THE EFFECT OF SUPPLEMENTATION WITH β -HYDROXY- β -METHYLBUTYRIC ACID (HMB) TO PREGNANT SOWS ON THE MUCOSAL STRUCTURE, IMMUNOLOCALIZATION OF INTESTINAL BARRIER PROTEINS, VIP AND LEPTIN IN THE LARGE INTESTINE IN THEIR OFFSPRING*

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Abstract

The large intestine epithelium plays an important role in water absorption and participates in fluid, acid-base and electrolyte balance, and the removal of waste products. The large intestine is rich in microorganism-presented enzyme activity. Apart from energy supply, the colon also participates in the synthesis of trophic factors and the modulation of the immune system and the systemic inflammatory response. The current study investigated the effects of dietary HMB administration to pregnant sows on the postnatal development of the colon in their offspring, at weaning. From the 70th to the 90th day of gestation, sows received either a basal diet (n = 12) or the basal diet supplemented with HMB (n = 12) at a dose of 0.2 g/kg of body weight/day. Maternal HMB treatment increased serum IgG and glucose concentrations and decreased serum urea concentration in the piglets. Basal histomorphometric analysis of offspring large intestines showed that prenatal HMB treatment led to a reduction in the thickness of the mucosa, submucosa and both types of myenterons, as well as reduced crypt thickness. The immunoreaction performed to mark T0 lymphocytes and total T lymphocytes in the colon wall showed that prenatal HMB treatment decreased the number of both types of lymphocytes. Greater expression for cadherin was found in the colon of piglets delivered by the HMB-treated sows. The expression of both tight junction proteins (occludin and claudin-3), as well as that of leptin, was stronger in the HMB-treated group. Vasoactive intestinal peptide (VIP) expression was stronger in the submucosal plexuses in the HMB maternal treated piglets, while no changes were observed in the myenteric plexuses. The results obtained indicate that the administration of HMB to pregnant sows significantly influenced the expression of leptin, VIP and some proteins of the intestinal barrier in their offspring, with less influence on large intestine basal morphology.

Key words: offspring, pigs, colon, pregnancy, weaning, HMB

The chyme that passes through the small intestine into the large intestine is initially quite fluid. Consequently, the large intestine epithelium plays an important role in water absorption and participates in fluid, acid-base and electrolyte balance, and the removal of waste products. The large intestine is also the site for the synthesis and absorption of the B-vitamins. Even though enzymatic digestion of nutrients does not take place in the large intestine, the large intestine is rich in microorganism-derived enzyme activity, which leads to the formation of VFAs (volatile fatty acids), which in turn possess neuroactive properties (Pascale et al., 2018). Acetate, propionate, and butyrate are the main metabolites produced by the colon through the bacterial fermentation of dietary fibres and

resistant starch. These are readily absorbed in the large intestine, especially in the colon, and provide a sufficient amount of energy to assist with the nutrient requirements of the intestinal epithelium. Apart from energy supply, the colon also participates in the synthesis of trophic factors and the modulation of the immune system and the systemic inflammatory response (Fung et al., 2017; Liu, 2015; Ostaszewski et al., 1998). At least 16 different etiologic agents, including bacteria, viruses, and parasites, can cause primary intestinal disease within the colon. Intestinal inflammation can be accompanied by the loss of plasma proteins or RBCs, resulting in hemorrhagic diarrhea. Colon hypersecretion observed in enterotoxic colibacillosis, porcine proliferative enteritis or *Clostrid-*

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ium difficile enteritis leads to intestinal loss of water and electrolytes, resulting in osmotic diarrhoea, followed by the accumulation of solutes in the gut lumen, finally concluding in malabsorption (Novotný et al., 2016).

Weaning is a particularly arduous period in swine breeding, where stress can have significant implications on nutrient assimilation and utilization, as a result of intestinal inflammation. Major physiological changes which occur upon weaning, involving alterations in mucin secretion and the expression of tight junction proteins, lead to an impaired intestinal morphology and barrier function. For this reason, it is important to develop strategies that improve offspring colon integrity and function, preventing water and electrolyte imbalances and impairment during inflammatory conditions.

Evidence suggests that one such strategy could include the prenatal programming of intestinal morphology and function by the supplementation of pregnant sows with dietary factors (Dobrowolski et al., 2021). Many studies present the beneficial effect of amino acids, their precursors or metabolites on body weight gain and the gastrointestinal tract or skeletal system (Tomaszewska et al., 2018, 2019; Mou et al., 2019; Duan et al., 2021). β -hydroxy- β -methylbutyrate (HMB), a metabolite of the essential branched-chain amino acid L-leucine, has been demonstrated to have benefits with regards to animal performance, as well as antimicrobial activity in monogastric animals including pigs. It has been proven that HMB has anticatabolic and anabolic activity. Furthermore, HMB increases the concentration of growth hormone, insulin-like growth hormone and the activity of bone alkaline phosphatase, leading to an increase in bone mass (Tatara et al., 2007; Blicharski et al., 2017; Tomaszewska et al., 2019). It is presumed that HMB administration increases the activation of the GH-IGF-1 axis, modulating IGF-1 expression in the muscle and thus leading to enhancement of the mTOR pathway activity (Baptista et al., 2013). This in turn may influence the general growth and acceleration of the development of many systems and organs. Thus, it can be assumed that the administration of HMB, considered to be an ergogenic nutraceutical, to pregnant sows could exert a beneficial effect on colon development in their offspring (Partanen et al., 2001; Dibner and Buttin, 2002; Cheng et al., 2020).

To verify the above hypothesis, the current study was carried out to investigate the effects of dietary HMB administration to pregnant sows on the postnatal development of the gastrointestinal tract in their offspring at weaning, with particular emphasis on the colon. In doing so, the current study examined (i) the integrity of the intestinal barrier and basal morphology of the colon, and (ii) the effects of HMB supplementation. In addition, changes in immunolocalization of vasoactive intestinal polypeptide (VIP), leptin and tight junction proteins (occludin and claudin-3) were studied after HMB supplementation. Basal blood morphology and biochemical serum analyses were also performed. Together these results should provide additional fundamental information

concerning the outcomes of HMB supplementation to pregnant sows, with regards to the development of the large intestine of their offspring.

Material and methods

Ethical approval

All experimental procedures were approved by The Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland and complied with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Throughout the experimental period, the health status of the pregnant sows and piglets was regularly monitored by a veterinarian.

Animals, breeding and experimental design

The study was performed on 24 piglets (12 males and 12 females) born from 12 clinically healthy sows of the Large White Polish breed. To avoid the effect of the number of pregnancies on the birth weight of the offspring, only primiparous sows, ca. 10 months old, were used. They were singly housed in separate cages, under standard rearing conditions (controlled temperature, humidity and 12:12-h light-dark cycle) and were sired during the third oestrus by the same boar.

To investigate the effects of prenatal treatment with HMB on the postnatal development, morphology and function of the colon, in weaned piglets, HMB (Lonza, Basel, Switzerland) was administered at a dose of 0.2 g/kg of body weight/day to $n = 6$ randomly selected sows, together with their morning meal, from the 70th to 90th day of gestation; the remaining $n = 6$ sows were fed a basal diet only. The HMB dose and the supplementation period was selected based on previous studies (Świetlicka et al., 2016; Tomaszewska et al., 2017). A two-phase feeding system for pregnant sows was introduced and the composition and amounts of feed were controlled during each phase of gestation. During late gestation a balanced standard commercial feed for pregnant and lactating sows was used in an amount of 3.0 kg of feed mixture per sow, water was given *ad libitum* (Grela and Skomial, 2015). The feed mixture used for sows during pregnancy and lactation contained in 1 kg: 13.2 MJ ME, 162 g of crude protein, 9.5 g of lysine and the scheme of the main essential amino acids proportion was lysine 100%: methionine+cysteine 60%: threonine 65%: tryptophan 22%.

Gestation length was not different between the control and HMB-treated sows. All piglets born by physiological partum had no congenital defects. There were no stillbirths. Females and males used in the study were treated in the same manner. Piglets born from sows fed the control diet belonged to the CONT group and piglets born from HMB-treated sows belonged to the HMB group. All offspring were housed with their mothers without translocation between sows and fed naturally with colostrum

and then with sow's milk. At the age of 35 days, $n = 12$ randomly chosen piglets in each group (one male and one female from each sow) were euthanized by intravenous injections of lethal doses of pentobarbitalum natricum (Morbital, Biowet, Puławy, Poland).

Basal blood morphology

The haematological analysis of blood samples (white blood cell number, WBC; red blood cell number, RBC; haematocrit value, PCV; haemoglobin concentration, Hb) was performed using the Advia 2120 (Siemens Healthcare, Erlangen, Germany) automatic haematology analyser.

Blood serum biochemical analyses

The blood serum, immediately separated by centrifugation, was stored at -80°C for further analysis. The serum concentrations of selected hormones were determined using commercial porcine-specific enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Wuhan, China): leptin (E-EL-P0063), insulin-like growth factor I (IGF-I, E-EL-P0853), interleukin 6 (IL-6, E-EL-P0020), immunoglobulin G (IgG, E-EL-P0004) and immunoglobulin M (IgM, E-EL-P2269), using a Benchmark Plus (Bio-Rad Laboratories, Hercules, CA, USA) microplate spectrophotometer. Serum total cholesterol, triglycerides, low-density lipoproteins (LDL), high-density lipoproteins (HDL), total bilirubin, urea, creatinine, uric acid, total protein, glucose, albumin, alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP), Mg, P, K, Cl, and Na were assessed using a Mindray BS-120 (Bio-Medical Electronics, Shenzhen, China) biochemistry analyser.

Tissue collection and histomorphometry analysis

Ten mm long segments of the large intestine from the colon (50% of the total intestinal length of the colon) were collected from each piglet. Tissues were fixed in 4% buffered formaldehyde (pH 7.0) for 24 h, dehydrated in a graded series of ethanol, cleared with a nonpolar solvent, embedded in paraffin and cut into $4\text{ }\mu\text{m}$ thick cross-sections, using a microtome (Microm HM 360, Wall-dorf, Germany). Sections were stained with Masson's trichrome or PicroSirius red (PSR). Stained slides were observed in brightfield (Masson's trichrome) and polarized light (PSR) using a CX43 (Olympus, Tokyo, Japan) microscope. Microscopic observations from Masson's trichrome stained sections were used for the identification and assessment of the structure and morphology of colon samples. The thickness of the mucosa, submucosa and both myenterons, as well as the total, active and inactive crypts and crypt depth and width were determined. PSR staining was used to differentiate collagen fibres in examined tissue samples, where type I (thicker, mature) collagen fibres appear orange or red, and type III (thinner, immature) fibres appear green in polarized light (Suvanna et al., 2013).

Immunohistochemistry

Immunohistochemical staining was performed according to the protocols provided by the producer of the antibodies (Abcam, Cambridge, UK). The following porcine-specific primary antibodies were used: rabbit polyclonal anti-claudin-3 (ab15102), rabbit polyclonal anti-E-cadherin (ab15148), rabbit polyclonal anti-occludin (ab222691), rabbit polyclonal anti-leptin (ab16227, Abcam, Cambridge, UK, dilution 1:200), rabbit monoclonal anti-CD3 (ab16669), rabbit polyclonal anti-VIP (ab22736), rabbit polyclonal anti-Ki67 antibody (ab15580), mouse monoclonal anti-neurofilament heavy polypeptide antibody (ab187374), rat monoclonal anti-null T cells SWC6 (T0 cells) antibody (ab23726), mouse monoclonal anti-active caspase-3 (ab208161).

In neurofilament immunostained sections the intestinal nervous plexuses (myenteric and submucosal) were analysed, as well as the cross-sectional area, perimeter and circularity (Tomaszewska et al., 2012). For Ki-67, caspase-3, CD3, T0 immunostained sections, the proliferating cell index and apoptotic cells index and the number of CD3 and T0 lymphocytes per mm^2 , respectively, were determined. The measurements were made on at least ten different areas of each section, and the mean value of calculated parameters for each piglet was subjected to statistical analysis.

The intensity of immunoexpression was measured by determining the number or percentage of cells with a positive response (Ki67, CD3, T0 cells, caspase 3) and by the quantitative comparison of mean pixel intensity in the photomicrographs (VIP, claudin-3, E-cadherin, occludin, leptin), which were first converted into negatives and then into grey-scale 8-bit digital images, with a scale from 0 (white pixel) to 255 (black pixel), where the higher the pixel value, the higher the intensity of the immunohistochemical reaction. The intensity of the immunoreaction in each of the analysed digital images was measured in 10 randomly selected areas of the positive signal (Puzio et al., 2021).

All collected microscopic images of stained and immunostained sections were examined blindly by an associate who was not aware of the treatment using graphical analysis software: Olympus cellSens (Olympus, Tokyo, Japan) and ImageJ (Schneider et al., 2012).

Statistical analysis

Data are presented as means $\pm$ standard deviation. In all analyses, a single pig was considered as an experimental unit. A Shapiro-Wilk normality test was applied to test the normality of the data; the homogeneity of variances was assessed using Levene's test. Normally distributed variables were analysed using a two-tailed Student's t-test or t-test with Welch's correction, when normally distributed data lacked equal variances; non-parametric data were analysed using a Mann-Whitney U test. For all tests, a P-value <0.05 was established as statistically significant. All statistical procedures were performed using Statistica 13.0 (Tibco, Palo Alto, CA, USA) software.

Results

Maternal HMB treatment increased serum concentrations of IgG (Table 1) and glucose and decreased serum urea concentrations in piglets at the age of 35 days (Table 2). No other changes in basal blood morphology (Table 3) or serum biochemical parameters were observed.

Basal histomorphometric analysis showed that prenatal HMB treatment led to a reduction in the thickness of the mucosa, submucosa and both types of myenterons (Figure 1 A). The thickness of the colon's crypts was reduced and the number of both types of lymphocytes was decreased in the HMB group (Table 4). There was no influence of HMB on the morphology of the intestinal plexuses (Table 5). The content of immature collagen was higher in the HMB group than in the CONT group (Table 4, Figure 1 B).

Greater cadherin expression was observed in the colon of piglets delivered by the HMB-treated sows. The E-cadherin expression was similar and continuous in both groups and located on the basal layer of the epithelium, with stronger expression on the top of epithelium and weaker expression on the bottom (Figure 2). The expression of both tight junction proteins (occludin and claudin-3) and that of leptin was stronger in the HMB group (Figure 2). Analysis of the VIP immunoreaction showed that VIP expression was stronger in the submucosal plexuses of the HMB maternal treated piglets, while no changes in VIP expression were observed in the myenteric plexuses (Figure 3).

The immunoreaction performed to mark T0 lymphocytes and total T lymphocytes in the colon's wall showed that HMB given to pregnant sows in the middle of their pregnancy decreased the number of both types of lymphocytes (Table 4).

Table 1. The concentrations of leptin, insulin like growth factor 1 and interleukin 6, immunoglobulins G and M in 35-old-day piglets, following maternal HMB treatment

Item	CONT	HMB	Sign.
Leptin (ng/ml)	3.66±0.80	3.63±1.27	NS
IGF-1 (pg/ml)	539±124	526±98	NS
IL-6 (pg/ml)	205±21	219±63	NS
IgG (ng/ml)	313±29	471±68	***
IgM (ng/ml)	17.70±1.87	17.10±2.16	NS

IGF-1: insulin-like growth factor I; IL-6: interleukin 6; IgG: immunoglobulin G; IgM: immunoglobulin M. Data are presented as mean ± standard deviation (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); NS – not significant; ***P< 0.001.

Table 2. The effect of maternal HMB treatment on blood serum biochemistry of piglets at the age of 35 days

Item	CONT	HMB	Sign.
AST (U/L)	65.0±3.1	64.4±10.88	NS
ALT (U/L)	51.8±2.03	54.5±2.37	NS
ALP (U/L)	476±8	498±25	NS
Total protein (g/L)	58.76±0.11	60.92±0.45	NS
Albumin (g/dL)	33.24±11.22	39.15±6.11	NS
Glucose (mmol/L)	2.99±0.09	7.96±0.79	***
Urea (mmol/L)	6.01±0.15	4.68±0.48	***
Creatinine (μmol/L)	92.1±4.1	88.3±4.5	NS
Uric acid (mmol/L)	0.001±0.0001	0.003±0.001	NS
Total bilirubin (μmol/L)	5.25±0.03	4.53±0.39	NS
Total cholesterol (mmol/L)	3.21±0.92	4.11±1.11	NS
Triglycerides (mmol/L)	0.45±0.53	0.68±0.91	NS
HDL (mg/dL)	1.29±0.74	1.80±0.78	NS
LDL (mg/dL)	0.018±0.005	0.021±0.001	NS
Mg (mmol/L)	0.89±0.02	0.95±0.17	NS
P (mmol/L)	2.66±0.02	2.69±0.11	NS
K (mmol/L)	4.61±0.11	4.78±0.49	NS
Na (mmol/L)	139±5.3	148±7.3	NS
Cl (mmol/L)	108.1±0.6	115.8±2.7	NS

AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase; HDL: high-density lipoproteins; LDL: low-density lipoproteins. Data are presented as mean ± standard deviation (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); NS – not significant; ***P< 0.001.

Table 3. The effect of maternal HMB treatment on basal blood morphology of piglets at the age of 35 days

Item	CONT	HMB	Sign.
RBC ($10^{12}/L$)	6.24 $\pm$ 1.01	6.44 $\pm$ 0.68	NS
WBC ($10^9/L$)	9.75 $\pm$ 0.82	10.98 $\pm$ 1.52	NS
Hb (mmol/L)	9.12 $\pm$ 0.32	9.78 $\pm$ 0.49	NS
PCV (%)	32 $\pm$ 3.0	36 $\pm$ 2.6	NS

RBC: red blood cell number; WBC: white blood cell number; Hb: haemoglobin concentration; PCV: haematocrit value. Data are presented as mean $\pm$ standard deviation (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); NS – not significant.

Table 4. Basal histomorphometry of intestinal wall and crypts, and the total number of lymphocytes and T0 in 35-old-day piglets following maternal HMB treatment

Item	CONT	HMB	Sign.
Mucosa thickness (μm)	377 $\pm$ 71	298 $\pm$ 67	*
Submucosa thickness (μm)	109 $\pm$ 37	92 $\pm$ 30	NS
Thickness of the inner muscle layer (μm)	151.0 $\pm$ 43	92 $\pm$ 35	**
Thickness of the outer muscle layer (μm)	61.5 $\pm$ 20.1	35.3 $\pm$ 13.2	**
Crypts depth (μm)	175 $\pm$ 80	149 $\pm$ 60	NS
Crypts width (μm)	73.3 $\pm$ 14.2	57.5 $\pm$ 15.2	*
Total number of crypts (/mm)	17.4 $\pm$ 5.8	18.8 $\pm$ 6.5	NS
Number of open crypts (/mm)	4.14 $\pm$ 1.92	3.64 $\pm$ 3.04	NS
Number of closed crypts (/mm)	13.6 $\pm$ 4.9	15.2 $\pm$ 6.1	NS
Apoptotic cells index (%)	9.51 $\pm$ 1.57	8.44 $\pm$ 1.65	NS
Ki index (%)	63.1 $\pm$ 8.7	53.2 $\pm$ 11.2	NS
Immature collagen (%)	10.5 $\pm$ 2.3	3.9 $\pm$ 2.2	***
Number of T0 lymphocytes (/mm ²)	14.6 $\pm$ 4.5	6.31 $\pm$ 2.6	***
Number of CD3 lymphocytes (/mm ²)	133 $\pm$ 58	56 $\pm$ 36	***

Data are presented as mean $\pm$ standard deviation (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); NS – not significant; *P<0.05; **P<0.01; ***P<0.001.

Table 5. Basal morphological parameters of intestinal plexuses in 35-old-day piglets following maternal HMB treatment

Item	CONT	HMB	Sign.
Myenteric plexus			
The cross-section area (μm^2)	2992 $\pm$ 2734	2015 $\pm$ 1527	NS
Perimeter (μm)	330 $\pm$ 212	254 $\pm$ 165	NS
Circularity	0.11 $\pm$ 0.09	0.09 $\pm$ 0.09	NS
Submucosal plexus			
The cross-section area (μm^2)	2979 $\pm$ 2320	1643 $\pm$ 990	NS
Perimeter (μm)	292 $\pm$ 169	204 $\pm$ 99	NS
Circularity	0.15 $\pm$ 0.17	0.20 $\pm$ 0.17	NS

Data are presented as mean $\pm$ standard deviation (n = 12). Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); NS – not significant.

Figure 1. Representative photomicrographs of the Masson's trichrome (A) and PSR (B) stains of the colon from control (CONT) and prenatally HMB-exposed (HMB) piglets. Black scale bars (Masson) represent 200 μ m, while white (PSR) scale bars represent 100 μ m

Figure 2. Representative photomicrographs of the immunohistochemical reactions for E-cadherin, occludin, claudin-3, and leptin in the colon of control (CONT) and prenatally HMB-exposed (HMB) piglets. All the scale bars represent 100 μ m. Bar graphs show the intensity of expression of the analysed proteins in the colon, by comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale – the higher the pixel value, the higher the intensity of the immunohistochemical reaction. Graphs show mean $\pm$ standard deviation. Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); * $P < 0.01$; *** $P < 0.001$

Figure 3. Representative photomicrographs of the immunohistochemical reactions for vasoactive intestinal (poly)peptide (VIP) in the submucosal and myenteric plexuses (arrows) in the colon of control (CONT) and prenatally HMB-exposed (HMB) piglets. All the scale bars represent 50 μ m. Bar graphs show the intensity of expression of VIP in plexuses measured by the comparison of the pixel brightness value in the microscopic images converted to an 8-bit grayscale – the higher the pixel value, the higher the intensity of the immunohistochemical reaction. Graphs show mean $\pm$ standard deviation. Significance was established using a two-tailed Student's t-test (normally distributed data), Welch's test (normally distributed data with unequal variances) or Mann-Whitney test (for pairwise comparisons with at least one non-normally distributed dataset); NS – not significant; *P<0.01

Discussion

The main role of the colon is to collect and excrete faeces. Moreover, the colon participates in the absorption of water and electrolytes, which starts in the small intestine. Water and electrolyte balance of an organism is controlled by the endocrine system. Thus, it may be assumed that the colon participates in the regulation and maintenance of overall organism homeostasis. In the colon, the epithelium covering the mucosa and crypts excretes mucus and intestinal juice, rich in potassium, sodium and carbohydrate ions. Mucus is excreted in response to the chyme reaching the colon. This process is not under neural or hormonal control. However, the visceral nerves should not be overlooked, since they may assist with the process, through the additional secretion of mucus (Barrett et al., 2019). The secretion of colonic juice is stimulated by bile, mechanical factors and parasympathetic innervation. Apart from its external innervation, the large intestine also possesses its own internal innervation (myenteric and submucosal plexuses), similar to other parts of the intestinal tract.

Our study showed that maternal HMB treatment did not influence the morphological parameters of the nerve plexuses belonging to the enteric nervous system, however the HMB treatment did influence the colonic epithelium and enhanced the maturation of collagen. Our results are difficult to explain and compare with other studies, due to the fact that there is a lack of similar stud-

ies presented in the literature. There are very few studies related to the morphology or function of the colon in maternal HMB treated offspring. One such available study shows that HMB supplementation attenuates intestinal injury, improves intestinal mucosal energy status and affects gut microbiota composition in LPS-challenged piglets (Zheng et al., 2020). Other available data is related to the skeletal system, the composition of sows milk or the development of muscle fibres (Wan et al., 2017; Tomaszewska et al., 2019). HMB is known to exert its effects via the mTOR signalling pathway or by influencing the activity of the GH/IGF-1 axis. The pathway by which HMB could influence, for example, smooth muscle development in the intestinal tract (Cieřlak and Nieradko-Iwanicka, 2018) is yet to be determined.

Our study showed that the reduced colonic mucosa thickness is linked with a decrease in the intestinal myenteron. Intestinal smooth muscle layers are responsible for intestinal contractions. Bowel movements include segmental and peristaltic contractions, which are similar to those observed in the small intestine. They not only help in the mixture of the chyme, but also in its further transfer through the large intestine, and they are vital for absorption. In the colon, apart from the two above-mentioned bowel type movements, there is a third type called mass movements, which include smooth muscle contractions covering a large area of the intestine which allows the chyme to mix. Colonic movement allows for an increase in local pressure and chyme distribution to

a larger area, compared to the muscle movements in the small intestine, which improves colonic absorption, depending on other local or general hormonal factors. The relationship between the decrease in colonic myenteron thickness and colonic function was not investigated in the current study. It may be surmised that there could have been difficulties observed with regard to colonic movement, but (1) it was not investigated and (2) it has been shown that maternal HMB treatment has a beneficial effect on general and skeletal development of newborns and weaned piglets through its anabolic action and (3) it has been proven that the gut-bone axis exists, thus bone development is dependent on gut function (Tomaszewska et al., 2018, 2020; Muszyński et al., 2020). Moreover, diarrhoea or other intestinal disorders were not observed in the piglets from birth until weaning. Apart from the narrower myenteron, changes in the intensity of the VIP immune reaction in the enteric nervous system were also observed. The enteric nervous system controls secretion, barrier function, and the movement of fluid across the epithelial lining. It is also a key regulator of local blood flow, interaction with the immune and endocrine systems of the gut and intestinal motility (Abot et al., 2018). VIP decreases the frequency of action potentials and lowers the contractile activity of the intestines, without influencing intestinal basic rhythm (Konturek, 1985). Our study showed that maternal HMB treatment did not influence VIP expression by the myenteric plexuses, however VIP expression in the submucosal plexuses was increased.

VIP is also responsible for intestinal excretion (water and electrolytes) and acts stronger than glucagon. VIP stimulates lipolysis and glycogenolysis resulting in an increase in blood glucose. This effect was observed in our piglets. Moreover, VIP stimulates pancreatic function in pigs (Holst et al., 1984; Fahrenkrug, 1993). VIP is important for the bacterial metabolite induced activation of intestinal gluconeogenesis, and in turn, its expression is modulated by the microbiota-derived compounds produced by bacterial fermentation. It is suggested that the local release of VIP is involved in a key mechanism by which nutrients or microbial metabolites are involved in the activation of intestinal gluconeogenesis and glucose metabolism (Abot et al., 2018). It may be that the general improvement in piglet development observed following maternal HMB treatment was due to the effects of HMB on the gut-bone axis (Tomaszewska et al., 2019).

Leptin is another factor which has significant influence on the enteric nervous system. Leptin expression was examined in the current study, and our results showed that maternal HMB treatment increased epithelial immune reaction for leptin. Leptin exerts its impact on enteric nitrergic neurons and intrinsic primary afferent neurons and is key in the regulation of food intake and energy homeostasis (Abot et al., 2018). Moreover, intestinal absorption of nutrients, mucus secretion, motility and inflammation, among other processes, are all influenced by leptin, which additionally participates in the NO release in the colon (Florian et al., 2013).

Colonic epithelium participates in the absorption of sodium, potassium and carbohydrate ions. Large amounts of sodium are absorbed in the colon, against an electrochemical gradient. The sodium content in faecal matter is lower than that in plasma in general. Active transport is present, but in the colon, this transport does not include other substances as observed in the small intestine. Sodium transport creates an electrochemical gradient, according to which potassium ions are excreted into the colonic lumen. Potassium excretion occurs via passive transport which is dependent on sodium transport. The sodium absorption occurring in the colon in turn depends on the chloride concentration, and it is kept unto a certain concentration of chloride, below which the excretion begins. On the other hand, the absorption of chloride is linked with the carbohydrate. The absorptive processes which take place in the colon, occur in the crypts, the activity and frequency of which is increased in intestinal disorders, like ulcerative enteritis (Konturek, 1985).

Taking into account that the intensity for VIP immune reaction was increased in the submucosal plexuses following maternal HMB treatment in the current study, one can assume that there was a risk of increased water and electrolyte excretion, leading to diarrhoea and dehydration of the animal. However, the basal biochemical serum analysis showed no changes in serum electrolyte concentrations. Moreover, our study also showed that prenatally HMB treated piglets had increased blood IgG concentrations (possible HMB effect on sows colostrum and milk content), and the colonic barrier was stronger after following maternal HMB treatment, compared to the control animals. In general, the capacity of the intestinal epithelium to prevent penetration into the mucosa by luminal bacteria and dietary allergens, which is considered as an intestinal barrier, was improved in the prenatally HMB treated weaned piglets.

The gut epithelium is not composed of functionally identical cells. Along the gastrointestinal tract, the epithelium varies and is composed of tight cell-cell junctions, which link epithelial cells. Claudin and occludin are necessary for the formation of cell-cell junctions and they bind to several other tight junction-associated proteins; and the cadherins mediate cell adhesion. Together they all play a fundamental role in normal development and participate in the maintenance of proper cell-cell contacts (Hollander and Kaunitz, 2019). Moreover, to maintain an optimal intestinal barrier, adherence of peripheral blood lymphocytes is needed. The CD3 antigen is present on between 68% to 82% of normal peripheral blood lymphocytes (T cells) and is never expressed on B or natural killer cells. The null T cells do not have markers of B or T cells but are included with the lymphocytes, which reach an amount of between 5% to 10% of whole blood lymphocytes. The current study showed that maternal treatment with HMB decreased lymphocyte number. It is possible that following the improvement in colonic epithelial integrity, the number of peripheral blood lymphocytes in the epithelium was decreased, with a reduced number of

null T cells. Activation of the intestinal immune system is associated with the synthesis of specialized signalling molecules, mainly pro-inflammatory cytokines, including IL-6 (Liu, 2015). An increase in serum IL-6 was not observed in the piglets after maternal HMB treatment.

The results obtained from the current study indicate that the administration of HMB to pregnant sows significantly influenced the expression of some proteins of the intestinal barrier in their offspring, with less influence on large intestine basal morphology. The possible mechanisms involved in the maintenance of the electrolyte balance observed in the piglets need to be further investigated.

Conflict of Interests Statement

The authors declare that they have no conflict of interests regarding the publication of this article.

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