

1 **Oral administration of a select mixture of *Bacillus* probiotics**
2 **affects the gut microbiota and goblet cell function in newly**
3 **weaned *MUC4* resistant pigs**
4 **following *Escherichia coli* challenge**

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16 **Supplementary Information**

17 **Content**

18 **1. Supplementary Figure S1.**

19 **2. Supplementary Figure S2.**

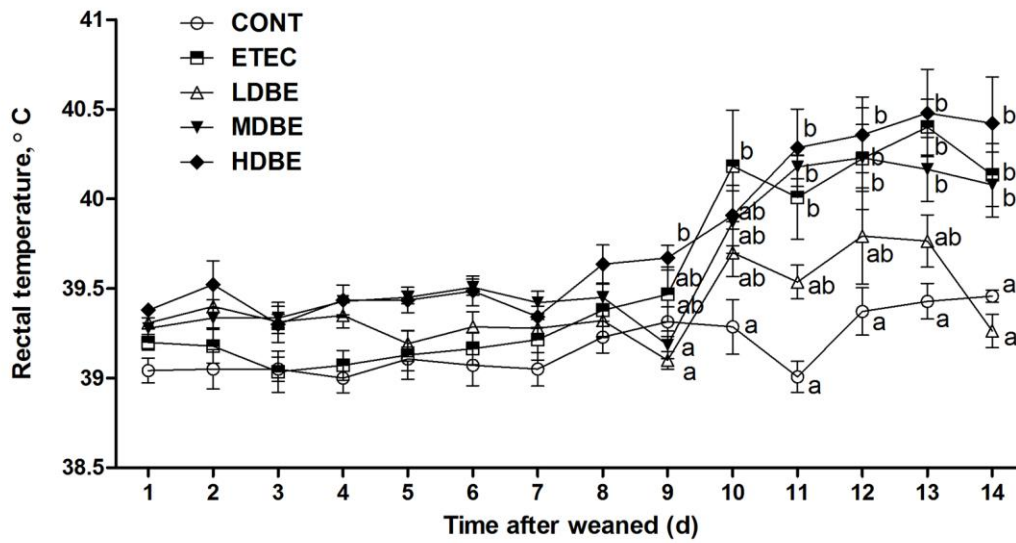
20 **3. Supplementary Figure S3.**

21 **4. Supplementary Table S1.**

22 **5. Supplementary Table S2.**

23 **6. Supplementary Table S3.**

24 **7. Supplementary Table S4.**



25

26 **Figure S1** Effects of BLS-mix on rectal temperature of newly-weaned piglets following F4⁺ ETEC challenge. The *MUC4*

27 RR pigs received sterile physiological saline orally (CONT), sterile physiological saline orally followed by F4⁺ ETEC

28 (1.0×10^{10} CFU/d, p.o.) challenge (ETEC), were pretreated with a low dose of BLS-mix (3.9×10^8 CFU/d, p.o.) for 1

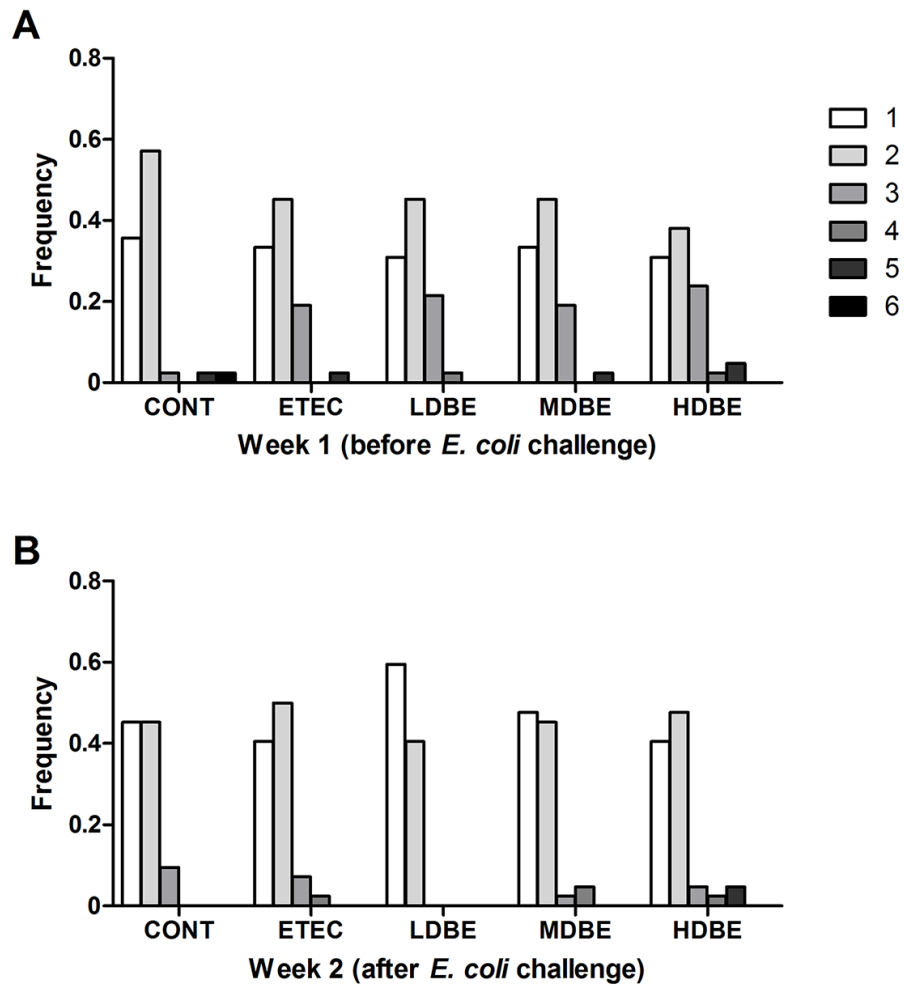
29 week followed by F4⁺ ETEC challenge (LDBE), were pretreated with a moderate dose of BLS-mix (7.8×10^8 CFU/d, p.o.)

30 for 1 week followed by F4⁺ ETEC challenge (MDBE), or were pretreated with a high dose of BLS-mix (3.9×10^9 CFU/d,

31 p.o.) for 1 week followed by F4⁺ ETEC (HDBE). Data are presented as means \pm SEM (n = 6 per group). Within the same

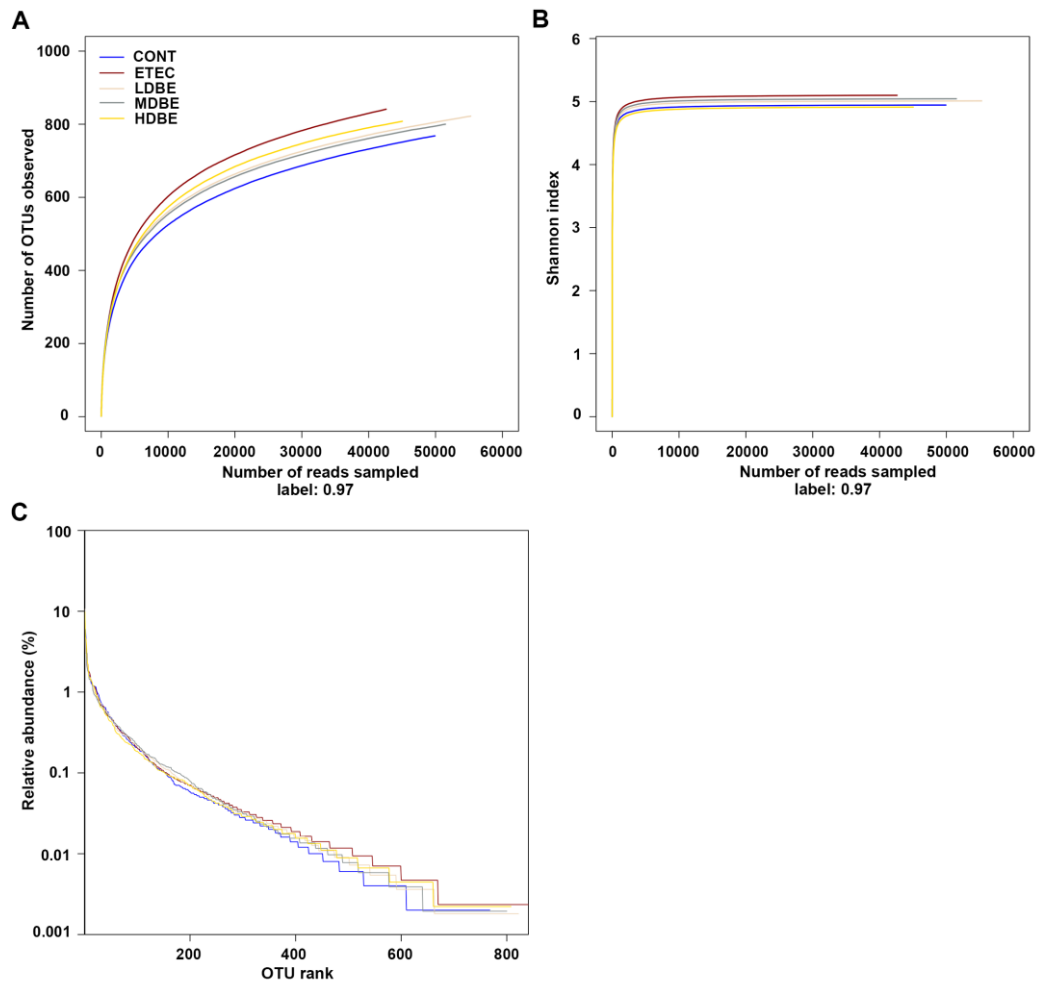
32 time, mean values with different superscript letters (a, b) are significantly different ($P < 0.05$).

33



34

35 **Figure S2** Effects of BLS-mix on diarrhea scores of newly-weaned pigs following F4⁺ ETEC challenge. The *MUC4* RR
 36 pigs received sterile physiological saline orally (CONT), sterile physiological saline orally followed by F4⁺ ETEC ($1.0 \times$
 37 10^{10} CFU/d, p.o.) challenge (ETEC), were pretreated with a low dose of BLS-mix (3.9×10^8 CFU/d, p.o.) for 1 week
 38 followed by F4⁺ ETEC challenge (LDBE), were pretreated with a moderate dose of BLS-mix (7.8×10^8 CFU/d, p.o.) for
 39 1 week followed by F4⁺ ETEC challenge (MDBE), or were pretreated with a high dose of BLS-mix (3.9×10^9 CFU/d,
 40 p.o.) for 1 week followed by F4⁺ ETEC (HDBE).



41

42 **Figure S3** Alpha diversity analysis of the colonic microbiota. Rarefaction curves for OTUs (A) and Shannon index (B), as

43 well as rank-abundance curves (C) of average reads within the same group (n = 6 per group) were calculated for reads

44 exhibiting $\geq 97\%$ sequence identity.

45

46 **Table S1** Effects of oral administration of BLS-mix on the incidence and duration of
 47 diarrhea in newly weaned pigs before and after F4⁺ ETEC challenge.

Group ^a /item	Pigs			Pig days			Significance of difference			
	At risk	With diarrhea		At risk	With diarrhea		ETEC	LDBE	MDBE	HDBE
	(n)	(n)	(%)	(n)	(n)	(%)				
Week 1										
CONT	6	2	33.33	42	2	4.76	0.084	0.032	0.084	0.020
ETEC	6	1	16.67	42	1	2.38		0.580	1.000	0.691
LDBE	6	1	16.67	42	1	2.38			0.580	0.544
MDBE	6	1	16.67	42	1	2.38				0.691
HDBE	6	2	33.33	42	2	4.76				
Week 2										
CONT	6	0	0	42	0	0	0.628	0.039	0.193	0.290
ETEC	6	1	16.67	42	1	2.38		0.058	0.630	0.558
LDBE	6	0	0	42	0	0			0.185	0.069
MDBE	6	2	33.33	42	2	4.76				0.445
HDBE	6	3	50	42	3	7.14				

48 ^a Piglets received sterile physiological saline orally (CONT), received sterile physiological saline orally followed by
 49 F4⁺ ETEC challenge (1×10^{10} CFU/d, per os [p.o.]) (ETEC), were pretreated with a low dose of BLS-mix (3.9×10^8
 50 CFU/d, p.o.) for 1 week followed by F4⁺ ETEC (LDBE), were pretreated with a moderate dose of BLS-mix (7.8×10^8
 51 CFU/d, p.o.) for 1 week followed by F4⁺ ETEC challenge (MDBE) or were pretreated with a high dose of BLS-mix
 52 (3.9×10^9 CFU/d, p.o.) for 1 week followed by F4⁺ ETEC challenge (HDBE).
 53 n = 6 pigs per group; Pearson's chi-squared test.

54 **Table S2** Effects of oral administration of BLS-mix on the growth of newly weaned pigs
 55 before and after F4⁺ ETEC challenge.

Item ²	Treatment ¹					SEM	P-value
	CONT	ETEC	LDBE	MDBE	HDBE		
Week 1 (days 0 to 8, pre-challenge)							
ADG, g/d	127	136	133	111	112	18	0.724
ADFI, g/d	159	146	145	147	138	13	0.872
G:F, g/g	0.80	0.93	0.92	0.76	0.81	0.08	0.963
Week 2 (days 8 to 15, post-challenge)							
ADG, g/d	287 ^a	106 ^b	233 ^{ab}	234 ^{ab}	214 ^{ab}	17	0.018
ADFI, g/d	310 ^a	218 ^b	318 ^a	324 ^a	302 ^a	12	0.003
G:F, g/g	0.93	0.49	0.73	0.72	0.71	0.06	0.821

56 ¹ Piglets received sterile physiological saline orally (CONT), received sterile physiological saline orally followed by
 57 F4⁺ ETEC challenge (1×10^{10} CFU/d, p.o.) (ETEC), were pretreated with a low dose of BLS-mix (3.9×10^8 CFU/d,
 58 p.o.) for 1 week followed by F4⁺ ETEC challenge (LDLE), were pretreated with a moderate dose of BLS-mix ($7.8 \times$
 59 10^8 CFU/d, p.o.) for 1 week followed by F4⁺ ETEC challenge (MDBE) or were pretreated with a high dose of
 60 BLS-mix (3.9×10^9 CFU/d, p.o.) for 1 week followed by F4⁺ ETEC challenge (HDBE). n = 6 per group.

61 ^{a, b} Within a row, least-square means without a common lowercase superscript differ ($P < 0.05$); Tukey's test.

62 ² ADG, average daily gain; ADFI, average daily feed intake; G: F, gain to feed ratio.

63

64 **Table S3** Relative abundance (%) of the predominant taxa in the colonic content microbiota of
 65 pigs.

Level	Treatment ^a				
	CONT	ETEC	LDBE	MDBE	HDBE
Phylum					
Bacteroidetes	61.00	52.60	59.60	59.20	57.80
Firmicutes	29.50	32.40	31.90	27.90	27.600
Spirochaetae	3.55	6.85	5.34	6.38	5.55
Tenericutes	2.45	1.83	0.78	0.75	1.23
Proteobacteria	1.41	3.48	1.30	3.56	4.22
Phylum. Class					
Bacteroidetes. Bacteroidia	60.50	50.80	59.20	57.40	57.50
Firmicutes. Clostridia	26.10	30.30	28.90	24.40	24.00
Spirochaetae. Spirochaetes	3.55	6.85	5.34	6.38	5.55
Tenericutes. Mollicutes	2.45	1.83	0.78	0.75	1.23
Firmicutes. Erysipelotrichia	1.72	0.98	2.09	1.22	1.47
Firmicutes. Negativicutes	1.38	0.92	0.64	1.75	1.69
Phylum. Class. Order					
Bacteroidetes. Bacteroidia. Bacteroidales	60.50	50.80	59.20	57.40	57.50
Firmicutes. Clostridia. Clostridiales	26.10	30.20	28.90	24.40	24.00
Spirochaetae. Spirochaetes. Spirochaetales	3.55	6.85	5.34	6.38	5.55
Tenericutes. Mollicutes. RF9	2.45	1.82	0.78	0.73	1.21
Firmicutes. Erysipelotrichia. Erysipelotrichales	1.72	0.98	2.09	1.22	1.47
Firmicutes. Negativicutes. Selenomonadales	1.38	0.92	0.64	1.75	1.69
Class. Order. Family					
Bacteroidia. Bacteroidales. Prevotellaceae	47.40	35.90	43.50	39.70	41.10
Clostridia. Clostridiales. Ruminococcaceae	14.20	17.10	15.40	14.10	12.90
Clostridia. Clostridiales. Lachnospiraceae	9.96	9.22	9.87	7.63	7.49
Bacteroidia. Bacteroidales. Rikenellaceae	5.63	2.06	5.20	4.02	1.18
Bacteroidia. Bacteroidales. S24-7	4.08	6.00	6.23	5.73	7.75
Spirochaetes. Spirochaetales. Spirochaetaceae	3.55	6.85	5.34	6.38	5.55
Bacteroidia. Bacteroidales. Porphyromonadaceae	2.53	4.54	2.30	4.60	6.04
Erysipelotrichia. Erysipelotrichales. Erysipelotrichaceae	1.72	0.98	2.09	1.22	1.47
Negativicutes. Selenomonadales. Acidaminococcaceae	1.23	0.73	0.56	1.39	1.43

Order. Family. Genus					
Bacteroidales. Prevotellaceae. <i>Prevotella</i>	29.14	23.48	27.70	28.14	29.84
Bacteroidales. Prevotellaceae.	14.85	8.66	12.71	9.34	8.72
uncultured					
Clostridiales. Ruminococcaceae. uncultured	6.16	7.38	6.62	5.35	5.18
Bacteroidales. Rikenellaceae.	5.62	1.88	5.19	3.98	1.11
<i>RC9_gut_group</i>					
Bacteroidales. S24-7. norank	4.08	5.98	6.23	5.73	7.75
Bacteroidales. Porphyromonadaceae.	2.52	4.32	2.21	4.57	5.91
<i>Parabacteroides</i>					
Spirochaetales. Spirochaetaceae. <i>Spirochaeta</i>	2.45	3.75	3.50	2.70	3.09
Selenomonadales. Acidaminococcaceae.	1.22	0.73	0.56	1.39	1.43
<i>Phascolarctobacterium</i>					
Clostridiales. Ruminococcaceae.	1.22	1.02	1.33	1.21	1.10
<i>Faecalibacterium</i>					
Clostridiales. Ruminococcaceae.	1.16	1.66	1.67	1.11	1.01
<i>Incertae_Sedis</i>					
Spirochaetales. Spirochaetaceae. <i>Treponema</i>	1.10	3.09	1.86	3.62	2.50
Clostridiales. Ruminococcaceae.	1.06	1.41	1.02	1.64	1.24
<i>Subdoligranulum</i>					
Clostridiales. Ruminococcaceae. <i>Oscillospira</i>	1.00	0.90	0.66	1.20	0.88

66 ^a Piglets received sterile physiological saline orally (CONT), received sterile physiological saline orally followed by F4⁺
67 ETEC challenge (1×10^{10} CFU/d, p.o.) (ETEC), were pretreated with a low dose of BLS-mix (3.9×10^8 CFU/d, p.o.) for
68 1 week followed by F4⁺ ETEC challenge (LDLE), were pretreated with a moderate dose of BLS-mix (7.8×10^8 CFU/d,
69 p.o.) for 1 week followed by F4⁺ ETEC challenge (MDBE) or were pretreated with a high dose of BLS-mix (3.9×10^9
70 CFU/d, p.o.) for 1 week followed by F4⁺ ETEC challenge (HDBE).

71 **Table S4** Order.family.genus taxa alteration of the colonic microbiota in response to F4⁺ ETEC infection and BLS-mix intervention (relative
 72 abundance, %).

Bacterial taxa	Mean ±SEM					One-way analysis of variance	Tukey's multiple comparison test									
	CONT	ETEC	LDBE	MDBE	HDBE		C	C	C	C	E	E	E	L	L	M
	(C)	(E)	(L)	(M)	(H)		vs. E	vs. L	vs. M	vs. H	vs. L	vs. M	vs. H	vs. M	vs. H	vs. H
Bacteroidales.	0.0575	0.0953	0.3149	0.5772	0.1243	<i>P</i> = 0.017	NS	NS	0.023	NS	NS	0.040	NS	NS	NS	NS
RF16.	±	±	±	±	±											
Norank	0.0117	0.0306	0.1348	0.2032	0.0482											
Lactobacillales.	0.0758	0.0458	0.0916	0.4281	0.2800	<i>P</i> = 0.019	NS	NS	NS	NS	NS	0.034	NS	NS	NS	NS
Lactobacillaceae.	±	±	±	±	±											
<i>Lactobacillus</i>	0.0079	0.0086	0.0283	0.1639	0.0996											
Clostridiales.	0.7093	0.6249	1.7069	1.1423	0.8678	<i>P</i> = 0.021	NS	0.041	NS	NS	0.023	NS	NS	NS	NS	NS
Clostridiaceae.	±	±	±	±	±											
<i>Clostridium</i>	0.0880	0.1302	0.3242	0.2653	0.2671											
Clostridiales.	0.0025	0.0019	0.0082	0.0032	0.0018	<i>P</i> = 0.019	NS	NS	NS	NS	0.030	NS	NS	NS	0.027	NS
Lachnospiraceae.	±	±	±	±	±											
<i>Howardella</i>	0.0014	0.0006	0.0022	0.0011	0.0011											

Clostridiales.	0.0413	0.0111	0.0184	0.0204	0.0034	$P = 0.034$	NS	NS	NS	0.034	NS	NS	NS	NS	NS	NS
Lachnospiraceae.	±	±	±	±	±											
<i>Lachnospira</i>	0.0179	0.0045	0.0027	0.0043	0.0012											
Clostridiales.	0.8652	1.2573	0.5217	0.5338	0.6001	$P = 0.009$	NS	NS	NS	NS	0.015	0.018	0.036	NS	NS	NS
Lachnospiraceae.	±	±	±	±	±											
Uncultured	0.2132	0.2150	0.0392	0.1365	0.0362											
Clostridiales.	0	0.0017	0.0003	0	0	$P = 0.019$	0.035	NS	NS	NS	NS	0.035	0.035	NS	NS	NS
Ruminococcaceae.	±	±	±	±	±											
<i>Acetanaerobacterium</i>	0	0.0008	0.0003	0	0											
Clostridiales.	0	0.0039	0.0010	0.0010	0.0018	$P = 0.024$	0.014	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ruminococcaceae.	±	±	±	±	±											
<i>Sporobacter</i>	0	0.0012	0.0007	0.0007	0.0009											
Erysipelotrichales.	0.0011	0	0.0026	0	0.0006	$P = 0.002$	NS	NS	NS	NS	0.002	NS	NS	0.002	0.025	NS
Erysipelotrichaceae.	±	±	±	±	±											
<i>Turicibacter</i>	0.0005	0	0.0006	0	0.0006											

73 C, CONT; E, ETEC; L, LDBE, M, MDBE; H, HDBE; NS, no significance.