ORIGINAL RESEARCH ARTICLES

Effect of creep feed supplemented with *Bacillus subtilis* on growth performance, blood parameters, gut permeability and fecal microbiome in piglets

Efecto del alimento iniciador suplementado con Bacillus subtilis sobre el desempeño productivo, parámetros sanguíneos, permeabilidad del intestino y microbioma fecal en lechones

Efeito da ração pré-inicial suplementada com Bacillus subtilis sobre o desempenho produtivo, parâmetros sanguíneos, permeabilidade intestinal e microbioma fecal em leitões

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Abstract

Background: Creep feed and *B. subtilis* supplementation can promote the growth and health of pigs around weaning. **Objective:** This study was conducted to evaluate the effects of creep feed supplemented with *Bacillus subtilis* on growth performance, gut permeability, inflammation response, fecal score, and microbiome of pigs during pre- and post-weaning periods. **Methods:** A total of 12 litters were randomly allotted to 3 treatments based on breed, parity, and litter size at d 2-5 of age. Treatments were: 1) Control (CON): no creep feed, 2) CF: creep feed (24% crude protein) without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet. At weaning (22-25 d of age), pigs were moved to the nursery facility within original treatments and fed a common diet in 5 pens per treatment with 5 pigs per pen for 35 d. **Results:** There were no significant differences in the number of pigs weaned, pig and litter weaning weight, weight gain, and creep feed intake in the suckling period. In the nursery period, the CF group had lower body weight ($p=0.05$, tendency) and average daily gain ($p<0.05$) in d 0-21 postweaning compared to the CON group, with the CFB group showing no difference from either CON or CF group, while no difference was observed in the overall nursery period. No significant differences were observed in average daily feed intake, gain-to-feed ratio and fecal score among dietary treatments in the entire nursery period. There were no differences in fecal *E. coli* count, serum TNF-α, and diamine oxidase levels. Serum levels of IL-1β at weaning ($p<0.05$) and d-lactate at d 21 postweaning ($p=0.08$, tendency) were lower in the CFB group than the CON group, with the CF group showing no difference from either CON or CFB group. Serum urea nitrogen concentrations at weaning were greater in the CON group than the CF group ($p<0.05$). The CFB group had greater fecal microbial richness than the CON group at d 21 postweaning ($p<0.05$). **Conclusion:** Creep feed with high protein content did not improve postweaning growth, while *B. subtilis* supplementation had potential to reduce gut permeability and inflammation, and influence fecal microbiome.

Keywords: *Bacillus subtilis; creep feed; feces; feed intake; gut permeability; growth; inflammation; microbiome; pigs; pre-weaning; probiotics; urea nitrogen; weaning.*

Resumen

Antecedentes: El alimento iniciador y la suplementación con *Bacillus subtilis* pueden promover el crecimiento y la salud de los cerdos alrededor del destete. **Objetivo:** Este estudio se realizó para evaluar los efectos del alimento iniciador suplementado con *Bacillus subtilis* sobre el desempeño productivo, la permeabilidad intestinal, la respuesta inflamatoria, la consistencia fecal y el microbioma de los cerdos durante los periodos pre y posdestete. **Métodos:** Un total de 12 camadas fueron asignadas aleatoriamente a 3 tratamientos con base en la raza, la paridad y el tamaño de la camada entre los días 2 y 5 de edad. Los tratamientos fueron: 1) Control (CON): sin alimento iniciador, 2) CF: alimento iniciador (24% de proteína cruda) sin suplementación con *B. subtilis*, y 3) CFB: alimento iniciador con suplementación de *B. subtilis* a una concentración de $1{,}88\times10^{8}$ UFC/kg de dieta. Al destete (22–25 días de edad), los lechones fueron trasladados a las instalaciones de recría manteniendo los tratamientos originales y alimentados con una dieta común en 5 corrales por tratamiento con 5 lechones por corral durante 35 días. **Resultados:** No se observaron diferencias significativas en el número de lechones destetados, el peso al destete de los lechones y de las camadas, la ganancia de peso y el consumo de alimento iniciador durante la lactancia. En el periodo de recría, el grupo CF presentó menor peso corporal ($p=0{,}05$, tendencia) y menor ganancia diaria promedio ($p<0{,}05$) entre los días 0–21 posdestete en comparación con el grupo CON, mientras que el grupo CFB no mostró diferencias con respecto a CON ni CF; no obstante, no se observaron diferencias en el periodo total de recría. Tampoco se observaron diferencias significativas en el consumo diario promedio de alimento, la conversión alimenticia ni la consistencia fecal entre los tratamientos dietarios durante todo el periodo de recría. No hubo diferencias en el conteo fecal de *E. coli*, ni en los niveles séricos de TNF-α y diamina oxidasa. Los niveles séricos de IL-1β al destete ($p<0{,}05$) y de D-lactato al día 21 posdestete ($p=0{,}08$, tendencia) fueron menores en el grupo CFB que en el grupo CON, mientras que el grupo CF no difirió de CON ni de CFB. Las concentraciones séricas de nitrógeno ureico al destete fueron mayores en el grupo CON que en el grupo CF ($p<0{,}05$). El grupo CFB presentó mayor riqueza microbiana fecal

que el grupo CON al día 21 posdestete (p<0,05). **Conclusión:** El alimento iniciador con alto contenido proteico no mejoró el crecimiento posdestete, mientras que la suplementación con *B. subtilis* mostró potencial para reducir la permeabilidad intestinal y la inflamación, además de influir en el microbioma fecal.

Palabras clave: *alimento iniciador; Bacillus subtilis; cerdos; consumo de alimento; crecimiento; destete; heces; inflamación; microbioma; nitrógeno ureico; permeabilidad intestinal; predestete; probióticos.*

Resumo

Antecedentes: A ração pré-inicial e a suplementação com Bacillus subtilis podem promover o crescimento e a saúde dos suínos ao redor do desmame. **Objetivo:** Este estudo foi conduzido para avaliar os efeitos da ração pré-inicial suplementada com *Bacillus subtilis* sobre o desempenho produtivo, a permeabilidade intestinal, a resposta inflamatória, a consistência fecal e o microbioma de suínos durante os períodos pré- e pós-desmame. **Métodos:** Um total de 12 leitegadas foram distribuídas aleatoriamente em 3 tratamentos com base na raça, ordem de parto e tamanho da leitegada entre os dias 2 e 5 de idade. Os tratamentos foram: 1) Controle (CON): sem ração pré-inicial, 2) CF: ração pré-inicial (24% de proteína bruta) sem suplementação com *B. subtilis*, e 3) CFB: ração pré-inicial com suplementação de *B. subtilis* na concentração de $1,88\times10^8$ UFC/kg de dieta. No desmame (22–25 dias de idade), os leitões foram transferidos para a instalação de recria, mantendo-se os tratamentos originais, e alimentados com uma dieta comum em 5 baias por tratamento, com 5 leitões por baia, durante 35 dias. **Resultados:** Não foram observadas diferenças significativas no número de leitões desmamados, no peso ao desmame dos leitões e das leitegadas, no ganho de peso e no consumo da ração pré-inicial durante o período de lactação. No período de recria, o grupo CF apresentou menor peso corporal (p=0,05, tendência) e menor ganho diário médio (p<0,05) entre os dias 0–21 pós-desmame em comparação com o grupo CON, enquanto o grupo CFB não diferiu dos grupos CON e CF; contudo, não houve diferenças no período total de recria. Não foram observadas diferenças significativas no consumo diário médio de ração, na conversão alimentar nem na consistência fecal entre os tratamentos dietéticos durante todo o período de recria. Não houve diferenças na contagem fecal de *E. coli*, nem nos níveis séricos de

TNF-α e diamina oxidase. Os níveis séricos de IL-1β no desmame ($p<0,05$) e de D-lactato no dia 21 pós-desmame ($p=0,08$, tendência) foram menores no grupo CFB do que no grupo CON, enquanto o grupo CF não diferiu dos grupos CON e CFB. As concentrações séricas de nitrogênio ureico no desmame foram maiores no grupo CON do que no grupo CF ($p<0,05$). O grupo CFB apresentou maior riqueza microbiana fecal que o grupo CON no dia 21 pós-desmame ($p<0,05$). **Conclusão:** A ração pré-inicial com alto teor de proteína não melhorou o crescimento pós-desmame, enquanto a suplementação com *B. subtilis* demonstrou potencial para reduzir a permeabilidade intestinal e a inflamação, além de influenciar o microbioma fecal.

Palavras-chave: *Bacillus subtilis; consumo de ração; crescimento; desmame; fezes; inflamação; microbioma; nitrogênio ureico; permeabilidade intestinal; pré-desmame; probióticos; ração pré-inicial; suínos.*

Introduction

Weaning is one of the most stressful events in pig's life, resulting in issues such as low feed intake, postweaning diarrhea, and thus growth retardation (Varley and Wiseman, 2001; Kwon *et al.,* 2025). Therefore, it is crucial to prepare pigs for a smooth weaning transition as newly weaned piglets experience stress due to abrupt dietary changes, even when high-quality ingredients such as fish meal and animal plasma are included (Zheng *et al.,* 2021).

Creep feeding is a feeding management practice in the suckling period that provides additional nutrients to pigs and offers a chance for pigs to obtain familiarity with solid diets before weaning (Kuller *et al.,* 2007; Heo *et al.,* 2018). Previous studies have reported that high-complexity creep feed exhibited a positive effect in improving preweaning growth rate (Heo *et al.,* 2018) and enhancing feed efficiency in the early nursery period (Isensee *et al.,* 2020) compared with low-complexity feed, indicating the importance of creep feed composition in pre- and post-weaning growth of pigs. In addition, gut health and microbiota play significant roles in postweaning growth in pigs, as there are significant changes in gut integrity and microbiome at weaning (St-Pierre *et al.*, 2023). Choudhury *et al.* (2021) reported that pigs fed a customized fibrous diet from d 2 of age until weaning at d 28 of age showed accelerated gut microbiome maturation. Moreover, probiotic supplementation in creep feed may have positive effects for suckling pigs by reducing gut pH, enhancing the immune system, and reducing postweaning

diarrhea, thereby improving overall postweaning gut health and growth in pigs (Hou *et al.,* 2015).

However, previous studies for creep feeding have mainly focused on specific aspects of providing additional nutrients and improving feed familiarity, while studies for probiotic supplementation have primarily focused on pigs after weaning (Duddeck *et al.,* 2024). Although, it has been reported that *Bacillus*-based probiotic supplementation in creep feed improved weaning weight and fecal score and increased fecal total lactic acid bacteria count in weaning pigs (Mazur-Kuśnirek *et al.,* 2023), there is limited information on how creep feed influences gut health and growth of weaning pigs, and how incorporation of *B. subtilis* into creep feed could facilitate gut microbiome maturation and improve gut health and immunity of pigs around weaning.

Therefore, the objective of this study was to evaluate the effects of creep feed supplemented with *B. subtilis* on pre- and post-weaning growth, along with gut permeability, inflammation response, and fecal microbiome of piglets at weaning and d 21 post-weaning.

Materials and Methods

Ethical considerations

The experiment was conducted under protocols approved by the Institutional Animal Care and Use Committee of the University of Georgia (GA, USA; A2024 06-008-Y1-A1) and the University of Kentucky (KY, USA; 2019-3227).

Location

This experiment and sample collections were carried out in an environmentally controlled room of the Swine Research Unit of the University of Kentucky and the University of Georgia.

Animals, Experimental Design, and Housing

A total of 12 litters were randomly allotted to 3 treatments based on breed, parity, litter size, and age of pigs at d 2-5 of lactation. The treatments were: 1) Control (CON): no creep feed, 2) CF: creep feed without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet (0.015% of 1.25×10^9 CFU/g *B. subtilis*; Arm & Hammer Animal and Food Production, Waukesha, WI, USA). The treatments were administered

from d 2-5 of lactation to allow sufficient time for creep feeding and ensure adequate creep feed consumption (Muro *et al.,* 2023). A common starter basal diet was used as the creep feed for the CF treatment in the suckling period. This diet was formulated to meet or exceed the nutrient requirement of NRC (2012) for 7-11 kg pigs and contained 24% crude protein as a high-protein diet (Table 1, Phase 1 diet) to evaluate the potential impact of high dietary protein from increased soybean meal inclusion in creep feed and to maintain feed familiarity between the creep feed and the Phase 1 nursery diet. *B. subtilis* was supplemented to this diet for the CFB treatment. All creep feed diets were mixed with 0.5% Cr_2O_3 as a fecal indicator to confirm creep feed consumption. All sows and their pigs were housed in individual farrowing crates with free access to water and feed in the environmentally controlled farrowing facility. A common corn-soybean meal-based diet that meets or exceeds the nutrient requirements of NRC (2012) was fed to all lactating sows. At weaning (22-25 d of age; average 23.5 ± 1.09 d of age), the fecal color of each pig was assessed to identify creep feed consumption, and all pigs were confirmed as having consumed creep feed in the CF and CFB groups. Pigs were moved to the environmentally controlled nursery facility and allotted to pens based on body weight and sex within the original treatments to 5 pens per treatment with 5 pigs per pen. The CF diet was used as a common diet for the postweaning period until day 21 postweaning to maintain pig's familiarity to feed (Phase 1; d 0-21 postweaning). From d 21-35 postweaning (Phase 2), another common diet formulated to meet or exceed the nutrient requirement of NRC (2012) for 11-25 kg pigs was fed to all pigs.

Table 1. Diet formulation and calculated composition

Ingredient, %	Phase 1 (d 0-21 postweaning)[1]	Phase 2 (d 21-35 postweaning)
Corn	41.30	56.72
Soybean meal (dehulled)	32.00	31.50
Whey	15.00	5.00
Oats	2.50	-
Fish meal	1.00	2.00
Animal plasma	3.00	-
Soybean oil	2.39	2.00
L-Lysine·HCl	0.13	0.23
DL-Methionine	0.15	0.15
L-Threonine	0.08	0.13
Dicalcium phosphate	0.65	0.62
Limestone	1.07	0.92
Salt	0.50	0.50
Vitamin premix[2]	0.08	0.08
Mineral premix[3]	0.15	0.15
Total	100.00	100.00
Calculated chemical composition		
Metabolizable energy, kcal/kg	3,405	3,398
Crude protein, %	24.01	21.98
SID[4] Lys, %	1.37	1.23
SID Met+Cys, %	0.83	0.75
SID Thr, %	0.92	0.82
SID Trp, %	0.28	0.23
Toal Ca, %	0.80	0.70
STTD[4] P, %	0.40	0.33

[1] This diet was used as a basal diet for creep feed in suckling period.

[2] Vitamin premix supplied the following per kilogram of diet: 16,521 IU of vitamin A, 4,683 IU of vitamin D_3, 124 IU of vitamin E, 14 mg of vitamin K, 53 μg of vitamin B_{12}, 6.7 mg of riboflavin, 42 mg of pantothenic acid, 83 mg of niacin, 0.33 mg of folic acid, 8.3 mg of vitamin B_6, 2.3 mg of thiamin, and 0.46 mg of biotin.

[3] Trace mineral premix supplied the following per kilogram of diet: 33 mg of Mn as manganous oxide, 110 mg of Fe as ferrous sulfate, 110 mg of Zn as zinc sulfate, 16.5 mg of Cu as copper sulfate, 0.3 mg of I as Ca iodate, 0.3 mg of Se as sodium selenite.

[4] SID: standardized ileal digestible; STTD: standardized total tract digestible.

Data and Sample Collection and Analysis

In the suckling period, litter size was recorded at treatment allotment (d 2-5 of age) and weaning (d 22-25 of age). Creep feed remaining in the feeder was weighed daily, and fresh feed was added to calculate daily creep feed intake. Individual pigs were weighed, and feed disappearance was recorded at initial, weaning, d 7, 14, 21, 28, and 35 postweaning to calculate average daily gain (ADG) in both suckling and nursery periods and to measure average daily feed intake (ADFI), and gain to feed ratio (G:F) in the nursery period. Fecal score was recorded every day in the nursery period using a 4-point fecal scoring system (1=normal, 2=soft, looser than normal feces, slight diarrhea, 3=moderate diarrheic feces, and 4=liquid, severe diarrhea) by observing individual pigs in each pen and assessing signs of stool consistency in the pen.

At weaning and d 21 postweaning, blood samples were collected from the jugular vein of 1 or 2 pigs per pen (6 pigs per treatment), selected based on the treatment average body weight within each treatment. Blood samples were drawn into disposable vacutainer tubes containing the anticoagulant K_3 EDTA (Becton Dickinson, Franklin, NJ, USA). Serum was separated from the blood by centrifugation at 2,500 × g for 30 min at 4°C and stored at -80°C until further analysis. Serum urea nitrogen was analyzed at the UGA Veterinary Diagnostic Laboratories using Roche Cobas c501 clinical chemistry analyzer. Serum samples were analyzed for TNF-α (RayBiotech Life, Inc., Peachtree Corners, GA, USA), IL1-β (RayBiotech Life, Inc.), diamine oxidase (AFG Bioscience, Northbrook, IL, USA), and d-lactate (Novus Biologicals, LLC., Centennial, CO, USA) by using commercial enzyme-linked immunosorbent assay kits following the manufacturer's instructions.

Fecal samples were collected from 1 or 2 pigs per pen (6 pigs per treatment) at weaning and d 21 postweaning. Fecal donors were selected based on the treatment average body weight within each treatment, and samples were collected by rectal palpation. Collected feces was flash-frozen in liquid nitrogen and stored at -80°C until the analysis of microbiome was performed. Fresh fecal samples were analyzed for *E. coli* count at the Arm & Hammer Animal and Food Production Laboratory (Waukesha, WI, USA), following the procedures outlined in the Bacteriological Analytical Manual (USFDA, 1998).

For fecal microbiome analysis, deoxyribonucleic acid (DNA) was extracted from samples following procedures adapted from (Rothrock Jr. *et al.,* 2014), which used mechanical and enzymatic methods. Briefly, fecal samples (0.35 g) were transferred into a 2 mL lysing matrix E

tube (MP Biomedicals LLC, Irvine, CA, USA) and then sample mechanical disruption was accomplished using a FastPrep-24 5G homogenizer (MP Biomedicals, LLC, Irvine, CA, USA) at 6.0 m/s for 40 s, for two cycles, followed by incubation at 95°C for 5 min. A QIAamp Fast DNA Stool Mini Kit (QIAGEN, Venlo, The Netherlands) was used for enzymatic extraction and DNA purification. Final concentration was checked via fluorometry (Qubit; Thermo Fisher Scientific, Waltham, MA, USA). Samples with concentrations less than 5 ng/µL were discarded, and the DNA extraction process was repeated. Amplicon libraries were generated by two rounds of polymerase chain reaction (PCR) amplifications. The first round of PCR amplification targeted the V3 and V4 hypervariable regions of the 16S rRNA gene with the forward: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and reverse: S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAA TCC-3′) primer pairs (Klindworth *et al.,* 2013), followed by PCR clean-up using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). A second PCR step was performed to attach Illumina's indices and sequencing adapters (Nextera XT Index Kit; Illumina Inc., San Diego, CA, USA), followed by a second PCR clean-up step using AMPure XP beads. The final library was quantified via fluorometry (Qubit; Thermo Fisher Scientific, Waltham, MA, USA). Samples were delivered to the Kelly Products Inc. sequencing lab (Covington, GA, USA) for 16S rRNA gene sequencing. Sequencing was performed using an Illumina MiSeq v3 2 × 300 bp kit (Illumina Inc., San Diego, CA, USA). A well-characterized bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA, USA) was used as a control for sequencing runs. Sequence data (FASTQ files) were demultiplexed and imported into QIIME2 v.2024.10 (Bolyen *et al.,* 2019). The DADA2 plugin was used to control sequence quality, merge forward and reverse reads, and remove chimeric sequences (Callahan *et al.*, 2016). The feature-classifier plugin, which utilized a Naïve Bayes classifier trained on the Greengenes2 reference database (McDonald *et al.,* 2024), was used for taxonomic classification. Taxonomic data was further cleaned and summarized using the mbX package in R (Lamichhane and Lourenco, 2025). Individual microbial taxa were summarized as relative abundance at different taxonomic levels ranging from phyla to species. All taxa that were less abundant than 0.1% were classified as 'Other.' In addition, samples were rarefied to a common sequencing depth for calculation of alpha diversity (microbial richness, diversity, and evenness) and beta diversity (Bray-Curtis dissimilarity and UniFrac distances).

Statistical Analysis

All data were analyzed following a randomized completely block design using the Proc Mixed procedure of SAS (ver. 9.4. SAS Inst. Inc., Cary, NC, USA). The experimental unit was a litter or pen for growth performance and fecal score, and an individual pig for blood and fecal parameters. The models included the treatment as a fixed effect and the replicate as a random effect. The least-square means were separated using the PDIFF option in SAS. Multiple comparisons were adjusted using Tukey's method. Statistical differences were established at $p<0.05$, and tendencies were established at $p<0.10$.

Results

In the creep feeding period, there were no significant differences in the number of weaning pigs, pig and litter body weights at weaning, pig and litter ADGs, and creep feed intake in the suckling period among dietary treatments (Table 2).

Table 2. Preweaning growth performance of pigs fed creep feed with or without *Bacillus subtilis* supplementation [1]

	Treatment[2]				
	CON	**CF**	**CFB**	**SEM**	**P-value**
Litter size, n					
Initial	9.25	8.75	9.67	0.46	0.45
Weaning	9.00	8.75	9.40	0.51	0.74
Weaning age	23.25	23.50	23.75	0.59	0.67
Pig body weight, kg					
Initial	1.93	2.16	1.89	0.12	0.42
Weaning	7.96	8.24	8.20	0.22	0.64
Pig weight gain, kg/d	0.302	0.307	0.310	0.013	0.89
Litter body weight, kg					
Initial	17.55	19.18	18.28	2.65	0.86
Weaning	71.71	71.69	76.45	3.81	0.65
Litter weight gain, kg/d	2.71	2.68	2.89	0.10	0.38
Creep feed intake, g					
By litter per day	-	38.29	43.00	6.23	0.63
By pig per day	-	4.33	5.09	0.10	0.62

[1] N=4 litters per treatment.

[2] Treatments: 1) CON: no creep feed, 2) CF: creep feed without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet.

During the nursery period (Table 3), no significant differences were observed among treatments in ADFI and G:F in each phase and the overall period. Pigs in the CF group tended to have lower (p=0.05, tendency) body weight at d 21 postweaning compared to those in the CON group, with the CFB group showing no difference from either CON or CF group. Although ADG did not differ among treatments during the individual weeks, the ADG in d 0-21 postweaning was greater (p<0.05) in the CON group than in the CF group, with the CFB group showing no difference from either CON or CF group, while no differences were observed in body weight and ADG among treatments in d 21-35 postweaning and overall period.

Table 3. Postweaning growth performance of pigs fed creep feed with or without *B. subtilis* supplementation[1]

	Treatment[2]				
	CON	**CF**	**CFB**	**SEM**	**P-value**
Body weight, kg					
d 0	8.21	8.25	8.24	0.32	0.93
d 7	10.05	10.17	9.97	0.31	0.50
d 14	13.30	13.02	13.04	0.35	0.45
d 21	18.17^{x}	17.55^{y}	17.67^{xy}	0.42	0.05
d 28	23.20	22.57	22.92	0.48	0.61
d 35	28.09	26.98	27.68	0.54	0.24
Average daily gain, kg/d					
d 0-7	0.263	0.274	0.248	0.013	0.35
d 7-14	0.464	0.408	0.439	0.018	0.15
d 14-21	0.697	0.647	0.661	0.021	0.22
d 21-28	0.718	0.717	0.751	0.043	0.83
d 28-35	0.698	0.630	0.679	0.042	0.53
d 0-21	0.475^{a}	0.443^{b}	0.449^{ab}	0.008	0.04
d 21-35	0.708	0.674	0.715	0.022	0.40
d 0-35	0.568	0.535	0.556	0.011	0.16
Average daily feed intake, kg/d					
d 0-7	0.356	0.344	0.328	0.013	0.29
d 7-14	0.629	0.598	0.610	0.015	0.40
d 14-21	1.012	0.963	0.921	0.027	0.12
d 21-28	1.209	1.157	1.203	0.036	0.55
d 28-35	1.144	1.118	1.112	0.028	0.64
d 0-21	0.666	0.635	0.619	0.015	0.14
d 21-35	1.176	1.137	1.157	0.025	0.42
d 0-35	0.870	0.836	0.835	0.017	0.28

G:F					
d 0-7	0.738	0.798	0.757	0.023	0.11
d 7-14	0.738	0.683	0.720	0.026	0.23
d 14-21	0.689	0.673	0.718	0.019	0.30
d 21-28	0.593	0.620	0.624	0.031	0.74
d 28-35	0.608	0.564	0.611	0.030	0.49
d 0-21	0.713	0.699	0.726	0.011	0.19
d 21-35	0.602	0.594	0.618	0.017	0.59
d 0-35	0.653	0.642	0.666	0.009	0.21

[a,b] Means in a row with different superscripts differ ($p<0.05$).

[x,y] Means in a row with different superscripts differ ($p<0.10$, tendency).

[1] N=5 pens per treatment with 5 pigs per pen.

[2] Treatments: 1) CON: no creep feed, 2) CF: creep feed without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* at 1.88×10^8 CFU/kg diet) supplementation.

There were no significant differences in fecal score in each phase and overall period and fecal *E. coli* count at weaning and d 21 postweaning (Table 4).

Table 4. Postweaning fecal score and *E. coli* count of pigs fed creep feed with or without *B. subtilis* supplementation

	Treatment[1]				
	CON	**CF**	**CFB**	**SEM**	**P-value**
Fecal score[2]					
d 0-7	1.51	1.70	1.46	0.11	0.24
d 7-14	2.07	2.24	2.29	0.12	0.47
d 14-21	1.93	1.90	1.97	0.09	0.84
d 21-28	1.40	1.63	1.51	0.09	0.26
d 28-35	1.57	1.66	1.53	0.12	0.48
d 0-21	1.84	1.95	1.90	0.07	0.51
d 21-35	1.49	1.65	1.52	0.10	0.30
d 0-35	1.70	1.83	1.75	0.07	0.35
Fecal *E. coli* count[3], log CFU/g					
Weaning	8.52	8.40	8.57	0.33	0.89
d 21	7.05	7.41	7.15	0.38	0.58

[1] Treatments: 1) CON: no creep feed, 2) CF: creep feed without *Bacillus subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet.

[2] N=5 pens per treatment with 5 pigs per pen.

[3] N=6 per treatment.

With regard to blood response parameters (Table 5), there were no significant differences observed in serum TNF-α and diamine oxidase levels. Serum urea nitrogen concentrations at weaning were greater ($p<0.05$) in the CON group than in the CF group with the CFB group showing no difference from either CON or CF group. Serum IL-1β levels at weaning were greater ($p<0.05$) in the CON group than in the CFB group, with the CF group showing no difference from either CON or CFB group. Serum d-lactate levels at d 21 postweaning tended to be greater ($p=0.08$) in the CON group than in the CFB group, with the CF group showing no difference from either CON or CFB group.

Table 5. Serum urea nitrogen, cytokines, and gut permeability parameters of pigs fed creep feed with or without *B. subtilis* supplementation [1]

	Treatment[2]				
	CON	**CF**	**CFB**	**SEM**	**P-value**
Urea nitrogen, mg/dL					
Weaning	8.67[a]	6.00[b]	7.00[ab]	0.54	0.02
d 21 postweaning	8.67	9.00	10.33	0.64	0.19
TNF-α, pg/ml					
Weaning	8.65	9.06	8.25	0.91	0.82
d 21 postweaning	8.29	8.85	8.60	1.32	0.96
IL-1β, pg/ml					
Weaning	57.18[a]	50.40[ab]	48.85[b]	1.33	0.02
d 21 postweaning	50.38	52.68	49.02	2.31	0.50
Diamine oxidase, ng/ml					
Weaning	11.59	10.54	9.43	1.94	0.74
d 21 postweaning	6.75	5.82	5.67	0.96	0.70
D-lactate, mM					
Weaning	1.655	1.550	1.685	0.162	0.68
d 21 postweaning	1.341[x]	0.892[xy]	0.869[y]	0.146	0.08

[a,b] Means in a row with different superscripts differ ($p<0.05$).

[x,y] Means in a row with different superscripts differ ($p<0.10$, tendency).

[1] N=6 per treatment.

[2] Treatments: 1) CON: no creep feed, 2) CF: creep feed without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet.

For the fecal microbiome analysis, a total of 553,455 raw reads were obtained from fecal samples collected from 16 pigs at weaning and d 21 postweaning. After quality control, combining

paired-end reads, and filtering chimeras, on average, 15,374 sequences per sample passed all the bioinformatics filters. There was an interaction of the day of postweaning × dietary treatment ($p<0.05$), in which the CFB group had lower richness (number of ASV) than the CF group at weaning (Figure 1B), but it had greater richness than the CON group at d 21 postweaning. There were day effects ($p<0.05$) in alpha diversity [number of observed amplicon sequence variants (ASV); Figure 1A] and beta diversity (Bray-Curtis dissimilarity; Figure 2) between weaning and d 21 postweaning.

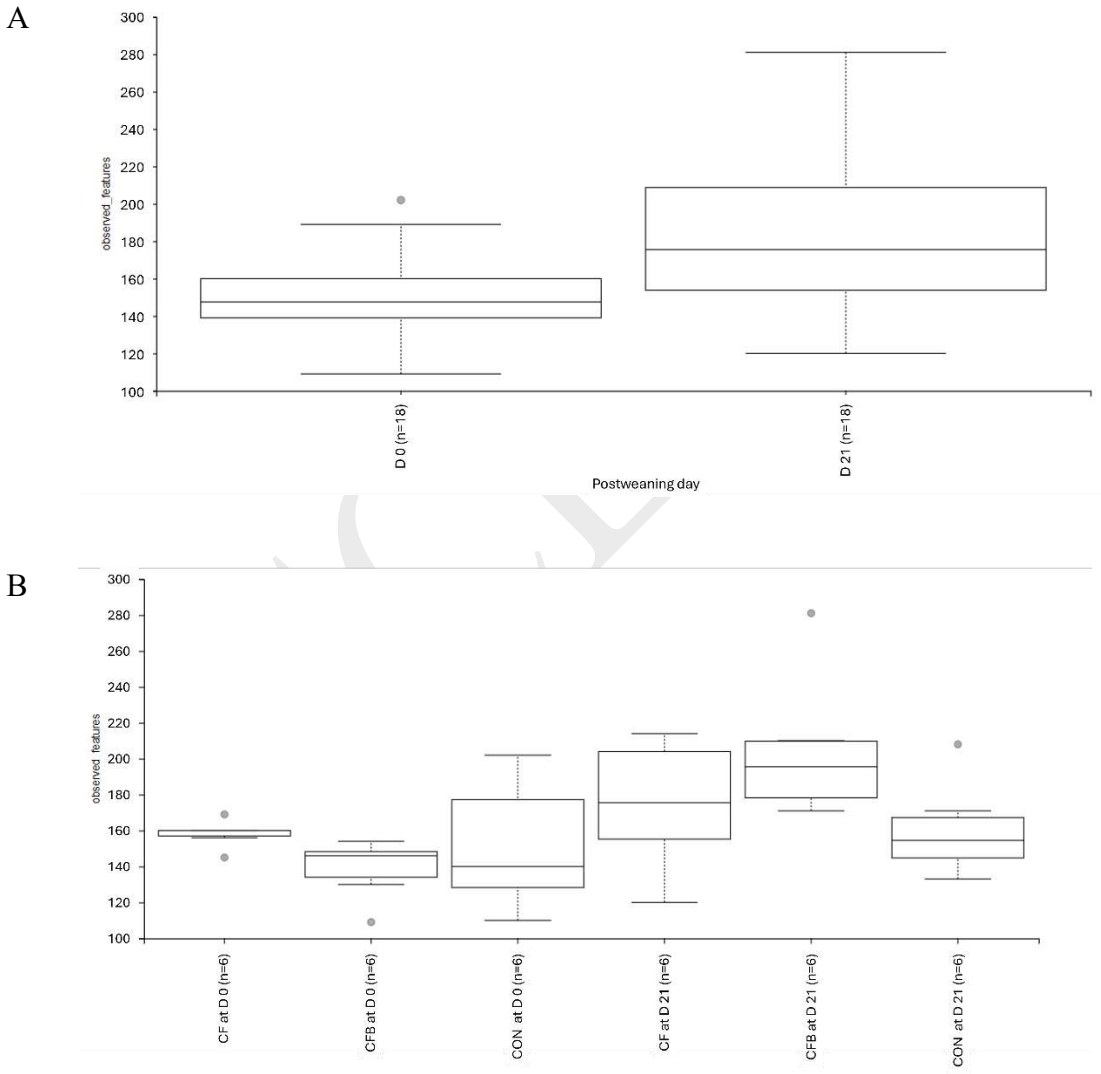

Figure 1. Alpha diversity [number of observed amplicon sequence variants (ASV)] in feces of pigs fed creep feed with or without *B. subtilis* supplementation at weaning and d 21 postweaning. Day effect (A; $p<0.05$, n=18 per day). Day × treatment effect (B; $p<0.05$, n=6 per treatment per

day). Treatments: 1) CON: no creep feed, 2) CF: creep feed without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet.

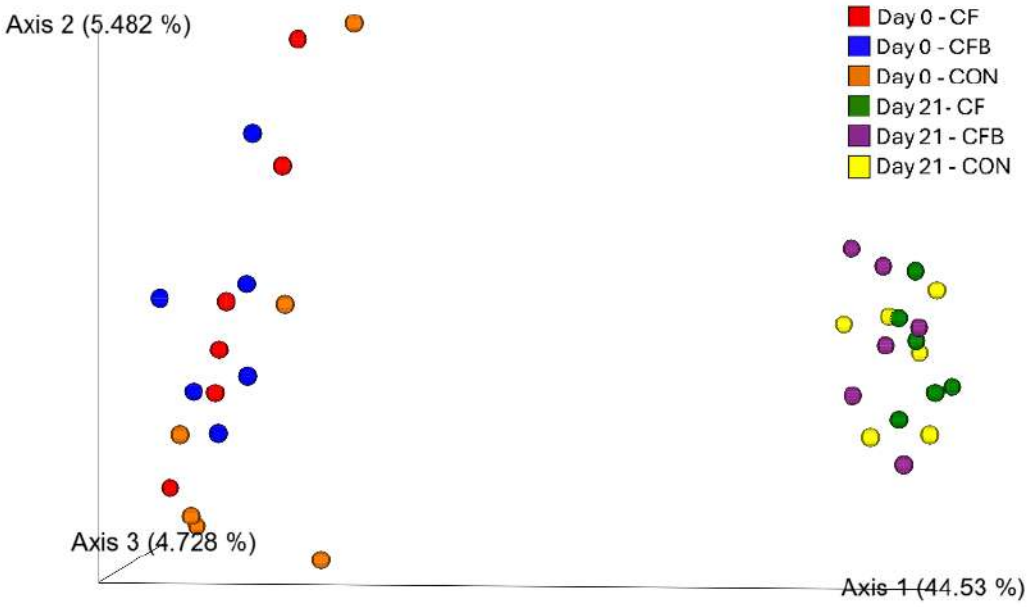

Figure 2. Beta diversity (Bray-Curtis dissimilarity) in feces of pigs fed creep feed with or without *B. subtilis* supplementation at weaning and d 21 postweaning. Day effect ($p<0.05$; n=18 per day). No interaction between day and treatment. Treatments: 1) CON: no creep feed, 2) CF: creep feed without *B. subtilis* supplementation, and 3) CFB: creep feed with *B. subtilis* supplementation at 1.88×10^8 CFU/kg diet.

Discussion

This study evaluated the effects of creep feed with or without *B. subtilis* supplementation on pre- and post-weaning growth performance along with blood biomarkers for gut health and immunity, fecal score, and microbiome at weaning and d 21 postweaning. Creep feeding in suckling period has been reported to accelerate gut microbiota maturation and promote preweaning growth, particularly when fed piglets with high-complexity creep feed (Heo et al., 2018). It may also enhance immune system and reduce the incidence of postweaning diarrhea (Hou *et al.*, 2015; Choudhury *et al.*, 2021). However, no significant differences in pig and litter body weight, weight

gain, and creep feed intake in the suckling period were observed among dietary treatments. This agrees with previous studies reporting no effect of creep feeding in pre-weaning growth rate and these results are likely due to low creep feed intake, which is a common limitation in evaluations of preweaning nutritional intervention (Sulabo *et al.,* 2010; Isensee *et al.,* 2020). In the current study, although creep feed was provided to pigs for 20 d in the suckling period, daily creep feed intake was lower than in previous studies (Sulabo *et al.,* 2010; Christensen and Huber, 2021) ranging from 52-132 g per day per litter. Sulabo *et al.* (2010) reported that increasing creep feeding duration from 2 d to 13 d increased total creep feed intake in the suckling period. However, Yan *et al.* (2011) reported that increasing creep feeding duration from 11 d to 16 d did not increase total creep feed intake in the suckling period. Although there is no direct comparison for creep feeding duration in the current study, this result suggests that providing creep feed in early life of pigs like d 2-5 of age does not enhance creep feed consumption and preweaning performance. In addition, although the relatively low average initial litter size of 9.2 piglets per sow in the current study might have influenced creep feed consumption, Muro *et al.* (2023) reported that litter size had no significant effect on total creep feed consumption.

In the nursery period, creep feeding in the suckling period did not affect postweaning feed intake and feed efficiency regardless of *B. subtilis* supplementation to the creep diets. This result agrees with previous studies reporting that postweaning feed intake and feed efficiency did not differ among pigs with no creep feeding, creep feed-eaters, and creep feed-non-eaters (Heo *et al.,* 2018; Isensee *et al.,*2020). Interestingly, the weight gain of pigs for d 0-21 postweaning was greater in the CON group than in the CF group. This result can be attributed to a slightly lower feed intake although there was no significant difference. This contrasts with previous studies, which showed that creep feeding could improve or had no influence in postweaning growth rate (Bruininx *et al.,* 2002; Heo *et al.,*2018). The creep feed used in the current study contained high protein levels (24%) fulfilled by using a higher level of soybean meal (32%) compared to the level typically used in starter diets (20%; Cemin et al., 2020) to evaluate the potential impact of high dietary protein from increased soybean meal inclusion in creep feed and to maintain feed familiarity between the creep feed and the Phase 1 nursery diet. Previous studies reported that high diet complexity of the creep feed using highly digestible feed ingredients could result in increased postweaning growth, feed intake and feed efficiency compared to the creep feed without high quality ingredients (Heo *et al.,* 2018; Isensee *et al.,* 2020). Young piglets have hypersensitivity to soybean allergens that can

increase inflammation responses and allergenic reactions (Li *et al.,*1990). Thus, too early exposure to high levels of soybean meal may not benefit postweaning growth in pigs as young pigs have an immature digestive capacity that limits their ability to process complex plant proteins (Lindemann *et al.,*1986). Additionally, their creep feed intake may be insufficient to enhance postweaning performance, which could typically be improved through creep feeding with higher quality ingredients than those used in the current study (Heo *et al.,* 2018; Isensee *et al.,*2020). Further studies are needed to demonstrate the effect of creep feed with *B. subtilis* supplementation and high protein content on pre- and postweaning growth performance in a larger-scale setting due to the low number of replicates in the current study.

However, *B. subtilis* supplementation to high-protein creep feed appears to show a partial mitigation of these effects, possibly through reduced inflammation responses (a reduction in serum IL-1β levels) and gut permeability (a reduction in serum d-lactate levels) as observed in the current study, suggesting a potential benefit of supplementing creep feed with *B. subtilis*. IL-1β is a pro-inflammatory cytokine; therefore, its reduction following *B. subtilis* supplementation suggests a decrease in inflammatory responses. Shim *et al.* (2005) reported that supplementing creep feed with oligofructose enhanced preweaning growth rate, duodenum crypt depth, while creep feed supplemented with probiotics had intermediate values. In addition, creep feed supplemented with oligofructose or probiotics had a higher count of ileum *Bifidobacteria* than the control group. Konieczka *et al.* (2023) reported that piglets fed creep feed supplemented with *Bacillus*-based probiotics had a thicker ileal mucosa, higher villi height, and greater Peyer's patches. Therefore, the results of the current study indicate that creep feed supplemented with *B. subtilis* may have potential to positively affect pig performance and health by reducing inflammation and gut permeability in the weaning transition period.

There were no significant differences in fecal scores and fecal *E. coli* count among the dietary treatment groups, which agrees with Middelkoop *et al.* (2020) reporting that creep feed provided from 2 d of age did not affect fecal score, severity, and duration of diarrhea in weaned pigs. Carstensen *et al.* (2005) also reported that postweaning diarrhea was not associated with creep feeding although it was associated with fecal shedding with pathogenic *E. coli*. Therefore, the results of the current study indicate that creep feeding and *B. subtilis* supplementation to creep feed had no influence on fecal consistency and diarrhea occurrence associated with *E. coli*. One possible explanation is that the pigs in the current study were not exposed to sufficient enteric

challenge to elicit a detectable effect of creep feed or *B. subtilis* supplementation. The absence of severe diarrhea across all groups suggests a generally healthy gut environment, which may have limited the observable benefits. Therefore, the lack of differences in fecal consistency and *E. coli* counts does not imply ineffectiveness, but rather that the pigs' healthy baseline status may have masked potential effects. Future studies under pathogen challenge are needed to clearly demonstrate their impact on gut health.

In the current study, serum d-lactate levels (a gut permeability parameter) at d 21 postweaning and IL-1β levels (a proinflammatory cytokine) at weaning were lower in the CFB group compared to the CON group with intermediate values in the CF group, indicating that creep feed with *B. subtilis* supplementation could reduce gut permeability and inflammation responses in pigs as serum d-lactate level is a blood marker of gut permeability, with elevated values indicating impaired gut barrier function (Tossou *et al.*, 2016; Ding *et al.*, 2024). In addition, the CF group had reduced serum urea nitrogen levels at weaning compared to the CON group, with intermediate values in the CFB group. This suggests that creep feeding may improve protein utilization efficiency, which agrees with Byrgesen *et al.* (2021) who reported that creep feed eaters tended to have lower blood urea nitrogen concentrations compared to non-eaters although the underlying mechanisms remain unclear. Weaning stress can increase intestinal permeability in pigs, primarily due to the stressors associated with weaning itself and the resulting elevation in proinflammatory cytokines (Hu *et al.,* 2013; Moeser *et al.,* 2017). *B. subtilis* supplementation for weaning pigs could reduce mRNA expression of IL1-β in ileal mucosa (He *et al.,* 2020a) and increased mRNA expression of tight junction protein when challenged with enterotoxigenic *E. coli* F18 (He *et al.,* 2020b), suggesting its beneficial effect on systemic immunity and gut barrier functions. Thus, these results suggest that creep feeding alone may slightly reduce inflammation and improve protein utilization in pigs during the suckling period, while the inclusion of *B. subtilis* in creep feed appears to further reduce inflammation and gut permeability. This indicates that pigs begin adapting to solid feed, develop stronger immune function during the suckling period, with *B. subtilis* contributing to improved gur barrier functions and reduced inflammation responses during the weaning transition.

Regarding the fecal microbiome, there was an effect of day observed in alpha and beta diversities. This indicates that pigs have different fecal microbiome composition in nursery period from weaning with a greater richness observed after weaning, which agrees with Saladrigas-García

et al. (2022) reporting that gut microbial richness increases with age. In addition, Lerch *et al.* (2023) reported that creep feeding from d 10 of age did not change alpha diversity in the stomach and cecum microbiome compared to pigs fed sow milk only, although it was changed by age before and after weaning. Their findings agree with the result of the current study, as the CON group showed similar alpha and beta diversities to both CF and CFB groups. At weaning, the pigs in the CFB group had lower microbial richness compared to those in the CF group. However, during the nursery period, the CFB group showed higher microbial richness than the CF group. This suggests that while *B. subtilis* supplementation in the creep feed may not immediately enhance fecal microbial richness at weaning, it could promote increased microbial diversity in the nursery period. Although the exact mode of action remains unclear, the delayed effect may be due to the initial microbial competition or immune modulation (Wang *et al.,* 2021). In addition, the immature gut environment in the suckling period may limit colonization by diverse microbial species despite *B. subtilis* supplementation, as the maturation of the gut ecosystem is not yet complete by the time of weaning (Everaert *et al.*, 2017). As pigs mature and undergo dietary and physiological changes associated with weaning (Wang *et al.,* 2021; Kwon *et al.,* 2025), *B. subtilis* may help establish a more favorable gut environment, enhance the fermentation of new dietary substrates, and support the proliferation of beneficial microbes, thereby contributing to greater microbial richness at later developmental stages (Duddeck *et al.*, 2024).

Overall, the effects of creep feeding on gut health, microbiome composition, and subsequent growth performance are complex and are primarily influenced by the intake and composition of creep feed. These factors, in turn, are affected by the sow's milking ability relative to the piglets' nutritional demands, which is associated with litter size and weaning age. These interrelated factors present the challenges in clearly identifying the specific effects of creep feeding and highlight the importance of well-controlled studies that account for these variables.

Conclusions

The results of the current study suggest that although early exposure to a high-protein creep feed slightly lower early postweaning growth in pigs, supplementation of *B. subtilis* helps mitigate these effects by reducing inflammation responses and gut permeability and increasing fecal microbial richness in the nursery period. Therefore, early dietary interventions and feed composition should be carefully matched to the digestive capacity and developmental stage of pigs.

B. subtilis may serve as a potential feed additive in creep feed to support digestive and immunological development in pigs.

Declarations

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Conflict of Interest

The authors declare that this study received partial funding from Arm & Hammer Animal and Food Production. They were involved in the study's conceptualization, experimental design, and laboratory analysis, and they provided the probiotic product used in this study. Enid T. McKinley and Samantha Hernandez are employees of Arm & Hammer Animal and Food Production and contributed to experimental design and fecal *E. coli* analysis. The remaining authors have no real or potential conflict of interest. All authors have read and approved the manuscript for submission.

Author Contributions

ESS, AKK, CHK, JAT, JHL, MDL, ETM, SH, JML, and YDJ were responsible for the design and conception of the study, collecting data, sample analysis, writing, reviewing, and critical reading of the paper. MDL and YDJ were responsible for administering the project and editing the paper.

Use of artificial intelligence (AI)

No AI or AI-assisted technologies were used during the preparation of this work.

Data availability

The data sets used in this study are available from the corresponding author upon request.

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