Effects of Glucagon-Like Peptide-2-Expressing *Saccharomyces cerevisiae* Not Different from Empty Vector^S

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Saccharomyces cerevisiae (*S. cerevisiae*) and glucagon-like peptide-2 (GLP-2) have been employed to improve the intestinal development of weaned animals. The goal of this study was to determine whether either exogenous *S. cerevisiae* or GLP-2 elicits major effects on fecal microbiotas and cytokine responses in weaned piglets. Ninety-six piglets weaned at 26 days were assigned to one of four groups: 1) Basal diet (Control), 2) empty vector-harboring *S. cerevisiae* (EV-SC), 3) GLP-2-expressing *S. cerevisiae* (GLP2-SC), and 4) recombinant human GLP-2 (rh-GLP2). At the start of the post-weaning period (day 0), and at day 28, fecal samples were collected to assess the bacterial communities via sequencing the V1-V2 region of the 16S-rRNA gene, and piglets' blood was also sampled to measure cytokine responses (*i.e.*, IL-1 β , TNF- α , and IFN- γ). This study revealed that, on the one hand, although *S. cerevisiae* supplementation did not significantly alter the growth of weaned piglets, it induced increases in the relative abundances of two core genera (*Ruminococcaceae_norank* and *Erysipelotrichaceae_norank*) and decreases in the relative abundances of two other core genera (*Lachnospiraceae_norank* and *Clostridiales_norank*) and cytokine levels (IL-1 β and TNF- α) ($p < 0.05$, Control vs EV-SC; $p < 0.05$, rh-GLP2 vs GLP2-SC). On the other hand, GLP-2 supplementation had no significant influence on fecal bacterial communities and cytokine levels, but it produced better body weight and average daily gain ($p < 0.05$, Control vs EV-SC; $p < 0.05$, rh-GLP2 vs GLP2-SC). Therefore, altered fecal microbiotas and cytokine response effects in weaned piglets were due to *S. cerevisiae* rather than GLP-2.

Keywords: *Sus scrofa*, weaned piglets, *Saccharomyces cerevisiae*, glucagon-like peptide-2, fecal microbiotas

Introduction

During the weaning stage, piglets are susceptible to infections, diarrhea, and a number of other disorders contributing to post-weaning problems, such as maldigestion and malabsorption, which are widespread health concerns in the swine industry [1, 2]. In addition to being subjected to dramatic changes in their social (*i.e.*, mixing with unfamiliar piglets) and physical environments (*i.e.*, transferring to an

unfamiliar pen), after weaning, piglets primarily suffer from being abruptly separated from sows and the subsequent significant shift in diet [3]. Because of this switch from highly-digestible milk to a less-digestible more complex feed, a significant decrease of circulating glucagon-like peptide-2 (GLP-2) concentration is observed in weaned piglets compared with suckling piglets of the same age, and this subsequent decrease in GLP-2 after weaning is a response to the marked decline in the piglets' nutrient

intakes [4]. Thus, this so-called 'weanling stress' can be accompanied by a dramatically decreased intake of GLP-2 [5]. Furthermore, the weaning process also results in dramatic shifts in intestinal structure and function toward post-weaning crypt elongation and villous atrophy [6, 7]. GLP-2 is primarily secreted from enteroendocrine L-cells in response to the ingestion of carbohydrates and fat, and this peptide can stimulate intestinal adaptation and growth [4]. Therefore, weaned piglets may be a potential target for GLP-2 treatment [4, 8]. Growing evidence has demonstrated that exogenous supplementation with GLP-2 can effectively stimulate the intestinal development of weaned rats and piglets [5, 8]. However, GLP-2 can be rapidly degraded by the intestinal enzyme dipeptidyl peptidase IV to form an inactive peptide metabolite [9]. For GLP-2 to elicit trophic effects on the gastrointestinal tracts (GIT) of weaned animals, it must survive digestive and cellular metabolic processes to maintain an effective concentration for binding the GLP-2 receptor, which subsequently stimulates the differentiation and growth of enterocytes [8]. Generally, the half-life of GLP-2 is only approximately eight minutes in animals, and the long-term maintenance of the circulation of GLP-2 at an effective concentration could be a potential strategy for decreasing weaning stress [9].

As a non-invasive and non-pathogenic eukaryote, *Saccharomyces cerevisiae* (*S. cerevisiae*) has been used as a common tool for protein expression in research, industry and medicine [10]. Recently, the potential of *S. cerevisiae* to generate fully biologically active epidermal growth factor (EGF) or other growth factors has been explored in some studies. For example, several recent studies have constructed the recombinant EGF-expressing *S. cerevisiae*, which can survive inside the GIT rather than outside, thus avoiding the risk of the genetically modified bacterium influencing the environment [2, 11]. As a dietary supplement for neonatal mammals during the transition from the weaning stage, the use of combinations of recombinant milk-borne growth factors (*i.e.*, EGF and GLP-2) and probiotics (*i.e.*, *S. cerevisiae* and *Lactobacillus* GG) has previously been evaluated for improving the intestinal development of weaned animals [5, 11, 12].

There is rather limited evidence to investigate the effects of the combination of recombinant milk-borne growth factors and probiotics on the intestinal microbiotas and cytokine responses in weaned piglets. Furthermore, it also has remained unclear whether either exogenous milk-borne growth factors or probiotics elicit major effects on intestinal development. Therefore, the goals of this study were to analyze the growth, serum cytokines, and fecal

microbiotas of weaned piglets that were fed GLP-2-expressing *S. cerevisiae*, and then determine whether either exogenous GLP-2 or *S. cerevisiae* elicits major effects on these parameters.

Materials and Methods

Production of a Recombinant *S. cerevisiae* Strain Expressing GLP-2

S. cerevisiae strains harboring an empty vector backbone or expressing recombinant GLP-2 were designated as EV-SC (empty vector) and GLP2-SC, respectively. The EV-SC and GLP2-SC strains were generated and cultured as described previously [5]. Briefly, the strains of EV-SC and GLP2-SC were fermented in 5-liter baths (SC-U medium supplemented with 2% D-glucose and 1 µg/ml ampicillin). Fermentations ran for 24 h in a fermentation system (Zhenjiang East Biotechnology Equipment Co., Ltd., China) filled with SC-U medium broth at 28°C with continuous agitation (120 rpm). Each fermentation batch was inoculated with a 50 ml preculture. The culture pH was allowed to drop naturally to 3.50, after which it was maintained at 5.50 and glucose was added at 5 ml/h. To regulate fermentation conditions, 2 N H₂SO₄, 10 N NH₄OH, and 50% glucose were used in this study. Precultures were all taken from one 24 h fermentation with an optical density of 4.00 at 600 nm, and frozen at -80°C prior to trial fermentations.

Animal Experiments

The animal procedures performed in this study were based on the guidelines of the China Animal Protection Association, and all of the work was approved by the Animal Care and Use Committee of West China Hospital, Sichuan University.

A total of 96 piglets weaned at 26 days of age were obtained from the Shenzhen Premix Inve Nutrition Co., Ltd., and randomly assigned to one of the following four treatments: 1) Basal diet supplemented with SC-U media (Control), 2) empty vector-harboring *S. cerevisiae* (EV-SC), 3) GLP-2-expressing *S. cerevisiae* (GLP2-SC), and 4) SC-U media with recombinant human GLP-2 (rh-GLP2) (Phoenix Pharmaceuticals, USA). Thus, 24 piglets were assigned to each group, with four pens (as an experimental unit) per group and six piglets per pen. Average initial body weight (BW) of the piglets in each pen was 6.35 ± 0.06 kg (means ± SEM).

The concentration of the GLP-2 protein expressed by *S. cerevisiae* was 1.35 mg/l according to a previously described study [5], and the count of live *S. cerevisiae* was 1.92 × 10⁹ per liter based on the plate-counting method. All treatments were manually mixed in with equal weights of solid feed before delivering the mix at 0800 h daily for four consecutive weeks. Briefly, throughout the 28-day trial, each kg of diet in the EV-SC and GLP2-SC groups was supplemented with 167 ml fresh cultures of the EV-SC and GLP2-SC strains, respectively. The control group was only given fresh SC-U media (the same volume), whereas the rh-GLP2 group, as the positive control, was additionally supplemented with the same dosage (1.35 mg/l) of the rh-GLP2 protein (Phoenix

Pharmaceuticals). Thus, each piglet in the treatment groups was given 1.44×10^8 live *S. cerevisiae* [0.45 kg of average daily feed intake (ADFI) * 0.167 L of fresh cultures per kg of solid diet * 1.92×10^9 /L live *S. cerevisiae*] and 100 µg GLP-2 (0.45 kg of ADFI * 0.167 L of fresh cultures per kg of solid diet * 1.35 mg/l of GLP-2) per day. The diet (Table S1) was formulated in powder form without any in-feed antibiotics in accordance with the guidelines of National Research Council [13]. The piglets had *ad libitum* access to water and feed, and remaining feed was weighed at 0800 h every day. ADFI and BW were recorded weekly to assess the feed-to-gain ratio (F/G) and average daily gain (ADG).

Sample Collection and Processing

On day 0 and day 28, one piglet from each pen was selected to collect fresh fecal samples using sterile plastic fecal loops, which were inserted into the rectum of these piglets for sampling. The collected fecal samples were subsequently placed into a sterilized 10 ml centrifuge tube and stored in liquid nitrogen until further processing.

Additionally, blood from the anterior vena cava of piglets was also sampled on days 0 and 28. The samples were subsequently centrifuged at 4,200 g for 15 min at 4°C to obtain the serum, which was stored at -80°C until further analysis.

Cytokine Assays

Serum concentrations of the porcine cytokines IL-1β, TNF-α, and IFN-γ were assessed using commercially available ELISA kits (Quantikine Porcine Immunoassays, R&D Systems, UK). The dynamic assay range of IL-1β, TNF-α, and IFN-γ was 39.10 to 2,500 pg/ml, 23.40 to 1,500 pg/ml, and 39.00 to 2,500 pg/ml, respectively. The minimum detectable dose of IL-1β, TNF-α, and IFN-γ was 13.60, 5.00, and 11.20 pg/ml, respectively.

DNA Extraction

Total DNA was extracted from fecal samples with an E.Z.N.A. Stool DNA Kit following the manufacturer's instructions. First, an approximately 200 mg stool sample was directly weighed into an Eppendorf tube (2 ml), and 200 mg glass beads (≤ 0.10 mm) and 540 µl SLX-Mlus buffer (see in the E.Z.N.A. Stool DNA Kit) were also added. Following vortex oscillation at maximum speed for 10 min, the subsequent steps were then performed according to the E.Z.N.A. Stool DNA Kit's instructions (Omega Bio-tek, Inc., USA). The quality of the obtained DNA was further verified via an electrophoresis analysis.

Amplification and High-throughput Sequencing

The V1-V2 region of the bacterial 16S rRNA gene was amplified from all of the fecal DNA samples obtained in this study and were sequenced on an Illumina MiSeq platform (Shanghai Personal Biotechnology Co., Ltd., China). Briefly, the V1-V2 of the 16S rRNA gene was PCR amplified using the previously described primer set 8F-338R [14]. The PCR reaction system, which was

purchased from Beijing TransGen Biotech Co., Ltd. (China), was carried out in 50 µl reaction volumes containing 25 ng DNA template, 0.40 mM primer (each), 2.50 U Pfu polymerase and 0.25 mM dNTPs. The PCR thermocycling conditions used were as follows: an initial denaturation at 94°C for 4 min, followed by 25 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (30 s), with a final extension at 72°C for 10 min. To avoid bias, we conducted three independent PCRs for each sample.

Bioinformatics and Statistical Analyses

Raw Fastq files were demultiplexed and quality filtered using QIIME (version 1.17) according to the following criteria [15]: 1) the 250-bp reads were truncated at any site receiving an average quality score less than 20 over a 10-bp sliding window; 2) specific barcodes were exactly matched; 3) truncated reads of less than 50 bp were removed; 4) reads containing ambiguous characters were removed; 5) allowed mismatching with primers was at most 1 bp; 6) reads that could not be assembled were discarded; and 7) only sequences that overlapped by more than 10-bp were further assembled.

Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) and chimeric sequences were identified and removed using UCHIME. Alpha diversity indices were determined using Mothur (version v.1.30.1, <http://www.mothur.org>). Community diversity was evaluated by the Shannon (<https://www.mothur.org/wiki/Shannon>) and Simpson indices (https://www.mothur.org/wiki/Simpson_similar). To select OTUs that exhibited significant structural segregation among groups tested, a parametric partial least squares discriminant analysis (PLS-DA) model was generated using Simca-P+12.0 (<http://www.umetrics.com/>). Additionally, the linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used to identify OTUs, and cladograms were produced using the online LEfSe tool (<http://huttenhower.sph.harvard.edu/galaxy/>).

The abundances of OTUs higher than 1.00% of the community were recorded as the dominant OTUs, which were subsequently sorted for comparing the differences among the treatment groups. The growth performances (*i.e.*, BW, ADG, ADFI, and F/G) were assessed using each pen as the experimental unit, which along with the compositions of bacterial communities from the phylum to the genus level were tested using one-factor ANOVA, with the different treatments used as one factor. The data was expressed as means $\pm$ standard error of the mean (SEM), and significant differences were declared when $p < 0.05$.

Results

Effects of the GLP-2-Expressing *S. cerevisiae* Strain on the Growth Performance of Weaned Piglets

By day 28, Table 1 displayed that GLP-2 supplementation increased the ADG and final BW of weaned piglets ($p <$

Table 1. Growth performance of weaned piglets.

Item	Control	EV-SC	GLP2-SC	rh-GLP2	P-value
	Means ± SEM (n = 4)				
Initial-BW (Day 0)	6.36 ± 0.14	6.35 ± 0.12	6.35 ± 0.13	6.36 ± 0.13	0.997
Final-BW (Day 28) (kg)	13.83 ± 0.23 ^a	14.36 ± 0.13 ^a	15.24 ± 0.39 ^b	15.05 ± 0.33 ^b	0.002
ADG (g)	267 ± 1.54 ^a	286 ± 1.77 ^a	318 ± 2.24 ^b	311 ± 2.07 ^b	0.002
ADFI (g)	440 ± 1.48 ^a	457 ± 1.99 ^{ab}	472 ± 1.21 ^b	468 ± 2.00 ^b	0.015
F/G	1.65 ± 0.09 ^a	1.60 ± 0.13 ^{ab}	1.49 ± 0.16 ^b	1.51 ± 0.10 ^b	0.016

BW: body weight, ADG: average daily gain, ADFI: average daily feed intake; F/G: feed-to-gain ratio, BW: body weight.

^{a,b}Mean values within a row with different superscript letters were significantly different ($p < 0.05$).

0.05, Control vs rh-GLP2; $p < 0.05$, EV-SC vs GLP2-SC). On the contrary, weaned piglets fed with *S. cerevisiae* had no significant influence in growth performance, including ADG, Final BW, ADFI and F/G (Control vs EV-SC; rh-GLP2 vs GLP2-SC). Taken together, compared with weaned piglets fed a diet supplemented with *S. cerevisiae*, GLP-2 showed better growth.

Operational Taxonomic Units (OTUs) and Alpha Diversity Analyses

The median of observed OTUs and Shannon and Simpson diversity indices were 1,188 (range: 812-1,661), 3.78 (range: 3.12-5.45), and 0.04 (range: 0.02-0.07), respectively (Figs. 1A–1C and Table S2).

By day 28, GLP-2 supplementation had no significant

Fig. 1. Fecal microbial diversities and bacterial compositions of weaned piglets.

(A–C) Fecal microbial diversities (normalized to sequence reads): The median of observed OTUs (A), Shannon diversity indices (B), and Simpson diversity indices (C). OTUs; (D) Bacterial compositions at genus level. Control group: basal diet; EV-SC group: basal diet supplemented with empty vector-harboring *S. cerevisiae*; GLP2-SC group: basal diet supplemented with GLP-2-expressing *S. cerevisiae*; rh-GLP2 group: basal diet supplemented with recombinant human GLP-2. OTUs, operational taxonomic units; Bars (mean $\pm$ SEM, $n = 4$) with different letters are considered significantly different ($p < 0.05$).

influence on the numbers of observed OTUs, Shannon diversity indices, and Simpson diversity indices (Control vs rh-GLP2; EV-SC vs GLP2-SC). Intriguingly, the results showed that weaned piglets fed with *S. cerevisiae* had significantly increased numbers of observed OTUs and Shannon diversity indices ($p < 0.05$, Control vs EV-SC; $p < 0.05$, rh-GLP2 vs GLP2-SC) (Figs. 1A–1C).

Effects of the GLP2-Expressing *S. cerevisiae* on the Fecal Bacterial Community of Weaned Piglets

Population dynamics from phylum level to family level were displayed in Table S3. To gain a deeper understanding of population dynamics, the bacterial community at genus level was then analyzed in this study. First, the OTUs (Fig. 1D and Table S4) were classified into 21 bacterial genera, although only the following 11 bacterial genera had $\geq 2.0\%$ of overall relative abundance: *Ruminococcaceae_norank* (17.61%), *Clostridiale_norank* (15.30%), *Lachnospiraceae_norank* (12.66%), *Erysipelotrichaceae_norank* (7.81%), *Lactobacillus* (7.70%), *Prevotella* (6.24%), *Bacteroidales_norank* (3.74%), *Clostridiaceae_norank* (3.06%), *Eubacterium* (3.02), *S24-7_norank* (2.55%), and *Catenibacterium* (2.24%). Less than 3.5 % of the sequences were not classified into bacterial genera.

On the basis of these 21 bacterial phyla, we further gained the most common core OTUs ($>1.00\%$ relative abundance in $\geq 80.00\%$ of piglets) (Table 2). By days 28, 10, 13, 16, and 10 core OTUs with $>1\%$ of the community at genus level could be identified in the Control, EV-SC, GLP2-SC and rh-GLP2 groups, respectively (Table 2). To compare the relative abundances of these core OTUs, we found that GLP-2 supplementation did not significantly change the following 9 core genera, including *Ruminococcaceae_norank*,

Clostridiale_norank, *Lachnospiraceae_norank*, *Erysipelotrichaceae_norank*, *Lactobacillus*, *Bacteroidales_norank*, *Clostridiaceae_norank*, *Eubacterium*, and *Catenibacterium* (Control vs rh-GLP2; EV-SC vs GLP2-SC) (Fig. 2). Notably, weaned piglets fed a diet supplemented with *S. cerevisiae* exhibited lower relative abundances of *Clostridiale_norank* and *Lachnospiraceae_norank* but higher relative abundances of *Ruminococcaceae_norank* and *Erysipelotrichaceae_norank* ($p < 0.05$, Control vs EV-SC; $p < 0.05$, rh-GLP2 vs GLP2-SC).

Compositional Differences of Bacterial Community

The PLS-DA, a supervised analysis method, allows for the detection of individual community differences that indicates distinctive fecal microbial communities among groups tested. By day 28, according to the relative abundance of bacterial taxa, the PLS-DA score plots among different groups showed a clear discrimination (Fig. 3). Specifically, the samples in GLP2-SC group were well separated from other groups based on the weighted UniFrac distances. Furthermore, the PLS-DA showed that the individuals fed with or without GLP-2 supplementation were also not associated with different microbiotas, but the individuals fed with or without *S. cerevisiae* supplementation clearly had different microbiotas (Fig. 3).

LEfSe, an effect size measurement method, was then utilized to identify dominant OTUs. By day 28, LDA (Fig. 4) showed that a total of 42 bacterial taxa differed in relative abundance ($\alpha = 0.01$, LDA score > 3.0) among tested groups. To further identify core OTUs, the probabilistic modeling with the LEfSe algorithm revealed 7, 10, 21, and 3 OTUs characteristic in the Control, EV-SC, GLP2-SC, and rh-GLP2 groups, respectively (Fig. 4). Specifically, at family

Table 2. Genus-level taxonomy of abundant OTUs* of fecal microbiotas in weaned piglets ($n = 4$).

Groups	Genera of the core OTUs* of fecal microbiotas (% of piglets with OTUs)
	Core microbiotas
Day 0	<i>Prevotella</i> (100), <i>Bacteroidales_norank</i> (100), <i>Clostridiale_norank</i> (100), <i>S24-7_norank</i> (100), <i>Ruminococcaceae_norank</i> (75), <i>Paraprevotellaceae</i> CF231 (100), <i>Oscillospira</i> (75)
Control (Day 28)	<i>Clostridiale_norank</i> (100), <i>Ruminococcaceae_norank</i> (100), <i>Catenibacterium</i> (100), <i>Lachnospiraceae_norank</i> (100), <i>Eubacterium</i> (100), <i>Clostridiaceae_norank</i> (100), <i>Coriobacteriaceae_norank</i> (100), <i>Lactobacillus</i> (100), <i>Prevotella</i> (75), <i>Dorea</i> (75)
EV-SC (Day 28)	<i>Ruminococcaceae_norank</i> (100), <i>Clostridiale_norank</i> (100), <i>Lachnospiraceae_norank</i> (100), <i>Lactobacillus</i> (100), <i>Prevotella</i> (75), <i>Clostridiaceae_norank</i> (100), <i>Dorea</i> (100), <i>Catenibacterium</i> (75), <i>Ruminococcus</i> (100), <i>Bacteroidales_norank</i> (100), <i>Ruminococcus</i> (75), <i>S24-7_norank</i> (75), <i>Oscillospira</i> (100)
GLP2-SC (Day 28)	<i>Ruminococcaceae_norank</i> (100), <i>Catenibacterium</i> (100), <i>Clostridiale_norank</i> (100), <i>Lachnospiraceae_norank</i> (100), <i>Blautia</i> (75), <i>Clostridiaceae_norank</i> (100), <i>Ruminococcus</i> (100), <i>Erysipelotrichaceae_norank</i> (75), <i>Lactobacillus</i> (100), <i>Eubacterium</i> (100), <i>Dorea</i> (100), <i>Coprococcus</i> (75), <i>Prevotella</i> (100), <i>Ruminococcus</i> (100), <i>RF39_norank</i> (100), <i>Coriobacteriaceae_norank</i> (75)
rh-GLP2 (Day 28)	<i>Clostridiale_norank</i> (100), <i>Ruminococcaceae_norank</i> (100), <i>Clostridiaceae_norank</i> (100), <i>Lachnospiraceae_norank</i> (100), <i>Prevotella</i> (100), <i>Lactobacillus</i> (100), <i>Bacteroidales_norank</i> (75), <i>Catenibacterium</i> (100), <i>Erysipelotrichaceae_norank</i> (100), <i>S24-7_norank</i> (75)

*Present at $>1\%$ relative abundance in $\geq 80\%$ of weaned piglets in each group.

Fig. 2. The core bacteria (at genus level) in fecal samples from weaned piglets. Bars (mean $\pm$ SEM, $n = 4$) with different letters are considered significantly different ($p < 0.05$).

level, a total of six taxa were more abundant in the GLP2-SC group (*Leuconostocaceae*, *Streptococcaceae*, *Lachnospiraceae*, *Enterococcaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae*; $p < 0.05$), three taxa were more abundant in the EV-SC group (*Streptococcaceae*, *Bifidobacteriaceae*, and *Syntrophomonadaceae*; $p < 0.05$), and two taxa were more abundant in the Control group (*Ruminococcaceae* and *Lactobacillales*; $p < 0.05$), while only one taxon was more abundant in the rh-GLP2 group (*Coriobacteriaceae*; $p < 0.05$). Based on above results, it suggested that weaned piglets fed a diet supplemented with *S. cerevisiae* had significantly increased the numbers of core OTUs.

Effects of the GLP-2-Expressing *S. cerevisiae* Strain on Cytokine Responses in Weaned Piglets

By day 28 (Fig. 5), GLP-2 supplementation had no significant effect on the levels of cytokines (*i.e.*, IL-1 β , TNF- α and IFN- γ) in weaned piglets (Control vs rh-GLP2; EV-SC vs GLP2-SC). However, weaned piglets fed a diet supplemented with *S. cerevisiae* showed decreased levels of IL-1 β and TNF- α ($p < 0.05$, Control vs EV-SC; $p < 0.05$, rh-GLP2 vs GLP2-SC), although no change was observed in the level of IFN- γ among these groups tested. Collectively, these results suggested that the altered cytokine response effect was due to *S. cerevisiae* rather than GLP-2.

Fig. 3. PLS-DA score plots based on the relative abundance of abundant OTUs (at a 97% similarity level).

Correlation between the Fecal Microbial Composition and the Cytokine Response

A Spearman’s correlation analysis was performed to assess the connection between the core genera and cytokines. As revealed in Table 3, the levels of IL-1 β and TNF- α were positively associated with the relative abundance of *Lachnospiraceae_norank* (IL-1 β : Spearman $\rho = 0.50$, $p < 0.01$; TNF- α : Spearman $\rho = 0.57$, $p = 0.02$) but negatively correlated with the relative abundances of *Ruminococcaceae_norank* (IL-1 β : Spearman $\rho = -0.79$, $p = 0.02$;

TNF- α : Spearman $\rho = -0.61$, $p < 0.01$) and *Erysipelotrichaceae_norank* (IL-1 β : Spearman $\rho = -0.42$, $p = 0.03$ TNF- α : Spearman $\rho = -0.25$, $p = 0.05$). On the contrary, positive or negative correlations between the level of IFN- γ and the relative abundances of these taxa were not observed in this study.

Discussion

Recently, the combination of milk-borne growth factor delivery and a micro-organism approach has piqued great interest among researchers focused on post-weanling diarrhea in piglets [2, 11, 16]. Indeed, our previous finding has confirmed a diet fed to weaned rats supplemented with the recombinant GLP-2-expressing *S. cerevisiae* strain promoted intestinal development and growth [5]. The present study further hypothesized whether either exogenous GLP-2 or *S. cerevisiae* elicits major effects on the intestinal microbiotas and cytokine responses in weaned piglets. Based on the results of this study, the altered fecal microbiotas and cytokine response effects in weaned piglets was really due to *S. cerevisiae* rather than GLP-2, but the latter displayed better growth. These aspects of the findings are further discussed below.

GLP-2 Supplementation Had Better Growth of Weaned Piglets but Not Fecal Bacterial Community and Cytokine Responses

GLP-2 supplementation has not yet been employed to modify intestinal microbiotas, but most studies have primarily focused on investigating the effects of GLP-2 on the regulation of intestinal growth and functions [5, 17, 18].

Table 3. Spearman’s correlation coefficients and relative p -values between the relative abundances of core bacteria (genus level) and serum cytokines.

	IL-1 β		TNF- α		IFN- γ	
	Correlation coefficient	p -value	Correlation coefficient	p -value	Correlation coefficient	p -value
Ruminococcaceae_norank	-0.79*	0.02	-0.61**	<0.01	0.22	0.14
Clostridiale_norank	-0.34	0.33	-0.22	0.31	0.55	0.29
Lachnospiraceae_norank	0.50**	<0.01	0.57*	0.02	0.73	0.25
Catenibacterium	-0.82	0.65	-0.74	0.36	0.13	0.49
Clostridiaceae_norank	-0.34	0.43	-0.08	0.51	0.84	0.22
Lactobacillus	0.74	0.15	0.52	0.12	0.50	0.18
Bacteroidales_norank	0.78	0.29	0.57	0.33	-0.42	0.23
Erysipelotrichaceae_norank	-0.42*	0.03	-0.25*	0.05	0.19	0.71
Dorea	-0.57	0.15	-0.40	0.17	0.28	0.32

The correlation is expressed with the r coefficient.
* $p < 0.05$; ** $p < 0.01$.

Fig. 4. Cladogram of bacterial biomarkers associated with phase of production (LEfSe).

Taxonomic representation of statistically and biologically consistent differences among different groups tested. Differences are represented by the color of the most abundant class. The diameter of each circle is proportional to the abundance of the taxon.

For weaned piglets, GLP-2 supplementation improved their growth, and promoted the development of intestinal morphology and the activity of digestive and absorptive enzymes [4, 8, 19]. Unexpectedly, we did not observe any positive effects of GLP-2 supplementation on fecal bacterial communities and cytokine responses, although it provided

for better growth. The lack of effects perhaps can be explained by GLP-2's functions and mechanisms of action. GLP-2 is a key stimulus for inducing the gene expression of gastrointestinal proglucagon and the synthesis and secretion of proglucagon-derived peptide in the intestine [20]. Perhaps GLP-2's functions associated with intestinal

Fig. 5. Serum concentrations of cytokines in weaned piglets.

^{a,b,c}Mean values with different letters were different ($p < 0.05$) (means $\pm$ SEM, $n = 8$).

development are located in an independent manner, and do not play a role in modifying intestinal microbial community. The results of this study were consistent with the earlier findings, which indicated that the addition of

other milk-borne growth factors, such as EGF, to the diet of weaned piglets also did not alter the observed overall microbial community structure and microbial diversity in the duodenum, but increased daily gain and body weight [21, 22]. Since the present study showed the lacking effect of different dosages of GLP-2 on the development of intestinal microbiotas in weaned piglets, further studies are still needed.

S. cerevisiae Supplementation Altered Fecal Bacterial Community in Weaned Piglets

As expected, *S. cerevisiae* supplementation exhibited altered fecal microbiota homeostasis with a reduced cytokine response in weaned piglets. To explain these positive effects, *S. cerevisiae* is potentially linked to the prevention of the intestinal dysfunction associated with weaning stress [23], thereby providing strong support for its use in the regulation of the intestinal dysfunction during the weaning stage [2, 24]. *S. cerevisiae* cell walls contain 10-15% protein and 85-90% oligosaccharide encompassing mannan oligosaccharides (MOS) and β -glucan [25]. MOS influences the microbial population in GIT, which is accomplished by the ability of MOS to attach to mannose-binding proteins on the cell surface of some strains of bacteria, such as *E. coli* and *Salmonella*, resulting in preventing these bacteria from colonizing the intestinal tract by interfering with the binding of carbohydrate residues on epithelial cell surfaces [26]. Recently, the use of *S. cerevisiae* as a dietary supplement has been shown to be an effective strategy for modifying the intestinal community, resulting in reducing the occurrence of diarrheal diseases [27]. The fermentation products derived from *S. cerevisiae* also decrease the ability of enterotoxigenic *E. coli* to adhere to the intestinal mucosa of young pigs by altering the bacterial diversity and richness in ileal digesta [23].

S. cerevisiae Supplementation Reduced Cytokine Responses in Weaned Piglets

Pro-inflammatory cytokines are not only primarily related to immune function, but also have the potential to alter intestinal integrity and epithelial function related to permeability and nutrient transport [28]. The overproduction of pro-inflammatory cytokines might cause pathological inflammatory response [29], therefore increased pro-inflammatory cytokines are associated with the occurrence of diarrhea induced by weaning stress [30]. To investigate the effects of *S. cerevisiae* on pro-inflammatory cytokines, the present study also observed that piglets fed *S. cerevisiae* had lower levels of IL-1 β and TNF- α . On the contrary,

other related studies demonstrated that the concentrations of IL-2 and IL-6 in the serum of piglets supplemented with *S. cerevisiae* were significantly increased compared with those in the control group [29]. Additionally, dietary β -glucan, as a major component of *S. cerevisiae*, also attenuated the increase of plasma IL-6 and TNF- α , and enhanced the increase of plasma IL-10 when pigs were challenged with lipopolysaccharide [31]. There are some possible explanations for this divergence, such as the dosage or the supplement strategies of *S. cerevisiae*, but we still need to further confirm it.

The Relationships between Cytokines and Core Genera

A Spearman's correlation test was used for investigating the relationships between cytokines and core genera, and we found that the levels of IL-1 β and TNF- α were positively associated with the relative abundance of *Lachnospiraceae_norank* but negatively correlated with the relative abundances of *Ruminococcaceae_norank* and *Erysipelotrichaceae_norank*. As for *Erysipelotrichaceae_norank* and *Ruminococcaceae_norank*, both were significantly increased in weaned piglets fed with *S. cerevisiae*. Associations between TNF- α and *Erysipelotrichaceae* were observed previously [32], and recent reports further documented a potential role of *Erysipelotrichaceae* in host physiology and/or inflammation-related disorders in the intestine [33]. In the intestine of piglets during the weaning stage, *Lachnospiraceae* was found to play positive roles in the regulation of cytokine responses by stimulating the production of butyrate [30]. In case of *Ruminococcaceae*, it was also suggested to be a potential beneficial component of the inflammatory response to weaning stress in piglets [30]. *Ruminococcaceae* is commonly found in the intestine of mammals and displays the ability to degrade hemicellulose and cellulose of plant material in solid diet [34]. In the ileum of piglets, the increase in the relative abundance of *Ruminococcaceae* was accompanied by decreases in the expression of pro-inflammatory genes (*i.e.*, IL-6, IL-8, and IFN- γ) and anti-inflammatory genes (*i.e.*, IL-10 and TGF- β) [35]. On the contrary, *Lachnospiraceae_norank* was significantly decreased in weaned piglets fed with *S. cerevisiae* in this study. *Lachnospiraceae*, a member of the phylum *Firmicutes* that includes major constituents of the intestinal microbiotas of mammals [36], has the ability to produce butyric acid to affect the evolution and energy metabolism in the intestine [37]. At 2-3 weeks post-weaning, significant decrease in the relative abundance of *Lachnospiraceae* contributed to the increases in the mRNA and protein expression of TNF- α and IFN- γ in piglets [30]. In addition to this, in the ileum of

pigs with rotavirus infection, increased mRNA expression of IFN- γ , IL-8 and IL-10 was also related to increased levels of *Lachnospiraceae* [38]. *Lachnospiraceae* is therefore known to have an impact on immune responses [30]. Considering the results together, the decrease of *Lachnospiraceae* and the increases in *Ruminococcaceae* and *Erysipelotrichaceae* would potentially be beneficial in the regulation of immune response in weaned piglets.

In conclusion, the altered fecal microbiotas and cytokine response effects in weaned piglets were due to *S. cerevisiae* rather than GLP-2, suggesting that the effects of GLP-2-expressing *S. cerevisiae* are not different from the empty vector.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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