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A field study of *Bacillus licheniformis*-fermented products on growth performance and faecal microbiota of weaning piglets

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Abstract

This study investigated the effects of *Bacillus licheniformis*-fermented products (BLFPs) on the growth performance, faecal microbiota, and antibiotic resistance gene (ARG) expression in weaning piglets on a commercial farm. Ninety-six weaning piglets were randomly assigned to four treatments as follows: basal diet as control (C), basal diet plus 30 mg/kg of antibiotics (bacitracin methylene disalicylate) (A), basal diet plus 1 g/kg of BLFPs (F), and basal diet plus 15 mg/kg of antibiotics and 0.5 g/kg of BLFPs (AF), with six replicate pens per treatment and four pigs per pen. Results showed that, similar to antibiotics, replacing all or half the antibiotics with BLFPs improved the feed conversion ratio of weaning piglets from 15–28 d. Microbiota analysis showed that microbial community composition in the faeces showed a clear separation between groups. Replacing all the antibiotics with BLFPs increased the abundance of the genus, *Streptococcus,* in the faeces compared with the other groups. Half replacement of antibiotics with BLFPs increased the chloramphenicol resistance gene levels in the faeces compared with the C group, whereas full replacement of antibiotics with BLFPs reduced the streptomycin resistance gene levels compared with the C group. A trend of decreased levels of formic acid and acetic acid was observed in the group treated with BLFPs in combination with antibiotics compared with the C group. In conclusion, the field study demonstrates that replacing all or half the antibiotics with BLFPs can improve feed conversion ratio, modulate faecal microbiota, and alter ARG expression in weaning piglets.

Keywords: feed additive, probiotic, pig, 16S rRNA gene sequencing, antibiotic resistance gene
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Introduction

Weaning has been considered as a critical period that affects piglet health and growth. Post-weaning diarrhoea caused by pathogens and environmental stress has an important economic impact on pig production worldwide (Rhouma *et al*., 2017). Gut microbiota dysbiosis is highly associated with a high incidence of diarrhoea in weaning piglets, resulting in poor growth rate (Dou *et al*., 2017). Antibiotic growth promoters have been widely used for the prevention of post-weaning diarrhoea and improving the growth of weaning piglets (Rhouma *et al*., 2017). Multidrug-resistant bacteria and antibiotic residues in animal products are becoming a major threat to human health since antibiotic growth promoters are overused in animal production. Therefore, the use of antibiotics as growth promoters in animal feeds has been prohibited in many countries (Maron *et al*., 2013). It is important to find potential alternatives to antibiotics to improve gut health and the growth of weaning piglets.

The establishment of a beneficial gut microbiota early in the life of piglets can improve health and growth (Gresse *et al*., 2017). Probiotics have been extensively used as a strategy for antibiotic alternatives in pig production and many studies suggest that probiotics exert beneficial effects on piglets through the modulation of gut microbiota (Guevarra *et al*., 2019). It has been demonstrated that probiotic

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supplementation protects weaned pigs against pathogenic bacteria and improves performance similar to antibiotics (Kritas & Morrison, 2005). *Bacillus licheniformis* is a spore-forming probiotic that promotes nutrient utilization and inhibits pathogen growth through the production of the digestive enzymes and antimicrobial peptides (Rozs *et al*., 2001; Thaniyavarn *et al*., 2003; Horng *et al*., 2019). Supplementation with *B. licheniformis* reduces diarrhoea incidence in weaning piglets (Zong *et al*., 2019). Recent studies demonstrate that, similar to antibiotic growth promoters, *B. licheniformis*-fermented products (BLFPs) can alleviate diarrhoea incidence and improve the growth performance of weaning piglets (Hung *et al*., 2019; Lin & Yu, 2020). BLFP supplementation in the diet of sows also improves the piglet body weight at weaning (Yu *et al*., 2020). In addition, microbial communities are different in terms of the caecal digesta or faeces between the weaning piglets treated with BLFPs and antibiotics (Hung *et al*., 2019; Lin & Yu, 2020).

It has been reported that the antibacterial activity with the combined use of antibiotics and probiotics is higher than when using the antibiotic or probiotic alone (Soleymanzadeh Moghadam *et al*., 2018). A 50% replacement of antibiotics with probiotics improves digestive enzyme activity, antioxidant activity, and growth performance in weaning piglets (Wang *et al*., 2017; Hu *et al*., 2018). Our previous study demonstrated that a 50% replacement of antibiotics with BLFPs was able to decrease the incidence of diarrhoea and modulate caecal microbiota composition in weaning piglets (Lin & Yu, 2020). To the best of our knowledge, little is known about the efficacy of parallel and combined supplementation with BLFPs and antibiotics in the diet of weaning piglets on growth performance and gut microbiota in the commercial farm. Furthermore, the effect of BLFPs on the abundance of antibiotic resistance gene (ARG) in the faeces of weaning piglets still remains to be evaluated. For practical application, these results can provide valuable information about the effect of BLFPs on weaning piglets for development as an alternative to antibiotics.

Therefore, the current study aimed at evaluating and comparing the effects of BLFPs and antibiotics, as well as their combination on growth performance, faecal microbiota, and ARG expression on a commercial pig farm.

Materials and Methods

Protocols for BLFP preparation in this study were performed as described in a previous study (Lin *et al*., 2019). Antibiotics (bacitracin methylene disalicylate) were purchased from Nice Garden Industrial (Taipei, Taiwan). The concentration of *B. licheniformis* spores in BLFPs was 5×10^{11} CFU/g. The detailed composition of BLFPs is listed in Table 1. All experiments were performed in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee of National Ilan University (IACUC, protocol number 109-4). The study was carried out from April to July 2020 on a commercial farrow-to-finish pig farm (Miaoli, Taiwan) with a breeding stock of 100 sows. The pig farm had its own feed mill. A total of 96, twenty-eight-day-old weaning pigs [Duroc × (Yorkshire × Landrace)] with an average body weight (BW) of 9.0 ± 0.03 kg were used in a 42-day trial. All pigs were randomly allotted to four experimental diets based on initial BW and sex (six replicate pens per treatment; two gilts, and two barrows/pen). Dietary treatments included: a basal diet as control (C), basal diet + 30 mg/kg of antibiotics (bacitracin methylene disalicylate) (A), basal diet + 1.0 g/kg of BLFPs (F), and basal diet + 15 mg/kg of antibiotics + 0.5 g/kg of BLFPs (AF). Diets (Table 2) were formulated to meet or exceed the nutrient requirements recommended by National Research Council (2012). In F and AF groups, the soybean meal in the basal diet was replaced with BLFPs equally. All the pigs were housed in an environmentally-controlled room with a slatted metal floor (2.5 m × 4.0 m). Each pen was equipped with a one-sided self-feeder and a nipple waterer to allow the pig *ad libitum* access to feed and water throughout the experimental period. The experimental period was 42 days. Room temperature during week 1 was maintained at 30 °C and was then gradually decreased and kept at 24°C until the end of the experiment. The photoperiod was controlled to provide 10 h of light and 14 h of dark in the shed throughout the experiment. The morbidity and mortality of piglets were monitored daily. The feed offered and refused was weighed daily to calculate the average daily feed intake (ADFI). The piglets were weighed weekly to calculate average BW and average daily weight gain (ADG). The feed conversion ratio (FCR) was calculated every week.

Table 1 Composition of BLFPs

Analysed chemical composition, g g^{-1}	
Calorie, kcal/kg	3.65
Crude protein	0.29
Crude fat	0.04
Carbohydrate	0.53
Ash	0.06
Water	0.08

Table 2 Composition of basal diets

Item	days 1 to 14	days 15 to 42
Ingredient, g kg^{-1}		
Corn, yellow	600	635
Soybean meal, 44% CP	270	280
Fish meal, 60% CP	50	25
Dried whey	25	0
Soybean oil	25	25
L-Lysine, 98%	5	5
$CaCO_3$, 38% Ca	8	10
$CaHPO_4$	10	12
Choline, 50%	1	1
Salt	3	4
Mineral and vitamin premix[1]	3	3
Analysed chemical composition, g kg^{-1}		
Crude protein	192.5	172.3
Crude fat	68.6	73.1
Crude fiber	37.0	49.4
Ash	61.0	59.2
Phosphorus	6.6	6.3
Calcium	8.1	7.4
Lysine	12.2	10.8
Methionine + Cystine	8.7	8.1
ME, kcal/kg	4079.4	4115.3

[1] Supplied per kg diet: Cu, 20 mg; Fe, 140 mg; I, 0.2 mg; Mn, 4 mg; Se, 0.1 mg; and Zn, 100 mg; vitamin A, 6000 IU; vitamin D, 900 IU; vitamin E, 30 IU; vitamin K3, 3 mg; vitamin B2, 6 mg; pantothenic acid, 18 mg; niacin, 60 mg; and vitamin B12, 30 μg

At the end of experiment (day 42), faeces from two piglets (male and female) per replicate were freshly collected and then pooled. Three replicates (n = 3) were used for faecal microbiota analysis. Faecal DNA was purified using the QIAamp DNA Stool Mini Kit (QIAGEN, Germany) and quantified on the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA).

The V3 and V4 hypervariable region of the 16S rRNA gene was amplified using a 341F–805R primer. PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN, Germantown, MD, USA). The library was constructed using TruSeq Nano DNA Library Prep kits (Illumina, San Diego, CA, USA). The constructed library was sequenced using the paired-end method on Illumina MiSeq platform (San Diego, CA, USA) after being subjected to DNA quantification and library testing. The low-quality part of the reads was cut, and in each sample, probes were split from the high-quality reads. The clean reads of all samples were clustered and classified into the same operational taxonomic unit (OTU) with an identity of 97% similarity using the UCHIME software (version 4.2) and Mothur software (version 1.39.5). The obtained sequences were aligned to the Genomes Online Database (gold.jgi.doe.gov) to determine the phylogeny of the OTUs. Taxonomic assignments, alpha diversity, and principal component analysis (PCA) were calculated by Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 1.9.1). Unweighted UniFrac metric and weighted principal coordinate analysis (PCoA) were performed using QIIME 2 software. A Venn diagram (version 1.6.17) was constructed to show the number of common and unique OTUs among groups. The R package, *corrplot* (version 0.84), was used for visualizing correlation matrices.

At the end of experiment (day 42), faeces from two piglets (male and female) per replicate were freshly collected and then pooled. Three replicates (n = 3) were used for ARG analysis. Faecal DNA was purified using the QIAamp DNA Stool Mini Kit (QIAGEN) and quantified on the Qubit 2.0 Fluorometer (Thermo Scientific). Quantitative polymerase chain reaction (qPCR) was performed using a MiniOpticon Real-Time PCR detection system (Bio-Rad, Hercules, CA) and KAPA SYBR FAST qPCR

Kit (Kapa Biosystems, Boston, MA). Primer pairs specific for each ARG were as follows: *sul1* (sulphonamide-resistant dihydropteroate synthase) forward: 5′-GGA TCA GAC GTC GTG GAT GT-3′, and reverse: 5′-GTC TAA GAG CGG CGC AAT AC-3′; *cat* (chloramphenicol acetyltransferase) forward: 5′-TCC ATG AGC AAA CTG AAA CG-3′, and reverse: 5′-GGG AAA TAG GCC AGG TTT TC-3′; *aadA* (aminoglycoside adenylyltransferase) forward: 5′-CAG CCC GTC TTA CTT GAA GC-3′, and reverse: 5′-GAT CTC GCC TTT CAC AAA GC-3′; *strA* (streptomycin phosphotransferase) forward: 5′-CCA GTT CTC TTC GGC GTT AG-3′, and reverse: 5′-ACT CTT CAA TGC ACG GGT CT-3′; *tetA* (tetracycline resistance determinant, class A) forward: 5′-CGA TCT TCC AAG CGT TTG TT-3′, and reverse: 5′-CCA GAA GAA CGA AGC CAG TC-3′; *tetB* (tetracycline resistance determinant, class B) forward: 5′-TAC AGG GAT TAT TGG TGA GC-3′, and reverse: 5′-ACA TGA AGG TCA TCG ATA GC-3′; *tetG* (tetracycline resistance determinant, class G) forward: 5′-GTG TTC CCG ATT CTG TTG CT-3′, and reverse: 5′-GAT TGG TGA GGC TCG TTA GC-3′. Amplification of the 16S rRNA gene (forward: 5′-GTG STG CAY GGY TGT CGT CA-3′, and reverse: 5′-ACG TCR TCC MCA CCT TCC TC -3′) was used as a reference to determine the total amount of DNA in each sample. After quantitative PCR, the relative expression of ARG in the total bacterial population was finally calculated using the formula $2^{-\Delta\Delta Ct}$.

At the end of experiment (day 42), faeces from two piglets (male and female) per replicate were freshly collected and then pooled. Three replicates (n = 3) were used for short-chain fatty acid analysis. Short-chain fatty acids from faeces were extracted and analysed using gas chromatography–mass spectrometry (Bruker GC-MS System, Burker Corp., Billerica, MA, USA), as described previously (Cheng *et al*., 2021).

Replicates were used as the experimental unit. The differences among the dietary treatment groups were analysed using one-way ANOVA followed by Tukey's honestly significant difference test using SAS (version 9.4, 2012; SAS Institute, Cary, NC, USA). A *P*-value between 0.05 and 0.1 was considered a trend, and a *P*-value of less than 0.05 was statistically significant. The PCoA and PCA were performed using UniFrac distances coupled with standard multivariate statistics.

Results

The piglets were healthy during the experimental period. No significant difference was found in BW, ADG, and ADFI between groups, except for FCR (Table 3). Antibiotic supplementation improved the FCR in weaning piglets at 15–28 days compared with the C group (P = 0.011) (Table 3). Similar to antibiotics, the FCR was improved at 15–28 days in the F and AF groups compared with the C group (P = 0.011) (Table 3).

Table 3 Effect of antibiotics and *Bacillus licheniformis*-fermented products on growth performance of weaning piglets

	C[1]	A	F	AF	SEM	*P* value
BW[2] (kg/head)						
1 d	9.00[3]	9.05	9.03	9.03	0.03	0.122
28 d	18.43	19.18	18.58	18.81	0.25	0.515
42 d	26.28	27.36	26.49	26.88	0.35	0.490
ADG (kg/d/head)						
1–14 d	0.32	0.29	0.27	0.28	0.01	0.376
15–28 d	0.36	0.44	0.41	0.41	0.01	0.209
29–42 d	0.56	0.58	0.57	0.58	0.01	0.278
1–42 d	0.41	0.44	0.42	0.43	0.01	0.532
ADFI (kg/d/head)						
1–14 d	0.42	0.39	0.37	0.41	0.01	0.264
15–28 d	0.56	0.63	0.59	0.60	0.02	0.644
29–42 d	0.89	0.95	0.91	0.91	0.02	0.431
1–42 d	0.62	0.66	0.62	0.64	0.01	0.759
FCR						
1–14 d	1.33	1.38	1.39	1.44	0.02	0.269
15–28 d	1.57[a]	1.43[b]	1.42[b]	1.44[b]	0.03	0.011
29–42 d	1.58	1.63	1.61	1.58	0.01	0.704
1–42 d	1.51	1.51	1.50	1.50	0.01	0.954

[1] C: Basal diet; A: Basal diet plus 30 mg/kg antibiotics; F: Basal diet plus 1 g/kg BLFPs; AF: Basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs; SEM: standard error of mean

[2] BLFPs: *Bacillus licheniformis*-fermented products; BW: Average body weight; ADG: average daily weight gain; ADFI: average daily feed intake; FCR: feed conversion ratio

[3] Data are mean values of six replicates per treatment

[a-b] Means in the row without common superscripts are significantly different (*P* <0.05)

After stringent quality trimming of raw data, the averages of high-quality reads from the faeces of C, A, F, or AF were 26951, 22360, 22133, and 22044, respectively (Table 4). Replacing all antibiotics with BLFP decreased OTUs in the faeces of weaning piglets compared with the C group (P = 0.022) (Table 4). No significant difference was observed in the bacterial species richness (Chao1 and Fisher alpha) and species evenness (Shannon and Enspie) between groups (Table 4).

Table 4 Microbial α-diversity in the faeces of weaning piglets

	C[1]	A	F	AF	SEM	*P* value
Effective reads	26950.7[2]	22360.3	22133.3	22043.7	965.10	0.228
Number of OTUs	11094.7[a]	8367.3[ab]	6861.7[b]	8244.0[ab]	546.32	0.022
Chao1	283.0	218.7	195.0	269.3	21.71	0.571
Fisher alpha	39.1	29.2	25.6	37.2	3.43	0.591
Shannon	5.4	5.3	4.8	5.1	0.12	0.415
Enspie	12.3	12.0	9.9	9.5	0.64	0.374

[1] C: Basal diet; A: Basal diet plus 30 mg/kg antibiotics; F: Basal diet plus 1 g/kg BLFPs; AF: Basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs; BLFPs: *Bacillus licheniformis*-fermented products; SEM: standard error of mean; OTU: operational taxonomic unit
[2] Data are mean values of three replicates per treatment
[a-b] Means in the row without common superscripts are significantly different (*P* <0.05)

The Venn diagram showed an overlap (199 OTUs, core) that was shared by four of the plotted groups (Figure 1). In total, 70, 19, 8, and 49 unique OTUs were found in the four aforementioned groups, respectively; 11 OTUs were found in both the C and A groups; 3 OTUs were found in both the C and F groups. By contrast, 134 OTUs were found in both the C and AF groups.

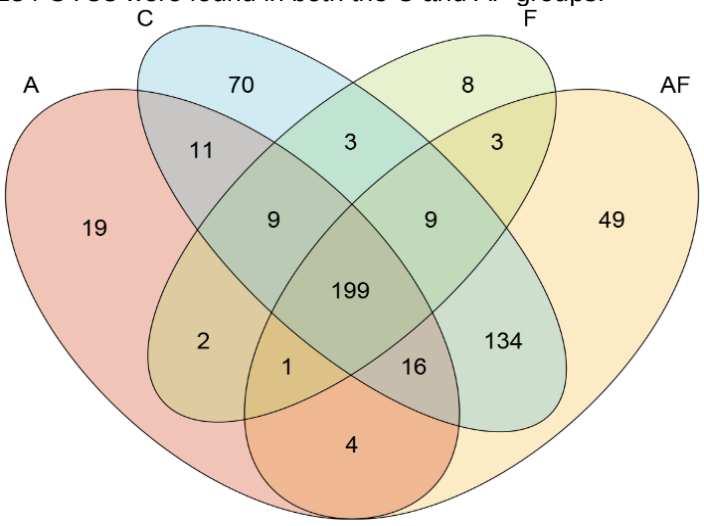

Figure 1 Venn diagram showing the distribution of operational taxonomic units (OTUs) between groups. Each ellipse represents one group. The overlapping regions between the ellipses represent the OTUs that are shared between the following: basal diet as the control (C), basal diet plus 30 mg/kg antibiotics (A), basal diet plus 1 g/kg *Bacillus licheniformis*-fermented products (BLFPs) (F), and basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs (AF) (n = 3). The value of each region represents the number of OTUs corresponding to the region.

Unweighted PCoA of qualitative traits indicated that the microbiota of faecal samples was not well-separated among the groups (Figure 2A). In contrast, weighted PCoA of quantitative traits and PCA revealed significant discrimination among the groups (Figure 2B and 2C).

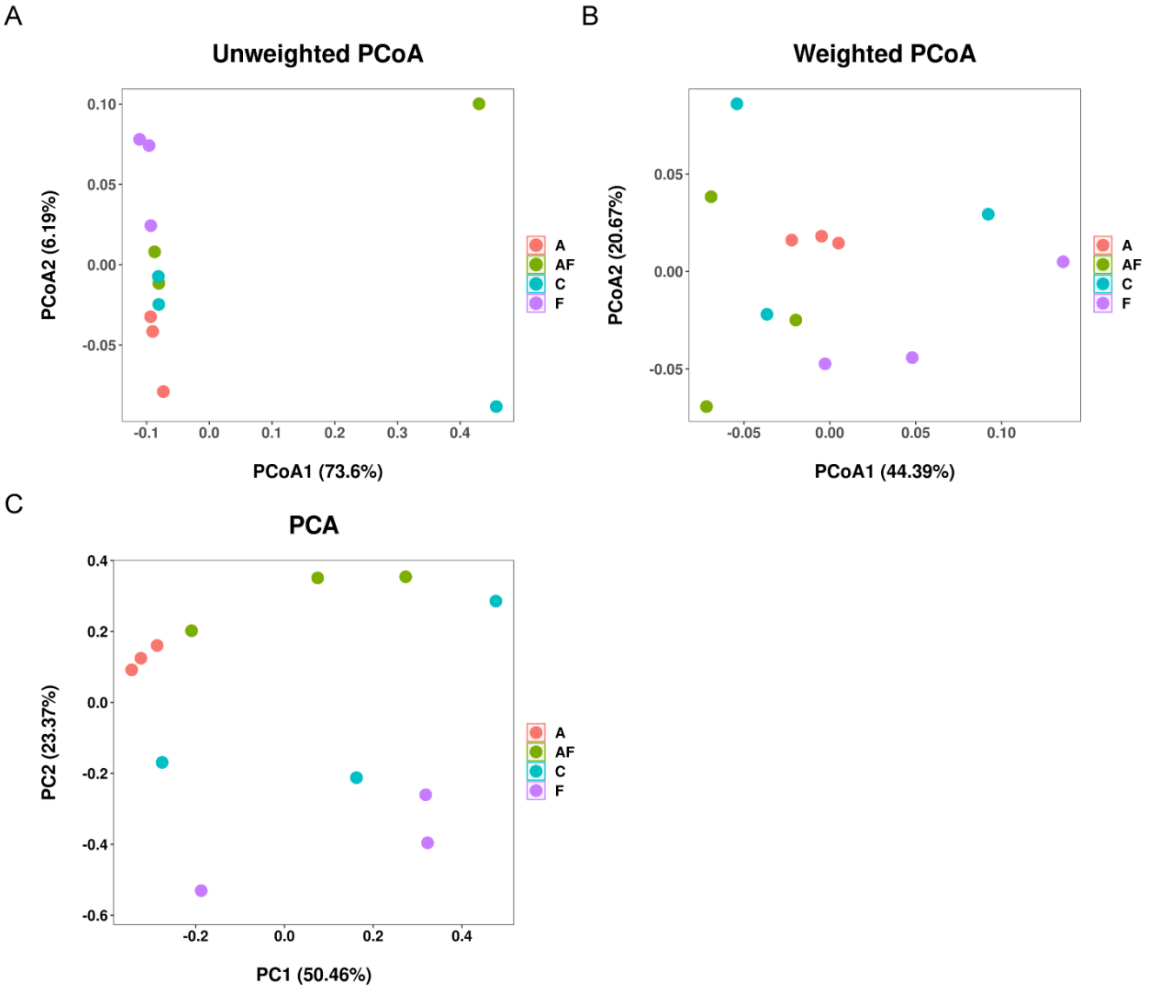

Figure 2 Microbial β-diversity in the faeces of weaning piglets (A) Unweighted principal coordinate analysis plots of qualitative traits and (B) weighted principal coordinate analysis plots of quantitative traits in the faeces of basal diet as the control (C), basal diet plus 30 mg/kg antibiotics (A), basal diet plus 1 g/kg *Bacillus licheniformis*-fermented products (BLFPs) (F), and basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs (AF) (n = 3). (C) Principal component analysis plots of the faecal bacterial communities from C, A, F, and AF (n = 3)

The result of bacterial taxonomy in the faeces of weaning piglets is shown in Table 5. No significant differences were observed in the abundance at the phylum level between groups. At the class level, the abundance of the class, Bacilli, was increased in the F group compared with the other groups (P = 0.007). The abundance of class, Methanobacteria, was higher in the AF group compared with the other groups (P = 0.002). At the order level, the abundance of the order, Lactobacillales, was higher in the F group compared with the other groups (P = 0.007). The abundance of the order, Methanobacillales, was increased in the AF group compared with the other groups (P = 0.002). Antibiotic supplementation increased the abundance of order, Spirochaetales, compared with the other groups (P <0.001). The abundance of order, Spirochaetales, was reduced in the F group compared with the AF group (P <0.001). At the family level, the abundance of the family, Streptococcaceae, was increased in the F group compared with the other groups (P <0.001). The abundance of the family, Ruminococcaceae, was lower in the F group compared with the A group (P = 0.05). Replacing all antibiotics or half the antibiotics with BLFPs decreased the abundance of the family, Lactobacillaceae, compared with the C and A groups (P = 0.002). The abundance of the family, Muribaculaceae, was increased in A and AF groups compared with the C and F groups (P = 009). Replacing all antibiotics with BLFPs increased the abundance of the family, Methanobacteriaceae, compared with the other groups (P = 002). The abundance of the family, Christensenellaceae, was decreased in the F group compared with other groups (P = 007). At the genus level, the abundance of the genus, *Streptococcus,* in the F group was higher compared with the other groups (P <0.001). Replacing all antibiotics or half the antibiotics with BLFPs decreased the abundance of the genus, *Lactobacillus,* compared with the C and A groups (P = 0.002). Antibiotic supplementation increased the abundance of the genera, *Lachnospiraceae_unclassified* and *Treponema 2*, compared with the other groups (P = 0.004 and

P <0.001). The abundance of the genus, *Blautia*, in the AF group was lower compared with the other groups (*P* = 0.046). Replacing all antibiotics with BLFPs decreased the abundance of the genus, *Christensenellaceae R-7 group*, compared with the other groups (*P* = 0.007). The abundance of the genus, *Treponema 2*, in the F group was lower compared with the A and AF groups (*P* <0.001). Replacing all antibiotics with BLFPs increased the abundance of the genus, *Methanosphaera*, compared with the other groups (*P* = 0.001).

Table 5 Effect of antibiotics and *Bacillus licheniformis*-fermented products on taxonomic assignment and ranking of faecal microbiota in weaning piglets

	Relative abundance (%)					
	C[1]	A	F	AF	SEM	*P* value
Phylum						
Firmicutes	72.4[2]	73.6	77.3	74.5	1.56	0.836
Bacteroidetes	18.6	19.6	19.4	15.7	1.38	0.782
Proteobacteria	3.3	0.8	0.8	2.2	0.66	0.588
Class						
Clostridia	53.0	53.8	52.4	56.3	0.97	0.660
Bacteroidia	18.4	19.6	19.4	15.6	1.40	0.769
Bacilli	11.6^{b}	11.2^{b}	16.8^{a}	9.1^{b}	0.93	0.007
Negativicutes	6.8	7.5	7.0	8.2	0.47	0.768
Methanobacteria	1.3^{b}	1.8^{b}	0.5^{b}	3.7^{a}	0.38	0.002
Gammaproteobacteria	2.4	0.7	0.7	1.5	0.44	0.557
Erysipelotrichia	1.0	1.1	1.1	0.9	0.06	0.805
Order						
Clostridiales	53.0	53.8	52.4	56.3	0.97	0.660
Bacteroidales	17.9	19.5	19.4	15.2	1.44	0.731
Lactobacillales	11.6^{b}	11.2^{b}	16.8^{a}	9.1^{b}	0.93	0.007
Selenomonadales	6.8	7.5	7.0	8.2	0.47	0.768
Methanobacteriales	1.3^{b}	1.8^{b}	0.5^{b}	3.7^{a}	0.38	0.002
Erysipelotrichales	1.0	1.1	1.1	0.9	0.06	0.805
Spirochaetales	0.6^{bc}	2.4^{a}	0.3^{c}	0.9^{b}	0.25	< 0.001
Family						
Clostridiaceae 1	29.8	29.0	31.4	34.1	0.92	0.327
Prevotellaceae	14.6	15.0	17.0	11.2	1.54	0.643
Streptococcaceae	7.1^{b}	5.7^{b}	14.3^{a}	6.9^{b}	1.07	< 0.001
Ruminococcaceae	7.6^{ab}	8.6^{a}	5.7^{b}	7.2^{ab}	0.44	0.050
Lachnospiraceae	7.0	8.0	7.1	6.0	0.28	0.111
Peptostreptococcaceae	6.7	6.1	7.2	7.0	0.20	0.306
Veillonellaceae	6.3	7.0	6.6	7.0	0.40	0.928
Lactobacillaceae	4.5^{a}	5.5^{a}	2.4^{b}	2.2^{b}	0.45	0.002
Muribaculaceae	1.7^{b}	2.4^{a}	1.7^{b}	2.4^{a}	0.12	0.009
Methanobacteriaceae	1.3^{b}	1.8^{b}	0.5^{b}	3.7^{a}	0.38	0.002
Christensenellaceae	1.3^{a}	1.5^{a}	0.6^{b}	1.4^{a}	0.11	0.007
Erysipelotrichaceae	1.0	1.1	1.1	0.9	0.06	0.805
Genus						
Clostridium sensu stricto 1	29.1	28.5	31.0	33.4	0.92	0.320
Streptococcus	7.1^{b}	5.7^{b}	14.3^{a}	6.9^{b}	1.07	< 0.001
Terrisporobacter	6.1	5.5	6.7	6.5	0.20	0.225
Prevotella 9	4.7	4.3	5.3	3.0	0.53	0.506
Megasphaera	3.5	4.2	4.1	3.5	0.27	0.742
Lactobacillus	4.5^{a}	5.5^{a}	2.5^{b}	2.2^{b}	0.45	0.002
Prevotella 7	2.2	3.0	4.0	2.2	0.37	0.220
Prevotellaceae NK3B31 group	2.4	2.8	2.6	2.2	0.18	0.739
Prevotellaceae_unclassified	2.3	1.4	2.3	1.5	0.37	0.800
Lachnospiraceae_unclassified	1.5^{b}	2.0^{a}	1.3^{b}	1.6^{b}	0.08	0.004
Blautia	1.6^{ab}	1.4^{ab}	1.8^{a}	1.2^{b}	0.08	0.046
Christensenellaceae R-7 group	1.3^{a}	1.5^{a}	0.6^{b}	1.4^{a}	0.11	0.007
uncultured Porphyromonadaceae bacterium	1.0	1.2	1.0	1.4	0.06	0.078
Treponema 2	0.6^{bc}	2.4^{a}	0.3^{c}	0.9^{b}	0.25	< 0.001
Methanosphaera	0.5^{b}	0.5^{b}	0.2^{b}	2.8^{a}	0.33	0.001

[1] C: Basal diet; A: Basal diet plus 30 mg/kg antibiotics; F: Basal diet plus 1 g/kg *Bacillus licheniformis*-fermented products (BLFPs); AF: Basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs; SEM: standard error of mean
[2] Data are mean values of three replicates per treatment
[a-c] Means in the row without common superscripts are significantly different (*P* <0.05)

The results of the heat map show that similar bacterial community clusters, such as genera *Ruminococcaceae UCG-008*, *Ruminococcaceae NK4A214 group*, *Christensenellaceae R-7 group*, and *Clostridium sensu stricto 6*, were observed between the C, A, and AF groups (Figure 3). Some bacterial community clusters were specifically decreased in the AF group, such as the genera, *[Eubacterium] hallii group*, *Blautia*, *Faecalibacterium*, and *Subdoligranulum* (Figure 3). Some bacterial community clusters were specifically increased in the A group, such as genera, *Turicibacter*, *Alloprevotella*, and *Lachnospiraceae_unclassified* (Figure 3). Replacing all antibiotics with BLFPs resulted in unique bacterial community clusters compared with other groups, such as the genera, *Streptococcus*, *Dialister*, and *Oribacterium* (Figure 3).

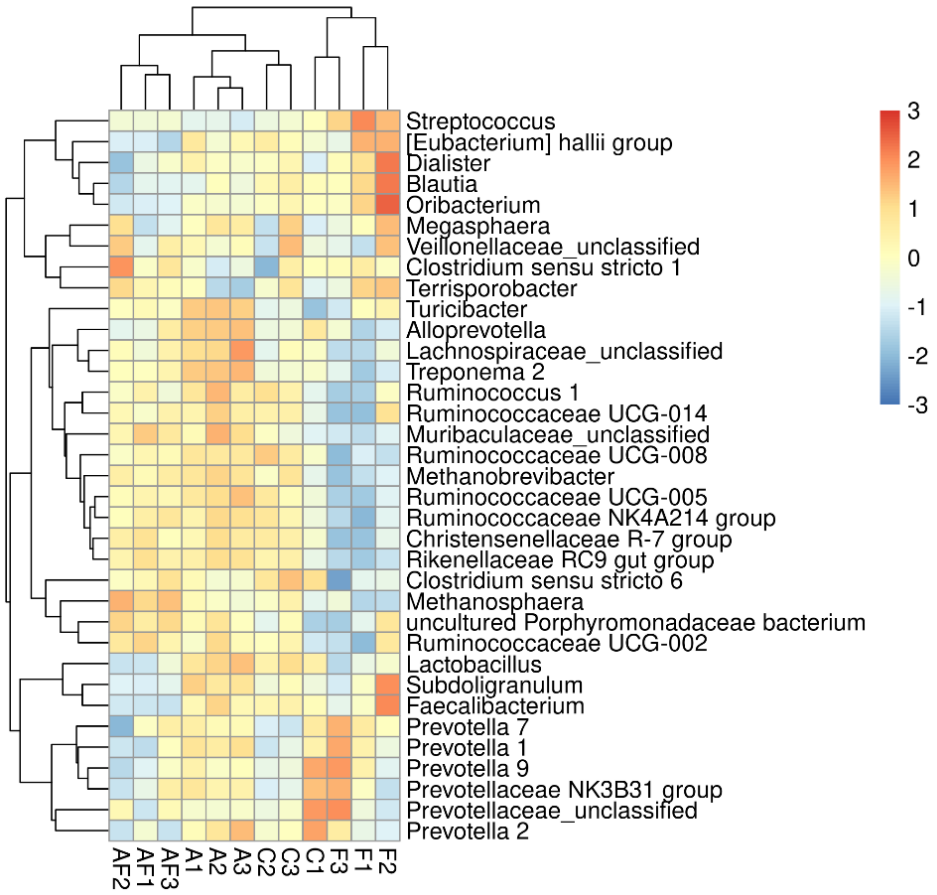

Figure 3 Taxonomic composition analysis of faecal microbiota. Heatmap indicating the dominant 35 genera (y-axis) across different treatment groups (x-axis) (n = 3)

No significant difference in the sulphonamide resistance gene (*sul1*) and aminoglycoside resistance gene (*aadA*) in the faeces was found among the groups (Figure 4). The chloramphenicol resistance gene (*cat*) levels were increased in the AF group compared with the C group (P <0.05) (Figure 4). Replacing all antibiotics with BLFPs decreased the levels of the streptomycin resistance gene (*strA*) in the faeces compared with the C group (P <0.05) (Figure 4). Two tetracycline resistance gene (*tetA* and *tetB*) levels in the faeces were not altered among the groups (Figure 4). The level of the *tetG* gene in the faeces of the A group was increased compared with the C group (P <0.05) (Figure 4). The result of short-chain fatty acid levels in the faeces of weaning piglets is shown in Table 6. Relative to the control group, a trend of the decreased levels of formic acid and acetic acid was observed (P = 0.084 and P = 0.067) in the group treated with BLFPs in combination with antibiotics.

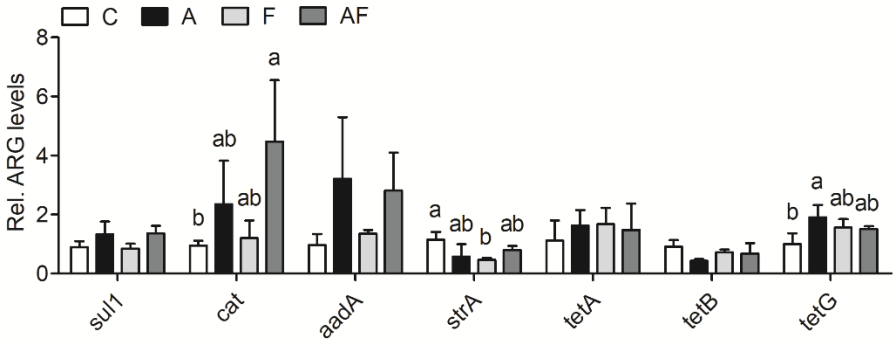

Figure 4 Expression of antibiotic resistance gene (ARG) in faeces. Effects of *Bacillus licheniformis*-fermented products (BLFPs) on sulphonamide resistance gene (*sul1*), chloramphenicol resistance gene (*cat*), aminoglycoside resistance gene (*aadA*), streptomycin resistance gene (*strA*), and tetracycline resistance genes (*tetA*, *tetB*, and *tetG*) of the faeces of basal diet as the control (C), basal diet plus with 30 mg/kg antibiotics (A), basal diet plus 1 g/kg BLFPs (F), and basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs (AF) (n = 3). Each bar represents mean ± standard deviation. Different superscripts indicate a significant difference between groups (P <0.05).

Table 6 Effect of antibiotics and *Bacillus licheniformis*-fermented products (BLFPs) on the faecal short-chain fatty acid levels of weaning piglets

	C[1]	A	F	AF	SEM	P value
Formic acid (μM)	23.7[2]	28.8	29.6	11.6	2.71	0.084
Acetic acid (μM)	4584.8	5113.2	4730.0	2998.0	324.40	0.067
Propionic acid (μM)	2928.2	3475.7	2307.9	2047.1	256.15	0.304
Butyric acid (μM)	1474.3	1492.2	1401.5	1283.6	69.30	0.755
Isobutyric acid (μM)	268.5	330.4	252.3	322.5	27.88	0.779
2-methylbutyric acid (μM)	140.2	166.6	180.5	159.2	15.02	0.866
3-methylbutyric acid (μM)	152.0	174.9	190.0	171.2	15.89	0.911
Pentanoic acid (μM)	273.8	334.0	351.3	244.8	25.09	0.402
4-methylpentanoic acid (μM)	1.9	1.4	1.1	1.3	0.23	0.645
Hexanoic acid (μM)	103.5	101.4	162.2	113.8	10.38	0.157

[1] C: Basal diet; A: Basal diet plus 30 mg/kg antibiotics; F: Basal diet plus 1 g/kg BLFPs; AF: Basal diet plus 15 mg/kg antibiotics and 0.5 g/kg BLFPs; SEM: standard error of mean
[2] Data are mean values of three replicates per treatment

Discussion

The application of probiotics or probiotic-derived functional metabolites for growth promotion has been investigated in weaning piglets (Hu *et al*., 2014; Cheng *et al*., 2019; Hung *et al*., 2019; Lin & Yu, 2020). A field study has reported that supplementation of *Bacillus* species-based probiotics (*B. licheniformis* and *B. subtilis*) in the diet of piglets can improve ADG and FCR at the weaning stage (Alexopoulos *et al*., 2004). Multi-strains of *Bacillus* species-based probiotics (*B. lichenformis*, *B. subtilis*, and *B. coagulans*) also ameliorate the ADG and feed efficiency of growing–finishing pigs (Balasubramanian *et al*., 2016). The combined use of *B. lichenformis* and zinc oxide in the diet of weaning piglets improves the ADG and FCR (Zong *et al*., 2019). Our previous study demonstrated that the replacement of half the antibiotics with BLFPs increased the ADG of weaning piglets (Lin & Yu, 2020). In this field study, similar to the effects of antibiotics, we further demonstrated that full replacement of antibiotics or half replacement of antibiotics with BLFPs improved the FCR of weaning piglets. Our previous studies have demonstrated that the antimicrobial peptides isolated from BLFPs exhibit antibacterial activity against pathogens, such as *Clostridium perfringens* and *Brachyspira hyodysenteriae* (Lin *et al*., 2019; Horng *et al*., 2019). Therefore, we speculate that BLFP-derived antimicrobial peptides may exert the antimicrobial effect of antibiotics in the gut of weaning piglets, thereby improving the growth of piglets. It has been reported that *B. licheniformis* spores are able to germinate in the gastrointestinal tract of pigs (Leser *et al*., 2008). Germination and outgrowth of *B. licheniformis* spores in the gut can reduce the pathogens by competitive exclusion (Xu *et al*., 2018). Thus, the *B. licheniformis* spores of fermented products may also eliminate the gut pathogens of weaning piglets by competitive exclusion in the present study. In addition, *B. licheniformis* can increase nutrient utilization by the production of digestive enzymes (Rozs *et al*., 2001). Taken together, these findings demonstrate that the dietary supplementation of BLFPs has beneficial effects on FCR in piglets. The mechanism of BLFPs on the improvement of FCR in weaning piglets may be different and

complicated compared to antibiotic supplementation.

The environmental and nutritional changes present huge challenges for the early life of pigs. The establishment of beneficial gut microbiota can ensure the health and growth of weaning pigs (Gresse *et al*., 2017). However, the weaning pigs are susceptible to pathogen infection since gut microbial composition is still developing in the weaning stage (Guevarra *et al*., 2019). Probiotics have been shown to have a broad range of beneficial effects in weaning pigs through the modulation of gut microbiota, thereby improving health and growth (Kritas & Morrison, 2005; Gresse *et al*., 2017; Guevarra *et al*., 2019). Our previous studies have demonstrated that gut microbiota imbalance is observed in broilers and weaning piglets in response to antibiotic prophylaxis (Hung *et al*., 2019; Chen & Yu, 2020; Lin & Yu, 2020). BLFP supplementation in the diet of weaning piglets can re-shape gut microbial composition and diversity and this microbiota modulation has a positive effect on health and growth (Hung *et al*., 2019; Lin & Yu, 2020). In this field study, BLFPs could modify the gut microbiota based on the PCA and weighted PCoA results, which is in agreement with previous studies (Hung *et al*., 2019; Lin & Yu, 2020). Interestingly, a differential bacterial community structure is also observed in the group treated with antibiotics and the group treated with BLFPs in combination with antibiotics in the present study, which is also in agreement with the previous study (Lin & Yu, 2020). The results imply that BLFP supplementation can still regulate the microbiota in the gut even though the antibiotics are simultaneously supplied. Further, simultaneous supplementation of BLFPs and antibiotics can ameliorate the FCR of weaning piglets in the present study. These findings demonstrate that BLFPs, alone or in combination with antibiotics, show a positive effect on the gut microbiota of weaning piglets.

The use of antibiotics as growth promoters in animal feeds has been banned in many countries, but livestock are more susceptible to pathogen infection, resulting in a negative impact on production. Developing a strategy to gradually replace antibiotics may be an effective and acceptable approach to maintain animal health and production. A previous study has reported that the combined use of *B. lichenformis* and antibiotics in the diet of weaning piglets does not promote growth performance (Collinder *et al*., 2003). BW and gut microbiota of weaning piglets were not improved by *Bacillus* species-based probiotics in combination with antibiotic supplementation (Poulsen *et al*., 2018). In contrast, a full replacement or half replacement of antibiotics with *B. amyloliquefaciens* ameliorated growth performance, digestive enzyme activity, and antioxidant capacity compared with the antibiotic-alone group (Wang *et al*., 2017; Hu *et al*., 2018). Our previous study demonstrated that half replacement of antibiotics with BLFPs can decrease the incidence of diarrhoea, regulate caecal microbiota composition, and improve ADG in weaning piglets (Lin & Yu, 2020). In this field study, we further confirmed that half or total replacement of antibiotics with BLFPs ameliorate the FCR of weaning piglets. Moreover, similar to the previous study (Lin & Yu, 2020), a clear separation of faecal bacterial communities between the groups treated with antibiotics alone, BLFPs alone, or both were also observed in this field study. These results imply that antibiotics alone, a full replacement or half replacement of antibiotics with BLFPs still have a different impact on the gut microbiota of weaning piglets although there is no significant difference in FCR among the groups (A, F, and AF). However, how these treatments differentially modulate microbiota in the gut and whether the microbiota regulated by these treatments have a direct impact on the health and growth of weaning piglets remains to be investigated.

Lactobacillus species have been considered as the major bacterial population found in the porcine gastrointestinal tract (Guevarra *et al*., 2019). Feeding of *Lactobacillus* species to weaning piglets resulted in an increased growth performance due to better FCR and improved gut health (Dowarah *et al*., 2016). It has been reported that the number of *Lactobacillus* species increased in the faeces of weaning piglets with an increasing dose of *B. subtilis* (Hu *et al*., 2014). However, low-doses of a *B. licheniformis* and *B. subtilis* mixture can increase the abundance of the genus, *Lactobacillus,* in the faeces of weaning piglets challenged with *Escherichia coli*, whereas high-doses of a *B. licheniformis* and *B. subtilis* mixture reduce the abundance of the genus, *Lactobacillus* (Zhang *et al*., 2017). In this field study, replacing all antibiotics or half the antibiotics with BLFPs decreased the abundance of the genus, *Lactobacillus,* in the faeces of weaning piglets. This finding is in agreement with the results of Hung *et al*. (2019), who observed that high-doses of BLFPs could reduce the abundance of *Lactobacillus* species in the faeces of weaning piglets. A previous study demonstrated that *Lactobacillus* species supplementation can promote the growth and utilization rate of the feed in weaning piglets (Guevarra *et al*., 2019). However, our previous results (Hung *et al*., 2019; Lin & Yu, 2020) and present field study demonstrate that the abundance of the genus, *Lactobacillus,* in the faeces is not strongly correlated with growth performance (BW, ADG, ADFI, and FCR). These findings may indicate that the effect of *Lactobacillus* species in the gut on the health and growth of weaning piglets remains to be confirmed. Furthermore, whether antagonistic interactions between *B. licheniformis* and *Lactobacillus* species occur in modifying the porcine gut microbiome also needs to be investigated. It has been

reported that the genus, *Streptococcus,* is enriched in the faeces of more feed-efficient pigs (Yang *et al*., 2017). Our previous study demonstrated that replacing all antibiotics with BLFPs increased the abundance of the genus, *Streptococcus,* in the cecal digesta of weaning piglets (Lin & Yu, 2020). The average abundance of the genus, *Streptococcus,* is negatively correlated with BW and the ADG (Lin & Yu, 2020). Similar to the previous study (Lin & Yu, 2020), we also confirmed that replacing all the antibiotics with BLFPs increased the abundance of the genus, *Streptococcus,* in the faeces and the abundance of the genus, *Streptococcus,* is negatively correlated with growth performance (BW, ADG, ADFI, and FCR). Furthermore, the combined use of *B. lichenformis* and antibiotics in the diet of weaning piglets can normalize the abundance of the genus, *Streptococcus,* in the faeces of weaning piglets. However, the effect of the genus, *Streptococcus,* in the gut on the health and growth of weaning piglets in response to BLFP supplementation remains to be elucidated in the future.

In the past, bacitracin was used widely as a growth promoter in pig feed and to control the spread of necrotic enteritis. It has been reported that bacitracin treatment can promote the resistance and virulence of *Streptococcus* species (Ma *et al*., 2019). Bacitracin-fed broilers have higher levels of bacitracin resistance genes and of vancomycin-resistant Enterococcaceae (Gupta *et al*., 2021). Antibiotic-resistant bacteria will remain in the gut of healthy pigs even when antibiotics are not used (Joyce *et al*., 2019). Probiotics have been considered as alternatives to antibiotics to prevent antibiotic resistance. In this study, the *tetG* (tetracycline resistance determinant, class G) gene expression was increased in the faeces of weaning piglets in response to bacitracin treatment, whereas full replacement or half replacement of antibiotics with BLFPs partially reduced the *tetG* gene expression. Interestingly, a full replacement of antibiotics with BLFPs decreased the *strA* (streptomycin phosphotransferase) gene expression in the faeces of weaning piglets. Taken together, BLFP supplementation is able to reduce ARG expression in the faeces of weaning piglets. A longitudinal study using metagenome sequencing on the effects of BLFPs on the dynamics of ARG in the faeces of weaning piglets is needed in the future.

Conclusion

Replacing all or half the antibiotics with BLFPs has beneficial effects on the FCR of weaning piglets from days 15 to 28. BLFPs and antibiotics differentially regulate the gut microbiota and ARG expression of weaning piglets. Therefore, based on our field study, BLFPs may be used as a natural alternative to antibiotics in weaning piglets.

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Authors' contributions

KHL and YHY collected the data, conducted the statistical analyses, collaborated in interpreting the results, wrote the initial draft of this manuscript, and finalized the manuscript. YHY developed the original hypothesis and designed the experiments. The authors have read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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