

**Butyrate enemas upregulate *Muc* genes expression but decrease
adherent mucus thickness in mice colon**

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ABSTRACT

Colonic mucosal protection is provided by the mucus gel, mainly composed of mucins. Several factors can modulate the formation and the secretion of mucins, and among them butyrate, an end-product of carbohydrate fermentation. However, the specific effect of butyrate on the various colonic mucins, and the consequences in terms of the mucus layer thickness are not known. Our aim was to determine whether butyrate modulates colonic *MUC* genes expression *in vivo* and whether this results in changes in mucus synthesis and mucus layer thickness. Mice received daily for 7 days rectal enemas of butyrate (100mM) versus saline. We demonstrated that butyrate stimulated the gene expression of both secreted (Muc2) and membrane-linked (Muc1, 3, 4) mucins. Butyrate especially induced a 6-fold increase in *Muc2* gene expression in proximal colon. However, butyrate enemas did not modify the number of epithelial cells containing the protein Muc2, and caused a 2-fold decrease in the thickness of adherent mucus layer. Further studies should help understanding whether this last phenomenon, i.e. the decrease in adherent mucus gel thickness, results in a diminished protective function or not.

KEYWORDS mucus, mucin, butyrate, colon, mice,

Abbreviations: *MUC*: human mucin gene; MUC human mucin protein; *Muc*: murine mucin gene; Muc: murine mucin protein.

INTRODUCTION

The mucus layer, covering the gastrointestinal mucosa, is considered as the first line of defence against mechanical, chemical, or microbiological aggressions arising from the luminal contents (Corfield, et al. 2000). Indeed, the break of the mucus barrier in inflamed colon has been shown to allow bacterial adherence to epithelial tissue (Swidsinski, et al. 2007), and the removal of the mucus layer favours the penetration of high molecular weight probes in mucosa (Iiboshi, et al. 1996, Khan, et al. 1999). In the colon, the mucus is mainly composed of the secreted mucin called MUC2, but other membrane-bound mucins are also expressed: mainly MUC1, MUC3 and MUC4 (Carraway, et al. 2003). The specific functions of the various mucins are still unclear, but they appear have other functions than only gel-forming. For example, it as been shown that Muc2 deficiency leads to inflammation of the colon and contributes to the onset and perpetuation of experimental colitis (Van der Sluis, et al. 2006). In addition, membrane-linked mucins, like MUC1 (Leroy, et al. 2006) and MUC4 (Rong, et al. 2005), exhibit specific functions in adhesion and cell signalling (Carraway, et al. 2003), and could take a crucial part in maintaining the integrity of the colonic barrier.

In the colon, the mucus layer is directly in contact with the short chain fatty acids (SCFA) produced by fermentation of indigestible carbohydrates by the resident microflora. These SCFA (mainly acetate, propionate and butyrate) are known to affect the colonic mucosa; more specifically, butyrate constitutes the major energy source for colonocytes (Roediger 1982); it is able to modulate both the epithelial cell proliferation (Blottiere, et al. 2003) and the intestinal immunity (Segain, et al. 2000), and thus plays a central role for colonic health (Roy, et al. 2006). However, the specific impact of butyrate has been studied only on the mucus layer and not on Muc gene expression (Kleessen, et al. 2003). SCFA, infused in colonic loop, have been shown to

stimulate mucin discharge in rats colon (Shimotoyodome, et al. 2000), and butyrate appeared to be the most effective SCFA in modulating mucin production and release *in vivo* (Barcelo, et al. 2000, Finnie, et al. 1995, Shimotoyodome, et al. 2000). In addition, butyrate could modulate mucin expression *in vitro* in human colonic cell lines both at the gene and protein level (Augenlicht, et al. 2003, Gaudier, et al. 2004, Willemsen, et al. 2003). But in all of these studies, Muc gene expression and mucin production were never related to the mucus layer, although the mucus layer thickness is considered as a main protective parameter of intestinal epithelium. Butyrate-producing carbohydrate fermentation was shown to increase the mucus layer thickness (Kleessen, et al. 2003), but in these experiments, it was not possible to discriminate the effects of butyrate production from the effects of the modifications in the colonic microflora that also take place during carbohydrate fermentation.

The aim of this study was to determine the effect of repeated instillation of butyrate *in vivo* on the expression of the different colonic mucins, and to relate it to possible modifications of the mucus layer thickness, which could result in a benefit for the colonic mucosa.

MATERIALS AND METHODS

Animals.

Male BALB/c mice (Janvier, Le Genest Saint Isle, France), 8 weeks of age, with an initial mean weight of 22.8 ± 0.2 g, were randomly housed by groups of six mice per cage, and maintained at 23°C in an animal room with a 12h light: dark cycle (light: 0700–1900 h). Food and water were consumed ad libitum. The diet followed AIN-93G standard formula (Reeves, et al. 1993) (INRA, Jouy-en-Josas, France). Body weight gain, food and water consumption were assessed every day

during the course of experiment. All experiments were carried out in accordance with the recommendations of the local Animal Care and Use Committee of Nantes (France).

Treatment with butyrate enemas.

At day 0, mice were randomly split in 2 groups (n=20 each) named control versus butyrate-treated. Every morning from day 0 to day 6, mice were mildly anaesthetised by intramuscular injection of 60 μ L of a mixture of 5 mg/ml of ketamin (Merial, Lyon, France) and 5 mg/ml of zoletyl (Virbac, Carros, France). 5 min after anesthesia, mice received rectal instillation of 100 μ l of saline or 100 mM butyrate (Sigma, Saint Quentin-Fallavier, France) by 2.4 cm steel canula for control mice or butyrate-treated mice respectively. Both solutions were adjusted to pH = 6.5 and osmolarity = 295 mosmol. Mice were kept in a vertical position head upside down for 10 min before returning to their cages, to avoid excretion of the enema solution.

Collection of digestive tissues and contents.

At the end of the 7 days experimental period, i.e. 24 h after the last butyrate instillation, the animals were killed by cervical dislocation. The colon length was first measured in a relaxed position without stretching. The proximal and distal colons were then removed; the split between proximal and distal colon was set at the half length of the colon. Colonic contents were aseptically collected and immediately used for SCFA concentration assessment (n=10 per group). Proximal and distal colonic tissues were carefully longitudinally opened and cleaned with sterile physiological serum. They were immediately frozen with 1ml of Trizol (Invitrogen Life Technologies, Cergy-Pontoise, France) in liquid nitrogen and stored at -70°C for RNA isolation (n=10 per group), or fixed in 4% formaldehyde in Dulbecco's phosphate buffered saline (Sigma), dehydrated and paraffin-embedded for histological observations (n=10 per group). In order to measure mucus layer thickness, small segments with their contents (n=10 per group) were taken

from the distal colon about 1 cm proximal to the anus, frozen in liquid nitrogen and stored at -70°C.

SCFA analysis.

Contents of proximal and distal colon were collected for SCFA analysis. After centrifugation of thawed samples (8000×g for 10 min), the supernatants were supplemented with 0.9ml of oxalic acid (0.5M) (Schafer 1995). SCFA were analysed by capillary gas-liquid chromatography (SGE BP21 capillary column: 25mx053mm, nitrogen as carrier gas : 17ml/min). The injector and detector temperature was maintained respectively at 250°C and 200°C, the oven temperature at 90°C. Samples (1µl) was introduced by splitless injection, with a split flow 50ml/min beginning 1min after injection. The concentration of butyrate was determined by comparing to a known concentration of butyrate solution (from 0.2mM to 2mM) analysed in the same chromatographic conditions.

Quantification of mucin gene expression by Real Time RT-PC

Total RNA of proximal and distal colon tissues was isolated by Trizol extraction and submitted to reverse transcription as previously described (Hoebler, et al. 2006). The amplification was performed with QuantiTect SYBR Green PCR Kit (Qiagen, Courtaboeuf, France)), using the following time and temperature profile: (i) 95°C for 5 min, (ii) 45 cycles of 1 min at 95°C, 10s at 60°C, 10s at 72°C, and (iii) a final extension at 72°C. The sequences of the primers used for *Muc1*, *Muc2*, *Muc3*, and *Muc4* gene amplification have been previously indicated (Hoebler, et al. 2006). Gene expressions in each segment of butyrate-treated mice were expressed by using calibrator gene (18s) and as relative values compared to values of saline-treated mice.

Analysis of Muc2 protein expression by immunohistochemistry.

The staining procedure was conducted using an automated immunostainer (ES, Ventana medical Systems, Strasbourg, France) and a three-step indirect process based on the biotin-streptavidin-peroxidase method. Tissue sections (8 μ m) were deparaffinized with xylene (Merck KGaA, Darmstadt, Germany) and rehydrated in graded ethanol solutions. After pressure cooker pre-treatment in citrate buffer (pH 6.0) for 1 minute 30, tissue sections were incubated for 20 minutes with fresh 3 % hydrogen peroxide in methanol to block endogenous peroxidase. Sections were then treated with periodic acid (20 mM in sodium acetate 0.05 M, pH 5.0) for 30 minutes and with glycine 1% for 30 sec to destroy glycotopes containing N-acetylgalactosamine or fucose as previously described (Bara, et al. 1992, Cao, et al. 1997). All sections were incubated for 32 min at 37°C with the primary antibody anti-Muc2 (1/20) (Muc2 goat polyclonal IgG, Santa Cruz Biotechnology, California, USA). After washing, the sections were incubated with the biotinylated secondary antibody (1/500) (Biotin-SP-conjugated AffiniPure Donkey anti-goat IgG, Jackson ImmunoResearch Laboratories, Soham, UK) for 8 minutes at 37°C, with streptavidine-peroxidase conjugate, and then developed with diaminobenzidine (Sigma) in 0.03 % hydrogen peroxide. Sections were then counterstained using hematoxylin and observed under Nikon microscope. For quantitative analysis of Muc2-positive cells, ten crypts were chosen in well-oriented sections of caecum, proximal and distal colonic sections of mice as previously described (Verburg, et al. 2000). The number of Muc2-positive cells found in proximal colon and distal colon of control mice and butyrate-treated mice were compared.

Histological morphometry of adherent mucus thickness in distal colon.

For the measurement of mucus layer thickness, transverse sections (20 μ m) of frozen distal colon including pellets were cut with a cryostat (Microm HM 500OM, Carl Zeiss, Oberkochen, Germany) and fixed on SuperFrost Plus Gold slides (Menzel-Glaser, Braunschweig, Germany)

(Szentkuti and Lorenz 1995). Sections were stained with AB pH 2.5-PAS (Jordan, et al. 1998). Microscope images (Nikon, Japan) of the stained sections were analysed with Lucia software (Laboratory Imaging Ltd, Praha, Cze Republic). The adherent mucus layer thickness was measured at five points of the circumference of six different sections for each mouse. The mean of these thirty measurements was considered as the adherent mucus thickness for each mouse.

Statistical analysis.

Statistical analysis was performed using the Statview 5.0 package (SAS Institute, Berkeley, CA). Student T-test was used to assess the effects of butyrate enemas versus saline enemas, with differences being considered as significant if $P < 0.05$. Data are expressed as mean \pm SEM.

RESULTS

Effect of butyrate enema

After 7 days of instillation of saline or butyrate, no difference was found between the 2 groups of mice in terms of food and water intake, as well as weight gain (1.28 ± 0.23 g in the saline group vs. 1.28 ± 0.12 g in the butyrate group).

Colonic characteristics were also not different between the 2 groups: colon length did not vary between saline- and butyrate-treated mice (9.0 ± 0.2 cm and 9.2 ± 0.2 cm, respectively), and SCFA amounts in the proximal colon were not affected by the rectal butyrate enema. More interestingly, SCFA concentrations in the distal contents were also not modified by the repetitive instillation of butyrate: indeed in butyrate-treated mice vs. saline treated mice, SCFA concentrations were 54 ± 10 vs 41 ± 6 $\mu\text{mol/g}$ of wet content of acetate, concentrations were 10 ± 2 vs 8 ± 1 $\mu\text{mol/g}$ of wet content of propionate and 5 ± 1 vs 4 ± 1 $\mu\text{mol/g}$ of wet content of butyrate.

Quantification of Muc gene expression in colon.

In distal colon, butyrate enemas significantly increased *Muc3* expression ($p=0.011$), and its stimulation of *Muc1* and *Muc2* was close to significance ($p=0.08$). Surprisingly, butyrate enhancing effects were greater in proximal colon than in distal colon, with three gene expressions significantly enhanced: *Muc1*, *Muc2* and *Muc4*, ($p=0.020$, $p=0.003$ and $p=0.008$, respectively) (Fig. 1). If we compare the four major Muc genes expressed in colon, butyrate enhanced preferentially *Muc2* expression, with a 6-fold increase and then *Muc4* expression, with 4.7-fold increase, both in the proximal colon. *Muc1* expression was enhanced by butyrate mainly in the proximal colon by a marked factor of 3.2, and *Muc3* expression was stimulated by butyrate only in the distal colon, by a moderate factor of 2.3.

Evaluation of Muc2 protein expression by immunohistochemistry.

Butyrate enemas caused no significant change in the number of Muc2-positive cells in mucosal crypts in both proximal and distal colon (Table 1 & Fig. 2), but we cannot assess that there was no changes in Muc2 protein.

Assessment of adherent mucus thickness in distal colon.

Butyrate enemas caused a significant 2.5-fold decrease in adherent mucus thickness in distal colon ($12 \pm 3 \mu\text{m}$), in comparison with saline enemas ($30 \pm 2 \mu\text{m}$) ($p=0.0001$) (Fig. 3).

DISCUSSION

The increase of *MUC* gene expression induced by butyrate has been always evaluated in *in vitro* conditions with colon cancer cell line (Augenlicht, et al. 2003, Gaudier, et al. 2004, Hatayama, et al. 2007). Moreover, the mucus layer of distal colon has been already shown to be increased by the fermentation of indigestible carbohydrate producing high concentration of

1 butyrate (Kleessen, et al. 2003). The aim of this work was to evaluate the specific *in vivo* effect of
2 butyrate on colonic mucin gene expression, and to relate it to the mucus layer thickness in mice.

3 We chose to administrate butyrate through rectal enemas, because it allowed a repeated
4 administration of butyrate for several days. The enema method has the disadvantage to inject free
5 water in the distal colon, but the volume of instillation was low (100 µl) and we did not observe
6 any diarrhoea in both groups of animals (butyrate *vs.* saline), which supports the absence of major
7 intestinal disturbance. We checked with an alcian-blue dye that the enema solution reached the
8 whole distal colon. However, as it was impossible to control the diffusion of butyrate in colonic
9 contents, we analysed the effect on mucin production in both proximal and distal segments. We
10 chose to use a 100 mM butyrate solution, because similar concentrations were used in rats studies
11 demonstrating a stimulation of mucin secretion by butyrate (Barcelo, et al. 2000,
12 Shimotoyodome, et al. 2000). This butyrate concentration is relatively high in comparison to
13 what was measured in colonic content (5mol/g of wet content). But, it is known that butyrate is
14 rapidly absorbed by the mucosa (Roediger 1982) and that the concentration determined in
15 contents is estimated to correspond only to a weak part of the total amount produced during the
16 fermentation (Mortensen and Clausen 1996). The fact that we do not recover an extra amount of
17 butyrate in colonic contents 24h after enemas suggests that the instilled dose of butyrate was low
18 enough to be fully absorbed by the mucosa.

19 We demonstrated for the first time *in vivo* that butyrate affects differently the expression
20 on the various colonic mucins. Gene expression of the secreted mucin *Muc2* was increased to the
21 highest extent (6-fold) in the colon. Among membrane-linked mucins, *Muc 1 and Muc 4* gene
22 expression was significantly stimulated mainly in proximal colon, while *Muc3* had the expression
23 slightly enhanced in distal colon. As butyrate is well known to be able to modulate proliferation
24 and differentiation of colonocytes (Frankel, et al. 1994), it can be wondered whether its effects

on mucin gene expression are simply resulting from such effects on the colonic epithelium. First, our results concerning *Muc* gene upregulation by butyrate cannot be attributed to a stimulation in cell proliferation because we quantified *Muc* gene expression in comparison with 18s RNA, which allows to assess the level of *Muc* mRNA independently of the number of cells. Second, the distinct effect of butyrate on the secreted Muc 2 (expressed only in goblet cells) vs. the membrane-bounds Muc1, 3 and 4 (expressed in both goblet cells and absorptive cells) could be due to selective stimulation of the differentiation into goblets cells. However, our immunohistochemistry results showed that butyrate enema did not increase the number of Muc2-positive goblet-cells per crypt. So we can interpret our results as an increase in the mucin gene expression per cell by butyrate, which is in accordance with previous *in vitro* results showing effects of butyrate on *MUC* gene expression (Gaudier, et al. 2004, Hatayama, et al. 2007).

Another striking result of our study was that the stimulation of mucin gene expression by butyrate was seen not only at the site of instillation (distal colon), but also in the proximal colon. Remote effect of butyrate has been shown for several functions (tropic effect, intestinal motility, gastric tonus) through enteric nervous system and hormonal mediation (Frankel, et al. 1994, Ropert, et al. 1996). Moreover, it is known that the stimulation of mucus secretion is also mediated by enteric nervous system, enterodocrine cells and mediators of the immune system (Plaisancie, et al. 1998). Thus, we can assume that local and remote stimulation of mucin secretion by butyrate probably operate at the same time, but the proportion of neurally-mediated indirect stimulation vs. direct stimulation is so far unknown.

However, the main question raised by our study is whether the striking stimulation of Muc2 gene expression by butyrate results in changes in the mucus layer and its protective functions. Regarding our absolute values of mucus layer thickness, it has to be mentioned that the histochemistry method can only measure the adherent mucus thickness and our values (30 μ m)

are similar to those found in distal colon of rats ($30 \pm 15 \mu\text{m}$) using the same histochemical method (Szentkuti and Lorenz 1995). Other studies have found higher mucus thickness values measuring both the adherent and the total colonic mucus layer in rat after overnight fasting and using *in vivo* system (miropipette technique and intravital microscopy) (Atuma, et al. 2001, Brownlee, et al. 2003). The observed upregulation of mucin gene expression caused by butyrate *in vivo* is consistent with the increase in mucin synthesis in human colonic biopsies demonstrated by Finnie et al. (1995) and the short-term effect of butyrate on the release of mucus from storage vesicles into the colonic lumen (Barcelo, et al. 2000, Sakata and Setoyama 1995, Shimotoyodome, et al. 2000). Surprisingly, the increase in *Muc* gene expression induced by repeated butyrate exposures, without changing the amount of Muc2-positive cell in the colonic mucosa, resulted in a decrease about 2 fold of the adherent mucus thickness. These results are not in agreement with the increase the adherent mucus gel thickness observed in the colon of rat fed diet rich in butyrate-producing indigestible carbohydrate (Kleessen, et al. 2003). The major difference in this intervention is that diet supplementation in fructans not only increases the production of butyrate, but also modifies the microflora composition by selectively stimulating the growth of lactic acid bacteria (Gibson and Roberfroid 1995), which could influence MUC genes expression (Mack, et al. 1999) and the mucin synthesis (Caballero-Franco, et al. 2007).

In our experiments, it is unclear why the stimulating effect of butyrate on Muc gene expression did not lead to an increase of the adherent mucus layer thickness. Although we cannot definitively exclude that 24 hours of delay after butyrate administration is too long and we were out of butyrate effect on mucus gel, two explanations can be raised: firstly, the increase in mucin gene expression could be accompanied with stimulation of the mucin secretion rate, which would prevent them from stabilizing and forming a gel; the mucins would be sloughed off in the colonic

contents by the transit. A second explanation could be that an increase in Muc gene expression would not lead to mucin synthesis or that an increase of mucin would also be accompanied by altered glycosylation. We previously found that the upregulation of *Muc* gene expression was not accompanied by marked changes in the expression of glycosyl-transferases (Gaudier, et al. 2004), which could result in alterations of mucin glycosylation, leading to changes in physico-chemical properties and gel-forming ability

Finally, it can be wondered whether the decrease in the adherent mucus layer thickness caused by butyrate is detrimental or beneficial for the colonic health. It is generally admitted that the mucus layer is a physical barrier protecting the mucosa (Sakamoto, et al. 2004) and it can thus be postulated that a thicker mucus is more protective to ensure efficient barrier diffusion (Iiboshi, et al. 1996). But mucus layer prevents the contact with epithelium through several mechanism, e.g. hydrophobicity of mucus layer (Lugea, et al. 2000), bacterial adherence (Vesterlund, et al. 2006). In view of our present results, another mechanism can be evoked: the mucus released in the colonic lumen and sloughed off with the transit could serve as a wash out for the mucosa, could take off with itself possible toxic compounds or pathogens microorganisms and prevent them from reaching the mucosa. It is then not excluded that an increase in secretion of soluble mucins possibly caused by butyrate could constitute a defence mechanism of the mucosa, all maintaining a stimulated gene expression and mucin production to keep a defense mucus layer.

In conclusion, this work for the first time demonstrated *in vivo* that butyrate differently stimulates the expression of various mucin genes in the colon. The expression of the secreted *Muc2* was enhanced to the largest extend, but membrane-linked *Muc1*, *Muc3* and *Muc4* were also moderately stimulated. Surprisingly, this resulted in no change in the number of *Muc2*-positive cells in the ceco-colonic mucosa and in a decrease in adherent mucus gel thickness. Further

studies should help understanding whether this last phenomenon, i.e. the decrease in adherent mucus gel thickness, results in a diminished protective function or not

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- 4

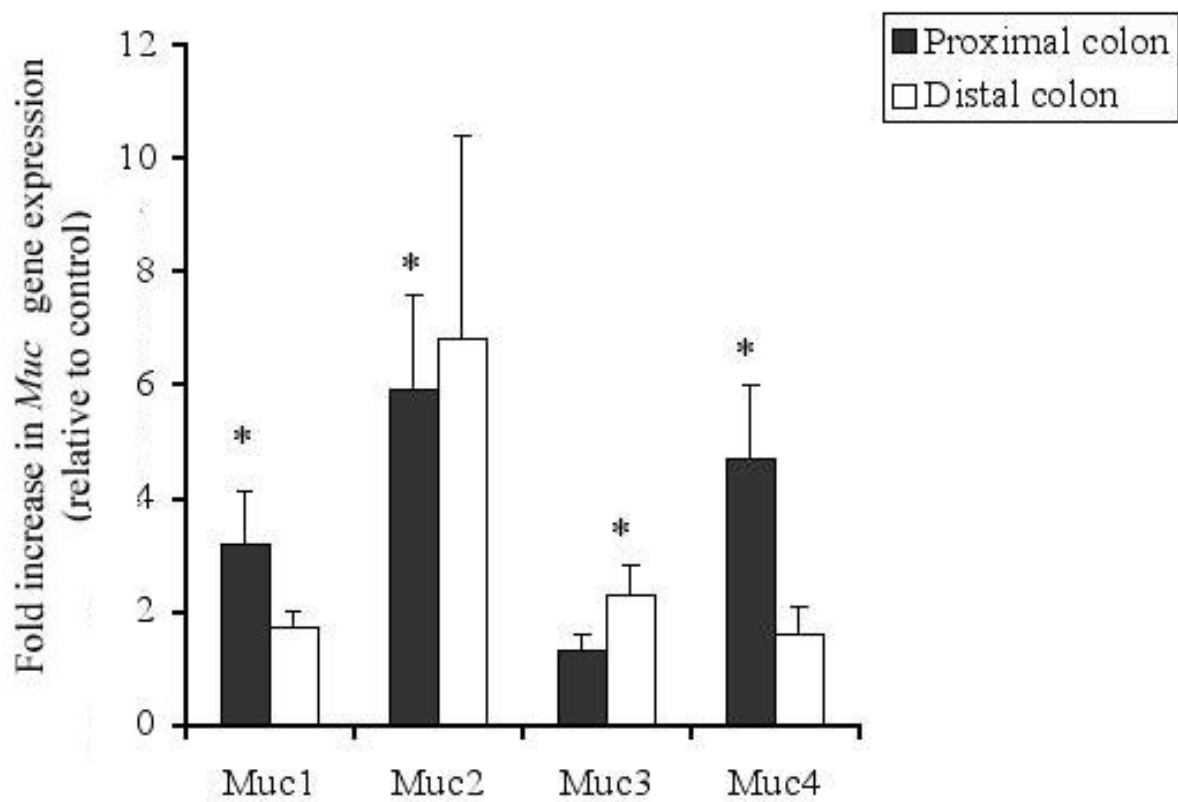
Table 1: *Number of Muc2-positive cells per crypt (immunohistochemistry) in the colon of mice who received saline enema versus butyrate enemas.*

	Saline	Butyrate	Student's T test
Proximal colon	4.8 ± 1.7	3.8 ± 2.2	NS
Distal colon	4.3 ± 1.3	4.1 ± 1.0	NS

Data are means ± SEM (n=10).

1 Fig 1: Muc (1,2,3,4) gene expression in the proximal colon and distal colon of mice treated with
2 saline vs butyrate enemas. Total RNA was isolated from colonic tissue. The expression of Muc
3 gene was determined by real-time quantitative PCR. Values are expressed as the relative gene
4 expression determined in each segment of butyrate-treated mice compared to saline-treated mice.
5 Values are means and SEM (n=10; significant difference by Student's t test, * : P<0.05).

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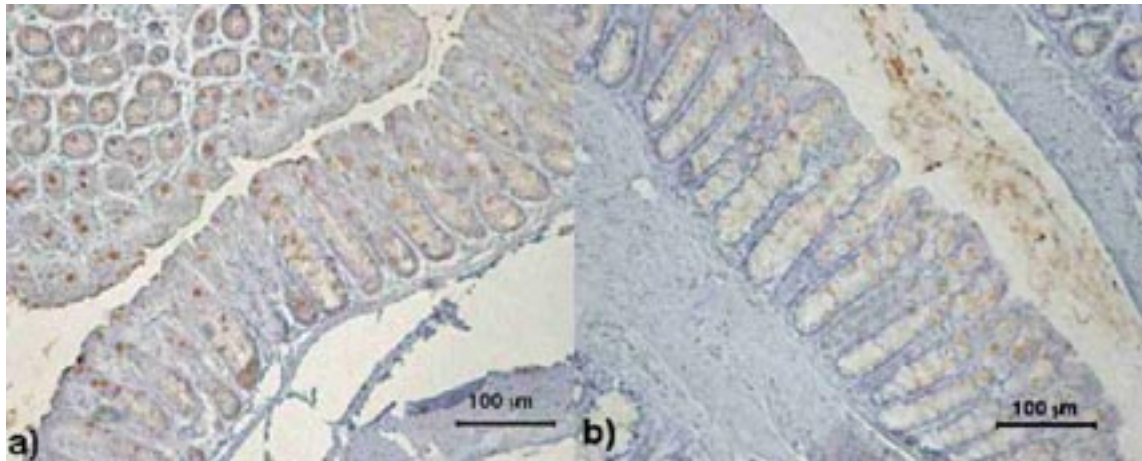
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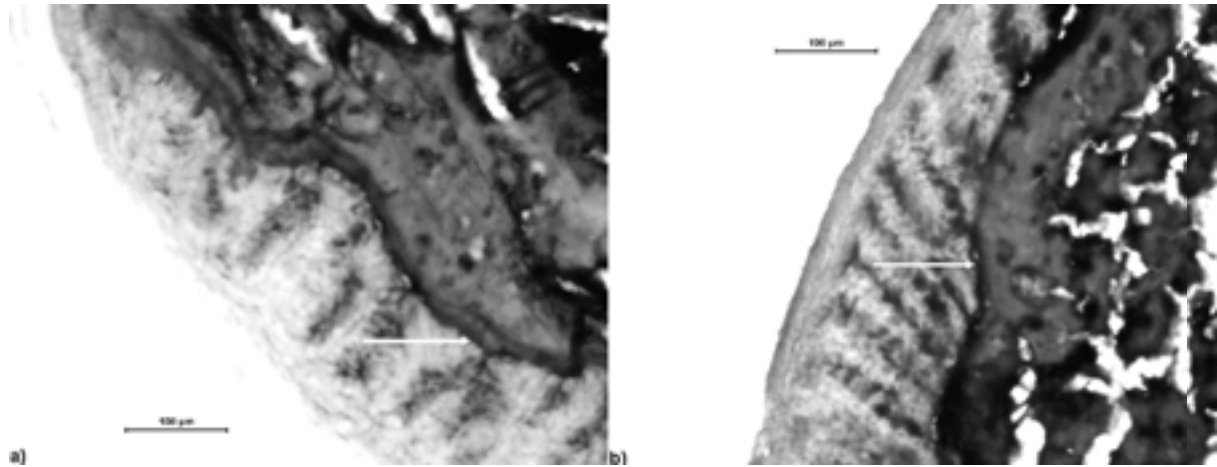
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1 Fig. 2: Goblet cell-specific expression of Muc2 in the distal colon of mice treated with saline (*a*)
2 versus butyrate enemas (*b*). Muc2 staining was performed on paraffin-embedded sections using an
3 anti-Muc2 polyclonal antibody (Santa Cruz Biotech) and hematoxylin as counterstained. Original
4 magnification: X20.



1

2 Fig. 3: PAS-Alcian blue stained sections of distal colon of mice treated with saline (*a*) vs butyrate
3 (*b*) enemas. White arrows point out the adherent mucus layer. Bar = 100 μ m. Original
4 magnification : X20.



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