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 × *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 (μg 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.

147 **Metabolic profile of blood serum**

148 A targeted metabolic technique, designed to quantify the concentration of 188 endogenous
149 metabolites from 5 different compound classes taken from 10 µL plasma, was performed
150 using the AbsoluteIDQ p180 Kit, (BIOCRATES, Life Science AG, Innsbruck, Austria).
151 Sample analyses were carried out on the API 4000 QTrap LC/MS/MS System (Applied
152 Biosystems, Foster City, CA,). Measurements were carried out on the same plate and
153 analyzed by MetIQ software packages, which are an integral part of the AbsoluteIDQ Kit.
154
155 **Statistical analysis**
156 Performance data were analyzed by ANOVA using the general linear model (GLM)
157 procedure of SAS (SAS Inst., Inc., Cary, NC) with a completely randomized design, two
158 blocks (time), sows within block and five dietary treatments. Degrees of freedom for the
159 dietary treatments were used to test the following orthogonal contrasts: CO *vs*. YEAST (PR,
160 CM, CU), PR *vs*. (CM and CU), CM *vs*. CU and CO *vs*. AB. However, for pre-challenge
161 observations, the CM and CU groups received the same diet and, thus, the contrasts were PR
162 *vs*. (CM+CU+CO), PR *vs*. AB and AB *vs*. CO.
163 $P < 0.05$ was statistically significant and $0.05 < P < 0.10$ was considered a trend .
164 For mortality data, Fisher's exact test were carried out comparing CO with each of the other
165 dietary treatments.
166 The metabolomic data were analyzed using linear mixed models (Pinheiro and Bates, 2009),
167 taking the concentration of a given metabolite as a dependent variable and including a
168 random effect for litter. Body weight at d 7 and fecal score were considered to be possible
169 confounding factors, the latter taken after centering with respect to the diet-specific mean
170 fecal score. In order to establish which of these factors should be included in the model for
171 each metabolite, a backward elimination procedure, based on bootstrap testing (Davison and

172 Hinkley, 1997) was carried out on the corresponding linear mixed model. The analysis was
173 focussed on diets AB, CO, CM and PR, and examined the following contrasts: AB *vs*. CO,
174 CM + PR *vs*. CO and CM *vs*. PR. For each null hypothesis, a Leave-One-Out (LOO)
175 procedure was implemented (Hastie et al., 2009) in order to account for the possible
176 presence of influential observations (Cook and Weisberg, 1982). The applied procedure
177 consisted of testing the given null hypothesis on 38 different datasets, each one obtained
178 after excluding one animal at a time; finally, the rejection of the null hypothesis was deemed
179 to be "most stable" when it occurred on each one of the 38 different LOO datasets.
180
181 **Results**
182 **Growth performances**
183 No difference in growth performance was observed among the experimental groups. The
184 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
185 7 (before the challenge), and 197, 188, 210, 204 and 202 g (SEM = 73.2), from d 7 to d 21,
186 for groups CO, AB, PR, CM and CU, respectively.
187
188 **Severity of diarrhea and mortality**
189 The *in vitro* tests confirmed the presence of specific receptors for ETEC on the intestinal
190 villi of all pigs.
191 Table 2 lists the number of pig deaths during the trial for each group. Mortality in the
192 CO group was significantly higher than in the AB group ($P < 0.01$) and a trend of reduction
193 was seen also for PR ($P = 0.089$). Figure 1 shows the time course of pig survival during the
194 trial. Twenty-four hours after infection (d 8), the first pig died in the CO group; in the PR
195 and CM groups, the first pig died on d 10. In the AB group, only one pig died on d 11.

196 Finally, in the CU group, even if the pigs started to die on d 10 as in the other yeast-treated
197 groups, the survival curve decreased faster than in the PR and CM groups.
198
199 **Fecal scores**
200 Table 3 shows the effect of the dietary supplementation with live yeast on the fecal scores of
201 weaned pigs challenged with ETEC at different times and doses. Before the challenge, the
202 maximum fecal score was 2.4, indicating that no diarrhea occurred and no differences
203 emerged among the groups. From 12 h to 144 h after infection, the CO group showed a
204 higher fecal score than the AB group (from $P = 0.01$ to $P < 0.001$). Conversely, the
205 administration of yeast significantly reduced the fecal score as compared with the CO diet
206 12 h and 48 h after infection ($P = 0.04$ and $P = 0.04$, respectively), and a tendency to reduce
207 this parameter against the same groups was seen 24 h post-challenge ($P = 0.08$). Moreover,
208 during the entire period of observation, no significant differences were observed among the
209 groups supplied with live yeast, even if 96 h after the infection, the PR group tended to
210 reduce the fecal score as compared with the CM and CU groups ($P = 0.08$).
211
212 **Immune response and *Escherichia coli* F4ac shedding in feces**
213 Table 4 shows the data related to the IgA concentration in the blood serum and to the fecal
214 excretion of ETEC. The total IgA never differed among the experimental groups at any of
215 the time points considered. Moreover, before the challenge, no difference was observed in
216 ETEC-specific IgA concentration among the experimental groups. At d 12, the ETEC-
217 specific IgA concentration was lower in the AB group than in the CO group ($P = 0.04$), and
218 the administration of live yeast tended to reduce the specific IgA concentration as compared
219 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

245 competitive and the curative; a group treated with the antibiotic colistin, frequently used
246 against Gram-negative enterobacteria, was also included as a positive reference.
247 Due to experimental design, the absence of differences for growth parameters is not
248 surprising. An experiment on a larger scale is necessary to evaluate growth performance
249 differences in susceptible challenged pigs fed live yeast.
250 However, in experimental challenge trials with ETEC, health parameters provided relevant
251 indications regarding the entire effect of testing feeding practices; of these, mortality was an
252 important parameter to be evaluated (Fairbrother et al., 2005). Moreover, a proper
253 evaluation of the sensitivity of the animals used in the trials is a prerequisite for avoiding
254 false negative responses. In the present study, specific receptors for ETEC on the intestinal
255 villi were present in all the piglets, strengthening the relevancy of the experimental results.
256 Furthermore, the ETEC strain used to infect the piglets was proven to be sensitive to the
257 antibiotic used here as a positive control. The low mortality rate of the pigs, the low
258 concentration of specific IgA against ETEC in the blood serum and the lowest diarrhea
259 score compared with the CO group confirm the effectiveness of the antibiotic. Only one pig
260 in the AB group died as a result of diarrhea immediately after weaning as a consequence of
261 the reduction in feed intake and the subsequent reduction in antibiotic ingestion. Between
262 the three feeding strategies studied in the trial supplying *S. cerevisiae* CNCM I-4407 in the
263 feed, the preventive method was the classic method of supplying probiotics to livestock feed
264 in order to protect animals against the risk of pathogenic infection. In the literature, there is
265 evidence of the preventive effect of *S. cerevisiae* spp. supplied in weaned pigs challenged
266 with lipopolysaccharide (LPS) from *E. coli* (Collier et al., 2011) in order to reduce the
267 inflammatory response and mortality in pigs. Moreover, a protective effect of *S. cerevisiae*
268 on porcine epithelial cell lines reducing the increased expression of genes related to
269 inflammation upon ETEC stimulation was observed (Badia et al., 2012). Furthermore, a

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

In the present trial, 70% of untreated piglets died after infection with ETEC while *S. cerevisiae* CNCM I-4407 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 *S. cerevisiae* CNCM I-4407 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 in order 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 *S. cerevisiae* CNCM I-4407 to compete with the pathogen. Furthermore, focusing on the potential therapeutic properties of *S. cerevisiae* CNCM I-4407 when diarrhea was already present was really challenging and innovative. The *S. cerevisiae* CNCM I-4407 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

295 intestinal tract, fermenting the sugar derived from the digestive process or provided by the
296 diet (Etienne-Mesmin et al., 2011). The ethanol concentration in the gut was not quantified
297 in this study. However, on the basis of the data of Bode et al. (1984), *S. cerevisiae* CNCM I-
298 4407 should be able to produce ethanol in the stomach by means of the fermentation of the
299 sugar provided by the milk-derived product supplied with the feed formula. This, in turn,
300 could have reduced the quantity of viable ETEC available to adhere to the intestinal
301 receptors and/or the gut sensitivity to the bacterial toxins, as demonstrated in macrophages
302 *in vitro* or in the liver of mice challenged with *E. coli* lipopolysaccharide (Nishiyama et al.,
303 2002). Moreover, the continuous supply of live yeast for an additional four days in the CM
304 group may have been responsible for containing the inflammation of the intestinal mucosa,
305 thereby reducing the consequences of the ETEC challenge (Zanello et al., 2011b).
306 Other studies in the scientific literature targeted to human gut health and therapy against
307 diarrhea suggest a curative approach using probiotics. In clinical trials on children,
308 *Lactobacillus rhamnosus* GG seems to shorten the duration of acute diarrhea (Shornikova et
309 al., 1997; Guandalini et al., 2000). On the other hand, *Saccharomyces* spp. are considered to
310 be broad-spectrum probiotics because they are not commonly found on or adherent to the
311 mucosa of the GIT in mammals (Blehaut et al., 1989). Thus, an interspecific effect is
312 conceivable, as suggested by the positive results obtained with the same yeast strain in
313 human and animal models (McFarland, 2010; Kurugöl and Koturoğlu, 2005). Our
314 therapeutic dose of *S. cerevisiae* CNCM I-4407 was one shot, four times more concentrated
315 than the dose used in the PR and CM groups, but the resulting health data did not show any
316 reduction in the detrimental effects of ETEC infection. This suggests that, when ETEC has
317 already exerted its pathogenicity adhering to the mucosa and producing its toxins, yeast is
318 not capable of interfering with the pathogenic mechanisms of ETEC. This finding partially
319 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 *S. cerevisiae* CNCM I-4407 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, two 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 (Flögel 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 upon stimulation (Robichaud et al., 2013). No specific reference to the two 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 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

369 action when ETEC has already adhered to its specific receptors but it is, however, effective
370 as preventive treatment.
371
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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 [1]Values were estimated by the EvaPig® database (Noblet et al., 2008); [2] Provided per
502 kilogram of diet: vitamin A, 9000 IU; vitamin D_3, 1500 IU; vitamin K_3, 2 mg; vitamin E, 50
503 mg; vitamin B_1, 2 mg; vitamin B_2, 4 mg; vitamin B_6, 4 mg; vitamin B_{12}, 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 $FeSO_4$, 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.

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	**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

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 x 10^{11} CFU

513 when the first diarrhea appears) + F4 challenge.

514

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

	Diet[1]										
Hours	**CO**	**AB**	**PR**	**CM**	**CU**	**SEM**	**AB *vs.* CO**	**YEAST *vs.* CO**	**PR *vs.* CM**	**PR *vs.* CM+CU**	**CM *vs.* 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

[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 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; [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]					SME	PR *vs.* CM+ CU+CO [3]	PR *vs.* AB	AB *vs.* CO	YEAST *vs.* CO [4]	PR *vs.* CM+CU [4]	CM *vs.* 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

[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 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; [2] Contrast before the challenge; [3] 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-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; [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]Shingomyeline-Ceramide; [8]Decadienyl-L-carnitine; [9]Affected 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).