

Antibiotic-free diet supplemented with live yeasts decreases inflammatory markers in the ileum of weaned piglets

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Abstract

Emerging bacterial resistance to antibiotics increases the need for effective alternatives to control intestinal inflammation and thus gut disorders in piglets. This study evaluated the effects of including *Saccharomyces cerevisiae* (strain 1026) and *Saccharomyces boulardii* (CNCM I-1079) as antibiotic alternatives in the starter diets of pigs on the concentrations of ileal inflammatory markers (nuclear factor- κ B (NF- κ B)), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF- α), and interleukin-12 subunit p40 (IL-12p40), and villus height. Forty piglets were assigned to four experimental diets, namely basal diet (C-), basal diet with antibiotics (C+), basal diet with *S. cerevisiae* (Sc), and basal diet with *S. boulardii* (Sb). At 7 and 14 days post weaning, five piglets per diet group were euthanized to quantify the inflammatory markers and to measure villus height. The C- group exhibited the highest concentration of inflammatory markers and the most atrophied villi. The Sc group had intermediate values for both variables. The C+ group had the lowest values for inflammatory markers and the highest villus height was similar to that of the Sb group, which showed low concentrations of inflammatory markers, although not so low as those of the C+ group. Both yeasts could be used as antibiotic alternatives to reduce the use of antibiotics in pig starter diets. However, *S. boulardii* CNCM I-1079 supplementation controls inflammation and preserves intestinal mucosa more effectively than *S. cerevisiae* strain 1026.

Keywords: cytokines, pigs, probiotic, *Saccharomyces*, weaning

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Introduction

Weaning is one of the most stressful events in a piglet's life and can contribute to intestinal and immune system dysfunction, resulting in intestinal bacterial proliferation (Dirkzwager *et al.*, 2005). This increase in bacteria can reduce the health, growth, and feed intake of pigs, particularly during the first week after weaning (Campbell *et al.*, 2013). Weaning of piglets is associated with an early and transient response in the gene expression of inflammatory cytokines in the gut (Pié *et al.*, 2004). The synthesis and release of cytokines are stimulated by bacteria, viruses, parasites, fungi, and numerous non-infectious stimulants such as allergens, transient anorexia, and other proinflammatory cytokines, including interleukin-1 (IL-1), tumour necrosis factor alpha (TNF- α), and interleukin-6 (IL-6), which induce the production of acute phase proteins (McCracken *et al.*, 1999; Naugler & Karin, 2013). Interleukin 12 subunit p40 (IL-12p40) increases during chronic inflammation (Trinchieri *et al.*, 2003; Abdi *et al.*, 2014; Zundler & Neurath, 2015).

Bacterial antigens in the intestinal lumen generate an acute inflammatory response through the action of proinflammatory cytokines, which activate a signalling cascade, leading to the release of nuclear factor- κ B (NF- κ B), which translocates to the nucleus and serves as a transcription factor for the synthesis of IL-6 and TNF- α (Lawrence, 2009; Fernández-Jiménez *et al.*, 2014). The proinflammatory cytokine TNF- α acts as an acute inflammation mediator that increases the production of IL-1 and IL-6 (Sánchez-Muñoz *et al.*, 2008). The inflammation mediated by IL-12p40 is activated through the Janus kinase/signal transducers and

activators of transcription (JAK/STAT), in which it is dimerised and phosphorylated, allowing its translocation to the nucleus where IL-12p40 is transcribed.

Early control of intestinal inflammation is important for the effective management of post-weaning gut disorders in piglets (Pié *et al.*, 2004). Traditionally, these disorders have been controlled with various methods including the administration of antibiotics. However, with the recent rise in bacterial resistance to antibiotics, several countries have restricted the use of antibiotics in animal feed. Recent research has focused on animal nutrition, aiming to find alternatives to antibiotics (Nyachoti *et al.*, 2006; Stein & Kil, 2006). One suggested alternative is the use of probiotics, which are defined by the World Health Organization (WHO) as live microorganisms, which, when administered in adequate amounts, confer health benefits to their host, and have potential to improve human health (WHO, 2001).

Probiotic yeasts act by interrupting the signalling pathways involved in the inflammatory response (Czerucka & Rampal, 2002; McFarland, 2010), thereby helping to restore intestinal balance and the health of the piglets. *Saccharomyces cerevisiae* and *Saccharomyces boulardii* are probiotic yeasts that affect their hosts differently, owing to their own morphophysiological differences (Hudson *et al.*, 2016). *Saccharomyces cerevisiae* has been used for many years as a probiotic in pigs owing to its effect on growth and reproductive performance and the reduction of morbidity and mortality, particularly in young animals (Pérez-Sotelo *et al.*, 2005). *Saccharomyces boulardii* is a nonpathogenic probiotic yeast that has been used for decades to protect against intestinal injury and inflammation (Chen *et al.*, 2013). Many studies have evaluated the effects of *S. cerevisiae* on the reduction of intestinal inflammation in pigs challenged with pathogens (e.g. Martins *et al.*, 2011; Trevisi *et al.*, 2016). *Saccharomyces boulardii* has also been evaluated in several studies, mostly conducted in humans and rodents (Dalmaso *et al.*, 2006; Sougioultzis *et al.*, 2006; Martins *et al.*, 2010).

The aim of the present study was to determine whether *S. boulardii* strain CNCM I-1079 and *S. cerevisiae* strain 1026 could be used as alternatives to antibiotics in the starter diets of piglets. Supplementation of these yeasts in starter diets for piglets was hypothesized to diminish the concentration of inflammatory markers (NF- κ B, IL-6, TNF- α , and IL-12p40) in ileal tissue and improve villus height. It was further hypothesized that *S. boulardii* strain CNCM I-1079 would have greater efficacy than *S. cerevisiae* strain 1026.

Material and Methods

All experimental procedures were reviewed and approved by the Bioethical Committee of the Faculty of Natural Sciences of the Autonomous University of Queretaro (approval number: 43FCN2016). Piglets were handled according to the guidelines of the Mexican Official Norm NOM-062-ZOO-1999 for production, care, and use of animals for experimentation (Diario Oficial de la Federación, 2001) and guidelines of the International Guiding Principles for Biomedical Research Involving Animals (CIOMS-ICLAS, 2012).

In this study the authors utilized forty hybrid piglets (Genetiporc, Fertilis 25 \times G Performance 8), weaned at 20 ± 1.6 days and weighing 6.5 ± 0.3 kg, and four experimental diets. The weaned piglets were assigned to one of the four diets according to litter origin and bodyweight, and were immediately housed in two pens per diet, each with five piglets, making a total of 10 piglets per diet. To prepare the four experimental diets, first the authors formulated a basal diet without antibiotics or live yeast (Table 1) or negative control (C-). Subsequently, the authors added 0.05% of antibiotics, 0.8 % of *S. cerevisiae*, and 0.1% of *S. boulardii* to the basal diet at the expense of maize to establish the three other diets, namely positive control (C+), Sc and Sb, respectively. The antibiotic that was used was LincoSpectin (lincomycin + spectinomycin 1:1) from Zoetis (Parsippany, NJ, USA); the *S. cerevisiae* was Yea-Sacc[®] strain 1026 from Alltech (Nicholasville, KY, USA); and the *S. boulardii* was Levucell SB[®], CNCM I-1079 from Lallemand Animal Nutrition (Montréal, Canada). The doses of yeast in diets were recommended by the manufacturer.

Pens were equipped with feeders and nipple drinkers in an environmentally controlled weaning room (30 °C and 28 ± 2 during the first and second post-weaning weeks, respectively). The piglets had free access to water and were fed thrice per day at 8h00, 12h00, and 16h00. Faecal consistency was monitored. Piglets were individually weighed at 0, 7, and 14 days post weaning (PWD), and the average daily gain (ADG) per postweaning week was calculated. Average daily gain and faecal consistency were considered indicators of pig health.

All piglets of one pen per diet were euthanized at 7 PWD, and the remaining five piglets per diet were euthanized at 14 PWD. Piglets were tranquilized with 20 mg/kg azaperone (Sural[®], Chinoin, Mexico City, Mexico) and then euthanized with an overdose of sodium pentobarbital (Pisabental[®], PiSA Agropecuaria, Hidalgo, Mexico). Ileal tissue sections (10 cm) were sampled from 5 cm before the ileocecal valve, gently washed with distilled water, and then dissected and fixed in 10% buffered formalin solution.

Ileal sections were embedded in paraffin, cut into 5- μ m slices, and stained with haematoxylin-eosin. To determine the average villus height (from apex–base) and crypt depth (from the villi base to the bottom of

the crypt) (Nabuurs *et al.*, 1993; Makkink *et al.*, 1994), 10 villi were examined under an optical microscope with a 10× objective (Primo Star, Carl Zeiss, Oberkochen, Germany) and analysed using ZEN software (Carl Zeiss, Jena, Germany).

The other 5-µm slices were used to quantify NF-κB, IL-6, TNF-α, and IL-12p40 using an immunofluorescence technique adapted from that of Rivera-Zavala *et al.* (2017). Ileal slices were deparaffinized for 2 hours at 60 °C in a dry-heat oven and then rehydrated in 100% xylol (10 min), 100% ethanol (5 min), 96% ethanol (5 min), 80% ethanol (5 min), and deionized water (10 min), in this order. Ileal slices were then soaked in saline-sodium citrate buffer concentrate 1× (Sigma-Aldrich, Saint Louis, MO, USA) in a water bath at 80 °C for 25 min. Then, ileal slices were blocked with 1% non-fat milk for 1 hour, washed three times for 5 min each with 0.05% TRIS-buffered saline plus Tween (TBST), and incubated for 2 hours at 4 °C with the following antibodies diluted in TBST: i) rabbit anti NKIRAS2 (MBS 631230) (Mybiosource, San Diego, CA, USA) at 1:50 dilution; ii) rabbit anti TNF-α (ab 6671) (Abcam, Cambridge, MA, USA) at 1:200 dilution; iii) rabbit anti IL-12p40 (ab 106270) (Abcam) at 1:100 dilution; and iv) incubated rabbit anti IL-6 (ab 6672) (Abcam) for 16 hours at 1:400 dilution.

Ileal slices were then washed three times for 5 min each with 0.05% TBST buffer and incubated for 2 hours with the secondary antibody Alexa Fluor 488 chicken anti-rabbit IgG (H+L) (A-21441) (Invitrogen Molecular Probes Inc., Eugene, OR, USA) at 1:500 dilution. The intensity of the fluorescence (arbitrary fluorescence units (AFU)) was evaluated with the positive (primary and secondary antibodies) and negative (secondary antibodies only) controls considering the same size of area in ileal tissue under both conditions (Rivera-Zavala *et al.*, 2017). For the analysis, 10 intestinal villi per piglet were visualized and photographed at 40× magnification (Olympus CX31, Olympus Instruments, Tokyo, Japan) and then 10 sections from each intestinal villi were selected per photo to obtain 100 measurements per piglet. Fluorescence was visualized and measured with a fluorescence microscope (Eclipse E600, Nikon, Minato, Japan) and Image-Pro Plus version 6.1 (Media Cybernetics, Silver Spring, MD, USA).

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

Ingredients	%	Chemical composition [∞]	
Maize	47.05	Dry matter, %	89.50
Soybean meal	15.00	Crude protein, %	21.80
Fish Menhaden meal	9.23	Neutral detergent fibre, %	12.30
Whey dried	24.69	Metabolizable energy, kcal/kg	3,300
Maize oil	1.49	Lysine, %	1.70
Lysine	0.25	Methionine, %	0.49
Aminogut ¹	0.80	Tryptophan, %	0.28
Threonine	0.10	Threonine, %	1.05
Tryptophan	0.05		
Dicalcium phosphate	0.90		
Vitamins ²	0.08		
Minerals ³	0.12		
Biocoline	0.04		
Sodium butyrate	0.20		

¹Aminogut: L-glutamine and L-glutamic acid (1:1), (Ajinomoto, Japan)

²Vitamins per kg of diet, A: 10,200 UI, D: 1980 UI, E: 60 UI, K: 1.20 mg, biocoline: 38 mg; niacin: 36 mg, pantothenate: 17 mg, riboflavin: 7.2 mg, B₁₂: 38 µg, thiamine: 0.3 mg, pyridoxine: 0.31 mg, biotin: 0.08 mg, folate: 0.75 mg

³Minerals per kg of diet, copper: 14.4 mg, iodine: 800 mg, iron: 105 mg, manganese: 36 mg, selenium: 0.3 mg, zinc: 144 mg

Average daily gain, villus height, crypt depth, and inflammatory marker concentrations were analysed using a completely randomized design, and each piglet was considered an experimental unit (Steel & Torrie, 1997). ANOVA was used to analyse the differences between diet groups (SAS, 1994). Means were compared with the Student-Newman-Keuls test using the GLM procedure of SAS, and differences were considered statistically significant at $P < 0.05$.

Results and Discussion

During the first week post weaning, no differences ($P >0.05$) were observed in ADG (Table 2). However, in the second post-weaning week, the highest ADG ($P <0.001$) was observed for the animals fed the C+ diet compared with the piglets fed the other diets. The ADG was similar between piglets fed the Sc and Sb diets, and the lowest for those in the C- group compared with the other groups ($P <0.001$) (Table 2).

Table 2 Mean (\pm SEM) average daily gain (g) of four starter diets with or without probiotics for piglets

	Experimental diets				P-value	SEM
	C+	Sc	Sb	C-		
ADG1	130	67	10	63	0.1834	18.58
ADG2	300 ^a	214 ^b	211 ^b	157 ^c	0.0001	7.34

ADG1 and ADG2: average daily gain in 1 and 2 post-weaning weeks; C+: diet with antibiotics; Sc; diet with *Saccharomyces cerevisiae*; Sb; diet with *Saccharomyces boulardii*; C-: diet without antibiotics or probiotics; P: statistical significance; ^{a,b,c} Row means with different superscripts differ significantly at $P <0.05$; SEM: standard error of mean

Faecal consistency indicated that all piglets had mild diarrhoea throughout the experimental period (14 days). However, no animals required medication, nor were any euthanized for health reasons.

At 7 and 14 PWD the longest villi were observed in piglets fed the Sb and C+ diets, while those fed the Sc diet had intermediate villus height. The shortest villi were observed in animals fed the C- diet ($P <0.001$) (Figure 1). Pronounced villous atrophy was observed in the piglets fed the C- diet because they were 55% and 57% (at 7 and 14 days post weaning, respectively) shorter than the villi of piglets fed the other diets (Figure 1). Regarding crypt depth at 7 and 14 PWD, no differences were observed ($P >0.05$) among diets. However, on day 7 after weaning, there was a tendency ($P <0.085$) for animals to have deeper crypts when fed the Sb diet (122 and 104 μm for Sb and other treatments, respectively).

The concentrations of NF- κ B, IL-6, TNF- α , and IL-12p40 in the ileal villi of weaned piglets were affected ($P <0.001$) by diet at 7 and 14 PWD (Figures 2–5). The piglets fed the C- diet had the highest concentrations of all inflammatory markers. Those fed the Sc diet showed intermediate concentrations, and the C+ and Sb groups had the lowest concentrations ($P <0.001$) (Figures 2 - 5). There was the highest AFU signal for IL-12p40 compared with the other inflammatory markers in piglets fed all experimental diets, but particularly in those fed the C- diet (Figure 5).

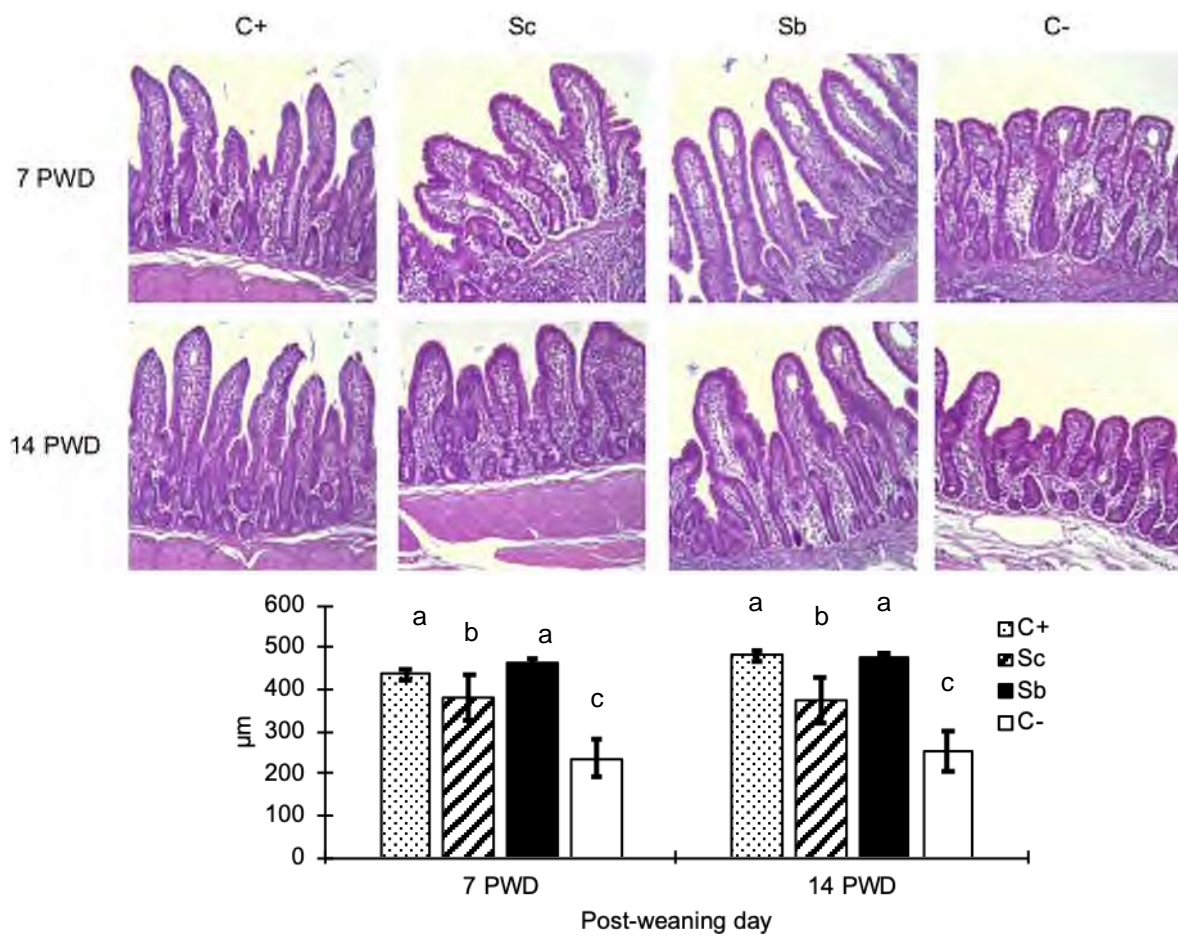


Figure 1 Haematoxylin-eosin staining of villi at 7 and 14 days post weaning (PWD).

Bars with different letters differ significantly at $P < 0.001$. Data are presented as mean \pm SD

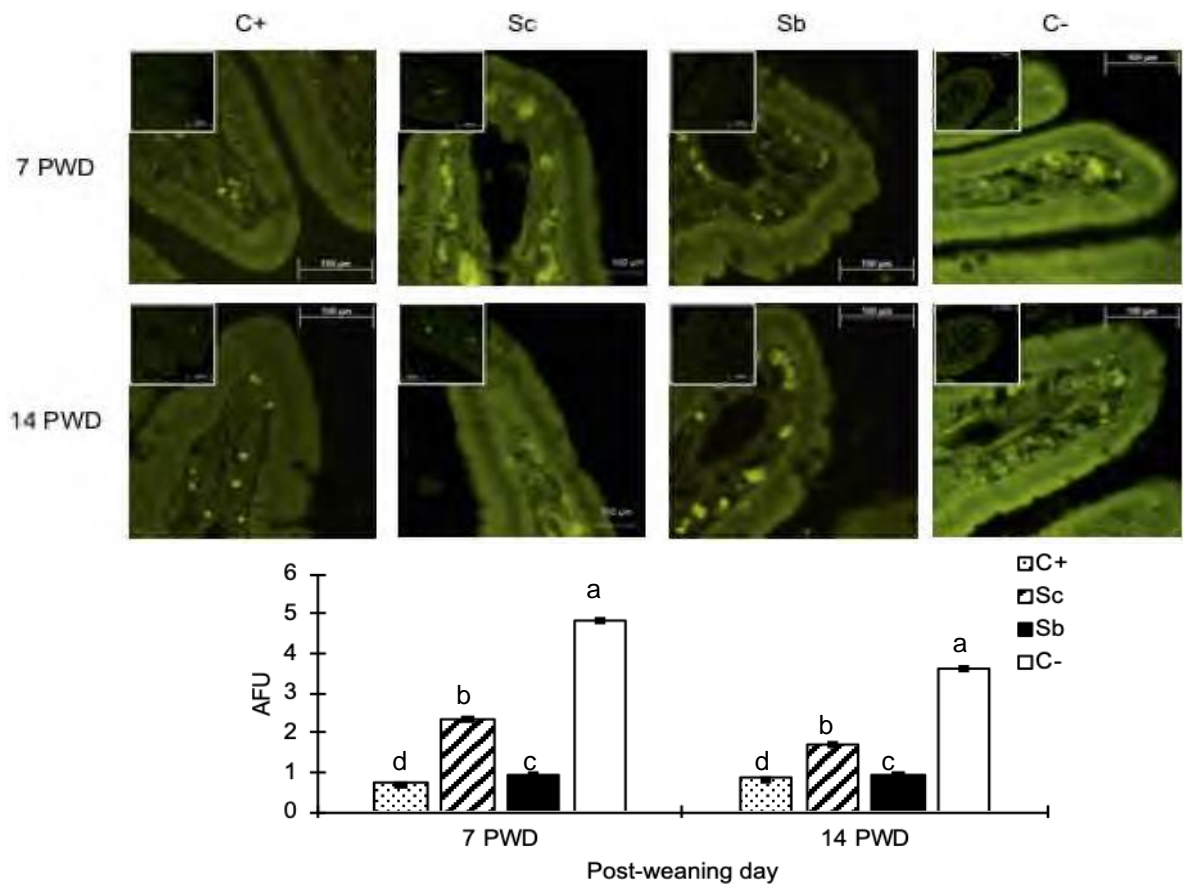


Figure 2 Concentration of NF- κ B in the ileum of piglets fed with different experimental diets highlighted by immunofluorescence staining

The inset boxes show representatives of the controls without the primary antibody. Bars with different letters differ significantly at $P < 0.001$. Data are presented as mean \pm SD

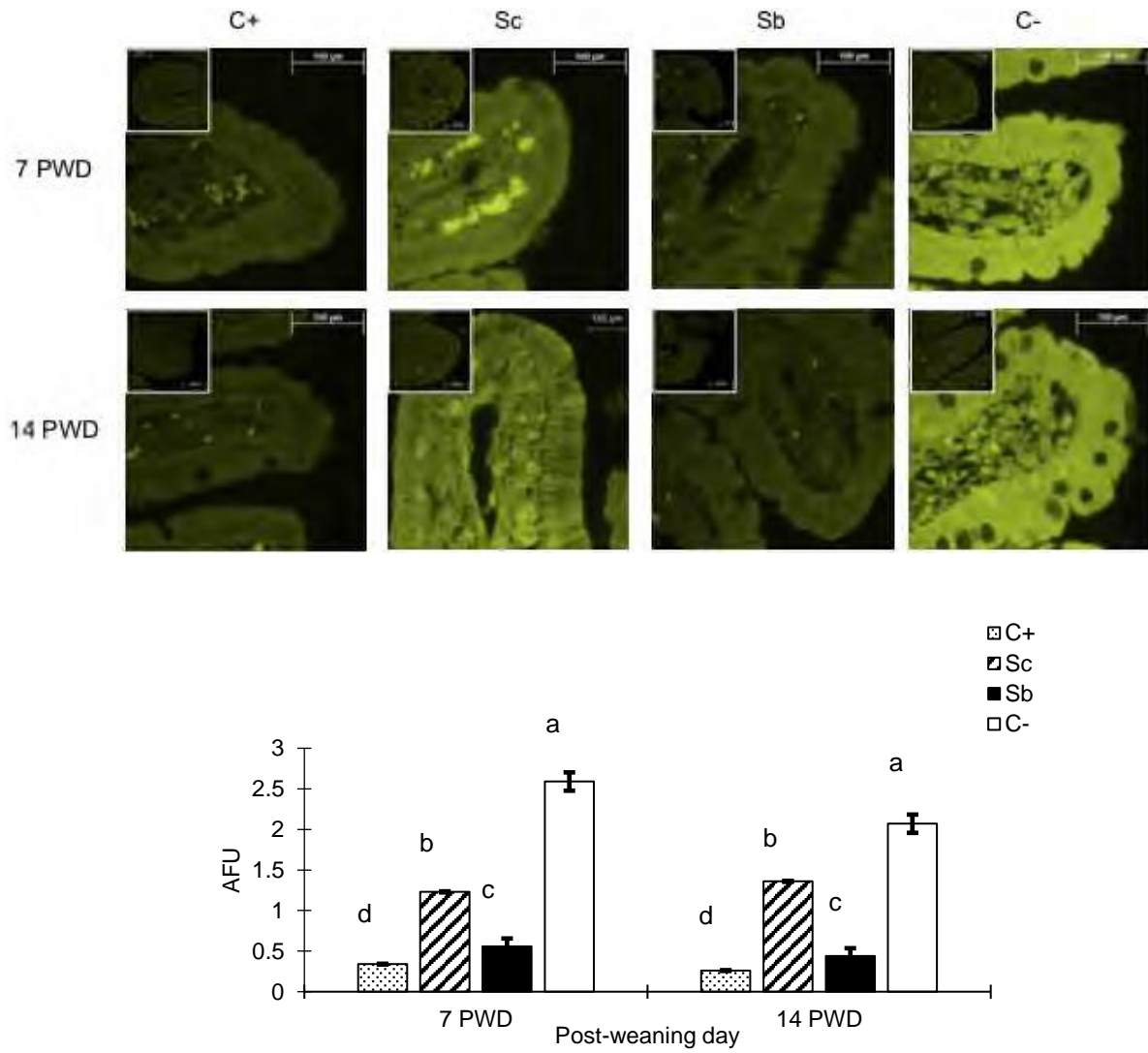


Figure 3 Concentration of IL-6 in the ileum of piglets fed different experimental diets highlighted by immunofluorescence staining

The inset boxes show representatives of the controls without the primary antibody. Bars with different letters differ significantly at $P < 0.001$. Data are presented as mean \pm SD

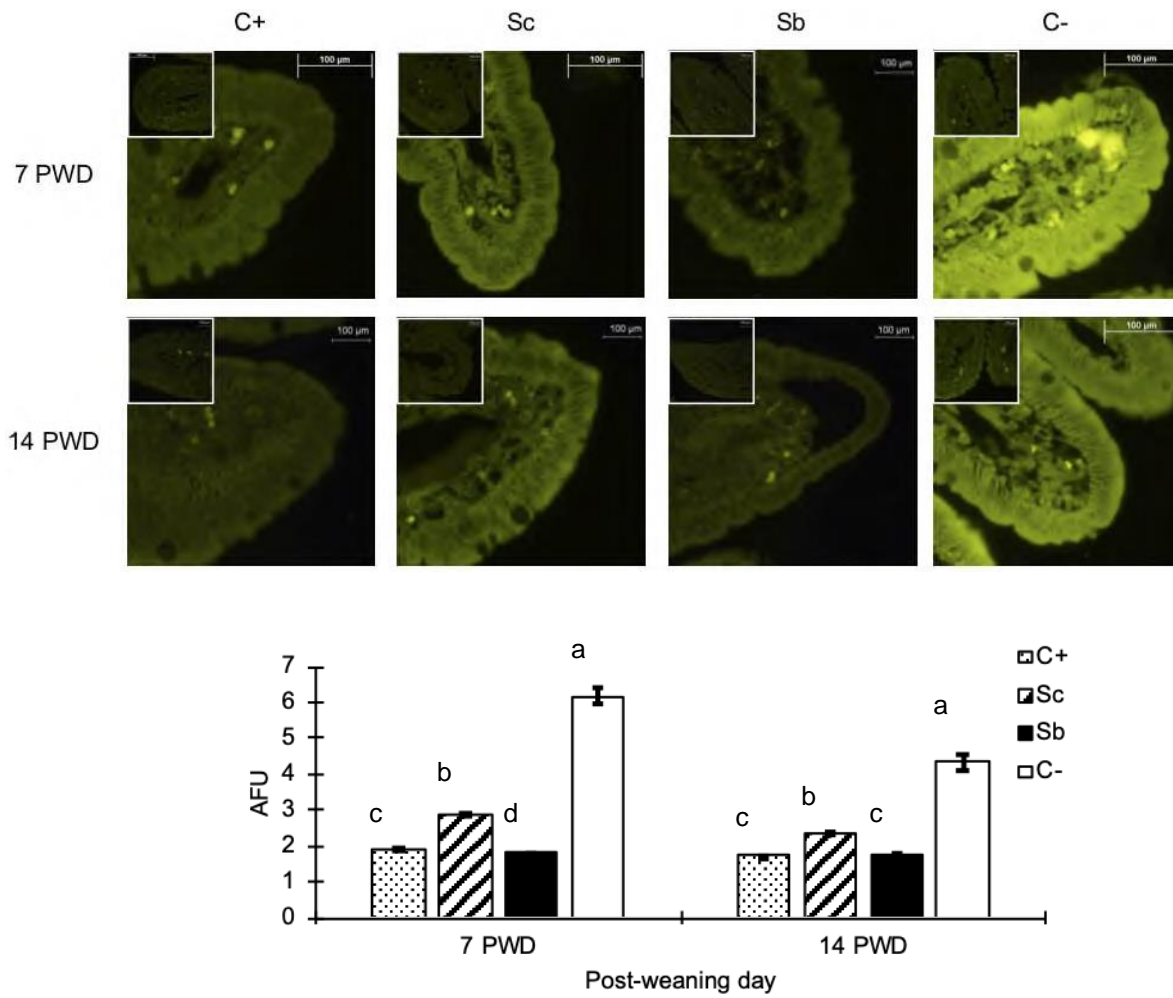


Figure 4 Concentration of TNF- α in the ileum of piglets fed different experimental diets highlighted by immunofluorescence staining

The inset boxes show representatives of the controls without the primary antibody. Bars with different letters differ significantly at $P < 0.001$. Data are presented as mean \pm SD

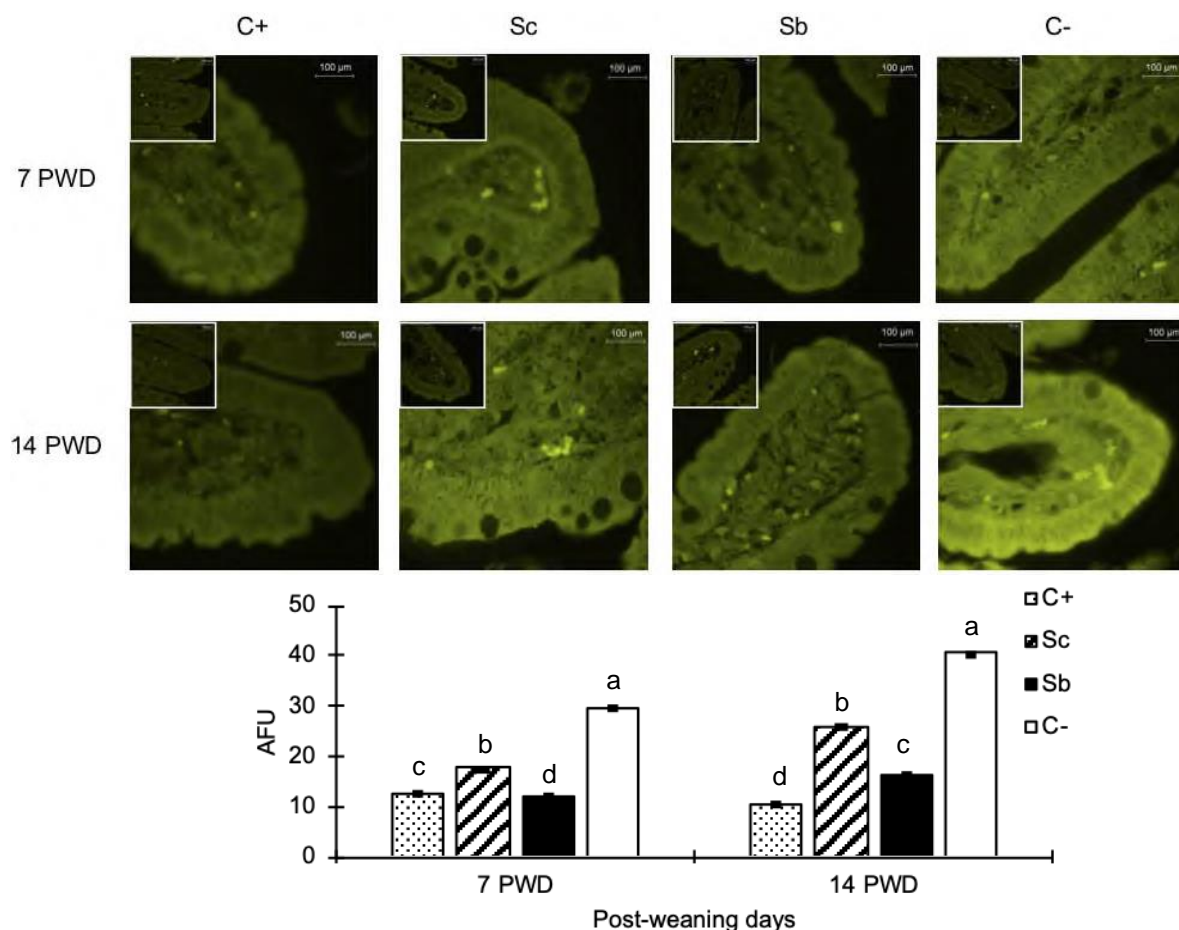


Figure 5 Concentration of IL-12p40 in the ileum of piglets fed different experimental diets highlighted by immunofluorescence staining

The inset boxes show representatives of the controls without the primary antibody. Bars with different letters differ significantly at $P < 0.001$. Data are presented as mean \pm SD

The low ADG observed in the first week after weaning can be explained by the stress of weaning, which provokes deleterious physiological and immunological changes and generalized poor development (Campbell *et al.*, 2013). In the second post-weaning week, the piglets in the C+, Sc, and Sb diet groups had an ADG within the parameters usually reported for piglets of the same age group on the experimental farm (Escobar *et al.*, 2015), while those in the C- diet group were below these parameters. Weight gain and faecal consistency indicated that the animals were in good health, in accordance with the post-weaning phase and the diet consumed. These issues were observed to be at their worst in animals fed antibiotic-free diets without yeast (C-). Moreover, the highest daily weight gain (Table 2) was observed during the second week post weaning week in piglets fed the diet with antibiotics (C+), which was probably owing to the growth promoter effect of the antibiotics (Cromwell, 2002; Gaskins *et al.*, 2002). However, in the present study, the yeasts did not show a similar effect to antibiotics to stimulate growth performance during the second week post weaning, and the animals in the Sc and Sb groups demonstrated good levels of recovery in this week. However, further studies are required with a greater number of animals to elucidate fully the effect of the yeast, especially *S. boulardii*, on piglet performance.

The findings of the present study corroborate these of previous observations (Czerucka & Rampal, 2002; Buts, 2009; McFarland, 2010; Palma *et al.*, 2015), in that the highest concentrations of inflammatory markers were observed in animals fed diets without antibiotics or yeasts (C-). This inflammatory process affected villus height because the animals fed the C- diet had the shortest villi at 7 and 14 PWD, as reported

by Pié *et al.* (2004) and Nyachoti *et al.* (2006). These authors found that weaning coupled with diets without antibiotics or probiotics caused intestinal dysbiosis that changed the intestinal environment and led to an inflammatory response that caused cell death, resulting in atrophied villi and an increase in apoptosis and a decrease in the replacement of enterocytes within the crypts (Jiang *et al.*, 2015).

Previous work had suggested that the results from the immunological markers in the ileum confirmed that cytokines play a central role in cell immunity and participate in maintaining tissue integrity (Pié *et al.*, 2004). Changes in the concentrations of intestinal cytokines were expected for weaning piglets because the abrupt modifications in dietary components and stress factors experienced during this stage lead to important morphological and functional adaptations in the intestine (McCracken *et al.*, 1999; Pié *et al.*, 2004). The imbalance caused by changes in the intestinal mucosa induces an inflammatory process that activates NF- κ B and subsequent production of proinflammatory cytokines (Czerucka & Rampal, 2002; Buts, 2009; McFarland, 2010; Palma *et al.*, 2015), and villous atrophy occurs (Stein & Kil, 2006; Lallès *et al.*, 2007). These responses are accentuated when newly weaned animals are fed antibiotic-free diets (Jiang *et al.*, 2015).

Dietary supplementation of antibiotics or yeasts (C+, Sc, and Sb) modulated the production of inflammatory markers, which were maintained at low concentrations (Liu *et al.*, 2018), and increased villus height. The downregulation of cytokines by antibiotics was confirmed by the current results, since all measured markers decreased in piglets fed the C+ diet. The suppressant effect of antibiotics on the ileal bacterial population (Cromwell, 2002; Gaskins *et al.*, 2002) probably inhibited the signalling pathway that induces the release of NF- κ B, which was slightly marked by the primary antibody used in the current study for immunofluorescence staining (Figure 2). IL-6 concentration also decreased because the activation of NF- κ B is required for its synthesis (Saavedra *et al.*, 2011). Furthermore, the antibiotics in the C+ diet decreased IL-12p40 synthesis, and thus concentration, probably owing to the reduced stimulus generated by bacterial pathogens and to the inhibition of the pathway mediated by JAK/STAT (Trinchieri *et al.*, 2003). Therefore, dietary antibiotic supplementation regulates inflammatory response and indirectly protects the intestinal mucosa, decreasing the detrimental effects of weaning on the intestinal villi (Stein & Kil, 2006; Thacker, 2013). This finding was further evidenced by the longer villi observed in piglets fed the C+ diet, avoiding villous atrophy.

The control of inflammatory markers in ileal mucosa owing to *S. cerevisiae* strain 1026 was expected because *S. cerevisiae*-supplemented diets modulate signalling pathways, preventing NF- κ B translocation to the nucleus and inhibiting the expression of genes involved in IL-6 and TNF- α synthesis (Martins *et al.*, 2011; Zanello *et al.*, 2011). Regarding IL-12p40, *S. cerevisiae* probably inhibits both NF- κ B and the JAK/STAT complex, preventing IL-12p40 synthesis, and thereby reducing inflammation. The regulation of inflammation resulting from the action of *S. cerevisiae* strain 1026 in the ileum was sufficient to avoid considerable villous atrophy at 7 and 14 PWD. The presence of *S. cerevisiae* strain 1026 in the intestine likely promoted competitive bacterial exclusion, short-chain fatty acid production, and growth factor synthesis leading to environmental conditions that are less damaging to the intestinal mucosa, limiting cell loss, and favouring villous and mucosal integrity (Kiarie *et al.*, 2012).

The current results support the assertion that *S. boulardii* can modulate the levels of intestinal cytokines and reduce villous atrophy (Czerucka & Rampal, 2002). Piglets fed diets supplemented with *S. boulardii* strain CNCM I-1079 (i.e. Sb) had high levels of inflammatory markers that were comparable with those of piglets fed the C+ diet. They also had reduced NF- κ B and IL-6 concentrations at 7 and 14 PWD. The immunomodulatory effect of *S. boulardii* strain CNCM I-1079 at the intestinal level is due to the inhibition of signalling pathways like NF- κ B, JAK/STAT complex, and mitogen-activated protein kinase pathways, causing this yeast to function as an "immunobiotic" by inhibiting the synthesis of proinflammatory cytokines (Pothoulakis, 2009; Martins *et al.*, 2013). These findings agree with those of previous studies (Sougioultzis *et al.*, 2006; Czerucka *et al.*, 2007), which reported a decrease in TNF- α and IL-6 expression in animals fed diets supplemented with this yeast. Another benefit observed with the addition of *S. boulardii* strain CNCM I-1079 to antibiotic-free diets was the control of chronic inflammation associated with the increase in IL-12p40 (López-Hernández *et al.*, 2015) at 14 days post weaning. This was 143% and 278% of that in Sc and C- fed piglets, respectively. The efficiency of *S. boulardii* strain CNCM I-1079 in controlling acute and chronic intestinal inflammation observed in the present study had a positive effect on villus morphology in the Sb-fed piglets. This was a similar effect to that of the antibiotics in the C+ diet, indicating that *S. boulardii* strain CNCM I-1079 is very efficient at preserving intestinal integrity, as reported in previous studies (Bontempo *et al.*, 2006). This is probably owing to polyamine production, which favours epithelial cell renewal, enterocyte differentiation and maturation, allowing villi to recover (Budiño *et al.*, 2005; McFarland, 2010; Rajput *et al.*, 2013; Liu *et al.*, 2018).

The differences observed between piglets fed Sc and Sb diets in the present study can probably be explained by the metabolic and physiological differences between *S. boulardii* and *S. cerevisiae*. These

differences result in different behaviours, particularly in temperature resistance and the response to stress generated by the gastric acid environment, which are important characteristics for a microorganism to function as a probiotic (Hudson *et al.*, 2016). In addition, several factors could influence yeast probiotic effects, such as cell wall thickness, resistance to pH changes, gastrointestinal tract viability, and the ability to compete with other intestinal microorganisms (Fietto *et al.*, 2004; Edwards-Ingram *et al.*, 2007; Hudson *et al.*, 2016).

Conclusions

Inclusion of both *S. cerevisiae* strain 1026 and *S. boulardii* CNCM I-1079 in antibiotic-free diets can improve intestinal health in the post-weaning period. Furthermore, *S. boulardii* CNCM I-1079 was able to maintain a healthy villus height and helped to reduce the inflammatory response of the measured pro-inflammatory markers, mainly in the second week after weaning. Its effects were similar to those of the antibiotic-supplemented diet. Therefore, *S. boulardii* CNCM I-1079 has high potential to be used as a feed additive in antibiotic-free diets. However, further studies are necessary to elucidate the molecular mechanism underlying the regulation of intestinal immunity and the function of *S. boulardii*.

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Authors' Contributions

Conceptualization, TRS and SBM; methodology, TRS, CMA and SBM; validation, TRS and GML; formal analysis, SBM and KEG; investigation, SBM and GML; resources, TRS and MDM; writing original draft preparation, SBM, TRS and KEG; writing, review and editing, SBM, TRS, CMA, GML, KEG and AAB; visualization, SBM and TRS; supervision, TRS; project administration, TRS; funding acquisition, TRS.

Conflict of Interest Declaration

The authors declare that they have no conflicts of interest.

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