Effect of exogenous butyrate on the gastrointestinal tract of sheep. I. Structure and function of the rumen, omasum, and abomasum¹

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ABSTRACT: The aim of this study was to determine the effect of exogenous butyrate on the structure and selected functions of the stomach in sheep. Eighteen rams (30.8 ± 2.1 kg; 12 to 15 mo of age) were allocated to the study and fed a diet for 14 d without (CTRL) or with sodium butyrate (BUT; 36 g/kg of offered DM). Neither DMI nor initial BW differed between treatments ($P \geq 0.61$), but final BW was greater for BUT compared with CTRL ($P = 0.03$). Butyrate concentration in the reticuloruminal fluid and abomasal digesta was greater for BUT compared with CTRL ($P \leq 0.01$), but total short-chain fatty acids (SCFA) concentration, as well as concentration of other SCFA, did not differ between treatments ($P \geq 0.07$). Relative to BW, reticuloruminal tissue mass tended ($P = 0.09$) to be greater and omasal digesta was less ($P = 0.02$) for BUT compared with CTRL. Dietary

butyrate did not affect ruminal papillae length, width, and density nor did it affect ruminal epithelium thickness ($P \geq 0.12$) in the ventral sac of the rumen. However, the DM of ruminal epithelium (mg/cm^2) tended ($P = 0.06$) to be greater for BUT compared with CTRL. Omasal and abomasal epithelium thicknesses were greater ($P \leq 0.05$) for BUT compared with CTRL. Mitosis-to-apoptosis ratio in the abomasal epithelium was less for BUT compared with CTRL ($P = 0.04$). Finally, the mRNA expression of peptide transporter 1 in the omasal epithelium was less ($P = 0.02$) and mRNA expression of monocarboxylate transporter 1 in the abomasal epithelium tended ($P = 0.07$) to be greater for BUT compared with CTRL. It can be concluded that exogenous butyrate supplementation affected not only the rumen but also omasum and abomasum in sheep.

Key words: absorption, digestion, gastrointestinal tract, ruminant, stomach

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INTRODUCTION

Butyrate is widely recognized as an important factor affecting both structure and functions of the rumen (Penner et al., 2011; Kowalski et al., 2015; Górka et al., 2018a). Promotion of its microbial production in the rumen or direct infusion into the rumen was shown, among other results, to increase rumen papillae dimensions and density (Shen et al., 2005; Malhi et al., 2013), expression of genes encoding transporters

mediating short-chain fatty acids (SCFA) absorption from the rumen (Malhi et al., 2013; Dengler et al., 2014b) and blood flow through ruminal epithelium (Storm et al., 2011). However, about 15% of SCFA that are released in the rumen flow with digesta to the omasum (Peters et al., 1990; López et al., 2003), and, due to incomplete SCFA absorption in this region of the gastrointestinal tract (GIT), also continue to flow to the abomasum and small intestine (Rupp et al., 1994). When butyrate was infused directly into the rumen, this resulted in greater butyrate flow to the duodenum (Górka et al., 2017b). Furthermore, butyrate infusion into the abomasum, likely resulting in greater butyrate flow to the intestine, affected expression of the number of genes in the duodenal epithelium (Foote et al., 2017). Hence, ruminal butyrate may affect not only the rumen but also lower regions of the GIT, including the omasum, abomasum, and proximal small intestine.

In this study, we hypothesized that exogenous butyrate supplementation, as a means of increasing ruminal butyrate, will affect not only the structure and functions of the rumen but also the omasum and abomasum. The specific aims of this study were to determine the effect of exogenous butyrate on ruminal, omasal, and abomasal tissue and digesta mass; omasal and abomasal epithelium thickness; mitotic and apoptotic indices in the omasal and abomasal epithelium; and the expression of mRNA of SCFA transporters (monocarboxylate transporter 1, 2, and 4), SCFA receptors (G-protein coupled receptor 41 and 43), glucose transporters (sodium/glucose co-transporter 1 and facilitative glucose transporter 2), and peptide transporter

(peptide transporter 1) in the ruminal, omasal, and abomasal epithelium. Impact of butyrate on selected blood indicators was also investigated to hasten the scientific hypothesis verification.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

The experimental protocol was reviewed and approved by the local ethics committee (Krakow, Poland) before study initiation (Protocol No. 190/2015). Eighteen Wrzosówka rams (30.8 ± 2.1 kg; 12 to 15 mo of age) were used for the study. The rams were transported to the research barn in 4 blocks of 6, 4, 4, and 4 rams at least 2 wk prior to the study. Within each block, the rams were paired by BW, and within pairs, randomly allocated to 1 of 2 treatments (9 rams/treatment) and fed a diet without (CTRL) or with supplemental butyrate (BUT). The diet consisted of chopped meadow hay (65%), concentrates (19.5%), and ensiled ground high-moisture corn grain (15.5%, DM basis; Table 1) and was fed in 2 equal meals at 0700 and 1500. Dry matter intake (DMI) was limited to 2.75% of initial BW of each block, with final adjustment on the first day of the experimental feeding protocol. For BUT, sodium butyrate was mixed with concentrates and ensiled high-moisture corn grain prior to feed allocation. High-moisture corn grain was used in the diet to add moisture to the feed and hasten sodium butyrate binding with the feed. Furthermore, in order to assure complete intake of butyrate, a mixture of concentrates and high-moisture corn grain with or without sodium

Table 1. Chemical composition of experimental feeds

Item	DM, %	Ash, % DM	CP, % DM	NDF, % DM	ADF, % DM	Energy ¹ , MJ ME/kg DM	Na ² , % DM
Hay	85.6	9.9	8.0	63.6	39.5	7.7	0.02
Concentrate ³	90.0	14.4	28.5	10.4	4.5	11.9	1.66
Ensiled high-moisture corn grain	70.0	1.6	7.9	11.5	10.5	12.9	0.01
Diet ⁴	84.2	9.4	12.0	45.1	28.1	9.3/9.7 ⁵	0.34/1.18 ⁵

¹Metabolizable energy in hay, concentrate and ensiled corn grain was calculated from INRA (2007) and subsequently used for estimation of ME in the diet.

²Dietary Na content in hay, ensiled high-moisture corn grain and components of concentrate mixture were taken from NRC (2001) and used for estimation of Na content in the diet.

³Consisted of (on DM basis) 24.6% barley, 14.9% corn, 50.1% soybean meal, 7.6% vitamin-mineral supplement (Polfamix OK, Trouw Nutrition, Grodzisk Mazowiecki, Poland; as fed: 24% Ca, 12% P, 6% Na, 6.5% Mg, 300 mg Mn, 2500 mg Zn, 50 mg I, 15 mg Co, 3 mg Se, 300 000 IU vitamin A, 30 000 IU vitamin D₃, 1 500 IU vitamin E), 2.8% NaCl.

⁴Consisted of (on DM basis) 65% hay, 15.5% ensiled ground high-moisture corn grain, and 19.5 % concentrate, and 0 or 36 g of additional sodium butyrate/kg of offered DM/day.

⁵Estimated contents for CTRL (lower value) and BUT (higher value), respectively. Higher values for BUT include additional energy and Na intake with supplemental sodium butyrate. Metabolizable energy for BUT was calculated using GE of sodium butyrate (20.0 MJ/kg DM; evaluated using bomb calorimeter) based on assumption that GE and ME contents in sodium butyrate are the same.

butyrate was fed 30 min prior to hay feeding. Sodium butyrate was fed at the rate of 36 g/kg of offered DM that corresponded with the doses of butyrate used in previous studies in cattle (Huhtanen et al., 1993) and sheep (Wilson et al., 2012; Wiese et al., 2013; Górka et al., 2017b). The experimental diets were fed for 14 d.

Sample Collection

The feeds were sampled weekly and stored at room temperature (hay and concentrates) or frozen (ensiled corn, -20°C) prior to analysis. On the last day of the study, 3 h after the morning feeding, rams were killed by captive bolt stunning and exsanguination. Immediately after killing, GIT was removed, and reticulorumen, omasum, and abomasum were separated, weighed full, and emptied from the digesta. The digesta from the reticulorumen and abomasum was emptied into designated containers, whereas the digesta from the omasum was discarded. From the cranial ventral sac of the rumen, 2 pieces of whole-tissue thickness (4×4 cm) were taken, weighed, and rinsed in ice-cold PBS (pH 7.4) until clear. Ruminal epithelium from 1 sample was manually stripped of the muscle layer, cut into small pieces (1×1 cm) using sterile forceps and scissors, and stored in RNAlater solution (Ambion, Austin, TX) according to the manufacturer's instruction. A second sample was stored in 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO) for 24 h and then in 70% ethanol (POCH, Gliwice, Poland). From the omasum, a tissue sample (1×1 cm) from the tip of the large order lamina was collected using sterile forceps and scissors, rinsed repeatedly in ice-cold PBS, and stored in RNAlater. In addition, tissue samples from the central portion of 5 large-order omasal laminae (from the tip to the bottom, 2-cm thick) were taken, rinsed in ice-cold PBS, weighed, stored in formalin solution for 24 h, and then in 70% ethanol. From the abomasum, epithelium from the pyloric region (5 cm from the pylorus) was clipped using sterile forceps and scissors, rinsed in ice-cold PBS, and placed in RNAlater. Correspondingly, a whole-thickness abomasum tissue sample (4×4 cm) was collected, weighed, rinsed in ice-cold PBS, and preserved in formalin solution for 24 h and then stored in 70% ethanol. Reticuloruminal digesta was strained through 2 layers of cheesecloth, the pH of the resulting fluid was measured (pH meter N517, Meratronic, Warszawa, Poland), and 5 mL of fluid was preserved with 1 mL of metaphosphoric acid (25% wt/vol) and stored frozen (-20°C) for SCFA

determination. Abomasal digesta pH was also recorded and 5 mL of whole digesta was collected and preserved as described above. After collection of the samples, the remaining reticuloruminal, omasal, and abomasal tissues were washed repeatedly with tap water, pressed by hand and shaken off 2 times, and weighed individually. Two pieces of whole-thickness tissue (10×10 cm) were collected from the ventral sac of the rumen, blotted dry using paper towel, and stored frozen (-20°C) for tissue (epithelium and muscle) DM mass determination. Digesta mass in the reticulorumen, omasum, and abomasum was calculated by the difference between full reticulorumen, omasum, and abomasum mass (tissue plus digesta) and corresponding tissue mass, respectively.

Just before killing, blood samples were taken from the jugular vein of the rams into tubes containing lithium heparin (16 IU/mL of blood; SARSTEDT, Nümbrecht, Germany). The tubes were immediately placed on ice, cooled down, and centrifuged at $1,800 \times g$ for 10 min. The plasma was harvested, frozen, and stored (-20°C) for later analysis of concentrations of plasma glucose, β -hydroxybutyric acid (BHBA), insulin, and insulin-like growth factor 1 (IGF-1).

Sample Analyses

Samples of the feeds were composited for each block of animals, dried at 55°C for 48 h, ground to pass through a 1-mm screen, and analyzed for DM, ash, CP, NDF, and ADF as described previously by Górka et al. (2017b). Gross energy (GE) in sodium butyrate was determined using bomb calorimeter (KL-10, Precyzja-Bit, Bydgoszcz, Poland), whereas GE and ME in feeds were calculated from INRA (2007). GE in feeds was also determined using bomb calorimeter and it was similar to GE estimated based on chemical composition of feeds (difference $\pm 1.02\%$). For sodium butyrate, ME was assumed to be equal to GE. Based on the chemical composition of the feeds, the diet composition was mathematically estimated (Table 1).

Ruminal and abomasal SCFA were determined by gas chromatography (3400 CX, Varian Star, Palo Alto, CA) equipped with a flame ionization detector using DB-FFAP column ($30 \text{ m} \times 0.5 \text{ mm}$, J&W Scientific, Folsom, CA) as described previously by Górka et al. (2017b). Argon was used as a carrier, the flow rate was set to 4 mL/min, and the temperature for the column, injector, and detector was set to 90 to 205, 200, and 240°C , respectively.

Tissue samples preserved in formalin and stored in ethanol were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Prior to embedding, tissue samples from the rumen and abomasum were divided into four 0.5- to 1-cm thick pieces. Each piece and each omasal lamina (5/animal) were embedded in a separate paraffin block. Six-micrometers thick sections were cut from each paraffin block and either stained with hematoxylin and eosin for morphometry or labeled for the determination of mitotic and apoptotic indices (described below). In the rumen, 8 well-oriented papillae were identified and epithelium (10× objective lens) and stratum corneum (20× objective lens) thicknesses were measured on the tip, middle point, and between rumen papillae using a light microscope (16× ocular; Zeiss Axio Imager M2, Zeiss, Göttingen, Germany), captured with a Canon PowerShot G10 video camera (Canon, Tokyo, Japan) and AxioVision software (Zeiss, Göttingen, Germany). Six measurements on the tip and in the middle point of the papillae and 4 measurements between the rumen papillae were performed, respectively, resulting in 128 measurements for each parameter and animal (e.g., epithelium thickness and stratum corneum thickness). Using the same microscope and objective lens, 6 pictures from the tip toward the bottom of each omasal lamina were taken, and in each picture 6 measurements for epithelium thickness and stratum corneum thickness were performed, resulting in 180 measurements for each parameter and animal. Initially, measurements from the tip to the bottom on 10 randomly selected omasal laminae were performed to determine whether epithelium thickness and stratum corneum thickness differ in the upper and lower regions of the lamina. From 8 to 15 pictures could be taken for the entire lamina and, on average, the results for the entire lamina differed from the results obtained from 6 pictures by $1.3 \pm 3.7\%$ and $5.5 \pm 5.5\%$ for epithelium and stratum corneum thickness, respectively. In the abomasum, 10 measurements for epithelium thickness and muscle layer thickness (2.5× objective lens) were performed on each slide, resulting in 40 measurements for each parameter and animal. Additionally, 1 cm² of rumen tissue was used to determine rumen papillae length, width and density, and rumen muscle thickness. All the papillae were cut off at the base using forceps and scissors under a SteREO Discovery.V12 Zeiss microscope with a PlanApo S 0.63× FWD 81-mm Zeiss lens and, subsequently, the length and width (middle point) of each papilla were measured using AxioVision software (Zeiss). The remaining portion

of the tissue was positioned on 1 edge and muscle layer thickness was also measured. The mucosa surface (mm²/cm²) was determined as the length × width × density × 2 (Malhi et al., 2013).

Expressions of Ki-67 and active caspase-3 were determined in the omasal and abomasal epithelium by scanning cytometry and used as markers for mitosis and apoptosis, respectively (Godlewski et al., 2009; Górka et al., 2011). Antigens were exposed through citrate buffer heating (pH = 6.0) for 5 min and then cooled for at least 20 min at room temperature. After washing with PBS-buffer (pH = 7.2), the tissue sections were covered for 45 min with bovine serum albumin (Sigma-Aldrich) to block nonspecific antibody binding. Individual slides were then incubated with antibodies: anti-Ki67 (mouse polyclonal anti-human Ki-67; 612472:FITC, BD Pharmingen, San Diego, CA; incubation for 180 min at room temperature; dilution 1:200), or anti-Active Caspase-3 (rabbit polyclonal anti-human active caspase-3; CPP32:FITC, BD Pharmingen; incubation for 180 min at room temperature; dilution 1:150). Finally, the nuclei were counterstained with HOECHST 33342 (Sigma-Aldrich). Afterwards, the samples were rinsed in PBS and the slides were mounted with fluoromount (Sigma-Aldrich) under coverslips. For quantitative evaluation of Ki-67 positive or active caspase-3 positive cell indices, process-related fluorescence was analyzed for all the tissue samples under a SCAN[^]R scanning cytometer (Olympus, Warszawa, Poland). The cells were identified using Hoechst 33342 fluorescence associated with the nuclei. Cell-related fluorescence was measured within an outline of +10 pixels around the contour of the nuclei. A standard mathematical protocol for tissues in the scanning cytometry, based on the average distance between the nuclei with and the lack of overlap between neighboring cells, was used to eliminate cell aggregates and debris. Following the analyses, the results were presented as the indices of cells positive for the Ki-67 and active caspase-3 fluorescence.

Frozen 10- × 10-cm ruminal tissue samples were thawed out overnight. From each sample, a 5- × 5-cm piece was cut off and the epithelium was manually stripped from the muscle layers. Both the epithelium and muscle layers were dried in a forced-air oven at 105 °C for 5 h to determine epithelium and muscle DM (Górka et al., 2017a). An average from 2 samples was used for statistical analysis.

Total RNA was isolated from tissue samples stored in RNAlater according to the method described by Chomczyński and Sacchi (1987) and

subsequently subjected to reverse transcription reaction. Validation of the quality and quantity of RNA, as well as the reverse transcription reaction procedure, was performed as described previously (Górka et al., 2017b). Obtained cDNA was stored at -20°C until analyzed for expression of sodium-dependent glucose co-transporter 1 (SGLT1), glucose transporter 2 (GLUT2), peptide transporter 1 (PepT1), monocarboxylate transporter 1 (MCT1), monocarboxylate transporter 2 (MCT2), monocarboxylate transporter 4 (MCT4), G-protein coupled receptor 41 (GPR41), G-protein coupled receptor 43 (GPR43), and 3 reference genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH], Na/K-ATPase, and β -actin [ACTB]). Gene expression was determined in duplicate and each reaction was performed as previously described (Górka et al., 2017b) using specific primers (Supplementary Table S1). NormFinder was used to determine the most optimal reference gene or genes (Andersen et al., 2004), and geometric mean of GAPDH and Na/K-ATPase was chosen for the normalization of the target genes. The interassay coefficient of variance for internal control was $0.44 \pm 0.31\%$. To ensure a correct result of analysis, the product of reaction for each primer was sequenced and the homology between the target and the product sequence was verified. Relative expression of the investigated genes was presented as ΔC_T , where ΔC_T = threshold cycle (C_T) for target gene – C_T for reference. Therefore, a higher ΔC_T value represented lower mRNA expression and vice versa.

Plasma glucose and BHBA were determined by a commercial laboratory (WDL, Gietrzwałd, Poland) using an automatic chemical analyzer (Hitachi 902, Hitachi, Japan) as previously described by Górka et al. (2017b). Plasma insulin and IGF-1 were determined by RIA (gamma counter WIZARD 2470, Perkin-Elmer, MA) using a commercial set of reagents (INS-IRMA and IGF-1-RIA-CT for insulin and IGF-1 determination, respectively; DIAsource, Louvain-La-Neuve, Belgium). Intra-assay and inter-assay CV were 1.80 and 6.30% and 4.23 and 6.55% for insulin and IGF-1 determination, respectively.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (version 9.2, Cary, NC). For statistical analysis, data for histometric measurements (e.g., papillae length and epithelium thickness) were pooled by animal and region of the GIT to obtain a mean value for each animal. Furthermore, measurements done on the tip, middle point, and between

rumen papillae were also pooled to obtain a mean value for stratum corneum, living strata, and total epithelium thickness representative for each animal. The model included the fixed effect of treatment and the random effect of the block and the pair of animals within a block. The results are presented as least square means and their corresponding standard errors. Significance was declared at $P \leq 0.05$ and tendencies were discussed when $0.05 < P \leq 0.10$.

RESULTS

The nutrient composition of the feeds and diet is presented in Table 1. The feeds were consumed well and only a slight aversion to the feed was observed for some BUT animals on the first day of the experimental feeding protocol. However, all the feed allocated on that day had been consumed by morning feeding the following day. In general, concentrates were consumed within 15 min after feeding, resulting in all the butyrate consumed before hay allocation.

Neither DMI nor initial BW differed between treatments ($P \geq 0.61$; Table 2); however, final BW was greater for BUT compared with CTRL ($P = 0.03$). Plasma BHBA was greater ($P < 0.01$) and plasma insulin tended ($P = 0.06$) to be greater for BUT compared with CTRL. Plasma glucose and serum IGF-1 did not differ between treatments ($P \geq 0.39$).

In the reticuloruminal fluid, total SCFA concentration tended ($P = 0.07$) to be greater and butyrate concentration was greater ($P < 0.01$) for BUT compared with CTRL (Table 3). In the abomasal digesta, butyrate concentration was greater ($P = 0.01$) for BUT compared with CTRL, whereas isobutyrate tended ($P = 0.07$) to be greater for CTRL compared with BUT.

Rumen tissue samples (10- × 10-cm pieces) from the first block of sheep were accidentally lost; thus, the presented results refer to 6 animals/treatment. Reticuloruminal tissue mass was greater ($P < 0.01$) and abomasal tissue mass tended ($P = 0.08$) to be greater for BUT compared with CTRL; however, when expressed relative to BW (g of tissue/kg of BW), reticuloruminal tissue mass tended ($P = 0.09$) to be greater for BUT compared with CTRL and abomasal tissue mass did not differ ($P = 0.16$) between treatments (Table 4). The dry mass of ruminal epithelium in the ventral sac of the rumen tended ($P = 0.06$) to be greater for BUT compared with CTRL. Omasal tissue mass was not affected by treatment ($P \geq 0.26$), but omasal digesta mass tended ($P = 0.06$) to be less and was less ($P = 0.02$)

Table 2. Effect of dietary sodium butyrate on dry matter intake, body weight, and selected blood indicators

Item	Treatment ¹		SEM ²	P-value
	CTRL	BUT		
Dry matter intake, g/d ³	862	864	2.0	0.82
Body weight, kg				
Initial	30.8	31.1	0.85	0.61
Final	32.2	33.4	0.90	0.03
Plasma concentration				
Glucose, mmol/L	5.27	5.16	0.105	0.39
BHBA, ⁴ mmol/L	0.58	0.98	0.040	<0.01
Insulin, uIU/mL	23.5	31.0	2.70	0.06
IGF-1, ⁵ ng/mL	58.2	62.3	5.58	0.61

¹Sheep were fed diet without (CTRL) or with 36 g of sodium butyrate/kg of offered dry matter (BUT).

²Standard error of the mean.

³Does not include sodium butyrate intake.

⁴ β -hydroxybutyric acid.

⁵Insulin-like growth factor 1.

Table 3. Effect of dietary sodium butyrate on pH and short-chain fatty acids concentration in the reticulorumenal fluid and abomasal digesta

Item	Treatment ¹		SEM ²	P-value
	CTRL	BUT		
Reticulorumen SCFA ³ , mmol/L				
Total	116	129	4.6	0.07
Acetate	80.6	83.3	3.46	0.56
Propionate	21.1	21.0	0.76	0.89
Butyrate	11.7	20.7	1.18	<0.01
Valerate	1.24	1.33	0.069	0.11
Isobutyrate	0.51	0.56	0.047	0.41
Isovalerate	0.86	0.81	0.053	0.31
Reticulorumen pH	6.25	6.14	0.079	0.34
Abomasum SCFA, mmol/L				
Total	22.2	22.4	1.68	0.95
Acetate	17.5	17.6	1.33	0.95
Propionate	2.96	2.61	0.234	0.30
Butyrate	1.31	2.03	0.164	0.01
Valerate	0.09	0.10	0.018	0.87
Isobutyrate	0.14	0.07	0.025	0.07
Isovalerate	0.15	0.11	0.019	0.18
Abomasum pH	3.24	3.21	0.181	0.49

¹Sheep were fed diet without (CTRL) or with 36 g of sodium butyrate/kg of offered dry matter (BUT).

²Standard error of the mean.

³Short-chain fatty acids.

when expressed as g of digesta/kg of BW for BUT compared with CTRL.

The rumen papillae length, width, and surface area of the ruminal epithelium, as well as the ruminal epithelium thickness in the ventral sac of

Table 4. Effect of dietary sodium butyrate on stomach tissue and digesta mass and epithelium and muscle mass in the ventral sac of rumen

Item	Treatment ¹			P-value
	CTRL	BUT	SEM ²	
Reticulorumen				
Full, g	7001	6955	259.9	0.86
Tissue, g	659	713	21.4	<0.01
Digesta, g	6342	6246	246.1	0.70
Full, g/kg BW	217	209	4.9	0.25
Tissue, g/kg BW	20.4	21.3	0.36	0.09
Digesta, g/kg BW	192	188	3.9	0.41
Dry epithelium mass, ^{3,4} mg/cm ²	15.9	19.1	1.25	0.06
Dry muscle mass, ^{3,4} mg/cm ²	18.4	18.3	1.30	0.96
Omasum				
Full, g	155	140	9.8	0.26
Tissue, g	66.2	67.4	4.33	0.84
Digesta, g	89.3	73.8	6.79	0.06
Full, g/kg BW	4.8	4.2	0.29	0.10
Tissue, g/kg BW	2.1	2.0	0.12	0.79
Digesta, g/kg BW	2.8	2.2	0.21	0.02
Abomasum				
Full, g	582	627	48.8	0.53
Tissue, g	146	162	7.2	0.08
Digesta, g	436	465	45.7	0.66
Full, g/kg BW	18.2	18.7	1.34	0.79
Tissue, g/kg BW	4.6	4.9	0.21	0.16
Digesta, g/kg BW	13.6	14.7	1.19	0.53

¹Sheep were fed diet without (CTRL) or with 36 g of sodium butyrate/kg of offered dry matter (BUT).

²Standard error of the mean.

³*n* = 6/treatment.

⁴Collected from ventral sac of the rumen.

the rumen, did not differ between treatments ($P \geq 0.12$; Table 5), with the exception of greater stratum corneum thickness on the tip of the papillae for CTRL compared with BUT (36 vs. 28 μm , $P = 0.03$; data not presented). Both omasal and abomasal epithelium thicknesses were greater for BUT than for CTRL ($P \leq 0.05$). Mitotic and apoptotic indices did not differ between treatments with the exception of lower mitosis-to-apoptosis ratio in the abomasal epithelium for BUT compared with CTRL ($P = 0.04$). Representative histograms of treatments can be found in [Supplementary Figures F1 and F2](#) (see online supplementary material for a color version of this figure).

The mRNA expression of the investigated genes in the omasal epithelium for 1 ram was very low ($C_T > 30$ for most of the analyzed genes). As a result, data for gene expression in the omasal epithelium for 1 pair of animals were excluded from the statistical analysis. Expression of *PepT1* mRNA in the omasal epithelium was less ($P = 0.02$), whereas expression of *MCT1*

Table 5. Effect of dietary sodium butyrate on ruminal, omasal, and abomasal epithelium morphology, mitotic and apoptotic indices of the omasal and abomasal epithelium

	Treatment ¹		SEM ²	P-value
Item	CTRL	BUT		
Ventral rumen sac				
Papillae length, mm	2.48	2.82	0.220	0.28
Papillae width, mm	1.81	1.86	0.075	0.57
Density, <i>n</i>	67	60	5.1	0.26
Surface area, mm ² /cm ²	636	630	70.5	0.96
Total epithelium thickness, µm	157	156	4.2	0.79
Living strata thickness, µm	34.4	29.6	2.71	0.12
Stratum corneum thickens, µm	115	119	3.8	0.47
Omasum				
Total epithelium thickness, µm	125	138	3.3	< 0.01
Living strata thickness, µm	110	123	4.6	0.01
Stratum corneum thickness, µm	15.3	15.2	1.54	0.90
Mitotic index, %	5.15	4.91	0.620	0.61
Apoptotic index, %	3.06	2.78	0.381	0.61
Mitosis-to-apoptosis ratio	2.48	2.29	0.352	0.68
Abomasum				
Epithelium thickness, µm	571	622	37.6	0.05
Muscle layer thickness, µm	1082	1078	63.6	0.97
Mitotic index, %	4.68	3.17	0.889	0.11
Apoptotic index, %	3.86	4.75	0.676	0.36
Mitosis-to-apoptosis ratio	1.67	0.90	0.248	0.04

¹Sheep were fed diet without (CTRL) or with 36 g of sodium butyrate/kg of offered dry matter (BUT).

²Standard error of the mean.

mRNA in the abomasal epithelium tended ($P = 0.07$) to be greater for BUT compared with CTRL. Expression of GLUT2 in the ruminal, omasal, and abomasal epithelium was very low (C_T value > 30 for most of analyzed samples) and thus was considered not present in those regions of the GIT.

DISCUSSION

In this study, ruminal butyrate was hypothesized to affect not only the rumen but also the omasum and abomasum. To verify this hypothesis, exogenous butyrate was supplemented in the diet in order to increase ruminal butyrate and its flow with digesta to the lower regions of the GIT. Greater butyrate concentration in the reticuloruminal and abomasal digesta for BUT animals proves that the model used in the present study was successfully induced, and confirms that increased ruminal butyrate results in greater exposure to this molecule of the subsequent regions of the GIT. In line with the results of the present study, butyrate infusion into

the rumen increased butyrate concentration not only in the reticuloruminal but also in the duodenal digesta and tended to increase butyrate flow to the duodenum (Górka et al., 2017b). Although butyrate concentration in the omasal digesta was not determined in the current study, increased butyrate concentration in the digesta of the preceding (reticuloruminal) and following (abomasum) region of the GIT clearly indicates that omasum was also exposed to the increased concentration of butyrate.

Prior to the discussion of results, weak points of the experimental model used in the current study have to be mentioned. This includes additional energy and sodium intake when sodium butyrate was supplemented in the diet. Assuming that GE and ME content in sodium butyrate is the same, its supplementation in the diet could increase energy intake by 4%. This could contribute to the observed greater final BW of BUT animals. However, not only positive, but also negative impact of high ruminal butyrate on nutrient digestion and absorption in sheep has been shown (Wilson et al., 2012; Górka et al., 2018b), meaning that increased ruminal butyrate may also decrease the amount of energy available for the animal. Therefore, the impact of greater intake of energy with supplemental butyrate on the results of the present study is difficult to discuss; however, it should be taken into account when interpreting the data. On the other hand, exogenous sodium butyrate supplementation increased sodium intake over 3 times (roughly from 3 to 10 g/d, though it is still much less than 24 g/d [60 g of salt] which is considered an upper limit negatively affecting feed intake, and thus likely GIT function in sheep; Digby et al., 2011). The authors are unaware of any study showing a negative impact of sodium intake within a range resembling that in the present study on ruminal, omasal, and abomasal epithelium function. Sodium supplementation exceeding 0.4% of DM is quite common in practical nutrition of growing sheep (this includes ad libitum access to salt licks) and has no negative impact on sheep performance (Wilson et al., 2012). In turn, low sodium concentration or blockage of sodium uptake by the epithelial cells was shown to impair omasal epithelium functions ex vivo (Ali et al., 2006; Caushi and Martens, 2018). In fact, large amounts of sodium are secreted with saliva into the rumen and mechanisms of sodium absorption and recycling into the rumen must be operating very effectively in ruminants in order to ensure efficient SCFA absorption (Leonhard-Marek et al., 2010). Furthermore, flux rates of sodium across the ruminal epithelium may vary greatly depending on the diet composition (Schurmann et al., 2014).

It is also worth mentioning that estimated sodium secretion into the rumen with saliva in sheep ranges between 28 and 30 g/d (Kay, 1960), whereas amount of sodium absorbed in the omasum may be as low as 2 to 3 g/d (Engelhardt and Hauffe, 1975). This low contribution of omasum to sodium absorption in sheep is reflected by lower mass of this region of the GIT (relative to BW) compared with other ruminants (McSweeney, 1988). In consequence, the reticulorumen seems to be a predominant place of sodium absorption in sheep. On the other hand, in the abomasum sodium is mostly secreted (Yang and Thomas, 1965). Taking into account that the main aim of the present study was to determine the impact of exogenous sodium butyrate on the omasum and abomasum, we speculate that observed differences between treatments can be referred mostly to the action of butyrate, not sodium.

In contrast to the results of previous studies on cattle and goats (Shen et al., 2005; Malhi et al., 2013; Kowalski et al., 2015), in the present study, greater ruminal butyrate did not translate into greater dimensions of rumen papillae and, consequently, greater surface area for nutrient absorption, at least in the ventral sac of the rumen. However, reticuloruminal tissue mass (both absolute and relative to BW) and epithelial DM mass (but not muscle mass) in the ventral sac of the rumen at least tended to be greater for BUT compared with CTRL, indicating that the methodological approach used in the present study was not without impact on the ruminal epithelium. Furthermore, stratum corneum on the tip of the ruminal papillae was thicker for CTRL than BUT animals (data not presented). It was shown in previous studies that butyrate (particularly in high concentrations) may accelerate transformation of stratum corneum and sloughing of cornified cells (Greco et al., 2018). Thus, it can be speculated that the period of study was too short for rumen papillae growth to occur. However, the results of numerous studies suggest that rumen papillae growth in response to dietary change may be rapid (Bannink et al., 2008; Dieho et al., 2016a). On the other hand, not only butyrate, but also other SCFA, play an important role in the stimulation of ruminal epithelium growth (Sakata and Tamate, 1979; Mentschel et al., 2001). To support this, Zitnan et al. (2005) showed no correlation between ruminal butyrate and rumen papillae development in calves, and the results of the study of Dieho et al. (2016a and 2016b) suggest the even greater importance of propionate than butyrate for the stimulation of ruminal epithelium growth and function in dairy cows.

It should also be mentioned that in the current study, DMI was limited, in contrast to some (Malhi et al., 2013; Kowalski et al., 2015) but not all (Shen et al., 2005) studies reporting the stimulatory impact of butyrate on ruminal epithelium growth. The restriction of DMI ensured that differences in nutrient intake between treatments did not confound the results. In line with observations of the present study, a high-grain diet (resulting in increased ruminal SCFA production, including butyrate) had no effect on ruminal papillae dimensions when feed intake was limited (Metzler-Zebeli et al., 2013; Schurmann et al., 2014). Furthermore, Wilson et al. (2012), using a very similar experimental model, except that the animals were fed mostly grain, showed a negative effect of supplemental butyrate on butyrate absorption across ruminal epithelium *ex vivo*. As a result, the effect of butyrate on ruminal epithelium growth, rumen papillae dimensions, and also ruminal epithelium functions may depend on the level of feed intake and feed composition, and all of these should be taken into account when interpreting the data.

Although the ruminal and omasal epithelium are very similar in terms of microstructure (Liu et al., 2013; Liu et al., 2014), some of their functions differ. For example, nearly no secretion occurs in the omasum whereas intensive absorption of bicarbonate, minerals, and also, most likely, water is observed (Prins et al., 1972; Martens and Gäbel, 1988; Ahvenjärvi et al., 2000). Therefore, the impact of butyrate on the epithelial cells of these 2 regions of the GIT does not necessarily have to be analogous. Nevertheless, the results of the current study indicate that butyrate has a trophic effect on the epithelium of the omasum, similar to its impact on the ruminal epithelium (Shen et al., 2005; Malhi et al., 2013). Both the ruminal and omasal epithelium preferentially metabolize butyrate to ketone bodies (Hird and Symons, 1961; Joyner et al., 1963). It was shown that less than 2% of butyrate is completely oxidized by the omasal epithelium, with the majority converted to ketone bodies (Hird and Symons, 1959; Hird and Symons, 1961). In line with this direction of butyrate metabolism by the epithelium of the forestomach, greater plasma BHBA concentration was observed in BUT animals, as also shown in other studies when exogenous butyrate was infused directly into the rumen or supplemented in the feed (Huhtanen et al., 1993; Wilson et al., 2012; Herrick et al., 2017). Since ketogenesis does not result in a relevant amount of ATP formation (Kristensen and Harmon, 2004), the stimulatory impact of butyrate on omasal epithelium

growth cannot be explained solely by the increased availability of energy for the cells; however, the ruminal epithelium poses metabolic flexibility and shifts toward more extensive butyrate oxidation when butyrate availability increases (Wiese et al., 2013). Increased butyrate flow to the omasum could have had a similar impact on the omasal epithelium metabolism. Increased omasal epithelium thickness, together with greater ruminal epithelium mass, could also be needed to prevent potential side effects of butyrate entering the blood, such as its potential toxicity or inhibition of propionate use for glucose synthesis in the liver (Krehbiel et al., 1992; Gäbel et al., 2002). It is likely that more efficient butyrate conversion to ketone bodies occurred with the increase of the epithelial cells population.

Independently of the mechanisms of butyrate action on omasal epithelium growth, it could be expected that greater omasal epithelium thickness should translate into greater omasal tissue mass in BUT animals, at least regarding the absolute tissue mass. A lack of such difference indicates that the overall size of the organ, size of the laminae, or muscle thickness was reduced, independently of the omasal epithelium growth. Lack of difference in the omasal tissue mass between treatments could be at least partially explained by less digesta content in the omasum when butyrate was supplemented in the diet. Less digesta in the omasum could simply decrease the requirement for omasal tissue. It is currently difficult to speculate whether butyrate affected omasal digesta mass by affecting its motility and digesta entrapment between the omasal laminae and its movement to the following parts of the GIT, or by affecting omasal microbes and efficiency of nutrient digestion, or both. Alternatively, butyrate could enhance digestion processes in the reticulorumen, thereby reducing the amount of digesta passing to the lower regions of the GIT. However, exogenous butyrate was shown to decrease fiber digestion in the stomach (e.g., reticulorumen, omasum, and abomasum) of sheep (Górka et al., 2017b). Additionally, in the aforementioned study and in the present study, exogenous butyrate decreased cellulolytic activity in the reticuloruminal digesta (Górka et al., 2017b; Górka et al., 2018b). Therefore, it is more likely that ruminal butyrate affects omasal digesta mass mostly by affecting omasum motility and digesta passage (or entrapment between the omasal laminae) than by stimulating microbial activity and nutrient digestion in the omasum. The impact on the microbial activity and associated nutrient digestion within the omasum should be very high in order to cope

with an increased fiber flow out of the reticulorumen. Collectively, in the present study, a substantial impact of exogenous butyrate on the structure of the omasum was shown. However, further studies are necessary to explain the mechanisms of butyrate action.

Greater epithelium thickness in the pyloric region of the abomasum of BUT animals is in line with the previously reported trophic effect of butyrate on the stomach tissue mass in monogastric animals (Kotunia et al., 2004; Mazzoni et al., 2008). It was also shown that butyrate is absorbed from the abomasum (Engelhardt et al., 1968; Williams et al., 1968). When absorbed, butyrate is most likely completely oxidized by the abomasal epithelium (Guilloteau et al., 2009; Guilloteau et al., 2010a). As opposed to the epithelium of the forestomach, the abomasal epithelium does not convert butyrate to ketone bodies (Pennington, 1952). Therefore, butyrate may promote abomasal epithelium growth by serving as a readily available energy substrate for proliferating cells. Nevertheless, butyrate may also indirectly affect GIT structure and functions, including the abomasum, via the impact on the secretion of GIT peptides and hormones such as gastrin or CCK, or via stimulation of the vagal nerve (Guilloteau et al., 2009; Guilloteau et al., 2010a; Guilloteau et al., 2010b). In line with the results of other studies (Shen et al., 2005; Herrick et al., 2017), in the current study exogenous butyrate did not affect plasma IGF-1 but increased plasma insulin. Based on the trophic effect of insulin on the GIT tissue growth and function, both in monogastrics and ruminants (Baldwin, 1999; Sukhotnik et al., 2008), at least part of the response observed in the current study could be associated with the indirect impact of butyrate on the GIT.

Although omasal and abomasal epithelium thickness increased following dietary butyrate supplementation, this was not associated with the increase in the mitotic nor a decrease in apoptotic indices within the epithelium. Moreover, in this study mitosis-to-apoptosis ratio in the abomasal epithelium was less for animals fed the diet with the addition of butyrate, mostly due to lower mitotic index in BUT animals. This observation contradicts the stimulatory effect of butyrate on mitosis and the suppressive impact on apoptosis of the epithelial cells of the rumen and small intestine (Sakata and Tamate, 1978; Mentschel et al., 2001; Górka et al., 2011). At least 2 explanations can be provided for this observation. Firstly, enhanced proliferation or reduced apoptosis of epithelial cells, or both, could be observed only shortly after initiation of butyrate

supplementation. Butyrate infusion into the rumen, as well as dietary changes leading to greater SCFA production in the rumen, was shown to increase ruminal epithelial cells proliferation for 5 d after initiation of experimental protocols (Sakata and Tamate, 1978; Goodlad, 1981). Later, proliferation rate of the epithelium decreased or even returned to the rate close to the base level, most likely to prevent excessive tissue growth (Sakata and Tamate, 1978). Taking into account the fact that greater tissue mass of the reticulorumen and abomasum was observed for BUT animals, this mode of action of butyrate on GIT tissue growth seems to be the most probable. Secondly, a low concentration of butyrate stimulated cell proliferation whereas a high concentration inhibited it (Inagaki and Sakata, 2005; Peng et al., 2007). In the current study, a dose of dietary butyrate (≈ 26 g/d or 290 mmol/d) even doubled butyrate production in the rumen of sheep (Bergman et al., 1965; Bergman, 1990), as well as nearly doubled butyrate concentration in the abomasal digesta. Therefore, at least shortly after feed (and butyrate) intake, abomasal epithelial cell proliferation could be inhibited due to the sharp increase of butyrate concentration in the digesta. It also cannot be excluded that GIT epithelial cell proliferation and apoptosis change during the day in sheep, and butyrate could affect this circadian variation. The latter one can be supported by data suggesting that cyclic variation of butyrate is needed to initiate its proliferative action (Malhi et al., 2013). If true, a single sampling point in the present study could simply have missed the true impact of butyrate on the abomasal epithelium mitotic index.

In the present study, mRNA expressions of MCT1, MCT2, MCT4, GPR41, and GPR43 in the omasal and abomasal epithelium were used as markers of the local impact of butyrate flowing out of the reticulorumen on the omasal and abomasal epithelium. In the rumen, MCT1 was shown to mediate the transport of SCFA and products of their metabolism across the stratum spinosum and stratum basale and most likely basolateral membrane of those epithelial cells (Muller et al., 2002; Kirat et al., 2006; Dengler et al., 2014a). There is evidence that MCT2 and MCT4 also mediate SCFA absorption from the GIT of the ruminants (Kirat et al., 2007; Kirat et al., 2013; Malhi et al., 2013). On the other hand, G-protein coupled receptors, GPR41 and GPR43, are believed to mediate SCFA action on the epithelium of the forestomach, particularly the rumen (Wang et al., 2009). It was shown that expression of these receptors in the ruminal epithelium was increased in

response to the intake of highly fermentable feeds, promoting SCFA production (particularly propionate and butyrate) in the rumen (Schurmann et al., 2014; Lu et al., 2015). Although gene expression at the mRNA level should be interpreted with caution, as it may not reflect final protein expression or protein functionality, only MCT1 mRNA expression in the abomasal epithelium tended to be greater for BUT compared with CTRL. In the acidic pH of the abomasum, butyrate is expected to be protonated, and thus absorbed via passive diffusion, based on the lipophilic character of the protonated SCFA (Gäbel et al., 2002); however, MCT1, MCT2, and MCT4 may enhance butyrate absorption from the stomach, including the abomasum (Iwanaga et al., 2006; Kirat et al., 2007). In ruminants, MCT1 was suggested to mediate SCFA absorption across both the apical and, especially, basolateral membrane of abomasal epithelial cells (Kirat et al., 2007). The results of the present study support the involvement of MCT1 in SCFA absorption from the abomasum and provide evidence for the impact of butyrate on these processes. The effect of butyrate on the function of the stomach epithelium was also shown in monogastric animals, manifested by a greater number of parietal, enteroendocrine, and somatostatin positive cells in sodium butyrate-supplemented piglets (Mazzoni et al., 2008). On the other hand, passive diffusion of SCFA in the omasum is considered as the most important pathway of their absorption (Ali et al., 2006; Leonhard-Marek et al., 2010) and increased ruminal butyrate may further increase passive diffusion of butyrate across the epithelium of the forestomach (Schurmann et al., 2014). As a result, upregulation of the expression of monocarboxylate transporters in the omasal epithelium for BUT animals might not be needed. It should also be mentioned that increased ruminal butyrate concentrations resulted in greater thickness (and likely mass) of the abomasal and omasal epithelium. This should translate into greater overall capacity of protein mediated transport, despite a lack of differences in the expression of the investigated transporters per 1 unit of tissue (Gäbel et al., 2002; Dieho et al., 2017), and additionally explains a lack or only minor (tendency) impact of butyrate on the mRNA expression of MCT1, MCT2, and MCT4 in the omasal and also the abomasal epithelium (Table 6).

Despite a lack of differences in the mRNA expression of MCT1, MCT2, MCT4, GPR41, and GPR43 in the omasal epithelium, increased ruminal butyrate and butyrate flow to the omasum

Table 6. Effect of dietary sodium butyrate on sodium-glucose transporter 1 (SGLT1), glucose transporter 2 (GLUT2), peptide transporter 1 (PepT1), monocarboxylate transporter 1, 2, and 4 (MCT1, MCT2 and MCT4, respectively), and G-protein coupled receptor 41 and 43 (GPR41 and GPR43, respectively) mRNA expression in the ruminal, omasal, and abomasal epithelium

Item ¹	Treatment ²		SEM ³	P-value
	CTRL	BUT		
SGLT1				
Rumen	3.34	3.61	0.328	0.51
Omasum	2.22	2.20	0.236	0.94
Abomasum	4.03	3.86	0.739	0.88
PepT1				
Rumen	8.55	8.49	0.658	0.95
Omasum	5.75	9.14	1.101	0.02
Abomasum	9.06	10.20	0.803	0.32
MCT1				
Rumen	-0.60	-0.54	0.306	0.69
Omasum	-1.17	-1.05	0.197	0.55
Abomasum	0.97	0.67	0.279	0.07
MCT2				
Rumen	7.18	7.86	0.315	0.13
Omasum	5.22	5.07	0.282	0.66
Abomasum	2.43	2.38	0.434	0.92
MCT4				
Rumen	6.92	7.84	0.803	0.30
Omasum	6.45	6.92	0.336	0.34
Abomasum	7.28	6.77	0.304	0.27
GPR41				
Rumen	10.1	10.0	0.61	0.83
Omasum	12.2	11.9	0.33	0.52
Abomasum	7.50	7.69	0.535	0.78
GPR43				
Rumen	9.43	9.55	0.195	0.67
Omasum	10.8	10.7	0.50	0.95
Abomasum	7.33	6.51	0.961	0.29

¹Gene expression is presented as relative gene expression (ΔC_T), where ΔC_T = threshold cycle (C_T) for target gene - C_T for reference; higher ΔC_T value represents lower mRNA expression and vice versa.

²Sheep were fed diet without (CTRL) or with 36 g of sodium butyrate/kg of offered dry matter (BUT).

³Standard error of the mean.

resulted in the lower mRNA expression of PepT1. The expression of SGLT1, GLUT2, and also PepT1 in the forestomach epithelium are generally lower than in the intestine (Matthews et al., 1996; Aschenbach et al., 2000; Poole et al., 2003). Nevertheless, absorption of both glucose and peptides from the forestomach was shown (Matthews and Webb, 1995; Aschenbach et al., 2000). The former authors have shown that the absorption of amino acids and peptides across the omasal

epithelium was even greater than across the epithelium of the rumen. So far, butyrate was reported to increase the expression of PepT1 in the intestinal epithelial cells in vitro (Dalmasso et al., 2008). Thus, lower PepT1 expression for BUT animals may suggest negative impact of butyrate on peptides absorption from the omasum. This may then represent an important aspect of butyrate impact on the functioning of the omasal epithelium.

In the current study, exogenous butyrate supplementation—besides affecting reticulorumenal tissue and ruminal epithelium mass—increased omasal and abomasal epithelium thickness, decreased omasal digesta mass, the mitosis-to-apoptosis ratio in the abomasal epithelium, and PepT1 mRNA expression in the omasal epithelium, and tended to increase MCT1 mRNA expression in the abomasal epithelium. Taken together, the results of the current study showed that increased ruminal butyrate affects not only the rumen but also the omasum and abomasum.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

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