Effect of Supplementing a Bacillus Multi-Strain Probiotic to a Post-weaning Diet on Nutrient Utilisation and Nitrogen Retention of Piglets.

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Article

Effect of Supplementing a *Bacillus* Multi-Strain Probiotic to a Post-Weaning Diet on Nutrient Utilisation and Nitrogen Retention of Piglets

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Simple Summary: There is a global need to produce pork that is free of antimicrobials and has a minimum impact on the environment. Probiotics (i.e. live microorganisms) are suggested to not only modulate piglets' health but also enhance nutrient utilization. This study demonstrated that the use of the multi-strain *Bacillus* probiotic (*B. amyloliquefaciens* and *B. subtilis*) was able to improve the nutrient efficiency of weaned piglets and may contribute to a reduced N pollution into the environment.

Abstract: Probiotics are suggested to improve pig health, nutrient utilisation, performance, and may reduce nitrogen (N) pollution. However, the effectivity of a single-strain might be different from a multi-strain. The study was conducted to investigate the effect of a novel *Bacillus* multi-strain on nutrient digestibility, energy utilisation, and N retention in weaned piglets. The experiment consisted of a control diet (CD) and a supplemented diet (SD). The probiotic used for SD consisted of *B. amyloliquefaciens* - 516 and *B. subtilis* - 541. A total of 8 boars/ treatment were weaned (day 0; 8.5 kg bodyweight). Until day 10, piglets were fed *ad libitum* and were housed in pairs; from day 11, piglets were fed semi *ad libitum* and were housed individually. From day 14, faecal and urine were collected twice daily. Piglets were humanely euthanised at day 19 (15.0 kg bodyweight) after which the jejunum, ileum, and colon content were collected. In faeces the apparent total tract digestibility (ATTD) of, amongst others, DM, organic matter (OM), crude protein (CP), non-starch polysaccharides (NSP), and subsequently net energy (NE) were calculated using titanium dioxide as an indigestible marker. In jejunum and ileum, the apparent digestibility of CP was estimated and in ileum the apparent AA digestibility. In urine, N content was measured to determine N retention. The volatile fatty acid (VFA), branched-chain fatty acid (BCFA), and lactic acid content were analysed in colon and faeces. Apparent CP digestibility in jejunum and ileum were not affected by treatment (P>0.05) and no effect was observed on apparent ileal digestibility of AA (P>0.05). Supplementation with the multi-strain probiotic improved ATTD of DM (P=0.01; +1.3%) and OM (P=0.02; +1.2%) and tended to improved ATTD of CP (P=0.10; +2.2%) and NSP (P=0.07; +1.9%). The multi-strain probiotic also improved the NE value (P=0.02; +0.2 MJ/kg DM) and improved N retention (P=0.05; +1.6%). Supplementation did not influence VFA, BCFA, and lactic acid content in the faeces (P>0.05). However, in colon, supplementation did influence lactic acid content (lower; P=0.01) and tended to influence valeric acid content (higher; P=0.09). In conclusion, results from the current study suggests that the multi-strain probiotic has the potential to contribute to improve nutrient efficiency in weaned piglets. More research needs to be done to identify the impact of the improved nutrient utilization on gut health in post-weaned pigs as well as environmental pollution.

Keywords: *Bacillus amyloliquefaciens; Bacillus subtilis;* energy utilisation; ileum digestibility; volatile fatty acids

1. Introduction

The various stressors at weaning have a great impact on piglet performance and health. This, along with restrictions with respect to the use of antimicrobials, have led to a significant shift in the

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development of bioactive alternatives. One of such alternatives may be probiotics (i.e. live microorganisms), which are suggested to not only modulate piglets' health [1–3] but to also enhance nutrient utilisation [4,5] and ultimately performance [2]. Especially, the *Bacillus*-based probiotic has gained interest due to its superior characteristics to withstand harsh environmental conditions [2,4,6]. Furthermore, *Bacillus* strains have been suggested to produce various extracellular enzymes [6], which may influence nutrient utilisation. In particular, *Bacillus* strains *B. amyloliquefaciens*, *B. subtilis*, and *B. mojavensis* are suggested to have strong probiotic potentials [7]. Whether a probiotic is effective depends on the diet, strain, dose, and several pig factors including health status and age [8]. Additionally, the mechanism of action of a single-strain probiotic might be different from that of a multi-strain [5]. It is furthermore speculated that probiotics cannot only play a role in digestion, but also in absorption and propulsion in the gastrointestinal tract, which may influence nutrient retention [9]. Due to sustainability concerns, besides the effects on animal health and performance, there is interest in the role of probiotics in reducing nitrogen (N) pollution, but studies evaluating this are lacking.

The objective of the current study was to evaluate whether a novel multi-strain probiotic consisting of *B. amyloliquefaciens* and *B. subtilis* could alter nutrient digestibility, energy utilisation, amino acid (AA) digestibility, and N retention in weaned piglets. *Bacillus amyloliquefaciens* can synthesize protease, whereas *B. subtilis* is able to produce α-amylase [4,5], therefore, it was hypothesized that the multi-strain probiotic improves nitrogen and energy efficiency in weaned piglets. This is important as limiting excess nutrients passing the distal intestine is key to avoid osmotic diarrhoea as a result of harmful end-products produced by the fermentation of proteins (reviewed by Huting et al. [10]). The *Bacillus* strains of interest were already individually tested in a protein and amino acid digestibility trial in pigs [4]. However it is unknown whether mixing strains would result in an additive, synergistic, or even a reduced efficiency. It was therefore of interest to evaluate the effectivity of the strains combined on amongst others nutrient utilization in weaned piglets.

2. Materials and Methods

The study was conducted at the research facility of Schothorst Feed Research B.V. (SFR, Lelystad, the Netherlands). The protocol of the experiment (AVD24600202010384) was approved by the Animal Care and Use committee of SFR (Lelystad, the Netherlands) and in accordance with the Dutch law on animal experimentation, which complies with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.1. Experimental design and diets

The experiment consisted of two dietary treatments; a control diet (CD) and a supplemented diet (SD). The CD was mainly based on wheat (35%), barley (20%), soybean meal (11%), maize (10%), and wheat middlings (10%) and was formulated to meet the Foundation Central Bureau for Livestock Feeding (CVB) recommendations for essential nutrients of weaned piglets (see Table 1). The SD diet was prepared as the CD basis but supplemented with the *Bacillus* multi-strain probiotic SOLPREME® (Chr. Hansen A/S; Hoersholm, Denmark) at 1.1x109 CFU per kg diet (actual dose 0.04%). The multi-strain probiotic (minimal 2.75x109 CFU per g product) consisted of the viable spores *Bacillus amyloliquefaciens* - 516 and *Bacillus subtilis* - 541 and was supplied using a $CaCO_3$ carrier. Therefore, less limestone (-4.7%) was added to the SD to obtain similar Ca levels to the CD. The experimental diets did not contain antibiotics, acidifiers, polysaccharides, or phytase; but contained zinc and copper supplements at levels according with the European law for weaned piglets [11,12]. Titanium dioxide (0.5%) was added to the experimental diets as an indigestible marker to calculate nutrient digestibility.

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Table 1. Diet composition and calculated nutrient values of the experimental diets including the control diet (CD) and the supplemented diet (SD).

	Unit	CD	SD
Wheat	%	35.0	35.0
Barley	%	20.0	20.0
Soybean meal	%	11.3	11.3
Maize	%	10.0	10.0
Wheat middlings	%	10.0	10.0
Soybean oil	%	2.70	2.70
Potato protein	%	2.50	2.50
Whey protein delactose	%	2.00	2.00
Soy protein concentrate	%	1.00	1.00
Molasses	%	1.00	1.00
Monocalcium phosphate	%	1.06	1.06
Salt	%	0.64	0.64
Lysine HCL (79%)	%	0.45	0.45
Methionine L/DL (99%)	%	0.15	0.15
Threonine L (98%)	%	0.14	0.14
Tryptophane L (98%)	%	0.03	0.03
Calcium formate	%	0.10	0.10
Titanium dioxide	%	0.50	0.50
Limestone	%	0.89	0.85
Vitamin/ mineral premix[1]	%	0.50	0.50
Copper sulphate (99%)	%	0.05	0.05
Probiotic	%	0.00	0.04
Moisture	g/kg	117	117
Ash	g/kg	59.3	59.3
Crude protein	g/kg	178	177
Crude fat (acid hydrolysis)	g/kg	52.9	52.9
Crude fibre	g/kg	31.8	31.8
Starch (enzymatic)	g/kg	393	393
Sugar	g/kg	43.2	43.2
NSP	g/kg	156	156
Net Energy (NE)	MJ/kg	9.81	9.81
SID Lys	g/kg	11.0	11.0
SID LYS/ NE	ratio	1.15	1.15
SID Met/SID Lys	ratio	0.36	0.36
SID M+C/SID Lys	ratio	0.59	0.59
SID Thr/SID Lys	ratio	0.63	0.63
SID Trp/SID Lys	ratio	0.20	0.20
SID Val/SID Lys	ratio	0.67	0.67
SID Ile/SID Lys	ratio	0.57	0.57
SID Leu/SID Lys	ratio	1.06	1.06
SID Arg/SID Lys	ratio	0.83	0.83
SID His/SID Lys	ratio	0.35	0.35
SID Phe/SID Lys	ratio	0.69	0.69

[1] Vitamin and mineral premix was added to provide the following nutrients per kg of diet: Vitamin A: 10,000 IU; Vitamin D_3: 2,000 IU; Vitamin B_1: 1.0 mg; Vitamin B_2: 4.0 mg; Niacin: 30 mg; D- pantothenic acid: 15 mg, Vitamin B_6: 1.5 mg; Choline chloride: 150 mg; Biotin: 0.05 mg; Folic acid: 0.4 mg; Vitamin B_{12}: 20 μg; Vitamin E: 40 mg; Vitamin K_3: 1.5 mg; Cu: 20 mg; Fe: 100 mg; Mn: 30 mg; Zn: 70 mg; I: 0.7 mg; Se: 0.25 mg.

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The experimental diets were produced in the specialised feed mill of Research Diet Services (Wijk bij Duurstede, The Netherlands) and pelleted at a diameter of 3 mm. The temperature during diet production ranged between 75-76 °C for the pellet press. The CFU recovery was analysed in the mash and pelleted diets prior the experiment (Chr. Hansen A/S; Hoersholm, Denmark). As a method of blinding, numerical coding was used. Each pen was identified with a diet code. Numerical-coded feed bags and pens matched treatment numerical codes.

2.2. Animals and Animal Housing

A total of 16 piglets (Tempo x TN70) were weaned at around 30 days of age (29.8 ± 1.11 days) with a weight of 8.48 ± 0.272 kg. Piglets were ear tagged at birth, and no teeth clipping, tail docking or castration were performed. The newly born piglets were injected with injectable iron (1 ml, Iron-ject®, Dopharma, The Netherlands) at 3-4 days of age. Piglets were not vaccinated pre- or post-weaning, but the progenitors (gilts and sows) were vaccinated according to the manufacturer's vaccination scheme with an inactivated vaccine against neonatal colibacillosis and Clostridium infections (SUISENG®, HIPRA, Amer, Girona, Spain). Piglets received creep feed from approximately one week of age until weaning. The selected weaned piglets were free from signs of injury or illness. Only boars were used in this trial to ease the collection of urine. Each experimental treatment consisted of 8 replicates boars, which were randomly allocated on the basis of weaning weight to the different treatments and pens.

Health status of piglets was checked and recorded daily. Piglets were inspected at least once a day by an animal caretaker. If an animal was in poor condition, it was observed more frequently. If deemed unlikely to recover or survive, the animal was humanely euthanized. In case (antibiotic) treatment was necessary, the individual pig number, the kind of treatment and treatment duration were recorded. In case of mortality, the cause of death was recorded. Faecal consistency (see for protocol Guan et al. [13]) was determined daily at pen level between day 0-11 post-weaning and at individual level between day 11-19 post-weaning.

2.2.1. Animal Housing

Piglets were housed, fed, and managed according to directive 2010/63/EU for the protection of animals used for scientific purposes. Weaned piglets were housed in pairs in a total of 8 pens (2.00 × 1.00 m) from weaning (day 0) till day 11 post-weaning. The pen floor was partly slatted and partly covered with a rubber mat. Each pen was equipped with two feeding troughs (with 1-2 feeder places), two drinking nipples, a metal chain with MS pig play material (horizontal or vertical bars; MS Schippers, Hapert, The Netherlands), and a cotton rope (MS Schippers, Hapert, The Netherlands). At day 11 post-weaning, piglets were individually weighed and subsequently separated (i.e. individually housed) through the placement of a transparent wall in each pen. Piglets were housed individually for the remaining experimental period (until day 19 post-weaning; around 49 days of age). Final pen dimensions at individual housing were 1.0 × 1.0 m. Room temperature and relative humidity were recorded daily and were mechanically controlled by a climate computer following a temperature curve targeted at 29 °C at the day of weaning (27.9 ± 0.10 °C) to 25°C at 19 days post-weaning (24.7 ± 0.33 °C). The rooms were ventilated using outdoor air. Humidity in the rooms was dependent of outdoor humidity and ventilation rate (humidity 63.0 ± 6.12%: range 51.3-76.6%; the trial was executed in June). Artificial lights were provided from 6.30 till 18.00h.

2.2.2. Feeding Scheme

From weaning till day 10 post-weaning piglets were fed the experimental diet *ad libitum*. Piglets were individually weighed at day 11 post-weaning for the estimation of the feeding portion. From day 11 post-weaning onwards, piglets were fed following a semi *ad libitum* feeding scheme (3.2x metabolic body weight) which was calculated by:

$$\text{Feeding portion} = \frac{0.419\ x\ BW^{0.75}\ x\ 0.7\ x\ 3.2}{NE} \quad (1)$$

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where 0.419 is the energy for maintenance per kg metabolic body weight (BW); $BW^{0.75}$ is the metabolic body weight; 0.7 is the factor to calculate net energy (NE) from metabolic energy (ME); and 3.2 is the feeding level used in this case 3.2 x maintenance level for NE. The feeding portion increased daily by approximately 3% on an estimated growth curve. The average daily feed intake during individual housing was 672 ±72.7 g/day.

The feed was spread over 2 feeding portions (i.e. morning and afternoon) from day 11 until day 16 post-weaning. On day 17 and 18 post-weaning, the total feeding portion was split in 6 smaller portions and was fed every 2.5 hours (between 06:00 till 18:30) in order to reach a steady state. At the day of euthanasia, piglets were fed half the daily feed portion spread over 3 smaller portions: 1/6 portion 6 hours before euthanasia, 1/6 portion 3.5 hours before euthanasia, and 1/6 portion 1 hour before euthanasia in order to ensure all parts of the digestive system were filled with sufficient content. Drinking water was available *ad libitum* throughout the trial.

2.3. Sampling and Analytical Methods

During the last 5 days of the experimental period (from day 14 post-weaning onwards), faecal samples were collected twice daily (8.30 and 15.00 h) from the pens. Urine was collected via a funnel from the tray underneath the pen into a bucket (total collection). Hydrochloric acid was added to urine after each sampling time (5 ml/ time; adjusted to the actual volume of urine that was collected). Faecal and urine samples were stored at 4°C during the collection period. Faeces and urine were stored at -20°C and freeze-dried for further analysis. Piglets were weighed and humanely euthanized via intracardiac injection with T61® (MSD Animal Health, Boxmeer, The Netherlands) after sedation with Zoletil® (Virbac, Barneveld, The Netherlands) to facilitate the collection of the digesta at day 19 post-weaning (15.2 ±1.43 kg). During dissection, the entire small intestine was spread on a table. The last 2 m from 2/3 of the small intestine was considered to represent the jejunum and the distal 2 m from the small intestine was considered to represent the terminal ileum. The mentioned segments were dissected to collect its content by gentle stripping. The digesta content was homogenised by manual mixing and pH was immediately measured with a portable pH meter (Mettler-Toledo B.V., Tiel, The Netherlands). Digesta samples were frozen at −20°C and freeze-dried for further analysis. The dried faecal and digesta samples were milled through a 1 mm sieve prior to chemical analyses.

Fresh colon (i.e. 1 g collected 1 m from the cecum) and faecal samples (i.e. 1 g fresh material) were collected and stored at -20°C for further analysis. Volatile fatty acids (VFA: the sum of acetic acid, propionic acid, and butyric acid), valeric acid, branched-chain fatty acids (BCFA: the sum of Iso-butyric, 2-Methyl-butyric, and Iso-valeric), and lactic acid content in the colon and faecal samples was analysed in mmol/kg by BaseClear B.V. (Leiden, The Netherlands). Lactic acid, VFA, and BCFA were derivatized to the respective phenyl esters by using phenyl chloroformate reagent. Resulting esters were analysed by Agilent GC-FID. Matrix-matched internal standard calibration with butyric-d7 - and acetic-d3 acids was used in quantitation.

The chemical analyses were performed in duplicate. Dry matter (DM) content was determined by drying to constant weight at 103˚C (NEN-ISO 6496:1999). Crude protein (CP; nitrogen × 6.25; NEN-EN-ISO 16634-2:2016) was determined by combustion according to the Dumas principle; crude fat (CFat) content was determined using ether extraction after hydrolysis with hydrochloric acid under heating (NEN-ISO 6492:1999); crude ash was measured gravimetrically after ashing the sample for 3 h at 550 °C (NEN-ISO 5984:2003); starch content was determined enzymatically according to NEN-ISO 15914:2005EN; sugar was measured according to ANAL-10138 (NutriControl B.V.); amino acid (AA) determination was according to ANAL-10018 (NutriControl B.V.), tryptophan content in according to ANAL-10017 (NutriControl B.V.), and titanium as described by Short et al. [14]. The titanium (Ti) content as found in the experimental diets was as expected (ranges between 2.95-3.01 g/kg; expected content was 3.00 g/kg), the titanium content as found in the jejunum was 4.55 g/kg (SD = 1.059), which was 7.92 g/kg in the ileum (SD = 1.819), and 17.2 in the faecal contents (SD = 1.03).

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2.4. Calculations and Statistical Analysis

The organic matter (OM) content and non-starch-polysaccharides (NSP) were calculated in accordance with CVB [15]. The digestibility coefficients (DC, %) of nutrients were calculated using the following equations:

$$\text{Marker\_ratio} = \text{Ti\_diet} / \text{Ti\_excreta} \quad (2)$$

$$\text{DC\_nutrient} = (1 - \text{Marker\_ratio} \times \text{Excreta\_nutrient} / \text{Diet\_nutrient}) \times 100\% \quad (3)$$

where marker is the indigestible marker content (measured as Ti in g/kg DM); excreta is defined as faecal, proximal jejunal digesta, mid jejunal digesta, or ileum digesta; DC is the apparent digestibility coefficient (in %); nutrient is the content of AA, CP, CFat, OM, DM, or Ash (in g/kg DM) in the diet and the excreta. For the calculations of the jejunal digesta and ileum digesta everything was expressed in g/kg. The NE value was calculated in accordance with CVB [15] following the apparent total tract digestibility (ATTD) coefficients as found in current study. For starch a 100% ATTD was assumed and for sugar the enzymatically digestible sugar (in %) content of the experimental diets was estimated based on CVB [15]. The nitrogen (N) retention and biological value (following [4]) was calculated using the following equations:

$$\text{N\_retention (in g)} = (\text{N\_intake} \times \text{ATTD\_N}) - (\text{N\_urine} \times \text{Total\_urine}) \quad (4)$$

$$\text{N\_retention\_coefficient (in \%)} = (\text{N\_retention} / \text{N\_intake}) \times 100 \quad (5)$$

$$\text{Biological value (in \%)} = (\text{N\_retention\_coefficient} / (\text{N\_intake} - \text{N\_faeces})) \times 100 \quad (6)$$

where N_intake is the total N intake (in g); ATTD_N is the apparent total tract digestibility of N; N_urine is the N content in urine (in g/kg); Total_urine is the total urine production (in kg); and N_faeces is the amount of N that was found back in the faeces (undigested).

Based on the sample size calculation with GenStat® for Windows Version 21 (VSN International Ltd, Hemel Hempstead, UK) using the ASAMPLESIZE procedure with a significant level of α <0.05 and a power of 0.80, a total of 8 piglets per treatment were needed. The Shapiro-Wilk Test was used to test for normal distribution of residuals (WSTATISTIC procedure) and Bartlett's Test was used to test for homogeneity of variances (VHOMOGENEITY procedure). The ABOXCOX procedure was used in case data was not normally distributed and needed to be transformed. For presentation purposes the calculated means were back transformed and are presented together with the 95% confidence interval (CI, using Bonferroni inequality) instead of SEM. The experimental results were analysed using a one-way analysis of variance (ANOVA) by GenStat®. For all parameters except performance between day 0 and 11 post-weaning, the experimental unit was piglet (n = 8); for performance data between day 0 and 11 post-weaning, pen was the experimental unit (n = 4). Replicate was used as random block effect. For the digestibility coefficients data, an extra quality check was performed: if the dietary OM digestibility of the animal differed more than 2.5 times the standard deviation from the average, the piglet was considered an outlier (based on CVB protocol for digestibility studies, [16]). If this was the case, all the digestibility data (e.g. also at intestinal level) of this animal was excluded from the analysis. Besides, all data were screened for outliers; data were identified as outlier if the residual (fitted – observed value) differs > 2.5 × standard error on the residuals of the data set. In addition, if this was the case for CP digestibility or CFat digestibility, the digestibility coefficient for NSP and NE value was also regarded as outlier [16]. Missing values were estimated through GenStat® (using least square estimates). Treatment means were compared using Least Significant Differences (LSD, Fisher's LSD method). A T-probability of P≤0.05 was considered

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statistically significant, while 0.05 <P ≤0.10 a near-significant trend. The data is presented as means ± SEM.

3. Results

The analysed nutrient contents of the experimental diets are presented in Supplementary Table S1. The CFU recovery was analysed in the mash and pelleted diets and were in line to expected values (Supplementary Table S2).

3.1. Exclusion of Animals

No pen or piglet was considered outlier on the basis of performance data. All piglets passed the quality check following CVB [16] protocol which means that the individual dietary ATTD of OM did not differ more than 2.5 times the standard deviation from the average of the particular treatment. Piglets from replicate 2 (i.e. one piglet from the CD and one piglet from the SD treatment) had an ileum pH content that was > 2.5 times lower than the standard error of the residuals and were therefore considered outlier. Replicate 2 from treatment SD did not have enough ileal content to perform the AA analysis, therefore this piglet was treated as missing value in subsequent analysis.

3.2. Animal Performance and Health

Piglets weighed on average 11.3 kg (SD = 1.04) at day 11 post-weaning, and 15.2 kg (SD = 1.43) at day 19 post-weaning. Experimental treatment did not influence piglet performance or faecal score (Supplementary Table S3). No piglets died during the course of the trial. One piglet from the CD treatment was treated with painkillers (i.e. Ketoprosol 10%, AST Farma, Raamsdonksveer, The Netherlands) for lameness at day 12 and day 17 post-weaning.

3.3. Apparent Small Intestine Digestibility Coefficients

The effect of dietary treatment on apparent nutrient digestibility in the small intestine can be found in Table 2. Apparent CP digestibility in both jejunum and ileum were not affected by dietary treatment (P = 0.18 and P = 0.32, respectively). The effect of dietary treatment on apparent ileal digestibility (AID) of AA and digesta pH can be found in Supplementary Table S4 and Supplementary Table S5, respectively. The results indicate that dietary treatment did not significantly influence AID of AA nor pH of the digesta content.

3.4. Apparent Total Tract Digestibility Coefficients and NE-value

The effect of dietary treatment on ATTD coefficients and NE-value can be found in Table 2. Supplementation with the *Bacillus* multi-strain probiotic did not improve ATTD of CFat (P = 0.60) and Ash (P = 0.17). Supplementation with the *Bacillus* multi-strain probiotic influenced ATTD coefficients of DM (P = 0.01) and OM (P = 0.02) and tended to influence ATTD coefficients of CP (P = 0.10; Δ 2.2%) and NSP (P = 0.07; Δ 1.9%). Weaned piglets fed the supplemented diet had a 1.3% higher ATTD coefficient of DM and a 1.2% higher ATTD coefficient of OM. Next to the improvements in nutrient digestibility, supplementation with *Bacillus* multi-strain also increased the NE value with 0.2 MJ/ kg DM (P = 0.02) compared with the CD treatment.

Table 2. The effect of experimental treatment (i.e. control diet = CD; supplemented diet = SD) on apparent nutrient digestibility.

Parameter	CD	SD	SEM	*P*-value
Small intestine, %				
Jejunal CP	37.0	44.0	3.34	0.18
Ileal CP	69.1	59.7	6.22	0.32
Total tract, %				
DM	80.7[a]	82.0[b]	0.26	0.01

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Ash	53.0	54.4	0.66	0.17
OM	83.3[a]	84.5[b]	0.27	0.02
CP	80.9[x]	83.1[y]	0.79	0.10
CFat	72.5	73.2	0.96	0.60
NSP	52.1	54.0	0.63	0.07
NE value, MJ/kg DM	10.8	11.0	0.03	0.02

a-b Values within a row with different superscripts differ significantly at $P < 0.05$. x-y Values within a row with different superscripts tended to differ at $P < 0.10$.

3.5. N Retention

The effect of dietary treatment on N retention can be found in Table 3. Supplementation with the multi-strain probiotic did not influence N intake ($P = 0.81$); faecal N content ($P = 0.11$); digestible N content ($P = 0.95$), urinary N ($P = 0.79$), N retention when expressed in g ($P = 0.99$), or the biological value ($P = 0.76$). However, supplementation with the multi-strain probiotic improved N retention ($P = 0.05$), with the SD treatment resulting in a 1.6% higher N retention coefficient than the CD treatment.

Table 3. The effect of experimental treatment (i.e. control diet = CD; supplemented diet = SD) on nitrogen (N) retention.

Parameter	CD	SD	SEM	*P*-value
N intake, g	112	109	6.5	0.81
Faecal N, g	21.2	18.3	1.09	0.11
Digestible N, g	90.5	91.9	5.81	0.95
Urinary N, g	7.69	8.15	1.171	0.79
N retention, g	82.8	82.9	4.91	0.99
N retention coefficient, %	74.2[a]	75.8[b]	0.47	0.05
Biological value, %	83.3	86.3	6.34	0.75

[a-b] Values within a row with different superscripts differ significantly at $P < 0.05$.

3.6. VFA, BCFA, and Lactic Acid Content in the Colon digesta and Faecal Material

The effect of dietary treatment on VFA, BCFA, and lactic acid content in the colon and faeces can be found in Table 4. No effects ($P > 0.05$) were found at faecal level with respect to VFA, BCFA, and lactic acid content. However, in colon, dietary treatment did influence lactic acid content ($P = 0.01$) and tended to influence valeric acid content ($P = 0.09$). Supplementation with the *Bacillus* multi-strain probiotic resulted in a higher colonic valeric acid content (+1.74 mmol/kg) and a lower lactic acid content (-0.21 mmol/kg).

Table 4. The effect of experimental treatment (i.e. control diet = CD; supplemented diet = SD) on the colonic and faecal SCFA, VFA, and BCFA content.[1]

Parameter[2]	CD	SD	SEM	*P*-value
Colon, mmol/kg				
VFA content				
Acetic acid	93.0	92.6	0.90	0.77
Propionic acid	40.6	36.9	1.69	0.17
Butyric acid	16.6	14.3	1.05	0.16
Total VFA	150	144	2.5	0.11
BCFA content				
Valeric acid	3.18[x]	4.92[y]	0.628	0.09
Total BCFA	7.17	8.55	0.599	0.15
Lactic acid	8.17[b]	7.96[a]	0.039	0.01
Total	166	160	2.4	0.17
Faecal, mmol/kg				

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VFA content				
Acetic acid	74.4	74.9	2.95	0.90
Propionic acid	34.3	31.2	1.55	0.20
Butyric acid[3]	16.2 (-25.9 - 34.5)	14.8 (-26.7 - 33.9)	-	0.46
Total VFA	124	121	5.0	0.62
BCFA content				
Valeric acid	3.61	3.55	0.151	0.79
Total BCFA	11.9	11.8	0.52	0.86
Lactic acid	8.89	8.76	0.053	0.15
Total	145	141	5.1	0.61

[a-b] Values within a row with different superscripts differ significantly at $P < 0.05$. [x-y] Values within a row with different superscripts tended to differ at $P < 0.10$. [1] VFA = volatile fatty acid and consists of the sum of acetic acid, propionic acid, and butyric acid; BCFA = branched chain fatty acids consisting of the sum of Iso-butyric, 2-Methyl-butyric, Iso-valeric, and valeric acid; SCFA = short chain fatty acid. [2] The experimental unit was piglet (n = 8). Replicate (1 to 8) was used as random effect. The experimental results were analysed using a two-way analysis of variance (ANOVA) by GenStat®. [3] This parameter was considered not normally distributed in its original form (i.e. Shapiro Wilk $P<0.05$). Transformation suggestions were made by the "ABOXCOX" procedure in Genstat. The butyric acid values in the faeces were transformed using X^2. For presentation purposes the calculated means were back transformed and are presented together with the 95% confidence interval (CI, using Bonferroni inequality) instead of SEM.

4. Discussion

This paper describes a study in which the effect of the multi-strain probiotic consisting of *B. amyloliquefaciens* and *B. subtilis* on nutrient digestibility, energy utilisation, and AA digestibility in weaned piglets is evaluated. It was hypothesized that the *Bacillus* multi-strain could improve nitrogen utilization by *B. amyloliquefaciens* that synthesize protease, and energy efficiency by *B. subtilis* that synthesize, amongst others, α-amylase and fibre degrading enzymes [4,5]. An improved N utilization is important for piglets' health [10], but also for reducing N pollution. Increasing N utilisation, reduces urinary and total N excretion, further reducing N pollution. This can be manipulated through the diet by for instance reducing the CP content, using highly digestible feedstuffs, synthetic amino acids, or zootechnical additives that can improve the utilization of CP [17]. To understand the potential of the *Bacillus* multi-strain in reducing N excretion, N balance was evaluated in this trial.

The current study was not set-up to evaluate the effect of the *Bacillus* multi-strain on post-weaning performance. The 4 pens (with only 2 piglets/pen) per treatment during the *ad libitum* feeding period was not expected to provide enough power to find differences in performance. However, the 8 piglets/ treatment were enough statistical power to detect differences in nutrient utilization.

Part of the large between animal variation as observed in current study and perhaps the lack of significant effects at small intestinal level might be explained by the method used. In the current study the samples were obtained using the slaughter technique, in which samples can only be obtained once compared with, for instance, the T-cannula where samples are obtained for a prolonged period of time [18]. Nonetheless, to limit the shedding of the intestinal cells and mucus into the digesta the piglets were euthanised under sedation and squeezing of the intestinal tract was avoided [19]. Using canulated growing-finishing pigs, the supplementation of *B. amyloliquefaciens*, but not *B. subtilis*, improved the AID digestibility of some dispensable and indispensable AA, though in current study no such effects were found [4]. Also, Lewton et al. [5] found no improvement after administration of probiotics on the AA digestibility in the ileum and only found significant effects in the jejunum (approximately 8 m proximal from the cecum). It was suggested that as *Bacillus* is a member of the firmicutes phyla, it may have helped to restore the microbiota composition of the jejunum generally being *Firmicutes*-dominated [5] and therefore may have contributed to an

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increased nutrient utilization in the jejunum. Although the results of current study did not show significant effects, it suggests that the *Bacillus* multi-strain was able to numerically improve apparent CP digestibility in the proximal intestine (i.e. +7%).

The observation that supplementation with the *Bacillus* multi-strain probiotic tended to increase the ATTD of CP (+ 2.2%) and resulted in a numerically lower faecal N content (-2.8 g), is in agreement with other studies using nursery pigs [20–22]. It is speculated that the improvement in N utilization might be a result of: 1) the metabolites produced by the probiotic that enhances nutrient digestibility; 2) improved gut development supporting digestion and absorption of nutrients; and 3) its effect on gut microbiome composition which alters gut health [22]. The absence of a significant effect may be amongst others a result of differences in the diet composition. Probiotics may have a greater potential when the inherent digestibility of the diet is already relatively high [23], when the CP content of the diets are low [5], or when supplementing low energy diets [24]. In the studies of Giang et al. [20], Lee et al. [21], and Cai et al. [22], the CP content of the experimental diets was >20%, whereas in the current study this was considerably lower (< 18%); the NE content was not different between studies (i.e. around 10-11 MJ/kg DM). The diet in current study mostly consisted of cereal (by-products) and soybean meal (75% and 11% respectively), whereas in the other studies [20,22] the inclusion of highly digestible protein sources was higher, such as milk products (e.g. whey powder, sweet whey, milk replacer), soy protein concentrate, and processed feedstuffs (i.e. extruding or fermentation). Nonetheless, the AID and ATTD of CP of the control diet in the current study was respectively 69% and 81%; this was slightly lower than Giang et al. [20] (71-75% and 83-84%, respectively), but higher than the ATTD of CP that was found by Cai et al. [22] (78%), and Lee et al. [21] (72%). Thus, differences in diet composition cannot entirely explain the results, but perhaps also factors like the strain and the dose used may play a role. Additionally, it has to be noted that piglets in the current study were on average 30 days of age at weaning with a weaning weight of 8.5 kg, whereas in the study of Lee et al. [21] the piglets were 21 days old and 6.4 kg, this was 24 days and 6.8 kg, respectively for Cai et al. [22], and 27 days and 7.7 kg, respectively for Giang et al. [20]. It is therefore speculated that the piglets used in current study might be more robust (i.e. older and heavier at weaning) and had a less challenging diet with respect to CP level [10], than the piglets used in the other studies. This may have contributed to the lack of significant effect. For instance, it has been suggested that probiotics are more effective in animals with an impaired GIT (e.g. unstable microbiota) and not so much for older pigs when pigs are better capable to resist intestinal disorders like for instance during the finisher phase [24].

Organic matter is the calculated fraction of CP, CFat, and carbohydrates (i.e. the sum of sugars, starch, and NSP). The significant improvement of ATTD coefficients of OM as found in current study as a result of the multi-strain probiotic might therefore be mostly a result of the numerical higher CP digestibility (+2.2%) and NSP digestibility (+1.9%). The fact that a higher ATTD of DM (+1.3%) was found for weaned piglets fed the diet containing the multi-strain probiotic might be a result of the significant improvement of ATTD of OM (+1.2%) and the numerically higher ATTD of Ash (+1.4%). Although the differences in ATTD DM seems rather small compared with other studies that found a ATTD DM improvement ranging between 1.9-3.5% [17,21,23]. Furthermore, in current study the multi-strain probiotic improved NE utilization. Other studies [4] found an increased energy utilization in pigs after supplementation with *Bacillus* strains. The difference in energy utilization in current study may come from the numerical improvements in ATTD of CP and NSP. On the other hand, it is suggested that some strains of *Bacillus* can synthesize α-amylase and fibre-degrading enzymes [4]. The enhanced fermentation of dietary fibre can increase the production of volatile fatty acid (VFA) and subsequently increase energy utilization [25]. It is suggested that colonic fermentation of NSP results in VFA and lactic acid contributes to approximately 20% of the total dietary energy utilization in adult pigs [26]. The VFA molar proportion (i.e. acetate: propionate: butyrate) of the colon content and faecal content in the current study (i.e. 63:26:10 and 61:27:12, respectively) was in line with Jaworski et al. [25]. However, similarly to Jaworski et al. [25], the addition of the multi-strain probiotic did not influence the total VFA content. The absence of effect might be a result of the absorption of VFA in the cecum, which is rather efficient, and therefore measuring the VFA content

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in the colon and faeces might not be accurate [27] and using *in-vitro* methods might be more applicable [26]. On the other hand, in the present study weaned piglets fed a diet with *Bacillus* strains tended to have a higher colonic valeric acid content and a significant lower colonic lactic acid content. The slightly higher colonic valeric acid content in the supplemented treatment may suggest a higher hindgut fermentation of protein [19]. However: 1) the total BCFA content which are formed by the fermentation of branched-chain amino acid (BCAA) was not significantly affected; and 2) AID of CP and BCAA (i.e. valine, leucine, and isoleucine) were not affected by dietary treatment suggesting that no more undigested protein entered the large intestine to be fermented. These results can therefore not explain the higher colonic valeric acid content after supplementation, and it is questionable whether the observed differences are large enough to have a biological relevance. The effects of *Bacillus* strains on organic acid concentrations (including lactic acid) in the intestinal lumen are inconsistent [20], but too high lactic acid contents may in fact be harmful for the pig [1]. The absence of differences in VFA production might also explain why in current study no differences in digesta pH at the different segments along the GIT were found. It is also worth to mention that it cannot be ruled out that supplemented pigs may have had a reduced maintenance energy requirement [25] which may have contributed to the improved energy efficiency.

The observation that N retention expressed in g was not significantly influenced by experimental treatment was probably a result of the relatively large SEM and variation in feed intake among piglets; for which the N retention coefficient was corrected. These results are in contrast to Blavi et al. [4] where no effects of single-strain supplementation of *B. amyloliquefaciens* and *B. subtilis* were observed on N retention. This might suggest a synergistic effect of the two strains in N retention or that the probiotic is more effective in younger piglets Though more research is necessary to evaluate the effect of the multi-strain on N retention in weaned piglets.

5. Conclusions

In conclusion, results from the current study suggests that the multi-strain probiotic consisting of the viable spores *B. amyloliquefaciens* and *B. subtilis* have the potential to improve nutrient efficiency in weaned piglets. More research needs to be done to identify the impact of the improved nutrient utilization on gut health in post-weaned pigs as well as environmental pollution.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Analysed dietary nutrient content of the experimental diets (i.e. control diet = CD; supplemented diet = SD), in g/kg ; Table S2: CFU recovery analysis of the experimental diets (i.e. control diet = CD; supplemented diet = SD) ; Table S3: The effect of experimental treatment (i.e. control diet = CD; supplemented diet = SD) on piglet performance; Table S4: The effect of experimental treatment (i.e. control diet = CD; supplemented diet = SD) on apparent ileal digestibility of amino acids; Table S5: The effect of experimental treatment (i.e. control diet = CD; supplemented diet = SD) on pH of the digesta content.

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Institutional Review Board Statement: The experiment was conducted according to the guidelines of the Animal and Human Welfare Codes/Laboratory practice codes in the Netherlands. The protocols were approved by the Ethics Review Committee.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to its proprietary nature.

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Conflicts of Interest: LHBH is employed by Chr. Hansen Holding A/S, funding this research. LHBH participated in conceptualization of the study design, decided to publish the results and reviewed this manuscript. AMSH, LVL, and FM declare that they have no competing interest.

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