; **CM:** Competitive administration of live yeast (5×10^{10} CFU/kg of feed from d 7 to d 11) + F4 challenge; **CU:**
519 Curative administration of live yeast (1 shot of 2×10^{11} CFU when the first diarrhea appears) + F4 challenge; ² CM and CU were combined with

520 CO because the pigs had not yet been given yeast;³ YEAST includes PR and CM only while CU was not considered in the contrast;⁴ Contrast
521 before the challenge.

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

| Diet ¹ | | | | | | PR | vs. | PR | AB | YEAST | PR | CM |
|---------------------------------|------|------|------|------|------|--------------------|------|------|-----------------|--------------------|-----------------|------|
| | | | | | | CM+ | | vs. | vs. | vs. | vs. | vs. |
| | SME | | | | | CU+CO ³ | AB | CO | CO ⁴ | CM+CU ⁴ | CU ⁴ | |
| | | | | | | | | | | | | |
| CO | AB | PR | CM | CU | | | | | | | | |
| Total IgA (mg/L) | | | | | | | | | | | | |
| d7 ² | 400 | 391 | 439 | 344 | 371 | 51 | 0.25 | 0.70 | 0.90 | - | - | - |
| d12 | 801 | 717 | 666 | 1045 | 711 | 102 | - | - | 0.61 | 0.96 | 0.28 | 0.11 |
| d21 | 1090 | 1312 | 1203 | 1298 | 1269 | 227 | - | - | 0.53 | 0.60 | 0.76 | 0.93 |
| Specific IgA against ETEC (UI) | | | | | | | | | | | | |
| d7 ² | 0.5 | 0.21 | 0.18 | 0.14 | 0.33 | 0.12 | 0.13 | 0.56 | 0.12 | - | - | - |
| d12 | 88.3 | 13.2 | 11.7 | 26.3 | 66.1 | 21.4 | - | - | 0.04 | 0.10 | 0.16 | 0.26 |
| d21 | 182 | 45 | 340 | 228 | 210 | 114 | - | - | 0.23 | 0.97 | 0.93 | 0.98 |
| ETEC fecal counts (log10 CFU/g) | | | | | | | | | | | | |

| | | | | | | | | | | | | |
|------------------|-----|-----|-----|-----|-----|-----|---|---|------|------|------|------|
| d10 ⁵ | 8.9 | 8.4 | 7.3 | 8.2 | 7.7 | 0.5 | - | - | 0.52 | 0.05 | 0.45 | 0.54 |
|------------------|-----|-----|-----|-----|-----|-----|---|---|------|------|------|------|

525

526 ¹ **CO**: no live yeast + F4 challenge; **AB**: antibiotic + F4 challenge; **PR**: Preventive administration pattern of live yeast (5×10^{10} CFU/kg of feed
527 from d 0 to d 21) + F4 challenge; **CM**: Competitive administration of live yeast (5×10^{10} CFU/kg of feed from d 7 to d 11) + F4 challenge; **CU**:
528 Curative administration of live yeast (1 shot of 2×10^{11} CFU when the first diarrhea appears) + F4 challenge; ² Contrast before the challenge; ³
529 CM and CU were combined with CO because the yeast had not yet been given to the pigs; ⁴ Contrast after the challenge; ⁵ Four days post-
530 challenge.

Table 5. Effect of *Saccharomyces cerevisiae* CNCM I-4407 on blood metabolic profile metabolites 24 h after infection with 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 ^{6,9} | <0.01 | CO ↑ |
| CM+PR vs. CO | | |
| SM_C18:0 ^{7,9} | 0.02 | CM+PR ↓ |
| C10:2 ⁸ | 0.02 | CM+PR ↑ |
| CM vs. PR | | |
| C10:2 ⁸ | <0.01 | CM ↑ |

¹ **CO:** no live yeast + F4 challenge; **AB:** antibiotic + F4 challenge; **PR:** Preventive

administration pattern of live yeast (5×10^{10} CFU/kg of feed from day 0 to day 21) + F4

challenge; **CM:** Competitive administration of live yeast (5×10^{10} CFU/kg of feed from d7

to d11) + F4 challenge; ²Dodecenoyl-L-carnitine; ³Glutaryl-L-carnitine / Hydroxyhexanoyl-

L-carnitine; ⁴Phosphatidylcholine diacyl C 40:1; ⁵Phosphatidylcholine diacyl C 40:6; ⁶alpha-

amino adipic acid; ⁷Shingomyeline-Ceramide; ⁸Decadienyl-L-carnitine; ⁹Affected by the

confounding factor “fecal score”

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 ETEC (····· **CO**: no live yeast + F4 challenge; — — **AB**: antibiotic + F4 challenge; —·—· **PR**: Preventive administration pattern of live yeast (5×10^{10} CFU/kg of feed from d 0 to d 21) + F4 challenge; — — — **CM**: Competitive administration of live yeast (5×10^{10} CFU/kg of feed from d 7 to d 11) + F4 challenge; ——— **CU**: Curative administration of live yeast (1 shot of 2×10^{11} CFU when the first diarrhea appears) + F4 challenge).

