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Issue: *Barriers and Channels Formed by Tight Junction Proteins***Microbial butyrate and its role for barrier function in the gastrointestinal tract**Svenja Plöger,<sup>1</sup> Friederike Stumpff,<sup>1</sup> Gregory B. Penner,<sup>2</sup> Jörg-Dieter Schulzke,<sup>3</sup> Gotthold Gäbel,<sup>4</sup> Holger Martens,<sup>1</sup> Zanning Shen,<sup>5</sup> Dorothee Günzel,<sup>6</sup> and Joerg R. Aschenbach<sup>1</sup>

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**Butyrate production in the large intestine and ruminant forestomach depends on bacterial butyryl-CoA/acetate-CoA transferase activity and is highest when fermentable fiber and nonstructural carbohydrates are balanced. Gastrointestinal epithelia seem to use butyrate and butyrate-induced endocrine signals to adapt proliferation, apoptosis, and differentiation to the growth of the bacterial community. Butyrate has a potential clinical application in the treatment of inflammatory bowel disease (IBD; ulcerative colitis). Via inhibited release of tumor necrosis factor  $\alpha$  and interleukin 13 and inhibition of histone deacetylase, butyrate may contribute to the restoration of the tight junction barrier in IBD by affecting the expression of claudin-2, occludin, cingulin, and zonula occludens proteins (ZO-1, ZO-2). Further evaluation of the molecular events that link butyrate to an improved tight junction structure will allow for the elucidation of the cofactors affecting the reliability of butyrate as a clinical treatment tool.**

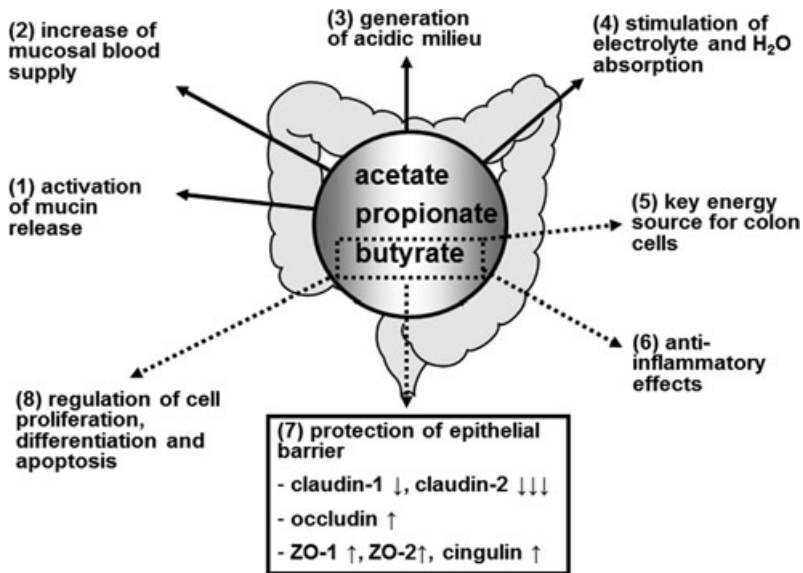
**Keywords:** butyrate; colon; inflammatory bowel disease; rumen; tight junction

**Introduction**

Acetate, propionate, and butyrate are the three major short-chain fatty acids (SCFA) produced during bacterial carbohydrate fermentation.<sup>1,2</sup> Butyrate is the least abundant but also the most dynamic of these three acids, varying from ~5% to more than 20% of total fermentation acids.<sup>3</sup> Maximum butyrate fermentation is achieved when degradable fiber and degradable starch coincide in a balanced way (see next section), whereby butyrate might be considered a signal molecule for balanced bacterial growth. The butyrate signal is apparently received and utilized by the mammalian host to adapt gastrointestinal functions to the growth of the bacterial community (Fig. 1).<sup>4,5</sup>

Some of the gastrointestinal effects of butyrate have clinical implications. For example, butyrate induces epithelial proliferation in normal intestinal tissue<sup>6,7</sup> but decreases cell proliferation, in-

creases apoptosis, and stimulates cell differentiation in colonic cancer cells,<sup>8–12</sup> which may minimize the incidence and progression of colon cancer.<sup>4,9,13</sup> Butyrate also stimulates NaCl absorption in the rat distal colon<sup>14,15</sup> and inhibits the prosecretory action of several cAMP-generating secretagogues, which can be beneficial in the treatment of diarrheal disorders.<sup>8,14</sup> Finally, butyrate may improve the barrier function of gastrointestinal epithelia<sup>16,17</sup> and thus ameliorate those diarrheal disorders that are sustained by barrier failure, for example, inflammatory bowel disease (IBD).<sup>8,18</sup> Due to the great importance of IBD, this last aspect of butyrate action is receiving increasing attention and will be the focus of this review. The main intention is to analyze the current knowledge on the effect of butyrate on the paracellular tight junction (TJ) barrier and the molecular events that link butyrate generation by luminal microbes to an improved TJ structure.



**Figure 1.** Effects of butyrate in the gastrointestinal tract. Acetate, propionate, and butyrate are produced by microbes in fermentative organs. All three acids have beneficial effects for gut health by stimulating mucin release in mucosecretory organs (1), increasing mucosal blood flow (2), the generation of an acidic milieu (3), and also by stimulation of electrolyte and water absorption (4). While butyrate is already more potent than acetate and propionate for some of these effects (e.g., 2 and 4), it has additional beneficial effects that are either attributed to the specific utilization of butyrate for epithelial cell metabolism (5) or to butyrate-inhibited histone deacetylation. The latter effect is involved in the regulation of cell proliferation, differentiation, and apoptosis (8) that is critical in the prevention of colon cancer, as well as the anti-inflammatory (6) and barrier-preserving actions (7) that are critical in the prevention and therapy of IBD.

In an attempt at interspecies comparison, the analysis not only includes butyrate effects in the large intestine but also butyrate generation and effects in the forestomach of ruminants. The rumen is interesting for comparative research because partly different concepts of butyrate action have been postulated for this gastrointestinal compartment.

### Fermentation conditions favoring microbial butyrate production

#### *Microbial and biochemical presuppositions of butyrate fermentation*

Acetate is the dominating SCFA under almost all fermentation conditions and is always present in the highest concentration relative to other SCFA in ruminal fluid<sup>19,20</sup> and intestinal contents.<sup>19,21</sup> The percentage of acetate is highest when the rate of carbohydrate degradation is slow.<sup>19</sup> In situations where there is abundant supply of rapidly fermentable carbohydrates, such as starch or sugars, fermentation end-product accumulation shifts toward propionate, and if such excessive fermentation conditions persist, including increased

acidity, lactate production and accumulation is promoted.<sup>22</sup>

Butyrate production and accumulation, on the other hand, seems to rise when high-fiber degradability and high availability of nonstructural carbohydrates coincide. This may be related to the use of acetate as a precursor for butyrate synthesis under conditions where lactate fermentation could occur.<sup>23</sup> Under those conditions, lactate-producing bacteria like *Butyrivibrio fibrosolvens* may utilize acetate directly for butyrate production via butyryl-CoA/acetate-CoA transferase rather than through the conversion of two acetyl-CoA molecules to acetoacetyl-CoA.<sup>24</sup> The replenishing of NAD<sup>+</sup> via the butyryl-CoA/acetate-CoA pathway has significant energetic benefits for the involved bacteria.<sup>25</sup> These bacteria are strictly anaerobic, Gram-positive firmicutes and include *Faecalibacterium prausnitzii*, *Butyrivibrio fibrosolvens*, *Eubacterium rectale*, *Roseburia faecis*, and *Eubacterium hallii*.<sup>25–27</sup>

#### *Nutritional modulation of butyrate fermentation*

While the biochemistry of butyrate production is rather well understood,<sup>25</sup> achieving elevated

butyrate concentrations in digesta contents is not as easily achieved. In this regard, the rumen is a comparatively simple model because nutrients are directly introduced into this compartment without prior modulation by small intestinal absorption. An analysis of a large data set for ruminal metabolism showed positive correlations of butyrate production with the sugar and fermentable neutral detergent fiber contents of the diet.<sup>28</sup> Dietary lactose addition appears to be a particularly efficient measure to increase the molar proportion of butyrate in ruminal fluid.<sup>29,30</sup>

In monogastric species, dietary sugars usually do not reach the large intestine due to small intestinal digestion and absorption. Alternatively, soluble fiber in combination with slowly degradable starch (e.g., corn starch) appears to promote large intestinal butyrate production. Rehman *et al.* fed chicks a basal diet (control) primarily consisting of corn and soya combined with either additional inulin, sucrose, or the combination of inulin and sucrose. Their study revealed that providing inulin or inulin with sucrose increased cecal butyrate concentration relative to the control, while sucrose alone failed to increase butyrate concentration.<sup>31</sup> Similarly, Metzler-Zebeli *et al.* observed increasing butyrate concentrations in the stomach, caecum, and colon of young pigs when supplementing a corn starch-based diet with high amounts of  $\beta$ -glucan.<sup>32</sup> These results underline that the coincidence of easily fermentable fiber and non-structural carbohydrates are a common prerequisite for high rates of butyrate fermentation. However, the nutritional strategy to achieve such conditions has to consider the site of digesta fermentation.

## Role of butyrate for barrier function of mucosecretory gastrointestinal mucosa

### *Butyrate and IBD*

Beneficial effects of butyrate have been suggested in acute gastroenteritis, cholera, congenital chloride diarrhea, and, most often, in IBD.<sup>8</sup> The special consideration of butyrate in IBD could be linked to the prominent involvement of TJ lesions in this disease complex. The two types of IBD, Crohn's disease (CD) and ulcerative colitis (UC), are characterized by leak flux diarrhea with loss of plasma/interstitial fluid into the gut lumen due to an insufficient TJ. The main feature of UC is a reduction in the number of TJ strands,<sup>33</sup> while CD additionally involves TJ breaks.<sup>34</sup>

The ongoing inflammation has been suggested to be a crucial factor triggering TJ leakiness in IBD.<sup>35</sup> On the other hand, it is well known that butyrate enemas can alleviate inflammation in patients with UC.<sup>36</sup> In an attempt to verify the positive effects of butyrate on the colonic barrier in IBD, Venkatraman *et al.* harvested colonic epithelia from the rat model of IBD, dextran sulfate sodium (DSS)-induced colitis. Butyrate treatment of those isolated colonic sheets led to a recovery in transepithelial resistance ( $R^t$ ), which was ascribed to a preservation of TJ integrity and inhibition of TNF- $\alpha$  release.<sup>37</sup> Other studies supported this assumption by demonstrating that butyrate inhibits the release of TNF- $\alpha$ <sup>38</sup> and IL-13, the latter being a key effector cytokine of UC.<sup>39</sup> Common to both TNF- $\alpha$  and IL-13 is their ability to upregulate the expression of the TJ molecule claudin-2.<sup>40</sup> Claudin-2 is a cation-selective pore<sup>41</sup> that is also upregulated in patients with IBD, where it explains at least part of the disturbances in barrier function.<sup>35</sup> One major effect of butyrate is to downregulate claudin-2 expression, as shown by microarray analysis in butyrate-treated colonic epithelial cells.<sup>42</sup> This suggests a relationship between the alleviation of inflammation by butyrate, the downregulation of claudin-2, and the improvement of barrier function.

Another study demonstrated a decrease of butyrate absorption in DSS mice and patients with IBD. This was mediated by a reduced expression of the butyrate transporter, monocarboxylate transporter (MCT)-1. A similar reduction in MCT-1 expression could be induced by TNF- $\alpha$  in HT-29 cells.<sup>43</sup> These findings support an alternative concept in which inflammation during IBD initially targets on MCT-1 to decrease butyrate uptake. The low intracellular availability of butyrate would, subsequently, result in a loss of the regulatory depression of claudin-2 expression by butyrate. However, further experiments are needed to clearly delineate the sequence of events that underlie the effects of butyrate on the TJs, especially on claudin-2 expression.

## Influence of butyrate on barrier function and TJ integrity

While many studies see the merit of butyrate treatment on TJ integrity mainly in its immune-modulatory and anti-inflammatory action, *in vitro* studies showed that barrier function

of intestinal epithelial cells can also be enhanced by butyrate in the absence of concurrent immune stimulation. Nevertheless, the results are equivocal. While butyrate promoted a higher  $R^t$  and a reduced mannitol permeability in Caco-2 cells,<sup>16</sup> a study by Suzuki *et al.* showed a short-term increase in  $R^t$  induced by a mix of SCFA, but not of butyrate alone.<sup>44</sup> Those discrepancies may depend on other modulating factors and on the dose of butyrate. With regard to the latter, Peng *et al.* observed an increased  $R^t$  in Caco-2 cells after treatment with 2 mmol/L butyrate and attributed this effect to a reorganization of the TJ molecules ZO-1 and occludin via activation of the AMP-activated protein kinase (AMPK).<sup>17</sup> However, higher concentrations of butyrate (8 mmol/L) decreased  $R^t$ , which was associated with a marked increase in the apoptosis level of Caco-2 cells.<sup>45</sup>

The ability of butyrate to modulate TJ protein expression was also demonstrated in noncolonic cell models. For example, Harten *et al.*<sup>46</sup> showed a restored epithelial barrier after sodium butyrate treatment in a tumor suppressor gene *VHL*-defective renal epithelial cell line that normally shows disrupted TJ structure. The protein concentrations of E-cadherin, occluding, and claudin-1 were increased after butyrate treatment, and TJ labeling for ZO-1, occludin, and claudin-1 was restored. These effects were assumed to be linked to a decreased HIF-1 $\alpha$  expression and an ability of butyrate to act as histone deacetylase inhibitor (HDACi).<sup>46</sup> Furthermore, butyrate also upregulated cingulin, ZO-1, and ZO-2 in Rat-1 fibroblasts, cingulin in COS-7 cells, and cingulin and occludin in HeLa cells.<sup>47</sup> The authors also attributed the results to an HDACi action of butyrate.<sup>47</sup>

### *Regulation of cell processes by butyrate acting as HDACi*

As already indicated in the previous section, butyrate may influence cell processes based on its action as an HDACi.<sup>12,47,48</sup> In many cases, this leads to hyperacetylation of histones that is followed by increased gene expression. On the other hand, a decrease in histone acetylation by butyrate, especially in downregulated genes, could also be demonstrated.<sup>49</sup> Either way, gene regulation by butyrate is very often the result of changes in histone acetylation. For example, in the gastric cancer cell line HGC-27, butyrate and the known HDACi trichostatin A (TSA) both enhanced vitamin D-induced

apoptosis by upregulating the gene PTEN. This was based on an increased histone acetylation level at the PTEN-promoter, which promoted the binding of the transcription factor Egr-1.<sup>50</sup>

Transcriptional regulation of gene expression by butyrate also plays an important role in cell differentiation. Gaschott *et al.* showed a synergism between butyrate and vitamin D receptor (VDR) activation on cell differentiation in the colon cancer cell line Caco-2.<sup>51,52</sup> Cell differentiation implies the expression of TJ proteins in intestinal epithelial cells.<sup>53,54</sup> So far, however, no direct link has been established between the expression of the vitamin D receptor and the expression of TJ proteins in the intestine. Nonetheless, butyrate action as HDACi seems to play a role in the regulation of TJ protein expression. For example, claudin-1 overexpression induced by increased histone deacetylation is linked to dedifferentiation and increased invasion in colon cancer. Claudin-1 overexpression can be reversed by butyrate or TSA, which decrease claudin-1 mRNA half-life time through their HDACi action.<sup>55</sup> Moreover, the downregulation of claudin-2 by butyrate mentioned earlier in this review<sup>42</sup> depends on a reduced binding affinity of transcription factors within the claudin-2 promoter.<sup>56</sup> It has been recently shown that TSA and butyrate similarly decreased claudin-2 expression in an intestinal cell line, which points to the possibility that this butyrate effect may also be dependent on altered histone acetylation.<sup>56</sup>

### **Role of butyrate for barrier function of cutaneous gastrointestinal mucosa**

#### *The forestomach as a model*

Although current research on the interaction of butyrate with epithelia is focused largely on the colon, far larger quantities of SCFA are produced in certain species that ferment forages in a forestomach, with production of butyrate alone estimated at approximately 5–10 mol/day in high-yielding dairy cows.<sup>2,3,19</sup> Accordingly, both the transepithelial absorption of SCFA and the intraepithelial metabolism of butyrate were first discovered in the forestomachs of ruminants.<sup>2,57,58</sup> Comparative research on forestomach epithelia may thus lead to valuable clues in understanding the general principles of butyrate action.

In contrast to the colon, the forestomachs are covered with multilayered stratified squamous

epithelia, the proliferation of which leads to increases in papillae length and in the number and composition of cell layers.<sup>59–61</sup> The influence of butyrate on epithelial growth and differentiation can be observed in milk-fed ruminants, who are born with a rumen that is nonfunctional and initially bypassed until transition to a grain or forage based diet occurs. Butyrate was identified as a key stimulus to initiate growth and differentiation of the rumen into the mature absorptive organ,<sup>62–64</sup> however, a number of hormonal mediators (glucagon-like peptide 2, insulin, insulin-like growth factor 1) appear to be involved as cofactors.<sup>60,65–69</sup> Interestingly, the mediation of butyrate effects via hormonal mediators has been intensively discussed for the rumen but has rarely been considered for the large intestine.<sup>7</sup> *In vitro* and without any hormonal stimulus, butyrate itself has antiproliferative action on primary cultures of ruminal epithelial cells.<sup>65,66,68</sup>

### *Butyrate and the forestomach barrier*

A current limitation on attempts to delineate the effects of butyrate on the forestomach barrier is that knowledge of its functional organization is far less advanced than that concerning monolayered gastrointestinal epithelia. Due to the multilayered structure of forestomach epithelia, a separation into a clearly distinct apical and basolateral domain appears too simplistic.<sup>70</sup> Instead, at least two functional barriers emerge. The first barrier is that of the uppermost stratum corneum, the keratinocytes of which are thought to form a loose physical boundary that may serve as an apical microclimate,<sup>71</sup> with cells possibly loosely interconnected by claudin-7.<sup>72</sup> The second barrier is that of the stratum granulosum, which clearly shows the highest incidence in morphological correlates of occluding junctions,<sup>70,73,74</sup> while staining for claudin-1, claudin-4, ZO-1, and occludin can be observed.<sup>70,72</sup> Staining intensity for claudin-4 drops sharply within the stratum spinosum toward the basal layer, whereas claudin-1 and occludin persist down to the stratum basale. Notably, and in contrast to the findings in monolayered epithelia, attempts to demonstrate the presence of the leaky claudin-2 via immunohistochemical staining, PCR, or Western blot in tissues and cultured cells of the rumen and other forestomach compartments have so far been unsuccessful.<sup>72</sup> Possibly both due to a lack of claudin-2 and due to the peculiarities of their multilayered organization,

forestomach epithelia appear to be relatively tight, allowing the absorption of ions against formidable gradients.<sup>75–77</sup> There is no indication that SCFA in general,<sup>78–80</sup> or butyrate in particular (Penner *et al.*, unpublished data), can upregulate what is already a formidable barrier, although they may enhance the absorptive capacity of the epithelium<sup>79,81,82</sup> and its ability to withstand osmotic challenges<sup>78</sup> and to metabolize butyrate.<sup>80,83,84</sup>

Instead, and in conjunction with decreasing luminal pH, rising concentrations of butyrate are suspected to be a detrimental factor for the forestomach barrier in a condition known as ruminal acidosis.<sup>81</sup> Clinicians expect symptoms ranging from decreased food intake to severe ruminal inflammation with liver abscesses and immunological manifestations.<sup>85,86</sup> Histologically, rapid structural changes of the epithelium are observed with a decline in cellular junctions, sloughing of the stratum corneum and a thinning of all underlying epithelial layers.<sup>87</sup> Chronic exposure leads to a thickening of the stratum corneum at the expense of the stratum granulosum,<sup>88,89</sup> suggesting profound alterations in barrier structure in this pathological situation. While butyrate is thus essential for the differentiation and maturation of the forestomachs into organs of impressive absorptive capacity, there is considerable reason to assume that excessive amounts of butyrate may be toxic, especially when coinciding with low luminal pH.

### **Future perspectives**

Butyrate is an important metabolic signal in the gastrointestinal tract with a proven role for the tightness of the epithelial barrier. The latter makes it a promising tool in the treatment of gut disorders like IBD. However, butyrate effects are variable and may depend on the butyrate concentration, pH, the differentiation state of affected cells and on confounding indirect effects of butyrate due to altered profiles of (or sensitivity to) hormones, growth factors, and inflammatory mediators. A better understanding of these complex interactions and the related molecular events, as well as a better understanding of the conditions favoring microbial butyrate production, are necessary to improve the therapeutic usability of butyrate. To this end, comparative research in the forestomach of ruminants is suggested to be of value for delineating primary butyrate actions from cofactor-dependent effects of butyrate.



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## Conflicts of interest

The authors declare no conflicts of interest.

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