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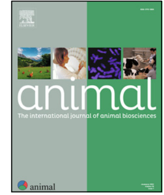

Yeast mixture supplementation modulates faecal microbiota and ileum morphology of weaning pigs

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ABSTRACT

Different yeast strains benefit postweaning piglets by promoting intestinal health. The objective of this study was to investigate the effect of a yeast mixture containing *Kluyveromyces marxianus fragilis*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae* (Vetoquinol italia s.r.l., Italy) on gut health parameters and growth performance traits of weaned piglets. Forty-eight postweaning castrated male piglets (27 ± 1.7 days, 7.19 ± 0.54 kg) were randomly allocated to two homogeneous experimental groups and involved in a 28-day trial. Both the groups received a basal diet with (yeast mixture, **YM**) or without (control, **CTR**) the inclusion of 0.8% yeast mixture during weeks 1 and 2, and 0.6% during weeks 3 and 4. Individual BW and box feed intake were determined on days 0, 14, and 28, and average daily gain and Gain:Feed ratio were subsequently calculated for each administration period (0–14, 14–28). Individual faecal samples were collected for microbiota analysis on days 4, 14, 21, and 28, and faecal score was evaluated on the same days. At the end of the trial, 12 piglets for each group were sacrificed, and ileal tissue was sampled for morphological analysis and the evaluation of mucins profile, using Alcian-Blue/Periodic Acid-Shiff (**PAS**) staining. On ileum samples, dividing and differentiated epithelial cells were also identified using proliferating cell nuclear antigen and alkaline phosphatase expression, respectively. Differences in the means between the experimental groups were determined by ANOVA, while the metataxonomics analyses were performed by sequencing for V3 and V4 hypervariable regions of the 16S rRNA gene. Growth performance traits were not different among the two experimental groups when considering the whole trial period, while treated animals showed increased faecal consistency on weeks 1 and 4 (P = 0.036 and 0.021, respectively). Yeast mixture administration increased the abundance of *Bifidobacterium* (P = 0.006) and *Coprococcus 2* (P = 0.015), and decreased *Clostridium Sensu Stricto 1* (P = 0.019) at all the considered timepoints. Ileum villous height, villous width, and crypt depth were significantly increased by yeast mixture supplementation (P = 0.019; P = 0.013; P = 0.036, respectively), while no differences were observed for the villous:crypt ratio among the groups. The mucin profile showed no differences among experimental groups for acid and neutral glycoconjugates. However, a higher presence of PAS-positive mucins was highlighted in the villi of YM piglets (P < 0.001) compared to CTR. Overall, the administration of a yeast mixture to postweaning piglets showed positive effects on gut health when compared to piglets not receiving the tested product, improving beneficial genera and intestinal morphology.

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Implications

The use of yeasts and yeast-derived products in pig farming is reported to improve gut health and growth performance traits via the modulation of gut microbiota and morphology. In the present work, we evaluated the potential of a yeast mixture containing *Kluyveromyces marxianus fragilis*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae* on the faecal microbiota and ileum morphology of weaning piglets. Our results show that the administration of the yeast mixture can decrease potentially harmful bacteria, improving some beneficial genera, as well as improving ileum

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morphology, therefore suggesting better gut health that might benefit the overall health and performance of postweaning piglets.

Introduction

Over the past years, there has been extensive research on the use of yeasts and yeast-derived products in pig farming to improve animal health and performance. Yeast can be added to a pig's diet in the form of a live probiotic (Xia et al., 2022) or as an inactive whole cell or its components, acting as a prebiotic (Thayer et al., 2022; Agazzi et al., 2020). From a nutritional perspective, intact yeast cells are rich in CP and low in crude fat and fibre. However, they also contain significant amounts of dietary fibre, such as mannan-oligosaccharide and β-glucans, which are found in the yeast cell wall and have a prebiotic and immunomodulatory effect (Patterson et al., 2022).

Yeast prebiotics have been reported to support the gut health and production performance of pigs through different modes of action. There exist multiple mechanisms through which they can impede the adherence of pathogens to the gastrointestinal mucosa. Mannan-oligosaccharides bind to pathogenic bacteria and inhibit their attachment to the mannan residues on intestinal epithelia (Roto et al., 2015). Similarly, β-glucans perform a comparable action and are also accountable for regulating the immune response. They activate phagocytes, natural killer cells, and B and T lymphocytes (Jensen et al., 2008). In parallel, yeast prebiotics also exert their activity by regulating the intestinal microbiota, promoting the growth of beneficial bacteria and maintaining a healthy balance of microorganisms (De Vries et al, 2020).

In swine production, the administration of yeast prebiotics can be carried out during different stages of growth, from gestating sows to weaned piglets to growing-finishing pigs. However, there is evidence suggesting that adding yeast to the diet of young animals is more effective, as they have not yet developed a stable intestinal microbiota (Lee et al., 2021; Boontiam et al., 2020). Promoting beneficial microbiomes, contrasting harmful bacteria, and supporting gut development during postweaning is then of primary importance to guarantee piglet health.

Dietary yeasts previously showed positive effects on the gut health of postweaning piglets. The administration of *Saccharomyces cerevisiae* has been shown to promote the gut environment by stimulating the growth of beneficial bacteria, ameliorating the intestinal epithelial morphology, and leading to an improvement in growth performance traits (Kiros et al., 2018; Boontiam et al., 2020). However, there are still some contrasts in the literature reporting that the dietary supplementation of *S. cerevisiase* did not provide any significant effect (Jiang et al., 2015; Molist et al., 2014).

Besides the historically consolidated use of *Saccharomyces* spp., some other yeasts gained increasing attention in recent years, such as *Kluyveromyces* spp. and *Pichia guilliermondii*. *Kluyveromyces* spp. showed to benefit the intestinal morphology of postweaned piglets, improving villi height and villi:crypt ratio, thanks to its amino acid and nucleotide content, which is higher compared to *Saccharomyces* spp. (Keimer et al., 2018). Although at the present moment, the effects of *Pichia guilliermondii* have not been tested in postweaning piglets yet, recent studies in poultry showed its ability to modulate the gut microbiota and bind enteric pathogens (Fathima et al., 2023). Additionally, the administration of whole-cell inactivated yeast of *Pichia guillermondii* to gestating and lactating sows resulted in improved reproduction performance, with a higher number of pigs born and weaned, and heavier weight at weaning (Thayer et al., 2022). Furthermore, *Pichia guillermondii* has a smaller cell surface area and stronger hydrophobic properties compared to *Saccharomyces cerevisiae*, thus possibly providing a stronger ability to inhibit pathogenic bacteria from binding to the gastrointestinal mucosa. (Peisker et al., 2017).

Taken together, it could be hypothesised that, thanks to their different properties, the combination of *Saccharomyces cerevisiae*, *Kluyveromyces fragilis* and *Pichia guilliermondii* has the potential to benefit the gut health of postweaning piglets by modulating the gut microbiota, decreasing the presence of harmful bacteria, and improving gut morphology. However, there is a lack of studies addressing the gut health of piglets when feeding a combination of yeasts. Hence, the study aimed to evaluate the potential of supplementing a mixture containing *Kluyveromyces marxianus fragilis*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae* (Vetoquinol italia s.r.l., Italy) on gut health and growth performance traits of weaned piglets. Preliminary results of the present study have been previously published in abstract form (Sandrini et al., 2023).

Material and methods

Animals and housing

A total of forty-eight commercial hybrid postweaning castrated male piglets (TOPIGS 40 X TOPIGS FOMEVA), homogeneous for age (27 ± 1.7 days) and BW (7.19 ± 0.54 kg) were selected from a commercial pig farm (Arioli-Sangalli, Genzone, PV, Italy) and transferred to the facilities of the Animal Production Research and Teaching Centre of the Department of Veterinary Medicine and Animal Sciences, University of Milan (Lodi, Italy). Upon arrival at the experimental facilities, the piglets were included in a completely randomised design based on BW and distributed into two groups of 24 animals, housed in a box of two piglets each, for a total of 12 replicates per treatment (two piglets/replicate).

The experimental animals were all housed in a single room, with electronically controlled temperature and humidity. The room temperature was maintained at 27 °C for the first week of the trial, with a ventilation rate of 10 m^3/h per head. Starting on the second week of the experiment, room temperature was gradually reduced by 1 °C per week, to a final temperature of 24 °C at the end of the trial. Each box measured 0.96 m x 0.93 m for a total surface of 0.89 m^2 and was equipped with a grid floor, feeders, and two nipple troughs. To improve animal welfare, environmental enrichments were provided for the entire trial period (i.e., rubber balls in each pen).

Experimental groups and dietary treatments

The experimental groups were defined according to the dietary treatments adopted, as follows: (a) Control (**CTR**), fed commercial basal diets; (b) Yeast Mixture (**YM**), fed the same basal diet with the supplementation of a yeast mixture. The basal diet was formulated to meet or exceed the nutrient requirements of weaned piglets according to the National Research Council Requirements (NRC, 2012) (Table 1), and was provided in meal form by a commercial supplier (Agricom International, Pognano, BG, Italy).

The tested product was a commercial mixture of three inactivated yeasts (*Kluyveromyces marxianus fragilis*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae;* Levustim B0399, provided by Vetoquinol italia s.r.l., Bertinoro (FC), Italy) and was mixed in the feed at a concentration of 0.8% of complete feed for weeks 1 and 2; then, the inclusion level was decreased to 0.6% for weeks 3 and 4, following the producer recommendations. For the duration of the trial, piglets in both the experimental groups had *ad libitum* access to feed and water.

Table 1
Ingredient composition (%, as fed) and analysed nutrient composition (%, DM), unless otherwise mentioned) of the basal postweaning piglet diet used during the experiment.

Items	Inclusion rate
Ingredient	
Barley meal	17.99
Wheat	16.67
Barley flakes	10.00
Sweet whey	10.00
Wheat flakes	8.00
Corn flakes	7.50
Soy protein concentrate	7.00
Corn meal	5.75
Corn gluten meal	2.00
Soy oil	2.00
Potato protein concentrate	2.00
Beet pulp	2.00
Wheat bran	1.75
Coconut oil	1.50
Pregelatinised starch	1.50
Cellulose	1.00
Dicalcium phosphate	0.89
L-Lysine	0.60
Benzoic acid	0.50
Calcium carbonate	0.40
L-Threonine	0.30
Oligo-Vitamins[1]	0.26
DL-Methionine	0.18
Sodium chloride	0.16
Tryptophan	0.05
Nutrient composition	
DM	89.30
CP	18.44
Crude fat	5.92
Crude fibre	4.39
Metabolisable energy, kcal/kg	3 741.00
Net energy kcal/kg	2 838.00
Lysine	1.33
Met + Cyst	0.82
Threonine	1.00
Tryptophan	0.26
Calcium	0.76
Total Phosphorus	0.58

[1] The piglet premix provided the following quantities of vitamins and minerals per kilogram of feed: Vitamin A: 10 000 IU; Vitamin D3: 1 000 IU; Vitamin E: 50 mg; Vitamin B1: 1.0 mg; Vitamin B2: 3.0 mg; Vitamin B12: 0.02 mg; Vitamin B6: 3,0 mg; Pantothenic acid: 10 mg; Nicotinic acid: 15 mg; Biotin: 0.06 mg; Vitamin PP: 0,35 mg; Folic acid: 0,99 mg; Vitamin K3: 2 mg; Colin: 300 mg; Fe: 100 mg; Cu: 20 mg; Co: 0.75 mg; Zn: 100 mg; Mn: 10 mg; I: 0.75 mg; Se: 0.4 mg.

Growth performance traits and faecal consistency

Individual BW and box feed intake were determined on days 0, 14, and 28 by electronic scale (Ohaus ES100L, Pine Brook, New Jersey; sensitivity ± 0.02 kg). The feed intake was determined as the difference between the total amount of feed provided in the two periods (0-14 d and 14-28 d) and the refusals determined on days 14 and 28, respectively. Piglets were daily fed an increasing amount of the experimental diets considering 10% daily orts. Average Daily Gain (**ADG**), Average Daily Feed Intake (**ADFI**), and Gain: Feed ratio were subsequently calculated. Daily evaluation of faecal consistency was performed using a 5-point scale as reported by Jiang et al. (2015) where: 1 = constipation status, 2 = pasty consistency, 3 = optimal condition, 4 = creamy consistency, 5 = watery consistency. Faecal consistency data are presented as average scores by week.

Faecal microbiota

Sample collection and 16S rRNA gene sequencing

On days 4, 14, 21, and 28, individual faecal samples were collected by spontaneous defecation in sterile tubes. An aliquot from each tube was then promptly transferred in cryovials, snap-frozen in liquid nitrogen, and subsequently stored at −80 °C for faecal 16S rRNA gene sequencing. For the 16S rRNA gene sequencing, faecal samples from 12 selected piglets for each group were used. The analysis of 16S rRNA was performed on faecal samples from the same piglets (n = 12/group), selected for ileum morphology evaluation (for the selection criteria of the animals, refer to the section on Ileal morphological analysis and mucin profile, below).

DNA was extracted from each faecal sample using the QIAmp DNA Stool kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The faecal samples were dissolved in 1 mL Buffer ASL and shaken at 1 000 rpm (Mixing Block MB-102, CaRlibiotech S.r.l. Rome, Italy), continuously until the stool samples were homogenised. The quality and quantity of the DNA were checked using a spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The isolated DNA was then stored at −20C until use. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified by using the primers (Forward: CCTACGGGNGGCWGCAG; Reverse: GACTACHVGGGTATCTAATCC) described in the literature (Caporaso et al., 2011). For library preparation and sequencing, the protocol described in Cremonesi et al. (2022) was followed. Briefly, the DNA was amplified by using KAPA HIFI Master Mix 2 × (Kapa Biosystems, Inc., MA, USA) in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific). Amplicons were then cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA), and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). After library quantification, a pool was prepared and sequenced in one MiSeq (Illumina) run with 2 × 250-base paired-end reads. A total of 23 794 504 sequences were selected for microbiota analysis. Sequence data were processed with Qiime v. 1.9 (Caporaso et al., 2011) using a pipeline adapted from Biscarini et al. (2018). Paired-end reads were joined (SeqPrep) and filtered or quality based on the following parameters: (1) a maximum of three consecutive low-quality base calls (Phred < 19); (2) no "N"-labelled (unidentified) bases allowed; (3) at least 75% of consecutive bases in a read with Phred > 19. Filtered reads were aligned against the SILVA database v. 132 for closed-reference Operational Taxonomic Unit (**OTU**) identification and quantification, with 97% cluster identity.

Faecal alpha and beta diversity

The diversity of the intestinal microbiota was evaluated within sample (alpha diversity) and between samples (beta diversity). All alpha and beta diversity indices were estimated from the OTU table. The OTU-table data were normalised using Cumulative Sum Scaling normalisation and filtered by a minimum number of counts (n = 10) and a minimum number of samples (n = 2). Variability within the sample (alpha diversity) was evaluated using the following indices: Chao1, ACE for richness, while Shannon, Simpson, Fisher's alpha, Observed OTU, Simpson E, and Equitability for evenness. The faecal microbiota between-sample diversity (beta diversity) was quantified by calculating Bray-Curtis dissimilarities. Among dietary groups, Bray-Curtis dissimilarities were evaluated non-parametrically, using the permutational ANOVA approach (999 permutations). Details on the calculation of the mentioned alpha- and beta-diversity indices can be found in Biscarini et al. (2018, S2 Appendix).

Ileal morphological analysis and mucin profile

At the end of the trial (day 28), all the piglets were transferred to a commercial slaughterhouse after 12 h of starvation. All the piglets were electrically stunned and bled following the common commercial slaughtering practices. At slaughtering, ileum samples were collected from 12 piglets for each group by selecting,

within each pen, the piglet whose BW at d28 was closer to the average BW of the relative experimental group (14.76 kg CTR, 14.77 kg YM). From each of the selected piglets, a 2 cm section of distal ileum was collected, promptly fixed in 10% neutral buffered formalin, and processed for routine histology. In brief, samples were dehydrated in decreasing alcohol concentrations, cleared with xylene, and embedded in paraffin. After dewaxing and rehydration, sections of 5 μm thickness were stained with hematoxylin–eosin to assess the morphology of the intestinal wall, as well as, the general mucosa architecture. Hematoxylin–eosin transversal stained sections were used to perform morphometric measurements. In particular, villi height (**VH**, measured from the villous apex to the villous crypt-junction), villi width (estimated as average among three measurements: base, middle, and apex), crypt depth (**CD**, measured from the villous-crypt junction till the deepest region of the crypt) and the VH:CD ratio were evaluated to assess the effects of the diet regimens on the gut health. A total of 10 villi and 10 crypts were analysed for each specimen.

Samples were observed under a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with a Ds-fi2 camera and the NIS-Elements software package (Nikon). Images were observed at continuous magnification between 200 and 400. The ileum mucin profile was determined by staining sections with Alcian blue (**AB**) pH 2.5- combined with periodic acid Schiff (**PAS**), which reveals neutral (PAS-reactive) and acid (AB-reactive) glycoconjugates. The total number of goblet cells, the number of goblet cells with different types of mucins as distinguished by AB 2.5/PAS staining, were determined by enumeration, taking as reference five points for nine ileal sections per experimental group.

Ileal immunohistochemistry and immunofluorescence

Alkaline phosphatase for differentiated cells

Fully differentiated epithelial cells were detected through the identification of Alkaline phosphatase. In brief, sections were brought to distilled water and immersed a Tris HCL (pH 9.5) to produce an alkaline environment. Subsequently, slides were exposed to BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Vector Laboratories, SK-4500 USA), which in the presence of the alkaline phosphatase enzyme assumes an indigo colour. Sections were then rapidly rinsed in tap water, dehydrated, counterstained with Mayer's hematoxylin, and permanently mounted.

Immunolocalisation of proliferating cell nuclear antigen

Proliferating cell nuclear antigen (**PCNA**) was used as a marker of actively dividing cells. Proliferating cell nuclear antigen expression was detected through indirect immunofluorescence. In brief, antigen retrieval was performed by incubating slides in a pressure cooker containing antigen retrieval solution (10 mM Tris-base, 1 mM EDTA solution, 0.05% tween20, pH 9 for 1 min). Aspecific bindings were avoided by incubating slides in 10% goat serum in PBS. Thereafter, samples were incubated with Mouse monoclonal anti-PCNA antibody (Millipore Corporation, MAB424, Darmstadt, Germany) diluted 1:1200 in 4% BSA in PBS for 60 min at room temperature in a humid chamber. Subsequently, slides were incubated with the adequate secondary antibody Alexa Fluor™ 594 goat anti-mouse IgG 1:250 (Life Technologies Corporation, A11005 Willow Creek Road, OG, USA) in PBS for 30 min at room temperature. Sections were counterstained with 4,6-diamidino-2-phenylindole and mounted with Pro- Long™ Gold Antifade Mountant (ThermoFisher Scientific, Waltham, MA, USA). Secondary antibody controls were performed by omitting the primary antibody.

Quantitative analysis

We quantified different aspects of the mucosal surface extension examining histological sections under a Zeiss AXION Zoom. V16 stereomicroscope equipped with an Axiocam 506 colour camera and the ZEN 2 version 2.0 package software or with a Leica DMR microscope equipped with a Nikon DS-Ri2 camera and the NIS-Elements D software, version 5.20. Images were observed at continuous magnifications between 6 and 400. The length of the differentiated cells compartment was measured over the extension of the absorptive tract, identified by alkaline phosphatase$^+$ cells (blue border). To avoid any staining interferences, sections for quantitative measurements were stained omitting the nuclear stain. The proliferation rate of the ileum epithelium has been determined by quantifying the number of PCNA+cells per crypt. At the same time, alkaline phosphatase has been used as a marker to identify fully differentiated epithelial cells. To identify the proliferative rate, we also measured the ratio between PCNA$^+$ cells and the total number of cells.

Statistical analysis

Data normality was verified using Shapiro–Wilk test (SAS version 9.4, SAS Institute Inc., Cary, NC, USA), and no outliers were detected. Growth performance traits (BW, ADG, ADFI, and Gain: Feed ratio) were analysed using a linear model (MIXED procedure for repeated measurements, SAS version 9.4, SAS Institute Inc., Cary, NC, USA). The model adopted was as follows:

$$y_{ijknp} = \mu + treat_j + time_k + (treat \times time)_{jk} + pig_n/box_p + e_{ijkn/p}$$

where y_{ijkn} is the i_{th} phenotypic record (BW, ADG, ADFI, and Gain: Feed ratio) from pig_n /box_p with treatment $_j$ (yeast mixture or not) at time $_k$ (days on treatment), while $e_{ijkn/p}$ is the residual. Treatment and time were considered fixed effects, while pigs or boxes were treated as random effects. The covariance structure was as follows:

Var(y) = Var(animal/box) + Var(e), with Var(pig/box) = $I^*\sigma^2_{pig/box}$ and Var(e) = $R^*\sigma^2_e$, where I is the identity matrix and R is a covariance matrix that allocates records to the corresponding pig/box.

From 16S rRNA gene sequencing data, alpha and beta diversity, and OTU counts, were obtained and analysed within days: the piglet represented the experimental unit, and the linear model applied to analyse between-treatment differences was as follows:

$$y_{ij} = \mu + treatment_i + e_{ij}$$

where y_{ij} is the alpha diversity index value or OTU counts at each specific day (d 4, 14, 21, and 28) for pig $_i$ belonging to treatment group $_j$; μ is the intercept, $treatment_j$ is the systematic effect of treatment $_j$ within the days, and e_{ij} is the residual.

Alpha diversity indices and Bray-Curtis dissimilarities (beta diversity) were estimated using the Python-based Qiime 1.9 software suite. Statistical comparisons between treatments (including PERMANOVA) were carried out using the R environment for statistical programming.

Faecal score, ileal histomorphology and immunohistochemistry data were subjected to ANOVA, using the GLM procedure of SAS (SAS version 9.4, SAS Institute Inc., Cary, NC, USA) applying the following model:

$$y_{ij} = \mu + treat_i + e_{ij}$$

where y_{ij} is the i_{th} phenotypic record (individual or box average), $treat_j$ is the effect of treatment and e_{ij} is the residual. The piglet represented the experimental unit for all the parameters that could be individually determined (i.e., BW, ADG, faecal consistency, and ileum morphology and immunohistochemistry). The box represented the experimental unit for ADFI and Gain:Feed ratio since

the animals were not housed individually. Differences between groups were considered statistically significant at $P < 0.05$.

Results

Growth performance traits

The effects of yeast mixture administration on growth performance traits and faecal consistency of postweaning piglets are shown in Table 2. No significant effect of the treatment was observed on BW, ADG, ADFI, and Gain:Feed ratio, during each administration period and over the entire experimental period (0-28 d). Faecal consistency was significantly improved in YM during the first ($P = 0.036$) and the last week of the trial ($P = 0.021$), as well as during the whole experimental period ($P = 0.006$).

Faecal microbiota

Alpha and beta diversity

The overall treatment effect did not show any significant differences ($P > 0.05$) in the number of OTUs nor for all the considered diversity indices (Table 3). However, when considering the effect of the treatment within each time point, YM was found to increase Chao (Estimate compared to control=+347.48, $P = 0.034$) and ACE (Estimate compared to control=+323.85, $P = 0.045$) richness indices on day 21. At the same time point, the same trend was observed for fisher_alpha (Estimate compared to control=+130.56, $P = 0.062$).

Fig. 1 shows the first two dimensions from the Non-metric Multidimensional Scaling for beta-diversity of Bray-Curtis distances over time for the two experimental groups. Samples were grouped by experimental units: by treatment, by days, and by treatment and days. PERMANOVA (999 permutations) showed that the beta diversity, as the diversity of microbiota between piglets, was not affected by the yeast mixture supplementation ($P = 0.297$), while the between-days P was equal to 0.007, and the treatment-by-days interaction P-value was 0.146.

Taxonomy

The CTR and YM relative abundances of the faecal microbial community at different taxonomic levels are reported in Supplementary Figure S1. The obtained results showed that Firmicutes were the predominant phylum throughout the trial period in both groups, with a constant increase until day 21 (d4: CTR: 65.06%, YM: 65.43%; d14: CTR 69.06%, YM: 68.46%; d21: CTR: 70.71%, YM: 71.41%), and a slight decrease at day 28 (CTR: 66.89%, YM: 68.04%), while Bacteroidetes and Proteobacteria phyla were respectively the second (~20%) and third (~2%) predominant phyla at all the days.

When comparing CTR and YM groups, no significant differences were observed among the two most highly expressed phyla ($P > 0.05$), while 15 genera were found significantly different (Fig. 2). At the class level, Actinobacteria showed a greater relative abundance in YM ($P = 0.02$) during the whole experimental period. Moreover, within the Actinobacteria class, a higher abundance of the order Bifidobacteriales ($P = 0.01$), the family *Bifidobacteriaceae* ($P = 0.01$), and genus *Bifidobacterium* ($P = 0.01$) in the YM group were evidenced ($P = 0.01$). At the genus level, the relative abundance of *Coprococcus 2* was higher in yeast-fed piglets ($P = 0.01$), while the *Clostridiaceae 1* family and the *Clostridium Sensu Stricto 1* genus were lower than CTR ($P = 0.01$; $P = 0.02$, respectively).

Ileal morphology and mucin profile

Morphological evaluation of ileum sections (Supplementary Figure S2) revealed no histopathological aspects in the epithelium of either CTR or YM piglets. The intestinal mucosa was regularly organised in intestinal villi and crypts in both control and yeast-supplemented piglets. Table 4 shows the effects of the yeast mixture on villous height, villous width, crypt depth, and villous

Table 2
Growth performance and faecal consistency of postweaning piglets fed a yeast mixture (n = 24)[1] (*Kluyveromyces marxianus fragilis, Pichia guilliermondii,* and *Saccharomyces cerevisiae*) or not (n = 24) for 28 consecutive days.

Items	CTR	YM	SEM	*P*-value[2]
BW (kg)			0.21	0.936
day 0	7.19	7.19		
day 14	9.47	9.38		
day 28	14.76	14.77		
ADG (g/day)			20.23	0.986
days 0–14	155	156		
days 14–28	382	380		
days 0–28	265	265		
ADFI (g/day)			24.61	0.562
days 0–14	301	308		
days 14–28	621	646		
days 0–28	457	470		
Gain:Feed ratio			0.02	0.524
days 0–14	0.51	0.49		
days 14–28	0.62	0.58		
days 0–28	0.58	0.56		
Faecal consistency				
days 0–7	3.70^{a}	3.51^{b}	0.06	0.036
days 7–14	3.13	3.09	0.03	0.268
days 14–21	3.09	3.06	0.03	0.374
days 21–28	3.04^{a}	3.00^{b}	0.16	0.021
days 0–28	3.24^{A}	3.17^{B}	0.02	0.006

Abbreviations: CTR=control; YM=yeast mixture; ADG=average daily gain; ADFI=average daily feed intake.
a-b Values within a row with different superscripts differ significantly at $P < 0.05$.
A-B Values within a row with different superscripts differ significantly at $P < 0.01$.
1 The tested product was included in the feed at a concentration of 0.8% of complete feed from 0 to 14 days, and 0.6% from 15 to 28d.
2 *P*-values refer to the treatment effect over the entire experimental period. Time and Treatment*Time effects were not reported because not significant.

Table 3
Alpha diversity indices and significance of differences between postweaning piglets fed (n = 12) or not (n = 12) a yeast mixture[1] (*Kluyveromyces marxianus fragilis*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae*) for 28 consecutive days.

	Day 4			Day 14			Day 21			Day 28		
Indices	CTR	YM	*P*-value	CTR	YM	*P*-value	CTR	YM	*P*-value	CTR	YM	*P*-value
Chao1	1 227.25	1 365.06	0.108	1 335.67	1 368.47	0.789	1 132.27	1 479.75	0.034	1 356.76	1 201.92	0.150
Ace	1 230.94	1 354.93	0.115	1 344.31	1 383.97	0.736	1 163.63	1 487.47	0.045	1 369.27	1 224.17	0.157
Fisher_alpha	556.62	610.33	0.110	590.18	606.35	0.727	527.48	658.03	0.062	606.13	548.38	0.156
Observed_otus	961.58	1 031.75	0.121	981.08	978.16	0.959	880.36	1 027.91	0.110	1 013.66	935.16	0.298
Shannon	9.58	9.67	0.143	9.58	9.57	0.941	9.30	9.64	0.197	9.63	9.52	0.275
Simpson	0.99	0.99	0.171	0.99	0.99	0.891	0.99	0.99	0.271	0.99	0.99	0.294
Equitability	0.96	0.96	0.706	0.96	0.96	0.927	0.96	0.96	0.169	0.96	0.96	0.198
Simpson_e	0.66	0.66	0.337	0.64	0.63	0.719	0.66	0.63	0.165	0.64	0.65	0.113

Abbreviations: CTR=control; YM=yeast mixture.
[1] The tested product was included in the feed at a concentration of 0.8% of complete feed from 0 to 14 days, and 0.6% from 15 to 28 days.

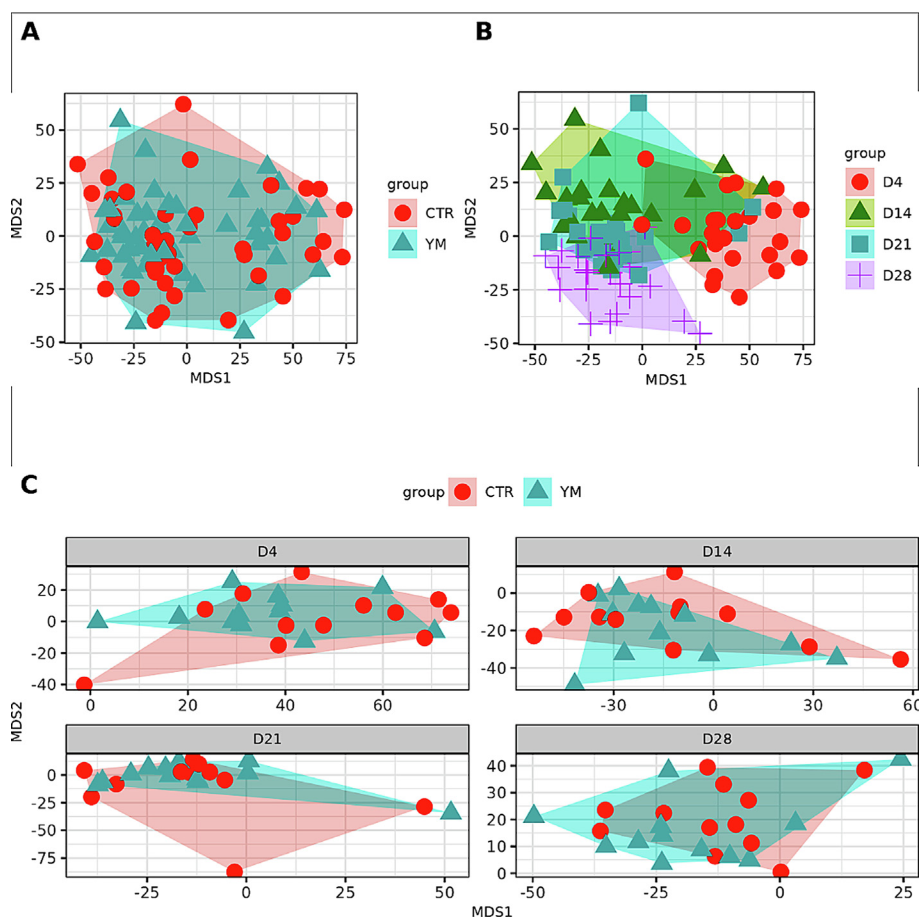

Fig. 1. Non-metric Multidimensional Scale of Bray-Curtis distances between samples of faecal microbiota of weaning piglets fed a yeast mixture (YM; n = 12) (*Kluyveromyces marxianus fragilis, Pichia guilliermondii,* and *Saccharomyces cerevisiae*) or not (CTR; n = 12) for 28 consecutive days. Data are shown according to treatment (A), time point (B), and treatment-by-time point (C). The legends indicate the colour codes and symbols used for the different sample groups [red circles and shading control (CTR) group; turquoise triangles and shading, yeast mixture (YM) group] and time points (red circle, T4; green triangle, T14; turquoise square, T21; purple cross, T28).

height/crypt depth ratio. Supplementation of yeasts in postweaning piglets significantly affected villous morphology, improving villous height ($P = 0.019$) and crypt depth ($P = 0.036$), and decreasing villous width ($P = 0.013$) in the ileum, while no differences were detected for villous height:crypt depth ratio concerning CTR group.

The ileum mucin profile showed that goblet cells of the ileum villi and crypts from both experimental groups produced mostly a mixture of neutral and acidic mucins (magenta-purple or blue-purple colour, Fig. 3 A and B). Supplementary Figures S3 and S4 show individually neutral (magenta) and acidic (blue) mucins, respectively. Neutral mucins were extended over the epithelial surface and the upper crypts, while acidic mucins were predominant in the bottom of ileum crypts. A higher presence of neutral mucins was observed in villi of YM piglets ($P < 0.001$) (Supplementary Table S1).

Ileal immunohistochemistry and immunofluorescence

In our study, immunodetection of PCNA revealed that cells were proliferating in the crypts of animals from both the experimental groups and, although at a lower frequency, some PCNA+cells were also present along the villi surface (Fig. 4, A and B). No differences

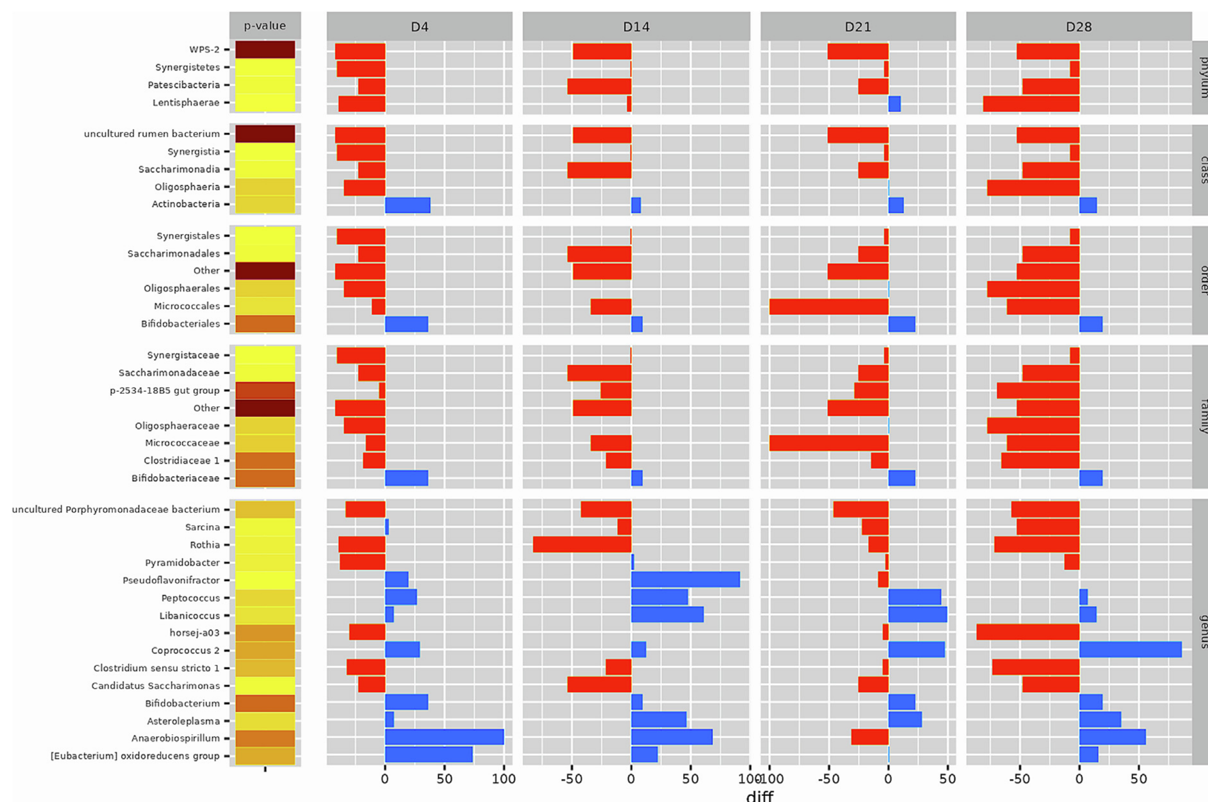

Fig. 2. Significantly different Operational Taxonomic Units (OTUs) in the faecal microbiota of weaning piglets fed a yeast mixture (YM; n = 12) (*Kluyveromyces marxianus fragilis, Pichia guilliermondii*, and *Saccharomyces cerevisiae*) or not (CTR; n = 12) for 28 consecutive days. The results are reported for the different time points and are presented as a heatmap where red indicates a decrease and blue indicates an increase of the normalised operational taxonomic unit value in the YM group vs the control group for each taxon at the different time points. The different colours in the *P*-value column mean different values of significance. The darker the colour, the more significant the *P*-value.

Table 4
Villous height, villous width, crypt depth, and VH:CD ratio in the ileum of postweaning piglets fed a yeast mixture (n = 12)[1] (*Kluyveromyces marxianus fragilis, Pichia guilliermondii*, and *Saccharomyces cerevisiae*) or not (n = 12) for 28 consecutive days.

Items	CTR	YM	SEM	*P*-value
Villous height (μm)	325.60[b]	388.42[a]	18.87	0.019
Villous width (μm)	134.30[a]	121.84[b]	3.51	0.013
Crypt depth (μm)	235.92[b]	256.85[a]	7.00	0.036
VH:CD ratio	1.55	1.71	0.12	0.341

Abbreviations: CTR=control; YM=yeast mixture; VH=villous height; CD=crypt depth.
[a–b] Values within a row with different superscripts differ significantly at $P < 0.05$.
[1] The tested product was included in the feed at a concentration of 0.8% of complete feed from 0 to 14 days, and 0.6% from 15 to 28d.

were observed in the distribution of proliferating cells along the ileal epithelium accounting for the effect of yeast administration ($P > 0.05$) (Supplementary Table S2). At the same time, the total length of differentiated epithelium appears similar in both experimental groups (Fig. 5). Furthermore, the balance between proliferating and differentiated cells was shifted in favour of proliferation (T = 62.02% PCNA+cells vs 41.66% AP+cells; C = 50.28% PCNA+vs 35.57% AP+; Supplementary Table S2).

Discussion

Yeast supplementation in weaning piglets is reported to modulate the intestinal environment by improving microbial diversity and stimulating the growth of beneficial microbial populations, such as the phylum Actinobacteria and Firmicutes, the family Ruminococcaceae, and the genus Bifidobacterium (Kiros et al., 2018). Although in our study, the administration of a yeast mixture had no or minor effect on the diversity of the microbial community, we observed an increase in the relative abundance of class Actinobacteria, order Bifidobacteriales in YM piglets at all the considered time points.

This result was further confirmed at lower levels of the taxonomic classification, where the family *Bifidobacteriaceae*, and *Bifidobacterium* genus, were found to be more abundant in YM compared to CTR at all the considered time points. The higher presence of the Actinobacteria class and *Bifidobacteriaceae* family in YM piglets is particularly of interest since they respectively play a crucial role in maintaining homeostasis and promoting beneficial effects on the gut barrier (Scott et al., 2014). Specifically, the *Bifidobacteriaceae* family was reported to increase protection from enteropathogenic infections (Fukuda et al., 2012) through the production of short-chain fatty acids and showed to be involved in the development of the immune system, through the stimulation of mucosal immunoglobulins production by the intraepithelial lymphocytes (Maynard et al., 2012). We also observed an increase in the relative abundance of *Coprococcus* 2 in YM piglets. *Bifidobacteriaceae* and *Coprococcus 2* are both involved in butyrate production,

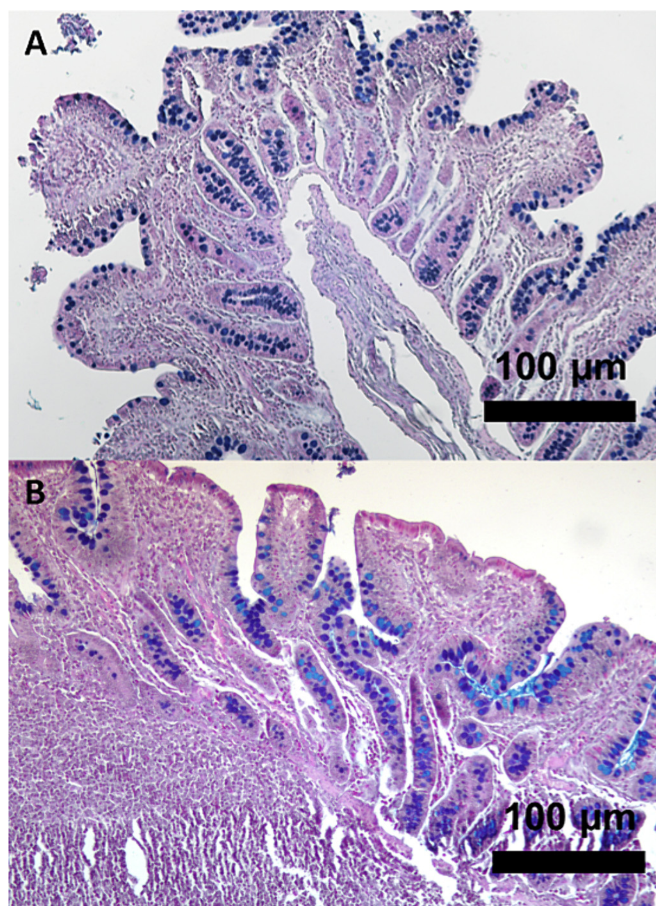

Fig. 3. Ileum staining with Alcian Blue-PAS of postweaning piglets fed a yeast mixture (A; n = 12) (*Kluyveromyces marxianus fragilis, Pichia guilliermondii,* and *Saccharomyces cerevisiae*) or not (B; n = 12) for 28 consecutive days. Most of the goblet cells exhibit both PAS and AlcianBlue positivity. Scale bar: 100 µm. Abbreviations: PAS=Periodic Acid-Shiff.

which is recognised to play a crucial role in regulating the functional integrity of the gastrointestinal tract, stimulating mucin secretion and serving as an energy source for epithelial cells and certain beneficial bacteria (Liang et al., 2022; Liu et al., 2023). Therefore, although we did not directly investigate the effect of YM on the intestinal barrier, it could be speculated that some of the beneficial effects observed at the ileum level in our study could be explained by such mechanisms of action.

Besides the increased relative abundance of the beneficial microbes in YM piglets, our trial highlighted a significant decrease in harmful bacteria such as *Clostridiaceae* family and, more specifically at the genus level, in *Clostridium sensu stictu 1* according to Cremonesi et al. (2022). *Clostridium sensu stricto1* plays a significant role in the occurrence of intestinal inflammation (Fan et al., 2017). Therefore, a lower relative abundance in YM piglets suggests that the administration of the yeast mixture used in our trial may have a protective role, reducing the growth of potentially harmful bacteria in the gut of treated animals.

As the first interface between the lumen and the intestinal epithelium, the mucous layer protects the intestinal mucosa against potential pathogens, preserving the gastrointestinal tract's structural integrity (Liu et al., 2014). In the present trial, the ileum mucin profile revealed that intestinal goblet cells contained both neutral and acid mucins, as previously reported by Bontempo et al. (2006). However, our results show that yeast administration determined a prevalence of acid mucins than both neutral and basic, with a higher presence of PAS-positive glycoconjugates in the villi of YM piglets, suggesting that acid glycoconjugates could be predominant in the lower crypts area as previously shown by Liu et al (2014). These finding suggest a possible greater resistance to bacterial infection in the gut since it is reported that acidic glycoconjugates are more involved in supporting the intestinal mucosa to counteract microorganisms and resist bacterial enzymes (Montagne et al., 2004).

In our study, yeast administration did not either enhance the renewal rate of intestinal mucosa or its differentiated part. PCNA is a favourable indicator of the state of cell proliferation, well-characterised also in pig intestine (Verdile et al., 2019, 2022),

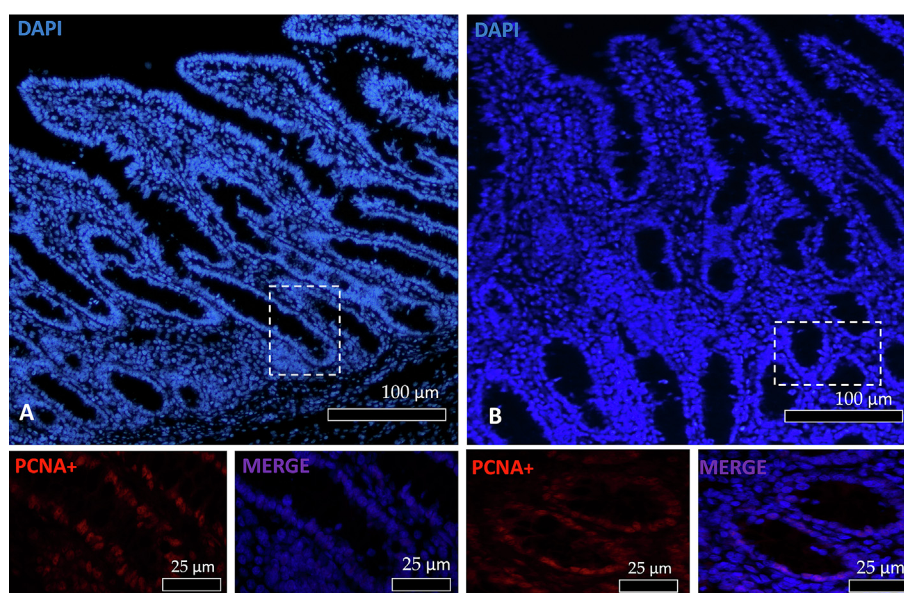

Fig. 4. Immunolocalisation of Proliferating Cell Nuclear Antigen (PCNA) in ileal crypts of piglets fed a yeast mixture (A; n = 12) (*Kluyveromyces marxianus fragilis, Pichia guilliermondii,* and *Saccharomyces cerevisiae*) or not (B; n = 12) for 28 consecutive days. Scale bars: 100 µm. Insets: 25 µm. Abbreviations: DAPI=Sections counterstained with 4,6-diamidino-2-phenylindole.

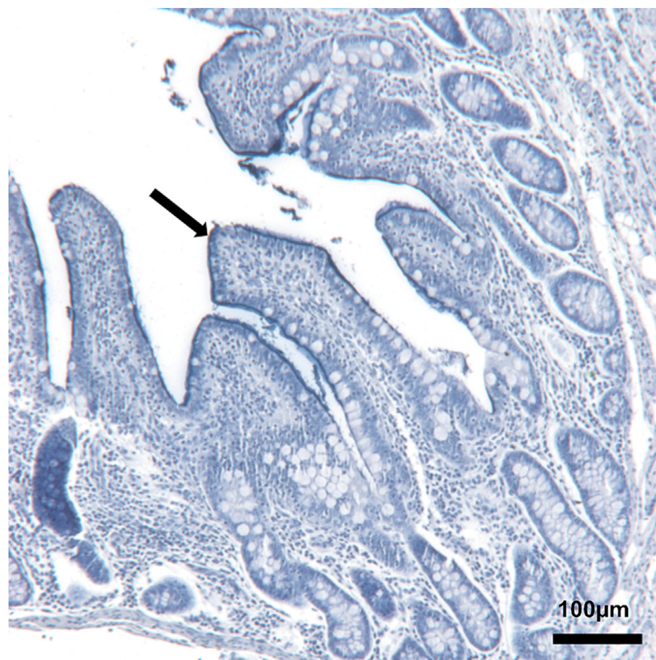

Fig. 5. Alkaline phosphatase enzyme assay for differentiated cells of piglet ileum fed a yeast mixture (YM; n = 12) (*Kluyveromyces marxianus fragilis, Pichia guilliermondii,* and *Saccharomyces cerevisiae*) or not (CTR; n = 12) for 28 consecutive days. Arrow, length of the blue-border to measure the differentiated part of the ileum epithelium. Scale bar: 100 µm.

while, among the brush–border enzymes, Intestinal Alkaline Phosphatase is considered a marker for epithelium differentiation (Alpers et al., 1995). Particularly, we observed a similar number of PCNA+cells, as well as the total length of differentiated ileum epithelium, in both experimental groups. On the contrary, Domeneghini et al. (2004) found a positive effect on the proliferation rate of ileal epithelial cells following L-glutamine and/or nucleotide administration to piglets.

The postweaning period is often associated with modification of the intestinal morphology, characterised by villous atrophy, crypt hyperplasia, decreased digestive and absorptive capacity, and impaired intestinal barrier and immune defence function (Stokes, 2017). The ileum is reported to be highly susceptible to pathogens and its functional and structural changes after nutraceutical administration can be a predictive tool for intestinal responsiveness (Domeneghini et al., 2004). Furthermore, Marinho et al. (2007) reported greater villous high in the ileum of prebiotic-fed piglets as a result of the higher level of energy absorbed originating from volatile fatty acids formed by the degradation of xylooligosaccharides. In our study, villous height, villous width, and crypt depth were enhanced by YM supplementation. These findings agree with Qin et al. (2019) who found an improvement in intestinal development via increasing villous height and decreasing villous width in the ileum when *Saccharomyces cerevisiae* glycoproteins (800 mg/kg) were administrated to piglets. Likewise, other investigators reported improved duodenum and jejunum morphology in piglets supplemented with different forms of dietary yeast due to the high content of glutamine and nucleotides as building blocks for enterocyte proliferation (Boontiam et al., 2020; Namted et al., 2022).

The observed beneficial results on gut health in terms of faecal microbiota, mucin profile, and ileum morphology in YM piglets were expected to turn into improved faecal consistency and growth performance. Faecal consistency was indeed found to be improved by YM administration during the first and last week of the trial, as well as over the entire experimental treatment, which is in line with the results previously reported in the literature (Lee et al., 2021). On the contrary, the supplementation of the yeast mixture did not affect the growth rates in the 28 days after weaning. Currently, the available literature still reports contrasting results on performance when yeasts are supplemented to postweaning piglets. While Molist et al. (2014) and Jiang et al. (2015) did not observe any significant improvement in growth rates following the inclusion of different forms of *Saccharomyces cerevisiae*, Boontiam et al. (2020) outlined significant improvements in ADG, ADFI, and Gain:Feed ratio when feeding hydrolysed yeasts. The observed lack of a positive effect on growth performance traits in our study may be explained by the fact that yeast supplementation seems to be more effective when animals are subjected to suboptimal experimental conditions or environmental challenges (Waititu et al., 2017), which however were missing in the present trial.

Conclusions

In this study, the administration of a yeast mixture (*Kluyveromyces marxianus fragilis*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae*) to postweaning piglets was able to improve the relative abundance of some beneficial microbial populations in the faeces, such as *Bifidobacterium* and *Coprococcus 2*, while decreasing potentially harmful pathogens such as *Clostridium sensu stricto1*. The administration of YM improved the villous height and width in the ileum but had only minor effects on the mucin profile. No significant differences were observed in growth performance traits. These results suggest that YM supplementation may have a beneficial effect on the intestinal environment of postweaning piglets. However, further investigation is needed, including an immunological challenge, to better understand the effectiveness of the product that was tested.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101275.

Ethics approval

The experiment followed the ARRIVE animal experimentation guidelines. All procedures adopted in the present research were reviewed and approved by the Animal Care and Use Committee of the University of Milan (OPBA n°96/21). The animals were raised and treated according to European Union Directive 2010/63/EU covering the protection of animals used for experimental or other purposes and according to the recommendation of Commission 2007/526/CE covering the accommodation and care of animals used for experimental and other scientific purposes.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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