Wheat bran fermented by mixed fungal strains improves the digestibility of crude fiber and may benefit the gut health without impacting the growth performance in weaned pigs

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1. Supplementary Materials and Methods

1.1 The fermentation of wheat bran

Wheat bran (WB) used in the current study was purchased from Xi'an Hangcheng Flour Company Limited (Shanxi, China). The raw WB was sampled using a quarter method and crushed with a 0.45mm sample sieve. The crushed sample was then collected into an erlenmeyer flask and autoclaved at 121°C for 20min for the following fermentation. Four fungal strains, *Aspergillus niger* GDM 3.576, *Neurospora crassa* BNCC 336675, *Trichoderma viride* GDM 3.141 and *Candida tropicalis* GDM 2.6, were used in the current study as inocula. The optimum fermentation parameters were determined based on pre-trials. Briefly, a total of 5-g $(NH_4)_2SO_4$, 1-g KH_2PO_4, 0.6-g $CaCl_2$, 0.5-g $MgSO_4·7H_2O$, 0.5-g $FeSO_4·7H_2O$, 0.5-g $ZnSO_4$, 0.5-g $MnSO_4$ and 0.5-g $CoCl_2·6H_2O$ were added into 1 L of deionized water and autoclaved to prepare the mineral solution for each strain. The ratio of WB to mineral solution (g/v) was 1:1.2, and the ratio of inoculum to substrate was 5% (start culture volume/wheat bran weight). The solid-state fermentation of WB was performed as follows:

(a) The autoclaved WB (1kg) with 5% inocula (compound fungi, *Aspergillus niger*, *Neurospora crassa* and *Trichoderma viride*) and mineral solution (1.2 L) were well mixed and fermented for 2 days at 27°C to get the first-step product.

(b) The first-step product and 5% *Candida tropicalis* was well mixed and fermented for 1 day at 27°C to get the second-step product.

(c) The second-step product was dried at 60°C to get the fermented WB (FWB).

The content of dry matter (DM), gross energy (GE), crude protein (CP), crude fiber (CF), neutral-detergent fiber (NDF) and acid-detergent fiber (ADF) in FWB and feed samples were measured according to the methods described by AOAC (1995). The concentration of soluble dietary fiber (SDF) and Insoluble dietary fiber (IDF) in WB and FWB was analyzed using the Megazyme kit K-TDRF (Wicklow, Ireland) based on the AOAC Method 991.43 and AACC Method 32-07.01 (Yin *et al.* 2004). Both raw WB and FWB were sampled and sent to Wuhan Servicebio Technology Co., Ltd. (Hubei, China) to observe the morphology using a scanning electron microscopy (SEM).

1.2 The determination of T lymphocyte subsets in blood and spleen

The determination of T lymphocyte subsets in jugular blood and spleen was described before (Liu et al., 2013). In the current study, the following four controls were used for each sample: a blank control without any fluorescent antibodies and three positive controls with CD3 (FITC-Mouse Anti-Pig CD3, Cat. nos. 559582), CD4 (PerCp-Cy5.5-mouse anti-pig CD4a, 561474) and CD8 (Alexa Fluor® 647-mouse anti pig CD8α, 561475) monoclonal fluorescent antibodies (BD Biosciences, Franklin Lakes, NJ, USA). After fixing in phosphate buffer saline (PBS) buffer containing 2% formaldehyde for 30 min, 10,000 events (lymphocytes or Peripheral blood mononuclear cell) for each sample were collected using a BD Verse flow cytometer (BD Biosciences, San Jose, CA) and data was analyzed using Flow Jo software version 8.7 (Tree Star Inc., Ashland, OR, USA).

2. Supplementary Tables

Supplementary Table S1 The sequences of primers using in the current study

Gene/Bacteria	Primer sequence (5’-3’)	Reference
β-actin	F:TCTGGCACCACACCTTCT R:TGATCTGGGTCATCTTCTCAC	Luo *et al.* 2019
GAPDH	F:TTTGCGTCAGTGTCATCG R:TGCTCTGCCTTGGGTAAT	Wang *et al.* 2019
18s rRNA	F:CTGCCTTCCTTGGATGTG R:GCGGCTTTGGTGACTCTA	Wang et al. 2012
MUC-1	F:GTGCCGCTGCCCACAACCTG R:AGCCGGGTACCCCAGACCCA	Luo et al. 2019
pBD1	F:TGCCACAGGTGCCGATCT R:CTGTTAGCTGCTTAAGGAATAAAGGC	Pu et al. 2018
Occluding	F:CTACTCGTCCAACGGGAAAG R:ACGCCTCCAAGTTACCACTG	Luo *et al.* 2019
ZO-1	F:TGGCATTATTCGCCTTCATAC R:AGCCTCATTCGCATTGTTT	Chen *et al.* 2013
TLR4	F:CAGATAAGCGAGGCCGTCATT R:TTGCAGCCCACAAAAAGCA	Bao *et al.* 2012
MyD88	F:GATGGTAGCGGTTGTCTCTGAT R:GATGCTGGGGAACTCTTTCTTC	Chen *et al*. 2012
NF-kb	F:AGTACCCTGAGGCTATAACTCGC R:TCCGCAATGGAGGAGAAGTC	Wang *et al.* 2019
IL-1β	F:GCATGTGCTGAGCCTTTGTA R:CCTGGTCCTCCCAAGATTGT	Chen *et al.* 2013
IL-6	F:GGCTGCTTCTGGTGATGC R:AGAGATTTTGCCGAGGATGTA	Wang *et al.* 2019
IL-10	F:GACCAGTGGGCGACTTCTT R:ACGGCCTTGCTCTTGTTTTC	Wang *et al.* 2019
Total bacteria	F:CGGTGAATACGTTCYCGG R:GGWTACCTTGTTACGACTT	Suzuki *et al.* 2000
Lactobacillus group	F:AGCAGTAGGGAATCTTCCA R:CACCGCTACACATGGAG	Kanno *et al.* 2009
Bifidobacterium	F:TCGCGTC(C/T)GGTGTGAAAG R:CCACATCCAGC(A/G)TCCAC	Matsuki *et al.* 2004

*Clostridium*cluster XIVa	F:AAATGACGGTACCTGACTAA R:CTTTGAGTTTCATTCTTGCGAA	Matsuki *et al.* 2002
Clostridium cluster IV	F:GCACA GCAGTGGAGT R:CTTCCTCCGTTTTGTCAA	Matsuki *et al.* 2002
Escherichia coli	F:GCTACAATGGCGCATACAAA R:TTCATGGAGTCGAGTTGCAG	Lee *et al.* 2008
Enterobacteriaceae family	F:CATTGACGTTACCCGCAGAAGAAGC R:CTCTACGAGACTCAAGCTTGC	Segata *et al.* 2011
Samongella spp.	F:GTGAAATTATCGCCACGTTCGGGCA R:TCATCGCACCGTCAAAGGAACC	Xu *et al.* 2008
Streptococcus	F:GAAGAATTGCTTGAATTGGTTGAA R:GGACGGTAGTTGTTGAAGAATGG	Collado *et al.* 2009
Heat-stable toxin I(STa)	F:ATGAAAAAGCTAATGTTGGC R:TACAACAAAGTTCACAGCAG	Han *et al.* 2007
Heat-stable toxin II(STb)	F:AATATCGCATTTCTTCTTGC R:GCATCCTTTTGCTGCAAC	Han *et al.* 2007
astA	F:TGCCATCAACACAGTATATCC R:TCAGGTCGCGAGTGACGGC	Ewers *et al.* 2005
Fecalibacterium prausnitzii	F: GGAGGATTGACCCCTTCAGT R: CTGGTCCCGAAGAAACACAT	Balamurugan *et al.* 2008
Eubacterium rectale	F: AAGGGAAGCAAAGCTGTGAA R: TCGGTTAGGTCACTGGCTTC	Balamurugan *et al.* 2008
Clostridium butyricum	F: TCAATTAGAAGGCAGAGTACC R: CTAAAACTGACTGTGGCATT	Yi *et al*. 2020
Anaerostipes caccae	F: GCGTAGGTGGCATGGTAAGT R: CTGCACTCCAGCATGACAGT	Veiga *et al*. 2010

Supplementary Table S2 The level of main nutrients in WB and FWB

Ingredients,%	WB	FWB
DM	88.87	94.37
GE, kcal/kg	3973.76	4119.08
EE	4.31	4.46
CP	17.95	19.33
CF	9.42	7.11
NDF	41.25	36.27
ADF	29.05	22.18
SDF	4.85	7.90
IDF	43.20	39.76

DM, dry matter; CP, crude protein; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber; SDF, soluble dietary fiber; IDF, insoluble dietary fiber; GE, gross energy; EE, ether extract.

Supplementary Table S3 The growth performance of piglets in different groups[1]

Items[2]	C	WB	FWB	SE	*P*-value
IBW, kg	7.18	7.16	7.18	0.08	0.99
FBW, kg	24.34	24.65	24.44	0.49	0.75
ADFI, g/d	704.02	693.50	709.93	12.38	0.96
ADG, g/d	428.86	437.63	435.22	13.69	0.97
F/G	1.58	1.60	1.63	0.02	0.65

[1] Data are presented as means, n = 7. C, control; WB, wheat bran; FWB, fermented wheat bran; SE, standard error of mean.

[2] IBW, initial body weight; FBW, final body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain ratio.

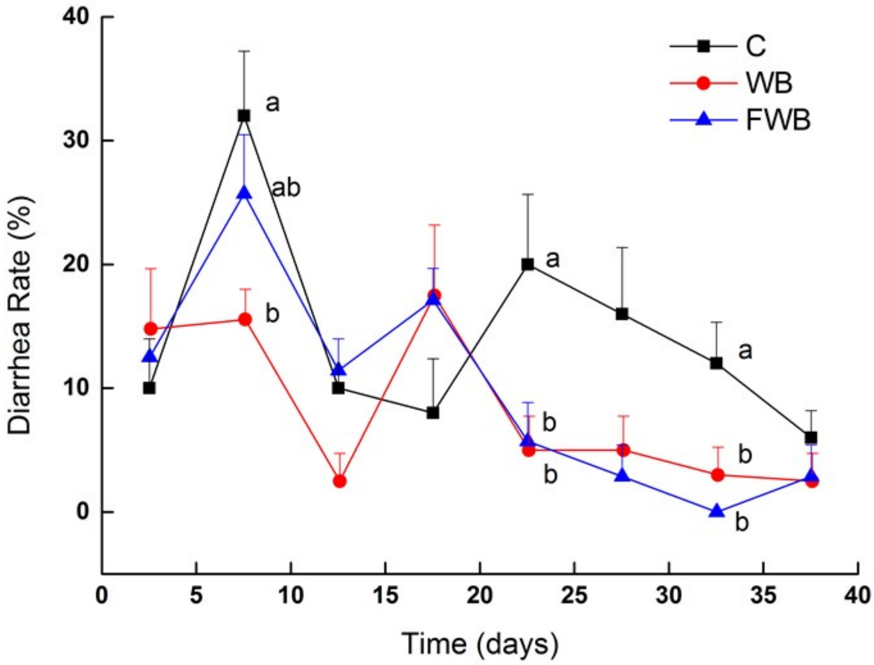
Supplementary Table S4 The percentage of T lymphocyte subsets in the spleen and blood of piglets in different groups (%)[1]

Item[2]	Group				*P*-value			
	C	WB	FWB	SE	Group	C vs WB	C vs FWB	WB vs FWB
Spleen								
$CD3^+$	60.61^{a}	53.74^{b}	58.37^{a}	1.13	0.02	0.01	0.6	0.04
$CD4^+$	27.49	24.45	28.1	1.21	0.47	NS	NS	NS
$CD8^+$	38.44	34.16	30.58	1.66	0.57	NS	NS	NS
$CD4^+/CD8^+$	0.62	0.62	1.03	0.13	0.94	NS	NS	NS
Blood								
$CD3^+$	41.16^{a}	46.19^{a}	62.56^{b}	2.54	< 0.01	0.26	< 0.01	< 0.01
$CD4^+$	14.26^{ab}	11.47^{a}	18.55^{b}	1.05	0.02	0.22	0.07	< 0.01
$CD8^+$	43.22	40.11	34.25	1.57	0.38	NS	NS	NS
$CD4^+/CD8^+$	0.68	0.59	0.95	0.08	0.53	NS	NS	NS

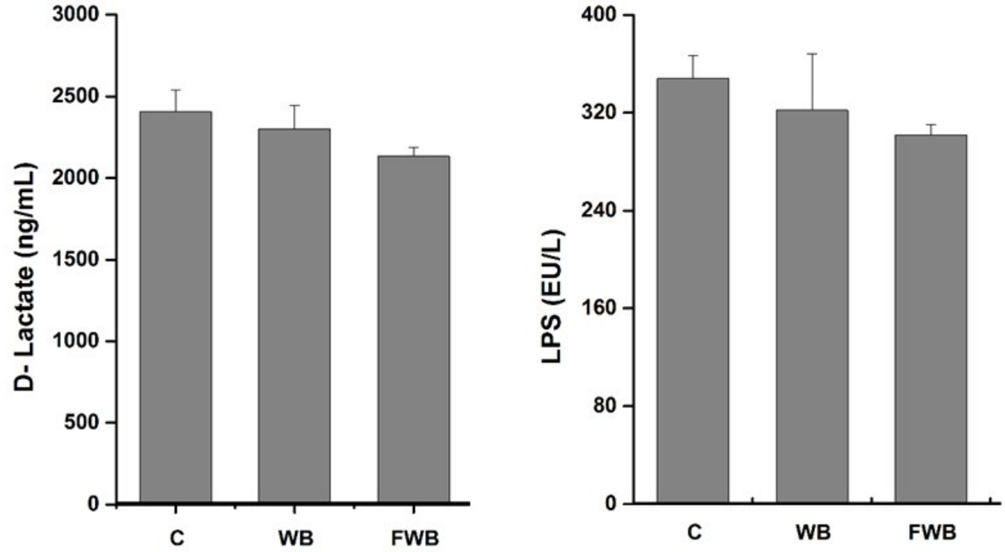
[1]The different alphabetical superscripts in the same row indicate significant difference ($P < 0.05$, n = 7). Normally distributed data are presented as means, while non-normally distributed data are presented as median. Of all data, $CD4^+/CD8^+$ in the blood of piglets is non-normally distributed. C, control; WB, wheat bran; FWB, fermented wheat bran; SE, standard error of mean; NS, not significant.

[2]$CD3^+$, percentage of $CD3^+$T lymphocytes; $CD4^+$, percentage of $CD4^+$T lymphocytes; $CD8^+$, percentage of $CD8^+$T lymphocytes; $CD4^+/CD8^+$, ratio of $CD4^+$T lymphocytes to $CD8^+$T lymphocytes.

3. Supplementary Figures



Supplementary Figure S1 The change of diarrhea rate in the piglets from different groups (n = 7). C, control; WB, wheat bran; FWB, fermented wheat bran. Superscripts of lowercase letters represent significant differences (*P*< 0.05).



Supplementary Figure S2 The D-lactate and LPS concentration of blood in the different treatments.

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