Tributyrin differentially regulates inflammatory markers and modulates goblet cells number along the intestinal tract segments of weaning pigs

# Tributyrin differentially regulates inflammatory markers and modulates goblet cells number along the intestinal tract segments of weaning pigs

The corrections made in this section will be reviewed and approved by a journal production editor.

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## Abstract

Butyric acid is widely used in pig production as feed additive to improve growth performance at weaning, based on its intestinal health-promoting action. The aim of this study was to evaluate the intestinal architecture and expression of inflammatory cytokines and tight junctions (**TJ**) markers in weaning piglets fed with tributyrin, either free or microencapsulated, as dietary source of butyric acid. One hundred and eight weaned piglets were divided into pens (4 piglets/pen, ~~n~~ $n = 9$) and received either a basal diet (control) or the basal diet supplemented with tributyrin at 1750 mg/kg (f-TB) or its encapsulated form (m-TB) at 3800 mg/kg (providing 1750 mg/kg of tributyrin equally to f-TB). Growth performance was recorded until d21 when 7 pigs/group were euthanized to collect intestinal samples (duodenum, jejunum, ileum, colon) for histomorphology, cytokines, and TJ markers gene expression analysis. Data were analyzed with 1-way ANOVA. Growth performance was not affected. Compared with control pigs, m-TB pigs tended to induce deeper crypts in the duodenum ($P = 0.09$); goblet cells number tended to be reduced by f-TB treatment in duodenum villi ($P = 0.09$), by both f-TB and m-TB treatments in ileum villi ($P = 0.06$), and by m-TB treatment in colon crypts ($P = 0.08$). Compared with control animals, f-TB pigs showed higher tumor necrosis factor α (**TNF-α**) expression in duodenum (trend with $P = 0.06$), and higher interferon γ (**IFN-γ**) in the jejunum ($P = 0.04$), whereas in the colon m-TB pigs tended to down regulate IFN-γ ($P = 0.06$). Claudin-1 mRNA in the jejunum was reduced in m-TB group compared to control group ($P = 0.05$). Occludin mRNA in the ileum was reduced in

both groups treated with tributyrin compared to control group ($P < 0.01$). In the colon occludin mRNA was downregulated both in f-TB and m-TB pigs compared to control pigs ($P < 0.05$). Overall, the supplementation of tributyrin in the diet reduced the mucous-secreting goblet cells in a tract-specific way and modulated the expression of inflammatory cytokines and TJ components along the intestinal tract of weaning piglets, without any negative effect on growth performance.

**Keywords**: Microencapsulation; Pig; Tributyrin; Weaning; Tight junctions

# 1 Introduction

Tributyrin (**TB**) is a triester of butyric acid that can be hydrolyzed by intestinal lipases into two molecules of butyric acid and monobutyrin, rapidly absorbed by the enterocyte (Leonel et al., 2013). Tributyrin is regarded as a precursor of butyric acid, which is well-known for its multiple properties as intestinotrophic, anti-inflammatory, anti-oxidant, anti-apoptotic, and protective of the epithelial barrier integrity (Guilloteau et al., 2010). The treatment with TB itself has been associated with various beneficial effects at intestinal and systemic level: TB protected the gut integrity through an anti-inflammatory action in a murine ethanol-induced intestinal injury model (Cresci et al., 2014) but also in dextran sodium sulfate (DSS)-induced colitis in mice and in acetic acid-induced colitis in pigs (Leonel et al., 2013; Hou et al., 2014). In addition, TB showed a metabolic effect protecting the liver from a LPS-mediated damage in rats (Miyoshi et al., 2011) and reducing inflammation and insulin resistance associated to diet-induced obesity in mice (Vinolo et al., 2012). Concerning pig production, previous studies showed that TB at 5000 mg/kg for 4 weeks improved growth performance, intestinal morphology, and digestive enzyme activity in weaning piglets (Hou et al., 2006). In addition, TB in combination with lactitol, as precursor and fermentable source of butyric acid, improved the trophic status of the mucosa and reduced histamine levels in nursery piglets (Piva et al., 2002).

Based on these literature findings, we hypothesized that dietary TB could beneficially impact gut health, as measured by architecture of the various intestinal segments and local inflammation response in weaning pigs. Moreover, as TB is rapidly hydrolyzed and absorbed in the small intestine, a microencapsulated form of TB was also tested to verify any additional benefit more distally in the hindgut.

# 2 Material and methods

The study was conducted at the research facilities of the Research centre for Animal Production and Environment (CERZOO), which is a Good Laboratory Practices-certified facility and operates in compliance with the Directive 2010/63/UE covering the protection of the animals used for experimental or other scientific purposes, adopted by the Italian legislation in D. Lgs 4th March 2014, No. 26, and according to the Recommendation 2007/526/CE.

## 2.1 Experimental design and sample collection

One hundred and eight piglets (Landrace × Large White, barrows and gilts, 6.67 (SE 0.07) kg of BW) weaned at 28 days of age were divided in 27 pens (4 piglets/pen) and randomly assigned to one of the

following experimental groups (9 pens/group): the basal diet (control); the basal diet added with 1750 mg/kg of free TB (f-TB); the basal diet supplemented with lipid microencapsulated TB (m-TB) at 3800 mg/kg, providing 1750 mg/kg of tributyrin equally to f-TB (both free and microencapsulated TB were supplied by Vetagro S.p.A., Reggio Emilia, Italy).

The basal diet was formulated to meet or exceed requirements for weaned piglets recommended by the NRC (2012). Chemical analysis of the diet was performed and DM, CP, ether extract, crude fiber, ash, and starch contents of the ~~feed~~basal diet were determined according to the AOAC (2000) methods. The composition of basal diet is provided in Table 1.

alt-text: Table 1

**Table 1**

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Composition and analyzed nutrient content of basal diet (as-fed basis).

| | **Nursery diet (0 to 21 d)** |
|---|---|
| Ingredients (%) | |
| Corn meal | 50.00 |
| Soybean meal (48%) | 20.53 |
| Barley meal | 13.84 |
| Milk serum | 8.00 |
| Potato proteins | 2.50 |
| Soybean oil | 1.72 |
| Calcium carbonate | 1.08 |
| Vitamin and mineral premix[1] | 0.50 |
| Dicalcium phosphate | 0.50 |
| L-Lys HCl | 0.51 |
| DL-Met | 0.21 |
| L-Thr | 0.19 |
| L-Trp | 0.08 |
| NaCl | 0.34 |
| | |

| | |
|---|---|
| DM (%) | 89.12 |
| Nutrients (% DM) | |
| CP | 18.87 |
| Ether extract | 4.19 |
| Crude fiber | 3.64 |
| Ash | 4.64 |
| Starch | 38.50 |
| Digestible energy[2], kcal/kg | 3427 |
| Net energy[3], kcal/kg | 2545 |

**Table Footnotes**

[1] Providing per kilogram of premix: vitamin A, 3,000,000 UI; vitamin D3, 400,000 UI; vitamin E, 12,000 mg; vitamin B1, 500 mg; vitamin B2, 1200 mg; vitamin B6, 1000 mg; vitamin B12, 6 mg; vitamin C, 15,000 mg; vitamin K, 500 mg; vitamin PP, 5000 mg; D-pantotenic acid, 4000 mg; choline cloride, 69,200 mg; folic acid, 300 mg; biotin, 40 mg; Mn, 8000 mg; Fe, 40,000 mg; Cu, 20,000 mg; Co, 80 mg; I, 300 mg; Zn, 24,000 mg; Se, 20 mg; sepiolite as anti-caking agent 50,000 mg; calcium carbonate, 38.61%; wheat midding 15%).

[2] According to the equation proposed by Whittemore (1987).

[3] According to the equation proposed by Noblet et al. (1994).

Pigs health was monitored throughout the study. Piglets were individually weighed at the beginning of the study (d0) and at the end of the study (d21). Feed was provided *ad libitum* and average daily gain (**ADG**), average daily feed intake (**ADFI**) and gain to feed ratio (**G:F**) were calculated between 0 and 21 d. After 21 d, 7 pigs per treatment (with individual ADG closest to the group mean ADG) were euthanized by penetrating captive bolt followed by exsanguination to collect intestinal samples (duodenum, jejunum, ileum, and colon). Intestinal samples were cut longitudinally to expose the mucosa and washed with phosphate buffered saline (PBS) to remove mucus and digesta. Then the mucosa was scraped gently with a glass slide, vacuum-packed, immediately frozen in liquid $N_2$ and stored at −80 °C until gene expression analysis was conducted. Samples for histological investigation were instead collected and fixed in formalin until staining.

## 2.2 Intestinal histomorphometry

Intestinal samples (duodenum, jejunum, ileum, and colon) were formalin-fixed and paraffin-embedded and routinely stained (hematoxylin-eosin). Slides were coded, and morphometric measures and goblet cells counting were carried out blindly by three skilled operators. All images were acquired with an optical microscope Leica DMLB, coupled to a Leica DFC camera (Leica Microsystems GmbH, Wetzlar, Germany). The acquired images were 2088 × 1550 pixel, in Jpeg format, and the digital image analysis was conducted with ImageJ 1.46 (http://rsbweb.nih.gov/ij/download.html).

Hematoxylin-eosin stained slides were used for measurements of villous height/crypt depth by means of histomorphometry of intestinal segments. Light microscope photographs were used to measure 10 of the

tallest, best oriented villi from villous tip to crypt mouth, and 10 crypts from crypt mouth to base, adjacent to submucosa (Obj. 10X). The villi height to crypt depth ratio (V:C ratio) was calculated (King et al., 2008).

The hematoxylin-eosin stained slides were also used for goblet cells counting. In crypts, goblet cells were counted in the 5 best oriented crypts/intestinal tract, from crypt mouth to base (adjacent to submucosa). Number of goblet cells is expressed as mean number per crypt per tract and mean number of goblet cells/100 μm of crypts (mean data of crypt length). This was determined in order to supply the number of cells/crypt (anatomo-functional unit), flanked by number of goblet cells/unit length of epithelium (linear density), that is more comparable with bibliographic data (Obj. 40X), (King et al., 2008). The equation to determine the number of goblet cells/100 μm was: goblet cells/100 μm = number of goblet cells × 100/(crypt depth × 2), (King et al., 2008). In villi, goblet cells were counted in the 5 best oriented villi/intestinal tract, from villous tip to base (adjacent to crypts mouth). Number of goblet cells is expressed as mean number/villous per tract and mean number of goblet cells/100 μm of villi epithelium (mean data of villous height). This was determined in order to supply the number of cells/villous (anatomo-functional unit), flanked by number of goblet cells/unit length of epithelium (linear density), that is more comparable with bibliographic data (Obj. 40X), (King et al., 2008). The equation to determine the number of goblet cells/100 μm was: goblet cells/100 μm = number of goblet cells × 100/(villi height × 2), (King et al., 2008).

### 2.3 Inflammatory cytokines and tight junctions (TJ) components gene expression profiling

Gene expression analysis of intestinal mucosa scrapings (duodenum, jejunum, ileum and colon) was performed with a Real-Time PCR method according to Grilli et al. (2015a). Gene expression was normalized using two housekeeping genes coding for portions of porcine ribosomal subunit 60S, such as ribosomal protein L35 (**RPL35**) and ribosomal protein L4 (**RPL4**). Average $C_T$ was determined for each gene of interest (**GOI**), and geometric average was calculated for housekeeping genes (**HKs**), assuming $C_T$ as number of cycles needed to reach a fixed arbitrary threshold. Delta $C_T$ was calculated as $C_T$ GOI−$C_T$ HKs, then the $2^{-\Delta\Delta C_T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (Livak and Schmittgen, 2001).

The sequences, expected product length, accession number in the EMBL database/GenBank and references of porcine primers are shown in Table 2. Primers were obtained from Life Technologies.

alt-text: Table 2

**Table 2**

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Primers used for gene expression analysis by Real-Time PCR.

| Gene | Primer Sequence (5′ → 3′) | Product length (bp) | Product Tm (°C) | Accession number | Reference |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| **Occludin** | F: ATCAACAAAGGCAACTCT<br>R: GCAGCAGCCATGTACTCT | 157 | 83 | NM_001163647.2 | Zhang and Guo, 2009 |
| **Claudin-1** | F: TCCAGTGCAAAGTCTTCGACTCCT<br>R: ATGCCAACAGTGGCCACAAAGATG | 121 | 85.5 | NM_001244539.1 | Grilli et al., 2016 |
| **TNF-α** | F: GCCCACGTTGTAGCCAATGTCAAA<br>R: GTTGTCTTTCAGCTTCACGCCGTT | 99 | 87 | NM_214022.1 | Grilli et al., 2015b |
| **IFN-γ** | F: GGCCATTCAAAGGAGCATGGATGT<br>R: TGAGTTCACTGATGGCTTTGCGCT | 149 | 83.5 | NM_213948.1 | Grilli et al., 2015b |
| **IL-1β** | F: GGCCGCCAAGATATAACTGA<br>R: GGACCTCTGGGTATGGCTTTC | 70 | 83 | NM_214055.1 | Arce et al., 2010 |
| **RPL35** | F: AACCAGACCCAGAAAGAGAAC<br>R: TTCCGCTGCTGCTTCTTG | 146 | 87.5 | NM_214326.2 | Alexander et al., 2012 |
| **RPL4** | F: CAAGAGTAACTACAACCTTC<br>R: GAACTCTACGATGAATCTTC | 122 | 84 | XM_003121741.3 | Alexander et al., 2012 |

Tm = melting temperature; *F* = forward primer; *R* = reverse primer; TNF-α = tumor necrosis factor α; IFN-γ = interferon-γ; IL-1β = interleukin 1 beta; RPL35 = ribosomal protein L35; RPL4 = ribosomal protein L4.

### 2.4 Statistical analysis

Animals were grouped in a completely randomized design and data were analyzed with one-way ANOVA, followed by Tukey post hoc test (Graph Pad Prism 5 version 5.03; GraphPad Software, Inc, San Diego, CA). The pen was the experimental unit for growth performance, whereas the pig was the experimental unit for histomorphometry and for gene expression analysis. Differences were considered significant at $P < 0.05$ and trends for $0.05 < P \leq 0.10$.

## 3 Results

Growth performance was not affected by the treatments and the results are presented in Table 3.

alt-text: Table 3

**Table 3**

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Effect of feeding free or microencapsulated tributyrin on growth performance in weaning piglets.

| Treatment | | | | | |
|---|---|---|---|---|---|
| | control | f-TB | m-TB | SEM | *P* |
| BW (kg) | | | | | |
| d0 | 6.66 | 6.69 | 6.66 | 0.07 | 0.92 |
| d21 | 11.92 | 11.34 | 11.75 | 0.35 | 0.50 |
| | | | | | |
| 0 to 21 d | | | | | |
| ADG (g/d) | 263 | 232 | 255 | 18 | 0.45 |
| ADFI (g/d) | 439 | 416 | 442 | 22 | 0.68 |
| G:F | 0.60 | 0.56 | 0.58 | 0.02 | 0.26 |

Data are presented as means (9 pens/treatment). Control = basal diet (control, 0 mg/kg of tributyrin); f-TB = basal diet + free tributyrin at 1750 mg/kg; m-TB = basal diet + microencapsulated tributyrin at 3800 mg/kg (providing 1750 mg/kg of tributyrin); ADG = average daily gain; ADFI = average daily feed intake; G:F = gain to feed ratio.

## 3.1 Intestinal histomorphometry

Table 4 summarizes histometrical results and goblet cells counts. In the duodenum, the villi length was not affected by the treatments, whereas the crypts tended to be deeper in pigs fed microencapsulated tributyrin compared to controls (*P* = 0.09). However, no differences in V:C ratio were observed. In the jejunum, no effects on villi length, crypt depth or V:C ratio were evident. In the ileum and in the colon, there were no differences between groups.

alt-text: Table 4

**Table 4**

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Effect of feeding free or microencapsulated tributyrin on intestinal histomorphometry in weaning piglets.

| Treatment | | | | | |
|---|---|---|---|---|---|
| | control | f-TB | m-TB | SEM | *P* |
| Duodenum | | | | | |
| Villous height (μm) | 234.1 | 252.2 | 286.7 | 28.3 | 0.43 |

| | | | | | |
|---|---|---|---|---|---|
| Crypt depth (μm) | 356.7 | 376.8 | 427.8 | 20.8 | 0.09 |
| V:C | 0.66 | 0.69 | 0.62 | 0.08 | 0.83 |
| Goblet cells/100 μm villous | 1.02 | 0.43 | 1.22 | 0.25 | 0.09 |
| Goblet cells/100 μm crypt | 2.92 | 2.40 | 2.18 | 0.29 | 0.35 |
| Jejunum | | | | | |
| Villous height (μm) | 279.5 | 231.1 | 294.1 | 24.6 | 0.17 |
| Crypt depth (μm) | 253.9 | 257.3 | 276.1 | 25.9 | 0.83 |
| V:C | 1.12 | 0.93 | 0.99 | 0.09 | 0.36 |
| Goblet cells/100 μm villous | 0.15 | 0.28 | 0.55 | 0.18 | 0.62 |
| Goblet cells/100 μm crypt | 2.49 | 2.15 | 2.69 | 0.33 | 0.53 |
| Ileum | | | | | |
| Villous height (μm) | 213.6 | 240.2 | 226.7 | 19.6 | 0.64 |
| Crypt depth (μm) | 221.3 | 223.2 | 267.0 | 24.4 | 0.34 |
| V:C | 0.97[ab] | 1.19[a] | 0.89[b] | 0.09 | 0.04 |
| Goblet cells/100 μm villous | 2.25 | 1.24 | 1.03 | 0.34 | 0.06 |
| Goblet cells/100 μm crypt | 4.77 | 5.39 | 4.24 | 0.56 | 0.37 |
| Colon | | | | | |
| Crypt depth (μm) | 349.5 | 347.1 | 370.0 | 27.2 | 0.82 |
| Goblet cells/100 μm crypt | 4.42 | 4.09 | 3.08 | 0.28 | 0.08 |

Data are presented as means (7 pigs/treatment).
Control = basal diet (control, 0 mg/kg of tributyrin); f-TB = basal diet + free tributyrin at 1750 mg/kg; m-TB = basal diet + microencapsulated tributyrin at 3800 mg/kg (providing 1750 mg/kg of tributyrin); V:C = villi height to crypts depth ratio.
[a,b] Values within a row with different superscripts differ significantly at $P < 0.05$.

In the duodenum, goblet cells were not different in the crypts but in the villi tended to be reduced in the f-TB group compared to control and m-TB groups (2.4 fold less, P = 0.09). In the jejunum, no effects were observed on goblet cells numbers in villi or crypts. In the ileum, goblet cells tended to be lower in the villi of both f-TB and m-TB groups than in the control group (1.8 and 2.2 fold less, respectively; P = 0.06). In the crypts of the colon, goblet cells number tended to be reduced in m-TB group compared to both control and f-TB groups (P = 0.08).

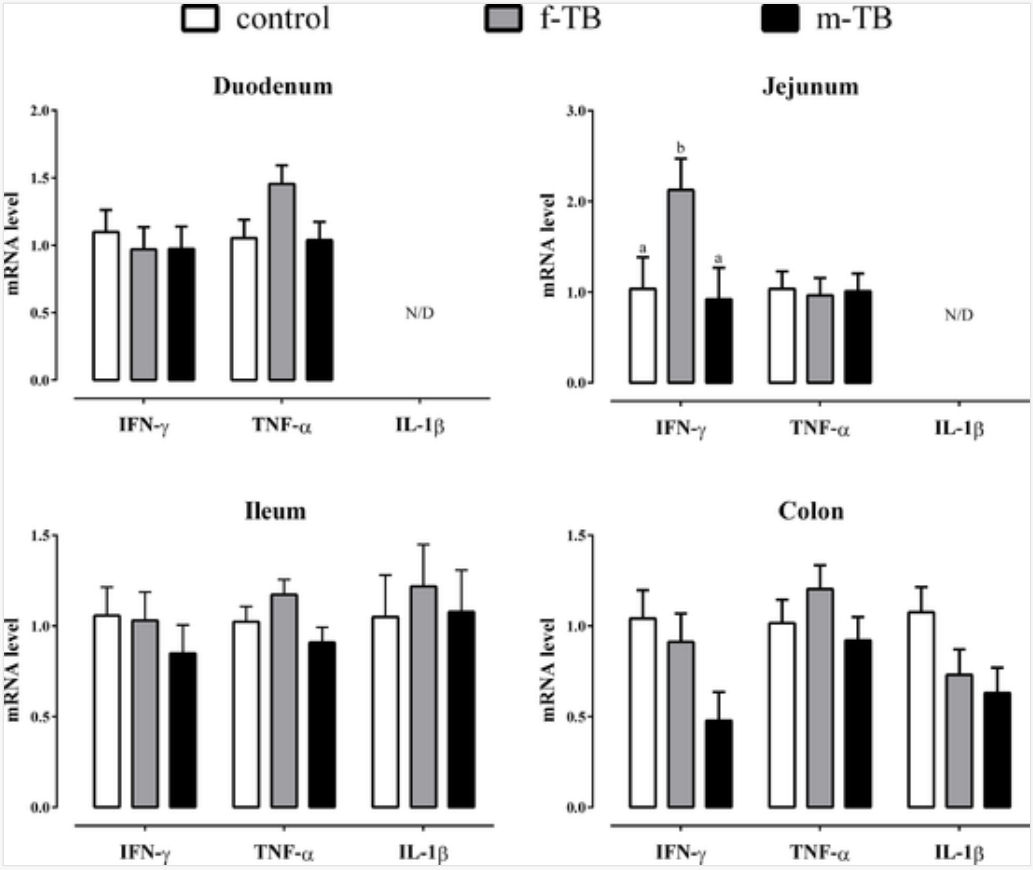
### 3.2 Inflammatory cytokines and TJ markers gene expression analysis

Inflammatory cytokines mRNA levels in ileum, duodenum, jejunum, and colon are summarized in Fig. 1. In the duodenum, IFN-γ expression was not influenced by the treatments while TNF-α tended to be higher in f-

TB group compared to control and m-TB groups (~~P~~ P = 0.06); IL-1β was not detected. In the jejunum, IFN-γ mRNA level was higher in f-TB pigs compared to both control and m-TB pigs (~~P~~ P = 0.04); TNF-α expression was not affected and IL-1β was not detected. In the ileum, no effects were observed on IFN-γ, TNF-α, and IL-1β expression. In the colon, IFN-γ level tended to be lower in m-TB pigs compared to control pigs (2.2 fold, ~~P~~ P = 0.06) while IL-1β level was not affected. TNF-α expression in the colon was not affected by treatments.~~Fig. 1~~

alt-text: Fig. 1

~~Figure~~ Fig. 1



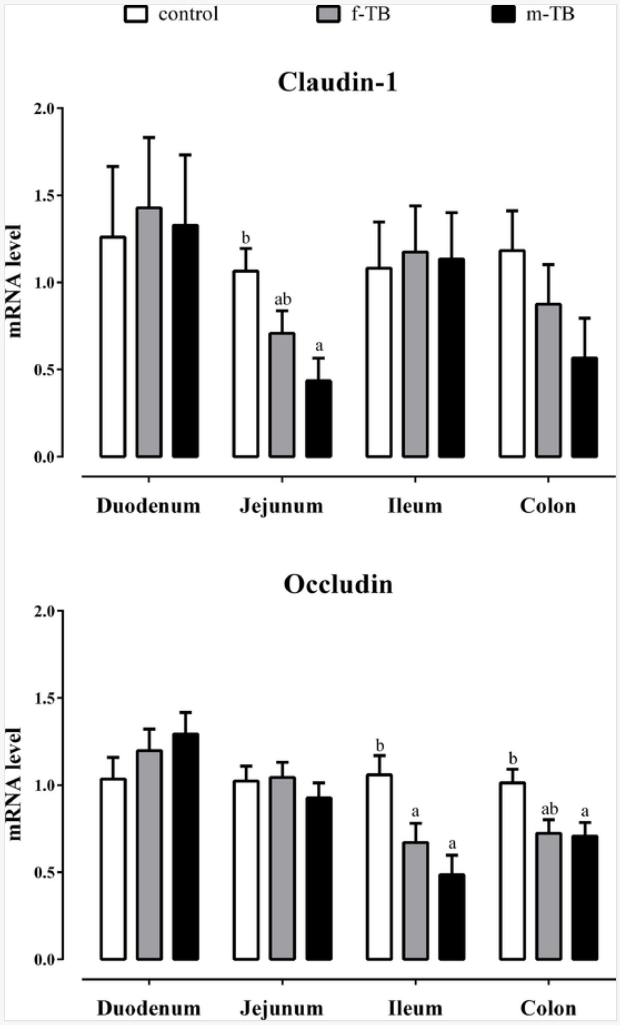

Effect of feeding free or microencapsulated tributyrin on gene expression of inflammatory cytokines in intestinal mucosa of weaning piglets. Data are expressed as means (~~n~~ n = 7 pigs/group) and SEM represented by vertical bars. [a,b] Within a gene, mean values with unlike letters were significantly different ($P < 0.05$). The $2^{-\Delta\Delta C_T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control) (Livak and Schmittgen, 2001). White bars for control; gray bars for f-TB (free tributyrin at 1750 mg/kg); black bars for m-TB (microencapsulated tributyrin at 3800 mg/kg providing 1750 mg/kg of tributyrin). N/D = not detected, TNF-α = tumor necrosis factor α; IFN-γ = interferon-γ; IL-1β = interleukin 1 beta .

Fig. 2 shows mRNA expression of some TJ components. Claudin-1 gene expression was not affected by the treatments in the duodenum and in the ileum. In the jejunum, claudin-1 mRNA level was reduced in m-TB group compared to control group (2.4 fold; $P \leq$ ~~<~~ 0.05), whereas f-TB group had intermediate value (1.5 fold less than control). No effects were evident on occludin gene expression in the duodenum and in the jejunum.

In the ileum, occludin mRNA level was reduced in both groups receiving TB compared to control group (1.6 and 2.2 fold, for f-TB and m-TB, respectively; $P \leq$ ~~<~~ 0.01). In the colon, m-TB pigs had lower occludin level than control pigs (1.4 fold, $P \leq$ ~~<~~ 0.05), whereas f-TB pigs had intermediate value.

alt-text: Fig. 2

~~Figure~~ Fig. 2




Effect of feeding free or microencapsulated tributyrin on gene expression of tight junctions markers in intestinal mucosa. Data are expressed as means (~~n~~ $n$ ~~=~~ = 7 pigs/group) and SEM represented by vertical bars. [a,b] Within an intestinal tract, mean values with unlike letters were significantly different ($P \leq$ ~~<~~ 0.05). The $2^{-\Delta\Delta C_T}$ method was used to analyze the relative expression (fold changes), calculated relative to the control group (control) (Livak and Schmittgen, 2001). White bars for control; gray bars for f-TB (free tributyrin at 1750 mg/kg); black bars for m-TB (microencapsulated tributyrin at 3800 mg/kg providing 1750 mg/kg of tributyrin .

# 4 Discussion

Butyric acid salts have been proposed as nutritional tools to control weaning-associated intestinal dysfunction and subsequent growth failure in pig production, as a result of well-known beneficial properties on the intestinal mucosa (Guilloteau et al., 2010). Despite the widespread use of these supplements, their efficacy as

growth promoters has not always been consistent, being highly dependent on several factors such as dose, physical form, supplementation duration, intestinal tract observed, and age of piglets (Lallès et al., 2009).

TB is considered to be a valid alternative to butyric acid salts given its physical and chemical stability as well as local and systemic effects in murine models and nursery/weaning pigs (Miyoshi et al., 2011; Cresci et al., 2014; Dong et al., 2016; Gu et al., 2017). Thus, the first aim of this study was to verify TB specific anti-inflammatory, barrier-improving and trophic properties along the intestinal tract of pigs at weaning. In addition, we introduced a comparison between free TB and encapsulated TB to evaluate whether it is possible to further extend potential positive effects of TB to the hindgut, by microencapsulating it in a lipid matrix.

In this study, the gene expression analysis for inflammatory cytokines showed an opposite effect of TB along the intestine: free TB was pro-inflammatory in the upper part, increasing TNF-α and IFN-γ in the small intestine (duodenum and jejunum, respectively), while microencapsulated TB tended to reduce IFN-γ distally in the colon. The anti-inflammatory action of TB in the colon, when provided in the encapsulated form, is consistent with the protective effects previously described for TB in experimentally induced colitis both in mice and pigs (Leonel et al., 2013; Hou et al., 2014) and also with the well-documented anti-inflammatory properties of butyrate (Guilloteau et al., 2010). Conversely, the stimulation of inflammatory cytokines induced by free TB in the upper gut is definitely less expected based on literature. Indeed, only very few studies reported a pro-inflammatory effect for butyrate, as indicated by an over-expression of inflammatory cytokines in human intestinal Ca~~CO~~co-2 cells or in human leukocytes treated with butyrate (Saegusa et al., 2007; Mirmonsef et al., 2012). Consistently with the present study, we found a similar result in a previous trial where butyric acid fed to weaning piglets induced an overexpression of inflammatory cytokines only in the duodenum, followed by a down-regulation more distally in the ileum and colon (Grilli et al., 2016). One possible explanation for the opposite effect of butyric acid and TB on the inflammatory status in the upper and lower intestine may be related to the different physiological role and occurrence of butyric acid along the intestine. Indeed, short chain fatty acids (SCFA) are end-products of microbial fermentations occurring mostly in the large intestine of hindgut fermenter animals such as humans and pigs. Usually SCFA are found at a molar ratio of approximately 60:25:15 for acetate:propionate:butyrate and butyrate concentrations are different through the intestinal tract, with low levels in the small intestine (3~~-~~–6 mM) and high levels in the colon (ranging from 12 to 45 mM), depending on animal species and diet (Lawhon et al., 2002; Diao et al., 2019). In our study, dietary TB might have induced an abnormal response in the upper sites of the intestine, such as the duodenum and the jejunum, normally not used to such a high load of butyrate. In the above-mentioned study by Grilli ~~and colleagues~~et al. (2016), the peak in pro-inflammatory cytokines induced by butyrate in the duodenum was also associated with a higher expression of the butyrate transporter monocarboxylate transporter 1, MCT-1, thereby suggesting an enhanced absorption of butyrate (Grilli et al., 2016). However, it remains to be established the exact role of butyrate on the inflammatory status in the small intestine and further investigations are needed.

In the present study we also looked at the impact of TB on the intestinal architecture of weaned piglets. Despite any relevant effect on the morphology (villi length, crypts depth, V:C), our most intriguing result was observed in the mucous-secreting goblet cells number where the dietary supplementation with TB tended to induce a reduction in the cells counts in a tract-specific way: free TB affected the upper intestine while the

microencapsulated form impacted the lower gut. In this regard, microencapsulation allowed TB to be gradually released along the intestinal tract and reach the large intestine, whereas the free TB appeared to be sooner available in the small intestine. This result is consistent with previous findings where slow release and targeted delivery of bio-active compounds was obtained with lipid microencapsulation (Piva et al., 2007; Grilli et al., 2010). Few studies investigated the effects of butyrate on mucous-secreting cells and an increase in goblet cells number has been reported and generally associated with improved intestinal health following the supplementation of fiber-rich diets to both pigs and rats, or after the direct dietary inclusion of sodium butyrate to pigs or broiler chickens (Tsukahara et al., 2003; Manzanilla et al., 2006; Paturi et al., 2012; Wu et al., 2018). From a functional standpoint, the reduction in the mucous-secreting cells number observed in this study may appear negative as they are a defensive mechanism, playing a pivotal role for the intestinal homeostasis. Goblet cells show a highly dynamic response to different stimuli, being either hyperactivated by several enteric infections (mainly bacterial and parasitic) or, conversely, down-regulated in case of prolonged bacterial infections, viral attacks, or chronic inflammatory diseases such as inflammatory bowel disease in humans ( Jung and Saif, 2017; Allaire et al., 2018). Particularly in case of severe bacterial infections, it has been suggested that the goblet cells depletion and consequent reduced mucous thickness may be a beneficial response as it strategically implies a reduction of mucins that could be used as nutrients by invading microbes ( Grilli et al., 2013; Allaire et al., 2018). In this study, the reduction in mucous-secreting cells number induced by TB was not correlated with any other significant change in the intestinal morphology or general health issues and growth impairment. Therefore, it seems unlikely that TB might have damaged the intestinal tissue causing goblet cells depletion. In the last decades, butyrate has been thoroughly studied for its role in modulating intestinal stem cells niche as well as colonocyte differentiation, promoting the development of an absorptive phenotype (Yu et al., 2012; Kaiko et al., 2016). Interestingly, butyrate has been shown also to inhibit *in vitro* the full differentiation into goblet cells of pre-committed cell lines, by repressing the expression of MUC2 gene, a differentiation marker of the secretory goblet cell lineage (Augenlicht et al., 2003). In this view, considering the ability of butyrate to modulate cell proliferation, it can be hypothesized that TB could somehow enhance the intestinal mucosa turnover, likely stimulating enterocytes proliferation at the expense of goblet cells.

Concerning the intestinal integrity, we found that dietary treatment with TB modulated gene expression of TJ components along the gastro-intestinal tract, reducing occludin and claudin-1 expression. Both occludin and claudin-1 are essential components of TJ, as they are trans-membrane proteins that anchor two epithelial cells and seal the inter-cellular space, thus regulating the para-cellular permeability (Turner, 2009). A reduction in TJ markers may suggest an intestinal barrier dysfunction although few previous studies showed the ability of TB to improve intestinal integrity by enhancing TJ markers expression in murine and pig models of intestinal damage (Cresci et al., 2014; Hou et al., 2014, ~~;~~ 2017). Butyrate was shown to exert a dual effect on the intestinal epithelial permeability: low doses of butyrate enhanced TJ and decreased intestinal permeability *in vitro* and *in vivo* in pigs, while high concentrations were increasing permeability both in some intestinal cell lines and in murine models (Hamer et al., 2008). It was suggested that butyrate like other nutrients (monosaccharides, amino acids, SCFA, and medium chain fatty acids), once absorbed by the intestinal cell, can stimulate a transient opening of the TJ to further allow its uptake through the paracellular route ( Luciano et al., 1984; Turner and Madara, 1995; Mariadason et al., 1999). Whether the reduced mRNA

expression of occludin and claudin-1 in our study is indicative of a transient increase in TJ permeability due to higher uptake of butyrate released from TB, still remains unclear but this mechanism can't be excluded.

To our knowledge this is the first study addressing the effect of ~~tributyrin~~TB along the whole intestinal tract of pigs and it can be concluded that TB can modulate the goblet cells number and the expression of inflammatory cytokines and TJ components, without affecting growth performance of weaning piglets. Nevertheless, these findings suggest that further in-depth studies to better disclose the physiological response of the upper intestinal tract to high doses of ~~tributyrin~~TB are needed.

## Author statement

All of the authors contributed in an equal way to the realization of this paper.

## Declaration of Competing Interest

Andrea Piva serves as professor at the University of Bologna and is a member of the board of directors of Vetagro S.p.A.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2020.103996.

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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## Highlights

- Tributyrin can be a dietary source of butyric acid for weaning pigs.
- Tributyrin modulates inflammatory cytokines and goblet cells number.
- Lipid microencapsulation allows to extend tributyrin effects to the hindgut.

## Appendix Supplementary materials

Multimedia Component 1

alt-text: Image, application 1

## Queries and Answers

**Query:** Please confirm that givennames and surnames have been identified correctly.

**Answer:** Yes