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PAPER

Hydrolysed yeast from *Kluyveromyces fragilis* improves plasma antioxidant efficiency and immunoglobulin concentration, and faecal microbiota of weaned piglets

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ABSTRACT

The aim of this study was to evaluate the effect of hydrolysed yeast from *Kluyveromyces fragilis* (HK) on growth performance, diarrhoea incidence, plasma antioxidant efficiency and immune status, and faecal microbiota of weaned piglets. A total of 100 weaned piglets with BW (7.03 ± 0.14 kg) and age (25 ± 1 days) were randomly allotted to 4 groups with 5 replicates in a 21-day experiment. Piglets were fed with basal diet (NC), NC + 2 g/kg zinc oxide (PC), NC + 7.5 g/kg HK (HK1), or NC + 10 g/kg HK (HK2). Blood and faecal samples were collected on day 21. Significant differences were pointed out in the PC and HK2 piglets compared to the NC group as to the diarrhoea incidence from day 0 to 21 ($p < 0.001$; $p = 0.032$), the activity of plasma superoxide dismutase ($p = 0.019$; $p = 0.003$) and the concentration of plasma malondialdehyde ($p = 0.042$; $p = 0.010$). Moreover, significant differences were pointed out in the HK2 piglets compared to the NC group as to the content of plasma immunoglobulin A ($p = 0.005$), the Ace index and Chao1 index ($p = 0.023$; $p = 0.018$,) and the relative abundance of Campylobacterota and *Escherichia-Shigella* ($p = 0.023$; $p = 0.032$). In conclusion, dietary HK at 10 g/kg alleviated diarrhoea incidence of piglets that might be attribute to the improved plasma antioxidant efficiency and immune status and the regulated faecal microbial community, and could be alternative to the high dose ZnO.

HIGHLIGHTS

- Dietary HK improved plasma antioxidant and immune status and reduced the diarrhoea incidence in weaned piglets.
- The dosage of 10 g/kg HK showed better effect than that of 7.5 g/kg.
- The supplementation of 10 g/kg HK to post-weaning diet may be an alternative to the high-dose ZnO.

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Antioxidant efficiency; faecal microbiota; hydrolysed yeast; *Kluyveromyces fragilis*; weaned piglets

Introduction

High dose zinc oxide (2 g/kg) has been considered as alternative to antibiotics and used in weaned piglet diets for a long time due to its significant effect on reducing diarrhoea incidence, improving health status and promoting growth performance of piglets (Hu et al. 2012). However, a large amount of zinc in excrement would cause serious pollution to the environment (Vahjen et al. 2015). The maximum dosage of zinc as ZnO for piglet diets during the first two weeks post weaning has been permitted at 1.6 g/kg (approximately 2 g/kg for ZnO) in China since 2018, and the use of ZnO in pharmacological doses (3 g/kg) has been banned in the European Union since 2022. Therefore, in order to ensure the production efficiency and sustainable development of pig industry, it is urgent to find the safe and high-efficiency feed additives to replace antibiotic and high dose zinc oxide.

Yeast hydrolysate is the hydrolyzate of yeast cells, which is mainly obtained by yeast cell autolysis or

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external enzymatic hydrolysis (Amorim et al. 2016). Yeast hydrolyzate is rich in protein, nucleotides, polysaccharide, small peptides, vitamins, biological enzymes, zymosan and other nutrients, which has plenty of biological functions such as improving growth, enhancing immunity, increasing antioxidant efficiency, and regulating intestinal health of weaned piglets (Melanie and John 2010; Boontiam et al. 2022). Recently, products containing *Kluveromyces* cerevisiae have received increasing attention due to their superior physiological properties and economic value over *Saccharomyces* cerevisiae (Keimer, Kröger et al. 2018). These properties include faster growth rate, heat tolerance, ability of absorbing various sugars, and hydrolases secretion (Fonseca et al. 2008; Melanie and John 2010). The cell wall of *Kluyveromyces* contains high concentrations of glucan, which have potential immunomodulatory and serum cholesterol lowering functions (Nguyen et al. 1998). Studies have shown that cell wall α-D-mannan derived from *Kluyveromyces* can scavenge hydroxy radicals, and induced nitric oxide production in macrophages, suggestive of their immunostimulatory effects. (Galinari et al. 2018).

Previous studies demonstrated that diets with 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis* (HK) could positively influence feed intake and intestinal morphology and increase innate immune response of piglets in the early postweaning period. However, the effect of HK especially low dose (less than 10 g/kg) on systemic antioxidant and immune and intestinal microbiota has not been reported in literature yet. Therefore, the purpose of this experiment was to evaluate the effect of HK comparing the high dose zinc oxide (2 g/kg) on diarrhoea incidence, plasma antioxidant efficiency and immunoglobulin, and faecal microbiota of weaned piglets and to test the efficacy of 2 different doses (7.5 and 10 g/kg) of HK supplementation, which may provide theoretical foundation for the deeply research and application of HK in pig industry.

Materials and methods

The animal protocol for this research was approved by the Animal Care and Use Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences (IFR-CAAS-20210705). The trial was conducted at the Tianpeng experimental farm, located in Langfang, from July to August 2021.

Experimental design and animal management

A total of 100 healthy weaned barrows (Duroc × Landrace × Yorkshire), with similar initial body weight (BW, 7.03 ± 0.14 kg) and age (25 ± 1 days) were randomly assigned to 4 treatment groups, balanced for BW and litter of origin. Animals were purchased from a Langfang commercial farm and housed in a nursery room. The negative control group (NC) was fed a basal diet, the positive control group (PC) was fed a basal diet supplemented with 2 g/kg zinc oxide, and the HK groups (HK1 or HK2) were fed a basal diet supplemented with 7.5 or 10 g/kg HK, respectively. There were 5 replicates pens per treatment and 5 piglets per replicate. The experiment lasted for 21 days. The hydrolysed yeast from *Kluveomyces fragilis* was provided by Prosol S.p.A, (Madone, Italy). It contains 56.14% crude protein, 6.34% acid soluble protein, 0.14% calcium, 0.84% phosphorus, 0.22% crude fat, 5.48% MOS and 9.66% β-glucanase. During the experiment, the diet for the piglets was formulated meeting the National Research Council (2012) nutrient requirements, and the composition and nutrient levels of the basal diet are shown in Tables 1 and 2. The basal diet did not contain

Table 1. Ingredient and calculated nutrient composition of the basal diet (as fed basis).

Item	Amount, %
Ingredients	
Corn	16.45
Extruded corn	32
Soybean meal	14
Extruded soybean	11.5
Fish meal	5.6
Whey	15
Soybean oil	1
Dicalcium phosphate	0.4
Limestone ($CaCO_3$)	0.75
Salt	0.3
Choline chloride (60%)	0.05
L-Lysine HCl	1.2
DL-Methionine	0.09
Threonine	0.27
Tryptophan	0.02
Phytase	0.02
Acidifier	0.35
Vitamin and mineral premix[a]	1
	100
Calculated nutrient content	
ME, MJ/kg	14.23
Lysine, %	1.3
Methionine, %	0.38
Threonine, %	0.76
Tryptophan, %	0.21

[a]Premix supplied per kg of diet: niacin, 38.4 mg; calcium pantothenate, 25 mg; folic acid, 1.68 mg; biotin, 0.16 mg; vitamin A, 35.2 mg; vitamin B1, 4 mg; vitamin B_2, 12 mg; vitamin B_6, 8.32 mg; vitamin B_{12}, 4.8 mg; vitamin D_3, 7.68 mg; vitamin E, 128 mg; vitamin K_3, 8.16 mg; zinc ($ZnSO_4 \cdot H_2O$), 110 mg; copper ($CuSO_4 \cdot 5H_2O$), 125 mg; selenium (Na_2SeO_3), 0.19 mg; iron ($FeSO_4 \cdot H_2O$), 171 mg; cobalt ($CoCl_2$), 0.19 mg; manganese ($MnSO_4 \cdot H_2O$), 42.31 mg; iodine ($Ca(IO_3)_2$), 0.54 mg.
ME: metabolizable energy.

Table 2. Analysed nutrient composition of diets (as fed basis).

	NC	PC	HK1	HK2
Crude protein, %	19.78	19.53	19.84	20.15
Calcium, %	0.80	0.83	0.86	0.82
Phosphorus, %	0.58	0.60	0.61	0.60
Fat, %	4.17	4.17	4.19	4.20
Ash, %	5.49	5.64	5.36	5.40

NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.

any antibiotic growth promoters and the form of diet was mash.

Piglets were housed in slatted floor pens (1.7 *m* × 1.5 m) and had *ad libitum* access to feed and water. The initial room temperature of the pigpen was 28 °C and was reduced by 1 °C per week to a final temperature of 26 °C. The room used a combination of daylight and artificial light, and ventilation was achieved through the use of speed-controlled fans. Each column was equipped with two nipple drinking fountains and stainless steel adjustable trough. Disinfection procedures and vaccinations were carried out according to farm routine.

Growth performance and diarrhoea incidence

Body weight (BW) was recorded individually at the beginning and the end of the trial. Any culling or mortality was recorded daily and feed consumption corrected for accordingly. Growth performance was evaluated by calculating the average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed ratio (G:F) for each pen. To determine the incidence of diarrhoea, faecal scores was monitored daily by visually appraising each subject using the following five-point faecal consistency scoring system: 1 = hard, dry pellet; 2 = firm, formed stool; 3 = soft, moist stool that retains its shape; 4 = soft, unformed stool; and 5 = watery liquid that can be poured. A liquid consistency (score 4–5) was considered indicative of diarrhoea (Jiang et al. 2015). The incidence of diarrhoea (%) was calculated as a percentage of the number of piglet days (piglets × days of faecal scoring) with diarrhoea divided by the total number of piglet days of faecal scoring in each treatment.

Plasma antioxidant and immune parameters

At the end of the experiment (day 21), one piglet with body weight close to the pen average was selected from each pen. Blood was collected *via* the jugular vein into the heparinised tubes and centrifuged at 3,000 rpm for 10 min after standing for 30 min. The plasma was separated and stored at −20 °C for analysis.

Antioxidant markers include superoxide dismutase (SOD: A001-3, dilution of the samples 1:1), glutathione peroxidase (GSH-Px: A005-1, dilution of the samples 1:0) activity and malondialdehyde (MDA: A003-1, dilution of the samples 1:0) content. Briefly, SOD activity was measured by non-enzymatic NBT assay, which measured the inhibitory effect of superoxide anion free radical formation. The free radical can reduce the content of nitrocyantetrazole in the sample, and the change in absorbance at 450 nm was recorded. GSH-Px activity was measured by 5, 50-disulfide bis-p-nitrobenzoic acid, recording absorbance changes at 412 nm. MDA concentration was measured with 2-thiobarbituric acid, and the change of absorbance at 532 nm was recorded. The change in absorbance at 450 nm was recorded. MDA concentration was analysed with 2-thiobarbituric acids, and the change in absorbance was read at 532 nm.

Immune indicators include Immunoglobulin A (IgA: H108, dilution of the samples 1:0) and Immunoglobulin G (IgG: H106, dilution of the samples 1:4). ELISA kit was used to determine the plasma immunoglobulin (Ig) A and G. According to different dilution concentrations of recombinant porcine immunoglobulin, ELISAcalc (Shanghai, China) was used to fit logistic curve (four parameter equation) and calculate the content. The results were expressed in mg/mL.

The kits for the determination of the above indicators were purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China), and the specific operation steps were performed according to the kit instructions.

Faecal microbial diversity

On day 21, pooled faecal samples from each pen (approximately 20 g) were collected *via* rectal massage. The samples were frozen at −80 °C, and subsequently subjected to DNA extraction and 16S rRNA PCR amplification. The faecal samples were sent to Major bio (Shanghai, China); 16S rRNA gene sequencing was performed on the faeces by high-throughput sequencing technology. DNA concentrations were measured using a Nanodrop-1000 instrument (Thermo Scientific, USA), and DNA quality assessed by agarose (0.8%) gel electrophoresis. The V3-V4 hypervariable region of the 16S rRNA gene was amplified with the barcode fusion primers (338 F: 5-ACTCCTACGGGAGGCAGCAG-3′, 806 R: 5-GGACTACHVGGGTWTCTAAT-3) with 56 °C annealing

temperature. After purification, PCR products were used for constructing libraries and sequenced on an Illumina MiSeq platform (Illumina, USA) at Major bio (Shanghai, China). Illumina sequencing was used (OTU sequence similarity: 0.97, species taxonomy database: silva138/16s_bacteria, Classification confidence: 0.7), all sequences were divided into operational taxonomic units (OTUs) according to the level of similarity, and statistical analysis was performed with OTUs with a similar level of 97%. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP388365). The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7 (Magoč and Salzberg 2011) with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching.

Statistical analysis

Data were analysed as a randomised complete block design using the GLM procedure of SAS 9.2 (SAS Institute 2009). Multiple comparisons were performed by Tukey HSD test, and the chi-square test was used to analyse the piglet diarrhoea incidence and culling/-mortality. The model included the treatment effect, and the pen represented the experimental unit for growth performance and faecal microbiota, while individual piglets were the experimental unit for diarrhoea incidence, culling/mortality, plasma antioxidant and immunoglobulin. Bioinformatic analysis of the faecal microbiota was carried out using the Majorbio Cloud platform (https://cloud.majorbio.com). Based on the OTUs information, alpha diversity indices including observed Ace index, Chao1 index, Shannon index and Simpson index were calculated with Mothur v1.30.1 (Schloss et al. 2009) (http://www.mothur.org/wiki/Calculators). PCoA analysis (principal coordinate analysis) based on bray-curtis distance algorithm was used to test the similarity of microbial community structure between samples. Venn diagram can be used to count the number of common and unique species (such as OTU) in multiple groups or samples, which can show the composition similarity and overlap of species (such as OTU) in environmental samples more intuitively. In general, samples of OTU or other taxonomic levels with a similar level of 97% were selected for analysis and R language (version 3.3.1) software was used for statistics and mapping. The linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/LEfSe) was performed to identify the significantly abundant taxa (phylum to genera) of bacteria among the different groups (LDA score >2, $p < 0.05$). The Kruskal-Wallis rank-sum test was used in the differential species analysis was a nonlinear model (GLM model: generalised linear model). The difference was considered to be significant when $p < 0.05$, and the difference was considered to have a trend when $0.05 \leq p < 0.10$.

Results

Growth performance and diarrhoea incidence

Table 3 shows the effects of dietary HK supplementation on growth performance and diarrhoea incidence of weaned piglets. No significant difference was observed in growth performance between all the experimental groups ($p > 0.05$).

As to diarrhoea incidence, PC evidenced a significant incidence decrease from day 0 to 21 with respect to the other groups ($p < 0.001$), while the higher HK supplementation showed a lower diarrhoea incidence when compared to the NC group ($p = 0.032$).

Table 3. Effect of hydrolysed yeast from *Kluyveromyces fragilis* (HK) supplementation on body weight, growth performance and diarrhoea incidence (DI) of weaned piglets overall the experimental period (0-21d)[A].

Treat	NC	PC	HK1	HK2	SEM	*P*-value
BW, kg						
Day 0	7.03	7.03	7.03	7.03	0.67	0.999
Day 21	10.84	10.77	10.79	10.77	1.13	0.999
ADG, g	181	178	179	178	26	0.999
ADFI, g	339	347	355	334	29	0.915
G:F ratio	0.536	0.498	0.501	0.529	0.041	0.886
DI, %	19.72[a]	5.01[c]	15.80[ab]	14.55[b]	–	<0.01
Culling/Mortality, %	4.00	0.00	4.00	4.00	–	0.794

ADG: average daily gain; BW: body weight; DI: diarrhoea incidence; G:F: gain to feed ratio;
NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.
[A]$n = 20$ (5 replicates per treatment and 5 piglets per replicate).
[a-b]\Means listed in the same row with different superscripts are significantly different ($p < 0.05$).

Table 4. Effect of hydrolysed yeast from *Kluyveromyces fragilis* (HK) supplementation on plasma antioxidant efficiency of weaned piglets[A].

Item	NC	PC	HK1	HK2	SEM	*P*-value
SOD, U/mL	25.33[b]	31.73[a]	29.82[ab]	33.5[a]	1.33	0.004
MDA, nmol/mL	3.15[a,x]	2.36[b]	2.47[ab,y]	2.17[b]	0.16	0.012
GSH-Px, U/mL	348	345	363	372	11	0.333

NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; SOD: superoxide dismutase; MDA: malondialdehyde; GSH-Px: glutathione peroxidase.
[A]$n = 20$ (5 replicates per treatment and 1 piglet per replicate).
[a,b]Means listed in the same row with different superscripts are significantly different ($p < 0.05$).
[x,y]Means listed in the same row with different superscripts have tendency to be different ($0.05 \leq p < 0.10$).

Table 5. Effect of hydrolysed yeast from *Kluyveromyces fragilis* (HK) supplementation on plasma immunoglobulin of weaned piglets[A].

Item	NC	PC	HK1	HK2	SEM	*P*-value
IgA, mg/mL	1.32[b,y]	1.76[ab,x]	1.41[b]	1.96[a]	0.10	0.003
IgG, mg/mL	33.86	46.00	44.59	46.50	4.80	0.285

NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; IgA: Immunoglobulin A; IgG: Immunoglobulin G.
[A]$n = 20$ (5 replicates per treatment and 1 piglet per replicate).
[a,b]Means listed in the same row with different superscripts are significantly different ($p < 0.05$).
[x,y]Means listed in the same row with different superscripts have tendency to be different ($0.05 \leq p < 0.10$).

Plasma antioxidant efficiency

The effect of hydrolysed yeast from *Kluyveromyces fragilis* (HK) supplementation on plasma antioxidant efficiency of weaned piglets is presented in Table 4. Compared with the NC group, dietary supplementation with 2 g/kg ZnO and 10 g/kg HK enhanced the activity of SOD ($p = 0.019$ and 0.003, respectively) and decreased the concentration of MDA in the plasma of piglets ($p = 0.042$ and $p = 0.010$, respectively). In addition, dietary HK1 tended to decreased plasma MDA content compared to the NC group ($p = 0.089$). There was no significant difference in plasma GSH-Px activity among all groups ($p > 0.05$).

Plasma immunoglobulin

Table 5 shows the effects of dietary HK supplementation on plasma immunoglobulins of weaned piglets. Compared with the NC group, the dietary supplementation with 10 g/kg HK significantly increased the concentration of IgA in weaned piglets ($p = 0.005$), and PC group tended to increase the plasma IgA level ($p = 0.059$). In addition, there was no significant difference in plasma IgG content among all groups ($p > 0.05$).

Faecal microbial community

Venn diagrams were drawn according to the OTUs in the faeces on day 21 (Figure 1), which can be used to count the number of unique and shared OTUs in different samples and can intuitively reflect the similarity and overlap of the composition of OTUs in the samples. Faecal bacterial community of four groups shared 533 OTUs (approximately 52%), and the unique OUTs in the NC, PC, 7.5 g/kg HK and 10 g/kg HK were 37, 14, 22 and 94, respectively.

Compared with the NC group, the Ace index and Chao1 index in the 10 g/kg HK group were significantly increased ($p = 0.023$ and 0.018, respectively; Table 6). In addition, dietary supplementation with 10 g/kg HK significantly increased the Chao1 index ($p = 0.049$) and tended to increase the Ace index compared to the PC group ($p = 0.075$). Compared with NC group, the diets of 7.5 g/kg HK and 10 g/kg HK groups had no significant effects on Shannon index and Simpson index ($p > 0.05$).

β-diversity analysis explores the similarity or difference of community composition among different groups of samples by comparing and analysing the species diversity among different habitats or microbial communities. The more similar the community composition of the samples is, the closer they are in the PCoA map. According to Figure 2, compared with the NC group, the PC group showed obvious separation, indicating that the microbial community composition between the two groups was significantly different. However, the 7.5 g/kg HK and 10 g/kg HK groups were close to the NC group, indicating that the microbial community composition among the three groups was similar.

At the phylum level, the faecal microbial community of weaned piglets was dominated by Firmicutes and Bacteroidota, accounting for approximately 90% of the total microbial population (Figure 3(A)). Among them, the relative abundances of Firmicutes in the NC, PC, 7.5 g/kg HK and 10 g/kg HK groups were 68.31%, 67.63%, 65.08% and 71.76%, and the relative abundances of Bacteroidota were 18.46%, 28.03%, 25.08% and 22.21%. The relative abundance of Firmicutes was the highest in the 10 g/kg HK group, and the relative abundance of Bacteroidota was the highest in the PC group.

At the genus level, the relative abundances of *Lactobacillus* in the NC, PC, 7.5 g/kg HK and 10 g/kg HK groups were 20.68%, 3.43%, 6.65% and 13.22%, and the relative abundances of *Escherichia-Shigella* were 9.76%, 0.47%, 5.56% and 3.07%, and the relative abundances of *Clostridium_sensu_stricto_1* were 3.18%,

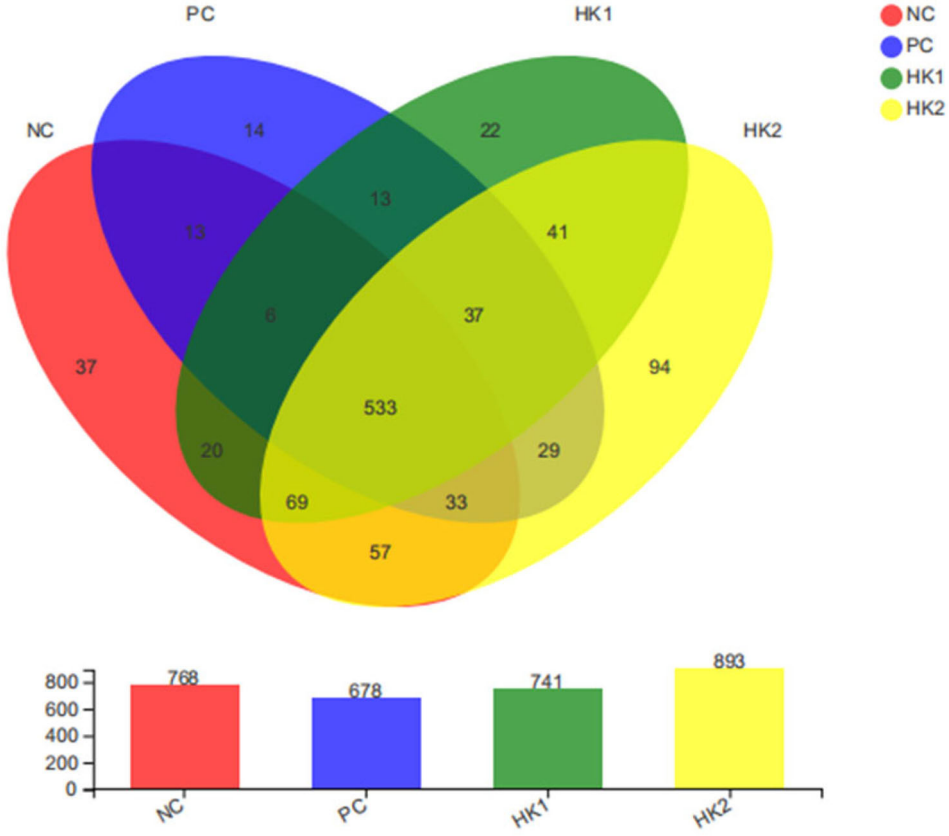

Figure 1. OTU Venn diagram. NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.

Table 6. Effect of hydrolysed yeast from *Kluyveromyces fragilis* (HK) supplementation on faecal microbiota α diversity of weaned piglets[A].

Item	NC	PC	HK1	HK2	SEM	*P*-value
Ace index	413[b]	456 [ab,y]	481[ab]	631[a,x]	29	0.045
Chao1 index	418[b]	455[b]	493[ab]	653[a]	31	0.040
Shannon index	3.64	4.03	4.34	4.48	0.15	0.267
Simpson index	0.10	0.06	0.03	0.03	0.02	0.375

NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.
[A]$n = 20$ (5 replicates per treatment and 1 piglet per replicate).
[a-b]Means listed in the same row with different superscripts are significantly different ($p < 0.05$).
[x,y]Means listed in the same row with different superscripts have tendency to be different ($0.05 \leq p < 0.10$).

20.12%, 7.37% and 7.41%. The relative abundances of *Lactobacillus* and *Escherichia-Shigella* in the NC group were higher than those in the others three groups, and the relative abundance of *Clostridium_sensu_stricto_1* in the PC group was higher than that in the others three groups (Figure 3(B)).

The phylum-level and genus-level differentiation of species composition is presented in Figure 4. At the phylum level, the relative abundance of Campylobacterota in the NC group was significantly higher than that in the others three groups ($p = 0.023$; Figure 4(A)). At the genus level, there were differences in the relative abundances of *Lactobacillus*, *Clostridium_sensu_stricto_1*, *Escherichia-Shigella*, *norank_f__Erysipelotrichaceae* among the treatment groups (Figure 4(B)). Compared with the NC group, the PC and 7.5 g/kg HK groups significantly decreased the *Lactobacillus* relative abundance ($p = 0.014$), and three treated groups significantly decreased the abundance of *Escherichia-Shigella* ($p = 0.032$). Compared with the PC group, the abundance of *Clostridium_sensu_stricto_1* in the other three groups was significantly decreased ($p = 0.028$).

Discussion

The present study allowed observing that dietary supplementation of 10 g/kg HK improved plasma antioxidant efficiency and immune status and reduced the diarrhoea incidence in weaned piglets. Early weaning can cause severe oxidative stress in piglets. Oxidative stress is an imbalance between the production and scavenging of reactive oxygen species, leading to irreparable oxidative damage, which in turn triggers a series of related diseases and affects the function of the entire body (Valko et al. 2007; Li et al. 2012).

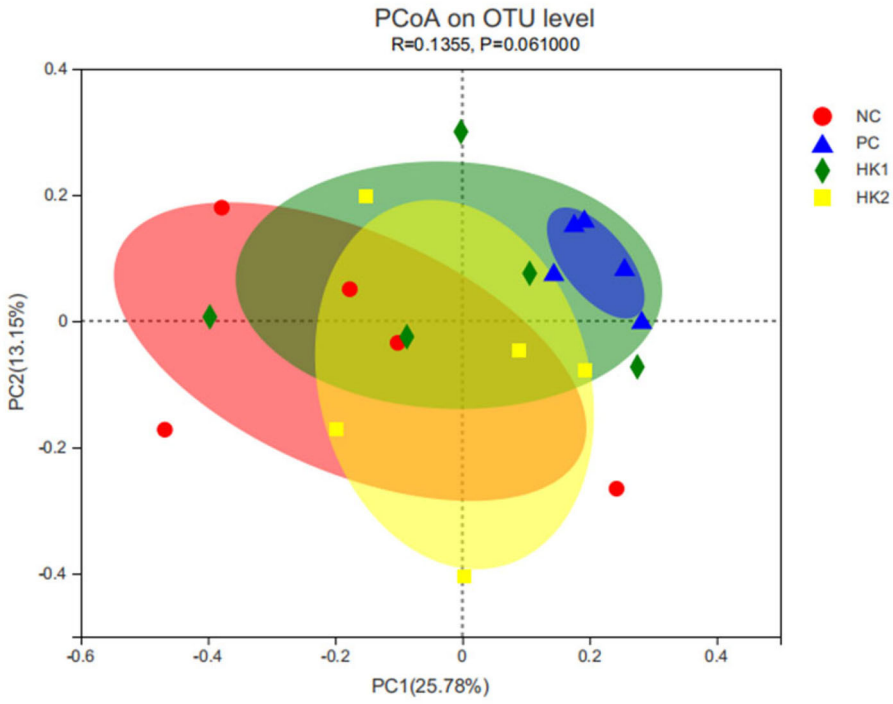

Figure 2. Faecal microbial β diversity (OTU level). NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.

Previous studies have found that weaning can disrupt the oxidative balance in the body, destroy the normal metabolism of free radicals, and reduce the antioxidant efficiency of weaned piglets (Yin et al. 2014). MDA is one of the main products of lipid peroxidation, which directly reflects the degree of lipid oxidative damage (Pirinccioglu et al. 2010). The enzymatic antioxidant system is the first defense line to protect the body from harmful pro-oxidants. SOD and GSH-Px are the endogenous enzymatic antioxidants, which are important enzymes in the system and reflects the strength of the body's antioxidant efficiency (Bazhin et al. 2016; Chen et al. 2020). Previous studies have found that yeast hydrolysate supplementation can increase the activities of SOD and GSH-Px in serum of broilers aged 21 days (Wang et al. 2022). Supplementation of 1.0 and 1.5 g/kg yeast hydrolysate in the diets of weaned piglets reduced the concentration of MDA in blood (Boontiam et al. 2020). Adding yeast mannan oligosaccharide to the diet of gestation sows could improve the activity of SOD in their progeny piglets (Czech et al. 2009). Our study observed that the supplementation of 10 g/kg HK or 2 g/kg ZnO to the diet increased the plasma SOD activity and reduced the MDA concentration in weaned piglets, indicating that 10 g/kg HK and ZnO have similar effects on antioxidant efficiency.

IgA and IgG in plasma play an important role in animal immune response and are the main antibodies against infection (Sun et al. 2010; Han et al. 2018). He et al. (2020) reported that supplementation of *Saccharomyces cerevisiae* to the diet increased IgA and IgG concentrations in the blood of weaned piglets. The addition of yeast hydrolyzate to the diet of weaned piglets was reported to increase plasma IgA concentration of weaned piglets (Boontiam et al. 2020; 2022). In our study, adding 10 g/kg HK could increase the plasma IgA concentration, while the addition of ZnO showed no significant effect, indicating that 10 g/kg HK might be more effective than high dose ZnO in improving the immunity of weaned piglets.

The intestinal microbiota is closely related to the health of the organism and the occurrence of diseases. The intestinal microbiota and the body are interdependent and restrict each other (Liu et al. 2021). The diversity of intestinal microbes affects gut health and therefore the overall health of animal (Marchesi et al. 2016). Da et al. (2021) found that adding 2.5 g/kg ZnO to the diet reduced the abundance of caecal microbiota, and tended to decrease the Chao1 index. Yu et al. (2017) found that adding 3 g/kg ZnO to the diet reduced the microbiota diversity of colonic digesta. Addition of live yeast additive can reduce the number of intestinal microbial pathogens in piglets (Gustavo et al. 2022). Many studies demonstrated that *Saccharomyces cerevisiae* supplementation regulated microbial diversity in the caecal contents of suckling piglets (Kiros et al. 2019) and regulated sow faecal

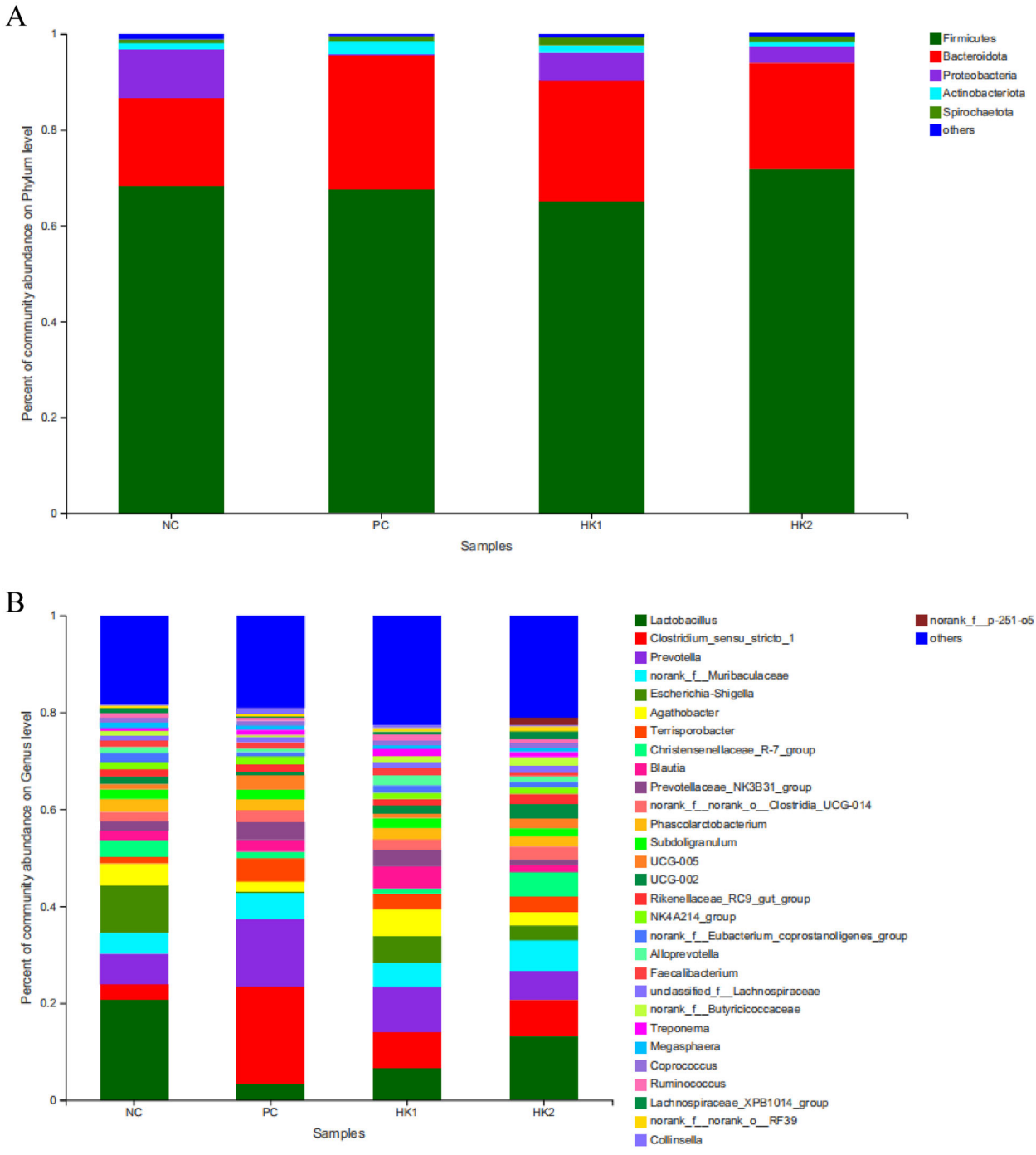

Figure 3. Relative abundance of species at the phylum (A) and genus (B) levels. The Y-axis represents the average relative abundance, the X-axis represents the different groups. NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.

microbiota (Zhao et al. 2022). In our study, we found an increase in total and unique OTUs in the 10 g/kg HK group compared to the other three groups. The Ace and Chao1 indexes are positively related to the relative abundance of species (Wang et al. 2020). In this experiment, PC group increased the Chao1 index, while supplementation with 10 g/kg HK increased the Ace and Chao1 indexes. Related studies showed that for intestinal microbiota β diversity, two different clusters were formed in dietary samples with and without zinc oxide, indicating the strong effect of zinc oxide on intestinal microbiota (Da et al. 2021). This experimental study showed that compared with the NC group, the PC group showed obvious outlier clusters

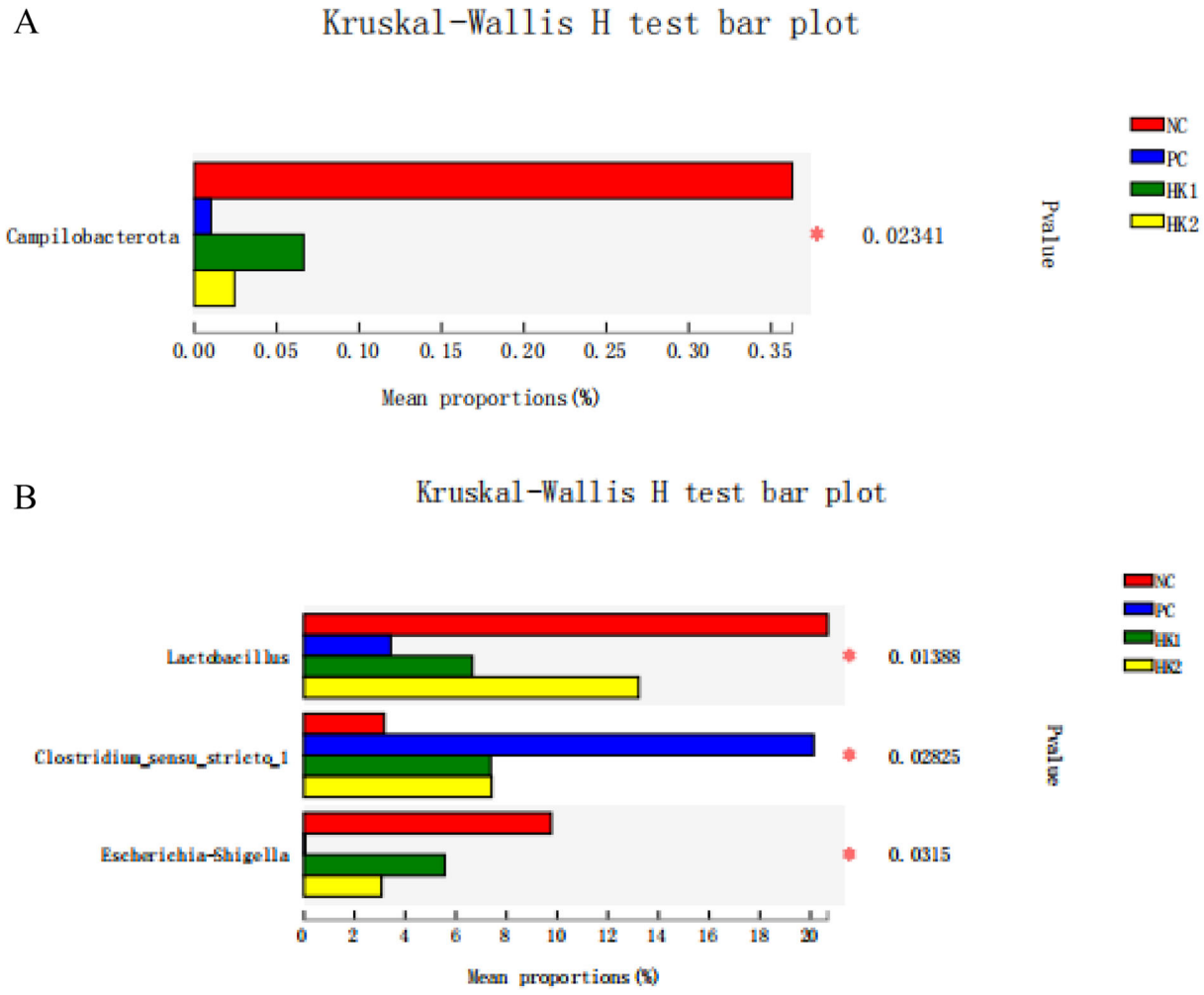

Figure 4. Significance test of difference between treatments at phylum (A) and genus (B) levels. The Y-axis represents the species names at a certain taxonomic level, the X-axis represents the average relative abundance in different groups of species, and the columns with different colours represent different groups; the far right is the *p*-value, $^*p < 0.05$. NC: basal diet without additive; PC: NC + 2 g/kg zinc oxide; HK1: NC + 7.5 g/kg hydrolysed yeast from *Kluyveromyces fragilis*; HK2: NC + 10 g/kg hydrolysed yeast from *Kluyveromyces fragilis*.

for β diversity analysis, while the 7.5 g/kg HK and 10 g/kg HK groups had no obvious separation. We suggest that high dose ZnO may have an effect on microbiota balance. Thus, the addition of HK with 10 g/kg to the diet can increase the richness of faecal microbiota of weaned piglets.

The composition and structure of microbial community were greatly affected by dietary composition and environmental factors. Firmicutes has the highest abundance at the phylum level among gut microbes of piglets, followed by Bacteroidetes, Proteobacteria, Actinobacteria, and Spirochaetes (Kim et al. 2011). In our study, there were 5 major phyla in the faeces of piglets including Firmicutes, Bacteroidetes, Proteobacteria, Actinomycetes, and Spirobacterium. There was no significant difference between the HK groups and the NC group, indicating that HK could maintain the balance of faecal microorganisms at the phylum level. The addition of various forms of *Saccharomyces cerevisiae* supplements (heat-killed whole yeast or superfine yeast powders) to the diet reduced the number of *E. coli* in the ileal and caecal contents of weaned piglets (Zhu et al. 2017). This study showed that both PC group and HKs groups decreased *Escherichia-Shigella* abundance, but high dose ZnO supplementation increased *Clostridium_sensu_stricto_1* abundance and decreased *Lactobacillus* abundance. In addition, the addition of 7.5 and 10 g/kg HK in the diet reduced the abundance of *Escherichia-Shigella* at the genus level, and the abundance of other genera was the same as that of the NC group, indicating that the addition of HK in the diet can reduce harmful intestinal bacteria and maintain the health of intestine.

Our study confirmed that the addition of high dose zinc oxide to the diet could reduce the diarrhoea rate

of weaned piglets, which is consistent with the previous studies (Shelton et al. 2011; Yu et al. 2017). Yeast hydrolyzate has a variety of beneficial bioactive substances (yeast nucleotides and yeast cell wall polysaccharides, etc.) and has the ability to improve growth performance and reduce the diarrhoea incidence of piglets after weaning (Boontiam et al. 2020). Our current study confirmed that adding 10 g/kg HK to the diet could reduce the diarrhoea incidence of weaned piglets that might be attribute to the improved health status and microbial community. Keimer, Pieper et al. (2018) observed that dietary HK with 10 g/kg had better growth performance compared to the piglets fed with 30 and 50 g/kg HK. In our study, the addition of HK with 7.5 and 10 g/kg to the diet had no significant effect on growth performance of piglets, which may be due to the limited experimental period, enriched rearing environment and good physical condition of weaned piglets, and it could also be that the energy was used for growth but for immunity and oxidative stress in treated piglets, thus further experiments are needed to verify the dietary effect of HK on growth performance extending the feeding period or under the challenge condition.

Conclusion

The addition of 10 g/kg HK to the diet, could be alternative to the high dose ZnO, reduced the diarrhoea incidence of weaned piglets, which might be related to the improvement of the antioxidant efficacy and immune status in the plasma and the regulation of microbial community in the faeces. In addition, the addition of 10 g/kg HK had more efficacious than 7.5 g/kg HK, which was suggested as the optimal dosage.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

The current study was carried out according to the regulations approved by the Animal Care and Use Committee of the Institute of Feed Research of the Chinese Academy of Agricultural Sciences with an approved number IFR-CAAS-20210705.

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Data availability statement

Datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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