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Effects of Maternal Probiotics and Piglet Dietary Tryptophan Level on Gastric Function Pre- and Post-Weaning

Dillon. P. Kiernan [1], John V. O'Doherty [2], Marion T. Ryan [1] and Torres Sweeney [1,*]

[1] School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland; dillon.kiernan@ucdconnect.ie (D.P.K.); marion.ryan@ucd.ie (M.T.R.)
[2] School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland; john.vodoherty@ucd.ie
* Correspondence: torres.sweeney@ucd.ie (T.S.); Tel.: +353-17166244

Abstract: Knowledge of how novel antigens or dietary stimuli affect stomach development and function in pigs remains limited. This study aimed to investigate stomach characteristics, parietal cell numbers, and the expression of genes essential to the functioning of the fundic and pyloric gland regions at weaning compared to seven days post-weaning and to examine whether maternal probiotic supplementation or piglet dietary tryptophan (Trp) levels influence these stomach parameters. This study has a 2 × 3 factorial design, with 48 sows assigned to one of two diets: basal or basal supplemented with *Bacillus subtilis* and *Bacillus amyloliquefaciens*. Their litters received creep diets containing 0.22, 0.27, or 0.33% standardized ileal digestible (SID) Trp. In total, 96 pigs were sacrificed for gastric sampling, 48 on the day of weaning and 48 on day 7 post-weaning. At 7 days post-weaning, pigs had an increased number of parietal cells and expression of parietal cell activity and digestive enzyme (*PGA5* and *CHIA*) genes in the fundic gland region ($p < 0.05$), although the expression of signaling molecules involved in the regulation of acid secretion was unchanged in the fundic gland region ($p > 0.05$) and reduced in the pyloric gland region ($p < 0.05$), compared to the day of weaning. Overall, maternal probiotic supplementation had a significant impact on gene expression in the fundic gland region of the offspring, elevating several genes related to parietal cell activity (*CLIC6*, *HRH2*, *KCNE1*, *KCNQ1*, *CHRM3*, *CCKBR*, and *SSTR2*) ($p < 0.05$). Additionally, there were time × maternal interactions, where certain acid secretion pathway (*ATP4A* and *HDC*), chitinase enzyme (*CHIA*), and ghrelin (*GHRL*) genes were increased in offspring from probiotic sows compared to control sows at weaning ($p < 0.05$), but not at 7 days post-weaning ($p > 0.05$). Maternal probiotic supplementation did not influence growth performance pre-weaning or during the 7-day post-weaning period. There was a limited effect of creep Trp level or maternal × creep interactions on performance, gene expression, or parietal cell counts. Low pre-weaning creep intake may have confounded this analysis. In conclusion, maternal probiotic supplementation accelerated the maturation of the offspring's stomach, particularly in terms of the expression of genes linked to acid secretion from parietal cells.

Keywords: fundic gland region; pyloric gland region; immunohistochemistry; parietal cells; oxyntic mucosa; maternal transmission.

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1. Introduction

To increase sow productivity and meet the economic demands on commercial pig farms, weaning typically occurs prematurely at three to four weeks of age, a time when the piglets' gastric acid and enzyme secretory capacity is still developing [1–3]. The acidic environment in the stomach plays a dual role, serving as one of the initial lines of defense against ingested pathogens, as well as aiding the activation of enzymes for the digestion of proteins. In suckling pigs, the fermentation of milk-derived lactose by stomach bacteria produces lactic acid and acetate, which play a crucial role in maintaining a low gastric pH [4]. At weaning time, the pig's diet is abruptly and prematurely switched from sow's milk to a plant-based feed, resulting in a dramatic reduction in dietary lactose [5], with direct consequences on stomach acidity. This increases the susceptibility of the post-weaned pig to pathogens and reduces the activity of proteolytic enzymes, which are pivotal for effective protein digestion [6].

Strategies to mitigate the challenges of the immature stomach at weaning time have typically focused on lowering post-weaning dietary pH using weak organic acids [7–9]. Furthermore, there is a renewed focus on minimizing the acid-binding capacity of the post-weaned pig diet to reduce the neutralizing effect of the feed on gastric pH [10–12]. While both dietary strategies will likely play a key role in supporting the pig in the post-weaning period, an additional strategy is to advance the development of the pig's stomach pre-weaning. This strategy has been relatively underexplored to date, with only a few studies focusing on the characterization, functioning, and development of the regions of the stomach [13–18] or on the effects of dietary modulation on its functioning [1,19–26].

The acidic environment created via lactose fermentation pre-weaning can delay the maturation of gastric acid secretory capacity in the pig [4]. Interestingly, providing piglets access to creep feed during the suckling phase reduces the concentrations of lactate and acetate in the gastric digesta [27] and enhances the maximum acid output of their stomachs [19]. It is possible to hypothesize that reduced stimulation of the suckling pig's acid secretory pathway could contribute to a delay in maturation, revealing the stimulation of this pathway as a potential mechanism with which to enhance acid secretory capacity pre-weaning. The calcium-sensing receptor (CaSR) is suggested to be a central receptor involved in the regulation of acid secretion in response to the luminal pH [28]. The CaSR is activated to stimulate acid secretion by divalent and trivalent metals, such as Ca^{2+}, Mg^{2+}, and Gd^{3+}, and certain amino acids, particularly Trp and phenylalanine [29–32]. Interestingly, both Trp and phenylalanine, acting via the CaSR, trigger the secretion of gastrin and somatostatin while elevating H^+K^+-ATPase activity in *in vitro* samples of pig stomach tissue [30]. This suggests that acid secretion can be stimulated by specific dietary ingredients, independent of luminal pH stimulation. Therefore, supplementation with dietary components that stimulate the process of acid secretion, like Trp, could be a means of promoting stomach development and enhancing acid secretory capacity in pigs pre-weaning.

An additional strategy that may improve stomach function is to modulate the piglet's early-life microbial exposure. The complexity of microbial exposure in the initial stage of life can impact the functional maturation of the fundic gland region in the young pig [24]. In recent years, the role of probiotics as beneficial feed additives has been continuously highlighted [33,34]. However, the potential effects of probiotic supplementation on the stomach have received limited attention. Supplementation with *Lactobacillus acidophilus* or *Bifidobacterium breve* and *Bifidobacterium animalis* increases the number of endocrine cells in the stomach at days 7, 14, 21, 28, and 35 of life [35]. Supplementation with sodium butyrate, a derivative of the bacterial metabolite butyrate, increases the number of parietal cells in the stomach [21]. Furthermore, when supplement-

ed in post-weaning diets, but not pre-weaning diets, it increases gastric mucosal thickness and the number of somatostatin-positive cells [21]. These studies offer preliminary evidence for the potential of probiotics, or their metabolites, to interact with the stomach mucosa. Given that the sow is a major microbial source in early life [36,37], maternal probiotic supplementation could modulate the offspring's early-life microbial exposure and thereby influence the functional development of the stomach.

Within the stomach, the fundic gland region plays a crucial role in acid and digestive enzyme secretion, while the pyloric gland region is the primary site for the secretion of gastrin and somatostatin, which are key regulators of gastric acid secretion [18]. Parietal cells, located in the fundic region, are specifically involved in acid secretion within the stomach. The selection of genes for analysis, in both the fundic and pyloric gland regions in the current study, was based on their functional role in the acid, digestive enzyme, and immune pathways, as previously determined in the different regions of the stomach by Kiernan et al. [18]. Therefore, the objectives of this study were twofold: to investigate stomach characteristics, parietal cell numbers, and the expression of genes essential to the functioning of the fundic and pyloric gland regions at weaning compared to seven days post-weaning and to examine whether maternal probiotic (*Bacillus subtilis* and *Bacillus amyloliquefacien*) supplementation or piglet dietary Trp levels influence these gastric parameters.

2. Materials and Methods

All experimental procedures described in the present study were approved under the University College Dublin Animal Research Ethics Committee (AREC-2022-ODoherty, AREC-2202-ODoherty) and were conducted in accordance with Irish legislation (SI no.543/2012) and the EU directive 2010/63/EU for animal experimentation.

2.1. Experimental Design and Animal Management

The experiment was conducted as a 2 × 3 factorial design, in which there were two maternal diets and three piglet creep diets, forming six dietary experimental groups.

2.1.1. Sow Dietary Groups and Management

A total of 48 crossbred sows (Large White x Landrace genetic lines) were blocked based on parity (mean parity 4.3 ± 2.5) and expected farrowing date. The sow parity distribution was as follows: 20% of sows were in their 1st parity, 40% were in their 2nd–4th parity, and 41% were in their 5th parity or beyond. The sows were then allocated to one of two dietary groups (n = 24 sows/group): (1) basal diet (control) and (2) basal diet supplemented with a probiotic blend (*Bacillus subtilis*–541 and *Bacillus amyloliquefaciens*–516). The probiotic product (SOLPREME®) was provided by Chr. Hansen A/S (Hørsholm, Denmark). The feeding period spanned from day 83 (±1.8 days) of gestation to weaning at day 26 (±1.8 days) of lactation. The ingredient composition and analysis of the lactation and gestation diets are presented in Tables 1 and 2. Diets were formulated to meet or exceed the National Research Council (NRC) recommendations (NRC 2012).

Table 1. Ingredient and chemical composition of gestation, lactation and creep diets.

Ingredients (g/kg)	Gestation Sow Diet [a]	Lactation Sow Diet [a]	Creep		
			0.22% Trp [b]	0.27% Trp [b]	0.33% Trp [b]
Wheat	-	380	472	472	472
Barley	750	250	100	100	100
Maize	-	-	120	120	120
Soya bean meal	90	170	-	-	-
Soya bean 50	-	-	90	90	90
Full-fat soya	-	80	90	90	90
Soycomil	-	-	30	30	30
Whey protein	-	-	40	40	40
Soya oil	12	25	30	30	30
Soya hulls	120	10	-	-	-
Beet pulp	4	10	-	-	-
Pollard	-	40	-	-	-
Vitamin and mineral premix [c]	1.5	1.5	3	3	3
Salt	4	5	2	2	2
Monocalcium phosphate	6	8	4.2	4.2	4.2
Limestone	9	12	4.5	4.5	4.5
Lysine-HCL 78.8%	2.2	4	5.8	5.8	5.8
Methionine	0.6	1.3	2.5	2.5	2.5
Threonine	0.7	2.5	2.8	2.8	2.8
Tryptophan	0	0.7	0.2	0.7	1.2

[a] Dietary groups: (1) basal diet, (2) basal diet supplemented with 400 g probiotic per tonne of feed. [b] Calculated from the tabulated nutritional composition [38]. [c] Vitamin and mineral premix (per kg diet): Sow diets: 100 mg of vitamin E as DL-α-tocopherol acetate; 500 mg of choline chloride; 80 mg of Zn as ZnO; 70 mg of Fe as $FeSO_4$; 60 mg of Mn as MnO; 25 mg of vitamin D3 as cholecalciferol; 15 mg of Cu as $CuSO_4$; 12 mg of nicotinic acid; 10 mg of pantothenic acid; 5 mg of folic acid; 5 mg of vitamin B2 as riboflavin; 3.4 mg of vitamin A as retinyl acetate; 3 mg of vitamin B6 as pyridoxine; 2 mg of vitamin K as phytyl-menaquinone; 2 mg of vitamin B1 as thiamine; 0.6 mg of I as calcium iodate on a calcium sulfate/calcium carbonate carrier; 0.2 mg Se as sodium selenite; 0.02 mg of biotin; 0.015 mg of vitamin B12 as cyanocobalamin. Creep diets: as reported in [25]: 250 mg choline chloride; 140 mg Fe; 112.5 mg Zn; 67 mg tocopherol; 47 mg Mn; 25 mg Cu; 12 mg nicotinic acid; 10 mg pantothenic acid; 4 mg menaquinone; 2 mg riboflavin; 2 mg thiamine; 1.8 mg retinol; 0.6 mg I; 0.3 mg S; 0.025 mg cholecalciferol; 0.015 mg pyridoxine; 0.01 mg cyanocobalamin.

Table 2. Analysis of chemical composition of diets (g/kg unless otherwise stated).

Ingredients g/kg	Gestation Sow Diet [a]	Lactation Sow Diet [a]	Creep		
			0.22% Trp [b]	0.27% Trp [b]	0.33% Trp [b]
Dry matter	870	870	900	880	900
Crude protein (N × 6.25)	141.5	170.3	180.5	177.0	182.5
Gross energy (MJ/kg)	15.9	16.0	16.6	16.8	16.8
Ash	50.5	52.6	50	60	50
Neutral detergent fiber	220.0	140.0	145.1	135.2	141.2
Crude oil	26.6	51.0	38.2	46.0	42.4
Arginine	9.3	11.0	10.7	11.4	10.6
Histidine	3.5	4.1	4.3	4.3	4.2
Isoleucine	5.1	7.2	7.4	7.8	7.5
Leucine	11.3	12.6	13.1	13.5	14.4
Lysine	7.5	11.3	13.9	14.0	14.1
Methionine	2.5	2.5	4.8	4.5	4.5
Phenylalanine	6.1	8.0	8.2	8.0	8.3
Threonine	5.7	6.9	8.9	8.5	9.1
Tryptophan	1.8	2.2	2.4	2.8	3.2
Valine	6.6	7.9	9.7	8.2	9.3

Trp, tryptophan. [a] Dietary groups: (1) basal diet, (2) basal diet supplemented with 400 g probiotic per tonne of feed. [b] Calculated from the tabulated nutritional composition [38].

From days 83 to 110 of gestation, the sows were managed in dynamic groups of six animals per pen based on their assigned diet. The temperature in the gestation room was maintained at 20 °C throughout the experiment. During this period, the sows received 3.1 kg/day of gestation feed. In the gestation room, the sows were fed in a shared trough (six sows per trough) with equal meals provided at 8 am and 2 pm.

On day 110, the sows were relocated to individual farrowing pens (2.4 m × 1.8 m) equipped with crates, slatted floors, and heat pads for piglets. From day 110 to day 113 of gestation, the sows received 2.9 kg/day of lactation feed. From day 113 until farrowing, the sows received 2.3 kg/day of lactation feed, and then the feed supply was increased by 0.7 kg/day until day 3 postpartum. Afterward, the sows were fed semi-ad libitum with the standard lactation diet, adjusted for each sow based on daily intake. In the farrowing room, the sows were fed in individual troughs in three equal meals provided at 6 am, 11 am, and 3 pm. The temperature in the farrowing room was maintained at approximately 24 °C during farrowing, gradually reducing to 20 °C by 7 days post-farrowing. The sows had ad libitum access to drinking water throughout the experimental period. The probiotic supplement contained 2.75×10^9 colony-forming units (CFU) per gram, consisting of viable spores of *Bacillus subtilis* (DSM 25841) and *Bacillus amyloliquefaciens* (DSM 25840). The probiotic was top-dressed on the feed to achieve a supplementation rate equivalent to 400 g of probiotic supplement per tonne of gestation/lactation feed consumed. The gestation and lactation feeds were top-dressed with the probiotic during the first feeding each morning to ensure consumption.

2.1.2. Farrowing, Piglet Management, and Piglet Dietary Groups

All farrowing's were supervised. Every piglet in each litter was individually weighed and tagged at birth. Four piglets (two male and two female), near the median birth weight, were selected per sow and excluded from cross-fostering. Cross-fostering occurred between 12 and 24 h postpartum within maternal dietary groups to equalize litter size (n = 14). All piglets received an intramuscular injection of iron (Uniferon, Pharmacosmos A/S, Holbæk, Denmark) on day 1 of life. On day 7 postpartum, each piglet was individually weighed. On day 8 postpartum, both maternal groups were sub-blocked into three groups based on parity, litter age, and litter size. Litters were then assigned to one of three creep diets: 0.22, 0.27, or 0.33% SID Trp, corresponding to 0.17, 0.21, and 0.25 SID Trp:Lys. The diets were formulated to have an SID lysine of 1.3%. The current NRC recommendation for pigs 7–11 kg is 0.22% SID Trp [39]. The 0.27% and 0.33% SID Trp levels were selected based on previous research [40–43]. There were minor discrepancies between the calculated and analyzed Trp and lysine contents of the diets. The analyzed Trp levels were 0.24% total Trp (1.39% total lysine), 0.28% total Trp (1.40% total lysine), and 0.32% total Trp (1.41 total lysine) corresponding to 0.17, 0.20, and 0.23 Trp:Lys. The ingredient composition and analysis of the creep diets are presented in Tables 1 and 2.

The two factors, maternal diet and creep diet, were arranged in a 2 × 3 factorial design, resulting in six experimental groups as follows: (T1)—BT (basal sows and piglets supplemented with 0.22% SID Trp); (T2)—BTT (basal sows and piglets supplemented with 0.27% SID Trp); (T3)—BTTT (basal sows and piglets supplemented with 0.33% SID Trp); (T4)—PT (probiotic sows and piglets supplemented with 0.22% SID Trp); (T5)—PTT (probiotic sows and piglets supplemented with 0.27% SID Trp); (T6)—PTTT (probiotic sows and piglets supplemented with 0.33% SID Trp) (see Figure 1).

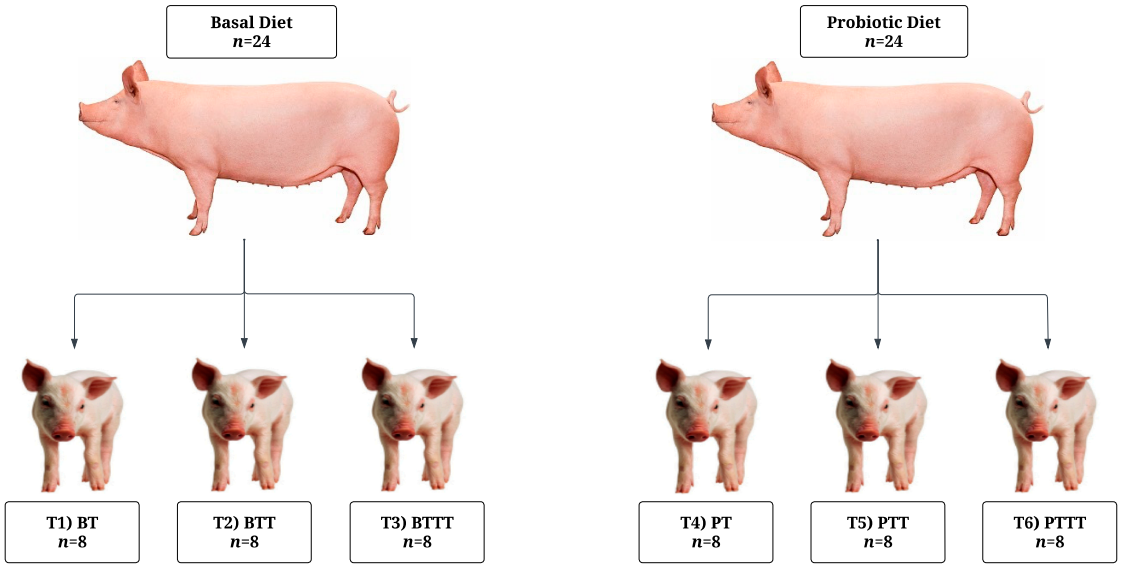

Figure 1. Experimental design overview.

2.1.3. Post-Weaning Period

At weaning, three piglets from the previously selected four piglets from each litter participated in the post-weaning trial. The 144 piglets with an average bodyweight of 7.75 ± 1.53 kg (26 ± 1.8 days of age) were housed with their litter mates in groups of three (1.68 × 1.22 m) and remained in the same dietary groups as pre-weaning. The experimental groups remained as outlined in Figure 1. The ambient environmental temperature within the house was thermostatically controlled at 30 °C for the first 7 days and then reduced by 2 °C/week. Humidity was controlled at 65%. Feed was provided in meal form in two-space feeders equipped with nipple drinkers for water. The feed intake was measured at 7 days post-weaning to calculate average daily feed intake (ADFI) and gain/feed (G:F). The average daily Trp intake was calculated by ADFI (kg/day) × total dietary Trp content (g/kg).

2.2. Sample Collection

A total of 96 pigs (2 pigs/litter of the previously selected 4 pigs/litter) were sacrificed, 48 on the day of weaning and 48 at 7 days post-weaning (n = 8/dietary group). At weaning and 7 days post-weaning, one pig/pen (n = 48) received a lethal injection with pentobarbitone sodium (Euthatal Solution, 200 mg/mL; Merial Animal Health) at a rate of 0.71 mL/kg bodyweight to the cranial vena cava to humanely sacrifice the animals. The pigs euthanized at weaning had an average bodyweight of 8.44 kg (SD = 1.65), while the pigs euthanized at 7 days post-weaning had an average bodyweight of 7.95 (SD = 1.78). The bodyweight of the pigs sacrificed at weaning was greater than that of pigs sacrificed at 7 days post-weaning due to selection design. When selecting the pig to be sacrificed at weaning, from the 4 pigs/litter selected at birth, the heaviest pigs were preferred to standardize the selection process. A similar selection process was performed for the sacrifice at 7 days post-weaning.

Euthanasia was completed by a competent person in a room separate from other piglets. The piglets were not fasted prior to sacrifice. The stomach was dissected from the gastrointestinal tract at the esophagus and the duodenum. The stomach was weighed (full stomach weight), and the pH of the stomach was measured by inserting a pH probe meter (HI-98190, Hanna Instruments, Padovana, Padua, Italy) into the center of the lumen. The stomach was dissected along the greater curvature, the contents were

removed, and the empty stomach was weighed (empty stomach weight). The stomach was gently rinsed with sterile PBS. For analysis of the expression of genes key to stomach functioning, one mucosal sample (1 cm^2) was collected from the fundic and pyloric gland regions, at location 1 and location 2 in Figure 2, respectively. The tissues were rinsed in PBS, stripped of the overlying smooth muscle, and stored in RNAlater® solution (5 mL) overnight at 4 °C. The RNAlater® was removed twenty-four hours later, and the samples were stored at −80 °C. For immunohistochemical staining of parietal cells, tissue was collected from the fundic gland region, at location A in Figure 2. The tissue was gently rinsed with PBS, pinned to a backboard to maintain a flat structure and orientation, and submerged in 10% neutral-buffered formalin until processing.

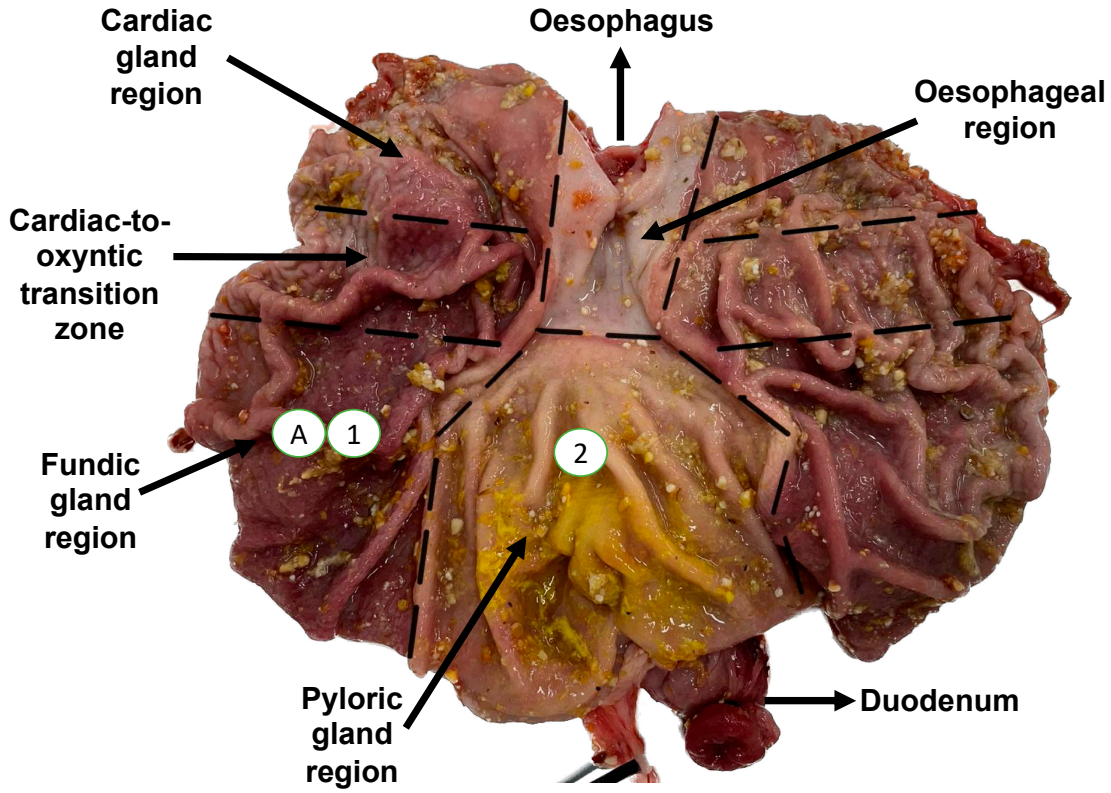

Figure 2. Labeled image of the pig's stomach exposed along the greater curvature of the stomach; image adapted from [18]. Sites 1 and 2 represent the mucosal sampling locations for gene expression analysis. Site A represents the fundic gland region mucosal sampling location for immunohistochemical analysis.

2.3. Gene Expression Analysis

2.3.1. RNA Extraction and cDNA Synthesis

RNA extraction from 100 mg of tissue was carried out using TriReagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Purification was performed with the E.Z.N.A ® Total RNA kit 1 including a DNase step using an on-column DNase 1 Digestion set (Omega Bio-Tek, Norcross, GA, USA). Purity and quantity were determined by the absorbance ratio at 260 nm and 280 nm on a Nanodrop-ND1000 (Thermo Scientific, Waltham, MA, USA). Reverse transcription of 2 µg total RNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) random primers in a 40 µL reaction. The resulting cDNA was then diluted to a final volume of 400 µL using nuclease-free water.

2.3.2. Quantitative PCR

The QPCR reaction mix (20 μL) included FastStart Universal QPCR Sybr Green Master Mix (Roche Diagnostics, Mannheim, Germany), forward and reverse primers (1.2 μL of a 5 μM mix for a final concentration of 300 nM/RXN), nuclease-free water (3.8 μL), and cDNA (5 μL equivalent to 25 ng total RNA). All reactions were performed in duplicate on the 7500 ABI Prism Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under cycling conditions of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers were designed with Primer Express™ (v 3.0.1) software (Applied Biosystems) and synthesized by Eurofins (Valiant Way, Wolverhampton, UK). Dissociation curves were generated to confirm PCR product specificity, and assay efficiencies were determined by plotting CT values from 4-fold serial dilutions of cDNA against their arbitrary quantities. Only assays with 90–110% efficiency and single products were included. The gene panel was selected based on [18] and further literature review, focusing on key genes functional within the fundic and pyloric gland regions (acid secretion, enzyme secretion, inflammation, and mucosal defense). Given the immune-homeostasis-promoting effects of Trp metabolites through the aryl hydrocarbon receptor (AhR) in the intestine [44] and the established link between AhR and IL-22 [45], both *AHR* and *IL22* were included in the gene panel for analysis. Optimal reference genes were selected using the GeNorm algorithm in qBase PLUS 2.0 software (Biogazelle, Ghent, Belgium), with the geometric mean of *ACTB* and *RPL27* used for normalizing target expression. Normalized relative quantities were obtained using qBase PLUS software, and accession numbers, primer sequences, and amplicon lengths are provided in Table 3.

Table 3. Primer sequences for QPCR analysis.

Target Gene	Gene Name	Accession No.	Forward Primer (5′-3′) Reverse Primer (5′-3′)	Amplicon Length (bp)
		Acid secretion		
ATP4A	H^+/K^+-ATPase Transporting Subunit Alpha	XM_021093570.1	F: GGACATGGCAGCCAAGATG R: TGTTCTCCAGCTTCTCCTTCCT	74
CLIC6	Chloride Intracellular Channel 6	XM_003358948.4	F: CGGAACCAGTCAGAAGAACGA R: TCCTACCGCCCAAGAAGCT	87
HRH2	Histamine Receptor 2	XM_003354192.4	F: CCAGCCTGGATGTCATGCCT R: CCGGTCGAGGCTGATCAT	65
KCNQ1	Potassium Voltage-Gated Channel Subfamily Q Member 1	XM_021082620.1	F: CGCGTCTACAACTTCCTCGAA R: CGATAAGGAAGACAGCAAAGTGGTA	73
KCNE1	Potassium Voltage-Gated Channel Subfamily E Regulatory Subunit 1	NM_214165.2	F: AGGTCCCCAGGCCATGA R: GCCCAGCACCATGAGAATGT	60
		Aryl hydrocarbon receptor		
AHR	Aryl Hydrocarbon Receptor	NM_001303026.1	F: GCAGCGCCAACATCACCT R: GGGATTGGCTTGACAGTTTTC	70
		Calcium-sensing receptor		
CASR	Calcium-sensing receptor	XM_021068447.1	F: GGTGGTGGCAGGATA R: TCGACACTGCTGATG	77
		Cholinergic receptor muscarinic 3		
CHRM3	Cholinergic Receptor Muscarinic 3	XM_021071815.1	F: TGGACGCTGCCACTTCTG R: GCTGTGGTCTTGGTCCATCTG	73
		Digestive enzymes		
CHIA	Chitinase Acidic	NM_001258377.1	F: GCCTTTTGTACCCACCTGGTCTA R: TCAGTGGTGGTGATCTCGTTGT	65
PGA5	Pepsinogen A5	NM_213872.2	F: CGGCAGCGTGGTGGTGTTGT R: GGAAACAGGCACCCAGTTCA	73
LCT	Lactase	XM_021076418.1	F: TGGTCCTACGAGCTG R:CAGAAGACAAATCAAGAGAGAGGAAGT	102
		Gastrin		
GAST	Gastrin	NM_001004036.2	F: TCCCAGCTCTGCAGTCAAGA R: CCAGAGCCAGCACATGGA	65

	Gastrin receptor		
CCKBR	Cholecystokinin B Receptor	XM_021062350	F: CATGGGCACGTTTATCTTTGG R: TCACAGACACCCCCATGAAGT	68
	Ghrelin production		
GHRL	Ghrelin	XM_013981924.2	F: AAGCTGGAAATCCGGTTCAA R: CGGACTGAGCCCCTGACA	64
	Histamine production		
HDC	Histidine Decarboxylase	XM_001925342.5	F: ATCTGCCAGTACCTGAGCACTGT R: GCAGGTAGCCAGGTCTCACATC	67
	Inflammation		
CXCL8	C-X-C Motif Chemokine Ligand 8	NM_213867.1	F: TGCACTTACTCTTGCCAGAACTG R: CAAACTGGCTGTTGCCTTCTT	82
IL22	Interleukin 22	XM_021091968.1	F: GATGAGAGAGCGCTGCTACCTGG R: GAAGGACGCCACCTCCTGCATGT	112
TNF	Tumor Necrosis Factor	NM_214022.1	F: TGGCCCCTTGAGCATCA R: CGGGCTTATCTGAGGTTTGAG	68
	Mucins		
MUC1	Mucin 1	XM_001926883.1	F: ACACCCATGGGCGCTATGT R: GCCTGCAGAAACCTGCTCAT	68
MUC5AC	Mucin 5AC	XM_021092583.1	F: GGATGTCGCCAGAGACTGAGTA R: CCCCCTCGTCTCCTTTTACC	71
MUC6	Mucin 6	XM_021082474.1	F: AAAACGTGGGCAGGATGTGT R: GCCATCCTCGCTCAGAAACT	77
	Mucosal defense		
PIGR	Polymeric Immunoglobulin Receptor	XM_021102216.1	F: GGGCTCGGTGACATTTGACT R: TTTAGCTGGCACAGAAATTTGG	72
	Somatostatin		
SST	Somatostatin	NM_001009583.1	F: CCCTGGAGCCTGAAGATTTG R: GCCGGGTTTGAGTTAGCTGAT	85
	Somatostatin receptor		
SSTR2	Somatostatin Receptor	XM_005668643.3	F: TTTTGTGGTCTGCATCATTGG R: GCGTAGCGGAGGATGACGTA	66
	Toll-like receptors		
TLR4	Toll-Like Receptor 4	NM_001293317.1	F: TGCATGGAGCTGAATTTCTACAA	140

			R: GATAAATCCAGCACCTGCAGTTC	
Reference genes				
ACTB	Beta Actin	XM_001927228.1	F: GGACATCGGATACCCAAGGA R: AAGTTGGAAGGCCGGTTAATTT	71
B2M	Beta-2-Microglobulin	NM_213978.1	F: CGGAAAGCCAAATTACCTGAAC R: TCTCCCCGTTTTTCAGCAAAT	83
RPL27	Ribosomal Protein L27	NM_001097479.1	F: GTCCTGGCTGGTCGCTACTC R: GGTCTGAGGTGCCATCATCA	70

2.4. Immunohistochemistry

2.4.1. Method

To count the number of parietal cells in the fundic gland region, formalin-fixed, paraffin-embedded (FFPE) porcine gastrointestinal tissue from the fundic gland region was immunohistochemically stained for hydrogen–potassium-ATPase beta (ATP4B). Consecutive sections were cut at 4 μM thickness. The staining was performed using the DAKO Link 48 Autostainer as per the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, USA) and the EnVision Flex kit (K8002, DAKO, Agilent Technologies). Antigen retrieval was performed using DAKO PTLink (Agilent Technologies) for 20 min at 97 °C in target retrieval buffer, pH6 (K8005, DAKO, Agilent Technologies). The tissue sections were blocked for endogenous peroxidase and non-specific binding by incubation with 30% H2O2 (H1009, Sigma-Aldrich, St. Louis, MO, USA) for 30 min and protein block (X0909, DAKO, Agilent Technologies) for 15 min. The optimal dilution of ATP4B (NB300-583, Novus Biologicals Biotechne, Centennial, CO, USA) was determined to be 1:200 for 30 min, using the porcine fundic gland region as a positive control tissue. The tissue sections were then washed and incubated with horseradish peroxidase (HRP) for 20 min. The chromogen 3,39-diaminobenzidine (DAB) was used for revelation (twice for 5 min each). Negative controls were run under identical conditions with diluent in place of primary antibody and an isotype control (IHC universal negative control reagent ADI-950-231-0025, Enzo Life Sciences, Farmingdale, NY, USA) in parallel with the experimental samples. The slides were counterstained with hematoxylin (K8008, DAKO, Agilent Technologies) and rinsed in deionized water. The slides were dried in an oven (58 °C) and permanently mounted.

2.4.2. Analysis

Once dry, all the slides were scanned and digitized using the Aperio AT2 Digital Slide Scanner (Leica Biosystems, Nußloch, Germany) at 20× magnification, and the images were reviewed using Aperio ImageScope 12.4 software (Leica Biosystems). Using the annotation tools in ImageScope, the area of analysis was outlined on all case slide images. To analyze ATP4B expression, various Aperio algorithms were trained to recognize and quantify the stained cells within the annotated areas. The Nuclear Algorithm was adapted to count the total cells in the annotated images. ATP4B is expressed in the cell cytoplasm, and the trained color deconvolution macro determined the percentage of ATP4B-positive staining and the area of analysis. Combining the results from both macros enables the number of ATP4B-positive cells per mm^2 porcine fundic gland region tissue to be calculated. The area of annotation was used to normalize the results across all treatments and cases.

2.5. Feed Analysis

Dry matter (AOAC.934.01), crude fiber (CF) (AOAC.962.09), and crude oil (AOAC.945.16) were determined according to the method of the Association of Official Analytical Chemists [46]. The gross energy content was measured with an adiabatic bomb calorimeter (Parr Instruments, Moline, IL, USA). Nitrogen content was determined using the LECO FP 528 instrument (Leco Instruments, Mansfield, UK). The neutral detergent fiber content was determined following the method described in [47] using an Ankom 220 Fiber Analyzer (Ankom™ Technology, New York, NY, USA). The dietary concentration of essential amino acids was determined by HPLC [48].

2.6. Statistical Analysis

The data were initially checked for normality using the univariate procedure (PROC UNIVARIATE) on Statistical Analysis Software (SAS) (v 9.4) (SAS Institute, Cary, NC, USA) and transformed if necessary. The pen was the experimental unit for growth parameters (bodyweight, daily gain, feed intake, and G:F). The performance, gastric pH, full stomach weight, empty stomach weight, gene expression (Bonferroni-adjusted $p < 0.05$), and parietal cell count data were analyzed using the general linear model procedure (PROC GLM) on SAS. To investigate the effect of diet on piglet performance, the data were analyzed as a 2 × 3 factorial. The model included the effects of maternal diet (control or probiotic) and piglet diet (0.22, 0.27, or 0.33% SID Trp creep) and their associated two-way interactions. To investigate the effect of time and diet on stomach physiological attributes, gene expression, and parietal cell counts, the data were analyzed as a 2 × 2 × 3 factorial. The model included the effects of time (weaning or 7 days post-weaning), maternal diet (control or probiotic), and piglet diet (0.22, 0.27, or 0.33% SID Trp creep) and their associated two- and three-way interactions. For the analysis of genes grouped by function, the average expression of the selected functionally similar genes was calculated for each pig, and this average value was then treated as a single entry in a PROC GLM analysis. The results are presented as least square means with their standard errors. The probability level that denoted significance was $p < 0.05$. The probability level that denoted a tendency was $p < 0.1$. Within R, the rcorr function from the Hmisc package [49] was used to compute the correlation matrix and p-values. The ggplot2 package [50] within R was then used to visualize the correlation matrix of selected genes with significance levels, while reshape2 was used to format the data for visualization.

A diagrammatic overview of the dietary regimen and analysis conducted in the study is presented in Figure 3.

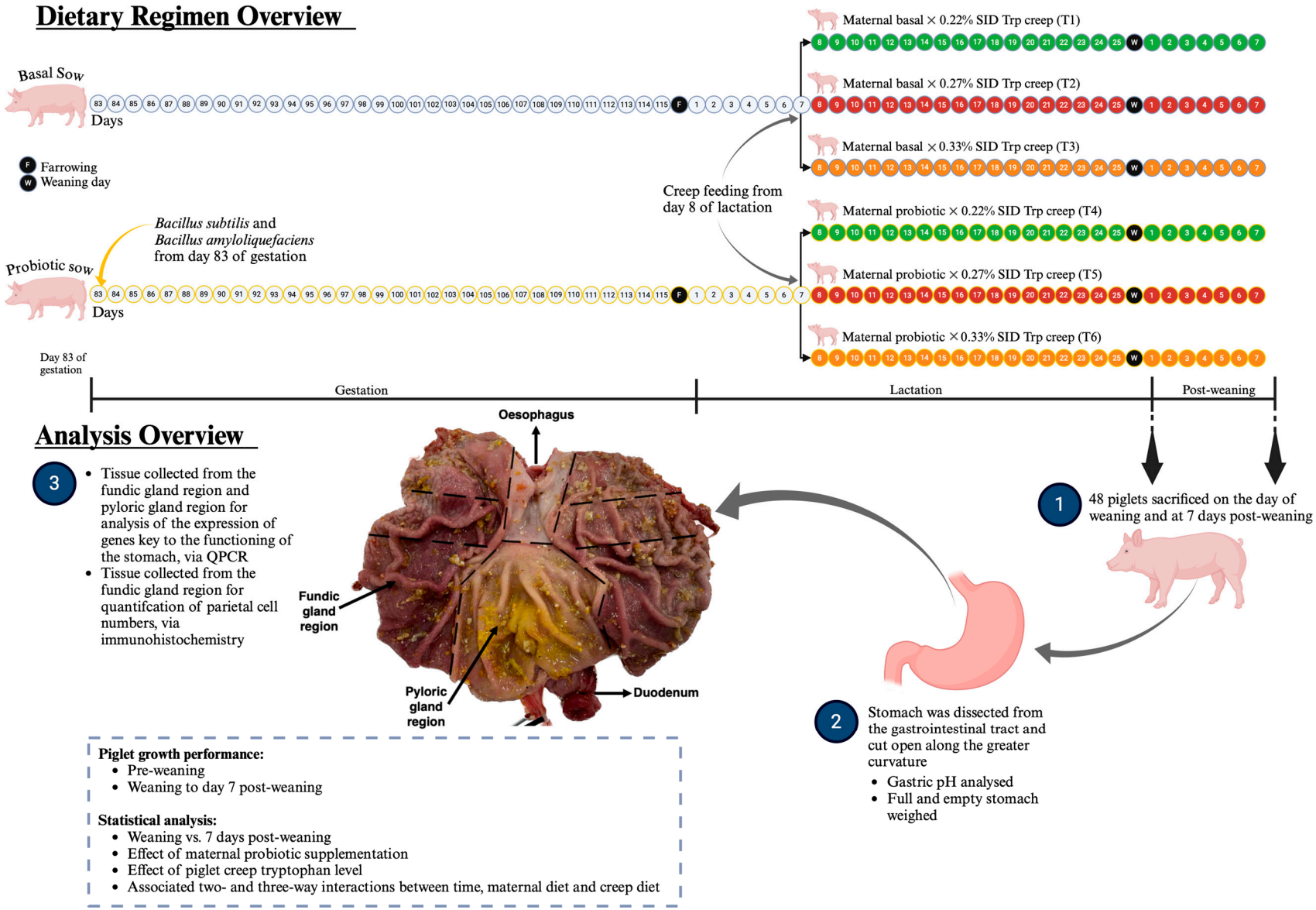

Figure 3. Diagrammatic overview of experiment. Created in BioRender. Kiernan, D. (2025) https://BioRender.com/y98r818.

3. Results

3.1. Performance

The effects of maternal probiotic supplementation and piglet dietary Trp level on the growth performance of all piglets during the pre-weaning phase and from day 0 to 7 days post-weaning are presented in Table 4. There was no effect of maternal diet, creep diet, or maternal × creep interaction on piglet bodyweight, daily gain, or feed intake at any timepoint ($p > 0.05$). There was no effect of maternal diet, creep diet, or maternal × creep interaction on G:F from day 0 to 7 days post-weaning ($p > 0.05$). The total Trp intake per day increased numerically with increasing Trp content of the creep diet (0.22% Trp = 0.63 g/day, 0.27% Trp = 0.72 g/day, 0.33% Trp = 0.82 g/day), but did not reach statistical significance ($p = 0.1287$).

Table 4. Effect of diet on piglet bodyweight, daily gain, feed intake and gain:feed in the pre-weaning and post-weaning periods (least square means with their standard errors).

	Maternal		SEM	Creep			SEM	*p*-Value [+]	
	Control	Probiotic		0.22% Trp	0.27% Trp	0.33% Trp		M	C
Piglet Bodyweight (kg)									
Pre-weaning									
Birth	1.46	1.51	0.04	1.50	1.51	1.45	0.05	0.4358	0.6432
Weaning	7.20	7.33	0.23	7.47	7.24	7.03	0.28	0.7417	0.5313
Post-weaning									
Initial weight	7.63	7.97	0.30	7.92	8.10	7.38	0.37	0.4149	0.3455
D7 post-weaning	8.03	8.18	0.39	8.24	8.50	7.57	0.47	0.7804	0.3479
Daily Gain (kg)									
Pre-weaning									
D0–D26	0.22	0.22	0.01	0.23	0.22	0.21	0.01	0.7946	0.5030
Post-weaning									
D0–D7	0.06	0.03	0.42	0.04	0.06	0.03	0.03	0.4168	0.7634
Feed Intake (kg)									
Pre-weaning litter intake									
Total	0.74	0.98	0.14	0.98	0.90	0.71	0.17	0.2272	0.5364
Post-weaning intake per pig D0–D7									
ADFI	0.26	0.27	0.02	0.29	0.27	0.25	0.03	0.6661	0.5304
Trp Intake (g/day)	0.70	0.74	0.05	0.63	0.72	0.82	0.06	0.6542	0.1287
Gain:Feed									
Post-weaning									
D0–D7	0.14	0.02	0.08	0.08	0.10	0.07	0.10	0.2695	0.9756

Trp tryptophan; M maternal; C creep; ADFI average daily feed intake. [+] There were no maternal × creep interactions.

3.2. Gastric pH and Stomach Weight

The effects of time, maternal probiotic supplementation, and piglet dietary Trp level on piglet bodyweight (sacrificed piglets only), gastric pH, full stomach weight, and empty stomach weight are presented in Table 5. There were no two-way or three-way interactions; hence, the results are presented as main effects only.

Table 5. Effect of diet and time on gastric pH and stomach physiological parameters (least square means with their standard errors).

	Time		SEM	Maternal		SEM	Creep			SEM	*p*-Value [+]		
	Pre	**Post**		**Control**	**Probiotic**		**0.22% Trp**	**0.27% Trp**	**0.33% Trp**		**T**	**M**	**C**
Bodyweight (kg) *	8.49	7.96	0.25	8.24	8.20	0.26	8.09	8.53	8.05	0.31	0.1418	0.9236	0.4668
Gastric pH	3.57	3.53	0.13	3.70	3.40	0.13	3.49	3.59	3.58	0.16	0.8322	0.0980	0.8875
Full stomach weight (g)	151.67	307.16	0.13	233.54	225.29	11.02	226.36	232.97	228.91	13.57	**<0.0001**	0.5953	0.9403
Empty stomach weight (g)	76.46	70.30	11.02	71.56	75.19	1.90	73.42	73.60	73.13	2.34	**0.0233**	0.1769	0.9894
Empty-stomach-to-bodyweight ratio (%)	0.93	0.90	0.03	0.88	0.95	0.03	0.94	0.87	0.93	0.03	0.3025	0.0998	0.2834

Trp, tryptophan; M, maternal; C, creep; Pre, pre-weaning; Post, post-weaning, T, time. * This is the average bodyweights of sacrificed pigs only. See Table 3 for bodyweights of full cohort. [+] There was no maternal × creep, time × maternal, time × creep, or time × maternal × creep interaction. Significant *p*-values are highlighted in bold.

3.2.1. Effect of Time on Gastric pH and Stomach Weight

There was no difference in the bodyweight of sacrificed pigs, gastric pH, or empty-stomach-to-bodyweight ratio between the pigs sacrificed on the day of weaning or at 7 days post-weaning ($p > 0.05$). The pigs sacrificed at weaning had numerically higher bodyweight than the group of pigs that were sacrificed at 7 days post-weaning (8.49 vs. 7.96 kg, respectively) ($p > 0.05$). Focusing on the group sacrificed at 7 days post-weaning, these pigs had an average bodyweight of 7.68 kg at weaning and gained an average of 0.27 kg between weaning and 7 days post-weaning (data not presented). Full stomach weight was greater in pigs at 7 days post-weaning compared to weaning; however, empty stomach weight was greater at weaning compared to 7 days post-weaning ($p < 0.05$).

3.2.2. Effect of Maternal Diet on Gastric pH and Stomach Weight

There was no significant effect of maternal diet on sacrificed piglet bodyweight, gastric pH, empty stomach weight, full stomach weight, or empty-stomach-to-bodyweight ratio ($p > 0.05$).

3.2.3. Effect of Creep Diet on Gastric pH and Stomach Weight

There was no significant effect of creep diet on sacrificed piglet bodyweight, gastric pH, empty stomach weight, full stomach weight, or empty-stomach-to-bodyweight ratio ($p > 0.05$).

3.3. Gene Expression

The effects of time and maternal diet on the expression of genes in the fundic and pyloric gland regions are presented in Table 6. Data on the maternal, creep, and maternal × creep effects on gene expression in the fundic and pyloric gland regions, split by timepoint (weaning and 7 days post-weaning), are presented in Appendix A (Tables A1 and A2, respectively).

Table 6. Effect of maternal diet and time on the gene expression in the fundic and pyloric gland regions (least square means with their standard errors).

Time		Pre-Weaning		Post-Weaning		SEM	*p*-Value [+]		
Maternal Diet		Control	Probiotic	Control	Probiotic		T	M	T × M
Function	Gene								
The fundic gland region									
Acid secretion	*ATP4A*	0.74 [a]	1.23 [b]	1.34 [b]	1.41 [b]	0.12	**<0.0001**	**0.0037**	**0.0262**
	CLIC6	0.81	2.02	1.84	2.05	0.29	0.0825	**0.0194**	0.0734
	HRH2	0.68	1.13	1.31	1.50	0.09	**<0.0001**	**0.0009**	0.1673
	KCNE1	0.69	1.02	1.37	1.37	0.12	**<0.0001**	**0.0111**	0.8952
	KCNQ1	0.62	1.12	1.40	1.64	0.10	**<0.0001**	**0.0004**	0.1891
Aryl hydrocarbon receptor	*AHR*	1.12	1.22	0.88	0.93	0.05	**<0.0001**	0.1112	0.6078
Calcium-sensing receptor	*CASR*	1.83	1.54	0.74	0.88	0.19	**<0.0001**	0.6850	0.2564
Cholinergic receptor muscarinic 3	*CHRM3*	0.73	1.02	1.21	1.30	0.06	**<0.0001**	**0.0047**	0.1053
Digestive enzyme	*CHIA*	0.56 [a]	0.93 [b]	1.89 [c]	1.84 [c]	0.11	**<0.0001**	0.1518	**0.0500**
	LCT	1.38	2.36	0.86	0.93	0.34	**0.0052**	0.1290	0.1849
	PGA5	0.70	0.96	1.58	1.65	0.10	**<0.0001**	0.1114	0.3509
Gastrin receptor	*CCKBR*	0.57	1.26	1.62	1.92	0.14	**<0.0001**	**0.0004**	0.1498
Ghrelin	*GHRL*	0.92 [a]	1.29 [b]	1.07 [b]	1.04 [b]	0.08	0.5348	**0.0408**	**0.0225**
Histamine production	*HDC*	0.87 [a]	1.47 [b]	1.07 [a]	1.07 [a]	0.09	0.2954	**0.0022**	**0.0024**
Inflammation	*CXCL8*	1.88	1.58	0.88	0.97	0.23	**0.0006**	0.6554	0.3851
	IL22	1.87	1.44	1.05	1.34	0.35	0.1993	0.8382	0.3069
	TNF	1.26	1.16	0.84	1.02	0.08	**0.0005**	0.6248	0.0722
Mucus production	*MUC1*	1.04	1.04	0.99	1.16	0.07	0.5814	0.2019	0.1857
	MUC5AC	1.35 [ab]	1.09 [a]	1.04 [a]	1.41 [b]	0.09	0.9747	0.5364	**0.0008**
	MUC6	0.97	1.59	1.46	1.85	0.19	**0.0494**	**0.0081**	0.5401
Somatostatin	*SST*	1.04	1.16	1.02	1.12	0.10	0.7343	0.2274	0.8843
Somatostatin receptor	*SSTR2*	0.66	1.17	1.24	1.49	0.11	**<0.0001**	**0.0007**	0.2324
Toll-like receptor	*TLR4*	1.27 [a]	1.09 [ab]	0.84 [c]	0.98 [bc]	0.05	**<0.0001**	0.7243	**0.0039**
The pyloric gland region									
Aryl hydrocarbon receptor	*AHR*	1.03 [a]	1.15 [b]	1.02 [a]	0.92 [a]	0.05	**0.0218**	0.9636	**0.0388**
Calcium-sensing receptor	*CASR*	1.52	1.61	0.91	0.73	0.11	**<0.0001**	0.6906	0.2046
Gastrin	*GAST*	1.49	1.94	0.85	0.83	0.16	**<0.0001**	0.1778	0.1471
Inflammation	*CXCL8*	1.41	1.48	1.24	1.19	0.23	0.3226	0.9888	0.7853

	TNF	1.05	1.17	1.11	0.99	0.10	0.5767	0.9853	0.2352
Mucosal defense	*PIGR*	0.86	1.10	1.20	1.71	0.15	**0.0019**	**0.0141**	0.3588
Mucus production	*MUC5AC*	0.93	0.92	1.17	1.22	0.07	**0.0002**	0.7673	0.6766
	MUC6	0.88	0.70	3.55	2.59	0.36	**<0.0001**	0.1151	0.2756
Somatostatin	*SST*	1.32	1.30	0.90	0.79	0.08	**<0.0001**	0.4367	0.5789
Toll-like receptor	*TLR4*	1.14	1.22	1.07	0.99	0.09	0.1080	0.9737	0.3856

M, maternal; T, time. [+] There were no time × creep, time × maternal × creep interactions. Significant *p*-values are highlighted in bold. [a,b,c] Values within a row with different superscript letters were significantly different.

Among the twenty-three genes analyzed in the fundic gland region, there was a time effect on the expression of sixteen genes, a maternal effect on the expression of eleven genes, and a time × maternal interaction on the expression of six genes ($p < 0.05$). There were no creep, maternal × creep, time × creep, or time × maternal × creep interactions on the gene expression in the fundic gland region ($p > 0.05$). Among the ten genes analyzed in the pyloric gland region, there was a time effect on the expression of seven genes and a maternal, creep, maternal × creep, and time × maternal effect on the expression of one gene each ($p < 0.05$). There were no time × creep or time × maternal × creep interactions on the gene expression in the pyloric gland region ($p > 0.05$).

3.3.1. Effect of Time on Gene Expression

In the fundic gland region, six genes had greater expression at weaning compared to 7 days post-weaning: *AHR*, *CASR*, *LCT*, *CXCL8*, and *TNF* ($p < 0.05$). Eight genes had greater expression at 7 days post-weaning compared to at weaning: *HRH2*, *KCNE1*, *KCNQ1*, *CHRM3*, *CHIA*, *PGA5*, *CCKBR*, *MUC6*, and *SSTR2* ($p < 0.05$).

In the pyloric gland region, three genes had greater expression at weaning compared to 7 days post-weaning: *CASR*, *GAST*, and *SST* ($p < 0.05$). Three genes had greater expression at 7 days post-weaning compared to at weaning: *PIGR*, *MUC5AC*, and *MUC6* ($p < 0.05$).

3.3.2. Effect of Maternal Diet and Interactions with Time on Gene Expression

In the fundic gland region, there was a maternal effect on the expression of eight genes; piglets from probiotic sows had upregulated expression of *CLIC6*, *HRH2*, *KCNE1*, *KCNQ1, CHRM3*, *CCKBR*, *MUC6*, and *SSTR2* compared to piglets from control sows ($p < 0.05$).

In the fundic gland region, there were seven time × maternal interactions: piglets from probiotic sows had upregulated *ATP4A* expression at weaning compared to piglets from control sows ($p < 0.05$), but not at 7 days post-weaning ($p > 0.05$). The expression of *ATP4A* was greater at 7 days post-weaning compared to at weaning in piglets from control sows ($p < 0.05$), but not in piglets from probiotic sows ($p > 0.05$); piglets from probiotic sows had upregulated *CHIA* expression at weaning compared to piglets from control sows ($p < 0.05$), but not at 7 days post-weaning ($p > 0.05$); piglets from probiotic sows had upregulated *GHRL* compared to piglets from control sows at weaning ($p < 0.05$), but not at 7 days post-weaning ($p > 0.05$); piglets from probiotic sows had upregulated *HDC* compared to piglets from control sows at weaning ($p < 0.05$), but not at 7 days post-weaning ($p > 0.05$). Furthermore, *HDC* expression was greater at weaning compared to 7 days post-weaning in piglets from probiotic sows ($p < 0.05$), but not in piglets from control sows ($p > 0.05$); piglets from probiotic sows had upregulated expression of *MUC5AC* compared to piglets from control sows at 7 days post-weaning ($p < 0.05$), but not at weaning ($p > 0.05$); *TLR4* expression was greater at weaning compared to 7 days post-weaning in piglets from control sows ($p < 0.05$), but not in piglets from probiotic sows ($p > 0.05$).

In the pyloric gland region, there was one time × maternal interaction: *AHR* expression was greater at weaning compared to 7 days post-weaning in piglets from probiotic sows ($p < 0.05$), but not in piglets from control sows ($p > 0.05$).

3.3.3. Effect of Creep Diet and Interactions with Time on Gene Expression

In the pyloric gland region, there was a creep effect on the expression of *PIGR* ($p < 0.05$); piglets fed 0.27 or 0.33% Trp creep had upregulated *PIGR* compared to pigs fed 0.22% Trp creep (1.38 and 1.36 vs. 0.91, respectively, SEM = 0.13) ($p < 0.05$). There was no

creep effect on gene expression in the fundic gland region, nor time × creep interactions on the gene expression in the fundic or pyloric gland regions ($p > 0.05$).

3.3.4. Maternal and Creep Diet Interactions on Gene Expression

In the pyloric gland region, there was a maternal × creep diet interaction on the expression of *GAST* ($p < 0.05$); *GAST* expression was upregulated in piglets from probiotic sows that were fed 0.33% Trp and from control sows fed 0.22% Trp creep compared to piglets from control sows that were fed 0.27% or 0.33% Trp creep (1.80 and 1.79 vs. 0.89 and 1.12, respectively, SEM = 0.19) ($p < 0.05$).

There were no time × maternal × creep interactions on the gene expression in the fundic or pyloric gland regions ($p > 0.05$).

3.4. Genes Grouped by Function

The effect of time and maternal diet on the expression of genes grouped by function in the fundic and pyloric gland regions is presented in Table 7. There were no creep, maternal × creep, time × creep, or time × maternal × creep interactions on the gene expression grouped by function in the fundic or pyloric gland regions ($p > 0.05$).

Table 7. Effect of diet and time on the expression of genes grouped by function in the fundic and pyloric gland regions (least square means with their standard errors).

Time		Pre-Weaning		Post-Weaning		SEM	*p*-Value [+]		
Maternal Diet		Control	Probiotic	Control	Probiotic		T	M	T × M
Function	**Gene**								
The fundic gland region									
Acid stimulation	*HRH2*, *CCKBR*, *CHRM3*, *HDC*	0.94	1.28	1.18	1.33	0.06	**0.0251**	**0.0003**	0.1217
Acid inhibition	*SST*, *SSTR2*	0.85	1.17	1.13	1.30	0.08	**0.0075**	**0.0018**	0.3545
Acid secretion	*ATP4A*, *CLIC6*, *KCNE1*, *KCNQ1*	0.72 [a]	1.35 [b]	1.49 [b]	1.68 [b]	0.11	**<0.0001**	**0.0003**	**0.0452**
Mucus production	*MUC1*, *MUC5AC*, *MUC6*	1.12	1.24	1.16	1.47	0.08	0.0809	**0.0068**	0.2292
The pyloric gland region									
Mucus production	*MUC5AC*, *MUC6*	0.90	0.81	2.36	1.91	0.18	**<0.0001**	0.1286	0.3131

[+] There was no effect of creep, maternal × creep, time × creep, or time × maternal × creep interactions. Significant *p*-values are highlighted in bold. [a,b] Values within a row with different superscript letters were significantly different.

3.4.1. Time Effect on Genes Grouped by Function

In the fundic gland region, piglets at 7 days post-weaning had greater acid stimulation and acid inhibition gene expression compared to piglets at weaning ($p < 0.05$). In the pyloric gland region, piglets at 7 days post-weaning had greater expression of mucus production genes compared to piglets at weaning ($p < 0.05$).

3.4.2. Maternal Effect and Interaction with Time on Genes Grouped by Function

Piglets from probiotic sows had upregulated acid stimulation, acid inhibition, and mucus production gene expression in the fundic gland region compared to piglets from control sows ($p < 0.05$). There was a time × maternal interaction on acid secretion gene expression; piglets from probiotic sows had upregulated acid secretion gene expression compared to piglets from control sows at weaning ($p < 0.05$), but not at 7 days post-weaning ($p > 0.05$). Furthermore, acid secretion gene expression was greater at 7 days post-weaning compared to at weaning in piglets from control sows ($p < 0.05$), but not in piglets from probiotic sows ($p > 0.05$).

3.5. Parietal Cell Counts

The effect of time on parietal cell numbers per mm² in the fundic gland region is presented in Figure 4. The number of parietal cells per mm² of fundic gland tissue was greater at 7 days post-weaning compared to at weaning (1250 vs. 913, SEM = 104) ($p < 0.05$). There was no effect of maternal, creep, maternal × creep, time × maternal, or maternal × creep × time interaction on the number of parietal cells at weaning or at 7 days post-weaning ($p > 0.05$; data not presented).

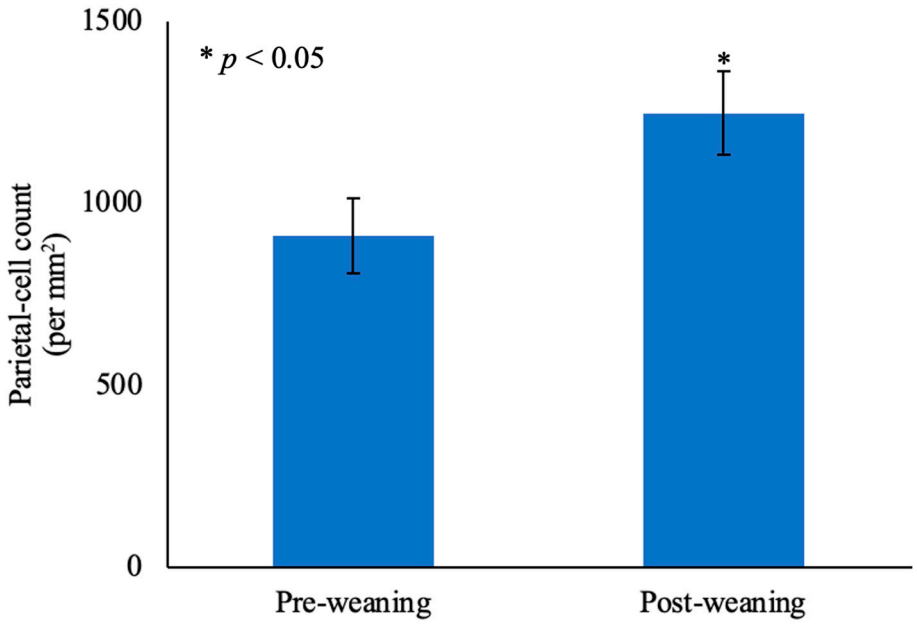

Figure 4. The number of parietal cells per mm² of fundic gland region tissue in pigs at weaning compared to 7 days post-weaning.

3.6. Correlation Analysis

Given the importance of the development of acid and enzyme secretion in the fundic gland region of the young pig, Pearson correlations were performed to assess co-expression patterns and correlations between pig physiological parameters and gene expression in the fundic gland region, both at weaning and at 7 days post-weaning. For the correlations, the data for the genes grouped by function were utilized for acid stimulation, acid secretion, acid inhibition, and mucus production rather than the individual gene expression values. Pearson correlations for the pyloric gland region at weaning and 7 days post-weaning are not discussed but are presented in Appendix A (Figures A1 and A2).

3.6.1. Weaning

Pearson correlations between gene expression in the fundic gland region, pre-weaning creep intake, sow parity, piglet age at weaning, full stomach weight, empty stomach weight, gastric pH, bodyweight, and parietal cell count at weaning time are presented in Figure 5.

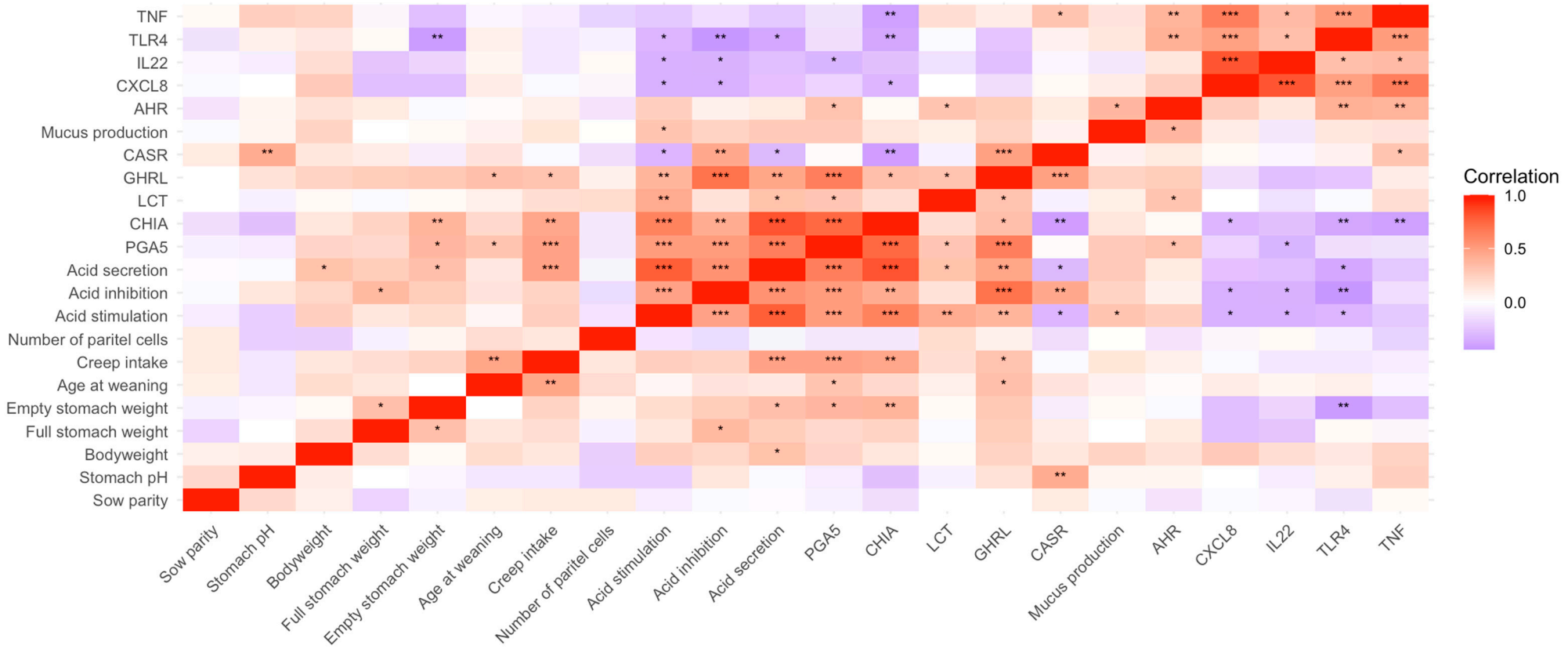

Figure 5. Correlation matrix illustrating Pearson correlations of fundic gland region gene expression and physiological parameters pre-weaning. Positive (red) and negative (blue) correlations are represented in color strength on a scale of −1 to 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Most notably, acid secretion (*ATP4A*, *CLIC6*, *KCNE1*, and *KCNQ1*), acid stimulation (*HRH2*, *CCKBR*, *CHRM3*, and *HDC*), acid inhibition (*SST* and *SSTR2*), digestive enzymes (*PGA5* and *CHIA*), and ghrelin (*GHRL*) expression were all positively correlated. Overall, stomach pH, sow parity, and bodyweight had minimal correlations with gene expression, parietal cell numbers, or selected production parameters: stomach pH was positively correlated to *CASR* expression; bodyweight was positively correlated to the expression level of acid-secretion-related genes, and sow parity had no correlations. Interestingly creep feed intake was positively correlated to acid secretion, the digestive enzymes *PGA5* and *CHIA*, and *GHRL* expression. As expected, full stomach weight and empty stomach weight were positively correlated, while empty stomach weight was also positively correlated to acid secretion, *PGA5*, and *CHIA* and negatively correlated to *TLR4* expression. The immune response genes *CXCL8*, *IL22*, *TNF*, and *TLR4* were positively correlated with each other, while *AHR* was positively correlated with *TLR4*, *TNF*, and mucus production expression. The expression levels of several immune response genes, *CXCL8*, *IL22*, and *TLR4*, were negatively correlated with acid stimulation and acid inhibition gene expression, while *TLR4* was also negatively correlated with acid secretion. *CASR* expression was positively correlated with stomach pH, acid inhibition, and *GHRL* expression; however, surprisingly it was negatively correlated to both the expression of acid stimulation and secretion. Moreover, the number of parietal cells ($ATP4B^+$) did not correlate with any gene expression in the fundic gland region or with any performance parameters.

3.6.2. Seven Days Post-Weaning

Pearson correlations between gene expression in the fundic gland region and piglet ADFI post-weaning, gastric pH, full stomach weight, empty stomach weight, bodyweight, and parietal cell count at 7 days post-weaning are presented in Figure 6.

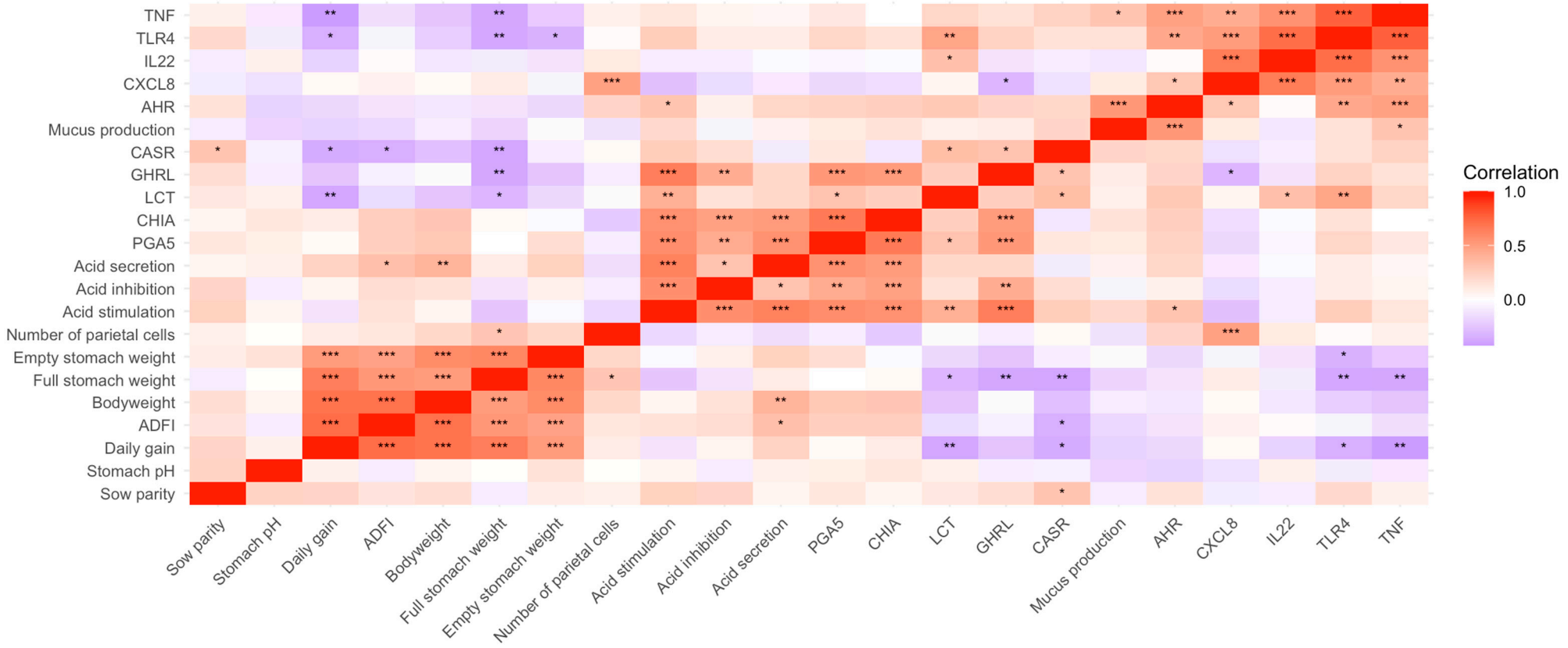

Figure 6. Correlation matrix illustrating Pearson correlations of fundic gland region gene expression and physiological parameters post-weaning. Positive (red) and negative (blue) correlations are represented in color strength on a scale of −1 to 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ADFI, average daily feed intake.

Notably, bodyweight, full stomach weight, empty stomach weight and ADFI were all positively correlated with each other. ADFI and bodyweight were also positively correlated to acid secretion gene expression. Interestingly, daily gain and full stomach weight were negatively correlated to *LCT*, *CASR*, *TLR4*, and *TNF* expression, while full stomach weight was also negatively correlated to *GHRL*. Like weaning, acid secretion, acid stimulation, acid inhibition, and the digestive enzymes *PGA5* and *CHIA*, were positively correlated to each other. Additionally, *GHRL* expression was positively correlated to acid stimulation, acid inhibition, and digestive enzyme *PGA5* and *CHIA* expression. The immune genes *CXCL8*, *IL22*, *TNF*, and *TLR4* were positively correlated with each other, while *AHR* was positively correlated with *CXCL8*, *TLR4*, *TNF*, and mucus production expression. However, unlike pre-weaning, where these immune response genes were negatively correlated to acid and enzyme secretion genes, the only negative gene expression correlation with an immune response gene was between *CXCL8* and *GHRL*. *CASR* expression was not correlated to any acid-secretion-related genes, but like pre-weaning, it did have a positive correlation with *GHRL*. Moreover, *CASR* was negatively correlated to ADFI and full stomach weight. The number of parietal cells ($ATP4B^+$) did not correlate to the acid secretion gene expression in the fundic gland region. The number of parietal cells was positively correlated with full stomach weight and the expression of *CXCL8*.

4. Discussion

The spatio-temporal development of gastric function in the stomach in relation to diet represents a relatively underexplored aspect of swine physiology [51]. In the current study, pigs had a more functionally mature stomach at 7 days post-weaning compared to at weaning, represented by a greater number of parietal cells, a greater expression of genes involved with parietal cell activity, and a change in the expression of digestive enzyme genes. The level of dietary Trp fed to piglets had a limited effect on growth performance, gastric pH, gene expression, or parietal cell counts in the pig's stomach. Overall, maternal probiotic supplementation had a significant impact on gene expression in the fundic gland region of the offspring, particularly upregulating the expression of several genes related to acid secretion from parietal cells. However, maternal probiotic supplementation did not influence growth performance pre-weaning or during the 7-day post-weaning period.

4.1. Effect of Time: The Day of Weaning vs. 7 Days Post-Weaning

Analyzing the changes in the pig's stomach at weaning compared to 7 days post-weaning offers valuable insights into the immediate effects of weaning on the stomach. Typically, the number of acid-secreting parietal cells increases steadily with age during the suckling period and is higher in pigs at 14 days post-weaning compared to pre-weaning [1]. In agreement, the number of parietal cells in the fundic gland region was greater in pigs at 7 days post-weaning compared to pigs at weaning in the current study. Consistent with this, the expression levels of several genes associated with parietal cell activity, such as acid secretion (*KCNE1*, *KCNQ1*) and cell receptors related to both acid secretion stimulation (*CCKBR*, *CHRM3*, *HRH2*) and inhibition (*SSTR2*), were all greater in the fundic gland region of pigs at 7 days post-weaning compared to the pigs at weaning. Gastric acid regulation involves a complex system of receptors, ligands, and signal cascades. Parietal cell acid secretion is regulated by paracrine (histamine), endocrine (gastrin/somatostatin), and neural (acetylcholine) pathways. The expression of the respective receptors for each of these pathways was greater in the fundic gland region of pigs at 7 days post-weaning compared to weaning. However, it is surprising that the expression of genes related to the production of the corresponding signaling molecules,

histamine and somatostatin, did not differ in the fundic gland region between pre- and post-weaning. Furthermore, the expression levels of gastrin and somatostatin in the pyloric gland region, the primary site for their production [18], were 2-fold and 1.5-fold higher, respectively, in pigs at weaning compared to pigs at 7 days post-weaning. In a study by Trevisi et al. [1], the expression of gastrin was not affected by age; however, there was a numerical decrease at 14 days post-weaning compared to at weaning. When the genes were grouped by function, acid stimulation and acid inhibition were greater in the fundic gland region at 7 days post-weaning compared to weaning. Furthermore, mucus production was greater in the pyloric gland region at 7 days post-weaning compared to weaning. Looking specifically at the weaning timepoint, maternal probiotic supplementation increased the expression of additional genes related to acid secretion in the fundic gland region of the offspring, which will be discussed in detail later. This increase resulted in a time × maternal interaction for certain genes, such as *ATP4A*, as well as for acid secretion genes when grouped by function. Notably, these genes had higher expression at 7 days post-weaning compared to weaning in offspring from control sows, whereas this pattern was not observed in offspring from probiotic-supplemented sows, due to their already elevated pre-weaning expression levels.

The expression of *CASR*, whose stimulation can lead to an increase in the activity of the acid pump H^+/K^+-ATPase and also the expression of gastrin and somatostatin [30,31,52], was higher in both the fundic and pyloric gland regions of the pre-weaned pigs compared to the post-weaned pigs. Given its suggested role in acid secretion, it is surprising that *CASR* expression did not align with that of acid secretion genes. The expression of digestive enzymes, *CHIA* and *PGA5*, was greater at 7 days post-weaning compared to at weaning, while the expression of the digestive enzyme lactase, *LCT*, was lower at 7 days post-weaning compared to at weaning. The reduction in *LCT* is not surprising given the reduction in dietary lactose in the post-weaning diet compared to that of the sows' milk pre-weaning. To the best of the authors' knowledge, this is the first study to document *LCT* gene expression in the stomach, as the literature has suggested its expression is limited to the intestine, with prior studies focusing on intestinal lactase activity [53] and gene expression [54]. While the intestine may indeed be the predominant site of its expression, this finding suggests that *LCT* is at least transcribed in the stomach.

The post-weaning period is typically associated with increased expression of inflammation and immune markers in the intestine [55,56]. However, in the current study, the expression of pro-inflammatory markers, *CXCL8* and *TNF*, was greater in the fundic gland region at weaning compared to 7 days post-weaning. Additionally, in piglets from control sows, *TLR4* was greater at weaning compared to 7 days post-weaning. However, in agreement with Trevisi et al. [15], the expression of polymeric immunoglobulin receptor, *PIGR*, a key component of the innate immune system, was greater in the pyloric gland region at 7 days post-weaning compared to at weaning.

Notably, the bodyweight of the group sacrificed at weaning was, on average, 0.53 kg heavier than the post-weaning group, though this difference was not statistically significant. At 7 days post-weaning, the full stomach weight was two-fold higher compared to at weaning, likely due to the increased presence of solid gastric contents. Despite this increase in full stomach weight, the empty stomach weight was lower at 7 days post-weaning compared to at weaning, which may possibly be linked to the difference in bodyweight between the two sacrificed groups. There was no difference in empty-stomach-to-bodyweight ratio or gastric pH between weaning and 7 days post-weaning.

4.2. Effect of Maternal Probiotic Supplementation

This study is the first study to examine the effects of maternal probiotic supplementation on the stomach of offspring in pigs. Intriguingly, offspring from sows supplemented with probiotics had a differential expression of several genes compared to offspring from control sows. Overall, in the fundic gland region, offspring from the probiotic sows had upregulated expression of genes associated with parietal cell activity, *KCNE1*, *KCNQ1*, *CLIC6*, *HRH2*, *CHRM3*, *CCKBR*, and *SSTR2*. Similarly, when the genes were grouped by function, offspring from probiotic sows had upregulated expression of genes related to acid stimulation and inhibition and mucus production in the fundic gland region compared to offspring from control sows. Furthermore, when the weaning and 7 days post-weaning timepoints were analyzed separately, offspring from probiotic sows had several additionally upregulated genes at weaning compared to offspring from control sows. These included digestive enzyme genes such as *CHIA* and *PGA5*; *GHRL*, which is involved in feeding behavior and energy balance at weaning [57,58]; *HDC*, which is involved in the stimulation of acid secretion from parietal cells; and, when grouped by function, acid secretion genes (see Appendix A: Table A1). Additionally, maternal probiotic supplementation reduced the expression of *TLR4* in the fundic gland region at weaning, which, given its role as a microbial-associated molecular pattern recognizer [59], may suggest a potential change in bacterial populations in the stomach at weaning. Several genes that were differentially upregulated in piglets from probiotic sows at weaning were numerically upregulated again at 7 days post-weaning. However, this effect did not reach statistical significance, potentially due to increased variation at 7 days post-weaning. This is evident in the fact that when the overall data were analyzed (combination of weaning and 7 days post-weaning), the statistically significant increase at weaning and numerical increase post-weaning led to significant overall effects for several genes, without significant time × maternal interactions.

In addition to the increase in the expression of parietal cell activity genes in piglets from probiotic sows, there was a tendency for reduced stomach pH in these piglets compared to piglets from control sows (3.4 vs. 3.7, SEM = 0.13). Furthermore, piglets from probiotic sows tended to have an increased empty-stomach-to-bodyweight ratio compared to piglets from control sows. Although these effects were only statistical tendencies, they provide additional evidence for the influence of maternal probiotic supplementation on the development of the offspring's stomach. There was no effect of maternal probiotics on the number of parietal cells or on the weights of the empty or full stomach. It is notable that the expression of genes associated with parietal cell activity was upregulated in piglets from probiotic-supplemented sows, while the number of parietal cells remained unchanged. This suggests that maternal probiotic supplementation enhances the functional activity of parietal cells rather than increasing their quantity.

Interestingly, piglets from probiotic sows had increased expression of mucus production genes in the fundic gland region compared to piglets from control sows when *MUC1*, *MUC5AC*, and *MUC6* were grouped together. Mucins are a key component of stomach mucus, protecting the epithelial layer from acidic gastric contents and harmful pathogens. Probiotic supplementation to pigs can enhance goblet cell numbers and mucus expression in the intestine [60–62]; however, to the best of the authors' knowledge, this is the first study demonstrating probiotics influencing mucus production in the stomach of pigs.

Looking specifically at the weaning timepoint, piglets from probiotic sows had upregulated expression of genes related to acid stimulation (*HRH2*, *CHRM3*, *CCKBR*, *HDC*), acid inhibition (*SST*, *SSTR2*), acid secretion (*ATP4A*, *CLIC6*, *KCNE1*, *KCNQ1*), digestive enzymes (*CHIA*, *PGA5*), *GHRL*, and *MUC6* in the fundic gland region. In contrast, *TLR4* expression was lower in piglets from probiotic sows compared to those from

control sows in the fundic gland region at weaning. An interesting comparison with the analysis of weaning versus 7 days post-weaning revealed strong similarities in gene expression patterns; at 7 days post-weaning, pigs had increased expression of the same genes related to acid regulation, digestive enzymes, *GHRL*, and *MUC6*, with a reduction in *TLR4* expression. These parallel patterns suggest that maternal probiotic supplementation accelerated the maturation of the offspring's stomach function prior to weaning.

Despite the advancement in stomach function in piglets from probiotic sows, this did not transform into improvements in performance in the pre-weaning or immediate post-weaning period. Growth performance is influenced by a complex interplay of multiple systems in the body, including the digestive and immune systems. Although improvements in stomach function were observed with maternal probiotic supplementation, these did not translate into measurable effects on performance over the short duration of the trial. However, the lack of improvement in pre-weaning growth performance is in contradiction to recent research utilizing this specific blend of *Bacillus subtilis* and *Bacillus amyloliquefaciens*, where supplementation increased offspring weight gain and weaning weights, both when supplemented solely to the sow [63] and in combination with direct piglet supplementation [64,65]. To the best of the authors' knowledge, the effect of maternal supplementation of this probiotic on post-weaning performance has yet to be documented in the literature. Extending the assessment period beyond the initial week post-weaning may provide further insight into the potential long-term benefits of maternal supplementation with this *Bacillus* probiotic blend on offspring performance and gastrointestinal health. Maternal probiotic supplementation may exert limited short-term effects but significantly influence the long-term performance of offspring. This is supported by work by Crespo-Piazuelo et al. [66], in which maternal supplementation with *Bacillus altitudinis* had no impact on offspring bodyweight at day 14, 28, or 56 post-weaning but did enhance bodyweight at day 105 and day 127 post-weaning.

Establishing a healthy microbiota that is diverse, with a high abundance of beneficial bacteria and a low abundance of potentially pathogenic bacteria, is the goal in terms of microbiota modulation for enhancing health and performance. The composition of the GIT microbiota in postnatal pigs can be influenced by various microbial sources, with the sow being one of the primary contributors [36,37,67–70]. Probiotics are defined as a "live microorganism which when administered in adequate amounts confers a health benefit on the host" [71]. A probiotic's general mode of action is to introduce beneficial microbial strains into the GIT environment which then fill environmental niches. This, in turn, leads to enhanced microbiota diversity, increased host resistance to pathogens, and increased production of host-health-promoting metabolites, thereby improving overall host health, as reviewed in [33]. The potential for maternal probiotic supplementation in sows has been reviewed in detail in [33]. Positive alterations to the composition of the maternal microbiota can benefit the sow's health and facilitate the transmission of beneficial microbes (via the sow feces or milk ingestion), including the supplemented probiotic strain, to the offspring [66,72]. However, to the best of the authors' knowledge, this represents the first study to analyze the effect of maternal probiotic supplementation on stomach characteristics, expression of genes key to the functioning of the stomach, or parietal cell counts in the offspring's stomach. The significant effects of maternal supplementation with *Bacillus subtilis* and *Bacillus amyloliquefaciens* on the offspring's stomach is an intriguing finding in the current study, although the precise mechanisms are unclear. The limited information in the literature on the interaction between early-life microbial exposure and the development of the stomach has been provided in two previous studies by Trevisi et al. [1,24]. In the first study, pigs delivered by cesarean section, removed from the sow and given an oral dose of simple starter bacteria (*Lactobacillus amylovorus, Clostridium glycolicum*, and *Parabacteroides* spp.) at birth, followed by an oral dose of di-

luted sow feces on day 3 of life, had enhanced functional maturation of the fundic gland region at two weeks of age compared to pigs who only received the oral dose of starter bacteria at birth [24]. This study highlights the potential impact of the composition of microbial exposure on the functioning of the stomach in young pigs. In the second study, Trevisi et al. [1] investigated the effects of maternal antibiotic treatment on the development of acid secretion, ghrelin regulation, and umami taste in offspring and concluded that, while umami taste and ghrelin regulation can be influenced by the maternal environment, the development of acid secretion is predominantly controlled by developmental processes. The results from the current study somewhat contradict this and suggest that the maternal environment does in fact affect the acid secretion pathway in the offspring's stomach. To better understand the mechanisms underlying the observed effects of maternal probiotic supplementation in this study, analyzing the alterations in sow microbiota composition and, more precisely, offspring stomach microbiota composition and gastric metabolite content would have been valuable and should be considered in future research.

4.3. Effect of Increased Piglet Dietary Tryptophan Level

The NRC-recommended Trp inclusion level for pigs weighing 5–7 kg and 7–11 kg is 0.25% SID Trp (SID Trp:Lys of 0.17) and 0.22% SID Trp (SID Trp:Lys of 0.16), respectively [39]. Due to insufficient data on biological relationships in pigs under 20 kg, the NRC model did not generate precise estimates for these pigs, relying instead on a simple mathematical approach and empirical estimates [39]. Interest in Trp inclusion rates has grown recently due to Trp's effects on immune response, as reviewed in [73,74], and its influence on GIT microbiota composition, as reviewed in [75]. However, regarding its impact on the stomach, only limited studies exist: one *ex vivo* study examined Trp's effect on acid secretion in finisher pig stomachs [30], and one *in vivo* study investigated its influence on stomach ghrelin expression in post-weaned pigs [76].

Recent research suggests that the current NRC-recommended Trp levels may not be optimal for post-weaning performance [41,43,77]. In a meta-regression analysis, Chae et al. [43] deemed that 0.21 SID Trp:Lys was optimal for feed efficiency in pigs weighing 7–11 kg. In a meta-analysis, Simongiovanni et al. [78] deemed that 0.22 SID Trp:Lys was optimal for daily gain and ADFI and 0.20 for feed efficiency, although model choice can have an impact. However, it is difficult to determine optimal inclusion rates as Trp requirements can differ depending on the environmental pathogen load and the degree of inflammation within the GIT [79,80]. The NRC-recommended inclusion levels for pigs weighing 5–7 kg and 7–11 kg are based on estimated daily intakes of 280 g/day and 493 g/day and equate to Trp intakes of 0.7 g/day and 1.2 g/day, respectively [39]. Average intakes can vary greatly from study to study, particularly during the immediate post-weaning phase. This is often overlooked in the literature, and therefore, it is beneficial to calculate and present the total Trp intake per day. The present study analyzed the effects of three different Trp inclusion levels (0.22 vs. 0.27 vs. 0.33% SID Trp, 1.3% SID lysine, correlating to SID Trp:Lys of 0.17, 0.21, and 0.25) in pre- and post-weaning diets on performance and stomach parameters at weaning and at 7 days post-weaning.

In the current study, increasing pre- and post-weaning Trp inclusion had no effect on growth performance pre-weaning or in the initial 7 days post-weaning. The average initial bodyweight for the post-weaning phase was 7.8 kg, and ADFI from day 0 to 7 post-weaning was 0.27 kg across all dietary groups. These intakes are comparable to other studies for the first week post-weaning [40,81]. There was no statistical difference in Trp intake per day in the post-weaning period between the dietary groups; however, Trp intake per day did increase numerically with increasing Trp dietary inclusion rates (0.22% Trp = 0.63 g/day, 0.27% Trp = 0.72 g/day, 0.33% Trp = 0.77 g/day). In the study by

Capozzalo et al. [40], there was no impact of increased Trp on performance from day 1 to 8 post-weaning; however, from day 8 to 15, increased Trp improved daily gain, ADFI, and FCR, with a level of 0.23 SID Trp:Lys, equating to around 0.31% SID Trp inclusion, optimizing both daily gain and FCR during the first two weeks post-weaning. Thus, further investigation into the effects of elevated post-weaning Trp beyond the initial week post-weaning is warranted.

There were no noteworthy effects of Trp inclusion level on the gene expression in the fundic or pyloric gland region or on the number of parietal cells in the fundic gland region, nor were there noteworthy maternal × creep diet interactions. There were extremely limited intakes of creep pre-weaning which may have hindered potential effects on the stomach at weaning, although there were reasonable intakes post-weaning and still no effect on the gene expression in the fundic or pyloric gland region or on parietal cell count. This is surprising as, in previous studies, Trp has stimulated the CaSR [30,82] and triggered gastrin and somatostatin secretion and elevated H^+/K^+-ATPase activity in *ex vivo* pig stomach tissue samples [30]. The lack of effects post-weaning may be due to several potential reasons, such as the inclusion levels utilized being too similar to observe differential expression between groups, or the complexity of the *in vivo* stomach environment, including large fluctuations in the types and levels of nutrients entering, may be a confounding factor.

Of note in the correlation analysis in the current study was the positive correlation between creep intake pre-weaning and the expression of acid secretion genes, digestive enzyme genes, and ghrelin in the fundic gland region at weaning. This aligns with an early study by Cranwell et al. [19], in which pre-weaning creep feeding increased acid and pepsin secretory capacity. Furthermore, at weaning time, there was a negative correlation between immune-related genes, particularly *CXCL8*, *IL22*, and *TLR4*, and the expression of fundic-gland-region-associated genes, such as acid- and enzyme-secretion-related genes. Similar findings were reported previously in post-weaned pigs [18]; however, in the current study, the correlations were only evident at weaning and not 7 days post-weaning. Surprisingly, the gene expression associated with parietal cell activity was not correlated to the number of parietal cells ($ATP4B^+$ cells) at weaning or at 7 days post-weaning. This may be because parietal cells can be present in the stomach in early life but are functionally immature. Trevisi et al. [1] noted that young pigs often present morphological signs indicating the immaturity of the fundic gland region, with one sign being an atypical form of the H^+/K^+-ATPase-immunoreactive cells.

Focusing on *CASR* expression correlations, *CASR* expression at weaning was positively correlated with stomach pH and fundic gland region acid secretion inhibition (*SST* and *SSTR2*) and *GHRL* expression. Furthermore, at weaning and 7 days post-weaning (see Appendix A: Figures A1 and A2), *CASR* was positively correlated with both *GAST* and *SST* gene expression in the pyloric gland region. This somewhat supports the role of CaSR in regulating acid secretion in response to luminal pH [28] and that somatostatin secretion is linked to the CaSR [30]. However, surprisingly, *CASR* expression was negatively correlated to acid stimulation (*HRH2*, *CCKBR*, *CHRM3*, *HDC*) and acid secretion (*ATP4A*, *CLIC6*, *KCNE1*, *KCNQ1*) gene expression at weaning. At 7 days post-weaning, the expression of *CASR* in the fundic gland region was, like at weaning, correlated to *GHRL* expression, but not with any acid-secretion-related genes. There was a positive correlation between sow parity and *CASR* in the fundic gland region post-weaning. Sow parity, particularly gilts compared to sows, is often cited as a factor affecting the development and performance of their offspring [83,84], so the current study included parity in the correlation analysis. However, sow parity was not correlated to any pre- or post-weaning performance parameters, while pre-weaning, sow parity only correlated with mucus production gene expression in the pyloric gland region.

5. Conclusions

Enhancing the development of the pig's stomach in early life is a crucial area of exploration, as it can reduce the pig's susceptibility to infection and improve digestive function, which is particularly important during the immediate post-weaning phase. The present work provides an insight into the changes in the fundic and the pyloric gland region of pigs at weaning compared to 7 days post-weaning. These changes were predominantly characterized by increased parietal cell number, increased expression of genes involved in parietal cell activity, and a change in the expression of digestive enzyme genes in the fundic gland region. However, the expression of signaling molecules involved in the regulation of acid secretion was unchanged in the fundic gland region and reduced in the pyloric gland region compared to the day of weaning. When analyzing the effect of diet, the level of Trp in the piglet diet had minimal effects on performance or the stomach, although the low pre-weaning creep intake may have limited any potential impact pre-weaning. Overall, maternal *Bacillus subtilis* and *Bacillus amyloliquefaciens* supplementation from day 83 of gestation had a significant impact on gene expression in the fundic gland region of the offspring by elevating the expression of several genes related to parietal cell activity and the acid secretion pathway. When genes were grouped by function, piglets from probiotic sows had upregulated expression of acid secretion and mucus production genes. These gene expression changes suggest that maternal probiotic supplementation accelerated the maturation of the offspring's stomach. However, maternal probiotic supplementation did not influence growth performance pre-weaning or during the 7-day post-weaning period. Further research is needed to better understand the mechanisms through which maternal probiotic supplementation affects the development and function of the offspring's stomach as well as the long-term implications on animal performance.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University College Dublin (AREC-2022-ODoherty, AREC-2202-ODoherty).

Data Availability Statement: All data presented and/or analyzed in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Effect of diet on the gene expression in the fundic and pyloric gland regions in the pig at weaning (least square means with their standard errors).

Maternal Diet		**Control**			**Probiotic**			**SEM**	***p*-Value**		
Creep Diet		**0.22% Trp**	**0.27% Trp**	**0.33% Trp**	**0.22% Trp**	**0.27% Trp**	**0.33% Trp**		**M**	**C**	**M × C**
Function	**Gene**										
The fundic gland region											
Acid secretion	*ATP4A*	0.80	0.68	0.75	1.10	1.31	1.28	0.17	**0.0013**	0.9186	0.6150
	CLIC6	1.03	0.50	0.90	1.87	2.28	1.93	0.51	**0.0067**	0.9931	0.6392
	HRH2	0.77	0.64	0.65	0.98	1.18	1.23	0.16	**0.0013**	0.9197	0.4533
	KCNE1	0.72	0.65	0.771	0.79	1.19	1.09	0.20	**0.0490**	0.6683	0.5093
	KCNQ1	0.63	0.59	0.64	0.95	1.27	1.14	0.17	**0.0007**	0.6822	0.5798
Aryl hydrocarbon receptor	*AHR*	1.09	1.12	1.14	1.16	1.26	1.25	0.08	0.1205	0.5894	0.9169
Calcium-sensing receptor	*CASR*	1.41	2.26	1.83	1.61	1.47	1.54	0.44	0.4297	0.7339	0.5490
Cholinergic receptor muscarinic 3	*CHRM3*	0.75	0.65	0.78	0.97	0.92	1.18	0.08	**<0.0001**	0.0681	0.5477
Digestive enzyme	*CHIA*	0.65	0.44	0.61	0.83	0.91	1.07	0.16	**0.0072**	0.5754	0.5841
	LCT	2.13	0.90	1.11	1.96	1.70	3.40	0.80	0.1465	0.4796	0.3081
	PGA5	0.73	0.71	0.65	0.89	0.90	1.08	0.11	**0.0074**	0.8385	0.3994
Gastrin receptor	*CCKBR*	0.66	0.56	0.50	1.24	1.24	1.31	0.22	**0.0006**	0.9735	0.8818
Ghrelin	*GHRL*	0.87	0.95	0.95	1.25	1.19	1.42	0.15	**0.0048**	0.6409	0.7508
Histamine production	*HDC*	0.82	0.91	0.88	1.69	1.38	1.33	0.18	**0.0002**	0.6851	0.4167
Inflammation	*CXCL8*	1.89	1.87	1.88	1.49	1.99	1.26	0.50	0.4733	0.7743	0.7544
	IL22	2.32	1.39	1.88	1.66	1.60	1.04	0.76	0.4869	0.7304	0.7615
	TNF	1.19	1.19	1.41	1.06	1.34	1.08	0.16	0.4430	0.6532	0.3498
Mucus production	*MUC1*	0.98	1.17	0.98	1.00	1.07	1.04	0.12	0.9750	0.5546	0.8048
	MUC5AC	1.33	1.49	1.22	1.09	1.22	0.97	0.19	0.1023	0.4074	0.9966
	MUC6	0.86	0.83	1.21	1.74	1.45	1.58	0.32	**0.0224**	0.7360	0.7291
Somatostatin	*SST*	0.93	1.10	1.08	1.19	1.09	1.21	0.18	0.3987	0.8874	0.7628
Somatostatin receptor	*SSTR2*	0.63	0.69	0.65	1.10	1.28	1.13	0.14	**<0.0001**	0.6714	0.8953
Toll-like receptor	*TLR4*	1.30	1.26	1.25	1.03	1.11	1.14	0.09	**0.0180**	0.9512	0.6416
The pyloric gland region											
Aryl hydrocarbon receptor	*AHR*	1.12	1.00	1.00	1.00	1.23	1.21	0.10	0.1947	0.7983	0.1305

Calcium-sensing receptor	*CASR*	1.65	1.52	1.38	1.53	1.56	1.73	0.23	0.6307	0.9747	0.5813
Gastrin	*GAST*	2.36	1.08	1.03	1.68	1.82	2.33	0.37	0.1268	0.2734	**0.0208**
Inflammation	*CXCL8*	1.85	1.19	1.21	1.35	1.69	1.40	0.31	0.7968	0.6422	0.2707
	TNF	1.13	0.88	1.15	0.87	1.25	1.38	0.13	0.2871	0.1191	0.0525
Mucosal defense	*PIGR*	0.69	0.97	0.94	0.53	1.30	1.46	0.17	0.1081	**0.0028**	0.1325
Mucus production	*MUC5AC*	0.99	0.96	0.82	0.82	0.93	1.00	0.10	0.9145	0.9175	0.2111
	MUC6	0.68	0.88	1.07	0.52	0.73	0.86	0.25	0.3882	0.3379	0.9918
Somatostatin	*SST*	1.35	1.24	1.38	1.25	1.32	1.35	0.18	0.8992	0.8916	0.9049
Toll-like receptor	*TLR4*	1.20	1.01	1.12	0.99	1.37	1.30	0.14	0.5289	0.8874	0.0750

Trp, tryptophan; M, maternal; C, creep. Significant *p*-values are highlighted in bold.

Table A2. Effect of diet on the gene expression in the fundic and pyloric gland regions in the pig at 7 days post-weaning (least square means with their standard errors).

Maternal Diet		**Control**			**Probiotic**			**SEM**	***p*-value**		
Creep Diet		**0.22% Trp**	**0.27% Trp**	**0.33% Trp**	**0.22% Trp**	**0.27% Trp**	**0.33% Trp**		**Maternal**	**Creep**	**M × C**
Function	**Gene**										
		The fundic gland region									
Acid secretion	*ATP4A*	1.38	1.25	1.39	1.31	1.58	1.34	0.15	0.5832	0.8838	0.3123
	CLIC6	1.66	1.79	2.07	1.48	2.44	2.08	0.47	0.6761	0.4611	0.6647
	HRH2	1.41	1.32	1.20	1.34	1.75	1.42	0.16	0.1581	0.3708	0.3121
	KCNE1	1.33	1.40	1.38	1.52	1.44	2.05	0.22	0.1027	0.3120	0.3248
	KCNQ1	1.34	1.37	1.52	1.51	1.84	1.57	0.18	0.1192	0.6020	0.4827
Aryl hydrocarbon receptor	*AHR*	0.82	0.99	0.82	0.87	0.99	0.93	0.09	0.4650	0.2263	0.8397
Calcium-sensing receptor	*CASR*	0.72	0.79	0.71	0.92	0.89	0.83	0.11	0.1274	0.8066	0.8908
Cholinergic receptor muscarinic 3	*CHRM3*	1.26	1.24	1.14	1.15	1.33	1.40	0.13	0.4631	0.8183	0.3937
Digestive enzyme	*CHIA*	1.71	1.96	2.01	1.62	1.95	1.95	0.21	0.7489	0.2864	0.9854
	LCT	1.07	0.70	0.80	0.65	1.00	1.13	0.20	0.6897	0.8174	0.1299
	PGA5	1.69	1.52	1.53	1.56	1.75	1.64	0.22	0.7088	0.9653	0.7087
Gastrin receptor	*CCKBR*	1.70	1.54	1.61	1.79	1.99	1.96	0.23	0.1237	0.9846	0.7437
Ghrelin	*GHRL*	1.10	1.05	1.05	1.14	1.06	0.94	0.14	0.8550	0.6633	0.8368
Histamine production	*HDC*	1.15	0.93	1.13	1.05	1.37	0.79	0.14	0.9853	0.4106	**0.0281**
Inflammation	*CXCL8*	0.50	1.27	0.85	0.99	0.89	1.04	0.22	0.6058	0.3466	0.1595
	IL22	0.77	1.22	1.17	0.92	1.01	2.10	0.41	0.3937	0.1634	0.3635
	TNF	0.74	1.03	0.74	1.11	1.02	0.91	0.10	**0.0295**	0.1206	0.1722

Mucus production	*MUC1*	1.02	1.04	0.91	1.06	1.22	1.21	0.10	**0.0474**	0.6783	0.4697
	MUC5AC	1.05	1.02	1.04	1.39	1.48	1.36	0.11	**0.0003**	0.9165	0.7843
	MUC6	1.02	1.71	1.63	2.31	1.81	1.43	0.32	0.1486	0.7673	0.0726
Somatostatin	*SST*	0.97	0.99	1.09	1.22	1.05	1.07	0.14	0.3790	0.8719	0.6121
Somatostatin receptor	*SSTR2*	1.27	1.14	1.32	1.22	1.66	1.61	0.22	0.1718	0.6134	0.4460
Toll-like receptor	*TLR4*	0.83	0.91	0.77	0.92	1.05	0.96	0.09	0.0831	0.4052	0.8786
The pyloric gland region											
Aryl hydrocarbon receptor	*AHR*	1.07	0.94	1.07	0.96	0.88	0.90	0.08	0.0945	0.3642	0.8229
Calcium-sensing receptor	*CASR*	1.07	0.82	0.85	0.76	0.65	0.80	0.11	0.0523	0.2523	0.4802
Gastrin	*GAST*	1.22	0.70	0.62	0.56	0.66	1.27	0.17	0.9064	0.2748	**0.0022**
Inflammation	*CXCL8*	1.14	1.69	0.92	1.36	1.18	1.03	0.46	0.8770	0.6094	0.6968
	TNF	1.09	1.24	1.00	0.99	1.08	0.94	0.19	0.4803	0.6047	0.9691
Mucosal defense	*PIGR*	1.07	1.26	1.27	1.34	1.98	1.81	0.31	0.0569	0.4034	0.7871
Mucus production	*MUC5AC*	1.27	1.24	1.13	1.27	1.30	1.10	0.15	0.6673	0.5603	0.7463
	MUC6	2.32	5.47	2.88	2.34	2.55	2.88	0.84	0.1682	0.1418	0.1421
Somatostatin	*SST*	0.94	0.89	0.88	0.79	0.73	0.85	0.10	0.1667	0.8246	0.7735
Toll-like receptor	*TLR4*	1.08	1.00	1.15	0.95	0.91	1.11	0.16	0.5487	0.5569	0.9621

Trp, tryptophan; M, maternal; C, creep. Significant *p*-values are highlighted in bold.

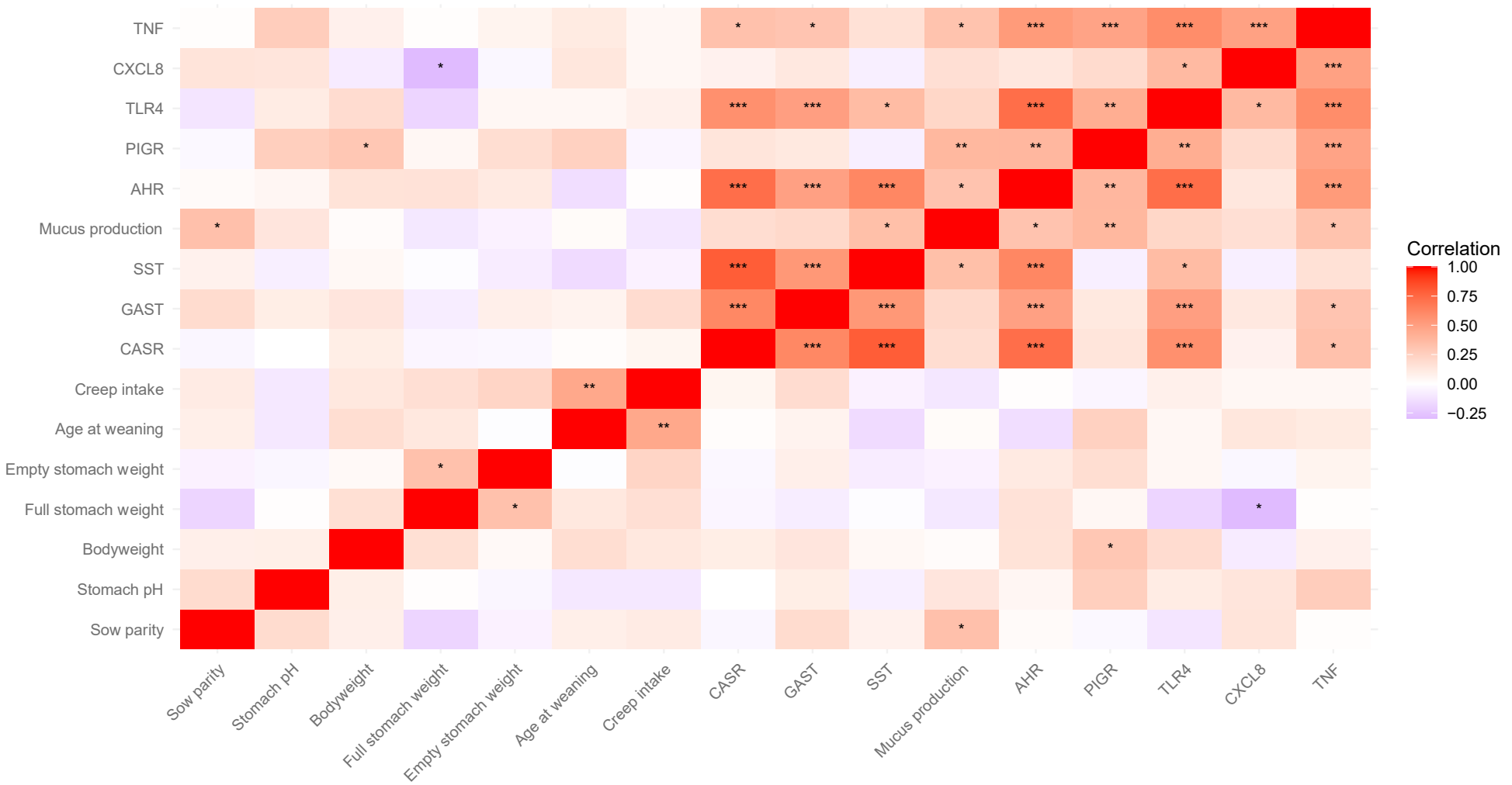

Figure A1. Correlation matrix illustrating Pearson correlations of pyloric gland region gene expression and physiological parameters at weaning. Positive (red) and negative (blue) correlations are represented in color strength on a scale of −1 to 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

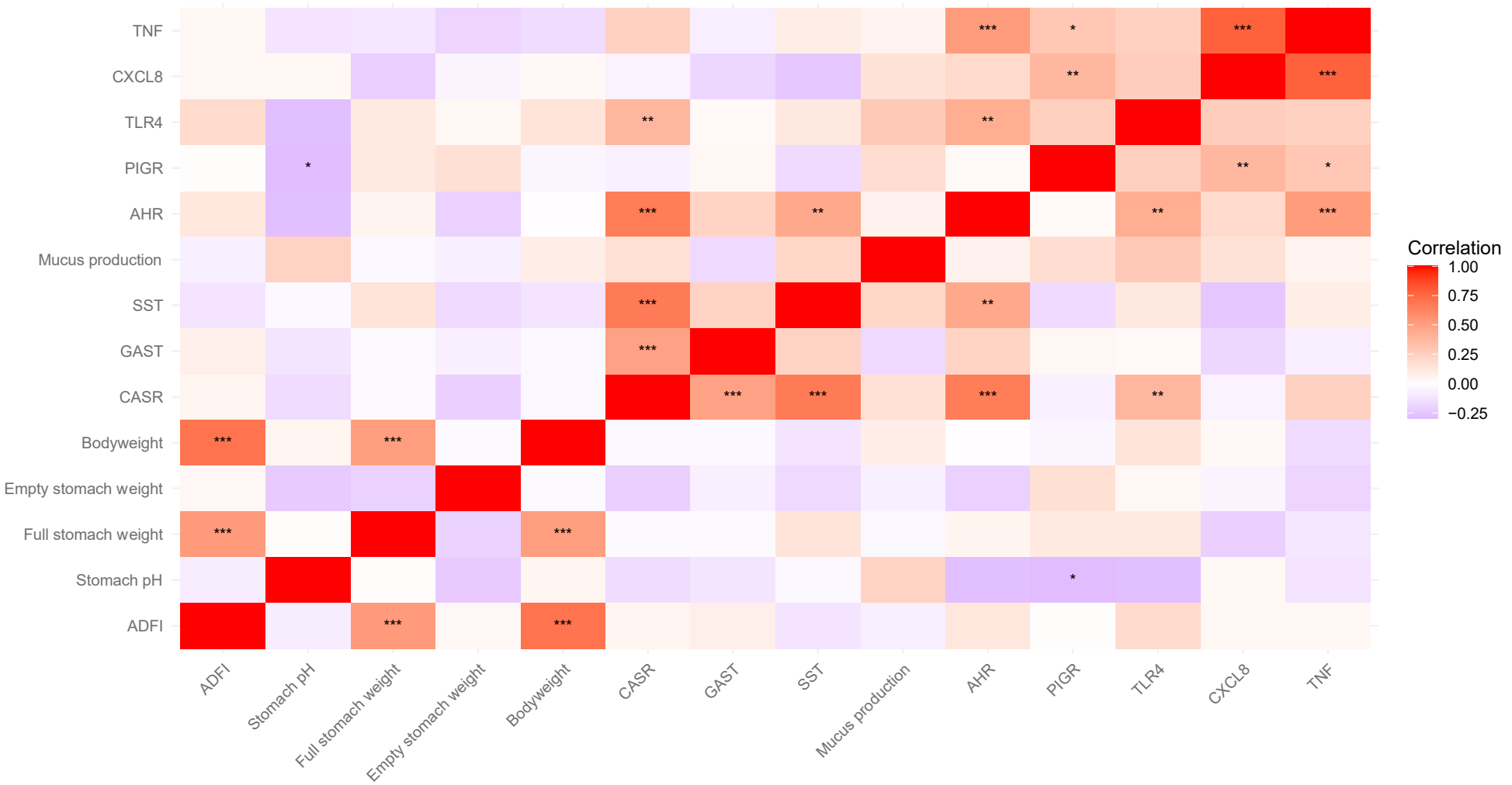

Figure A2. Correlation matrix illustrating Pearson correlations of pyloric gland region gene expression and physiological parameters post-weaning. Positive (red) and negative (blue) correlations are represented in color strength on a scale of −1 to 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ADFI, average daily feed intake.

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