BUTYRATE IN BROILER DIETS

Impact of butyrate presence in distinct gastrointestinal tract segments on digestive function, microbiota composition and immune responses

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Chapter 1

General introduction

BACKGROUND

Poultry production is facing a challenging future as there is a growing global demand for human-edible protein sources (Wu *et al.*, 2014). Poultry meat could play a role in fulfilling this demand due to its favourable hedonic value, its nutritive value, the absence of cultural or religious restrictions concerning its consumption and the efficiency of its production (Magdelaine *et al.*, 2008). As a consequence, intensive poultry production is regarded as one of the main provider of additional animal protein to meet the growing global demand (Mottet and Tempio, 2017). Policy makers are, however, concerned that a further increase in worldwide livestock production could lead to a competition for the use of human-edible ingredients between feed, fuel and food production activities (Banerjee, 2011). Such concerns can be mitigated by substituting cereals by local oilseeds by-products in livestock diets (Manceron *et al.*, 2014) and by achieving modest gains in feed efficiency (Mottet *et al.*, 2017). Both strategies are sometimes difficult to conciliate in the European context. For instance, complete substitution of overseas imported soybean meal by locally produced rapeseed meal can hamper growth performance of broilers (Qaisrani *et al.*, 2014). Thus, there is a need to find strategies that allow an extensive use of locally available by-products while maintaining or even improving growth performance of birds. Additionally, welfare of broilers and the use of antibiotics as growth promoters are developing concerns in Western societies (Smith, 2011). Taken together, these facts indicate that the poultry production sector is prompted to find strategies that can ensure improved growth performance, health and welfare while using new feed ingredients that decrease cereal use and soybean meal dependence as well as the use of antibiotics (Jez *et al.*, 2011).

A better understanding of digestive dynamics of macronutrients in the avian GIT (Liu and Selle, 2015) and their associated effects on growth performance and nutrient utilization (Liu *et al.*, 2016) may help address this challenging future. Gaining a fundamental understanding of unique features of the avian GIT such as digesta reflux may be of relevance in this context (Sacranie *et al.*, 2005). In addition, several technological advances maybe equally relevant: e.g. next-generation sequencing to further improve the health and growth potential of birds (Diaz-Sanchez *et al.*, 2013), enzymatic and processing technologies to upgrade the nutritional value of by-products (de Vries *et al.*, 2012) or feed additives modulating gut functions and immunity (Klasing, 2006). Among feed additives, butyrate has received much attention and has been proposed as a potential solution to help the poultry sector to address the aforesaid challenges (Ahsan *et al.*, 2016). This nutritional strategy is governed by the putative capacity of butyrate to trigger different mechanisms in the avian gastrointestinal tract (GIT) as presented in Figure 1.

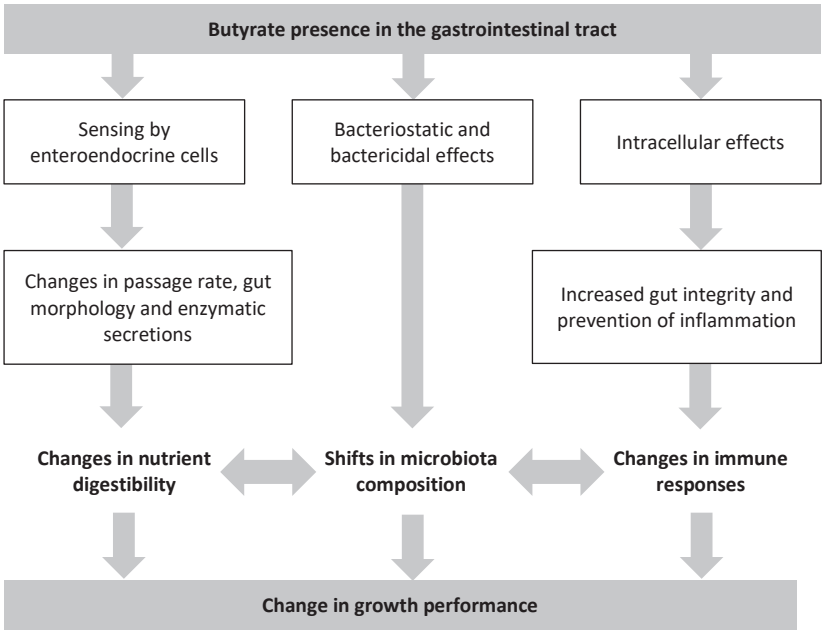

Figure 1. Putative modes of action of butyrate resulting in changes in the growth performance of broiler chickens

Dietary butyrate supplementation has however an inconsistent effect on the growth performance of broilers, with factors such as inclusion level, health status, age of the bird or diet composition being influential for the observed effects on growth performance (Cerisuelo *et al.*, 2014; Polycarpo *et al.*, 2017). Several mechanisms underlying the observed effects of butyrate on growth of livestock have been hypothesised (For review, see Guilloteau *et al.*, 2010). At present, a limited number of studies have investigated the existence of such mechanisms in poultry (Zhang *et al.*, 2011; Liu *et al.*, 2017). In addition, most of the poultry studies resorted to only one butyrate additive and did not report butyrate concentrations along the GIT (e.g. Czerwinski *et al.*, 2012; Cerisuelo *et al.*, 2014). Hence, it remains uncertain whether the effects elicited by butyrate are conditioned by the GIT segment wherein the molecule is present. This dearth of knowledge could limit the full use of butyrate supplements as a nutritional strategy to meet the challenges of the poultry industry.

RESEARCH QUESTIONS AND HYPOTHESIS

The present thesis aims to address the following research questions:

- What are the effects of supplementing broiler diets with butyrate on digestive function, microbiota composition and immune responses?
- Are such effects conditioned by the GIT segment wherein the molecule is present?

It is hypothesised that the presence of butyrate in the GIT may influence gut hormone levels and digestive functions (e.g. passage rate or enzymatic activities) in a manner that could influence nutrient digestibility. In addition, it is hypothesised that butyrate presence would also modify immune response (e.g. pro-inflammatory cytokines levels or specific antibody responses). Putative effects on digestive function and immune response may also add up to direct effects of butyrate on prokaryotic cells, resulting in changes in cecal microbiota composition. Finally, given the diversity of cell type and physico-chemical conditions encountered throughout the avian GIT, it was hypothesised that the effects elicited by butyrate would vary according to the GIT segment considered. Hence, the overall aim of this study was to gain insights in the mechanisms underlying the effects of dietary butyrate supplementation on growth performance of broiler chickens.

OUTLINE OF THE THESIS

This thesis describes the results of an *in vitro* experiment and two *in vivo* trials. Chapter 2 consists of a review of the literature concerning potential modes of action of butyrate in the avian GIT, highlighting a potential role of butyrate presence in the digesta of distinct GIT segments in triggering specific effects. In Chapter 3, an *in vitro* model was used to compare the release profiles of experimental and commercially available butyrate additives along a simulated poultry GIT. Based on the outcome of this *in vitro* screening, several additives were included in the trial described in Chapter 4, in order to investigate the effects of butyrate presence in the digesta of distinct GIT segments on digestive processes and amino acid digestibility. In Chapter 5, a gene expression approach was applied in an effort to associate observed changes in digestive processes to changes in the secretory activities of different enteroendocrine cells subsets. In addition, the relationship between mucosal butyrate concentration and the expression of genes related to butyrate metabolism was investigated. In Chapter 6, the intricate relationship between cecal microbiota composition, pre-cecal digestive processes, butyrate presence along the GIT and expression of immune effectors was explored. We investigated in Chapter 7 the effect of butyrate presence in the digesta of distinct GIT segments on immune responses after *in vivo* stimulations. Finally, Chapter 8 summarizes and discusses the results of the previous chapters.

LITERATURE CITED

Ahsan, U., Ö. Cengíz, I. Raza, M. F. A. Cacher, Z. Iqbal, S. Umar, and S. Çakir. 2016. Sodium butyrate in chicken nutrition: the dynamics of performance, gut microbiota, gut morphology, and immunity. Worlds Poult. Sci. J. 72:265-275.

Banerjee, A. 2011. Food, feed, fuel: transforming the competition for grains. Dev. Change 42:529-557.

Cerisuelo, A., C. Marin, F. Sanchez-Vizcaino, E. A. Gomez, J. M. de la Fuente, R. Duran, and C. Fernandez. 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and Salmonella counts in experimentally challenged broilers. Poult. Sci. 93:599-606.

Czerwinski, J., O. Hojberg, S. Smulikowska, R. M. Engberg, and A. Mieczkowska. 2012. Effects of sodium butyrate and salinomycin upon intestinal microbiota, mucosal morphology and performance of broiler chickens. Arch. Anim. Nutr. 66:102-116.

de Vries, S., A. M. Pustjens, H. A. Schols, W. H. Hendriks, and W. J. J. Gerrits. 2012. Improving digestive utilization of fiber-rich feedstuffs in pigs and poultry by processing and enzyme technologies: A review. Anim. Feed Sci. Technol. 178:123-138.

Diaz-Sanchez, S., I. Hanning, S. Pendleton, and D. D'Souza. 2013. Next-generation sequencing: The future of molecular genetics in poultry production and food safety. Poult. Sci. 92:562-572.

Guilloteau, P., L. Martin, V. Eeckhaut, R. Ducatelle, R. Zabielski, and F. Van Immerseel. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23:366-384.

Jez, C., C. Beaumont, and P. Magdelaine. 2011. Poultry production in 2025: learning from future scenarios. Worlds Poult. Sci. J. 61:105-114.

Klasing, K. C. 2006. Micronutrient supply: influence on gut health and immunity. Pages 210-223 in Avian gut functions in health and disease. G. C. Perry, ed. CAB International, Wallingford, UK.

Liu, S. Y. and P. H. Selle. 2015. A consideration of starch and protein digestive dynamics in chicken-meat production. Worlds Poult. Sci. J. 71: 297-310.

Liu, S. Y., P. H. Selle, D. Raubenheimer, D. J. Cadogan, S. J. Stephen and A. J. Cowieson. 2016. An assessment of the influence of macronutrients on growth performance and nutrient utilisation in broiler chickens by nutritional geometry. Br. J. Nutr. 116(12): 2129-2138.

Liu, J. D., H. O. Bayir, D. E. Cosby, N. A. Cox, S. M. Williams, and J. Fowler. 2017. Evaluation of encapsulated sodium butyrate on growth performance, energy digestibility, gut development, and Salmonella colonization in broilers. Poult. Sci. 96:3638-3644.

Magdelaine, P., M. P. Spiess, and E. Valceschini. 2008. Poultry meat consumption trends in Europe. Worlds Poult. Sci. J. 64:53-64.

Manceron, S., T. Ben-Ari, and P. Dumas. 2014. Feeding proteins to livestock: Global land use and food vs. feed competition. Oils. Crops Lipids 21(4):1-10.

Mottet, A., C. de Haan, A. Falcucci, G. Tempio, C. Opio, and P. Gerber. 2017. Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. Glob. Food Sec. doi https://doi.org/10.1016/j.gfs.2017.01.001

Mottet, A., and G. Tempio. 2017. Global poultry production: current state and future outlook and challenges. Worlds Poult. Sci. J. 73:245-256.

Polycarpo, G. V., I. Andretta, M. Kipper, V. C. Cruz-Polycarpo, J. C. Dadalt, P. H. M. Rodrigues, and R. Albuquerque. 2017. Meta-analytic study of organic acids as an alternative performance-enhancing feed additive to antibiotics for broiler chickens. Poult. Sci. 96:3645-3653.

Qaisrani, S. N., P. C. Moquet, M. M. van Krimpen, R. P. Kwakkel, M. W. Verstegen, and W. H. Hendriks. 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93:3053-3064.

Sacranie A., P. A. Iji and M. Choct. 2005. Reflux of digesta and its implication for nutrient digestion and bird health. Proc. Aust. Poult. Sci. Symp. 8:171-174. (Abstr.)

Smith, J. A. 2011. Experiences with drug-free broiler production. Poult. Sci. 90:2670-2678.

Wu, G., J. Fanzo, D. D. Miller, P. Pingali, M. Post, J. L. Steiner, and A. E. Thalacker-Mercer. 2014. Production and supply of high-quality food protein for human consumption: sustainability, challenges, and innovations. Ann. N. Y. Acad. Sci. 1321:1-19.

Zhang, W. H., Y. Jiang, Q. F. Zhu, F. Gao, S. F. Dai, J. Chen, and G. H. Zhou. 2011. Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. Br. Poult. Sci. 52:292-301.

Chapter 2

Importance of release location on the mode of action of butyrate additives in the avian gastrointestinal tract

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ABSTRACT

In the field of animal nutrition, butyrate is used as a feed additive and can be fed as an unprotected salt or in the form of protected additives such as butyrate glycerides or butyrate-loaded matrices. Dietary butyrate supplementation has been shown to improve growth performance and resilience of broiler chickens through distinct mechanisms operating on both eukaryotic and prokaryotic cells. Firstly, butyrate influences endogenous avian cells in multiple ways: it is an agonist of free-fatty acid receptors, an inhibitor of pro-inflammatory pathways, an epigenetic modulating agent and finally an energy source. Secondly, butyrate influences the microbiota residing in the avian gastrointestinal tract (**GIT**) as a result of its bacteriostatic properties. The responses, e.g. changes in growth performance, gut morphology, carcass traits or nutrient digestibility of chickens, to dietary butyrate supplementation are inconsistent with factors such as additive inclusion level, diet composition, age and health status of the bird modulating the effects of butyrate and its additives. For many additives, the precise GIT segment wherein butyrate is released is unclear. Release location may also affect the observed effects of butyrate given the diversity of cell types and pH conditions encountered throughout the gastrointestinal tract of poultry, and the differences in microbiota composition in the different gut segments. As a consequence, our understanding of the mode of action of butyrate is hampered. Characterization of existing additives and development of targeted-release formulations are, therefore, important to gain insight in the different physiological effects butyrate can elicit in broiler chickens.

Keywords: Butyrate, broiler, release location, mode of action

INTRODUCTION

Butyrate is used as a feed additive for broiler chickens and reported responses are mostly favourable in terms of maintaining optimal gut health and stimulating growth performance. Butyrate effects on growth performance, however, are sometimes inconsistent and its mode of action is not yet fully understood (Cerisuelo *et al.*, 2014). In human medicine, butyrate has been advocated as a promising molecule for the prevention or amelioration of a number of human diseases such as inflammatory bowel disease and colon cancer (Segain *et al.*, 2000; Sengupta *et al.*, 2006). However, butyrate has not always achieved all the expected multifarious, positive effects in a consistent manner. As a result, reviews related to this molecule often contain interrogative titles, e.g. "*The butyrate story: old wine in new bottles?*" (Scheppach and Weiler, 2004) and "*Butyrate: what is the future for this old substance?*" (Sossai, 2012). Regarding its use as a feed additive for broilers, factors such as inclusion level, diet composition, age and health status of the birds should be taken into account when assessing the effects of butyrate additives (Cerisuelo *et al.*, 2014). Several reviews have been published on this subject, describing endogenous production and effects on gastrointestinal epithelial cell function in mammals (Guilloteau *et al.*, 2010a), butyrate metabolism (Astbury and Corfe, 2012) and its modulating effects on immunity in humans (Meijer *et al.*, 2010).

The present review aims to describe the response of broiler chickens to dietary butyrate supplementation, with an emphasis on the importance of release location in relation to the various effects of this molecule. The first section of this review briefly describes the different butyrate additives currently used in the animal feed industry. Next, discrepancy in the literature regarding avian responses to dietary butyrate supplementation is discussed. Finally, proven modes of action in poultry of butyrate are discussed and putative modes of actions derived from research on mammals are presented. In this review, information from other species is used where there data on avian species is lacking. No distinction between endogenous synthesised or exogenous dietary butyrate are made in the present review, as the microbial or dietary origin of the molecule does not change its effect at the gut level. Butyrate can be present as an anion, as a salt or as an acid (Kato *et al.*, 2011). It is widely accepted that the anion form (CH_3-CH_2-CH_2-COO^-) is mediating the effects reported in literature (Guilloteau *et al.*, 2010a). The term "butyrate" used here encompasses the acid, salts and anion forms. Concerning the description of dietary inclusion rates in the present contribution, g/kg refers to g of butyrate, corrected for coating material, per kg of diet.

BUTYRATE ADDITIVES USED IN ANIMAL NUTRITION

Unprotected butyrate

Unprotected butyrate will be mainly present in a protonated form due to the low pH in the stomach of monogastric animals due to its pKa of 4.82. Butyrate is taken-up by non-ionic, passive absorption in the stomach (Ichikawa *et al.*, 2002). As such, supplementation of unprotected forms leads to a significant increase in butyrate concentration in the gastric region but not in the jejunal chyme of broilers (Hu and Guo, 2007) nor in the cecum, colon and rectum of pigs (Manzanilla *et al.*, 2006). As a consequence, unprotected butyrate is supposed to exert mainly effects on epithelial

cells and microbiota of the crop, proventriculus and gizzard of broiler chickens. It is unclear whether delivery at these segments of the gastrointestinal tract (**GIT**) is effective in eliciting effects on growth performance, gut morphology and immune system regulation. Much research has been conducted to 'protect' the butyrate to achieve release in the more distal GIT, where it is also produced by microbiota.

Mono-, di- and tributyrins

Butyrins include mono-, di- and tri-glycerides of butyrate. As chickens have very limited pre-duodenal lipolytic activity (Moreau *et al.*, 1988), it is assumed that butyrate will mostly be released in the small intestine under the action of pancreatic lipase. Pancreatic lipase can also be present in the gizzard of poultry due to reflux (Sklan *et al.*, 1978). It is, however, known that pancreatic lipase activity on tributyrin is compromised at low pH (Borgström, 1975). Endogenous lipases are solely able to cleave ester bonds of triacylglycerol at the *sn-1* and *sn-3* position (Doreau and Chilliard, 1997). As a consequence, part of the butyrate is inevitably absorbed as *sn-2* monobutyrin in the duodenum and jejunum.

Butyrate-loaded matrices

Butyrate can be embedded in a continuous matrix. Such matrices can be characterized by the type of embedding material used and by the average bead diameter. Although commonly referred to as fat coated or microencapsulated butyrate in the literature, these description are inaccurate as in microencapsulated or coated products, an outer shell can be distinguished from an inner core. Vegetable fat seems to be the most common embedding material for use in animal nutrition (Smulikowska *et al.*, 2009; Zhang *et al.*, 2011a; Czerwinski *et al.*, 2012; Jerzsele *et al.*, 2012). The used fat source and mean bead diameters of products are often not reported in the published research papers. In addition, the butyrate inclusion percentages in the matrix are variable. Most trials have been conducted with matrices loaded at a concentration of 30% w/w (e.g. Smulikowska *et al.*, 2009). The use of matrices loaded at 70% w/w has also been reported (Jerzsele *et al.*, 2012). Release kinetics can be affected by general parameters such as the type of embedding material used or inclusion level (De Brabander *et al.*, 2000; Vervaeck *et al.*, 2013). The aforementioned characteristics may vary from one feed additive supplier to the other. As a consequence, the GIT segment wherein butyrate is released is difficult to predict with currently used butyrate-loaded matrices. It is generally accepted that fat matrices provide a partial protection from gastric absorption, thus increasing the delivery of butyrate to the small intestine. Concerning the availability at colonic and caecal level, information is scarce. However, it has been shown that feeding vegetable-fat based matrices containing butyrate does increase the butyrate concentration in the colon of pigs (Mallo *et al.*, 2012). Similar results are observed in the caeca of broilers (Onrust, unpublished data).

EFFECTS OF BUTYRATE ON GROWTH PERFORMANCE, GUT MORPHOLOGY, CARCASS TRAITS AND NUTRIENT DIGESTIBILITY IN BROILERS CHICKENS

Effect on growth performance

As indicated in Table 1, butyrate can improve body weight gain, voluntary feed intake and feed efficiency of broiler chickens. Literature data, however, show large discrepancies in the effect of butyrate on growth performance, both among and within additives. It has been reported that health status of the animal, diet composition and environmental conditions influence the response of the broilers to butyrate supplementation, thereby explaining part of the inconsistencies (Cerisuelo *et al.*, 2014). In the case of unprotected butyrate supplementation, dose effects might also play a role. Indeed, Hu and Guo (2007) and Panda *et al.* (2009) used larger doses than Zhang *et al.* (2011b) and the latter authors could not find any effect of butyrate on growth performance of broilers. Concerning fat coated butyrate, discrepancies might also be due to differences in matrix characteristics. Fat coated butyrate used in published studies originated from various suppliers.

Effect on gut morphology

Long villi and short crypts are generally regarded as indicators of a healthy and functional small intestine. However, these parameters only provide partial information on the intestinal surface and function. Additional read-out parameters such as mucosal enzyme activity, mucus layer thickness and composition, or number and quality of goblet cells should be included to elaborate on gut health and digestive capacities (Van Dijk *et al.*, 2002). In this section, only villus height (**VH**) and crypt depth (**CD**) are used as indicators of intestinal health.

As shown in Table 2, effects of butyrate additives on gut morphology are inconsistent. Unprotected butyrate seems to improve duodenal as well as jejunal morphology when fed at levels equal to, or higher than, 2 g/kg during the 0-35 d period (Hu and Guo, 2007). Concerning fat coated butyrate, information originating from the literature is inconsistent. Jerzsele *et al.* (2012) as well as Czerwinski *et al.* (2012) found positive effects of supplementation on jejunal VH using 1.05 and 0.3 g/kg inclusion levels, respectively. Conversely, Smulikowska *et al.* (2009) did not find any significant effect of 0.3 g/kg supplementation on jejunal morphology. Butyrate glycerides also seem to show conflicting effects on gut morphology. Leeson *et al.* (2005) did not find any effect of butyrate glycerides on VH or CD. Antongiovanni *et al.* (2009) found that butyrate glycerides can significantly reduce ileal and jejunal VH while increasing ileal CD. Sayrafi *et al.* (2011) reported a significant increase in duodenal CD when butyrate glycerides were fed to broilers. Antongiovanni *et al.* (2009), also showed that high levels of butyrate glycerides supplementation significantly increased the number of villi per length unit and the number of jejunal microvilli.

Table 1. Effect of different butyrate additives on the growth performance of broiler chickens[1]

Supplementation form	References	Experimental set-up	Effects on performance parameters expressed as a percentage of respective controls
Unprotected butyrate	Hu and Guo (2007)	C: Control, T1: 0.5 g/kg B in SGF, T2: 1 g/kg B in SGF, T3: 2 g/kg B in SGF.	BWG was improved in T1 (+4.7%) and T3 (+4.7%) in comparison to C birds during the 0-21 d period. In T3, FCR (+4.6%) was influenced in a positive quadratic fashion during the 0-42 d period.
	Panda *et al.* (2009)	C: Control, T1: 0.5 g/kg furazolidone, T2: 2 g/kg B in SGF, T3: 4 g/kg B in SGF, T4: 6 g/kg B in SGF.	T3 (+3.4%) and T4 (+4.0%) had significantly higher BWG than control birds over the whole experimental period. FCR was significantly reduced by B supplementation (T1: -2.7%; T2: -5.9%; T3: -4.8%).
	Zhang *et al.* (2011b)	Exp. 1: C: Control, T1: 0.25 g/kg B in SGF, T2: 0.50 g/kg B in SGF, T3: 0.75 g/kg B in SGF. Exp. 2: 2 x 2 factorial arrangement with B (none or 1 g/kg in SGF) and challenge status (saline or endotoxin injection) as main factors.	No significant effect of B supplementation on performance parameters in comparison with the control in experiment 1. In experiment 2, B alleviated the reduction in BWG and VFI caused by LPS challenge.
Fat coated butyrate	Smulikowska *et al.* (2009)	2 x 3 factorial arrangement: Enzyme (None or 1 g/kg in SGF) and SCFA (None, 0.3 g/kg B in SGF, 1 g/kg of a SCFA blend in SGF) as main factors.	B supplementation significantly reduced FCR (-4.6%) in comparison to the control diet.
	Jerzsele *et al.* (2012)	C: Control, T1: 1.05 g/kg B in SGF, T2: 1 g/kg of an essential oils blend in SGF, T3: T1+T2 in SGF, T4: 10^9 CFU of a probiotic.	No significant effect of B supplementation on BWG of *C. Perfringens* challenged birds in comparison to challenged control.
	Hautekiet *et al.* (2011)	2 x 2 factorial arrangement: Dietary metabolisable energy and amino acid concentration (H: high or L: low) and B (none or 0.75 g/kg in S and 0.5 g/kg in G) as main factors.	B supplementation significantly increased VFI (H: +2.4%; L: +1.9%) and BWG (H: +1.5%; L: +3.4 %) in comparison to control over the whole experimental period.
Blend of mono-, di- and tri-butyrins	Leeson *et al.* (2005)	Exp.1: C: Control, T1: 11 pm virginiamycin in SGF, T2: 2 g/kg BB in SGF, T3: 4 g/kg BB in SGF. Exp. 2: C: Control, T1: 50 pm bacitracin in SGF, T2: 1 g/kg BB in SGF, T3: 2 g/kg BB in SGF.	Experiment 1: No effect of dietary treatments on BWG. T3 birds showed a reduced VFI (-7.7%) compared to control birds. Experiment 2: No effect of dietary treatments on performance parameters.
	Antongiovanni *et al.* (2009)	C: Control; T1: 2 g/kg BB in SGF; T2: 3.5 g/kg BB in SGF; T3: 5 g/kg BB in both SGF and T4: 10 g/kg BB in only S.	Birds from T1 (+3.1%), T3 (+1.7%) and T4 (+1.7%) showed a significantly higher slaughter weight than C birds.
	Mahdavi and Torki (2009)	C: Control, T1: 2 g/kg BB in S, T2: 2 g/kg BB in SG, T3: 2 g/kg BB in SGF, T4: 3 g/kg BB in S, T5: 3 g/kg BB in SG, T6: 3 g/kg BB in SGF.	No significant effect of dietary treatments on performance parameters.
	Aghazadeh and Taha Yazdi (2012)	2 x 4 factorial arrangement with two forms of wheat (whole vs. ground): C: Control; T1: 2.5 g/kg BB SGF; T2: 2.5 g/kg BB in S and 1 g/kg BB in GF; T3: 2.5 g/kg BB in S.	No significant effect of supplementation during starter, grower/finisher or whole period on BWG and FCR. T2 resulted in a significantly higher VFI (+1.5%).

[1]B: Butyrate; BB: Butyrins Blend; BWG: Body Weight Gain; Exp.: Experiment; F: Finisher feed; FCR: Feed Conversion Ratio; G: Grower feed; LPS: Lipopolysaccharide; S: Starter feed; SCFA: Short Chain Fatty Acid; T: Treatment; VFI: Voluntary Feed Intake.

Table 2. Effect of different butyrate additives on gut morphology of broiler chickens[1]

Supplementation form	References	Experimental set-up	Effects on performance parameters expressed as a percentage of respective controls
Unprotected butyrate	Hu and Guo (2007)	C: Control, T1: 0.5 g/kg B in SGF, T2: 1 g/kg B in SGF, T3: 2 g/kg B in SGF	Jejunal VH:CD ratio was significantly increased for T3 (+32.3%)
	Panda *et al.* (2009)	C: Control, T1: 0.5 g/kg furazolidone, T2: 2 g/kg B in SGF, T3: 4 g/kg B in SGF, T4: 6 g/kg B in SGF	Duodenal VH (T2: +7.25%; T3: +8.9%; T4: +8.1%) and CD (T2: +10.3%; T3: +18.1%; T4: +15.5%) were significantly increased by B supplementation
Fat coated butyrate	Smulikowska *et al.* (2009)	2 x 3 factorial arrangement: Enzyme (None or 1 g/kg in SGF) and SCFA (None, 0.3 g/kg B in SGF, 1 g/kg of a SCFA blend in SGF) supplementation.	No significant effect of B on VH, villus width, CD and tunica muscularis width in the jejunum or the ileum.
	Jerzsele *et al.* (2012)	C: Control, T1: 1.05 g/kg B in SGF, T2: 1 g/kg of an essential oils (EO) blend in SGF, T3: T1+T2 in SGF, T4: 10^9 CFU of a probiotic.	B significantly increased jejunal VH (+15.8%) but did not significantly affect VH:CD ratio.
	Czerwinski *et al.* (2012)	C: Control, T1: 0.3 g/kg B, T2: 0.06 g/kg salinomycin, T3: 0.3 g/kg B and 0.06 g/kg salinomycin.	B significantly increased jejunal VH (+5.7%) but did not significantly affect villus width, CD, VH:CD ratio or tunica muscularis width. None of these parameters was significantly affected by dietary treatments at ileum level.
Blend of mono-, di- and tri-butyrins	Leeson *et al.* (2005)	Exp. 1: C: Control, T1: 11 pm virginiamycin in SGF, T2: 2 g/kg BB in SGF, T3: 4 g/kg BB in SGF. Exp. 2: C: Control, T1: 50 pm batriacyin methylene disalicylate in SGF, T2: 1 g/kg BB in SGF, T3: 2 g/kg BB in SGF.	VH and CD were measured in experiment 2 but no significant effect of B was detected
	Antongiovanni *et al.* (2009)	C: Control; T1: 2 g/kg BB in SGF; T2: 3.5 g/kg BB in SGF; T3: 5 g/kg BB in both SGF and T4: 10 BB g/kg in only S.	VH was reduced by B in both jejunum and ileum (T1: NS and -22.1%; T2: -16.6% and -22.3%; T3: -10.7% and -36.7%; T4: -20.1% and -28.5% respectively). Number of villi per length unit was significantly increased by T4 (+19.3%) in the ileum but not affected by B in the jejunum. Jejunal microvillus length was improved in T1 (+57%) but unaffected in the ileum. Jejunal CD was significantly increased in T1 (+26.9%) and ileal crypts were significantly deeper in T1 (-24.1%), T2 (-19.3%) and T4 (-37.7%).
	Sayrafi *et al.* (2011)	C: Control, T1: 1 g/kg prebiotic, T2: 3 g/kg BB in S and 2 g/kg in G, T3: 0.5 g/kg bacitracin in S and 0.25 g/kg bacitracin in GF.	Duodenal, jejunal and ileal VH and width were unaffected by B. B induced deeper duodenal crypts (+25.4%) without changing VH in the jejunum or the ileum.

[1]B: Butyrate; BB: Butyrins Blend; CD: Crypt Depth; Exp.: Experiment; F: Finisher feed; G: Grower feed; S: Starter feed, SCFA: Short chain fatty acid; T: Treatment; VH: Villus Height.

Effects on carcass traits

Supplementation of a broiler chicken diet with 2, 4 and 6 g/kg of unprotected butyrate results in a significant reduction of abdominal fat percentage and in an increase of dressed carcass weight (Panda *et al.*, 2009). In another study using butyrins at inclusion levels ranging from 2 to 10 g/kg and measuring carcass traits such as dressing percentage, breast, thigh, drumstick and abdominal weights, no significant effects of supplementation was detected (Antongiovanni *et al.*, 2009). Similarly, Leeson *et al.* (2005) did not find any significant effect of the inclusion of 2 or 4 g/kg of butyrins in both starter and grower/finisher diets on breast meat percentage of 42 d old broilers. Zhang *et al.* (2011a) also reported that supplementation of broiler diets with 0.4 g/kg of fat coated butyrate did not result in significantly different carcass characteristics in comparison to non-supplemented diets. However, in the same experiment, butyrate supplementation reduced drip loss of the breast muscle. Based on these studies it can be concluded that carcass characteristics are inconsistently affected by butyrate supplementation. Carcass quality might be influenced by an interaction between the presence of nutritional, environmental and immunological stressors and butyrate supplementation, as explained below.

Effects on nutrient digestibility

Information is scarce regarding the effect of dietary butyrate supplementation on nutrient digestibility in broiler chickens. Smulikowska *et al.* (2009) reported that dietary inclusion of fat coated butyrate at 0.3 g/kg significantly increased apparent total tract digestibility of nitrogen and organic matter without affecting crude fat digestibility in broiler chickens. In the same study, nitrogen retention was improved while apparent metabolisable energy content of the diet was unaffected by butyrate supplementation. Improvements in nitrogen digestibility are in line with findings of Qaisrani (2014) who reported a trend for higher proventricular proteolytic activity in broilers fed the same butyrate additive. Finally, Swiatkiewicz *et al.* (2014) reported that inclusion of 0.7 g/kg of fat coated butyrate in broiler chicken diets containing high levels of dried distillers grains with solubles did not improve apparent total tract digestibility of the various measured nutrients. Collectively, these results indicate that butyrate might improve protein digestibility whereas improvements in energy digestibility are uncertain.

MODES OF ACTION OF BUTYRATE ADDITIVES ON THE AVIAN GASTROINTESTINAL TRACT

Figure 1 depicts putative and confirmed modes of action of butyrate on the GIT. It can be concluded that butyrate has effects on immune responses and gut integrity, elicits shifts in microbiota composition and might influence the endocrine control of digestive processes. Each category of effect will be discussed below.

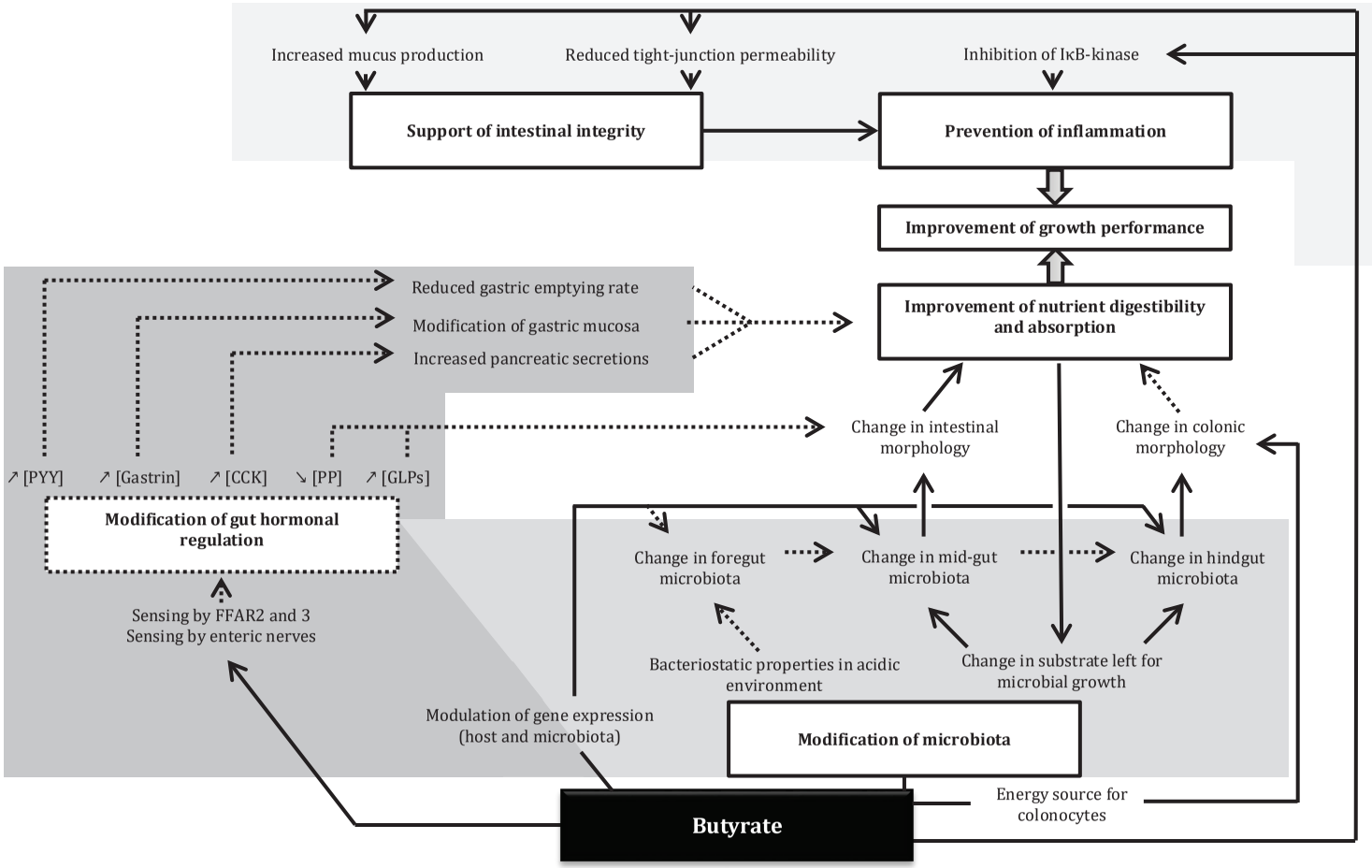

Figure 1. Putative (broken lines) and confirmed (solid lines) effects of butyrate on endocrine system, immune response, intestinal integrity and microbiota composition might contribute to improved growth performances

Immuno-modulatory properties

Butyrate down-regulates pro-inflammatory pathways by inhibiting cytoplasmic IκB kinase (**IKK**) (Moeinian *et al.*, 2013). IKK initiates the translocation of a transcription factor from the cytoplasm to the nucleus, the nuclear factor κB (**NF-κB**), resulting ultimately in the production of pro-inflammatory cytokines such as tumor necrosis factor α (**TNF-α**) or interleukins (**IL**) 1β, 2 and 6 (Barnes and Karin, 1997). In poultry, these pro-inflammatory cytokines induce a homeorhetic response that modifies nutrient portioning. During inflammatory responses, dietary nutrients are used to support immune responses and disease resistance via numerous metabolic processes while skeletal muscle deposition and growth are reduced (Klasing and Johnstone, 1991). Additionally, inhibiting NF-κB activation reduces the level of reactive oxygen species and up-regulate the expression of genes involved in antioxidant synthesis (Hamer *et al.*, 2009; Maa *et al.*, 2010; Moeinian *et al.*, 2013).

Such changes might protect the organism from DNA, lipid and protein damage induced by reactive oxygen species upon oxidative stress (Canani *et al.*, 2011). Butyrate might, therefore, contribute to an improved nutrient utilization while protecting the organism from oxidative stress.

Butyrate also appears to affect mammalian immune cell chemotaxis in a species, dose and immune cell-dependent manner (Meijer *et al.*, 2010). Until now, the influence of dietary butyrate supplementation on avian cell chemotaxis has never been studied. However, Sunkara *et al.* (2011) demonstrated that feeding 1 g/kg unprotected butyrate to broiler chicken leads to a significant increase in expression of genes encoding for antimicrobial peptides (**AMP**), which could subsequently induce chemotaxis of avian innate immune cells. These results should be nuanced by the fact that correlation between mRNA and protein abundances is sometimes poor (Maier *et al.*, 2009). Butyrate is an epigenetic modulating agent inhibiting nuclear histone deacetylase (**HDAC**) enzymes, resulting in hyperacetylation of histones (Smith *et al.*, 1998). Pre-treatment of human antigen-presenting cells with butyrate reduces their capacity to stimulate T-cell responses in a dose-dependent manner due to effects on cell adhesion and production of stimulatory molecules (Böhmig *et al.*, 1997). Modulation of T-cell function by dietary butyrate supplementation varies depending on the type of butyrate additive considered (Böhmig *et al.*, 1999). It can be concluded that butyrate can prevent inflammation and infiltration of immune cells in peripheral tissues by influencing cell adhesion and chemotaxis (Meijer *et al.*, 2010). Such changes are associated with differences in gene expression that could indicate that the observed effects are mediated through the known inhibitory activity of butyrate on HDAC.

Once converted in any of its intermediate metabolites in the mitochondria, butyrate cannot return to the cytosol. On the other hand, a sufficient intracellular concentration of butyrate is required to induce effects on gene expression (Astbury and Corfe, 2012) and NF-κB inhibition (Moeinian *et al.*, 2013). Current research on human colon cell carcinoma strongly suggests that mitochondrial oxidation of butyrate plays a crucial role in the regulation of its intracellular concentration (Andriamihaja *et al.*, 2009). As a consequence, both the favoured energy substrate of the cell type and type of metabolic substrates available might influence gene-modulating properties of butyrate. Chicken enterocytes

do not favour butyrate as an energy source and do benefit from the availability of other energy sources such as glucose, glutamate or glutamine (Watford *et al.*, 1979). In contrast, butyrate is known to be the primary energy substrate of colonocytes in mammals (Sunkara *et al.*, 2011). For these reasons, it can be hypothesised that the GIT segment wherein butyrate is released modulates the observed immuno-modulatory properties.

Literature about the ability of butyrate to regulate the innate immune response of broiler chickens is relatively scarce. Unchallenged broiler chickens given 1 g/kg unprotected butyrate had reduced serum TNF-α and IL-6 levels at 21 d of age while serum antioxidant indices were increased (Zhang *et al.*, 2011b). At 42 d of age, only IL-6 levels were significantly reduced while serum antioxidant indices were increased. When the entire growth period was considered, there was no significant effect of butyrate supplementation on growth performance. This is not unexpected, because any positive effect of butyrate on growth performance is likely to be more evident when chickens are subjected to a stressor. Loss of body weight induced by a subcutaneous injection of corticosterone at 42 d of age can be partially alleviated in broiler chickens fed 0.4 g/kg fat coated butyrate (Zhang *et al.*, 2011a). A shift from an antioxidant to a pro-oxidant system under stressor exposure is prevented by butyrate supplementation, thus illustrating the ability of butyrate to decrease catabolism and oxidative injury of tissues. Similarly, body weight gain and feed intake could be maintained in broilers challenged by LPS injection at 16, 18 and 20 d of age due to the prevention of a rise in pro-inflammatory cytokines (Zhang *et al.*, 2011b).

As indicated by Klasing and Johnstone (1991), pro-inflammatory cytokines such as IL-1, IL-6 or TNF-α have a pronounced effect on avian lipid metabolism. Immunological challenges are associated with fat carcasses (Klasing *et al.* 1987, cited by Klasing and Johnstone, 1991). Due to its ability to decrease TNF-α and IL-6 levels, butyrate might alleviate adipose tissue accumulation when broilers are subjected to environmental and immunological stressors. As indicated earlier, butyrate supplementation affects carcass characteristics in an inconsistent manner. Discrepancies might be explained by the occurrence of subclinical infections or mild environmental stressors in certain experiments.

Intestinal integrity support

In addition to its effects on immunity, butyrate is able to improve the integrity of the epithelial cell layer (Canani *et al.*, 2011; Ploger *et al.*, 2012). This epithelial barrier is essential for a normal intestinal function and impairment may lead to inflammation, because bacterial components will activate inflammatory signalling cascades after binding to Toll like receptor at the basolateral surface of the epithelial cells (Yilmaz *et al.*, 2005; Lewis *et al.*, 2010). Two structures are important to maintain intestinal integrity: the tight junctions (**TJ**), forming a seal between adjacent epithelial cells, and the mucus layer, covering the epithelial cell luminal surface (Canani *et al.*, 2011; Ulluwishewa *et al.*, 2011). Butyrate has been reported in the literature to affect barrier function by increasing the formation of mucin glycoproteins, which improves the protection against luminal agents (Willemsen *et al.*, 2003). Increased expression of MUC2 gene has been shown *in vitro* (Barcelo *et al.*, 2000) and the effect is dose dependent (Gaudier *et al.*, 2004). Mentschel and Clause (2003) described an increase in thickness of the mucous layer after administration of butyrate. AMP such as

cathelicidin, heat shock proteins and trefoil factors, produced by epithelial cells, are induced by butyrate, influencing the viscoelasticity of the mucous layer (Hamer *et al.*, 2008). Up-regulation of antimicrobial peptides by butyrate was also reported in chicken jejunal and caecal explants (Sunkara *et al.*, 2011). Like in piglets and humans, butyrate has also been reported in chickens to affect TJ (Peng *et al.*, 2009; Ma *et al.*, 2012; Wang *et al.*, 2012). Butyrate increases the expression of TJ proteins and, therefore, the intestinal epithelial integrity (Peng *et al.*, 2009). The permeability of TJ is measured by transepithelial electrical resistance (**TEER**) (Schneeberger and Lynch, 2004). In the presence of 2 mM butyrate, an increase in TEER could be measured in Caco-2 cell monolayers (Peng *et al.*, 2009). Wang and co-authors (2012) reported increased TEER and stimulation of the transcription of TJ protein claudin-1 by 4mM sodium butyrate in a cdx2-IEC cell monolayer. In summary, butyrate has beneficial effects in maintaining the intestinal integrity and barrier through its effect on TJ and mucin production. These phenomena in turn will reduce inflammation and possibly also microbial translocation (Lewis *et al.*, 2010). Increased mucin production, however, may lead to increased requirements of specific amino acids.

Shifts in microbiota composition

Butyrate is a weak acid with a pKa equal to 4.8 (Guilloteau *et al.*, 2010a). The foregut of broilers is acidic, with pH values ranging from 4.5 (crop) to 2.5 (gizzard) (Denbow, 2015). As a consequence, butyrate is mainly protonated rather than in an ionic form in the crop, proventriculus and gizzard of birds (Smith *et al.*, 2012). Cherrington *et al.* (1991) hypothesised that the dissociation of short chain fatty acids (**SCFA**) in a bacterial cytoplasm disrupts the proton motive force across the membrane and lowers the cytoplasmic pH, resulting in bacteriostatic or bactericidal effects. Nevertheless, some bacterial species have evolved to become acid-tolerant and are commonly characterized by an important internal potassium pool that can be used as a countercation to balance the effects of organic acids dissociation (Russell and Diez-Gonzalez, 1998).

The control exerted by butyrate on the microbial population in the proximal part of the GIT might sustain the development of a beneficial microbiota in the more distal segments, as suggested by studies in rabbits (Ribeiro *et al.*, 2012). Finally, it has been suggested that butyrate-induced enhancements of digestibility and absorption processes can affect microbial diversity in the cecum and distal colon of pigs by modulating the nature and quantity of substrate left for microbial growth (Castillo *et al.*, 2006).

Panda *et al.* (2009) found that supplementation of broiler diets with 4 and 6 g/kg unprotected butyrate is similar to the use 0.5 g/kg furazolidone in reducing *Escherichia coli* counts in the crop and small intestine. In addition, supplementation of broilers with 2 g/kg unprotected butyrate from 1 to 21 days of age significantly reduced *Lactobacillus spp.* counts in jejunal chyme (Hu and Guo, 2007). Supplementation of broilers with 3 g/kg fat coated butyrate from 1 to 30 days of age, however, did not result in significant changes in total numbers of bacteria nor in *Lactobacillus spp.* and *Enterococcus spp.* counts in ileal and caecal digesta (Czerwinski *et al.*, 2012). Butyrate is also known to down-regulate the expression of genes involved in the pathogenicity of *Salmonella typhimurium* (Lawhon *et al.*, 2002) and also to reduce caecal colonization and fecal shedding of *Salmonella enteritidis* (Van Immerseel *et al.*, 2005).

Most of the studies concerning the effects of butyrate on microbiota of the broiler are focused on the reduction of specific pathogenic bacteria. Literature data on the effect of butyrate on the entire microbiome composition using molecular methods are scarce. Supplementation of a rapeseed meal-based diet with 2 g/kg fat coated butyrate from 1 to 34 days of age had a significant effect on the broiler caecal microbiota as 16S rRNA analysis showed a reduced Shannon's diversity index (Qaisrani, 2014). Furthermore, analysis revealed that butyrate supplementation promotes the average relative contribution of *Clostridium lactifermentans* and *Ruminococcus bromii* while reducing the relative abundance of *Clostridium perfringens.* It is hitherto unclear what the contribution of these specific microbes to the overall beneficial effects of butyrate might be.

Modifications of endocrine control of digestive processes.

There are no publications addressing the effects of dietary butyrate supplementation on endocrine control of digestive processes in poultry. There is, however, a body of evidence indicating that butyrate can influence several key gut hormones in mammals, as indicated in Table 3.

Summarizing these data, butyrate presence in the GIT can lead to a rise in cholecystokinin (**CCK**), peptide YY (**PYY**) and glucagon-like peptide (**GLP**) 2 levels and to a decrease in pancreatic peptide (**PP**) level. Such changes could support intestinal and gastric mucosa development, increase gastric retention time and stimulate gastric and pancreatic secretions. As a consequence, nutrient digestibility and absorption could be improved. Such putative changes in endocrine regulation would be consistent with improvements in growth performance, intestinal morphology, proteolytic activity and nutrient digestibility observed in broiler chickens fed butyrate.

Table 3. Effect of butyrate on gut hormonal regulation[1]

Hormone	Main gut-related effects	References	Observations
CCK	Stimulation of gallbladder contractions Increased exocrine pancreas secretion Inhibition of gastric emptying	Kotunia *et al.* (2004)	Dietary supplementation with 3 g/kg unprotected butyrate significantly increased plasma CCK concentration in suckling piglets
		Guilloteau *et al.* (2010b)	Dietary supplementation with 3 g/kg unprotected butyrate significantly increased plasma CCK 150 min after the meal. Butyrate infusion (± 5.5 M at 10 ml.min^{-1} for 1 mn) in the duodenum of calves tended to increase CCK plasma concentration in the 1.5 hour period post-infusion.
PP	Antagonist of CCK Inhibition of gastric mucosa growth	Kotunia *et al.* (2004)	Dietary supplementation with 3 g/kg unprotected butyrate significantly decreased plasma PP concentration in suckling piglets
Gastrin	Increased gastric acid and pepsin secretions Stimulation of gastric mucosa growth	Kotunia *et al.* (2004)	Dietary supplementation with 3 g/kg unprotected butyrate did not significantly affect plasma gastrin concentration in suckling piglets
		Mazzoni *et al.* (2008)	Dietary supplementation with 3 g/kg unprotected tended to increase gastrin mRNA concentration in the pyloric mucosa of weaned piglets
		Guilloteau *et al.* (2010b)	Infusion of butyrate (± 5.5 M at 10 ml.min^{-1} for 1 mn) in the duodenum of calves did not significantly affect plasma gastrin concentration in the 1.5 hour period post-infusion
PYY	Inhibition of gastric emptying Inhibition of intestinal motility Increased water and electrolyte absorption	Plaisancie *et al.* (1996)	Luminal butyrate presence (0.5-5 mM) increased PYY release in a dose-dependent manner in isolated vascularly perfused rat colon
		Dumoulin *et al.* (1998)	Luminal butyrate (20 mM at 250 µl.min^{-1} for 30 mn) induced an early and transient increase in plasma PYY in isolated vascularly perfused rat ileum
		Vidrine *et al.* (2014)	Caecal butyrate derived from resistant starch fermentation increased plasma PYY concentration in rats while supplementation with 32 g/kg unprotected butyrate did not
GLP-1 and GLP-2	Trophic effect on intestinal cells Incretin	Plaisancie *et al.* (1995)	Luminal butyrate presence (5-100 µM) did not significantly affect GLP-1 secretion in isolated vascularly perfused rat colon
		Dumoulin *et al.* (1998)	Luminal butyrate (20 mM at 250 µl.min^{-1} for 30 mn) induced an early and transient increase of plasma GLP-1 in isolated vascularly perfused rat ileum
		Bartholome *et al.* (2004)	Total parenteral nutrition (continuous infusion based on requirements) supplemented with 9 or 60 mM butyrate increased GLP-2 concentration in jejunoileal resected piglets
		Kien *et al.* (2007)	Infusion of butyrate in the caeca (12.7 µmol.min^{-1} during 1 hour) of suckling piglets did not significantly influence plasma GLP-2 concentration

[1]CCK: Cholecystokinin; PP: Pancreatic Peptide, PYY: Peptide YY, GLP: Glucagon Like Peptide.

Butyrate has potential to affect neuro-hormonal control of the gut in different ways. It can bind to free fatty acid receptors (**FFAR**) 2 and 3 that are present at the apical membrane of several types of enteroendocrine cells (Sykaras *et al.*, 2012; Nohr *et al.*, 2013). It is worth noticing that FFAR can be expressed without necessarily inducing functional cellular responses to SCFA sensing (Liou, 2013). For instance, butyrate-induced regulation of GLP-1 appears to be FFAR3 independent (Lin *et al.*, 2012). Nevertheless, FFAR sensing pathways might influence hormone production and release. Secreted hormones can have multiple functions including paracrine functions on adjacent cells or reach general circulation to exert endocrine functions (Engelstoft *et al.*, 2008) as well as a direct influence on efferent nerve endings that express FFAR (Lal *et al.*, 2001; Nohr *et al.*, 2013). It is postulated that in rats the autonomic nervous system can transmit a butyrate-induced nervous signal from the caecum or colon to the central nervous system, resulting in the release of trophic signals such as gastrin (Sakata, 1989; Reilly *et al.*, 1995). Based on their experimental work, Vidrine *et al.* (2014) suggest that butyrate release location affects the release of gut hormones. This could be due to the localization of the various specialised enteroendocrine cells. It is also likely that butyrate-induced modifications of endocrine regulation are local and not systemic. SCFA (70 mM acetate, 20 mM butyrate, 20 mM butyrate at 1.5 ml/h) infusion in the colon of rats results in an increase in jejunal gastrin concentration (Frankel *et al.*, 1994; Reilly *et al.*, 1995) without significantly affecting plasma gastrin concentration (Reilly *et al.*, 1995). Similarly, Kotunia *et al.* (2004) could not find any effect of unprotected butyrate supplementation on plasma gastrin in piglets whereas Mazzoni *et al.* (2008), using a similar dose, reported a trend for increased gastrin mRNA in piglet gastric mucosa.

CONCLUSION

This review summarizes evidence-based results originating from recent poultry studies and proposes putative mechanisms derived from research in mammals. Improvements of growth performance observed in broilers fed butyrate are attributable to a lower pro-inflammatory response to nutritional, environmental and immune challenges, associated with improved digestibility and absorption of dietary nutrients. The latter can be explained by the modulating effects of butyrate on gut microbiota but also by assumed effects of butyrate on gut endocrine regulation. Most of the mechanisms are likely to be affected by the delivery site of butyrate within the GIT, for example bacteriostatic properties require an acidic environment. Additionally, an elevated intracellular butyrate concentration is required to observe modulating effects on gene expression and immune response. Thus, both the favoured energy substrate of the cell and type of energy available might have an influence on butyrate-induced effects. In addition, neuro-hormonal mechanisms might also play a role. Each enteroendocrine cell type is defined by its location within the GIT and by its response to FFAR stimulation. Therefore, neuro-endocrine responses to luminal butyrate presence may vary depending on the GIT segment considered. Factors such as inclusion level, diet composition, age and health status of the bird are known to modulate the effects of dietary butyrate supplementation. The GIT segment wherein butyrate is released may also modulates the observed effects. Unfortunately, commercially available butyrate additives offer poorly documented release kinetics. In order to better

understand the effect of butyrate on avian GIT physiology, development of targeted-release butyrate formulations as well as characterization of commercially available products appears to be an inevitable step.

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LITERATURE CITED

Aghazadeh, A. M., and M. Taha Yazdi. 2012. Effect of butyric acid supplementation and whole wheat inclusion on the performance and carcass traits of broilers. S. Afr. J. Anim. Sci. 42:241-248.

Andriamihaja, M., C. Chaumontet, D. Tome, and F. Blachier. 2009. Butyrate metabolism in human colon carcinoma cells:implications concerning its growth-inhibitory effect. J. Cell. Physio. 218:58-65.

Antongiovanni, M., A. Buccioni, F. Petacchi, S. Leeson, S. Minieri, A. Martini, and R. Cecchi. 2009. Butyric acid glycerides in the diet of broiler chickens: effects on gut histology and carcass composition. Ital. J. Anim. Sci. 6:19-25.

Astbury, S. M. and B.M. Corfe. 2012. Uptake and metabolism of the short-chain fatty acid butyrate, a critical review of the literature. Curr. Drug. Metab. 13:815-821.

Barcelo, A., J. Claustre, F. Moro, J.A. Chayvialle, J. C. Cuber, and P. Plaisancie. 2000. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut 46:218-224.

Barnes, P. J., and M. Karin. 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. 336:1066-1071.

Bartholome, A. L., D. M. Albin, D. H. Baker, J. J. Holst, and K. A. Tappenden. 2004. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in neonatal piglets. J. Parenter. Enteral. Nutr. 28:210-222.

Böhmig, G.A., P. M. Krieger, M. D. Saemann, R. Ullrich, H. Karimi, T. Wekerle, F. Muhlbacher, and G. J. Zlabinger. 1999. Stable prodrugs of n-butyric acid:suppression of T cell alloresponses in vitro and prolongation of heart allograft survival in a fully allogeneic rat strain combination. Transpl. Immunol. 7:221-227.

Böhmig, G.A., P.M. Krieger, M. D. Säemann, C. Wenhardt, E. Pohanka, and G. J. Zlabinger. 1997. n-Butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells:a potential mechanism for modulating T-cell responses by short-chain fatty acids. Immunol. 92:234-243.

Borgström, B. 1975. On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts. J. Lipid Res. 16:411-417.

Canani, R. B., M. D. Costanzo, L. Leone, M. Pedata, R. Meli, and A. Calignano. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J. Gastroenterol. 17:1519-1528.

Castillo, M., S. M. Martin-Orue, M. Roca, E .G. Manzanilla, I. Badiola, J. F. Perez, and J. Gasa. 2006. The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. J. Anim. Sci. 84:2725-2734.

Cerisuelo, A., C. Marin, F. Sanchez-Vizcaino, E. A. Gomez, J. M. De La Fuente, R. Duran, and C. Fernandez. 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and *Salmonella* counts in experimentally challenged broilers. Poult. Sci. 93:599-606.

Cherrington, C. A., M. Hinton, G. R. Pearson, and I. Chopra. 1991. Short-chain organic acids at ph 5.0 kill *Escherichia coli* and *Salmonella spp*. without causing membrane perturbation. J. Appl. Bacteriol. 70:161-165.

Czerwinski, J., O. Hojberg, S. Smulikowska, R. M. Engberg, and A. Mieczkowska. 2012. Effects of sodium butyrate and salinomycin upon intestinal microbiota, mucosal morphology and performance of broiler chickens. Arch. Anim. Nutr. 66:102-116.

De Brabander, C., C. Vervaet, L. Fiermans, and J.-P. Remon. 2000. Matrix mini-tablets based on starch/microcrystalline wax mixtures. Int. J. Pharm. 199:195-203.

Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Doreau, M., And Y. Chilliard. 1997. Digestion and metabolism of dietary fat in farm animals. Br. J. Nutr. 78 Suppl 1:S15-35.

Dumoulin, V., F. Moro, A. Barcelo, T. Dakka, and J. C. Cuber. 1998. Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. Endocrinology 139:3780-3786.

Engelstoft, M. S., K. L. Egerod, B. Holst, and T. W. Schwartz. 2008. A gut feeling for obesity: 7TM sensors on enteroendocrine cells. Cell Metab. 8:447-449.

Frankel, W.L., W. Zhang, A. Singh, D. M. Klurfeld, S. Don, T. Sakata, I. Modlin, and J.L. Rombeau. 1994. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. Gastroenterol. 106:375-380.

Gaudier, E., A. Jarry, H. M. Blottiere, P. De Coppet, M. P. Buisine, J. P. Aubert, C. Laboisse, C. Cherbut, and C. Hoebler. 2004. Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose. Am. J. Physiol. Gastrointest. Liver Physiol. 287:G1168-1174.

Guilloteau, P., L. Martin, V. Eeckhaut, R. Ducatelle, R. Zabielski, and F. Van Immerseel. 2010a. From the gut to the peripheral tissues:the multiple effects of butyrate. Nutr. Res. Rev. 23:366-384.

Guilloteau, P., G. Savary, Y. Jaguelin-Peyrault, V. Rome, L. Le Normand, and R. Zabielski. 2010b. Dietary sodium butyrate supplementation increases digestibility and pancreatic secretion in young milk-fed calves. J. Dairy Sci. 93:5842-5850.

Hamer, H. M., D. Jonkers, K. Venema, S. Vanhoutvin, F. J. Troost, and R .J. Brummer. 2008. Review article:the role of butyrate on colonic function. Aliment. Pharmacol. Ther. 27:104-119.

Hamer, H. M., D. M. Jonkers, A. Bast, S. A. Vanhoutvin, M. A. Fischer, A. Kodde, F. J. Troost, K. Venema, and R. J. Brummer. 2009. Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. J. Clin. Nutr. 28:88-93.

Hautekiet, V., P. Collet, V. Van Hamme, L. Goethals, And H. Schäublin. 2011. Influence of an additive based on encapsulated calcium butyrate in two nutritional profiles on growth performance of broiler chickens in Neuvièmes Journées de la Recherche Avicole. 9:247-250 (Abstr.)

Hu, Z., and Y. Guo. 2007. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. Anim. Feed Sci. Technol. 132:240-249.

Ichikawa, H., R. Shineha, S. Satomi, and T. Sakata. 2002. Gastric or rectal instillation of short-chain fatty acids stimulates epithelial cell proliferation of small and large intestine in rats. Dig. Dis. Sci. 47:1141-1146.

Jerzsele, A., K. Szeker, R. Csizinszky, E. Gere, C. Jakab, J. J. Mallo, and P. Galfi. 2012. Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and Bacillus amyloliquefaciens spore suspension against artificially induced necrotic enteritis in broilers. Poult. Sci. 91:837-843.

Kato, S., K. Sato, H. Chida, S.G. Roh, S. Ohwada, S. Sato, P. Guilloteau, and K. Katoh. 2011. Effects of Na-butyrate supplementation in milk formula on plasma concentrations of GH and insulin, and on rumen papilla development in calves. J. Endocrinol. 211:241-248.

Kien, C. L., R. Blauwiekel, J. Y. Bunn, T. L. Jetton, W. L. Frankel, and J. J. Holst, 2007. Cecal infusion of butyrate increases intestinal cell proliferation in piglets. J. Nutr. 137:916-922.

Klasing, K. C., and B. J. Johnstone. 1991. Monokines in growth and development Poult. Sci. 70:1781-1789.

Klasing, K. C., D. E. Laurin, R. K. Peng, and D. M. Fry. 1987. Immunologically mediated growth depression in chicks:influence of feed intake, corticosterone and interleukin-1. J. Nutr. 117:1629-1637.

Kotunia, A., J. Wolinski, D. Laubitz, M. Jurkowska, V. Rome, P. Guilloteau, and R. Zabielski. 2004. Effect of sodium butyrate on the small intestine development in neonatal piglets fed [correction of feed] by artificial sow. J. Physiol. Pharmacol. 55 Suppl 2:59-68.

Lal, S., A. J. Kirkup, A. M. Brunsden, D. G. Thompson, and D. Grundy. 2001. Vagal afferent responses to fatty acids of different chain length in the rat. Am. J. Physiol. Gastrointest. Liver Physiol. 281:G907-915.

Lawhon, S. D., R. Maurer, M. Suyemoto and C. Altier. 2002. Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. Mol. Microbiol. 46:1451-1464.

Leeson, S., H. Namkung, M. Antongiovanni, and E. H. Lee. 2005. Effect of butyric acid on the performance and carcass yield of broiler chickens. Poult. Sci. 84:1418-1422.

Lewis, K., F. Lutgendorff, V. Phan, J. D. Soderholm, P. M. Sherman, and D. M. Mckay. 2010. Enhanced translocation of bacteria across metabolically stressed epithelia is reduced by butyrate. Inflamm. Bowel Dis. 16:1138-1148.

Lin, H.V., A. Frassetto, E .J. Kowalik, A .R. Nawrocki, M. M. Lu, J. R. Kosinski, J. A. Hubert, D. Szeto, X. Yao, G. Forrest, and D. J. Marsh. 2012. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLOS ONE 7:e35240.

Liou, A. P. 2013. Digestive physiology of the pig symposium:G protein-coupled receptors in nutrient chemosensation and gastrointestinal hormone secretion. J. Anim. Sci. 91:1946-1956.

Ma, X., P. X. Fan, L. S. Li, S. Y. Qiao, G. L. Zhang, and D. F. Li. 2012. Butyrate promotes the recovering of intestinal wound healing through its positive effect on the tight junctions. J. Anim. Sci. 90 Suppl 4:266-268.

Maa, M. C., M. Y. Chang, M. Y. Hsieh, Y. J. Chen, C. J. Yang, Z. C. Chen, Y. K. Li, C. K. Yen, R. R. Wu, and T. H. Leu. 2010. Butyrate reduced lipopolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity. J. Nutr. Biochem. 21:1186-1192.

Mahdavi, R. and M. Torki. 2009. Study on usage period of dietary protected butyric acid on performance, carcass characteristics, serum metabolite levels and humoral immune response of broiler chickens. J. Anim. Vet. Adv. 8:1702-1709.

Maier, T., M. Güell, and L. Serrano. 2009. Correlation of mRNA and protein in complex biological samples. FEBS Lett. 583:3966-3973.

Mallo, J. J., A. Balfagon, M. I. Gracia, P. Honrubia, and M. Puyalto. 2012. Evaluation of different protections of butyric acid aiming for release in the last part of the gastrointestinal tract of piglets. J. Anim. Sci. 90 Suppl 4:227-229.

Manzanilla, E.G., Nofrarias, M., Anguita, M., Castillo, M., Perez, J.F., Martin-Orue, S.M., Kamel, C. And Gasa, J. 2006. Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. J. Anim. Sci. 84:2743-2751.

Mazzoni, M., M. Le Gall, S. De Filippi, L. Minieri, P. Trevisi, J. Wolinski, G. Lalatta-Costerbosa, J. P. Lalles, P. Guilloteau, and P. Bosi. 2008. Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. J. Nutr. 138:1426-1431.

Meijer, K., P. De Vos, and M. G. Priebe. 2010. Butyrate and other short-chain fatty acids as modulators of immunity:what relevance for health? Curr. Opin. Clin. Nutr. Metab. Care. 13:715-721.

Mentschel, J., and R. Claus. 2003. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. Metab. Clin. Exp. 52:1400-1405.

Moeinian, M., S. Farnaz Ghasemi-Niri, S. Mozaffari, and M. Abdollahi. 2013. Synergistic effect of probiotics, butyrate and L-carnitine in treatment of IBD. Med. Hypotheses. 7:50-53.

Moreau, H., Y. Gargouri, D. Lecat, J. L. Junien, and R. Verger. 1988. Screening of preduodenal lipases in several mammals. Biochim. Biophys. Acta 959:247-252.

Nohr, M. K., M. H. Pedersen, A. Gille, K. L. Egerod, M. S. Engelstoft, A. S. Husted, R. M. Sichlau, K. V. Grunddal, S. S. Poulsen, S. Han, R. M. Jones, S. Offermanns, and T. W. Schwartz. 2013. GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. Endocrinol. 154:3552-3564.

Panda, A. K., S. V. R. Rao, M. V. L. N. Raju, and G. S. Sunder. 2009. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. Asian. Australas. J. Anim. 22:1026-1031.

Peng, L., Z. R. Li, R. S. Green, I. R. Holzman, and J. Lin. 2009. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J. Nutr. 139:1619-1625.

Plaisancie, P., V. Dumoulin, J. A. Chayvialle, and J. C. Cuber. 1995. Luminal glucagon-like peptide-1(7-36) amide-releasing factors in the isolated vascularly perfused rat colon. J. Endocrinol. 145:521-526.

Plaisancie, P., V. Dumoulin, J. A. Chayvialle, and J. C. Cuber. 1996. Luminal peptide YY-releasing factors in the isolated vascularly perfused rat colon. J. Endocrinol. 151:421-429.

Ploger, S., F. Stumpff, G. B. Penner, J. D. Schulzke, G. Gabel, H. Martens, Z. Shen, D. Gunzel, and J. R. Aschenbach. 2012. Microbial butyrate and its role for barrier function in the gastrointestinal tract. Ann. N. Y. Acad. Sci. 1258:52-59.

Qaisrani, S. N. 2014. Improving performance of broilers fed lower digestible protein diets. PhD Diss. Wageningen Univ., Wageningen, NL.

Reilly, K. J., W. L. Frankel, A. M. Bain, and J. L. Rombeau. 1995. Colonic short chain fatty acids mediate jejunal growth by increasing gastrin. Gut 37:81-86.

Ribeiro, J., S. Gaspar, M. Pinho, J. P. B. Freire, and L. Falcão-E-Cunha. 2012. Sodium butyrate in growing and fattening diets for early-weaned rabbits. World Rabbit Sci. 20:199-207.

Russell, J. B. and F. Diez-Gonzalez. 1998. The effects of fermentation acids on bacterial growthAdv. Microb. Physiol. 39:205-234.

Sakata, T. 1989. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation of isolated and denervated jejunal segment of the rat. Scand. J. Gastroenterol. 24:886-890.

Sayrafi, R., F. Soltanalinejad, R. Shahrooz, and S. Rahimi. 2011. Comparative study of the effect of alternative and antibiotic feed additives on the performance and intestinal histomorphometrical parameters of broiler chickens. Afr. J. Agric. Res. 6:2794-2799.

Scheppach, W. and F. Weiler. 2004. The butyrate story:old wine in new bottles? Curr. Opin. Clin. Nutr. Metab. Care. 7:563-567.

Schneeberger, E. E. and R. D. Lynch. 2004. The tight junction:a multifunctional complex. Am. J. Physiol. Cell. Physiol. 286:C1213-1228.

Segain, J. P., D. Raingeard De La Bletiere, A. Bourreille, V. Leray, N. Gervois, C. Rosales, L. Ferrier, C. Bonnet, H.M. Blottiere, and J.P. Galmiche. 2000. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut 47:397-403.

Sengupta, S., J. G. Muir, and P. R. Gibson. 2006. Does butyrate protect from colorectal cancer? J. Gastroenterol. Hepatol. 21:209-218.

Sklan, D., B. Shachaf, J. Baron, and S. Hurwitz. 1978. Retrograde movement of digesta in the duodenum of the chick:extent, frequency, and nutritional implications. J. Nutr. 108:1485-1490.

Smith, D.J., A. Barri, G. Herges, J. Hahn, A. G. Yersin, and A. Jourdan. 2012. In vitro dissolution and in vivo absorption of calcium [1-^{14}C]butyrate in free or protected forms. J. Agric. Food Chem. 60:3151-3157.

Smith, J.G., W.H. Yokoyama, and J.B. German. 1998. Butyric acid from the diet:actions at the level of gene expression. Crit. Rev. Food. Sci. Nutr. 38:259-297.

Smulikowska, S., J. Czerwinski, A. Mieczkowska, and J. Jankowiak. 2009. The effect of fat-coated organic acid salts and a feed enzyme on growth performance, nutrient utilization, microflora activity, and morphology of the small intestine in broiler chickens. J. Anim. Feed Sci. 18:478-489.

Sossai, P. 2012. Butyric acid:what is the future for this old substance? Swiss Med. Wkly. 142:w13596.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R.G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLOS ONE 6:e27225.

Swiatkiewicz, S., A. Arczewska-Wlosek, and D. Jozefiak. 2014. Feed enzymes, probiotic, or chitosan can improve the nutritional efficacy of broiler chicken diets containing a high level of distillers dried grains with solubles. Livest. Sci. 163:110-119.

Sykaras, A.G., C. Demenis, R.M. Case, J. T. Mclaughlin, and Smith C.P. 2012. Duodenal enteroendocrine I-cells contain mRNA transcripts encoding key endocannabinoid and fatty acid receptors. PLOS ONE 7:e42373.

Ulluwishewa, D., R. C. Anderson, W. C. Mcnabb, P. J. Moughan, J. M. Wells, and N. C. Roy. 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141:769-776.

Van Dijk, J., J. Huismam, and J. Koninkx. 2002. Structural and functional aspects of a healthy gastrointesinal tract. Pages 71-92 in Nutrition and health of the gastrointestinal tract. M. C. Bloc, H. A. Vahl, L. De Lange, A. E. Van De Braak, G. Hemke, and M. Hessing, eds. Wageningen Academic Publishers, Wageningen, NL.

Van Immerseel, F., F. Boyen, I. Gantois, L. Timbermont, L. Bohez, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2005. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. Poult. Sci. 84:1851-1856.

Vervaeck, A., L. Saerens, B.G. De Geest, T. De Beer, R. Carleer, P. Adriaensens, J.-P. Remon, and C. Vervaet. 2013. Prilling of fatty acids as a continuous process for the development of controlled release multiparticulate dosage forms. Eur. J. Pharm. Biopharm. 85:587-596.

Vidrine, K., J. Ye, R. J. Martin, K. L. Mccutcheon, A. M. Raggio, C. Pelkman, H. A. Durham, J. Zhou, R. N. Senevirathne, C. Williams, F. Greenway, J. Finley, Z. Gao, F. Goldsmith, and M.J. Keenan. 2014. Resistant starch from high amylose maize (HAM-RS2) and dietary butyrate reduce abdominal fat by a different apparent mechanism. Obesity 22:344-348.

Wang, H. B., P. Y. Wang, X. Wang, Y. L. Wan, and Y. C. Liu. 2012. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. Dig. Dis. Sci. 57:3126-3135.

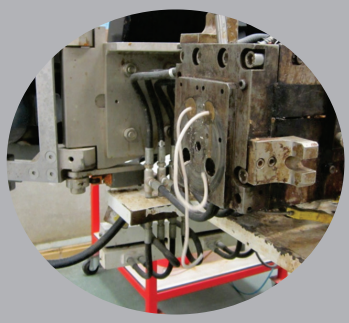
Watford, M., P. Lund, and H. A. Krebs. 1979. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589-596.

Willemsen, L.E., M. A. Koetsier, S. J. Van Deventer, and E. A. Van Tol. 2003. Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts. Gut 52:1442-1447.

Yilmaz, A., S. Shen, D. L. Adelson, S. Xavier, and J.J. Zhu. 2005. Identification and sequence analysis of chicken Toll-like receptors. Immunogenetics. 56:743-753.

Zhang, W. H., F. Gao, Q. F. Zhu, C. Li, Y. Jiang, S. F. Dai, and G. H. Zhou. 2011a. Dietary sodium butyrate alleviates the oxidative stress induced by corticosterone exposure and improves meat quality in broiler chickens. Poult. Sci. 90:2592-2599.

Zhang, W. H., Y. Jiang, Q. F. Zhu, F. Gao, S. F. Dai, J. Chen, and G.H. Zhou. 2011b. Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. Br. Poult. Sci. 52:292-301



Chapter 3

Development and *in vitro* evaluation of targeted and sustained release butyrate formulations for delivery in the avian gastrointestinal tract

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ABSTRACT

An *in vitro* model of the poultry gastro-intestinal tract (**GIT**) was used to compare the release profiles of experimental butyrate formulations. Two processes were applied: 1) melt-extrusion/grinding to produce four types of continuous protective matrices differing in the embedding materials and 2) wet-extrusion/spheronization to produce non-protective butyrate-loaded cellulose pellets that were subsequently coated with a pH-sensitive polymer in a fluidized bed reactor. Release profiles of these five new formulations and of five commercially available butyrate additives were evaluated in triplicate in a three-step (pre-gastric, gastric and enteric) *in vitro* digestion procedure. The pre-gastric step had a pH of 5.8, contained α-amylase and lasted for 35 min. The gastric step had a pH of 2.7, contained pepsin and lasted for 35 min. The enteric step had a pH of 6.5, contained pancreatin and bile salts and lasted for 10, 80 or 170 min if the digestion until the end of the duodenum, jejunum or ileum was simulated, respectively. Aliquots were drawn at the five stages of digestion representing the end of the crop, proventriculus-gizzard, duodenum, jejunum and ileum. The percentage of the initial butyrate dose solubilized was calculated for each stage. Significant differences were found among formulations for each digestion stage (P<0.001). Commercial fat-coated products showed heterogeneous release profiles, some products being non-protective whereas others displayed a sustained release profile. Wax matrices, with or without starch inclusion, showed a sustained release profile, being generally more protective than commercial fat-coated products. Tributyrin and coated pellets had a release profile targeting enteric segments. Behenic and stearic acid matrices released most of their content at the crop stage. Results of this study can aid to develop experimental contrasts suitable to study the effects of butyrate release location on avian digestive physiology.

Keywords: *In vitro*, poultry, butyrate, coating, matrix

INTRODUCTION

Butyrate elicits stimulatory, albeit inconsistent, effects on growth performance of broiler chickens (Cerisuelo *et al.,* 2014; Leeson *et al.,* 2005; Van Immerseel *et al.,* 2005; Zhang *et al.,* 2011). Release location may affect the observed effects of butyrate throughout the gastrointestinal tract (**GIT**) of broilers given the diversity of cell types and pH conditions encountered, and the differences in microbiota composition in the different gut segments (Moquet *et al.*, 2016).

Formulations which vary in their release profile of butyrate in the GIT are important tools to allow investigation of effects *in vivo*. Targeted release refers to the localized delivery of an active compound based on organ-specific characteristics such as pH value or enzymatic activity. This can be achieved using pH-dependent protections such as free fatty acid matrices or non-protective matrices coated with pH-sensitive polymers (Ji *et al.*, 2007; Vervaeck *et al.*, 2013). Also tributyrin, the triglyceride analogue of butyrate, can be considered as a targeted release formulation due to the low pre-duodenal lipolytic activity of broiler's GIT (Cherif *et al.*, 2006; Moreau *et al.*, 1988). Sustained release refers to the prolonged release of an active compound over time based on enzymatic or mechanic erosion and diffusion mechanisms. This can be achieved, for example, by wax matrices where inclusion of starch additives influences the release rate of the active molecule (Zhou *et al.*, 1996). Embedding butyrate in vegetable fat matrices is commonly used by the feed industry to offer a partial protection against gastric absorption (Moquet *et al.*, 2016) which leads to a sustained release of butyrate in the broilers' GIT (van den Borne *et al.*, 2015).

The release profile of butyrate in the GIT can be determined *in vivo* by measuring the content in the chyme at different locations (Sotak *et al.*, 2013) or by measuring the oxidation of labelled butyrate in respiratory chambers (Smith *et al.*, 2012; van den Borne *et al.*, 2015). The first technique does not allow discrimination between dietary and microbial derived butyrate, while the costs of the latter prohibits routine screening of formulations. *In vitro* models are interesting alternatives to the aforementioned techniques as they are relatively rapid, cost-effective, and the results are a not confounded by endogenous fermentation end-products or biological variation. Release profiles influenced by physical and chemical characteristics of the GIT, e.g. pH, temperature, enzymatic activity or retention time can be investigated through such an *in vitro* approach. Specific conditions encountered in the avian GIT are well documented in the literature (Long, 1967; Philips and Fuller, 1983; Żyła *et al.*, 1999; Denbow, 2015). Current *in vitro* models stimulating digestive processes in poultry, however, are not based on these specific conditions and as such are open for improvements.

The present work was undertaken to compare the release profiles of experimental and commercially available butyrate additives, using an improved *in vitro* poultry digestion simulation model. It was hypothesised that pH- and enzyme-sensitive protections would provide targeted release properties whilst wax and triglyceride matrices would provide sustained release profiles.

MATERIAL AND METHODS

Development of butyrate formulations

Preparation of continuous matrices by melt-extrusion and grinding. Different sodium butyrate (Adimix C, Nutri-Ad International NV, Dendermonde, BE) formulations were prepared according to Table 1. Behenic and stearic acid powders were provided by Chempri Oleochemicals (Raamsdonksveer, NL), hydrogenated palm fat flakes by Nutri-ad International NV, Luncera M wax beads by Füller GmbH (Lüneburg, DE) and S2004 soluble potato starch powder by Sigma-Aldrich (Saint-Louis, USA). Prior to mixing, palm fat flakes and wax beads were ground at 15,000 rpm with a ZM-1000 grinder (Retsch, Haan, DE) equipped with a 12-tooth rotor and without screen. Three kg batches of each formulation were mixed with a pedal mixer model 305 (Dinissen, Sevenum, NL) at 50 % of the maximum speed for 5 min and subsequently extruded with a twin screw extruder (M.P.F.50, Baker Perkins Ltd., Peterborough, UK). During a preliminary trial, extrusion settings were adjusted for each formulation in order to obtain homogenous, smooth-surfaced, non-melted extrudates. Screw configuration was identical to the one described by De Vries and coworkers (2014). No die face cutter was used. Final extrusion settings are indicated in Table 1.

Table 1. Composition and extrusion settings used for the production of continuous matrices

	Experimental Formulation			
	Behenic	Stearic	Wax	Wax + starch
Composition (g/kg)				
Behenic acid	700	-	-	-
Stearic acid	-	700	-	-
Lunacera M	-	-	700	600
Sodium butyrate	300	300	300	300
Potato starch	-	-	-	100
Extruder configuration				
Dies (n x mm)	2 x 3	1 x 3	1 x 3	2 x 3
Feeding rate (% of maximum rpm)	8	8	8	8
Screw speed (% of maximum rpm)	10	20	10	10
Load (%)	12	20	22-25	28-30
Temperature settings (°C)				
Zone 1	29	25	30	30
Zone 2	37	47	45	37
Zone 3	57	54	60	43
Zone 4	61	55	60	50
Zone 5	56	56	60	49
Zone 6	52	58	61	53
Zone 7	55	51	59	54
Zone 8	82	68	37	55
Zone 9	61	50	55	52
Product temperature at the die	49.4	48.2	52-53	41

Extrudates were cooled to room temperature overnight and subsequently ground at 10.000 rpm with a ZM-1000 grinder (Retsch) equipped with a 12-tooth rotor without screen. Ground extrudates were sieved manually in order to obtain the 1.2-1.4 mm fraction.

Preparation of microencapsulated pellets by wet-extrusion, spheronization and fluidized-bed reactor coating. Avicel PH-101 (850 g/kg; FMC BioPolymer, Philadelphia, USA) microcrystalline cellulose and sodium butyrate (150 g/kg; Admix C, Nutri-Ad International NV) were mixed with a commercial mixer (Kenwood Chef, Kenwood Belgium, Mechelen, BE). Demineralized water (720 ml/kg dry powder mix) was added to the dry powder mix in a drop-wise manner while mixing. The wet mixture was subsequently extruded with a dome granulator (model DG-L1, Fuji-Paudal, Osaka, JPN) and spheronized for 5 min (model 15, Sturminster Newton, UK). Microcrystalline cellulose butyrate pellets were dried overnight at room temperature and sieved in order to obtain the 800-1200 µm fraction. Wet powder formulation was determined on the basis of a preliminary trial aimed at obtaining homogenous, non-brittle, smooth-surfaced pellets. Eudragit® FS 30 D (Evonik Industries, Essen, DE) was applied as coating on the pellets using a fluidized-bed reactor (model GPCG1, Glatt GmbH, Binzen, DE) including a Wurster module. Eudragit® FS 30 D is a polymer that dissolves at pH > 7. Coating solution was prepared using glycerol monostearate as an anti-tacking agent according to the technical information provided by the supplier. Recorded weight gain was 20 % (w/w). The resulting product is referred to as microencapsulated pellets. The fluidized-bed reactors settings are provided in Table 2.

Table 2. Fluidized bed reactor settings used for the production of microencapsulated pellets

Nozzle diameter	0.8 mm
Tubing diameter	1.6 mm
Sprayed air pressure	0.8 bar
Microclimate	0.4 bar
Inlet air temperature	35 °C
Air flow	35 m^3/h
Product temperature during coating phase	28 °C
Drying phase temperature	40 °C
Spraying phase duration	30 min
Drying phase duration	120 min

In vitro evaluation of butyrate formulations

Experimental design. The four experimental matrices, microencapsulated pellets, tributyrin (T8626, Sigma-Aldrich) and four commercially available fat-protected butyrate additives: Adimix Precision (Nutri-ad International NV), Ding Su (Singao, Xiamen, China), Sodium Butyrate 300 and 500 (Vega Pharma Limited, Hangzhou, China) were evaluated in triplicate in the *in vitro* digestion procedure as one batch. All formulations were submitted to the butyrate content determination described below, except for tributyrin, for which the butyrate content was known.

Butyrate content determination procedure. Butyrate formulations were ground with a mortar and a pestle. Approximately 0.25 g ground product was accurately weighed in triplicate into 80 ml centrifuge tubes containing a stirring magnet and 20 ml of 1 M HCl solution was added to each tube. The centrifuge tubes were then closed with a rubber stopper and placed on multipoint stirrers in thermostatically-controlled heating chamber at 40 ± 0.1°C for 24 hours at a stirring rate of 360 rpm. Aliquots drawn at the end of this procedure were used to determine the average butyrate content of each formulation.

In vitro *digestion procedure.* The *in vitro* simulation of the digestive locations of poultry consisted of pre-gastric, gastric and enteric steps. The enteric step had three different durations, resulting in a total of five digestion stages. Approximately 0.25 g of sample was accurately weighed into an 80 ml centrifuge tube containing a stirring magnet. To each tube, 20 ml of a 1 M tris-HCl buffer solution (pH 5.8) containing 1 g/l NaCl and 0.5 g/l of α-amylase from porcine pancreas (10 U/mg solid; A3176, Sigma-Aldrich) was added. Thereafter the tube was closed with a rubber stopper and placed on a multipoint stirrer in a thermostatically-controlled heating chamber for 35 min. When only the pre-gastric stage was considered, the procedure was terminated by transferring the tube to ice. Otherwise, digestion was continued by adding 2 ml of a 0.085 M HCl solution containing 11 g/l of pepsin from porcine gastric mucosa (250 U/mg solid; P7000, Sigma-Aldrich). If necessary, pH was adjusted to 2.7 ± 0.05 with drops of 6 M HCl or 6 M NaOH. The second stage of digestion then proceeded for 35 min and the procedure was stopped when only the gastric digestion was considered by transferring the tubes to ice. Otherwise, 4 ml of a 1 M tris-buffer solution (pH 6.5) containing 1.875 g/l porcine pancreatin (8 × USP specifications; P7545, Sigma-Aldrich) and 0.7 g/l bile salts (48305, Sigma-Aldrich) was added to each tube. If necessary, pH was adjusted to 6.5 ± 0.05 with drops of 6 M HCl or 6 M NaOH. The digestion was terminated by placing the tubes on ice, 10, 80 or 170 min after addition of pancreatin for simulation of duodenal, jejunal or ileal digestion, respectively. Aliquots were drawn at the end of each digestion stage. The pH value at the end of the final digestion stage was recorded. A temperature of 40 ± 0.1°C and a stirring rate of 360 rpm were maintained throughout the different stages of digestion.

Chemical and statistical analysis

Butyrate determination. Aliquots were diluted with phosphoric acid containing iso-caproic acid as an internal standard. Samples were centrifuged for 5 min at 14,000 rpm at 5°C. Butyrate was separated by gas chromatography (Trace GC Ultra, ThermoFischer Scientific, Waltham, MA, USA) using an EM-1000 (30 m x 0.53 mm) column from Alltech (Deerfield, IL, USA), helium as the mobile phase and detected by flame ionization detector. Quantification was based on a chemical standard solution after internal standard correction.

Statistical analysis. Total amount of butyrate added to the assay was calculated by multiplying the sample weight by the butyrate content. Butyrate release was expressed as the percentage of the initial butyrate dose that was solubilized at a certain digestion stage. In case release values above 100% were recorded, the highest value was corrected to 100%, with all other values adjusted accordingly within formulation. Butyrate release values within simulated digestion stages and pH values at the simulated ileum were analysed by the PROC GLM procedure of SAS® version 9.2 (SAS Institute Inc., Cary, NC, USA), using the following model:

$$Y_i = \mu + F_i + e_i$$

where Y_i is the dependent variable, μ the overall mean, F_i the formulation effect (i= 1 to 10) and e_i the residual error of the model. Fisher's least significant difference test was used as a post-hoc test. Results were considered significant at a probability level below 5%.

RESULTS AND DISCUSSION

Table 3 presents the butyrate release values, expressed as the percentage of initial dose, solubilised at each digestion stage. The pH value observed at the simulated ileal digestion stage was significantly lower for tributyrin than for the other butyrate additives (P<0.05).

Table 3. *In vitro* butyrate release at different simulated gastrointestinal locations (% of the initial butyrate dose)

Item[1]	Gastrointestinal locations					Simulated ileal pH
	Crop	Gastric region	Duodenum	Jejunum	Ileum	
Commercial butyrate formulations						
Adimix Precision	54.1^{c}	73.6^{c}	77.9^{cd}	84.9^{bc}	89.1^{bc}	5.86^{c}
Vega 300	54.1^{c}	70.4^{c}	80.9^{bc}	87.2^{bc}	90.7^{ab}	5.97^{ab}
Vega 500	97.8^{a}	96.5^{ab}	95.2^{a}	96.5^{ab}	95.8^{ab}	5.97^{ab}
Ding Su	79.5^{b}	88.0^{b}	94.8^{a}	89.3^{ab}	89.8^{abc}	5.93^{abc}
Tributyrin	13.4^{d}	13.9^{f}	20.1^{f}	34.0^{e}	41.2^{e}	5.66^{d}
Experimental butyrate formulations						
Behenic	84.8^{b}	89.1^{ab}	84.3^{b}	94.8^{ab}	91.6^{abc}	5.97^{ab}
Stearic	98.0^{a}	97.6^{a}	94.4^{a}	99.0^{a}	96.1^{ab}	5.89^{bc}
Wax	51.3^{c}	61.6^{d}	76.4^{cd}	68.5^{d}	76.3^{d}	5.93^{abc}
Wax + starch	53.8^{c}	70.3^{c}	72.7^{d}	76.5^{dc}	84.6^{c}	5.97^{ab}
Micropellets	12.7^{d}	25.6^{e}	58.6^{e}	98.9^{a}	96.8^{a}	6.01^{a}
Statistical analysis						
SEM	2.2	2.9	2	3.9	2.5	0.03
P-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

^{a,b,c,d,e,f}Means within a column lacking a common superscript are significantly (P<.05) different.
[1]Averages of three replicates.

Fat-coated commercial butyrate formulations

Van den Borne and coworkers (2015) demonstrated in an *in vivo* study that fat-coated butyrate had an extended release profile in broiler chickens. In the present *in vitro* study, only half of the tested commercial fat-protected butyrate products (Adimix 30C and Vega 300) had a similar release profile. Differences can be attributed to variations in industrial processes as numerous technologies can be used to achieve spray chilling (Oxley *et al.*, 2012). Such variability of release profiles among products that had similar macroscopic shapes, colours and particle sizes could contribute to inconsistencies in performance data and intestinal morphology responses of broilers fed different fat-coated butyrate additives (Smulikowska *et al.*, 2009; Czerwinski *et al.*, 2012; Jerzsele *et al.*, 2012).

Tributyrin

With a simulated average release of 41.2 % for tributyrin at the ileum, results may contrast with findings of Schwarzer and Bjork (2015), who established *in vitro* and *in vivo* that two third of the butyrate contained in tributyrin is released in the very proximal part of the small intestine of pigs. In the present study, tributyrin digestion significantly reduced the pH in the ileum compared to the other additives (P<0.05). Cleavage of tributyrin's ester bonds by pancreatic lipase leads to the release of butyric acid. The resulting acidification is known to negatively affect lipase activity (Borgstrom, 1975), which could contribute to the lower release reported in the present study.

Experimental butyrate formulations

The novel butyrate additives assayed displayed diverse release profiles. Behenic and stearic acid matrices did not provide any gastric protection, in contrast to published *in vitro* work in humans (Vervaeck *et al.*, 2013). For such matrices, release is triggered by increased ionization rates occurring when chyme pH becomes higher than the pKa of the considered acid. Due to their respective pKa of 4.70 and 4.75, behenic and stearic acids were already ionized at the crop digestion stage (pH 5.8), resulting in an early butyrate release. Microencapsulated pellets showed a partial gastric protection followed by a rapid release in the enteric segments that is consistent with the type of coating used. Finally, the wax matrices showed a sustained release profile and appeared to be highly effective in releasing butyrate at the simulated ileum. Inclusion of starch in such matrices led to a higher (P<0.05) release at the ileum. This is in agreement with data in the literature and can be due to a change of matrix solubility and to an increased exchange surface resulting from starch digestion (Zhou *et al.*, 1996).

CONCLUSION

Several butyrate additives were shown to have potential for establishing experimental contrasts in butyrate release within the avian GIT. Several formulations had sustained release properties (Adimix 30 C, Vega 300, wax matrices), while others allowed to target enteric segments (tributyrin, microencapsulated pellets). Additionally, despite being macroscopically similar, commercial fat-coated butyrate products show variations in release properties *in vitro*. This could explain part of the inconsistencies observed *in vivo* in the scientific literature of these products.

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LITERATURE CITED

Borgstrom, B. 1975. On the interactions between pancreatic lipase and colipase and the substrate, and the importance of bile salts. J. Lipid Res. 16:411-417.

Cerisuelo, A., C. Marin, F. Sanchez-Vizcaino, E. A. Gomez, J. M. de la Fuente, R. Duran, and C. Fernandez. 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and *Salmonella* counts in experimentally challenged broilers. Poult. Sci. 93:599-606.

Cherif, S., N. Miled, M. Kammoun, H. Mejdoub, and Y. Gargouri. 2006. Purification and biochemical characterization of a turkey preduodenal esterase. Comp. Biochem. Physiol. B Comp. Biochem. Mol. Biol. 145:358-364.

Czerwinski, J., O. Hojberg, S. Smulikowska, R. M. Engberg, and A. Mieczkowska. 2012. Effects of sodium butyrate and salinomycin upon intestinal microbiota, mucosal morphology and performance of broiler chickens. Arch. Anim. Nutr. 66:102-116.

de Vries, S., A. M. Pustjens, M. A. Kabel, R. P. Kwakkel, and W. J. J. Gerrits. 2014. Effects of processing technologies and pectolytic enzymes on degradability of nonstarch polysaccharides from rapeseed meal in broilers. Poult. Sci. 93:589-598.

Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Jerzsele, A., K. Szeker, R. Csizinszky, E. Gere, C. Jakab, J. J. Mallo, and P. Galfi. 2012. Efficacy of protected sodium butyrate, a protected blend of essential oils, their combination, and *Bacillus amyloliquefaciens* spore suspension against artificially induced necrotic enteritis in broilers. Poult. Sci. 91:837-843.

Ji, C., H. Xu, and W. Wu. 2007. In vitro evaluation and pharmacokinetics in dogs of guar gum and Eudragit FS30D-coated colon-targeted pellets of indomethacin. J. Drug Target 15:123-131.

Leeson, S., H. Namkung, M. Antongiovanni, and E. H. Lee. 2005. Effect of butyric acid on the performance and carcass yield of broiler chickens. Poult. Sci. 84:1418-1422.

Long, J. F. 1967. Gastric secretion in unaesthetized chickens. J. Physiol. 212:1303-1307.

Moquet, P. C. A., L. Onrust, F. Van Immerseel, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72:61-80.

Moreau, H., Y. Gargouri, D. Lecat, J. L. Junien, and R. Verger. 1988. Screening of preduodenal lipases in several mammals. Biochim. Biophys. Acta 959:247-252.

Oxley, J. D. 2012. 5 - Spray cooling and spray chilling for food ingredient and nutraceutical encapsulation. Pages 110-130 in Encapsulation technologies and delivery systems for food ingredients and nutraceuticals. Garti, N., and J. D. McClements, eds. Woodhead Publishing, Cambridge, UK.

Philips, S. M., and R. Fuller. 1983. The activities of amylase and a trypsin-like protease in the gut contents of germ-free and conventional chickens. Br. Poult. Sci. 24:115-121.

Schwarzer, K., and J. Bjork. 2015. Tributyrin: biological effects to stimulate animal performance and the integrity of of the intestinal tract in pigs, in: 47e Journées de la Recherche Porcine, INRA, Paris, pp. 133-134.

Smith, D. J., A. Barri, G. Herges, J. Hahn, A. G. Yersin, and A. Jourdan. 2012. *In vitro* dissolution and *in vivo* absorption of calcium [1-^{14}C] butyrate in free or protected forms. J. Agric. Food Chem. 60:3151-3157.

Smulikowska, S., J. Czerwinski, A. Mieczkowska, and J. Jankowiak. 2009. The effect of fat-coated organic acid salts and a feed enzyme on growth performance, nutrient utilization, microflora activity, and morphology of the small intestine in broiler chickens. J. Anim. Feed Sci. 18:478-489.

Sotak, K., M. Song, H. Stein, and S. Moreland. 2013. Disappearance of butyrate in the digestive tract of weanling and growing pigs fed diets containing different sources of butyrate. J. Anim. Sci. 91(2):582-583.

van den Borne, J. J. G. C., M. J. W. Heetkamp, J. Buyse, and T. A. Niewold. 2015. Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. Livest. Sci. 175:96-100.

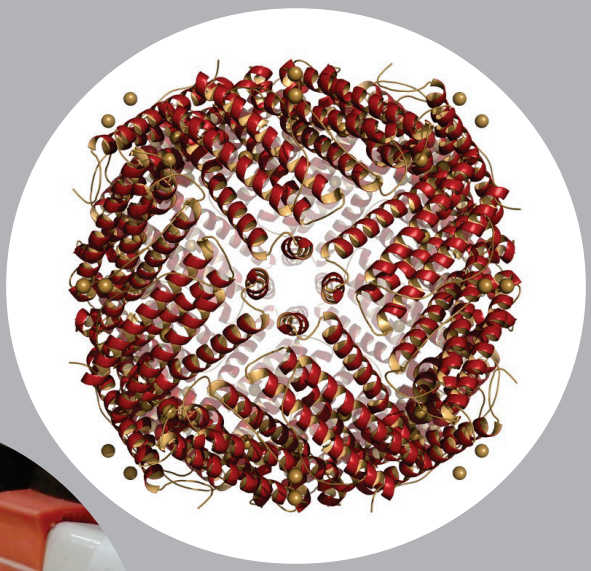
Van Immerseel, F., F. Boyen, I. Gantois, L. Timbermont, L. Bohez, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2005. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. Poult. Sci. 84, 1851-1856.

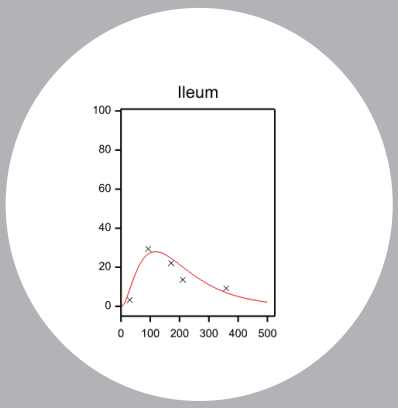
Vervaeck, A., L. Saerens, B. G. De Geest, T. De Beer, R. Carleer, P. Adriaensens, J. P. Remon, and C. Vervaet. 2013. 2013. Prilling of fatty acids as a continuous process for the development of controlled release multiparticulate dosage forms. Eur. J. Pharm. Biopharm. 85:587-596.

Zhang, W. H., Y. Jiang, Q. F. Zhu, F. Gao, S. F. Dai, J. Chen, and G. H. Zhou. 2011. Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. Br. Poult. Sci. 52:292-301.

Zhou, F., C. Vervaet, and J. P. Remon. 1992 Matrix pellets based on the combination of waxes, starches and maltodextrins. Int. J. Pharm. 133:155-160.

Żyła, K., D. Gogol, J. Koreleski, S. Świątkiewicz, and D. R. Ledoux. 1999. 1999. Simultaneous application of phytase and xylanase to broiler feeds based on wheat: in vitro measurements of phosphorus and pentose release from wheats and wheat-based feeds. J. Sci. Food Agric. 79:1832-1840.





Chapter 4

Butyrate presence in distinct gastro-intestinal tract segments modifies differentially digestive processes and amino acid bioavailability in young broiler chickens

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ABSTRACT

The hypothesis was tested that butyrate presence in the digesta of distinct gastrointestinal tract (**GIT**) segments of broilers leads to differential effects on digesta retention time, gut morphology and proteolytic enzymatic activities, ultimately resulting in differences in protein digestibility. A total of 320 male day-old Ross 308 broilers were randomly assigned to 5 dietary treatments: 1. control (no butyrate), 2. unprotected butyrate (main activity in the crop and gastric regions), 3. tributyrin (main activity in the small intestine), 4. fat-coated butyrate (activity in the whole GIT) and 5. unprotected butyrate combined with tributyrin, each replicated 8 times. Rapeseed meal was used in combination with a fine dietary particle size in order to challenge the digestive capacity of young broilers. Birds were dissected at 22, 23 and 24 d of age and samples of digesta at various GIT locations as well as tissues were collected. Butyrate concentration varied significantly across GIT segments depending on treatment, indicating that the dietary contrasts were successful. The apparent ileal digestibility of methionine tended to increase when butyrate and/or propionate was present in colonic and cecal contents, possibly due to modifications of GIT development and digesta transit time. Butyrate presence in the digesta of the crop, proventriculus and gizzard, on the contrary, tended to decrease the apparent ileal digestibility of several amino acids (**AA**). In addition, butyrate presence beyond the gizzard elicited anorexic effect that might be attributable to changes in intestinal enteroendocrine L-cells secretory activities. The present study demonstrates that, in broilers, effects of butyrate on digestive processes are conditioned by the GIT segment wherein the molecule is present and indicates its influence on digestive function and bioavailability of AA.

Key words: Butyrate, broiler, digestibility, digesta retention time, location effect

INTRODUCTION

The short-chain fatty acid (**SCFA**) butyrate is commonly used as a feed additive to enhance growth performance of broiler chickens. The mode of action of butyrate is not fully understood and broiler performance response to dietary butyrate supplementation is inconsistent (Cerisuelo *et al.*, 2014). Mammalian research indicates that luminal butyrate elicits changes in the endocrine regulation of digestive processes (Dumoulin *et al.*, 1998; Kotunia *et al.*, 2004; Mazzoni *et al.*, 2008; Vidrine *et al.*, 2014). These changes could influence digesta transit time, gut morphology and digestive enzymes activities in a manner where it can affect nutrient digestibility. Significant improvements in nitrogen digestibility and trends for higher proventricular proteolytic activities in broilers fed butyrate are consistent with putative changes in digestive physiology (Smulikowska *et al.*, 2009; Qaisrani *et al.*, 2015).

Subsets of enteroendocrine cells are characterized by their localization within the gastrointestinal tract (**GIT**) and by their response to luminal butyrate sensing. Therefore, it can be hypothesized that the GIT segment wherein butyrate is present modulates the activation of the enteroendocrine cells and their associated physiological effects. The use of different butyrate additives allows to test this hypothesis as unprotected butyrate is active in the crop, proventriculus and gizzard whereas tributyrin provides butyrate to the small intestine (Moquet *et al.*, 2016). In addition, several fat-coated butyrate additives display a sustained release profile, possibly increasing butyrate availability in the colon and ceca (Van den Borne *et al.*, 2015; Moquet *et al.*, 2016). Previous research has highlighted that a lesser digestible protein source, such as rapeseed meal, in association with a fine diet structure, is particularly challenging for the GIT development of broilers (Qaisrani *et al.*, 2014). Using such a feed challenge in young broilers could aid to distinguish possible effects of butyrate on protein digestibility and GIT development.

The present work was undertaken to investigate the effect of butyrate presence in distinct GIT segments on digestive processes of broilers and to attribute possible changes in protein digestibility to changes in gut morphology, digesta transit time or enzymatic activities. In this paper, the term "butyrate presence" is used to emphasize that it is not known whether the active molecule has an endogenous or exogenous origin. It was hypothesized that butyrate-mediated changes in gastric and enteric retention time, gut morphology and enzymatic activities would result in improvements of protein digestibility. In addition, it was hypothesized that the response of broilers to butyrate supplementation would differ depending on the GIT segment wherein the molecule is present.

MATERIAL AND METHODS

Experimental design

A completely randomized design consisting of 5 dietary treatments was used: 1. control (**CTR**; no butyrate), 2. unprotected butyrate (**UP**; butyrate mainly available in the crop/proventriculus/gizzard), 3. tributyrin (**TB**; butyrate mainly available in the small intestine), 4. fat-coated butyrate (**FCB**; butyrate available in the whole GIT) and

5. A 50:50 mixture of UP and TB (butyrate radical $C_4H_7O_2^-$ weight basis; **UPTB**). Butyrate-supplemented diets contained 1 g/kg of butyrate radical (as is basis). Ingredient constituents used as carrier in the butyrate additives such as palm fat or silica were present in similar amounts across diets. Sodium bicarbonate was used to maintain equal sodium supply across diets. Diets were formulated to meet or exceed the requirements of broiler chickens (CVB, 2007) and contained chromium sesquioxide (Cr_2O_3) as an indigestible marker of the solid phase fraction. Ingredient and analysed nutrient compositions of the diets are provided in Table 1.

Table 1. Ingredient and analysed nutrient compositions of the control diet (CTR) and diet containing unprotected butyrate (UP), tributyrin (TB), fat-coated butyrate (FCB) and a mixture of UP and TB (UPTB)

Item	CTR	UP	TB	FCB	UPTB
Ingredient (g/kg; as in basis)					
Corn	292.05	292.05	292.05	292.05	292.05
Wheat	200.00	200.00	200.00	200.00	200.00
Rapeseed meal	350.00	350.00	350.00	350.00	350.00
Fishmeal	75.00	75.00	75.00	75.00	75.00
Soybean oil	55.29	55.03	54.13	55.03	54.58
Premix[1]	5.00	5.00	5.00	5.00	5.00
Lime fine	9.00	9.00	9.00	9.00	9.00
Monocalcium phosphate	5.50	5.50	5.50	5.50	5.50
Salt	0.90	0.90	0.90	0.90	0.90
$NaHCO_3$	2.80	1.80	2.80	1.80	2.30
L-Lysine HCl	0.50	0.50	0.50	0.50	0.50
Fat-coated butyrate[2]	0.00	0.00	0.00	4.21	0.00
Tributyrin on silica support[3]	0.00	0.00	1.78	0.00	0.89
Compacted sodium butyrate[4]	0.00	1.40	0.00	0.00	0.70
Ground palm fat	2.95	2.95	2.95	0.00	2.95
Silica	0.62	0.62	0.00	0.62	0.31
Dextrin	0.14	0.00	0.14	0.14	0.07
Chromic sesquioxide	0.25	0.25	0.25	0.25	0.25
Calculated nutrient composition					
Crude protein (g/kg)	215.4	215.4	215.4	215.4	215.4
Metabolisable energy (MJ/kg)	12.36	12.36	12.36	12.36	12.36
Calcium (g/kg)	9.1	9.1	9.1	9.1	9.1
Available Phosphorus (g/kg)	4.0	4.0	4.0	4.0	4.0
Methionine (g/kg)	4.67	4.67	4.67	4.67	4.67
Lysine (g/kg)	12.16	12.16	12.16	12.16	12.16
Analysed nutrient composition (g/kg DM)					
Starch	333.6	326.1	327.4	314.6	325.6
Fat	96.8	98.8	98.4	99.1	99.1
Nitrogen	38.1	37.8	38.0	38.3	38.4
Crude fibre	67.6	66.6	71.1	65.5	66.0
Ash	63.5	64.8	64.5	64.3	64.5

[1]Provided per kg of diet: vitamin A, 12.000 IU; vitamin D_3, 2.500 IU; vitamin E, 50 mg; vitamin B_2, 7.5 mg; vitamin B_6, 3.5 mg; vitamin B_1, 2.0 mg; vitamin K_3, 1.5 mg; vitamin B_{12}, 20 µg; choline chloride, 460 mg; antioxidant (oxytrap PXN),125 mg; niacin, 35 mg; pantothenic acid, 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg; Fe, 80 mg; Zn, 60 mg; Cu, 12 mg; I, 0.8 mg; Se, 0.15 mg.
[2]Adimix Precision (Nutriad International, Dendermonde, Belgium). Contains 700 g/kg palm fat; 237 g/kg $C_4H_7O_2^-$; 63 g/kg Na^+.
[3]Experimental Tributyrin (Nutriad International); contains 650 g/kg tributyrin; 350 g/kg silica.
[4]Adimix Easy (Nutriad International); contains 712 g/kg $C_4H_7O_2^-$; 188 g/kg Na^+, 100 g/kg dextrin.

Birds and experimental procedures

The experiment was conducted at the research farm Carus of Wageningen University. All the experimental procedures were approved by the Animal Care and Use Committee of Wageningen University. A total of 320 male, one-day-old broilers (initial BW 42.1 ± 3.3 g; Ross 308, Aviagen Group, Newbridge, United Kingdom) were obtained from a commercial hatchery (Kuikenbroederij Morren B.V., Lunteren, the Netherlands). Upon arrival, birds were wing-tagged, individually weighed and randomly assigned to one of the 40 floor pens in a single climate-controlled room. Each of the 5 treatment diets was fed to 8 replicate pens of 8 birds per pen. Each pen had a dimension of 1.85 × 1 m (L × W) and was enriched with a perch. Pelleted lignocellulose (Softcell; Agromed Austria GmbH, Kremsmünster, Austria) was used as a bedding material to prevent confounding effects of coarse litter ingestion on GIT development. Ambient temperature was maintained at 32°C until d 3 and thereafter gradually reduced to 22°C at d 23. A 23L:1D photoperiod was applied until d 3 and was changed thereafter to 16L:8D until the end of the study. Birds were allowed *ad libitum* access to feed and water. Individual and pen body weights, as well as pen feed intake, were recorded weekly. Each 20 kg bag of feed used in the experiment was sampled at the top, middle and bottom. Feed samples were pooled by treatment group and kept at 4°C.

At d 22 and 23, 5 birds per pen were euthanized by intravenous (**i.v.**) sodium pentobarbital injection. Feeding status was synchronized across pens by applying 3 hours of fasting followed by 3 hours of feed access and continuous light prior to euthanasia to allow later comparison of gene expression data. The feeding of birds was staggered such that birds were sampled at the same time after the start of feeding. The birds in half of the pens per treatment were sampled on one day and the remaining pens on the other day. Slaughter weights were recorded, the body cavity was carefully opened and the content of the following GIT segments were isolated using tie-wraps: crop, proventriculus, gizzard, duodenum (from the pyloric junction to the hepatopancreatic duct), jejunum (from the hepatopancreatic duct to Meckel's diverticulum), ileum (from Meckel's diverticulum to the ileocecal valve), colon and ceca. Ileal contents of the 5 birds per pen were gently flushed with ice-cold saline solution, pooled in a plastic bottle and frozen at -20°C. The bird with an individual BW closest to the pen average was sampled for transcriptomic analysis. For the 4 other birds of each pen, empty digestive organ weights were recorded, the empty proventriculus was collected and frozen at -20°C. Jejunal content was collected by gentle finger stripping, pooled per pen and frozen at -20°C. Finally, 5 mm pieces of the middle of the duodenum and jejunum were sampled, fixed in buffered formaldehyde solution (pH = 7.0; 9713, VWR International, Amsterdam, Netherlands) during 4 days and thereafter stored in demineralized water.

At d 24, 15 birds with an individual body weight at 14 d close to their respective pen average were selected per treatment group and dosed orally with 3 gel capsules containing 150 mg titanium (**Ti**) oxide each (equivalent to 90 mg of Ti; Apotheek Diergeneeskunde Utrecht University, Utrecht, Netherlands). Birds were euthanized by i.v. sodium pentobarbital injection at 5 different time points after dosing (30, 90, 180, 270 and 360 min; 3 birds per treatment group and time point) following the method developed by van Krimpen and coworkers (2011) to determine digesta mean retention

time. For each bird, digesta content of the crop, proventriculus-gizzard, small intestine (from the pyloric junction to the ileocecal valve) and colon-ceca were quantitatively collected by gentle finger stripping and frozen at -20°C. Differences in GIT development and digestive function are more susceptible to be observed during the starting period. As such, the experiment was conducted during the 0-24 d period.

Analytical methods

A representative fraction of the digesta content taken for mean retention time determination was analysed for dry matter (**DM**), Ti and SCFA contents (15 birds per treatment group, 4 GIT segments per bird). DM was measured according to standard methods (method ISO 6496; ISO, 1999), Ti according to the method described by Short and colleagues (1996) and SCFA contents were determined according to the method described by Qaisrani and coworkers (2014).

Feed and freeze-dried ileal samples were ground to pass a 1 mm sieve and analysed for DM (method ISO 6496; ISO, 1999) , ash (method ISO 5984; ISO, 2002), N (method ISO 5983-1; ISO, 2005a) and amino acids (**AA**) by HPLC after acid hydrolysis (method ISO 13903; ISO, 2005b) contents. The Cr_2O_3 content was determined in feed and ileal samples after ashing and acid hydrolysis as described by Williams *et al.* (1962). Apparent ileal digestibility of DM, AA, organic matter (**OM**) and N was calculated using Cr_2O_3 as a solid phase marker (Kotb and Luckey, 1972). Amino acid N and non-AA N were calculated using the N content of samples, molar weights of AA and assuming that Asp and Glu molecules have 1.5 N atoms. Diets were analysed for starch, crude fat and crude fibre contents according to standard methods (AOAC, 2012).

Duodenal and jejunal tissue samples were dehydrated in increasing concentrations of alcohol, embedded in paraffin and sectioned at 5 µm thickness. Six cross-sections per sampled organ per bird were stained with haematoxylin and eosin. Villus height (from tip to crypt mouth) and crypt depth (from crypt mouth to base) were measured using a Microphot-FXA light microscope (Nikon, Tokyo, Japan) equipped with a DP 50 digital camera (Olympus, Tokyo, Japan). Images were analysed using the AnalySiS Extended Pro 3.1 software (Soft Imaging System GmbH, Berlin, Germany). Averages represent 3 to 4 birds per pen; in each slide 10 well-oriented, intact villi and crypts were measured.

Proteolytic activity in the proventriculus was evaluated according to the method described by Qaisrani and coworkers (2015). Proteolytic activity in the jejunum was evaluated according to the method developed by Rada and coworkers (2016).

Statistical analysis

Rationale for the use of contrast analysis. Separated (UP, TB, FCB) and combined (UPTB) use of different additives may allow changes in butyrate concentration in distinct GIT segments while keeping butyrate inclusion level constant across diets. Assuming that such conditions are met, comparison of growth performance and digestive function data between appropriate treatment groups using pre-defined contrasts may reveal the effect of butyrate presence in specific GIT segments. Theoretical considerations underlying such statistical approach need, however, to be supported by observed changes in butyrate concentration along the GIT. Hence, a two-step analysis of experimental data was carried out. First an analysis of SCFA concentrations along the GIT was performed using a set of contrasts testing for overall butyrate and additives-specific effects, thereby addressing the validity of the experimental approach. Thereafter, growth and digestive function data were analyzed using a set of pre-defined contrasts testing for the effect of butyrate presence in different GIT segments.

Step one: Effects of butyrate additives on SCFA concentration along the GIT. SCFA data were analyzed separately for each GIT location using the PROC GLM of SAS (version 9.3, SAS Institute Inc., Cary, NC) using the following model:

$$Y_{ij} = \mu + D_i + \varepsilon_{ij}$$

where Y_{ij} is the observed butyrate concentration of the j^{th} replicate (j = 1 to 15) fed the i^{th} diet (i = CTR, UP, TB, FCB or UPTB), μ is the overall mean response, D_i is the i^{th} fixed diet effect and ε_{ij} the residual term associated with the j^{th} replicate fed the i^{th} diet. Model assumptions were checked by visual inspection of the residuals. When a significant dietary effect was detected, multiple pairwise comparisons were performed using the LSD test. Additionally, contrasts were used to assess the overall butyrate supplementation effect (But: CTR vs UP+TB+FCB+UPTB) and the specific effects of including different butyrate additives in butyrate supplemented diets (effect of unprotected butyrate Un-but: UP+UPTB vs TB+FCB, effect of tributyrin Tri-but: TB+UPTB vs UP+FCB, effect of fat-coated butyrate F at-but: FCB vs UP+TB+UPTB). Contrasts were not orthogonal, resulting in correlation among tests indicated in Table 2.

Table 2. Pearson's correlation coefficient (R) among contrasts in the statistical analysis of SCFA concentrations

Contrasts[1]	Butyrate	Un-but	Tri-but	Fat-but
Butyrate	1	0	0	0
Un-but	0.5	1	0	0.5
Tri-but	0.5	0	1	0.5
Fat-but	0	0	0	1

[1]Contrasts: Butyrate (overall effect of butyrate: CTR vs UP+TB+FCB+UPTB), Un-but (effect of unprotected butyrate: UP+UPTB vs TB+FCB), Tri-but (effect of tributyrin: TB+UPTB vs UP+FCB), Fat-but (effect of fat-coated butyrate: FCB vs UP+TB+UPTB).

Step two: Contrast analysis of growth performance and digestive function data. Body weight gain (**BWG**), average daily feed intake (**ADFI**), feed conversion ratio (**FCR**) and bodyweight variation coefficient (**CV**) were calculated and analysed using the PROC GLIMMIX of SAS using the following model:

$$Y_{ijk} = \mu + D_i + A_j + A \times D_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the observed response of the k^{th} replicate (k = 1 to 40) fed the i^{th} diet (i = CTR, UP, TB, FCB or UPTB) during the j^{th} measurement period (j = 1-7, 8-14, 15-21), D_i is the i^{th} fixed diet effect, A_j is the j^{th} random effect of measurement period, and $A \times D_{ij}$ the interaction effect between the diet and measurement period, and ε_{ijk} is the residual error term of the k^{th} replicate fed the i^{th} diet at the j^{th} measurement period. Visual inspection of the residuals indicated that the normal distribution fitted best the BWG data whereas ADFI, FCR and CV were analysed with a lognormal distribution. Contrasts were used to assess the overall effect of butyrate supplementation (But: CTR vs UP+TB+FCB+UPTB) and the effect of butyrate presence in different GIT segments (Crop, proventriculus and Gizzard, C+P+G: TB vs UPTB; Duodenum, jejunum and ileum, D+J+I: UP vs UPTB; Colon and ceca, Co+Ce: FCB vs UPTB). Contrasts were not orthogonal, resulting in correlations among tests indicated in Table 3.

Table 3. Pearson's correlation coefficients (R) among contrasts in the statistical analysis of performance parameters

Contrasts[1]	Butyrate	C+P+G	D+J+I	Co+Ce
Butyrate	1	0	0	0
C+P+G	0.25	1	0.25	0.25
D+J+I	0.25	0.25	1	0.25
C+C	0	0	0	1

[1]Contrasts: Butyrate (overall effect of butyrate: CTR vs UP+TB+FCB+UPTB); C+P+G (effect of presence in the crop, proventriculus and gizzard; TB vs UPTB); D+J+I (effect of presence in the duodenum, jejunum and ileum: UP vs UPTB); C+C (effect of presence in the colon and ceca: FCB vs UPTB).

Digestive tract organs relative weights, enzymatic activities, histological measurements and digestibility coefficients were analyzed with the same procedure as SCFA and the same contrasts as performance parameters. Digesta mean retention time was calculated using the model developed by van Krimpen and coworkers (2011) and analyzed using the same contrasts as performance parameters.

RESULTS

SCFA

Table 4 shows the effect of dietary treatments on SCFA concentrations in the content of the different GIT segments of broilers at 24 d of age. Significant diet effects were observed for butyrate concentration in all the GIT segments except the colon and ceca (P<0.001) and for propionate concentration in colonic and cecal contents (P=0.031). Contrast analysis indicated that butyrate concentration in crop, proventriculus, gizzard and small intestine contents were significantly lower in birds fed control diets compared to their butyrate-fed counterparts (P<0.001).

Table 4. Effect of dietary treatments on acetate, propionate and butyrate concentrations in the digesta of different gastrointestinal segments of broilers at 24 d of age

Fatty acid concentration	Dietary treatment means[1]					Pooled SEM	Diet effect	Contrasts[2]			
Location	CTR	UP	TB	FCB	UPTB			Butyrate	Un-but	Tri-but	Fat-but
Acetate (µmol/g DM)											
Crop	20.9	15.2	18.0	20.6	20.2	8.7	0.691	0.543	0.484	0.741	0.390
Proventriculus and gizzard	4.3	3.9	4.1	2.9	3.4	2.4	0.364	0.354	0.700	0.926	0.184
Small intestine	8.8	11.4	9.1	8.4	9.3	4.8	0.152	0.634	0.139	0.277	0.206
Colon and ceca	25.7	28.4	27.9	26.6	29.9	17.9	0.912	0.597	0.698	0.866	0.463
Propionate (µmol/g DM)											
Crop	0.45	0.57	0.47	0.44	0.55	0.12	0.146	0.153	**0.045**	094	**0.062**
Proventriculus and gizzard	0.14	0.22	0.17	0.10	0.13	0.16	0.118	0.664	0.209	0.589	**0.079**
Small intestine	0.43	0.61	0.48	0.42	0.55	0.39	0.363	0.377	**0.092**	0.908	0.167
Colon and ceca	1.5^{ab}	1.1^{b}	1.5^{ab}	1.8^{a}	1.4^{b}	1.3	**0.031**	0.820	**0.010**	0.423	**0.003**
Butyrate (µmol/g DM)											
Crop	0.9^{b}	10.2^{a}	8.3^{a}	8.8^{a}	10.5^{a}	0.9	**<0.001**	**<0.001**	**0.023**	0.504	0.012
Proventriculus and gizzard	0.29^{c}	4.56^{a}	3.28^{b}	2.37^{b}	3.08^{b}	0.42	**<0.001**	**<0.001**	**0.042**	0.941	0.381
Small intestine	0.31^{b}	0.35^{b}	1.79^{a}	0.40^{b}	1.46^{a}	0.14	**<0.001**	**<0.001**	0.177	**<0.001**	**<0.001**
Colon and ceca	20.2	21.1	21.6	27.9	25.4	4.3	0.676	0.432	0.890	0.818	0.204

[1]Dietary treatments groups: CTR (no butyrate supplementation); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg as in basis. Values are means of 15 birds per treatment.

[2]Contrasts used: Butyrate (overall effect of butyrate: CTR vs UP+TB+FCB+UPTB), Un-but (effect of unprotected butyrate: UP+UPTB vs TB+FCB), Tri-but (effect of tributyrin: TB+UPTB vs UP+FCB), Fat-but (effect of fat-coated butyrate: FCB vs UP+TB+UPTB).

Means within a row lacking a common superscript differ (P<0.05).

In addition, contrast analysis showed that UP significantly increased propionate concentration in the crop content (*P*=0.045) in comparison to the other butyrate additives. A similar trend was observed in the small intestine (*P*=0.092) whereas propionate concentration in colonic and cecal contents was reduced by UP supplementation (*P*=0.010) in comparison to the other additives. The UP additive also significantly increased the butyrate concentration in the crop (*P*=0.023) and in the proventriculus-gizzard (*P*=0.042) content in comparison to the other additives. The TB additive did not affect acetate or propionate concentration along the GIT but significantly (*P*<0.001) increased butyrate concentration in the small intestine content compared to the other additives. The FCB additive tended to reduce propionate concentration in the crop (*P*=0.062) and in the proventriculus-gizzard (*P*=0.079) content while significantly (*P*=0.003) increasing propionate concentration in colonic and cecal contents compared to the other butyrate additives. The FCB additive numerically increased the butyrate concentration in colonic and cecal contents compared to the other butyrate additives (+22.9 %; *P*=0.204).

Performance responses

Table 5 shows the effect of butyrate presence in distinct GIT segments on growth performance of broiler chickens during the 1-21 d period. Addition of butyrate to the diet tended (*P*=0.090) to decrease feed intake compared to the control group. In butyrate-supplemented birds, feed intake tended (*P*=0.075) to be higher when butyrate was present in the crop, proventriculus and gizzard whereas presence in the colon and ceca tended (*P*=0.086) to reduce the FCR.

Table 5. Effect of dietary treatments on performance parameters of broiler fed a control diet or the same diet supplemented with 4 butyrate preparations during the 0-21 d period

	Dietary treatment[1]					SEM	Contrasts[2]			
Item	CTR	UP	TB	FCB	UPTB		But	C+P+G	D+J+I	Co+Ce
Initial BW, g	42.3	41.8	42.1	42.2	42.2	-	-	-	-	-
Performance parameters (0-21d)[3]										
Mortality, %	4.7	1.6	3.1	4.7	0	-	-	-	-	-
WG, g/bird	679	676	644	669	674	6	0.514	0.242	0.946	0.831
FI, g/bird	975	938	906	913	976	6	***0.090***	***0.075***	0.191	0.214
BW CV	11.2	12.3	11.0	12.8	11.4	0.1	0.497	0.835	0.436	0.432
FCR, g:g	1.44	1.39	1.41	1.37	1.45	0.03	0.101	0.777	0.154	***0.086***

[1]Dietary treatments groups: CTR (no butyrate supplementation); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg as in basis. Means are averages of 8 replicate pens per treatment.

[2]Contrasts used: But (overall effect of butyrate: CTR vs UP+TB+FCB+UPTB); C+P+G (effect of butyrate presence in the crop, proventriculus and gizzard; TB vs UPTB); D+J+I (effect of butyrate presence in the duodenum, jejunum and ileum: UP vs UPTB); Co+Ce (effect of presence butyrate and/or propionate in the colon and ceca: FCB vs UPTB).

[3]Performance parameters abbreviations: Weight gain (WG); Feed intake (FI); Body weight coefficient of variation (BW CV); Feed conversion ratio (FCR).

Digestive traits responses

The effects of butyrate presence in different GIT segments on several digestive trait parameters are presented in Table 6.

Table 6. Effect of dietary treatments on digestive tract development, digesta retention time, proteolytic activities and intestinal morphology of 22-23 d old broilers fed a control diet or the same diet supplemented with 3 butyrate preparations during the 0-23 d period

	Dietary treatment[1]					SEM[2]	Contrasts[3]			
	CTR	UP	TB	FCB	UPTB		But	C+P+G	D+J+I	Co+Ce
Digestive segment weight (% of slaughter weight)										
Crop	0.40	0.39	0.42	0.40	0.38	0.01	0.957	**0.038**	0.508	0.270
Proventriculus	1.42	1.43	1.38	1.40	1.49	0.03	0.782	0.930	0.984	0.794
Gizzard	1.53	1.55	1.62	1.52	1.54	0.04	0.686	0.181	0.865	0.805
Duodenum	1.07	1.05	1.1	1.07	1.02	0.02	0.810	**0.019**	0.397	0.127
Jejunum	2.04	1.96	2.04	2.06	1.92	0.04	0.299	**0.036**	0.444	**0.010**
Ileum	1.70	1.63	1.74	1.71	1.65	0.03	0.575	**0.030**	0.650	0.133
Ceca	0.24	0.24	0.25	0.24	0.24	0.01	0.603	0.272	0.138	0.545
Colon	0.33	0.32	0.36	0.34	0.34	0.01	0.537	0.117	0.950	0.791
Total GIT	7.94	7.77	8.15	7.98	7.72	0.11	0.790	**0.004**	0.741	**0.072**
Mean retention time (min)										
Crop	72	86	112	104	89	19	0.241	0.416	0.591	0.913
Prov+Gizz[4]	24	28	37	25	22	7	0.621	0.124	0.743	0.485
Small intestine	91	83	76	151	142	17	0.262	**0.020**	0.705	**0.032**
Colon/Ceca	4	6	9	13	16	5	0.258	0.383	0.672	0.235
Total	191	204	235	292	268	17	**0.010**	0.183	0.340	**0.022**
Proteolytic activity										
Proventriculus[5]	5.64	6.42	5.08	5.95	6.6	0.4	0.418	**0.012**	0.759	0.261
Jejunum[6]	0.23	0.22	0.23	0.20	0.23	0.02	0.645	0.839	0.730	0.213
Intestinal morphology (µm)										
Duodenum										
Villi length	1464	1466	1490	1447	1470	40.63	0.928	0.726	0.945	0.702
Crypt depth	313	314	329	303	300	12.65	0.916	0.109	0.417	0.858
Villi:Crypt	4.68	4.67	4.53	4.78	4.90	0.17	0.685	0.210	0.368	0.593
Jejunum										
Villi length	1050	968	1033	1050	1016	26.74	0.265	0.655	0.212	0.380
Crypt depth	211	205	206	210	204	6.47	0.458	0.846	0.895	0.544
Villi:Crypt	4.98	4.72	5.01	5.00	4.98	0.14	0.863	0.492	0.165	0.944

[1]Dietary treatments groups: CTR (no butyrate supplementation); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg as in basis. Means are values of 15 birds per treatment group for passage rate and 32 birds per treatment group for the other read-out parameters.
[2]Pooled standard error of the mean.
[3]Contrasts used: Butyrate (overall effect of butyrate: CTR vs UP+TB+FCB+UPTB); C+P+G (effect of butyrate presence in the crop, proventriculus and gizzard; TB vs UPTB); D+J+I (effect of butyrate presence in the duodenum, jejunum and ileum: UP vs UPTB); Co+Ce (effect of presence butyrate and/or propionate in the colon and ceca: FCB vs UPTB).
[4]Prov+Gizz: Proventriculus + gizzard.
[5]Expressed in mmol tyrosine released per min per g fresh organ.
[6]Expressed in difference in absorbance measured at 366 nm.

Dietary butyrate significantly (P=0.010) increased total tract retention time. Crop, duodenum, jejunum, ileum and total GIT relative weights were decreased by butyrate presence in the crop, proventriculus and gizzard (P=0.038, 0.019, 0.036, 0.030 and 0.004; respectively). Additionally, butyrate presence in the crop, proventriculus and gizzard significantly increased proteolytic activity of the proventriculus (P=0.012) and

small intestinal retention time (*P*=0.020). Butyrate presence in the small intestine did not affect any of the measured responses. Presence of butyrate and/or propionate in colonic and cecal contents increased jejunum relative weight (*P*=0.010), small intestinal (*P*=0.032) and total tract retention times (*P*=0.022) and tended (*P*=0.072) to increase total GIT relative weight. Measured intestinal morphology traits were unaffected by dietary butyrate supplementation.

None of the aforementioned changes resulted in significant differences in OM, DM or N apparent ileal digestibility coefficients, but the digestibility coefficient of several AA tended to be affected by the presence of butyrate in different GIT segments (Table 7). Increased butyrate concentration in the crop, proventriculus and gizzard tended to decrease the apparent ileal digestibility of Ile, Leu, Phe, His and Lys (*P*=0.080, 0.076, 0.061, 0.094 and 0.080; respectively). Increased butyrate and/or propionate concentration in the colonic and cecal contents tended to increase the apparent ileal digestibility of Met and His (*P*=0.079 and 0.069; respectively).

Table 7. Effect of dietary treatments on apparent ileal digestibility of nutrients of 22-23 d old broilers fed a control diet or the same diet supplemented with 3 butyrate preparations during the 0-23 d period

	Dietary treatment[1]					SEM	Contrasts[2]			
	CTR	UP	TB	FCB	UPTB		But	C+P+G	D+J+I	Co+Ce
Component (%)										
DM	53.1	54.5	54.5	54.6	53.7	1.3	0.433	0.667	0.653	0.643
OM	59.7	60.7	61.2	61.1	59.7	1.3	0.503	0.457	0.600	0.483
Nitrogen (N)	76.6	75.2	77.0	76.7	76.2	0.6	0.691	0.357	0.267	0.583
AAN[3]	79.8	81.0	82.0	80.2	80.6	1.2	0.377	0.410	0.792	0.833
NPN[4]	56.6	41.8	50.9	59.6	51.7	4.8	0.320	0.912	0.168	0.263
Amino acids (%)										
Cysteine	70.0	68.6	70.8	70.6	69.2	1.1	0.907	0.345	0.708	0.398
Methionine	89.5	89.5	89.9	90.1	88.5	0.6	0.971	0.103	0.256	***0.079***
Aspartic acid	76.7	75.3	77.5	77.1	75.9	0.9	0.817	0.206	0.614	0.345
Threonine	73.4	72.6	74.7	73.9	72.7	0.8	0.934	0.108	0.927	0.316
Serine	75.8	75.1	77.1	76.2	75.3	0.8	0.852	0.151	0.877	0.474
Glutamic acid	86.2	85.4	86.7	86.4	85.7	0.6	0.861	0.515	0.750	0.365
Proline	76.7	76	77.4	76.8	76.2	0.8	0.900	0.314	0.867	0.624
Glycine	76.7	75.6	77.7	77.5	75.7	0.9	0.907	0.131	0.953	0.175
Alanine	82.2	81.3	83.2	82.8	81.6	0.7	0.978	0.110	0.828	0.218
Valine	78.7	77.9	79.5	79.2	78.1	0.7	0.980	0.164	0.831	0.271
Isoleucine	80.1	79.1	81.2	80.5	79.4	2.6	0.981	***0.080***	0.764	0.25
Leucine	83.7	82.9	84.7	84.2	83.0	0.7	0.984	***0.076***	0.902	0.212
Phenylalanine	82.5	81.9	83.9	82.9	82.1	0.6	0.795	***0.061***	0.866	0.393
Histidine	80.1	79.2	80.5	80.7	78.8	0.6	0.738	***0.094***	0.659	***0.067***
Lysine	81.2	80.2	82.0	81.7	80.6	0.7	0.846	***0.080***	0.605	0.157
Arginine	85.5	84.3	86.1	85.9	84.5	0.5	0.737	0.119	0.871	0.155

[1]Dietary treatments groups: CTR (no butyrate supplementation); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg as in basis. Means are pooled samples of 4 birds per replicate pen.
[2]Contrasts used: Butyrate (overall effect of butyrate: CTR vs UP+TB+FCB+UPTB); C+P+G (effect of butyrate presence in the crop, proventriculus and gizzard; TB vs UPTB); D+J+I (effect of butyrate presence in the duodenum, jejunum and ileum: UP vs UPTB); Co+Ce (effect of presence butyrate and/or propionate in the colon and ceca: FCB vs UPTB).
[3]AAN: Amino acid N.
[4]NPN: Non protein N.

DISCUSSION

Presence of butyrate in distinct GIT segments

The current study aimed to unravel effects of butyrate presence in distinct GIT segments on digestive processes and protein digestion in young broilers. Differences in luminal butyrate concentration across GIT segments were achieved using three different dietary butyrate additives. Unprotected butyrate is taken up by passive absorption under acidic conditions as present in the proventriculus-gizzard (Ichikawa *et al.*, 2002; Denbow and Scanes, 2015). Logically, unprotected butyrate increased butyrate concentration in the crop and proventriculus-gizzard but not beyond this segment of the GIT in the current study. This is in agreement with other published results on broilers (Hu and Guo, 2007; Smith *et al.*, 2012). Tributyrin, the triglyceride of butyrate, only increased enteric butyrate concentration in the present study due to a low pre-duodenal lipolytic activity in the broiler GIT (Moreau *et al.*, 1988; Cherif *et al.*, 2006). Finally, fat-coated butyrate numerically increased butyrate concentration in the pooled colon and ceca contents when compared to birds fed other butyrate additives (+22.9 %; P=0.204). This is in agreement with the suggestion that fat-coating extends butyrate release beyond the small intestine in broilers (Van den Borne *et al.*, 2015). The lack of statistical significance of this result could be attributed to the large variation observed across individual birds when colonic and cecal butyrate concentrations were considered. In addition, propionate concentration in colonic and cecal contents was significantly higher in birds fed fat-coated butyrate compared to birds fed other butyrate additives (P=0.003). Collectively, these results indicate that the dietary contrasts in digesta butyrate concentration were successfully achieved in this experiment with the notable exception of the colon and ceca. For these distal GIT segments, the observed increase in butyrate concentration failed to reach statistical significance and was confounded with a higher propionate concentration. As a consequence, testing the effect of butyrate presence in distinct GIT segments by means of statistical contrasts is possible up until the ileum. In addition, a statistical contrast testing for the effect of butyrate and/or propionate presence in the colon and ceca is possible.

Anorexic effect of butyrate

In the current study, butyrate tended to have an anorexic effect. Such effect is unusual in broilers fed 1 g/kg of different butyrate additives (UP: Hu and Guo, 2007; FCB: Smulikowska *et al.*, 2009) but have been reported at higher supplementation levels (2 g/kg butyrins: Leeson *et al.*, 2005). Mammalian studies have demonstrated that colonic L-cells producing peptide YY (**PYY**) and glucagon like peptide 1 (**GLP-1**) are mediating the anorexic effect of luminal SCFA (Lin *et al.*, 2012; Chambers *et al.*, 2015). Such mechanism has never been studied in avian species but the possibility to extrapolate insights in gut endocrinology from rodents to production animals has been advocated recently (Bravo, 2015; Furness *et al.*, 2015). It is, however, worth mentioning that L-cells are distributed along the small intestine in chickens (Rawdon and Andrew, 1999; Monir *et al.,* 2014) whereas in rodents the distribution is extended to the colon (Hansen *et al.*, 2013). The latter is traditionally regarded as the main site for triggering anorexic effects (Chamber *et al.*, 2015). In addition, PYY has orexigenic properties in chickens and anorexic properties in rodents (Batterham *et al.*, 2002; Denbow and Cline,

2015). Such differences make the extrapolation between murine and poultry species challenging. Interestingly, anorexic effects tended to be reduced when butyrate was delivered to the crop, proventriculus and gizzard compared to more distal segments. This observation is congruent with a putative role of intestinal L-cells in mediating the anorexic effects of butyrate in poultry.

Amino acids digestibility and digesta retention time

Butyrate and/or propionate presence in the colon and ceca resulted in significantly heavier jejuna and longer small intestinal and total tract retention times. Feed efficiency is associated with longer total tract retention time and lighter jejuna in 29 d old broilers (Rougière and Carré, 2010). In the present study, observed changes have accumulated into a trend for lower FCR values. Discrepancies in the effect of intestinal weights on feed efficiency confirm that a consistent relationship between measured read-out parameters and intestinal functionalities is sometimes difficult to establish across broiler studies (Svihus, 2014). Improved feed efficiency could be related to the trend for higher digestibility of methionine, the first limiting AA in practical poultry diets (Scanes, 2015). The relationship between digesta transit time and rapeseed meal digestibility is scarcely discussed in the literature, as most of the strategies proposed to improve rapeseed meal digestibility rely on enzymatic treatment and physical processing (Kozłowski and Jeroch, 2014) rather than on modifications of digestive physiology. Digesta retention time in poultry is controlled by a set of complex and poorly understood neuroendocrine mechanisms (Denbow, 2015). It is, therefore, difficult to speculate on the exact mechanism underlying the observed effect of butyrate and/or propionate presence in colonic and cecal contents on transit time. In mammals, only one study has demonstrated a link between hindgut butyrate concentration and colonic motility (Soret *et al.*, 2010).

Butyrate and intestinal microbiota

Higher butyrate concentration in the crop, proventriculus and gizzard was associated with a trend for lower apparent ileal digestibility coefficients of Ile, Leu, Phe, His and Lys. Such changes were associated with a higher propionate concentration in the intestinal digesta which may indicate changes in microbiota activity. In chickens, intestinal microbiota dominated by *Lactobacillus* spp., a genus whose auxotrophy for the aforementioned AA is well documented (Morishita *et al.*, 1981; Ruiz-Barba and Jimenez-Diaz, 1994). Luminal AA taken up by intestinal microbiota can be incorporated into microbial protein that bypasses ileal digestion (Apajalahti and Vienola, 2016). In the present study, higher butyrate concentration in the crop, proventriculus and gizzard digesta was associated with significant increases in intestinal retention time and proteolytic activity in the proventriculus. In addition, up-regulation of antimicrobial peptides by butyrate has been reported in chicken jejunal and caecal explants (Sunkara *et al.*, 2011). Both digesta retention time and antimicrobial peptides are known to influence intestinal microbiota activity in chickens (Apajalahti and Vienola, 2016). Unfortunately, the absence of quantitative insights into intestinal microbiota composition in the present study hinders the complete understanding of the intricate relationship between intestinal microbiota and ileal AA bioavailability. Butyrate provision to the crop, proventriculus and gizzard also significantly reduced the relative weight of each intestinal segment. It is known that villous enterocytes

growth is primarily supported by luminal AA absorption rather than plasma AA (Fuller and Reeds, 1998). An overgrowth of *Lactobacillus* spp. could have exacerbated the competition between bacteria and host cells for the absorption of luminal AA, thus hampering intestinal development.

Future research work

Future work could be directed to an improved understanding of the possible endocrine effects and host-microbe interactions that mediated such changes. In addition, effects of butyrate presence in distinct GIT segments on other parameters, e.g. immune functions and microbiota composition, should be investigated.

CONCLUSION

The present study demonstrates that different butyrate additives can be used to create a contrast in luminal butyrate concentrations throughout the GIT of broilers. The effect of butyrate on several parameters such as GIT development, proteolytic activity or digesta retention time was found to vary depending on the GIT segment wherein the molecule was present. When butyrate and/or propionate was present in colonic and cecal contents, such effects tended to improve the digestibility of Met, the first limiting AA in practical poultry diets. In contrast, butyrate presence in the digesta of the crop, proventriculus and gizzard decreased the digestibility of several AA, possibly due to an increase in intestinal microbiota activity. In addition, butyrate presence beyond the gizzard elicited anorexic effects that might be attributable to changes in intestinal enteroendocrine L-cells secretory activities. The effect of butyrate on digestive functions and bioavailability of AA is therefore conditioned by the GIT segment wherein the molecule is present.

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LITERATURE CITED

AOAC. 2012. Official methods of analysis of AOAC international. 19th edition. AOAC international, Gaithersburg, MD.

Apajalahti, J., and K. Vienola. 2016. Interaction between chicken intestinal microbiota and protein digestion. Anim. Feed Sci. Technol. 221:323-330.

Batterham, R. L., M. A. Cowley, C. J. Small, H. Herzog, M. A. Cohen, C. L. Dakin, A. M. Wren, A. E. Brynes, M. J. Low, M. A. Ghatei, R. D. Cone, and S. R. Bloom. 2002. Gut hormone PYY3-36 physiologically inhibits food intake. Nature 418:650-654.

Bravo, D. M. 2015. Comparative gut physiology symposium. J. Anim. Sci. 93:482-484.

Cerisuelo, A., C. Marin, F. Sanchez-Vizcaino, E. A. Gomez, J. M. de la Fuente, R. Duran, and C. Fernandez. 2014. The impact of a specific blend of essential oil components and sodium butyrate in feed on growth performance and Salmonella counts in experimentally challenged broilers. Poult. Sci. 93:599-606.

Chambers, E. S., D. J. Morrison, and G. Frost. 2015. Control of appetite and energy intake by SCFA: what are the potential underlying mechanisms? Proc. Nutr. Soc. 74:328-336.

Cherif, S., N. Miled, M. Kammoun, H. Mejdoub, and Y. Gargouri. 2006. Purification and biochemical characterization of a turkey preduodenal esterase. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 145:358-364.

CVB. 2007. Tabellenboek veevoeding 2007. Voedernormen landbouwhuisdieren en voederwaarde veevoeders. Centraal Veevoederbureau, Lelystad, NL

Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Denbow, D. M., and M. A. Cline. 2015. Chapter 21 - Food intake regulation. Pages 469-485 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Dumoulin, V., F. Moro, A. Barcelo, T. Dakka, and J. C. Cuber. 1998. Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. Endocrinology 139:3780-3786.

Fuller, M. F., and P. J. Reeds. 1998. Nitrogen cycling in the gut. Annu. Rev. Nutr. 18:385-411.

Furness, J. B., J. J. Cottrell, and D. M. Bravo. 2015. Comparative gut physiology symposium: Comparative physiology of digestion. J. Anim. Sci. 93:485-491.

Hansen, C. F., N. Vrang, P. T. Sangild, and J. Jelsing. 2013. Novel insight into the distribution of L-cells in the rat intestinal tract. Am. J. Transl. Res. 5:347-358.

Horwitz, W. 2006. Official methods of analysis of AOAC International. AOAC International, Gaithersburg, MD.

Hu, Z., and Y. Guo. 2007. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. Anim. Feed Sci. Technol. 132:240-249.

Ichikawa, H., R. Shineha, S. Satomi, and T. Sakata. 2002. Gastric or rectal instillation of short-chain fatty acids stimulates epithelial cell proliferation of small and large intestine in rats. Dig. Dis. Sci. 47:1141-1146.

International organization for standardization (ISO). 1999. ISO 6496:1999. Animal feeding stuffs - Determination of moisutre and other volatile matter content. ISO, Geneva, CH.

International organization for standardization (ISO). 2002. ISO 5984:2005. Animal feeding stuffs - Determination of crude ash content. ISO, Geneva, CH.

International organization for standardization (ISO). 2005a. ISO 5983-1:2005. Animal feeding stuffs - Determination of crude protein content - Part 1: Kjeldahl method. ISO, Geneva, CH.

International organization for standardization (ISO). 2005b. ISO 13903:2005. Animal feeding stuffs - Determination of amino acid content. ISO, Geneva, CH.

Kotb, A. R., and T. D. Luckey. 1972. Markers in nutrition. Nutr. Abstr. Rev. 42:813-845.

Kotunia, A., J. Wolinski, D. Laubitz, M. Jurkowska, V. Rome, P. Guilloteau, and R. Zabielski. 2004. Effect of sodium butyrate on the small intestine development in neonatal piglets fed by artificial sow. J. Physiol. Pharmacol. 55(2):59-68.

Kozłowski, K., and H. Jeroch. 2014. Enhancing the nutritional value of poultry feedstuffs using the example of rapeseed products - A review. Ann. Anim. Sci. 14(2):245-256.

Leeson, S., H. Namkung, M. Antongiovanni, and E. H. Lee. 2005. Effect of butyric acid on the performance and carcass yield of broiler chickens. Poult. Sci. 84:1418-1422.

Lin, H. V., A. Frassetto, E. J. Kowalik Jr, A. R. Nawrocki, M. M. Lu, J. R. Kosinski, J. A. Hubert, D. Szeto, X. Yao, G. Forrest, and D. J. Marsh. 2012. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLOS ONE 7:e35240.

Mazzoni, M., M. Le Gall, S. De Filippi, L. Minieri, P. Trevisi, J. Wolinski, G. Lalatta-Costerbosa, J. P. Lalles, P. Guilloteau, and P. Bosi. 2008. Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. J. Nutr. 138:1426-1431.

Monir, M. M., K. Hiramatsu, K. Nishimura, C. Takemoto, and T. Watanabe. 2014. Distribution of glucagon-like peptide (GLP)-2-immunoreactive cells in the chicken small intestine: antigen retrieval immunohistochemistry. J. Vet. Med. Sci. 76:565-568.

Moquet, P. C. A., L. Onrust, F. Van Immerseel, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72:61-80.

Moreau, H., Y. Gargouri, D. Lecat, J. L. Junien, and R. Verger. 1988. Screening of preduodenal lipases in several mammals. Biochim. Biophys. Acta. 959:247-252.

Morishita, T., Y. Deguchi, M. Yajima, T. Sakurai, and T. Yura. 1981. Multiple nutritional requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. J. Bacteriol. 148:64-71.

Qaisrani, S. N., P. C. A. Moquet, M. M. van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, and W. H. Hendriks. 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93:3053-3064.

Qaisrani, S. N., M. M. van Krimpen, R. P. Kwakkel, M. W. A. Verstegen, and W. H. Hendriks. 2015. Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology and cecal fermentation characteristics in broilers. Poult. Sci. 94(9): 2152-2164.

Rada, V., M. Lichovníkova, M. Foltyn, and I. Šafařík. 2016. The effect of exogenous protease in broiler diets on the apparent ileal digestibility of amino acid and on protease activity in jejunum. Acta Univ. Agric. Silvic. Mendel. Brun. 64:1645-1652.

Rawdon, B. B., and A. Andrew. 1999. Gut endocrine cells in birds: an overview, with particular reference to the chemistry of gut peptides and the distribution, ontogeny, embryonic origin and differentiation of the endocrine cells. Prog. Histochem. Cytochem. 34:3-82.

Rougière, N., and B. Carré. 2010. Comparison of gastrointestinal transit times between chickens from D+ and D− genetic lines selected for divergent digestion efficiency. Animal 4:1861-1872.

Ruiz-Barba, J. L., and R. Jimenez-Diaz. 1994. Vitamin and amino acid requirements of Lactobacillus plantarum strains isolated from green olive fermentations. J. Appl. Microbiol. 76:350-355.

Scanes, C. G. 2015. Chapter 20 - Protein metabolism. Pages 455-467 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Short, F. J., P. Gordon, J. Wiseman, and K. N. Boorman. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Technol. 59:215-221.

Smith, D. J., A. Barri, G. Herges, J. Hahn, A. G. Yersin, and A. Jourdan. 2012. In vitro dissolution and in vivo absorption of calcium [1-$^{(14)}$C]butyrate in free or protected forms. J. Agric. Food Chem. 60:3151-3157.

Smulikowska, S., J. Czerwinski, A. Mieczkowska, and J. Jankowiak. 2009. The effect of fat-coated organic acid salts and a feed enzyme on growth performance, nutrient utilization, microflora activity, and morphology of the small intestine in broiler chickens. J. Anim. Feed Sci. 18:478-489.

Soret, R., J. Chevalier, P. De Coppet, G. Poupeau, P. Derkinderen, J. P. Segain, and M. Neunlist. 2010. Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. Gastroenterol. 138:1772-1782.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R. G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLOS ONE 6:e27225.

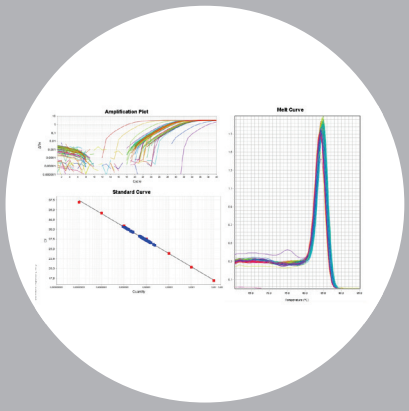
Svihus, B. 2014. Function of the digestive system. J. Appl. Poutl. Res. 23(2):306-314

Van den Borne, J. J. G. C., M. J. W. Heetkamp, J. Buyse, and T. A. Niewold. 2015. Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. Livest. Sci. 175:96-100.

Van Krimpen, M. M., R. P. Kwakkel, C. M. Van Der Peet-Schwering, L. A. Den Hartog, and M. W. Verstegen. 2011. Effects of dietary energy concentration, nonstarch polysaccharide concentration, and particle sizes of nonstarch polysaccharides on digesta mean retention time and gut development in laying hens. Br. Poult. Sci. 52:730-741.

Vidrine, K., J. Ye, R. J. Martin, K. L. McCutcheon, A. M. Raggio, C. Pelkman, H. A. Durham, J. Zhou, R. N. Senevirathne, C. Williams, F. Greenway, J. Finley, Z. Gao, F. Goldsmith, and M. J. Keenan. 2014. Resistant starch from high amylose maize (HAM-RS2) and dietary butyrate reduce abdominal fat by a different apparent mechanism. Obesity 22:344-348.

Williams, C. H., D. J. David, and O. Iismaa. 1962. The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. J. Agric. Sci. 59(03):381-385.



Chapter 5

Distinct effects of butyrate along the chicken gastrointestinal tract on the expression of uptake, metabolism, sensing and gut hormones related genes

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ABSTRACT

Butyrate is known to influence energy metabolism and endocrine regulation in the mammalian gastrointestinal tract (**GIT**). Such effects may also occur in broilers in a location-dependent manner and could condition intracellular butyrate concentration along the GIT. An experiment was conducted to investigate at the mRNA level the effect of providing butyrate to distinct GIT segments on energy metabolism and endocrine regulation of broilers. A total of 320 male one day-old Ross 308 broilers were randomly assigned to 5 dietary treatment groups, each replicated 8 times: one control group and four groups supplemented with 1 g/kg butyrate in the form of feed additives having markedly different butyrate release profiles. Diets were based on rapeseed meal, corn and wheat and were fed *ad libitum*. At 22-23 d of age, birds were sacrificed. The GIT of the bird having its BW the closest to the pen average was sampled at five different locations, i.e. proventriculus, pylorus, duodenum, ileum and colon. For each location, the control without added butyrate was compared to the butyrate-supplemented diet that resulted in the largest increase in digesta butyrate concentration. Expression levels of genes related to the uptake, sensing and metabolism of butyrate as well as hormonal regulation were quantified by qPCR. Mucosal and digesta butyrate concentrations were measured by gas chromatography. Our results suggest that butyrate uptake is a passive process in the gastric region while being facilitated by putative (sodium-dependent) monocarboxylate transporters in the small and large intestine. Increasing digesta butyrate concentration in the gastric region seemed to increase the β-oxidation of lipids. A similar effect was observed in the ileum and colon. Increasing digesta butyrate concentration in the duodenum seemed, however, to promote glycolysis. Butyrate has, therefore, a location-dependent effect on energy metabolism in the gut. Despite such specificities, intracellular butyrate concentration remained low across GIT segments and diets. This may prevent the negative effects of intracellular butyrate accumulation on gut cells functions. butyrate increased the expression of preprocholecystokinin in the duodenum. This may explain the anorectic effect of dietary butyrate supplementation in poultry.

Keywords: Butyrate, broilers, sensing, uptake, metabolism, hormones

INTRODUCTION

Research in the late 1970s demonstrated that the accumulation of butyrate in human cell cultures inhibited altered gene expression and cell functioning by inhibiting the chromatin-remodelling activity of histone deacetylases (**HDAC**) (Riggs *et al.*, 1977; Sealy and Chalkley, 1978). Two decades later, novel G protein-coupled receptors (**GPR**) were discovered (Sawzdargo *et al.*, 1997). Butyrate and other short chain fatty acids (**SCFA**) were subsequently identified as potent ligands activating GPR 41, 43 and 109a (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Singh *et al.*, 2014). G proteins-coupled receptors 43 and 41 were coined free fatty acid receptors (**FFAR**) 2 and 3, respectively, and their role in modulating the secretory activity of different enteroendocrine cells was established in mammals (Liou, 2013). Changes in the secretory activity of enteroendocrine cells may affect energy homeostasis at the gut and systemic levels (Murphy and Bloom, 2006). The capacity of butyrate to modulate endocrine regulation and energy metabolism in distinct gastrointestinal tract (**GIT**) segments through HDAC inhibition or GPR sensing is, therefore, well documented in mammals (e.g. Tabuchi *et al.*, 2006; Nohr *et al.*, 2013).

In contrast to mammals, such effects have been scarcely investigated in the avian GIT. Earlier research was focused on the effect of supplementing broiler diets with butyrate on intestinal host defence peptides production (Sunkara *et al.*, 2011; Sunkara *et al.*, 2012; Sunkara *et al.*, 2014) and, more recently, on hepatic lipid metabolism (Yin *et al.*, 2016). Little is, however, known about the intricate relationships existing between digesta butyrate concentration and the expression of genes regulating the sensing, uptake and metabolism of butyrate in the avian GIT. Such relationships have been speculated to vary in distinct GIT segments due to differences in cell metabolism and luminal pH along the GIT (Moquet *et al.*, 2016).

Besides, changes in the secretory activities of G-, I- and L-type enteroendocrine cells have been proposed as underlying mechanisms mediating the physiological changes associated with the presence of butyrate in the GIT of chickens, such as increased pepsin secretion (Onrust *et al.,* 2015; Moquet *et al.*, 2016). We demonstrated recently, using butyrate feed additives having markedly different release kinetics, that butyrate and/or propionate presence in the distal GIT (colon and ceca) of broilers resulted in alterations of digesta retention time and feed intake that were congruent with putative changes in the secretory activities of L-cells (Moquet *et al.*, 2018).

The present study is an extension of our previous work in which gut tissues collected during the cited experiment (Moquet *et al.*, 2018) are used to address a twofold objective: first, to study in five distinct GIT segments the effect of increasing digesta butyrate concentration on the expression level of genes associated with butyrate uptake and energy metabolism, and to assess the mucosal butyrate concentration. Second, to investigate the effect of increasing digesta butyrate concentration on the expression of genes related to the hormonal regulation of gut functions. It was hypothesised that butyrate catabolism would be more effective in the distal GIT (e.g. ileum or colon) compared to more proximal GIT segments as a result of differences in cell metabolism (Moquet *et al.*, 2016). We hypothesised that increasing digesta butyrate concentration in the distal GIT would result in an upregulation of genes

involved in butyrate catabolism and in a lower mucosal butyrate concentration compared to more proximal GIT segments (e.g. proventriculus or duodenum). It was further hypothesized that increasing digesta in distinct GIT segments would modulate the expression of FFAR2 and upregulate the expression of gut hormones that are produced by the different enteroendocrine cells.

MATERIAL AND METHODS

All the experimental procedures were approved by the Animal Care and Ethics Committee of Wageningen University & Research and conducted under the Dutch Law on Animal Experiments.

Experimental design, management and sample collection

The experiment described in the present manuscript was part of a larger study published by Moquet and colleagues (2018). A brief summary of this experiment is presented below.

Experimental design. The effect of providing butyrate to distinct GIT segments on pre-cecal protein digestibility of broilers was investigated using a complete randomized design. A total of 320 male day-old Ross 308 broilers were randomly assigned to 5 dietary treatment groups: 1) Control (**CTR**; no butyrate), 2) unprotected butyrate (**UP**; activity in the crop and gastric regions), 3) tributyrin (**TB**; activity in the small intestine), 4) fat-coated butyrate (**FCB**; activity in whole GIT) and 5) a 50:50 mixture of unprotected butyrate and tributyrin (**UPTB**; butyrate radical $C_4H_7O_2^-$ weight basis), each replicated 8 times. Butyrate radical was included to a rapeseed meal based diet at 1 g/kg, as fed basis. Ingredient and analyzed nutrient compositions of the diets are provided in Chapter 4, Table 1.

Management. Birds were allowed ad libitum access to feed and water. Each pen had a dimension of 1.85 × 1 m (L × W) and was enriched with a perch. Pelleted lignocellulose (Softcell; Agromed Austria GmbH, Kremsmünster, Austria) was used as bedding material.

Sample collection. At d 22 and 23, 5 birds per pen were euthanized by intravenous (**i.v.**) sodium pentobarbital injection. Feeding status was synchronized across pens to allow later comparison of gene expression data by applying 3 hours of fasting followed by 3 hours of feed access and continuous light prior to euthanasia. The feeding of birds was staggered such that birds were sampled at the same time after the start of feeding. The birds in half of the pens per treatment were sampled on one day and the remaining pens on the other day. From the bird having its BW the closest to the pen average, the following GIT tissue segments were sampled for gene expression analysis: proventriculus, pylorus, middle of the duodenum (halfway between the pyloric junction and the hepatopancreatic duct), proximal ileum (first third between Meckel's diverticulum to the ileocecal valve) and middle of the colon (halfway between the ileocecal junction and the cloaca). Collected tissues were gently rinsed with ice-cold 0.9% NaCl, placed in cryogenic vials, snap frozen in liquid nitrogen and stored at -80°C until further processing.

Remaining birds were euthanized and dissected at 24 d of age for the determination of SCFA in the digesta of different GIT compartments and for the determination of digesta retention time.

Analytical Methods

Rationale for the selection of tissues. Table 1 presents the changes in luminal butyrate concentration, expressed as percentage of the control, induced by dietary intervention.

Table 1. Effect of dietary treatments on butyrate concentration expressed as a percentage of the control (%; µmol/g DM basis) in the digesta of different gastrointestinal segments of broilers at 24 d of age
Data adapted from Moquet and coworkers (2018)

Location	Dietary treatment means[1]				Diet effect
	UP	TB	FCB	UPTB	
Crop	1033[a]	822[a]	878[a]	1067[a]	**<0.001**
Proventriculus and gizzard	1472[a]	1031[b]	717[b]	962[b]	**<0.001**
Small intestine	13[b]	477[a]	29[b]	371[a]	**<0.001**
Colon and ceca	4	7	38	26	0.676

[1]Dietary treatments groups: UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg on an as is basis. Values are means of 15 birds per treatment.
[a,b]Means within a row having a superscript differ significantly from the control (no butyrate supplementation). Means within a row lacking a common superscript differ significantly (P<0.05).

For each GIT segment sampled, it was decided to compare the control without added butyrate to the butyrate-supplemented diet that resulted in the largest increase in digesta butyrate concentration. Consequently, following treatment groups were selected for comparison: CTR and UP for the proventriculus and pylorus, CTR and TB for the duodenum, and CTR and FCB for the colon. For the distal part of the small intestine (ileum) it was decided to compare CTR and FCB despite the fact that CTR and TB showed a larger contrast in butyrate concentration in pooled intestinal digesta. This was decided because TB is known to release butyrate in the proximal part of the small intestine in pigs (Schwarzer and Bjork, 2015) and poultry (unpublished results from our lab) while FCB is known to extend the effect of butyrate to the entire small intestine in chickens (van den Borne *et al.*, 2015).

Short chain fatty acids determination in tissue samples. Frozen tissue samples were ground in liquid nitrogen with a pestle and mortar. A fraction of ground tissue was analysed for short chain fatty acids (**SCFA**) determination by gas chromatography according to the method described by Qaisrani and colleagues (2014).

RNA extraction and real time quantitative PCR. Total RNA was extracted from 1-2 mg fraction of ground tissue using Trizol reagent (ThermoFisher Scientific, Bleiswijk, Netherlands). Isolated RNA was subsequently subjected to an on-column DNase digestion to eliminate possible DNA contamination (NucleoSpin RNA II kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total nucleic acid concentration and purity were determined by optical density measurement using a NanoDrop ND-1000 (Thermofisher Scientific) while RNA integrity and size was assessed using a

Bioanalyzer 2100 and RNA 6000 Nano LabChip kit (Agilent, Santa Clara, California). The average RNA integrity number value was 9.9, ranging between 9.7 and 10. First-strand cDNA synthesis was performed with 250 ng of total RNA per 20-mL sample reaction with Superscript III reverse transcriptase (Thermofischer Scientific), deoxyribonucleotide triphosphate (Roche Diagnostics, the Netherlands), and random hexamer primers (Roche Diagnostics, the Netherlands) for 1 h at 50 °C, according to the manufacturer's protocol (Thermofischer Scientific). Real-time quantitative PCR was carried out with a ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, California) by using the SensiMix SYBR Low-ROX mix (Bioline UK Ltd., London, UK). Amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 35 s each. A final melting protocol of increasing temperature from 60°C to 95°C with 0.5°C increments of 5 s each was applied.

The primer sequences used in this study are listed in Table 2. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA template for corresponding genes. We designed primers for 5 genes, commonly used as reference genes in mammalian species, namely ACTB, EFF2, IPO8, PPIA and RPLPO. Analysis of the measured values with the Normfinder algorithm (Andersen *et al.*, 2004) revealed that IPO8 was the most suitable housekeeping gene across locations and diets.

Table 2. Primers used for real-time quantitative PCR

Gene[1]	Forward primer (5'-3')	Reverse primer (5'-3')
Normalization genes		
IPO8	ACCTCCGAGCTAGATCCTGT	GGCTCTTCTTCGCCAACTCT
ACTB	GCCCTGGCACCTAGCACAAT	GCGGTGGACAATGGAGGGT
RPLP0	TTGGGCATCACCACAAAGATT	CCCACTTTGTCTCCGGTCTTAA
PPIA	CCCGTCGTGTTCTTCGACAT	CCCTTGTAGCCAAATCCCTTCT
EEF2	CAGTTGGCTTTGGTTCTGGC	AAAGTATCTGTCTCCCCACAGC
Transporters		
SMCT1	GGCACACCTTCTGGACGATT	TAGAGGGACATTTTTGCGTGGA
SMCT2	TTGGGGGAACCTTTACGTGG	AAGTGCCATCTTTGCATGCTT
MCT1	CGGTGCTGTTTGAAACCCTG	GTCGTTGAGCTTACCGAGCA
MCT2	AGCCGACCAGTGATGATAGC	GGTCAAGGCAGGCTGTAAGT
MCT8	TCGTGAGCATCTTCACTGACC	CTTCCAGAGACTTGGTGAAGGAG
MCT9	TCAACAGGCTCAAGTGTTGGA	GGTCTCATTAGGCTGCCACA
MCT10	TTCTTTTGTAAGCACCATCGAGCC	TGCCCAAGGATGACCAAAGAT
MCT14	AGTAACAGCTGGCATTGGGA	CCAAACCCTGTTCCTGTGGT
Energy metabolism		
ACSM3	GTTGTCAGCAGTCCAGACCC	GCTCTCGAACAAACTCCATTTTCC
ACSM4	TGGTTTTAGACCCCTGTTATGTGA	GGAAGCTGCAGAGTGTATTTAGG
ACSM5	CAGTAGCAGGCACTGGATGAA	GCAGAAGACGGTGATAGGGT
CS	TTACCGCAACCTTTACCGGG	GAACTGCGGGTCGGTGTAA
DLAT	TTCGGAGGGTCATTGCTCAG	CGTAGCACCAGTACTTCTCCC
G6PC	TGGTCCTGCACCTCTTTGATG	ACATGAAGCTGCCCCTAGAC
GADPH	ATCCCTGAGCTGAATGGGAAG	AGCAGCCTTCACTACCCTCT
GPI	AAGGGAGGTCCACGTGTTTG	GGTGGTGAAGGTCTTTGATGC

[1]*IPO8*: Importin 8; *ACTB*: Actin beta; *RPLP0*: 60S acidic ribosomal protein P0; *PPIA*: Peptidylprolyl Isomerase A; *EEF2*: Eukaryotic elongation factor 2; *SMCT*: Sodium-coupled monocarboxylate transporter; *MCT*: Monocarboxylate transporter; *ACSM*: Acyl-CoA synthetase medium chain family member; *CS*: Citrate synthase; *DLAT*: Dihydrolipoamide S-acetyltransferase; *G6PC*: Glucose-6-phosphatase catalytic subunit; *GADPH*: Glyceraldehyde-3-phosphate dehydrogenase; *GPI*: Glucose-6-phosphate isomerase.

Table 2. (Continued)

Gene[1]	Forward primer (5'-3')	Reverse primer (5'-3')
Sensing and hormonal regulation		
FFAR2	GGCTACCACTGCTACGATGA	CCCCAAAGTTGACCATGGTG
preproCCK	CAAGCCCGGAAAGGTTCCA	GTATTCTTCAGCACTGCGGC
preproGastrin	GTTCCTCGGCCTCATCCTC	TAGAAGTGGTCGTGCAGGG
GCG-A	CCCTTTTTCTCTGCCTCTGTAGT	CTGGGAATGATCACCAGAGCA
GCG-B	TGGCTGCCAAAGAGTTCCTAA	TGGCAGTTGAGGCAGTGAAG
PC1/3	GCACTGATACCCAGCCTGAA	TGTGTGGTATGGTCCTCTGC
PC2	ACCCCCGCTATACAGATGACT	CGAATACCTGCCACCTTGGA

[1]; *FFAR*: Free fatty acid receptor; *preproCCK*: Preprocholecystokinin; *preproGastrin*: *CGC-A*: Proglucagon A; *GCG-B*: Proglucagon B; *PC1/3*: Proprotein convertase subtilisin/kexin type 1/3; *PC2*: Proprotein convertase subtilisin/kexin type 2

Statistical analysis

SCFA Data. SCFA data were analyzed separately for each GIT location using the PROC GLM of SAS (version 9.4, SAS Institute Inc., Cary, NC) using the following model:

$$Y_{ij} = \mu + D_i + \varepsilon_{ij},$$

where Y_{ij} is the observed butyrate concentration of the j^{th} bird (j = 1 to 8) fed the i^{th} diet (i = CTR, UP, TB, FCB or UPTB), μ is the overall mean response, D_i is the i^{th} fixed diet effect and ε_{ij} the residual term associated with the j^{th} bird the i^{th} diet. Model assumptions were checked by visual inspection of the residuals.

Gene expression Data. Differences in gene expression were tested for each gene and location with the Mann-Whitney U test using PROC NPAR1WAY of SAS 9.4.

RESULTS AND DISCUSSION

In the present paper, we refer to the genes using their abbreviated protein names. Abbreviated gene names are mentioned upon their first appearance in the text if they differ from the abbreviated protein name.

Butyrate uptake along the avian GIT

Monocarboxylate transporters. Astbury and Corfe (2012) summarized in an authoritative review the uptake and metabolism of butyrate in the mammalian GIT. Briefly, early research works reported that butyrate uptake in the mammalian colon increased with $HCO3^-$ gradient and was reduced by the presence of acetate and propionate (Mascolo *et al.*, 1991). This suggested the involvement of a carrier-mediated anion exchange process in the absorption of SCFA in the gut (Mascolo *et al.*, 1991). The monocarboxylate transporter 1 (**MCT1**, gene name SLC16A1) was later characterized in hamsters (Garcia *et al.*, 1994) and its role as HCO_3^-/butyrate exchanger was confirmed in humans (Harig *et al.*, 1996). Next to its function as HCO_3^-/butyrate exchanger, MCT1 was also characterized as a proton-linked cotransporter for SCFA, pyruvate, lactate and ketone bodies in mammals (Halestrap and Price, 1999). The MCT family contains fourteen members (Halestrap, 2013). In this family, there are three alternative SCFA transporters with characteristics overlapping those of MCT1, namely MCT2, 3 and 4 (gene names SLC16A7, SLC16A8 and SLC16A3, respectively; Halestrap, 2013). In mammals, MCT1 proteins are almost absent from the duodenal and jejunal

brush border membrane (**BBM**), but are highly present in the ileal and colonic BBM (Gill *et al.*, 2005; Welter and Claus, 2008). To date, there is no report describing the distribution, cellular localization and functions of the MCT family members along the avian GIT.

Sodium-coupled monocarboxylate transporters. After functional characterization of MCT1, additional butyrate transporters were discovered. Coady and coworkers (2004) demonstrated with frog oocytes that butyrate uptake could also be facilitated by the sodium-coupled monocarboxylate transporter 1 (**SMCT1**, gene name SLC5A8). Such features are shared with SMCT2 (gene name SLC5A12; Gopal *et al.*, 2007). MCT1 and SMCT1 share the same distribution and cellular localization in the mammalian GIT (Takebe *et al.*, 2005; Borthakur *et al.*, 2010). Because of its higher substrate affinity, SMCT1 is believed to play a more important role than MCT1 in the uptake of butyrate when digesta SCFA concentration is within the µM range (Astbury and Corfe, 2012). This view is also supported by the fact that Na^+ gradient is far greater than pH or $HCO3^-$ gradients in the GIT (Gupta *et al.*, 2006). The expression of several SLC5 genes has been investigated in the GIT of poultry (Garriga *et al.*, 2006; Gilbert *et al.*, 2007). Such research was, however, only focused on sodium-dependent glucose transporters. The distribution, cellular localization and functions of SMCT1 and 2 remain undocumented in the avian GIT.

Types of (S)MCT expressed in broilers GIT. We tried to address a part of this gap of knowledge by investigating the expression of SMCT1, SMCT2 and of the fourteen MCT family members in the chicken GIT. Complementary DNA samples were pooled across diets and locations and subsequently screened for the presence of mRNA of provisional avian SMCT1, SMCT2 and MCT family members using PCR. Gel electrophoresis confirmed the presence of amplicons coding for SMCT1, SMCT2, MCT1 and MCT2. In addition, we also detected MCT8, MCT9, MCT10 and MCT14 amplicons (Data not shown; gene names SLC16A2, SLC16A9, SLC16A10 and SLC16A14, respectively). The expression of detectable genes was quantified by qPCR at five GIT locations. For each location, the CTR without dietary butyrate supplementation was compared to the butyrate supplemented diet (UP, TB or FCB) that resulted in the highest increase in digesta butyrate concentration (Table 3).

Butyrate does not affect (S)MCT expression in the gastric region. Changes in digesta butyrate concentration in the gastric region (proventriculus and pylorus) did not influence significantly the expression of any of the measured (S)MCT genes. The pH of the digesta found in the gastric region is usually lower than the pKa of butyrate (Denbow, 2015). Consequently, butyrate exist mostly in a non-ionized (protonated) form in the gastric region of birds (Moquet *et al.*, 2016). Non-ionized butyrate can diffuse passively through the gastric wall (Walter and Gutknecht, 1984). This may explain why, in the present experiment, we observed no significant effect of butyrate concentration in the gastric digesta on the expression of putative butyrate transporters.

Table 3. Effect of dietary treatments[1] on expression of sodium-coupled monocarboxylate transporters (SMCT) and monocarboxylate transporters (MCT) genes in different gastrointestinal tract segments of broilers at 22-23 d of age

	Average gene expression[2]							
	SMCT		MCT					
	SMCT1	**SMCT2**	**MCT1**	**MCT2**	**MCT8**	**MCT9**	**MCT10**	**MCT14**
Proventriculus								
CTR	0.01	0.01	2.91	29.76	0.57	0.62	1.95	0.32
UP	0.02	0.01	3.00	30.54	0.61	0.61	2.09	0.41
P-value	0.418	0.479	0.479	0.793	0.713	0.479	0.437	0.396
Pylorus								
CTR	14.79	0.03	12.45	1.16	0.60	9.38	15.85	0.21
UP	14.05	0.03	14.20	1.29	0.64	9.03	13.60	0.25
P-value	0.500	0.215	0.282	0.114	0.282	0.437	***0.078***	0.135
Duodenum								
CTR	17.99	0.04	12.73	0.63	0.47	10.03	18.09	0.12
TB	25.26	0.08	18.95	1.04	0.74	15.98	29.89	0.24
P-value	0.247	**0.012**	**0.001**	**0.005**	**0.007**	**0.005**	**0.001**	**0.020**
Ileum								
CTR	3.61	100.00	13.95	1.43	0.73	12.02	12.98	0.20
FCB	3.46	86.39	12.80	1.62	0.67	10.70	10.87	0.21
P-value	0.215	0.247	0.159	***0.064***	0.159	**0.042**	0.114	0.396
Colon								
CTR	6.59	0.02	17.22	8.05	1.12	15.62	9.86	2.44
FCB	6.02	0.02	20.57	9.97	1.15	16.07	12.64	3.12
P-value	0.500	0.437	**0.033**	***0.078***	0.357	0.437	***0.078***	**0.007**

[1]Dietary treatments groups: UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.

[2]Arbitrary units. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. The highest average gene expression of the table was set to 100.

Butyrate upregulates various (S)MCT in the duodenum. Historically, intestinal SCFA absorption was thought to be mediated by passive diffusion mechanisms in mammals and poultry (Sudo and Duke, 1980; Walter and Gutknecht, 1984). Intestinal digesta has, however, a pH higher than the pKa of butyrate (Denbow, 2015). This means that butyrate exists mostly in a ionized form in this GIT segment (Moquet *et al.*, 2016). Transporters such as MCT1 or SMCT1 may, therefore, be required to move butyrate across the intestinal wall (Astbury and Corfe, 2012). Such transporters are, however, almost completely absent from the duodenal BBM of mammals fed diets without supplemented butyrate (Gill *et al.,* 2005; Takebe *et al.*, 2005; Welter and Claus, 2008; Borthakur *et al.*, 2010). Expression of (S)MCT in the BBM of the mammalian duodenum may be constitutively low and induced by the presence of butyrate in the lumen.

In the present experiment, we observed that increasing digesta butyrate concentration in the duodenum resulted in a significant increase in the expression of genes coding for MCT1, MCT2 and SMCT2 (*P*=0.001, *P*=0.005 and *P*=0.012, respectively). As stated earlier, butyrate is known to be a substrate for such transporter in mammals. In addition, we observed that increasing digesta butyrate concentration in the duodenum resulted in a significant increase in the expression of MCT8, 9, 10 and 14 genes (*P*=0.007, *P*=0.005, *P*=0.001 and *P*=0.020, respectively). In mammals, physiological roles of the latter MCT are related to the transport of thyroid hormones

and their metabolites (MCT8 and 10), of aromatic amino acids (MCT10 and 14) and of carnitine (MCT9) (Suhre *et al.*, 2011; Halestrap, 2013; Roshanbin *et al.*, 2016). It would be relevant to investigate in future research the cellular localization and exact functions of the avian (S)MCT that are reported in Table 3.

Butyrate modulates MCT expression in the ileum and colon. We observed that the expression of MCT9 was significantly reduced (P=0.042) when digesta butyrate concentration was increased in the ileum. This transporter is still considered as orphan in mammals (Halestrap, 2013) although a possible role in carnitine transport has been reported (Suhre *et al.*, 2011). The expression of other (S)MCT was not significantly affected by dietary intervention. We found that total (S)MCT expression was significantly higher in the ileum than in any other measured GIT segment (P<0.001; Data not shown). Overall, our results suggest that (S)MCT expression in the ileum was constitutively sufficient to cope with the changes in luminal butyrate concentration that were incurred by our dietary intervention.

Concerning the colon, our results indicated that the expression of MCT1 and 14 (P=0.033 and P=0.007) increased with digesta butyrate concentration. In mammals, the Michaelis constant ($\mathbf{K_m}$) of SMCT1 is far lower than the K_m of MCT1 (50-100 μM vs 10 mM; Coady *et al.*, 2004; Cuff *et al.*, 2002). Because of such difference, Astbury and Corfe (2012) speculated that SMCT1 has a lower maximum rate ($\mathbf{V_{max}}$) than MCT1 in mammals. Physiological concentration of butyrate reaches 50 mM in the colon of mammals (Astbury and Corfe, 2012; Hamer *et al.*, 2008). Under such circumstances, the V_{max} of SMCT1 is likely to be reached and, consequently, the uptake of butyrate via this transporter may be limited (Astbury and Corfe, 2012). This could explain why only MCT1 expression has been reported to be regulated by digesta butyrate concentration in the distal GIT of mammals (Borthakur *et al.*, 2010; Cuff *et al.*, 2002). The physiological concentration of butyrate in the digesta of the colon and ceca of broilers reaches the mM range (Moquet *et al.*, 2018). Provided that enzymes kinetics are conserved across phylogenetic classes, this would mean that the capacity of SMCT1 to facilitate the uptake of butyrate in the distal GIT of birds is more limited than the one of MCT1. This rationale may explain why several MCT but no SMCT were upregulated in the present experiment when butyrate concentration was increased in the ileum and in the colon.

Butyrate metabolism along the avian GIT

Key steps in butyrate metabolism. There is a gap of knowledge concerning the mechanisms mediating the transport of butyrate from the cytosol to the mitochondria of mammalian cells (Astbury and Corfe, 2012). Upon entry in the mitochondria, a five steps reaction allows the activation and catabolism of butyrate (Figure 1).

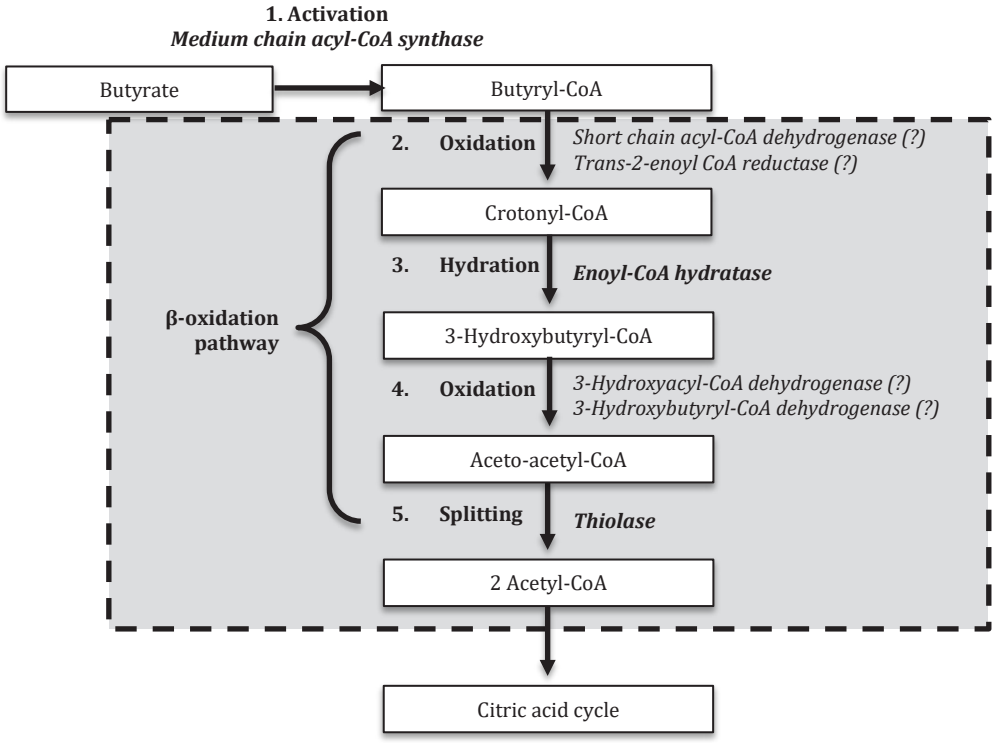

Figure 1. Summary of mechanisms and knowledge gaps for butyrate metabolism in the mitochondria

First, butyrate is ligated to acetyl-CoA to form butyryl-CoA (Step 1 in Figure 1). This initial activation step is conducted in the mitochondria by broadly specific (C4-11) medium chain acetyl-CoA synthetase enzymes (**ACSM**; Aas and Bremer, 1968). Following steps (Steps 2 to 5 in Figure 1) also take place in the mitochondria and correspond to the actual catabolism of butyrate in the β-oxidation pathway. There is little consensus in the literature concerning the enzymes carrying out the oxidation of butyrate intermediates (steps 2 and 4 in Figure 1; for review, see Astbury and Corfe, 2012). The hydration step is carried by enoyl-CoA hydratase, a broadly specific enzyme (C4-16; step 3 in Figure 1). The final splitting step is carried out by a thiolase that is identical in the β-oxidation of all fatty acid molecules (step 5 in Figure 1; Astbury and Corfe, 2012).

Types of medium chain acetyl-coa synthetase expressed in the avian GIT. Energy metabolism is primarily regulated at the transcriptional level in intestinal cells (Chang *et al.*, 2008). Messenger RNA are, therefore, good proxy to study energy metabolism in the gut. As mentioned previously, enzymes involved in the catabolism of butyrate are either well characterized (i.e. steps 1, 3 and 5 in Figure 1) or poorly defined (i.e. steps 2 and 4 in Figure 1). Among well characterized enzymes, ACSM family members have the most narrow substrate specificity. Hence, we decided to use the expression of ACSM family members as proxy for the catabolism of butyrate in the present study. Complementary DNA samples were pooled across diets and locations and subsequently screened for the presence of mRNA of provisional avian ACSM1, 2a, 2b, 3, 4 and 5 using

PCR. Gel electrophoresis confirmed the presence of amplicons coding for ACSM3, 4 and 5 (Data not shown). The expression of detectable genes was quantified by qPCR at five GIT locations. For each location, the CTR without dietary butyrate supplementation was compared to the butyrate supplemented diet (UP, TB or FCB) that resulted in the highest increase in digesta butyrate concentration (Table 4).

Table 4. Effect of dietary treatments[1] on the genetic expression of medium chain acetyl-CoA synthetase (ACSM), glucose metabolism and citric acid cycle enzymes in different gastrointestinal tract segments of broilers at 22-23 d of age

	Average gene expression[2,3]							
	ACSM			Glycolytic and citric acid cycle enzymes				
	ACSM3	**ACSM4**	**ACSM5**	**CS**	**DLAT**	**G6PC**	**GAPDH**	**GPI**
Proventriculus								
CTR	1.67	94.45	76.85	92.07	100.00	0.70	26.63	25.58
UP	6.71	100.00	100.00	94.07	94.97	0.97	28.35	25.28
P-value	***0.053***	0.772	0.159	0.437	***0.095***	0.159	0.282	0.479
Pylorus								
CTR	61.92	29.14	1.05	63.40	64.90	46.12	37.61	51.48
UP	41.06	n.d.	n.d.	63.86	63.14	36.13	35.83	45.01
P-value	0.247	0.191	0.183	0.282	0.396	***0.052***	0.479	**0.026**
Duodenum								
CTR	57.52	81.41	3.32	88.09	98.32	65.97	63.92	100.00
TB	100.00	n.d.	n.d.	100.00	93.30	100.00	100.00	95.07
P-value	0.282	-	0.191	***0.078***	0.215	**0.003**	**0.001**	0.159
Ileum								
CTR	21.47	n.d.	0.00	59.42	43.37	13.78	64.01	30.89
FCB	29.37	n.d.	2.55	59.80	39.29	8.46	56.98	26.59
P-value	0.247	-	0.191	0.318	**0.020**	**0.026**	**0.007**	**0.042**
Colon								
CTR	27.66	n.d.	4.28	52.37	39.90	1.75	65.57	20.10
FCB	27.77	n.d.	0.15	62.16	38.09	1.86	72.30	22.14
P-value	0.479	-	0.145	**0.012**	0.215	0.318	0.114	0.282

[1]Dietary treatments groups: UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.
[2]Arbitrary units. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. For each gene, the highest average gene expression across location and treatments was set to 100.
[3]n.d. : not detectable.

At the cellular level, energy substrate selection is regulated by the so-called Randle (1963) and McGarry cycles (1977). Because of such mechanisms, glucose and lipids compete with each other for oxidation in the mitochondria. Changes in ACSM activity may, therefore, influence indirectly glucose metabolism in the gut. Such indirect effects were assessed by measuring, in addition to ACSM, the expression of several genes related to glucose metabolism (Table 4).

Butyrate reduces glycolysis in the gastric region. We observed a tendency for a higher expression of ACSM3 (*P*=0.053) and for a lower expression of dihydrolipoamide S-acetyltransferase (**DLAT**; *P*=0.095) when digesta butyrate concentration increased in the proventriculus. As mentioned above, ACSM enzymes allow the ligation of coenzyme A to butyrate. DLAT is one of the three enzymes constituting the pyruvate dehydrogenase complex. This complex links the glycolysis to the citric acid cycle (**CAC**). The expression of citrate synthase (**CS**) was unaffected by the presence of butyrate in

the proventriculus and pylorus (P=0.437 and P=0.282, respectively). CS is a pace-making enzyme catalysing the first step of the CAC.

In addition, we observed a significant decrease of glucose-6-phosphate isomerase exprssion (**GPI**; P=0.026) and a tendency for a lower expression of glucose-6-phosphatase (**G6PC**; P=0.052) when digesta butyrate concentration was increased in the pylorus. Both GPI and G6PC are involved in glucose metabolism. The former is a glycolytic enzyme allowing the interconversion of glucose-6-phosphate and fructose-6-phosphate while the latter allows the conversion of glucose-6-phosphate to glucose, thereby affecting glucose homeostasis. Collectively, our results may suggest that the presence of butyrate in the gastric region shifted partly the origin of acetyl-CoA consumed in the CAC from glycolysis to β-oxidation, without significant effect on overall CAC activity. Hence, our results suggest that lipid oxidation, including butyrate oxidation, may have been increased by the presence of butyrate in gastric contents.

Butyrate increases glycolysis in the duodenum. We observed significantly higher expression levels of G6PC (P=0.003) and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**; P=0.001) when digesta butyrate concentration was increased in the duodenum. These results suggest that glycolytic activity increased when digesta butyrate concentration was increased in the duodenum. An increase in glycolytic activity leads to a reduction of the β-oxidation of lipids due to the mechanisms described in McGarry's cycle (1977). Hence, our results suggest that lipid oxidation, including butyrate oxidation, may have been reduced by the presence of butyrate in duodenal contents.

Butyrate reduces the apoptosis of healthy enterocytes while stimulating their differentiation and maturation (Guilloteau *et al.*, 2010). This is why butyrate increases, although inconstantly, villus length when fed to broilers (Moquet *et al.*, 2016). It is known that the expression of proteins related to glucose metabolism increases as enterocytes mature and move upwards along the crypt-villus axis (Chang *et al.*, 2008; Yang *et al.,* 2016). An increase in the maturation rate of enterocytes may explain why we observed an increase in glycolytic activity when butyrate was present in the duodenum.

The expression of DLAT was, however, unaffected by the presence of butyrate (P=0.215). This may suggest that the increase in glycolysis was not accompanied by a proportional increase in the transfer of pyruvate to the CAC via the pyruvate dehydrogenase complex. An uncoupling between glycolysis and CAC was further suggested by the rather limited increase in CAC activity (P=0.078) in comparison to the significant increase in G6PC and GAPDH expressions (P=0.003 and P=0.001, respectively). Such uncoupling may have led to the accumulation of pyruvate and, consequently, to the build-up of lactate via the anaerobic glycolysis pathway. Several MCT may also transport lactate and pyruvate in addition to SCFA, e.g. MCT1, 2, 3 and 4 (Halestrap, 2013). We observed a significant increase in MCT1 and 2 expression when butyrate was present in the lumen of the duodenum. Such changes may reflect lactate and pyruvate transport rather than butyrate uptake.

Butyrate reduces glycolysis in the ileum. We observed significantly lower expressions of DLAT (P=0.020), G6PC (P=0.026), GAPDG (P=0.007) and GPI (P=0.042) when digesta butyrate concentration increased in the ileum. Such changes clearly suggest a reduction of the glycolytic activity. Glycolytic activity can be reduced by an increase in β-oxidation activity due to the mechanisms described in Randle's cycle (1963). Hence, our results suggest that lipid oxidation, including butyrate oxidation, may have been increased by the presence of butyrate in the ileum. The lack of significant upregulation of ACSM enzymes may indicate that the ileum is constitutively able to metabolize butyrate.

Butyrate presence in the ileum reduced glycolysis, while an opposite effect was observed in the duodenum. Research indicates that the preference of enterocytes for various energy substrates varies according to their location along the GIT. In mammals, glucose, glutamate and glutamine are the favoured energy substrates of healthy enterocytes obtained indistinctly from the entire small intestine (Watford *et al.*, 1979). Butyrate is, however, the favoured energy substrate of healthy enterocytes obtained specifically from the distal ileum of mammals (Chapman *et al.*, 1995). In mammals, enterocytes located in the distal ileum have, therefore, the same favoured energy substrate as colonocytes (Roediger, 1982). Such location-dependent specificities in the energy metabolism of enterocytes may also apply to the avian GIT. This may explain the divergence in energy metabolism response observed when digesta butyrate concentration was increased in the duodenum or in the ileum during the present experiment.

Such divergence in energy metabolism response carries significant implications for the efficiency of broiler production. There is an asynchrony in starch and protein digestion rates in the GIT of broilers (Riesenfeld *et al.*, 1980; Sklan and Hurwitz, 1980; Osman, 1982). Such asynchrony results in glucose being less available than amino acids (AA) in the distal ileum of broilers (Liu and Selle, 2015). This situation is thought to impair feed efficiency by increasing AA catabolism (Weurding *et al.*, 2003). So far, corrective strategies aimed at either increasing the rate of AA digestion in the proximal GIT, e.g. by feeding exogenous proteases or synthetic AA (Liu and Selle, 2017), or at reducing the rate of starch digestion in the proximal GIT, e.g. by feeding slow digestible starch (Weurding *et al.*, 2003). Our work indicates similarities at the ileum level between mammalian and avian enterocytes, meaning that such cells may prefer butyrate over other energy substrates. Onrust and coworkers (2015) have formulated a series of dietary strategies that could promote butyrate production in the distal GIT of poultry. Such strategies may help to spare AA from catabolism in the distal GIT of poultry.

Butyrate increases citric acid cycle activity in the colon. We observed in the present study that CS expression was significantly higher when butyrate was present in the lumen of the colon (P=0.012). This result indicates an increase in CAC activity. There was, however, no significant change in the expression of enzymes related to glucose metabolism or butyryl-CoA formation. This could indicate that the colon is constitutively able to metabolize butyrate. This is in line with the observations of Watford and colleagues (1979), who reported that avian colonocytes use preferentially butyrate as energy substrate.

Butyrate does not accumulate in gut tissues. Dietary intervention resulted in important changes in digesta butyrate concentration at different GIT segments (Table 1). We investigated the relationship between digesta butyrate concentration and intracellular butyrate concentration. Our results indicate that intracellular butyrate concentration was not affected by variations in digesta butyrate concentration (Table 5).

Table 5. Effect of dietary treatments[1] on intracellular short chain fatty acid concentration in distinct gastrointestinal tract segments of broilers at 22-23 d of age

	Short chain fatty acids[2] (mM)					
	Acetate	**Propionate**	**Butyrate**	**Iso-butyrate**	**Valerate**	**Iso-valerate**
Proventriculus						
CTR	0.55	0.03	0.02	n.d.	n.d.	0.01
UP	0.58	0.06	0.03	n.d.	n.d.	0.01
P-value	0.710	0.181	0.172	-	-	1.00
Pylorus						
CTR	0.16	<0.01	0.01	n.d.	n.d.	n.d.
UP	0.22	0.02	0.01	n.d.	n.d.	n.d.
P-value	0.291	0.137	0.403	-	-	-
Duodenum						
CTR	5.54	1.73	0.03	0.06	0.05	0.08
TB	5.61	1.75	0.04	0.05	0.04	0.07
P-value	0.786	0.701	0.484	***0.096***	0.740	0.430
Ileum						
CTR	4.58	1.42	0.01	0.04	0.02	0.08
FCB	4.75	1.47	0.01	0.04	0.04	0.07
P-value	0.470	0.529	0.458	0.688	**0.017**	0.305
Colon						
CTR	4.93	1.38	0.08	0.05	0.04	0.08
FCB	5.16	1.25	0.11	0.05	0.03	0.08
P-value	0.668	0.173	0.605	0.843	0.577	0.429

[1]Dietary treatments groups: UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.
[2]n.d. : not detectable.

Intracellular butyrate concentration remained very low across GIT segments. This may indicate that intracellular butyrate concentration is tightly regulated. The importance of intracellular butyrate regulation is well established in colonocytes. Butyrate may act as a survival factor in healthy colonocytes or as an apoptotic agent in cancerous ones (Luciano *et al.*, 2002). This paradox is explained by differences in energy metabolism existing between healthy and cancerous cells. Most cancerous cells rely on aerobic glycolysis due to the so-called Warburg effect (van der Heiden *et al.*, 2009). This metabolic profile limits greatly the β-oxidation of lipids (McGarry *et al.*, 1977). As a consequence, butyrate accumulates in most of cancerous colonocytes, thereby inhibiting HDAC activity and acting as an apoptotic signal (Donohoe *et al.*, 2012). In contrast, butyrate is the primary energy source of healthy colonocytes (Roediger, 1982). Hence, healthy colonocytes do not accumulate butyrate but benefit from it as an energy substrate (Donohoe *et al.*, 2012). Intracellular butyrate concentration may be tightly regulated in the avian GIT to avoid possible apoptotic effects, as seen in human colonocytes.

Such regulation can be achieved in several ways. Butyrate can be catabolized, incorporated in lipids through *de novo* lipogenesis, or transported out of the cell. In the present paper, we observed at the mRNA level direct (i.e. increased ACSM and CS expression) or indirect (i.e. decreased glycolytic enzymes expression) clues indicative of lipid catabolism when digesta butyrate concentration increased in the GIT of broilers. This was true for all measured GIT segments, except for the duodenum. For the latter, a clear increase in glycolytic activity was observed. Such metabolic shift was accompanied by an upregulation of numerous putative butyrate transporters. This may suggest that butyrate is not oxidized in the duodenum, but rather transported out of it.

In birds, lipids absorbed in the gut are packaged in portomicrons and transported to the liver via the portal vein (Denbow, 2015). Unpublished results from our lab indicate that feeding 1 g/kg butyrate in the form of UP, TB or FCB does not influence significantly butyrate concentration in the peripheral blood of 39 d old broilers. This suggests that butyrate escaping oxidation in the gut mucosa may be metabolised in the liver. Yin and coworkers (2016) supplemented broiler diets with TB, a butyrate glyceride that increases digesta butyrate concentration in the duodenum and jejunum, and reported significant changes in the hepatic transcriptome that evoked an increase in lipid catabolism. Such changes are consistent with the decreases in relative abdominal fat weight reported when broilers diets are supplemented with butyrate glycerides (Bedford *et al.*, 2017a,b).

Collectively, these observations suggest that increasing digesta butyrate concentration in the proximal part of the intestines (i.e. duodenum and possibly jejunum) leads to the uptake and transport of butyrate to the liver, where it is oxidized. In contrast, increasing digesta butyrate concentration in the gastric region or in the distal part of the intestines (i.e. ileum and colon) leads to the uptake and oxidation of butyrate in the gut tissue itself. It remains unclear if the absorption of butyrate in the gastric region or distal part of intestines is followed by a complete oxidation in the gut or if a fraction of the absorbed butyrate escapes oxidation in the gut to reach the liver. Future studies may investigate the effect of increasing butyrate concentration in distinct GIT segment on gene expression in the liver.

Butyrate sensing and endocrine regulation

Types of butyrate receptors expressed in the Avian GIT. Three receptors are known to sense butyrate in mammals: FFAR2, FFAR3 and GPR109a (Brown *et al.*, 2003; Le Poul *et al.*, 2014). Sequences coding for FFAR2 have been identified in the avian genome (Meslin *et al.*, 2015). To date, the comparison of avian and mammalian genomes did not allow the identification of FFAR3 and GPR109a orthologs in chickens (Lagerström *et al.,* 2006; Meslin *et al.*, 2015).

We observed that FFAR2 was expressed in all GIT segments sampled (Table 6). In addition, FFAR2 expression was reduced by the presence of butyrate in the digesta of the pylorus and of the ileum (P=0.007 and P=0.026, respectively). A similar trend was observed in the proventriculus (P=0.078). Such results suggest that an excess of ligand may down regulate FFAR2 expression.

Table 6. Effect of dietary treatments[1] on the expression of genes related to the hormonal regulation of gut functions in different gastrointestinal tract segments of broilers at 22-23 d of age

	Average gene expression[2]						
	FFAR2	**CCK**	**GAST**	**CGC-A**	**CGC-B**	**PCSK1**	**PCSK2**
Proventriculus							
CTR	17.25	15.54	96.65	n.m.	n.m.	n.m.	n.m.
UP	16.06	28.79	100.00	n.m.	n.m.	n.m.	n.m.
P-value	***0.078***	0.318	0.479	-	-	-	-
Pylor							
CTR	52.61	35.02	49.13	n.m.	n.m.	n.m.	n.m.
UP	27.60	30.35	50.93	n.m.	n.m.	n.m.	n.m.
P-value	**0.007**	0.396	0.437	-	-	-	-
Duodenum							
CTR	50.33	59.44	80.63	7.13	6.69	100.00	43.12
TB	54.64	100.00	81.52	8.10	6.62	87.65	61.20
P-value	0.479	**0.026**	0.396	0.376	0.338	0.753	0.376
Ileum							
CTR	100.00	n.m.	n.m.	100.00	100.00	86.41	59.96
FCB	68.95	n.m.	n.m.	85.32	70.56	87.69	61.39
P-value	**0.026**	-	-	0.215	***0.052***	0.479	0.357
Colon							
CTR	63.06	n.m.	n.m.	3.12	0.85	65.38	94.89
FCB	62.76	n.m.	n.m.	4.21	1.04	84.11	100.00
P-value	0.437	-	-	0.435	0.282	0.157	0.318

[1]Dietary treatments groups: UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.

[2]Arbitrary units. n.m.: not measured. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. For each gene, the highest average gene expression across location and treatments was set to 100.

Expression of genes related to gut hormones. Butyrate is known to modulate the secretory activities of different enteroendocrine cells subsets in mammals, including gastrin-producing G-cells, cholecystokinin (**CCK**) producing I-cells and glucagon like peptides (**GLP**) producing L-cells in mammals (Guilloteau *et al.*, 2010; Kimura *et al.*, 2014) and, presumably, in birds (Onrust *et al.*, 2015; Moquet *et al.*, 2016; Moquet *et al.*, 2018). Numerous articles have described the distribution of G-, I-, and L-cells along the GIT of chickens (Larsson *et al.*, 1974; Rawdon and Andrew, 1999; Hiramatsu *et al.*, 2005; Monir *et al.*, 2014). We investigated in relevant GIT segments the effect of increasing digesta butyrate concentration on the expression of genes related to gut hormones (Table 6).

Our results indicate that the expression of preprogastrin (**preproGAST**) by G-cells was unaffected by digesta butyrate concentration in the proventriculus, pylorus and duodenum (*P*=0.479; *P*=0.437; *P*=0.396, respectively). The expression of preprocholecystokinin (**preproCCK**) by I-cells was increased when digesta butyrate concentration increased in the duodenum (*P*=0.003). Such effect was, however, not observed in the proventriculus or in the pylorus (*P*=0.318 and *P*=0.396, respectively). Most of the preproCCK is processed into CCK7 and 8 in the avian GIT (Jonson *et al.*, 2000). The biological functions of CCK8 in the avian GIT have been extensively studied while those of CCK7 remain undocumented (Denbow, 2015). Intravenous infusion of CCK8 inhibits gastric emptying (Savory *et al.*, 1981) while stimulating duodenal electric activity in a manner that suggests an increase in segmental contractions (Martinez *et*

al., 1993). Increased circulatory levels of CCK8 have been consistently associated with a reduction of voluntary feed intake in chickens (Honda *et al.*, 2017). The physiological functions of CCK8 seem, therefore, to be conserved in birds and mammals (Denbow, 2015). A change in CCK activity may explain the increase in total tract retention time and the associated reduction in voluntary feed intake reported when broiler diets are supplemented with various butyrate additives (Moquet *et al.*, 2018). Such change does not explain, however, the specific increase in intestinal retention time reported when digesta butyrate concentration is selectively increased in the colon and ceca (Moquet *et al.*, 2018). The latter is thought to be mediated by changes in the secretory activities of L-cells (Moquet *et al.*, 2018).

In L-cells, GLP1 and 2 are produced by the processing of preproglucagon (**preproCGC**) A and B by the prohormone convertase 1 enzyme (**PC1/3**, gene name PCSK1) (Lim and Brubaker, 2006). Processing of preproCGC A and B by the prohormone convertase 2 (**PC2**, gene name PCSK2) results, otherwise, in the production of other signalling peptides such as glucagon (Lim and Brubaker, 2006). We observed that increasing digesta butyrate concentration in the ileum tended to reduce the expression of preproCGC B (P=0.052). Such effect was not observed in the duodenum or colon (P=0.338 and P=0.282, respectively). Besides, the expression of preproCGC A, PC1/3 and PC2 remained unaffected by the changes in the digesta butyrate concentration in the duodenum, ileum and colon. Overall, our results indicate that digesta butyrate concentration had little direct effect on the regulation, at mRNA level, of GLP1 and 2 production. This is in contrast with the observations of Tappenden and colleagues (1998), who reported a positive effect of enteric SCFA on total preproGCG mRNA concentration in the ileum of rats.

Peptide hormone signalling involves several steps: expression of mRNA coding for the (pro)hormone, subsequent protein synthesis, post-translational modification of the (pro)hormone, secretion and finally binding to a receptor cognate (Denbow, 2015; Scanes, 2015). Gene expression of a (pro)hormone may be a poor proxy for the circulating levels of the said hormone due to the number of intermediary steps existing between mRNA expression and actual signalling effect. In the present experiment, we did not observe any clear relationship between digesta butyrate concentration and the regulation, at mRNA level, of gastrin, GLP1 and GLP2 production. Such relationship exists, however, at the protein level in mammals (Guilloteau *et al.*, 2010) and, presumably, in birds (Moquet *et al.*, 2016). Hence, additional insights on the effect of butyrate on the endocrine regulation of digestive functions may be seen at the protein level. Unfortunately, there are no commercially available antibodies directed against avian gut hormones to allow such investigation.

It was not possible to show the effect of butyrate presence in a given GIT segment on gene expression in a remotely distant GIT segment with the approach chosen in the present study (e.g. the effect of digesta butyrate concentration in the colon on gastrin expression in the pylorus). Based on the work of Reilley and coworkers (1995), such effects may be relevant. They reported that colonic SCFA increased jejunal gastrin concentration in rats. Such indirect effect may also exist in poultry and are beyond the scope of the present work.

CONCLUSION

The present study indicates that increasing digesta butyrate concentration in the gastric region of the avian GIT results in a non-ionic, passive uptake followed by a catabolism of butyrate in the gastric wall. In contrast, increasing digesta butyrate concentration in the duodenum increases the expression of numerous putative butyrate transporters and glycolytic enzymes. This suggests that, in the duodenum, butyrate is taken up by transporters but not metabolized in the tissue itself. Duodenal butyrate may, therefore, be metabolized in the liver. Besides, MCT are the only transporters that are upregulated by digesta butyrate concentration in the ileum and colon. This suggests that MCT are more effective than SMCT in transporting butyrate when digesta concentration reaches the mM range. Butyrate seems to be catabolised, to some extent, in the ileum and colon. It remains unclear if the absorption of butyrate in the gastric region or distal part of the intestines is followed by a complete oxidation in the gut or if a fraction of butyrate escapes oxidation in the gut to reach the liver. Irrespective of the GIT segment considered, there is no relationship between digesta butyrate concentration and intracellular butyrate concentration. The latter remains overall very low. Intracellular butyrate concentration seems, therefore, to be tightly regulated. This may protect gut cells from the adverse effects of high intracellular butyrate on HDAC activity. The butyrate receptor FFAR2 seems to be present along the entire GIT of poultry. Its expression can decrease when digesta butyrate concentration increases. There are indications at mRNA level that butyrate affects the activity of CCK producing I-cells in the duodenum. Such effects may underlie the observed anorectic effects of butyrate in poultry, as well as changes in total tract retention time associated with dietary butyrate supplementation. The present study investigated direct, local effects of increasing digesta butyrate concentration on the expression of a selected set of genes in gut tissues originating from distinct GIT segments. Future research could investigate indirect effects at the protein level as well as effects on hepatic metabolism.

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LITERATURE CITED

Aas, M., and J. Bremer. 1968. Short-chain fatty acid activation in rat liver a new assay procedure for the enzymes and studies on their intracellular localization. Biochim. Biophys. Acta Lipids Lipid Metab. 164:157-166.

Andersen, C. L., J. L. Jensen, and T. F. Orntoft. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64:5245-5250.

Astbury, S. M., and B. M. Corfe. 2012. Uptake and metabolism of the short-chain fatty acid butyrate, a critical review of the literature. Curr. Drug. Metab. 13:815-821.

Bedford, A., H. Yu, M. Hernandez, J. Squires, S. Leeson, Y. Hou, and J. Gong. 2017a. Response of Ross 308 and 708 broiler strains in growth performance and lipid metabolism to diets containing tributyrate glycerides. Can. J. Anim. Sci. 98:98-108.

Bedford, A., H. Yu, E. J. Squires, S. Leeson, and J. Gong. 2017b. Effects of supplementation level and feeding schedule of butyrate glycerides on the growth performance and carcass composition of broiler chickens. Poult. Sci. 96:3221-3228.

Borthakur, A., A. N. Anbazhagan, A. Kumar, G. Raheja, V. Singh, K. Ramaswamy, and P. K. Dudeja. 2010. The probiotic Lactobacillus plantarum counteracts TNF-α-induced downregulation of SMCT1 expression and function. Am. J. Physiol. Gastrointest. Liver Physiol. 299:G928-G934.

Brown, A. J., S. M. Goldsworthy, A. A. Barnes, M. M. Eilert, L. Tcheang, D. Daniels, A. I. Muir, M. J. Wigglesworth, I. Kinghorn, N. J. Fraser, N. B. Pike, J. C. Strum, K. M. Steplewski, P. R. Murdock, J. C. Holder, F. H. Marshall, P. G. Szekeres, S. Wilson, D. M. Ignar, S. M. Foord, A. Wise, and S. J. Dowell. 2003. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J. Bio. Chem. 278:11312-11319.

Chang, J., M. R. Chance, C. Nicholas, N. Ahmed, S. Guilmeau, M. Flandez, D. Wang, D.-S. Byun, S. Nasser, J. M. Albanese, G. A. Corner, B. G. Heerdt, A. J. Wilson, L. H. Augenlicht, and J. M. Mariadason. 2008. Proteomic changes during intestinal cell maturation in vivo. J. Proteom. 71:530-546.

Chapman, M. A., M. F. Grahn, M. Hutton, and N. S. Williams. 1995. Butyrate metabolism in the terminal ileal mucosa of patients with ulcerative colitis. Br. J. Surg. 82:36-38.

Coady, M. J., M. H. Chang, F. M. Charron, C. Plata, B. Wallendorff, J. F. Sah, S. D. Markowitz, M. F. Romero, and J. Y. Lapointe. 2004. The human tumour suppressor gene SLC5A8 expresses a Na+-monocarboxylate cotransporter. J. Physiol. 557:719-731.

Cuff, M. A., D. W. Lambert, and S. P. Shirazi-Beechey. 2002. Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. J. Physiol 539:361-371.

Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Donohoe, D. R., L. B. Collins, A. Wali, R. Bigler, W. Sun, and S. J. Bultman. 2012. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol. Cell 48:612-626.

Garcia, C. K., J. L. Goldstein, R. K. Pathak, R. G. Anderson, and M. S. Brown. 1994. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. Cell 76:865-873.

Garriga, C., R. R. Hunter, C. Amat, J. M. Planas, M. A. Mitchell, and M. Moretó. 2006. Heat stress increases apical glucose transport in the chicken jejunum. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290:R195-R201.

Gilbert, E. R., H. Li, D. A. Emmerson, J. K. E. Webb, and E. A. Wong. 2007. developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. Poult. Sci. 86:1739-1753.

Gill, R. K., S. Saksena, W. A. Alrefai, Z. Sarwar, J. L. Goldstein, R. E. Carroll, K. Ramaswamy, and P. K. Dudeja. 2005. Expression and membrane localization of MCT isoforms along the length of the human intestine. Am. J. Physiol. Cell. Physiol. 289:C846-C852.

Gopal, E., N. S. Umapathy, P. M. Martin, S. Ananth, J. P. Gnana-Prakasam, H. Becker, C. A. Wagner, V. Ganapathy, and P. D. Prasad. 2007. Cloning and functional characterization of human SMCT2 (SLC5A12) and expression pattern of the transporter in kidney Biochim. Biophys. Acta 1768:2690-2697.

Guilloteau, P., L. Martin, V. Eeckhaut, R. Ducatelle, R. Zabielski, and F. Van Immerseel. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23:366-384.

Gupta, N., P. M. Martin, P. D. Prasad, and V. Ganapathy. 2006. SLC5A8 (SMCT1)-mediated transport of butyrate forms the basis for the tumor suppressive function of the transporter. Life Sci. 78:2419-2425.

Halestrap, A. P. 2013. The SLC16 gene family – Structure, role and regulation in health and disease. Mol. Aspects Med. 34:337-349.

Halestrap, A. P., and N. T. Price. 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem. J. 343:281-299.

Hamer, H. M., D. Jonkers, K. Venema, S. Vanhoutvin, F. J. Troost, and R. J. Brummer. 2008. Review article: the role of butyrate on colonic function. Aliment. Pharmacol. Ther. 27:104-119.

Harig, J. M., E. K. Ng, P. K. Dudeja, T. A. Brasitus, and K. Ramaswamy. 1996. Transport of n-butyrate into human colonic luminal membrane vesicles. Am. J. Physiol. 271:G415-422.

Hiramatsu, K., A. Yamasaki, and T. Shioji. 2005. Immunohistochemical and morphometrical studies on the distribution of glucagon-like peptide-1 (GLP-1)-immunoreactive cells in the chicken intestine. J. Poult. Sci. 42:223-229.

Honda, K., T. Saneyasu, and H. Kamisoyama. 2017. Gut hormones and regulation of food intake in birds. J. Poult. Sci. 54:103-110.

Jonson, L., N. Schoeman, H. Saayman, R. Naude, H. Jensen, and A. H. Johnsen. 2000. Identification of ostrich and chicken cholecystokinin cDNA and intestinal peptides. Pept. 21:1337-1344.

Kimura, I., D. Inoue, K. Hirano, and G. Tsujimoto. 2014. The SCFA receptor GPR43 and energy metabolism. Front. Endrocrinol. 5:e85.

Lagerström, M. C., A. R. Hellström, D. E. Gloriam, T. P. Larsson, H. B. Schiöth, and R. Fredriksson. 2006. The G Protein–Coupled Receptor Subset of the Chicken Genome. PLOS Comput. Biol. 2:e54.

Larraufie, P., C. Martin-Gallausiaux, N. Lapaque, J. Dore, F.M. Gribble, F. Reimann, and H.M. Blottiere. SCFAs strongly stimulate PYY production in human enteroendocrine cells. Sci Rep. 2018 Jan 8;8(1):74. doi: 10.1038/s41598-017-18259-0.

Larsson, L. I., F. Sundler, R. Håkanson, J. F. Rehfeld, and F. Stadil. 1974. Distribution and properties of gastrin cells in the gastrointestinal tract of chicken. Cell and tissue research 154:409-421.

Le Poul, E., C. Loison, S. Struyf, J. Y. Springael, V. Lannoy, M. E. Decobecq, S. Brezillon, V. Dupriez, G. Vassart, J. Van Damme, M. Parmentier, and M. Detheux. 2003. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J. Bio. Chem. 278:25481-25489.

Lim, G. E., and P. L. Brubaker. 2006. Glucagon-Like Peptide 1 Secretion by the L-Cell. Diabetes 55:S70.

Liou, A. P. 2013. Digestive physiology of the pig symposium: G protein-coupled receptors in nutrient chemosensation and gastrointestinal hormone secretion. J. Anim. Sci. 91:1946-1956.

Liu, S. Y., and P. H. Selle. 2015. A consideration of starch and protein digestive dynamics in chicken-meat production. Worlds Poult. Sci. J. 71:297-310.

Liu, S. Y., and P. H. Selle. 2017. Starch and protein digestive dynamics in low-protein diets supplemented with crystalline amino acids. Anim. Prod. Sci. 57:2250-2256.

Luciano, L., S. Groos, R. Busche, W. Von Engelhardt, and E. Reale. 2002. Massive apoptosis of colonocytes induced by butyrate deprivation overloads resident macrophages and promotes the recruitment of circulating monocytes. Cell Tissue Res. 309:393-407.

Martinez, V., M. Jimenez, E. Gonalons, and P. Vergara. 1993. Effects of cholecystokinin and gastrin on gastroduodenal motility and coordination in chickens. Life Sci. 52:191-198.

Mascolo, N., V. M. Rajendran, and H. J. Binder. 1991. Mechanism of short-chain fatty acid uptake by apical membrane vesicles of rat distal colon. Gastroenterol. 101:331-338.

McGarry, J. D., G. P. Mannaerts, and D. W. Foster. 1977. A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Clin. Investig. 60:265-270.

Meslin, C., C. Desert, I. Callebaut, A. Djari, C. Klopp, F. Pitel, S. Leroux, P. Martin, P. Froment, E. Guilbert, F. Gondret, S. Lagarrigue, and P. Monget. 2015. Expanding duplication of free fatty acid receptor-2 (GPR43) genes in the chicken genome. Genome Biol. Evol. 7:1332-1348.

Monir, M. M., K. Hiramatsu, K. Nishimura, C. Takemoto, and T. Watanabe. 2014. Distribution of glucagon-like peptide (GLP)-2-immunoreactive cells in the chicken small intestine: antigen retrieval immunohistochemistry. J. Vet. Med. Sci. 76:565-568.

Moquet, P. C. A., L. Onrust, F. Van Immerseel, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72:61-80.

Moquet, P. C. A., S. A. Salami, L. Onrust, W. H. Hendriks, and R. P. Kwakkel. 2018. Butyrate presence in distinct gastrointestinal tract segments modifies differentially digestive processes and amino acid bioavailability in young broiler chickens. Poult. Sci. 97:167-176.

Murphy, K. G., and S. R. Bloom. 2006. Gut hormones and the regulation of energy homeostasis. Nature 444:854-859.

Nohr, M. K., M. H. Pedersen, A. Gille, K. L. Egerod, M. S. Engelstoft, A. S. Husted, R. M. Sichlau, K. V. Grunddal, S. S. Poulsen, S. Han, R. M. Jones, S. Offermanns, and T. W. Schwartz. 2013. GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. Endocrinol. 154:3552-3564.

Onrust, L., R. Ducatelle, K. Van Driessche, C. De Maesschalck, K. Vermeulen, F. Haesebrouck, V. Eeckhaut, and F. Van Immerseel. 2015. Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. Front. Vet. Sci. 2:75. doi: 10.3389/fvets.2015.00075.

Osman, A. M. 1982. Amylase in chicken intestine and pancreas. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 73:571-574.

Qaisrani, S. N., P. C. Moquet, M. M. van Krimpen, R. P. Kwakkel, M. W. Verstegen, and W. H. Hendriks. 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93:3053-3064.

Randle, P. J., P. B. Garland, C. N. Hales, and E. A. Newsholme. 1963. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 281:785-789.

Rawdon, B. B., and A. Andrew. 1999. Gut endocrine cells in birds: an overview, with particular reference to the chemistry of gut peptides and the distribution, ontogeny, embryonic origin and differentiation of the endocrine cells. Prog. Histochem. Cytochem. 34:3-82.

Reilly, K. J., W. L. Frankel, A. M. Bain, and J. L. Rombeau. 1995. Colonic short chain fatty acids mediate jejunal growth by increasing gastrin. Gut 37:81-86.

Riesenfeld, G., D. Sklan, A. Bar, U. Eisner, and S. Hurwitz. 1980. Glucose absorption and starch digestion in the intestine of the chicken. J. Nutr. 110:117-121.

Riggs, M. G., R. G. Whittaker, J. R. Neumann, and V. M. Ingram. 1977. n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature 268:462-464.

Roediger, W. E. 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterol. 83:424-429.

Roshanbin, S., F. A. Lindberg, E. Lekholm, M. M. Eriksson, E. Perland, J. Ahlund, A. Raine, and R. Fredriksson. 2016. Histological characterization of orphan transporter MCT14 (SLC16A14) shows abundant expression in mouse CNS and kidney. BMC Neurosci. 17:e43.

Savory, C. J., G. E. Duke, and R. W. Bertoy. 1981. Influence of intravenous injections of cholecystokinin on gastrointestinal motility in turkeys and domestic fowls. Comp. Biochem. Physiol. A Physiol. 70:179-189.

Sawzdargo, M., S. R. George, T. Nguyen, S. Xu, L. F. Kolakowski, and B. F. O'Dowd. 1997. A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1. Biochem. Biophys. Res. Commun. 239:543-547.

Scanes, C. G. 2015. Chapter 22 - Avian endocrine system. Pages 489-496 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Schwarzer, K., and J. Bjork. 2015. Tributyrin: biological effects to stimulate animal performance and the integrity of of the intestinal tract in pigs, in: 47e Journées de la Recherche Porcine, INRA, Paris, pp. 133-134.

Sealy, L., and R. Chalkley. 1978. The effect of sodium butyrate on histone modification. Cell 14:115-121.

Singh, N., A. Gurav, S. Sivaprakasam, E. Brady, R. Padia, H. Shi, M. Thangaraju, P. D. Prasad, S. Manicassamy, D. H. Munn, J. R. Lee, S. Offermanns, and V. Ganapathy. 2014. Activation of GPR109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immun. 40:128-139.

Sklan, D., and S. Hurwitz. 1980. Protein digestion and absorption in young chicks and turkeys. The J. Nutr. 110:139-144.

Sudo, S. Z., and G. E. Duke. 1980. Kinetics of absorption of volatile fatty acids from the ceca of domestic turkeys. Comp. Biochem. Physiol. A Physiol. 67:231-237.

Suhre, K., S.-Y. Shin, A.-K. Petersen, R. P. Mohney, D. Meredith, B. Wägele, E. Altmaier, CardioGram, P. Deloukas, J. Erdmann, E. Grundberg, C. J. Hammond, M. H. de Angelis, G. Kastenmüller, A. Köttgen, F. Kronenberg, M. Mangino, C. Meisinger, T. Meitinger, H.-W. Mewes, M. V. Milburn, C. Prehn, J. Raffler, J. S. Ried, W. Römisch-Margl, N. J. Samani, K. S. Small, H. E. Wichmann, G. Zhai, T. Illig, T. D. Spector, J. Adamski, N. Soranzo, and C. Gieger. 2011. Human metabolic individuality in biomedical and pharmaceutical research. Nature 477:54-60.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R. G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLOS ONE 6:e27225.

Sunkara, L. T., W. Jiang, and G. Zhang. 2012. Modulation of antimicrobial host defense peptide gene expression by free fatty acids. PLOS ONE 7:e49558.

Sunkara, L. T., X. Zeng, A. R. Curtis, and G. Zhang. 2014. Cyclic AMP synergizes with butyrate in promoting beta-defensin 9 expression in chickens. Mol. Immunol. 57:171-180.

Tabuchi, Y., I. Takasaki, T. Doi, Y. Ishii, H. Sakai, and T. Kondo. 2006. Genetic networks responsive to sodium butyrate in colonic epithelial cells. FEBS Lett 580:3035-3041. doi 10.1016/j.febslet.2006.04.048

Takebe, K., J. Nio, M. Morimatsu, S.-I. Karaki, A. Kuwahara, I. Kato, and T. Iwanaga. 2005. Histochemical demonstration of a Na+ coupled transporter for short-chain fatty acids (SLC5A8) in the intestine and kidney of the mouse. Biomed. Res. 26:213-221.

Tappenden, K. A., L. A. Drozdowski, A. B. Thomson, and M. I. McBurney. 1998. Short-chain fatty acid-supplemented total parenteral nutrition alters intestinal structure, glucose transporter 2 (GLUT2) mRNA and protein, and proglucagon mRNA abundance in normal rats. Am. J. Clin. Nutr. 68:118-125.

van den Borne, J. J. G. C., M. J. W. Heetkamp, J. Buyse, and T. A. Niewold. 2015. Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. Livest. Sci. 175:96-100.

Van der Heiden, M. G., L. C. Cantley, and C. B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324:1029-1033.

Walter, A., and J. Gutknecht. 1984. Monocarboxylic acid permeation through lipid bilayer membranes. J. Membr. Biol. 77:255-264.

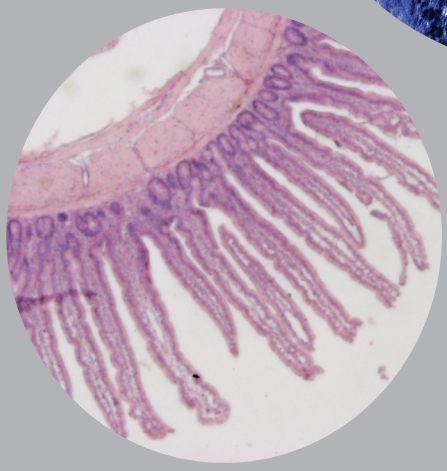
Watford, M., P. Lund, and H. A. Krebs. 1979. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589-596.

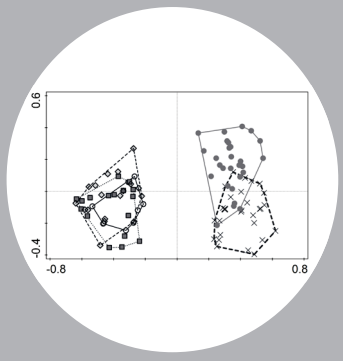
Welter, H., and R. Claus. 2008. Expression of the monocarboxylate transporter 1 (MCT1) in cells of the porcine intestine. Cell Biol. Int. 32:638-645.

Weurding, R. E., H. Enting, and M. W. Verstegen. 2003. The relation between starch digestion rate and amino acid level for broiler chickens. Poult. Sci. 82:279-284.

Yang, H., X. Wang, X. Xiong, and Y. Yin. 2016. Energy metabolism in intestinal epithelial cells during maturation along the crypt-villus axis. Sci. Rep. 6:31917.

Yin, F., H. Yu, D. Lepp, X. Shi, X. Yang, J. Hu, S. Leeson, C. Yang, S. Nie, Y. Hou, and J. Gong. 2016. Transcriptome analysis reveals regulation of gene expression for lipid catabolism in young broilers by butyrate glycerides. PLOS ONE 11:e0160751.





Chapter 6

Unprotected butyrate induces bacterial dysbiosis and inflammation in the distal gastrointestinal tract of broilers

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ABSTRACT

Dysbiosis is a condition related to an imbalanced gut microbiota composition that can negatively impact the health and growth performance of broilers. Butyrate has been shown to exert multiple effects on host-microbiota interactions in distinct gastrointestinal tract (**GIT**) segments which may influence the onset of dysbiosis. An experiment was conducted to study the effects of providing butyrate to distinct GIT segments on cecal microbiota composition and gut health of broilers. A total of 320 male day-old Ross 308 broilers were randomly assigned to 5 dietary treatment groups, each replicated 8 times: one control group and four groups supplemented with 1 g/kg butyrate in the form of feed additives having markedly different butyrate release profiles. Diets were based on rapeseed meal, corn and wheat and were provided *ad libitum*. At 22-23 d of age, birds were sacrificed. Cecal contents of 5 birds per pen were analysed for microbiota composition with Illumina HiSeq analysis of 16S ribosomal RNA gene fragments. Ileal and colonic tissues of one bird per pen were analysed for gut health markers by RT-qPCR. Unprotected butyrate, which is mostly active in the crop, proventriculus and gizzard, promoted cecal microbiota dysbiosis (e.g. decreased microbial alpha diversity and increased *Proteobacteria* relative abundance). This was associated with activation of TLR-4 and inflammation in the distal GIT. Feeding tributyrin, which increases selectively butyrate concentration in the small intestine, did not induce bacterial dysbiosis in the ceca *per se*, and yet promoted inflammation in the distal GIT. Fat-coated butyrate, which has a sustained release profile with partial protection of butyrate against gastric and enteric absorption, did not significantly modify cecal microbiota composition and did not induce inflammation in the distal GIT. Observed changes in microbiota composition may be attributable to observed modulatory effect of butyrate on the expression of host defense peptides and mucus genes.

Key words: Butyrate, broiler, cecal microbiota, dysbiosis, inflammation, location effect

INTRODUCTION

The avian gastrointestinal tract (**GIT**) harbours a complex microbiota that influences host nutrition and health (Pan and Yu, 2014). A balanced and stable gut microbiota provides the bird with additional nutrients such as short chain fatty acids (**SCFA**) as well as protection against pathogens through a competitive exclusion process (Gabriel *et al.*, 2006). Harmful alterations in gut microbiota composition -dysbiosis- may, however, arise in modern broiler production as a result of withdrawal of antimicrobial growth promoters, changes in diet composition or environmentally induced stress (Teirlynck *et al.*, 2009a,b; Teirlynck *et al.*, 2011; Wideman *et al.*, 2012). Dysbiosis has been associated with inflammation and reduced health in poultry (Kogut, 2013). This condition negatively affects the digestibility and utilization of nutrients, resulting in a reduction in growth performance of broilers (Kogut, 2013; Apajalahti and Vienola, 2016). As a consequence, establishing a balanced and stable gut microbiota is a key objective to ensure health and growth efficiency in modern broiler production.

Bacteria have to contend with numerous selection mechanisms exerted by the host in order to successfully colonize the GIT (Ley *et al.*, 2006). In poultry, such selection mechanisms include, among others, the secretion of mucus, digestive enzymes and host defence peptides (**HDP**) by the gut mucosa (Pan and Yu, 2014; Lee *et al.*, 2016). Such secretions are controlled by a succession of feedback loops between the host and the microbiota (Kogut, 2013; Pedroso and Lee, 2014). This crosstalk is thought to favour the onset of a healthy steady state (Lozupone *et al.*, 2012) and mediated at the mucosal level by microbe-associated molecular patterns as well as microbial metabolites such as SCFA (Wells *et al.*, 2011).

Among SCFA, butyrate has received much attention from poultry nutritionists. This molecule is commonly used as a feed additive to support intestinal health and enhance growth performance of broiler chickens (Moquet *et al.*, 2016). Supplementing broiler diets with butyrate has been reported to modulate cecal microbiota composition (Qaisrani, 2014; Bortoluzzi *et al.*, 2017;). Different underlying mechanisms may explain the effect of butyrate presence in the GIT on cecal microbiota composition. Firstly, butyrate is known to modulate the production of HDP and mucus in the GIT of birds and mammals (Mentschel and Claus, 2003; Sunkara *et al.*, 2011). Secondly, butyrate modifies digestive processes in the proximal GIT segments of birds, thereby affecting the substrate left for microbial growth in the distal GIT (Moquet *et al.*, 2018). The aforementioned effects of butyrate may influence the settlement of the microbiota in the ceca and, consequently, the occurrence of dysbiosis in poultry.

Until recently, it was unclear whether the presence of butyrate in the digesta of different segments of the GIT would differentially affect digestion and intestinal health. A recent study in our laboratory, however, showed that butyrate additives with markedly different release profiles differentially influenced growth performance and digestive processes of young broiler chickens (Moquet *et al.*, 2018). The present study is an extension of the previous work using cecal contents and gut tissues collected during the latter experiment to address a three-fold objective. The first objective was to investigate the effect of butyrate presence in the digesta of distinct GIT segments on cecal microbiota composition. The second objective was to investigate whether changes

in cecal microbiota composition may be associated with changes in inflammation and gut integrity markers in the distal GIT. The third objective was to relate observed changes in cecal microbiota composition to possible causative factors (e.g. SCFA concentration across the GIT, nutrient digestibility or passage rate) using exploratory multivariate analysis. It was hypothesised that supplementing broiler's diet with butyrate would modulate cecal microbiota composition and inflammation in the distal GIT. In addition, it was hypothesised that such effects would differ depending on the type of butyrate additive used.

MATERIAL AND METHODS

Experimental design

The experiment described in the present manuscript was part of a larger study published previously (Moquet *et al.*, 2018). The study investigated the effect of providing butyrate to distinct GIT segments on pre-cecal protein digestibility in broilers. A total of 320 male day-old Ross 308 broilers were randomly assigned to five dietary treatment groups: 1) control (**CTR**; no butyrate), 2) unprotected butyrate (**UP;** activity in the crop and gastric regions), 3) tributyrin (**TB;** activity in the small intestine), 4) fat-coated butyrate (**FCB;** activity in whole GIT) and 5) a 50:50 mixture of unprotected butyrate and tributyrin (**UPTB;** butyrate $C_4H_7O_2^-$ weight basis), each replicated 8 times. Butyrate was included in a rapeseed meal based diet at 1 g/kg, as fed basis. Ingredient and analyzed nutrient compositions of the diets are provided in Chapter 4, Table 1.

Performance parameters were monitored on a weekly basis at a pen level during the first three weeks of the experiment. At 22 and 23 d of age, five birds per pen were selected in such a manner that the average bodyweight (**BW**) of the selected birds was close to the average pen weight, and sacrificed. Dissections were staggered in such a manner that half of the birds in a pen was dissected on d 22 and the other half on d 23. Cecal contents of four birds per pen were collected by gentle squeezing, snap-frozen in liquid nitrogen and stored at - 20 ^{0}C pending further analysis. From the bird having its BW the closest to the pen average, the following GIT tissue segments were sampled for gene expression analysis: proventriculus, pylorus, middle of the duodenum (halfway between the pyloric junction and the hepatopancreatic duct), proximal ileum (first third between Meckel's diverticulum to the ileocecal valve) and middle of the colon (halfway between the ileocecal junction and the cloaca). Collected tissues were gently rinsed with ice cold 0.9% NaCl, placed in cryogenic vials, snap frozen in liquid nitrogen and stored at -80°C until further processing. In addition and as reported by Moquet and coworkers (2018), the relative weights of GIT segments, the average villi length and crypt depth in the duodenum and jejunum, the proteolytic activity in the proventriculus and jejunum and the apparent ileal digestibility of amino acids were measured. Remaining birds were euthanized and dissected at 24 d of age for the determination of SCFA in the digesta of different GIT compartments and for the determination of digesta retention time. The data presented by Moquet and coworkers (2018) were related to microbiota composition and gene expression data reported here.

Cecal microbiota composition

Microbial DNA extraction and purification. Total bacterial DNA was extracted by mixing 0.25 g of cecal content with 700 µL Stool Transport and Recovery lysis buffer (S.T.A.R. buffer, Roche Diagnostics Corporation, Indianapolis, IN), 0.5 g of 0.1 mm sterilized zirconia beads and 5, 2.5 mm sterilized glass beads in a sterile microfuge tube. Samples were homogenized in a FastPrep (MP Biomedicals, Santa Ana, CA) at 5.5 m·s-1 for 3 cycles of 1 min each, then shaken at 100 rpm for 15 min at 95 ºC and subsequently centrifuged for 5 min at 20,000 × g at 4 ºC. The supernatant was mixed with 300 µL S.T.A.R. buffer and subjected again to the aforementioned homogenization, shaking and centrifugation procedures. Bacterial DNA was purified from 250 µL of the final supernatant using a Maxwell 16® Total DNA system (Promega, Madison, WI) and associated purification kit (AS1220, Promega) according to the manufacturer's recommendations. Total nucleic acid concentration and purity were determined by optical density measurement using a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and subsequently adjusted to 20 ng·µL-1 with nuclease free water (Qiagen, Hilden, Germany).

PCR amplification and sequencing. For the 16S rRNA gene-based microbial composition profiling, barcoded amplicons from the V4 region of the 16S rRNA were generated using a barcoded primer PCR method. The PCR was performed in a total volume of 50 µL containing 20 ng of template DNA, 1 µL of 10 µM 515F (5´-GTGYCAGCMGCCGCGGTAA-3´) and 806R (5´-GGACTACNNGGGTATC TAAT-3´) barcoded-primers (10 µM/reaction), 10 µL of 5 × HF buffer (Finnzymes, Vantaa, Finland), 1 µL of a 10 µM dNTPs Mix (Roche Dianostics GmbH, Mannheim, Germany), 1 U Phusion® Hot start II DNA polymerase (Finnzymes, Vantaa, Finland) and 36.5 µL DNAse and RNAse free water (Qiagen). Thermocycling conditions included an initial denaturation step of 30 s at 98°C followed by 25 cycles consisting each of a 10 s long denaturation step at 98°C, followed by 20 s of annealing at 56°C and 20 s of final elongation at 72°C. The procedure was completed by a final extension step of 10 min at 72°C. The presence of PCR products was confirmed by agarose gel electrophoresis using the Lonza FlashGel® System (Lonza Group AG, Basel, Switzerland). High Pure PCR cleanup micro kit (MagBio Genomics, Alphen aan den Rijn, The Netherlands) was used for the purification of barcoded amplicons. Barcoded amplicons were quantified using the Qubit® dsDNA BR Assay Kit (Life Technologies, Leusden, Netherlands) and 70 samples, each labeled with a unique barcode, were equimolarly pooled. Pooled libraries were sequenced at GATC-Biotech AG (Konstanz, Germany) by Illumina Hiseq sequencing.

Sequence analysis. The 16S rRNA gene sequencing data was processed and analysed using the NG-Tax analysis pipeline (Ramiro-Garcia *et al.*, 2016). Libraries were filtered to contain only read pairs with matching barcodes used to separate reads per sample. Operational taxonomic units (OTU) were assigned using SILVA 111 16S rRNA database (Quast *et al.*, 2013). Alpha diversity was estimated based on the phylogenetic diversity (PD) whole tree metric using QIIME (Lozupone and Knight, 2005; Caporaso *et al.*, 2010). Rarefaction curves were constructed per dietary treatment group for PD whole tree index. Rarefaction curves reached a plateau at around 2000 reads for the PD whole tree

diversity index, thereby indicating that a cut-off point of 2500 reads per sample was sound (data not shown).

Analysis of gene expression at gut tissue level

Rationale for the selection of tissues. There was a twofold objective for the gene expression analysis of gut tissue samples. The first was to determine the effect of cecal microbiota composition on gut health in the distal GIT. It was, therefore, decided to compare the expression of genes related to immune system homeostasis, gut integrity, and oxidative stress metabolism in the ileum and colon of birds fed the CTR, UP, TB and FCB diets. It was decided to leave UPTB out of the analysis because preliminary observations indicated that feeding UP or UPTB diets resulted in similar microbiota compositions. The second objective was to determine the direct effect of butyrate presence in different GIT segment on the expression of mucus and host-defence peptides associated genes.

Table 1. Effect of dietary treatments on butyrate concentration expressed as a percentage of the control (%; µmol/g DM basis) in the digesta of different gastrointestinal segments of broilers at 24 d of age
Data adapted from Moquet and coworkers (2018)

Location	Dietary treatment means[1]				Diet effect
	UP	TB	FCB	UPTB	
Crop	1033[a]	822[a]	878[a]	1067[a]	**<0.001**
Proventriculus and gizzard	1472[a]	1031[b]	717[b]	962[b]	**<0.001**
Small intestine	13[b]	477[a]	29[b]	371[a]	**<0.001**
Colon and ceca	4	7	38	26	0.676

[1]Dietary treatments groups: UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg on an as is basis. Values are means of 15 birds per treatment.
[a,b]Means within a row having a superscript differ significantly from the control (no butyrate supplementation). Means within a row lacking a common superscript differ significantly (P<0.05).

For each GIT segment sampled, it was decided to compare the control (without added butyrate) to the butyrate-supplemented diet that resulted in the largest relative increase in digesta butyrate concentration (Table 1). Consequently, the following treatment groups were selected for comparison: CTR and UP for the proventriculus and pylor, CTR and TB for the duodenum, and CTR and FCB for the colon. For the distal part of the small intestine (ileum) it was decided to compare CTR and FCB, despite CTR and TB presenting a larger contrast in butyrate concentration in pooled intestinal digesta because TB is known to release butyrate in the very proximal part of the small intestine in pigs (Schwarzer and Bjork, 2015) and of poultry (Moquet, unpublished results) while FCB is known to extend the effect of butyrate to the entire small intestine in chickens (van den Borne *et al.*, 2015; Moquet *et al.*, 2018).

RNA extraction, reverse transcription and quantitative PCR. Total RNA was extracted from 1-2 mg fraction of ground tissue using Trizol reagent (ThermoFisher Scientific, Bleiswijk, Netherlands). Isolated RNA was subsequently subjected to an on-column DNase digestion to eliminate possible DNA contamination (NucleoSpin RNA II kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total nucleic acid concentration and purity were determined by optical density measurement using a NanoDrop ND-1000

(Thermofisher Scientific) while RNA integrity and size was assessed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip kit (Agilent, Santa Clara, California). The average RNA integrity number value was 9.9, ranging between 9.7 and 10. First-strand cDNA synthesis was performed with 250 ng of total RNA per 20-mL sample reaction with Superscript III reverse transcriptase (Thermofischer Scientific), deoxyribonucleotide triphosphate (Roche Diagnostics, the Netherlands), and random hexamer primers (Roche Diagnostics, the Netherlands) for 1 h at 50 °C, according to the manufacturer's protocol (Thermofischer Scientific). Real-time quantitative PCR was carried out with a ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, California) by using the SensiMix SYBR Low-ROX mix (Bioline UK Ltd., London, UK). Amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 35 s each. A final melting protocol of increasing temperature from 60°C to 95°C with 0.5°C increments of 5 s each was applied.

Primers were designed with Primer Express Software (ThermoFisher Scientific, Bleiswijk, Netherlands), and recommended primers sets spanning at least one intron were selected (Table 2). Messenger RNA was quantified by establishing a linear calibration using 10-fold serial dilutions of cDNA templates for corresponding genes. We designed primers for 5 genes, commonly used as reference genes in mammalian species, namely ACTB, EFF2, IPO8, PPIA and RPLPO. Analysis of the measured values with the Normfinder algorithm (Andersen *et al.*, 2004) revealed that IPO8 was the most suitable housekeeping gene across locations and diets.

Table 2. Primers used for real-time quantitative PCR

Gene[1]	Forward primer (5'-3')	Reverse primer (5'-3')
Normalization genes		
IPO8	ACCTCCGAGCTAGATCCTGT	GGCTCTTCTTCGCCAACTCT
ACTB	GCCCTGGCACCTAGCACAAT	GCGGTGGACAATGGAGGGT
RPLPO	TTGGGCATCACCACAAAGATT	CCCACTTTGTCTCCGGTCTTAA
PPIA	CCCGTCGTGTTCTTCGACAT	CCCTTGTAGCCAAATCCCTTCT
EEF2	CAGTTGGCTTTGGTTCTGGC	AAAGTATCTGTCTCCCCACAGC
Pathogen recognition receptors and associated signal transducing adaptor protein		
Dectin-1	GTCTGCAATGCGGGAAGAAC	TCAGCTGAGAGCTGCGTATC
TLR-2	CCAGGGAATGGTTTCTGCAC	CTCAGGGCTTGTTCTTCAGGG
TLR-4	GCTGAAATCCCAAACACCACC	TATGGATGTGGCACCTTGAAAGA
MyD88	AAAGAAGGTGTCGGAGGATGG	GAATCAGCCGCTTGAGACGA
Cell signalling molecules		
IL-10	GCTGAGGGTGAAGTTTGAGGA	TCTGTGTAGAAGCGCAGCAT
IL-12b	CCCAGATGCTGGCAACTACA	GAACGTCTTGCTTGGCTCTTT
IL-1b	GACATCTTCGACATCAACCAG	CCGCTCATCACACACGACAT
IL-6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT
COX2	ATTCCTGACCCACAAGGCAC	AGTCAACCCCATGGCCGTAA
Tight junction proteins		
CDH1	GGCAAGCCGTTTACCACATC	CATAATCCAGGCCCTTGGCTG
CLD3	TATGGGGCTGGAGATCGGT	ACCACGCAGTTCATCCACAG
JAM2	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA
ZO1	CCGCAGTCGTTCACGATCT	GGAGAATGTCTGGAATGGTCTGA

[1]*IPO8*: Importin 8; *ACTB*: Actin beta; *RPLP0*: 60S acidic ribosomal protein P0; *PPIA*: Peptidylprolyl Isomerase A; *EEF2*: Eukaryotic elongation factor 2; *TLR*: Toll-like receptor; *MyD88*: Myeloid differentiation primary response 88; *IL*: Interleukin; *b*: Beta; *COX2*: Cyclooxigenase 2; *CDH1*: Cadherin 1; *CLD3*: Claudin 3; *JAM2*: Junctional adhesion molecule 2; *ZO1*: Zona occludens 1.

Table 2. (Continued)

Gene[1]	Forward primer (5'-3')	Reverse primer (5'-3')
Reactive oxygen species metabolism		
CAT	GCCACATGGTGACTACCCTC	TGTTGCTAGGGTCATACGCC
NOS2	CTACCAGGTGGATGCATGGAA	ATGACGCCAAGAGTACAGCC
Host defence peptides		
AvBD1	ACCATTGTCAGCCCTGTGAAAA	CCCATATTCTTTTGCAGCAGAGGT
AvBD4	CATCTCAGTGTCGTTTCTCTGC	ACAATGGTTCCCCAAATCCAAC
AvBD5	CTGCCAGCAAGAAAGGAACCTG	TGAACGTGAAGGGACATCAGAG
AvBD6	AGGATTTCACATCCCAGCCGTG	CAGGAGAAGCCAGTGAGTCATC
AvBD7	CTGCTGTCTGTCCTCTTTGTGG	CATTTGGTAGATGCAGGAAGGA
AvBD10	TGGGGCACGCAGTCCACAAC	ATCAGCTCCTCAAGGCAGTG
AvBD12	CCCAGCAGGACCAAAGCAATG	GTGAATCCACAGCCAATGAGAG
CATH3	GCTGTGGACTCCTACAACCAAC	TGGCTTTGTAGAGGTTGATGC
Mucus		
MUC2	TGACATCAGGGCACACAGAT	ATTGAAGCCAGCAATGGTGT
MUC4	GACTTCAAGGTTTGGCAGCA	TGGCACTCACATGTTCCTCT
MUC5ac	AACCAAGCTCTGTGACAGCAA	GTTTGCCCTCCGCTTAGACT
MUC5b	AGGCGTACACTGGTGGAAAA	GGCAGTTGAACTCTGTTGCTG
MUC13	TCAGTAAGAGCCAAGCACAGT	GGTCTGGACTCTGGGGAAGA

[1]*CAT*: Catalase; *NOS2*: Nitric oxide synthase 2; *AvBD*: Avian beta defensin; *CATH3*: Cathelicidin 3; *MUC*: Mucin

Statistical analysis

Microbial alpha diversity index. PD whole tree index was analyzed using the PROC MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC), using the following model:

$$Y_{ijk} = \mu + D_i + P_j + \varepsilon_{ijk}$$

where Y_{ijk} is the observed response of the k^{th} chicken (k = 1 to 4) fed the i^{th} diet (i = CTR, UP, TB, FCB or UPTB) in the j^{th} pen (j = 1 to 8), μ is the overall mean response, D_i is the i^{th} fixed diet effect, P_j is the random pen effect and ε_{ijk} is the residual error term. Means were separated using Tukey's HSD.

Microbiota composition. The part of variation in microbiota composition attributable to the dietary treatments was visualized at both phylum and genus levels by redundancy analysis (RDA) in Canoco 5 (Microcomputer Power, Itahaca, NY). Differences were tested with an F-test. The effect of dietary intervention on cecal microbiota composition was further investigated at phylum and genus levels using permutation tests for the univariate analysis of variance. Tests were carried out using the lmPerm package of R (version 3.4.0; R Core Team, 2017). Means were separated using Tukey's HSD test when a significant diet effect was detected. Finally, the variation in microbiota composition at phyla level was summarized by principle component analysis (PCA) in Canoco 5. Functional traits measured in the experiment were used as supplementary variables to interpret the PCA summary. Functional traits were grouped in categories (Table 3).

Table 3. Categories of functional traits used to interpret the PCA summary of the variation in microbiota species at phylum level

Category	Traits	Unit	Observation levels
Performance	Slaughter weight	G	Individual
	FCR	g:g	Pen
Nutrient digestibility	N apparent ileal digestibility (AID)	%	Pen
	N-corrected OM AID	%	Pen
GIT[2] development	*Crop*, relative weight	% BW	Individual
	Proventriculus, relative weight	% BW	Individual
	Gizzard, relative weight	% BW	Individual
	Duodenum		
	Relative weight	% BW	Individual
	Villus length	mm	Individual
	Crypt depth	mm	Individual
	Villus length:Crypt depth ratio	-	Individual
	Jejunum		
	Relative weight	% BW	Individual
	Villus length	mm	Individual
	Crypt depth	mm	Individual
	Villus length:Crypt depth ratio	-	Individual
	Ileum, relative weight	% BW	Individual
	Colon, relative weight	% BW	Individual
	Ceca, relative weight	% BW	Individual
SCFA[1]	*Crop*	mmol/g DM	Treatment group
	Proventriculus and gizzard	mmol/g DM	Treatment group
	Small intestine	mmol/g DM	Treatment group
	Colon and ceca	mmol/g DM	Treatment group
Passage rate	*Crop*	min	Treatment group
	Proventriculus and gizzard	min	Treatment group
	Small intestine	min	Treatment group
	Colon and ceca	min	Treatment group

[1]Gastrointestinal tract
[2]Short chain fatty acids: acetate, propionate, butyrate, iso-butyrate and iso-valerate concentrations in digesta.

Gene expression data. Differences in the expression of genes related to tight junctions, innate immune system and oxidative stress were tested at ileal and colonic levels using Kruskal-Wallis test (4 treatment groups per location). Means were separated using Tukey's HSD test when a significant diet effect was detected. Differences in the expression of mucus and host-defence peptide genes were tested for each location using Mann-Whitney U test (2 treatment groups per location). All the tests were carried out using the PROC NPAR1WAY procedure of SAS 9.4.

RESULTS AND DISCUSSION

Feeding unprotected butyrate induces cecal microbiota dysbiosis.

Microbial alpha diversity and biomarkers of dysbiosis at phylum level. PD, an alpha diversity index, was significantly affected by dietary intervention ($P<0.001$; Table 4). Post-hoc analysis revealed that birds fed the UP and UPTB diets had significantly lower PD indices than their CTR, TB and FCB fed counterparts.

Table 4. Effects of dietary treatments[1] on cecal microbiota phylogenetic diversity and composition at phylum level of 22-23 d old broilers

	Dietary treatment					Pooled	Diet
	CTR	UP	TB	FCB	UPTB	SD	effect
Alpha diversity index							
PD[2]	4.8[a]	4.4[b]	5.0[a]	4.9[a]	4.4[b]	0.1	**<0.001**
Composition at phylum level (%)							
Actinobacteria	2.0	1.1	1.5	1.4	1.6	0.4	0.405
Bacteroidetes	8.3[ab]	6.5[b]	9.8[a]	7.8[ab]	7.4[ab]	0.8	**0.048**
Cyanobacteria	0.6	1.2	0.5	0.8	0.7	0.3	0.436
Firmicutes	86.4	86.1	84.8	86.5	85.9	1.0	0.777
Proteobacteria	0.5[c]	1.9[a]	0.9[bc]	0.6[bc]	1.4[ab]	0.2	**<0.001**
Tenericutes	1.7	1.9	1.8	2.0	1.6	0.3	0.885
Unknown	0.6[b]	1.3[a]	0.7[b]	0.8[b]	1.4[a]	0.1	**<0.001**

[1]Dietary treatments groups: CTR (Control, no butyrate added); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg on an as in basis. Values are means of a minimum of 30 birds per treatment.

[2]Phylogenetic diversity.

[a,b,c]Means within a row lacking a common superscript differ significantly (P<0.05).

Redundancy analysis indicated that dietary intervention had a significant effect on cecal microbiota composition at the phylum level (P=0.002, Figure 1, panel A) and accounted for 6.9% of the total observed variation. Plots derived from the RDA indicated that chickens could be grouped in two separate clusters based on the presence (UP, UPTB) or absence of unprotected butyrate in the diets (CTR, TB, FCB).

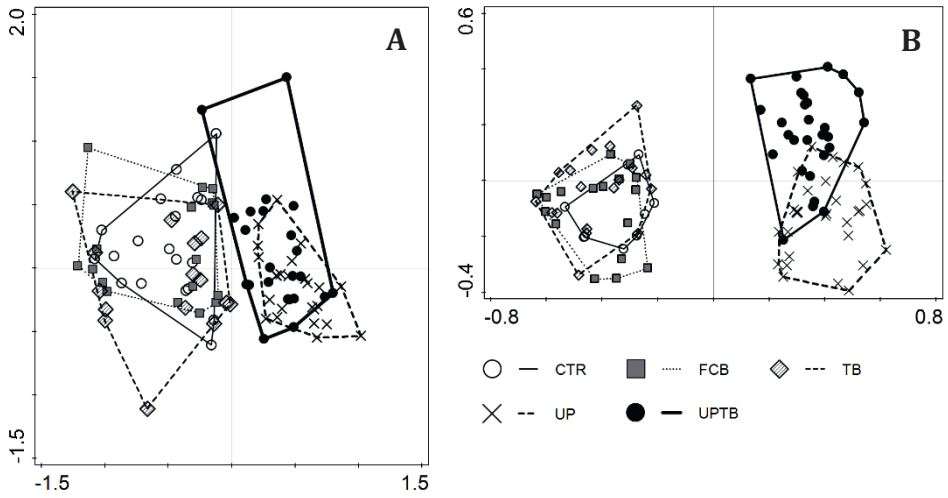

Figure 1. Correlation biplot based on a redundancy analysis depicting the relationship between the cecal microbiota composition at phylum (panel A) and genus (panel B) levels of 22-23 d old broilers and diets differing solely by the type of butyrate additive used (CTR: none, FCB: fat-coated butyrate, TB: tributyrin, UP: unprotected butyrate, UPTB: mixture of unprotected butyrate and tributyrin)

Prevalence of *Bacteroidetes* and *Proteobacteria* were significantly affected by dietary intervention (P=0.048, P<0.001 and P<0.001, respectively; Table 4). Post-hoc analysis indicated that *Proteobacteria* relative abundance was significantly higher in

birds fed UP diets than in birds fed CTR diets. Birds fed the UP diets had, in addition, a significantly lower *Bacteroidetes* relative abundance than their TB fed counterparts.

Increased prevalence of the *Proteobacteria* phylum is a consistent hallmark of dysbiosis in adult mice fed high-fat diets (Geurts *et al.*, 2011; Zhang *et al.*, 2012; Everard *et al.*, 2014). Similar results have been reported in obese human adults (Larsen *et al.*, 2010; Verdam *et al.*, 2013; Zhang *et al.*, 2013). Such consistent findings have led Shin and colleagues (2015) to suggest using *Proteobacteria* prevalence in the gut as a diagnostic tool for dysbiosis. Other biomarkers of dysbiosis have been reported in the literature, such as loss of microbiota diversity in poultry (Kogut, 2013). Our results indicate that birds fed the UP diet displayed all the aforementioned signs of dysbiosis.

Biomarkers of dysbiosis and of changes in hydrogen metabolism at genus level. Redundancy analysis indicated that dietary intervention had a significant effect on microbiota composition at a genus level (P=0.002) and explained 8.6% of the total observed variation (Figure 1, panel B). Plots derived from the RDA indicated that chickens could be grouped in two separate clusters based on the presence (UP, UPTB) or absence of unprotected butyrate in the diets (CTR, TB, FCB), in line with the separation observed when using phylum-level data as described above (Figure 1, panel B). Dietary intervention significantly influenced the relative abundance of 25 genera out of the 66 detected (Table 5). Interestingly, UP suppressed the strictly anaerobic *Alistipes* genus while promoting facultative anaerobes belonging to the *Escherichia-Shigella* group. Such changes have been associated with gut inflammation and dysbiosis in both mammals and avian species (Kogut, 2013; Verdam *et al.*, 2013; Jiang *et al.,* 2015).

Butyrate production and hydrogen removal are microbial activities that are restrictively performed in the gut by well-defined sets of bacterial families. Butyrate production is of importance as this molecule seems to be a key inter-kingdom signalling molecule with pleiotropic effects in both mammals (Guilloteau *et al.*, 2010) and poultry (Moquet *et al.*, 2016). Hydrogen removal is also of importance as it conditions the availability of oxidized pyridine nucleotides and, consequently, the progression of saccharolytic fermentation (Cummings and Branch, 1986).

Butyrate production is realized by members of the *Ruminococcaceae* and *Lachnospiraceae* families in broilers (Onrust *et al.*, 2015). Feeding UP had mixed effects on the relative abundance of these two families. Our results show that *Ruminococcaceae anaerotroncus* were suppressed while *Lachnospiraceae copprococus, Ruminococcaceae anaerofilum, R. incertae sedis* and *R. oscillospira* were promoted (Table 6). Overall, dietary intervention did not significantly affect the total relative abundance of *Ruminococcaceae* and *Lachnospiraceae* families in the present experiment (P=0.533 and P=0.364; respectively). As a consequence, it is difficult to infer any clear effect of UP on the butyrogenic capacity of the cecal microflora.

Table 5. Effects of dietary intervention[1] on cecal microbiota composition at genus level of 22-23 d old broilers

Order	Family	Genus	Average relative contribution[3] (%)					Diet effect
			CTR	UP	TB	FCB	UPTB	
Anaeroplasmatales	Anaeroplasmataceae	*Anaeroplasma*	0.22^{a}	0.01^{b}	0.37^{a}	0.26^{a}	0.01^{b}	**>0.001**
Bacteroidales	Rikenellaceae	*Alistipes*	8.15^{ab}	6.34^{b}	9.81^{a}	7.73^{ab}	7.34^{b}	**0.015**
Clostridiales	Christensenellaceae	N.D.[2]	0.73^{bc}	0.52^{c}	1.53^{a}	1.04^{b}	0.74^{bc}	**0.004**
Clostridiales	Eubacteriaceae	*Anaerofustis*	0.02^{a}	0.00^{b}	0.01^{ab}	0.01^{ab}	0.00^{b}	***0.081***
Clostridiales	Family XIII Incertae Sedis	N.D.	0.08^{bc}	0.00^{d}	0.13^{a}	0.10^{ab}	0.04^{cd}	**>0.001**
Clostridiales	Lachnospiraceae	*Blautia*	4.40^{b}	5.83^{ab}	4.21^{b}	3.87^{b}	7.41^{a}	**0.018**
Clostridiales	Lachnospiraceae	*Coprococcus*	0.38^{bc}	0.93^{a}	0.16^{c}	0.22^{bc}	0.54^{b}	**>0.001**
Clostridiales	Lachnospiraceae	N.D.	11.28^{a}	8.20^{c}	11.18^{ab}	10.98^{ab}	9.45^{bc}	**0.001**
Clostridiales	Lachnospiraceae	*Shuttleworthia*	0.18^{b}	0.17^{bc}	0.10^{cd}	0.09^{d}	0.25^{a}	**>0.001**
Clostridiales	N.D.	N.D.	1.21^{c}	3.04^{a}	2.34^{b}	1.88^{bc}	2.29^{b}	**>0.001**
Clostridiales	Peptococcaceae	N.D.	0.04^{a}	0.00^{b}	0.03^{a}	0.02^{ab}	0.00^{b}	**0.026**
Clostridiales	Peptostreptococcaceae	*Incertae Sedis*	0.33^{bc}	0.01^{c}	1.13^{a}	0.48^{b}	0.01^{c}	**>0.001**
Clostridiales	Ruminococcaceae	*Anaerofilum*	0.02^{b}	0.06^{a}	0.01^{b}	0.02^{b}	0.00^{b}	**0.007**
Clostridiales	Ruminococcaceae	*Anaerotruncus*	1.82^{a}	1.24^{b}	2.03^{a}	1.84^{a}	1.80^{a}	**>0.001**
Clostridiales	Ruminococcaceae	N.D.	11.95^{a}	8.98^{b}	12.38^{a}	12.02^{a}	9.13^{b}	**>0.001**
Clostridiales	Ruminococcaceae	*Oscillospira*	0.00^{b}	0.07^{a}	0.00^{b}	0.01^{b}	0.02^{b}	**>0.001**
Clostridiales	Uncultured	N.D.	1.47^{b}	2.60^{a}	2.26^{ab}	1.59^{b}	1.98^{ab}	**0.026**
Coriobacteriales	N.D.	N.D.	1.55^{a}	0.55^{c}	1.17^{ab}	1.09^{bc}	0.94^{bc}	**0.002**
Desulfovibrionales	N.D.	N.D.	0.00^{b}	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	**0.004**
Enterobacteriales	Enterobacteriaceae	*Escherichia-Shigella*	0.44^{c}	1.87^{a}	0.85^{bc}	0.57^{c}	1.31^{ab}	**>0.001**
Erysipelotrichales	Erysipelotrichaceae	*Incertae Sedis*	2.80^{ab}	2.42^{ab}	1.91^{b}	3.50^{a}	2.15^{b}	**0.042**
Erysipelotrichales	N.D.	N.D.	1.52^{a}	0.33^{c}	1.22^{ab}	1.11^{ab}	0.41^{bc}	**0.026**
Lactobacillales	Enterococcaceae	*Enterococcus*	0.02^{b}	0.17^{a}	0.06b	0.06^{b}	0.20^{a}	**>0.001**
Lactobacillales	Streptococcaceae	*Streptococcus*	0.04^{ab}	0.00^{b}	1.07^{a}	0.92^{a}	0.00^{b}	**0.001**
N.D.	N.D.	N.D.	0.65^{b}	1.32^{a}	0.67^{b}	0.83^{b}	1.38^{a}	**>0.001**

[1]Dietary treatments groups: CTR (no added butyrate); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate); UPTB (butyrate radical provided as a 50:50 w/w mixture of unprotected salt and tributyrin). Butyrate was provided at 1 g/kg as in basis. Values are means of minimum 30 birds per treatment.

[2]N.D.: Not determined.

[3]Only genera that were significantly affected by dietary treatments are presented in this table.

[a,b,c]Means within a row lacking a common superscript differ significantly (P<0.05).

Disposal of hydrogen in the hindgut through dissimilatory sulfate reduction is restrictively performed by bacteria belonging to the *Desulfobacterales* and *Desulfovibrionales* orders (Gibson *et al.*, 1993). The former was not detected in the present experiment while the latter was only associated with the UP diet. Feeding the UPTB diet, and, to a lesser extent the UP diet, increased the relative abundance of *Blautia*. This genus contains several species such as *Blautia hydrogenotrophica* (formerly called *Ruminococcus hydrogenotrophicus*) which are associated with hydrogenotrophic acetogenesis (i.e. Wood-Ljungdahl pathway) (Bernalier *et al.*, 1996; Liu *et al.*, 2008). Collectively, our results suggest that the relative abundance of microbial groups known for their ability to perform dissimilatory sulfate reduction and acetogenesis in cecal hydrogen removal was increased when UP was added to the diet. Dissimilatory sulphate reduction results in the production of hydrogen sulphide (**H_2S**) (Carbonero *et al.*, 2012). This metabolite inhibits butyrate oxidation in colonocytes (Pitcher *et al.*, 2000) and was, therefore, initially considered to be a toxic metabolite (Pitcher and Cummings, 1996). Later work indicated that low levels of H_2S could also support gut tissue repair, thereby, illustrating the dual nature of H_2S (Wallace *et al.*, 2007a,b). It is, therefore, difficult to state whether the presence of *Desulfovibrionales* in the ceca of birds fed TB was harmful or beneficial to the health of the host. Increased acetogenesis by member of the *Blautia* genus may, on the other hand, benefit the host by increasing energy harvest.

Feeding unprotected butyrate is associated with inflammation in the distal GIT

We reported previously that increasing digesta butyrate concentration in the gastric region of broilers, e.g. by feeding unprotected butyrate, resulted in multiple signs of intestinal microbiota overgrowth, e.g. reduced intestinal weight, impaired AA digestibility and skewed intestinal SCFA profile (Moquet *et al.*, 2018). Here, we quantified the expression of genes related to immune system homeostasis, gut integrity and oxidative stress metabolism in the ileum and colon to evaluate the intestinal health of birds fed different the control diet and the different butyrate additives (Table 6).

TLR-4 expression and inflammation. Several innate immune receptors, including Dectin-1, TLR-2 and TLR-4, are anchored at the surface of various immune cells populating the gut associated lymphoid tissue (Smith *et al.*, 2014). Fewer of these innate immune receptors (e.g. TLR-2 and TLR-4, but not Dectin-1) can also be found at the basolateral side of epithelial cells lining the gut wall (Beal *et al.*, 2006). In the present experiment, TLR-4 expression was significantly affected by dietary intervention in the ileum (P=0.006, Table 6). Interestingly, TLR-4 expression was significantly higher in birds fed the UP diet in comparison to their control counterparts for this GIT segment. A similar trend was observed in the colon (P=0.094, Table 6).

Sensing of bacterial lipopolysaccharide by TLR-4 triggers the recruitment of the adaptor protein MyD88 and, subsequently, the activation of the transcription factor NF-κB (Juul-Madsen *et al.*, 2014). Our results indicate that dietary intervention had a significant effect on MyD88 expression in the ileum and colon (P<0.001, Table 6). For these two locations, birds fed the UP and TB diets had significantly higher expression of MyD88 than birds fed the CTR and FCB diets.

Table 6. Effect of dietary treatments[1] gene expression in the ileum and colon of broilers at 22-23 d of age

	Ileum					Colon				
	Average gene expression[2]				*P*-value	Average gene expression[2]				*P*-value
	CTR	UP	TB	FCB		CTR	UP	TB	FCB	
Pathogen recognition receptors and associated signal transducing adaptor protein										
Dectin-1	59.4	44.9	63.6	72.7	0.173	100.0	62.0	63.0	66.9	***0.070***
TLR-2	60.7	64.1	75.7	100.0	0.444	59.1	69.2	73.0	55.4	0.184
TLR-4	60.3^{b}	92.6^{a}	84.2^{ab}	74.2^{ab}	**0.006**	81.2	100.0	83.7	83.9	***0.094***
MyD88	32.4^{b}	84.0^{a}	97.0^{a}	44.2^{b}	**<0.001**	42.3^{b}	100.0^{a}	84.7^{a}	42.8^{b}	**<0.001**
Cell signalling molecules										
IL-10	47.2	47.2	58.0	100.0	0.220	25.1	37.6	27.5	28.1	***0.055***
IL-12b	25.0	21.8	30.4	36.3	0.926	70.9	72.0	100.0	63.1	0.366
IL-1b	45.8	39.4	56.1	100.0	0.383	72.0	68.2	57.6	79.1	0.559
IL-6	7.7^{b}	22.0^{a}	32.4^{a}	4.9^{b}	**0.001**	50.7	87.6	100.0	66.1	***0.089***
COX2	17.2	27.9	28.3	29.0	***0.080***	74.6	100.0	88.1	81.4	0.128
Tight junction proteins										
CDH-1	55.9^{b}	64.2^{a}	71.5^{a}	51.2^{b}	**0.004**	68.9^{b}	99.0^{a}	100^{a}	71.5^{b}	**0.001**
CLD3	55.4	57.7	53.4	48.6	0.205	97.6^{a}	90.5^{a}	67.2^{b}	100.0^{a}	**0.006**
JAM2	44.0	50.0	66.5	63.4	***0.099***	89.5	100.0	91.0	90.8	0.218
ZO1	51.6^{c}	66.3^{b}	78.4^{a}	46.7^{c}	**<0.001**	56.6^{c}	88.0^{b}	100.0^{a}	57.6^{c}	**<0.001**
Reactive oxygen species metabolism										
CAT	42.7	93.8	79.3	33.6	***0.055***	17.2^{ab}	37.4^{a}	30.7^{ab}	9.6^{b}	**0.012**
NOS2	15.7^{b}	29.9^{a}	37.2^{a}	20.1^{b}	**<0.001**	32.9^{b}	100.0^{a}	74.1^{a}	34.6^{b}	**<0.001**

[1]Dietary treatments groups: CTR (no added butyrate); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.

[2]Arbitrary units. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. For each gene, the highest average gene expression across location and treatments was set to 100.

[a,b,c]Means within a row lacking a common superscript differ significantly ($P<0.05$).

In poultry, activation of the NF-κB pathway results in the release of the pro-inflammatory cytokines INF-γ, Il-1β and IL-6 (Kaiser and Stäheli, 2014). We observed that dietary intervention had a significant effect on IL-6 expression in the ileum (P=0.001, Table 6). A similar trend was observed in the colon (P=0.089, Table 6). Post-hoc analysis indicated that birds fed the UP and TB diets had significantly higher IL-6 expression in the ileum than birds fed the CTR and FCB diets. Biological functions of IL-6 are conserved across avian and mammalian species (Schneider *et al.*, 2001). The pivotal role of this pro-inflammatory cytokine in orchestrating chronic intestinal inflammation is clearly described for humans (For review, see: Mudter and Neurath, 2007; Neurath and Finotto, 2011). Specifically, IL-6 exerts anti-apoptotic effects on $CD4^+$ T-cells (Atreya *et al.*, 2000). This contributes to the accumulation of $CD4^+$ T-cells during chronic inflammatory diseases of the GIT such as Crohn's disease or ulcerative colitis in humans (Mudter and Neurath, 2007). Higher expressions of TLR-4, MyD88 and IL-6 in the ileum and colon of birds fed the UP diet confirms the pro-inflammatory effects in the ceca of broilers.

Our results also indicate that TB led to significant increases in MyD88 and IL-6 gene expression compared to the control in the ileum and colon. Such results are intriguing because there were no significant differences between CTR and TB fed birds concerning most of the cecal dysbiosis markers, e.g. *Proteobacteria* relative abundance and microbial alpha diversity. Tributyrin increases butyrate concentration in the small intestine of broilers (Moquet *et al.*, 2018). We compared the expression of the genes listed in Table 6 also in the duodenum of birds fed the CTR and TB diets to investigate the direct effect of butyrate presence on immune system regulation in the proximal intestine. These results showed that, in the duodenum, birds fed TB had lower IL-10 expression and higher COX2 expression than CTR birds (P=0.046 and P=0.002, respectively; data not shown).

Colonic expression of Dectin-1 tended to be suppressed when butyrate was added to broiler diets (P=0.070, Table 6). In addition, we observed changes in duodenal expression of both COX2, a prostaglandin-forming enzyme, and of IL-10 (data not shown), an alternatively activated macrophage cytokine, when birds were fed TB. Studies in mice indicate that reduced expression of Dectin-1, a major innate immune receptor of antifungal immunity, induces fungal dysbiosis and increases the susceptibility to colitis (Iliev *et al.*, 2012). Fungal dysbiosis increases plasma prostaglandin E2 in a manner that alters systemic immunity and, more precisely, macrophage polarization (Kim *et al.*, 2014). Fungal dysbiosis can also lead to mucosal inflammation (for review, see Iliev and Leonardi, 2017). Hence, our findings may suggest a role of fungal dysbiosis in TB-induced inflammation of the distal GIT of poultry. This should be further confirmed in future studies, adding analysis of abundance and composition of the intestinal mycobiota.

Gut integrity and oxidative stress metabolism. Interleukin-6 is known to induce a remodeling of epithelial tight junction (**TJ**) proteins assembly, which results in a leaky gut (Desai *et al.*, 2002; Yang *et al.*, 2003; Al-Sadi *et al.*, 2014). Gut integrity loss is mediated by increased expression and mislocalization of TJ proteins of the CLD and ZO families (Yang *et al.*, 2003; Al-Sadi *et al.*, 2014). Our results indicate that dietary intervention resulted in significant changes in the expression of several TJ proteins such

as CDH1, CLD3 and ZO1 at ileal or colonic level (Table 6). The UP diet was found to increase CDH1 and ZO1 expression compared to CTR in both the ileum and colon. Feeding TB resulted in a similar effect and, in addition, suppressed CLD3 expression in the colon. Hence, gut inflammation induced by IL-6 resulted in alterations of TJ proteins expression in the present experiment. Whether such alterations led to functional changes in terms of permeability of the gut was, however, not determined.

Interleukin-6 activates avian macrophages (Kaiser and Stäheli, 2014). Such activation results in the production of nitric oxide (**NO**) by the cytokine-inducible NOS2 enzyme (Klasing, 1998). We found that birds fed the UP and TB diets had significantly higher NOS2 expression in the ileum and colon than birds fed CTR or FCB diets (Table 6). Nitric oxide has a dual role in the gut. Constitutive NO production contributes to tissue homeostasis while increased NO production by cytokine-inducible NOS2 leads to oxidative stress (for review, see Barrachina *et al.,* 2001). Indeed, NO can react with superoxide to form peroxynitrite, a potent reactive nitrogen radical (Koppenol, 1998). We also found that birds fed UP and TB diets had significantly higher CAT expression in the colon than their CTR and FCB fed counterparts (P=0.012, Table 6). A similar trend was observed in the ileum (P=0.055, Table 6). The enzyme CAT catalyzes the decomposition of hydrogen peroxide, a potent reactive oxygen radical, in water and oxygen. The reaction catalyzed by CAT, therefore, protects cells against damage induced by oxidative stress. Concomitant increases in NOS2 and CAT expression indicate that the distal GIT of birds fed the UP and TB diet was subjected to oxidative stress.

Factors explaining changes in microbiota composition and growth performance

Changes in SCFA along the GIT correlate with the observed changes in microbiota. After observing that UP induced bacterial dysbiosis and inflammation in the distal GIT of broilers, we tried to unravel the mechanisms underlying the observed differences in microbiota composition. Variation in microbiota composition at phylum level was visualized using PCA. The first two PCA axes explained 40.8 and 24.9 % of the observed variation in cecal microbiota composition. Functional traits, summarized in Table 4, were used sequentially in an attempt to interpret the PCA summary.

Dietary treatment groups aligned along the second PCA axis, indicating that the relative abundance of unprotected butyrate in the UP and UPTB diets increased the relative abundance of cecal *Proteobacteria*. Butyrate additives used in the present study had markedly different release profiles (Moquet *et al.*, 2018). Unprotected butyrate is quickly absorbed in the proventriculus while TB is predominantly released in the proximal small intestine under the action of pancreatic lipase (Moquet *et al.*, 2016). Fat coated butyrate provides a partial protection against gastric and enteric absorption (van den Borne *et al.*, 2015; Moquet *et al.*, 2018). Hence, the alignment of dietary treatment groups along the second PCA axis indicates that butyrate presence in the crop and proventriculus increased cecal *Proteobacteria* relative abundance (Figure 2). This relationship was further confirmed by the use of a subset of SCFA data as supplementary variables (Figure 2). Such supplementary variables tended to be correlated with the second PCA axis (P=0.099; pseudo-canonical R^2: 0.24). Cecal *Proteobacteria* expansion was associated with the presence of butyrate in the crop and gastric regions, i.e. proventriculus and gizzard, and also with the presence of propionate in the small intestine. Reduced cecal *Proteobacteria* relative contribution was

associated with the presence of propionate in the colon and ceca. Nutrient digestibility, GIT development and passage rate data showed little relevance in interpreting the variation in microbiota composition at phylum level (data not shown).

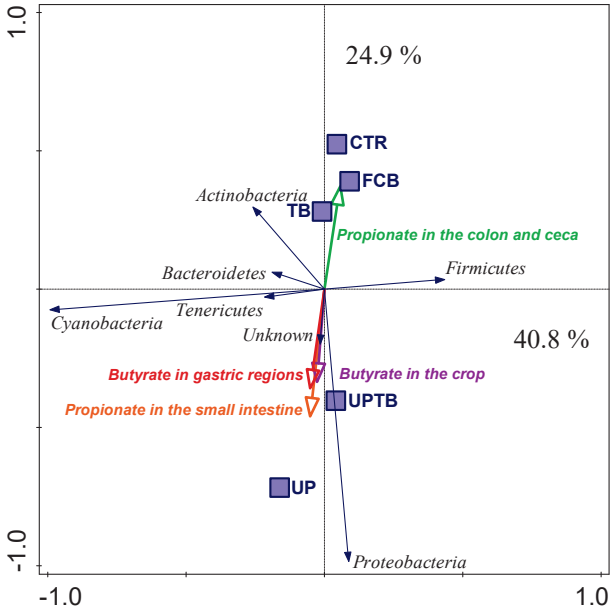

Figure 2. Correlation byplot based on principle component analysis (PCA) of the cecal microbiota composition at phylum level of 22-23 d old broilers. Axis one accounts for 40.8 % of the total variation while axis two accounts for 24.9 % of the observed variation. Interpretation of PCA axis is facilitated by short chain fatty acids data provided as supplementary variables.

Collectively, our findings demonstrate that dysbiosis induced by UP is independent from observed changes in digestive functions while being associated with changes in propionate and butyrate concentrations along the GIT. The presence of butyrate in the crop, proventriculus and gizzard may have triggered mechanisms at a mucosal level that influenced the settlement of the microbiota in the GIT. Differences in propionate concentration in the small intestine, colon and ceca may reflect resulting changes in microbiota composition.

Expression of HDP and Mucus Genes. Mucosal secretion of HDP is one of the mechanisms by which birds can affect microbiota composition in the gut (Pan and Yu, 2014). Butyrate modulates the expression of numerous avian HDP genes in both cultured HD11 macrophages and gut tissues explants of broilers (Sunkara *et al.*, 2011). We screened different GIT segments of birds (Proventriculus, pylorus, duodenum, ileum and colon) for the expression of known avian HDP and, for each GIT segment considered, we compared the expression of detectable HDP genes between the CTR diet and the butyrate-supplemented diet that resulted in the highest increase in local butyrate concentration. The results indicate that butyrate has a location-dependent effect on HDP gene expression, leading to either up- or downregulation of different HDP genes (Table 7).

Table 7. Effect of dietary treatments[1] on expression of host defence peptides and mucus genes in different gastrointestinal tract segments of broilers at 22-23 d of age

	Average gene expression[2,3]												
	Host defence peptides								Mucus				
	AvBD1	**AvBD4**	**AvBD5**	**AvBD6**	**AvBD7**	**AvBD10**	**AvBD12**	**CATH3**	**MUC2**	**MUC4**	**MUC5ac**	**MUC5b**	**MUC13**
Proventriculus													
CTR	100.0	100.0	98.4	41.0	45.0	0.5	37.7	95.0	n.d.	n.d.	89.8	100.0	2.5
UP	63.7	64.8	61.2	29.4	37.1	0.5	30.3	91.6	n.d.	n.d.	100.0	26.2	2.4
P-value	0.135	0.186	0.149	0.479	0.318	0.215	0.500	0.357	-	-	0.437	**0.020**	0.159
Pylor													
CTR	31.5	70.9	45.6	58.7	63.4	2.8	49.4	61.8	n.d.	n.d.	25.7	0.0	43.7
UP	49.4	86.1	58.9	100.0	100.0	3.5	27.8	80.6	n.d.	n.d.	40	0.0	33.4
P-value	0.135	0.357	0.282	***0.095***	0.282	***0.078***	0.301	0.318	-	-	***0.052***	0.247	***0.078***
Duodenum													
CTR	70.5	39.5	38.6	21.3	24.0	0.6	31.1	72.1	44.1	18.7	29.2	0.0	61.5
TB	46.2	54.7	58.1	22.5	30.3	0.6	63.6	60.5	45.2	76.8	46.1	n.d.	93.2
P-value	**0.042**	0.500	0.201	0.159	0.479	0.423	***0.056***	0.247	0.357	0.133	**0.005**	-	**0.024**
Ileum													
CTR	43.6	78.5	59.0	30.4	36.7	0.9	46.4	100.0	66.3	83.3	27.3	0.0	89.3
FCB	20.2	76.0	25.8	16.9	24.9	0.9	44.6	58.6	84.5	100.0	21.9	0.0	95.3
P-value	***0.052***	0.318	***0.064***	***0.095***	0.247	0.437	0.500	0.215	0.318	0.376	***0.064***	0.500	0.500
Colon													
CTR	24.1	62.7	76.0	21.3	21.6	55.5	100.0	53.1	86.1	83.3	27.3	0.0	78.6
FCB	26.8	78.3	100.0	21.3	34.8	100.0	81.0	70.3	100	100	21.9	0.0	100.0
P-value	0.247	0.136	0.186	0.479	**0.020**	0.318	0.415	**0.033**	***0.078***	***0.095***	0.247	0.159	**0.026**

[1] Dietary treatments groups: CTR (no added butyrate); UP (butyrate radical provided as unprotected salt); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.

[2]Arbitrary units. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. For each gene, the highest average gene expression across location and treatments was set to 100.

[3]n.d.: Not detectable.

The type of HDP genes detected in the present experiment differed from those previously reported by Sunkara and coworkers (2011). Such discrepancies may be attributable to age-dependent effects on HDP gene expression (Cuperus *et al.*, 2013).

Mucus secretion is another mechanism allowing the host to modulate microbiota composition (Pan and Yu, 2014). We screened different GIT segments of birds for the expression of known avian mucus genes and, for each GIT segment considered, we compared the expression of detectable mucus genes in the same way as HDP genes. Like HDP genes, expression of several mucus genes was found to be modulated by butyrate in a location-dependent manner (Table 7).

Bactericidal properties of avian HDP have been characterized for a few pathogenic bacteria but not for commensal ones (Cuperus *et al.*, 2013). Besides, the interaction between mucus and microbiota composition has not been studied in birds (Pan and Yu, 2014). It is, therefore, difficult to discuss the possible implications of observed changes in the expression of HDP and mucus genes. In addition, the present approach fails to capture the effect of butyrate presence in a given GIT segment on HDP and mucus gene expression in a remotely distant GIT segment (e.g. effect of FCB on HDP gene expression in the proventriculus). This prevents the use of multivariate analysis to explore the relationships between cecal microbiota composition and the expression of HDP and mucus genes. Our work demonstrates nonetheless that butyrate has location-dependent modulatory effects on the expression of HDP and mucus genes in the gut of broilers. Such effects may have contributed to the observed differences in cecal microbiota composition.

Association between cecal microbiota composition and growth performance. Using RDA, we found that microbiota composition at phylum level tended to explain a small part of the observed variation in growth performance (1.3 %; P=0.062). A similar trend was observed at genus level (0.9 %; P=0.096). Microbiota composition did not significantly explain the observed changes in feed conversion ratio. Overall, cecal microbiota composition had a rather limited influence on growth performance. Presence of butyrate and/or propionate in the distal GIT elicits changes in digestive processes that are positive for feed efficiency (Moquet *et al.*, 2018). Increased butyrate presence in the distal GIT can be achieved by feeding FCB (van den Borne *et al.*, 2015) or by steering endogenous butyrate production (Onrust *et al.*, 2015). Aforementioned dietary strategies may improve feed efficiency while triggering minimal gut inflammation.

A major limitation of the present study is that it only depicts an instant snapshot of the microbiota and gut health status of birds at 22-23 d old. It is well known that dynamic processes contribute to immune system homeostasis and microbiota stability in the gut of broilers. Bacterial communities are rudimental and very similar across GIT segments around hatching (Lu *et al.*, 2003). Distinct meta-communities develop gradually in later life (Pedroso and Lee, 2014). There is a critical immune window lasting a couple of days after hatching wherein birds develop oral tolerance to encountered bacteria (Klipper *et al.*, 2004). This critical window is followed by several weeks of intestinal immune system maturation, wherein different cytokines and Ig isotypes are successively expressed (Lammers *et al.*, 2010; Simon *et al.*, 2014). Future

research should include time series to better understand the effect of butyrate on gut microbiota colonization and immune system maturation.

CONCLUSION

The present study demonstrates that supplementing broiler diets with distinct butyrate additives leads to differential effects on microbiota composition and gene expression as related to gut health in the distal GIT. Unprotected butyrate, mostly active in the crop, proventriculus and gizzard of birds, promotes cecal microbiota dysbiosis. This is associated with the activation of TLR-4, leading to inflammation in the distal GIT. Feeding TB selectively increases butyrate concentration in the small intestine. This does not induce bacterial dysbiosis in the ceca *per se*, and yet TB still promotes inflammation in the distal GIT. The inflammatory effect of TB may be related to fungal dysbiosis. Inflammation of the distal GIT alters the expression of genes involved in TJ proteins and oxidative stress metabolism. Fat coated butyrate with its sustained release profile that allows partial protection of butyrate against gastric and enteric absorption, does not significantly affect cecal microbiota composition and does not induce inflammation in the distal GIT. The precise mechanisms underlying the observed changes in microbiota composition in the distal GIT are independent from changes in digestive function in the upper GIT but are related to the release kinetics of the additives. The presence of butyrate in various GIT segments modulated expression of mucus and HDP genes. This may explain the observed differences in microbiota composition.

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LITERATURE CITED

Al-Sadi, R., D. Ye, M. Boivin, S. Guo, M. Hashimi, L. Ereifej, and T. Y. Ma. 2014. interleukin-6 modulation of intestinal epithelial tight junction permeability is mediated by jnk pathway activation of Claudin-2 gene. PLOS ONE 9:e85345

Andersen, C. L., J. L. Jensen, and T. F. Orntoft. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64:5245-5250.

Apajalahti, J., and K. Vienola. 2016. Interaction between chicken intestinal microbiota and protein digestion. Anim. Feed Sci. Technol. 221:323-330.

Atreya, R., J. Mudter, S. Finotto, J. Mullberg, T. Jostock, S. Wirtz, M. Schutz, B. Bartsch, M. Holtmann, C. Becker, D. Strand, J. Czaja, J. F. Schlaak, H. A. Lehr, F. Autschbach, G. Schurmann, N. Nishimoto, K. Yoshizaki, H. Ito, T. Kishimoto, P. R. Galle, S. Rose-John, and M. F. Neurath. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat. Med. 6:583-588.

Barrachina, M. D., J. Panes, and J. V. Esplugues. 2001. Role of nitric oxide in gastrointestinal inflammatory and ulcerative diseases: perspective for drugs development. Curr. Pharm. Des. 7:31-48.

Beal, R. K., C. Powers, F. Davison, and A. L. Smith. 2006. Immunological development of the avian gut. Pages 85-104 in Avian gut function in health and disease. G.C. Perry ed. CAB International, Oxon, UK.

Bernalier, A., A. Willems, M. Leclerc, V. Rochet, and M. D. Collins. 1996. *Ruminococcus hydrogenotrophicus sp. nov*., a new H2/CO2-utilizing acetogenic bacterium isolated from human feces. Arch. Micobiol. 166:176-183.

Bortoluzzi, C., A. A. Pedroso, J. J. Mallo, M. Puyalto, W. K. Kim, and T. J. Applegate. 2017. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. Poult. Sci. 96(11):3981-3993.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

Carbonero, F., A. C. Benefiel, A. H. Alizadeh-Ghamsari, and H. R. Gaskins. 2012. Microbial pathways in colonic sulfur metabolism and links with health and disease. Front. Physiol. 3:448. doi: 10.3389/fphys.2012.00448.

Cummings, J. H., and W. J. Branch. 1986. Fermentation and the production of short-chain fatty acids in the human large intestine. Pages 131-149 in Dietary fiber: basic and clinical aspects. G.V. Vahouny and D. Kritchevsky eds. Springer US, Boston, MA.

Cuperus, T., M. Coorens, A. Dijk, and H. P. Haagsman. 2013. Avian host defense peptides. Dev. Comp. Immunol. 41(3):352-369.

Desai, T. R., N. J. Leeper, K. L. Hynes, and B. L. Gewertz. 2002. interleukin-6 causes endothelial barrier dysfunction via the protein kinase C pathway. J. Surg. Res. 104:118-123.

Everard, A., V. Lazarevic, N. Gaïa, M. Johansson, M. Ståhlman, F. Backhed, N. M. Delzenne, J. Schrenzel, P. François, and P. D. Cani. 2014. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. ISME J. 8:2116.

Gabriel, I., M. Lessire, S. Mallet, and J. F. Guillot. 2006. Microflora of the digestive tract: critical factors and consequences for poultry. Worlds Poult. Sci. J. 62:499-511.

Geurts, L., V. Lazarevic, M. Derrien, A. Everard, M. Van Roye, C. Knauf, P. Valet, M. Girard, G. G. Muccioli, P. Francois, W. M. de Vos, J. Schrenzel, N. M. Delzenne, and P. D. Cani. 2011. Altered gut microbiota and endocannabinoid system tone in obese and diabetic leptin-resistant mice: impact on apelin regulation in adipose tissue. Front. Microbiol. 2:149. doi: 10.3389/fmicb.2011.00149

Gibson, G. R., G. T. Macfarlane, and J. H. Cummings. 1993. Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34:437-439.

Guilloteau, P., L. Martin, V. Eeckhaut, R. Ducatelle, R. Zabielski, and F. Van Immerseel. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23:366-384.

Iliev, I. D., V. A. Funari, K. D. Taylor, Q. Nguyen, C. N. Reyes, S. P. Strom, J. Brown, C. A. Becker, P. R. Fleshner, M. Dubinsky, J. I. Rotter, H. L. Wang, D. P. McGovern, G. D. Brown, and D. M. Underhill. 2012. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 336:1314-1317.

Iliev, I. D., and I. Leonardi. 2017. Fungal dysbiosis: immunity and interactions at mucosal barriers. Nat. Rev. Immunol. 17:635.

Jiang, W., N. Wu, X. Wang, Y. Chi, Y. Zhang, X. Qiu, Y. Hu, J. Li, and Y. Liu. 2015. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. Sci. Rep. 5:8096.

Juul-Madsen, H. R., B. Viertlböeck, S. Härtle, A. L. Smith, T. W. Göbel, B. Kaspers, and P. Kaiser. 2014. Chapter 7 - Innate immune responses. Pages 121-147 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Kaiser, P., and P. Stäheli. 2014. Chapter 10 - Avian cytokines and chemokines. Pages 189-204 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Kim, Y.-G., K. G. S. Udayanga, N. Totsuka, J. B. Weinberg, G. Núñez, and A. Shibuya. 2014. Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE(2). Cell Host Microbe 15:95-102.

Klasing, K. C. 1998. Avian macrophages: regulators of local and systemic immune responses. Poult. Sci. 77:983-989.

Klipper, E., D. Sklan, and A. Friedman. 2004. Maternal antibodies block induction of oral tolerance in newly hatched chicks. Vaccine 22:493-502.

Kogut, M. H. 2013. The gut microbiota and host innate immunity: Regulators of host metabolism and metabolic diseases in poultry? J. App. Poult. Res. 22:637-646.

Koppenol, W. H. 1998. The basic chemistry of nitrogen monoxide and peroxynitrite. Free Radic. Biol. Med. 25:385-391.

Lammers, A., W. H. Wieland, L. Kruijt, A. Jansma, T. Straetemans, A. Schots, G. den Hartog, and H. K. Parmentier. 2010. Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. Dev. Comp. Immunol. 34:1254-1262.

Larsen, N., F. K. Vogensen, F. W. J. van den Berg, D. S. Nielsen, A. S. Andreasen, B. K. Pedersen, W. A. Al-Soud, S. J. Sørensen, L. H. Hansen, and M. Jakobsen. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLOS ONE 5:e9085.

Lee, M. O., H.-J. Jang, D. Rengaraj, S.-Y. Yang, J. Y. Han, S. J. Lamont, and J. E. Womack. 2016. Tissue expression and antibacterial activity of host defense peptides in chicken. BMC Vet. Res.12:231. doi 10.1186/s12917-016-0866-6

Ley, R. E., D. A. Peterson, and J. I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837-848.

Liu, C., S. M. Finegold, Y. Song, and P. A. Lawson. 2008. Reclassification of *Clostridium coccoides, Ruminococcus hansenii, Ruminococcus hydrogenotrophicus, Ruminococcus luti, Ruminococcus productus* and *Ruminococcus schinkii* as *Blautia coccoides gen. nov., comb. nov., Blautia hansenii comb. nov., Blautia hydrogenotrophica comb. nov., Blautia luti comb. nov., Blautia producta comb. nov., Blautia schinkii comb. nov.* and description of *Blautia wexlerae sp. nov*., isolated from human faeces. Int. J. Syst. Evol. Microbiol. 58:1896-1902.

Lozupone, C., and R. Knight. 2005. UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. Appl. Environ. Microbiol. 71:8228-8235.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature. 489:220-230.

Lu, J., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee. 2003. diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. 69:6816-6824.

Mentschel, J., and R. Claus. 2003. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. Metab. Clin. Exp. 52:1400-1405.

Moquet, P. C. A., L. Onrust, F. Van Immerseel, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72:61-80.

Moquet, P. C. A., S. A. Salami, L. Onrust, W. H. Hendriks, and R. P. Kwakkel. 2018. Butyrate presence in distinct gastrointestinal tract segments modifies differentially digestive processes and amino acid bioavailability in young broiler chickens. Poult. Sci. 97:167-176.

Mudter, J., and M. F. Neurath. 2007. Il-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. Inflamm. Bowel Dis. 13:1016-1023.

Neurath, M. F., and S. Finotto. 2011. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. Cytokine Growth Factor Rev. 22:83-89.

Onrust, L., R. Ducatelle, K. Van Driessche, C. De Maesschalck, K. Vermeulen, F. Haesebrouck, V. Eeckhaut, and F. Van Immerseel. 2015. Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. Front. Vet. Sci. 2:75. doi: 10.3389/fvets.2015.00075.

Pan, D., and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108-119.

Pedroso, A. A., and M. D. Lee. 2014. Chapter 2: The composition and role of the microbiota in chickens. Pages 21-50 in Intestinal health. Key to maximise growth performance in livestock. T. Niewold, ed. Wageningen Academic Publishers, Wageningen, NL.

Pitcher, M., E. Beatty, and J. Cummings. 2000. The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. Gut 46:64-72.

Pitcher, M. C., and J. H. Cummings. 1996. Hydrogen sulphide: a bacterial toxin in ulcerative colitis? Gut 39:1-4.

Qaisrani, S. N. 2014. Improving performance of broilers fed lower digestible protein diets. PhD dissertation. Wageningen University, Wageningen, NL.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:590-596.

R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, AT.

Ramiro-Garcia, J., G. Hermes, C. Giatsis, D. Sipkema, E. Zoetendal, P. Schaap, and H. Smidt. 2016. NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. F1000Res. 2016, 5:1791. doi: 10.12688/f1000research.9227.1

Schneider, K., R. Klaas, B. Kaspers, and P. Staeheli. 2001. Chicken interleukin-6. cDNA structure and biological properties. Eur. J. Biochem. 268:4200-4206.

Schwarzer, K., and J. Bjork. 2015. Tributyrin: biological effects to stimulate animal performance and the integrity of of the intestinal tract in pigs, in: 47e Journées de la Recherche Porcine, INRA, Paris, pp. 133-134.

Shin, N. R., T. W. Whon, and J. W. Bae. 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33:496-503.

Simon, K., G. de Vries Reilingh, B. Kemp, and A. Lammers. 2014. Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers. Poult. Sci. 93:3017-3027.

Smith, A. L., C. Powers, R. K. Beal, B. Kaspers, and P. Kaiser. 2014. Chapter 13 - The avian enteric immune system in health and disease. Pages 227-250 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R. G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLOS ONE 6:e27225.

Teirlynck, E., L. Bjerrum, V. Eeckhaut, G. Huygebaert, F. Pasmans, F. Haesebrouck, J. Dewulf, R. Ducatelle, and F. Van Immerseel. 2009a. The cereal type in feed influences gut wall morphology and intestinal immune cell infiltration in broiler chickens. The British journal of nutrition 102:1453-1461.

Teirlynck, E., M. D. Gussem, J. Dewulf, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Morphometric evaluation of "dysbacteriosis" in broilers. Avian Pathol. 40:139-144.

Teirlynck, E., F. Haesebrouck, F. Pasmans, J. Dewulf, R. Ducatelle, and F. Van Immerseel. 2009b. The cereal type in feed influences *Salmonella enteritidis* colonization in broilers. Poult. Sci. 88:2108-2112.

van den Borne, J. J. G. C., M. J. W. Heetkamp, J. Buyse, and T. A. Niewold. 2015. Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. Livest. Sci.175:96-100.

Verdam, F. J., S. Fuentes, C. de Jonge, E. G. Zoetendal, R. Erbil, J. W. Greve, W. A. Buurman, W. M. de Vos, and S. S. Rensen. 2013. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obesity 21:607-615.

Wallace, J. L., G. Caliendo, V. Santagada, G. Cirino, and S. Fiorucci. 2007a. Gastrointestinal safety and anti-inflammatory effects of a hydrogen sulfide-releasing diclofenac additive in the rat. Gastroenterol. 132:261-271.

Wallace, J. L., M. Dicay, W. McKnight, and G. R. Martin. 2007b. Hydrogen sulfide enhances ulcer healing in rats. FASEB J. 21:4070-4076.

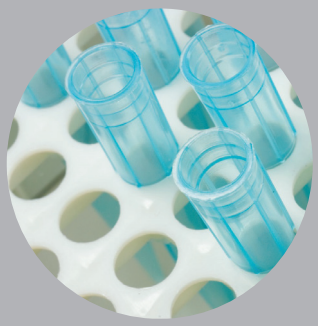
Wells, J. M., O. Rossi, M. Meijerink, and P. van Baarlen. 2011. Epithelial crosstalk at the microbiota–mucosal interface. Proc. Natl. Acad. Sci. USA 108:4607-4614.

Wideman, R. F., Jr., K. R. Hamal, J. M. Stark, J. Blankenship, H. Lester, K. N. Mitchell, G. Lorenzoni, and I. Pevzner. 2012. A wire-flooring model for inducing lameness in broilers: evaluation of probiotics as a prophylactic treatment. Poult. Sci. 91:870-883.

Yang, R., X. Han, T. Uchiyama, S. K. Watkins, A. Yaguchi, R. L. Delude, and M. P. Fink. 2003. IL-6 is essential for development of gut barrier dysfunction after hemorrhagic shock and resuscitation in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 285:621-629.

Zhang, C., M. Zhang, X. Pang, Y. Zhao, L. Wang, and L. Zhao. 2012. Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. ISME J. 6:1848-1857.

Zhang, X., D. Shen, Z. Fang, Z. Jie, X. Qiu, C. Zhang, Y. Chen, and L. Ji. 2013. Human gut microbiota changes reveal the progression of glucose intolerance. PLOS ONE 8:e71108.



Chapter 7

Effect of butyrate presence in distinct gastrointestinal tract segments on immune responses of broiler chickens after *in vivo* immune stimulations

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ABSTRACT

The hypothesis was tested that butyrate presence in the digesta of distinct gastrointestinal tract (**GIT**) segments leads to different immune responses when broilers are subjected to non-infectious immune challenges. A total of 240 male day-old Ross 308 broilers were randomly assigned to 6 dietary treatments: 1) control (no butyrate), 2) unprotected butyrate (main activity in the crop and gastric regions), 3) tributyrin (main activity in the small intestine), 4) fat-coated butyrate (activity in the whole GIT, higher release in the proximal GIT), 5) wax-coated butyrate (activity in the whole GIT, higher release in the distal GIT) and 6) cellulose-acetate-butyrate (main activity in the colon and ceca), each replicated 5 times. At 21 and 22 d post-hatch, 2 birds per pen received intratracheally a human serum albumin (**HuSA**) challenge. HuSA-specific as well as natural antibody (**NAb**) levels were measured at d 0 (pre), 3, 7 and 18 d post-challenge. Birds challenged with HuSA were dissected at 39 d and digesta were analysed for butyrate concentration. At 42 d post-hatch, remaining birds received an intraperitoneal lipopolysaccharide (**LPS**; 0.5 mg/kg BW) injection. Body weight, nitrogen retention and AME_n were measured in the 24 h post LPS challenge. Dietary intervention resulted in significant changes in butyrate concentration across the GIT until the end of the jejunum. Butyrate increased IgY NAb titers, in particular when present in the duodenum and jejunum. Birds subjected to the *in vivo* LPS challenge had growth and nutrient utilisation impairments that were not alleviated by any of the dietary interventions. The present study demonstrates that, in broilers, effects of butyrate on IgY NAb levels are conditioned by the GIT segment wherein the molecule is present.

Key words: Butyrate, broiler, immunization, natural antibody, lipopolysaccharide

INTRODUCTION

In avian species, activation of the nuclear factor κ-B (**NF-κB**) pathway stimulates the production of pro-inflammatory cytokines, which can result in anorexia, increased basal metabolism and shifts in nutrient partitioning (Klasing and Johnstone, 1991). These physiological changes are associated with a negative nitrogen balance and a reduction in growth performance (Klasing and Johnstone, 1991; Lochmiller and Deerenberg, 2000). The NF-κB pathway is down-regulated by the presence of butyrate, a short chain fatty acid (**SCFA**), in the gut mucosa (Yin *et al.*, 2001; Luhrs *et al.*, 2002). Consequently, butyrate modulated the expression of pro-inflammatory cytokines IL-1β and IL-6 in chickens in both *in vitro* (Zhou *et al.*, 2014) and *in vivo* (Li *et al.*, 2015; Bortoluzzi *et al.*, 2017) experiments. Dietary butyrate supplementation has also been shown to alleviate the negative effects of intraperitoneal (**i.p.**) injections of the potent NF-κB inducer *E. coli* lipopolysaccharide (**LPS**) on growth of broilers (Zhang *et al.*, 2011). The positive effect of dietary butyrate supplementation on growth performance of broilers may, therefore, be partly attributable to its anti-inflammatory properties (Moquet *et al.*, 2016b).

Immuno-modulatory properties of butyrate might extend beyond the sole dampening of gastrointestinal tract (**GIT**) inflammation in poultry. Dietary butyrate supplementation has been observed to improve specific antibody (**SpAb**) responses of broilers following immunization (Sikandar *et al.*, 2017), probably because a decrease in NF-κB activity shifts the bird's adaptive immunity from cell-mediated to humoral responses (Koutsos *et al.*, 2014). In mammals, the presence of butyrate in the GIT increases IL-10 levels (Kim *et al.*, 2014; Corrêa-Oliveira *et al.*, 2016). The functions of IL-10 are conserved in birds and mammals, e.g. promoting B cell survival, proliferation and antibody production (Kaiser and Stäheli, 2014). As a consequence, butyrate presence in the GIT may influence not only SpAb response following immunization but also natural antibodies (**NAb**) levels of chickens. NAb have a fundamental role in the first line of defence and maintenance of physiological homeostasis (Panda and Ding, 2015; Boes, 2000).

Immunomodulatory properties of butyrate presumably vary depending on the GIT segment wherein the molecule is present because of metabolic differences among cell types along the avian GIT (Moquet *et al.*, 2016b). Dietary supplementation with butyrate additives (e.g., unprotected sodium salt, tributyrin or fat coated butyrate) can have markedly different butyrate release patterns allowing contrasts in butyrate concentration in the digesta of broilers until the end of the small intestine to be created (Moquet *et al.*, 2018). Use of such additives, however, fails to significantly increase butyrate concentration in cecal digesta. Novel protection methods, such as embedding in a wax matrix or butyrylation to a glucose polymer, may help delivering butyrate to the more distal GIT (Zhou *et al.*, 1996; M'Sadeq *et al.*, 2015).

This study aimed to evaluate how increasing butyrate concentrations in the digesta of distinct GIT segments affect the immune responses of broilers. We hypothesised that the presence of butyrate in the GIT of broilers would decrease NF-κB activation and, subsequently, the production of pro-inflammatory cytokines in a manner that could improve specific humoral response (SpAb), influence innate humoral status (NAb) and

reduce general inflammatory response (LPS challenge). Human serum albumin (**HuSA**) immunization was used to evaluate SpAb response and was considered to be indicative of adaptive humoral immunity responsiveness. Changes in NAb levels were used as indicator of innate humoral status while decreased responsiveness towards a LPS challenge was considered to be a sign of reduced pro-inflammatory response. We further hypothesised that the immunomodulatory properties of butyrate would be conditioned by the GIT segment wherein the molecule was present.

MATERIAL AND METHODS

Experimental design and dietary treatments

Experimental design. A complete randomized block design consisting of 6 dietary treatment groups in 5 blocks was used to assess the effect of butyrate concentration in the digesta on immune responses of broiler chickens. A total of 30 pens were blocked on their location in the experimental room and pens within a block were randomly allocated to one of the 6 experimental diets. Dietary treatment groups were: control (CTR; no butyrate), unprotected butyrate (UP; main activity in the crop, proventriculus and gizzard), tributyrin (TB; main activity in the small intestine), fat-coated butyrate (FCB; activity in the whole GIT with a higher release in the proximal GIT), wax-protected butyrate (WAX; activity in the whole GIT with a higher release in the distal GIT) and cellulose-acetate-butyrate (CAB; main activity in the hindgut). These feed additives were chosen after in vitro and in vivo assessments (Moquet *et al.*, 2016a; Moquet *et al.*, 2018).

Feed Additives and experimental diets. The UP (Adimix Easy) and FCB (Adimix Precision) feed additives were provided by Nutri-Ad International NV (Dendermonde, BE). The TB was prepared by mixing 600 g/kg of tributyrin (T8626, Sigma-Aldrich, Saint-Louis, MO, US) and 400 g/kg silica powder (S5130, Sigma-Aldrich). The WAX additive was produced as follows: Lunacera M wax beads (Füller GmbH, Lüneburg, DE) were ground at 15,000 rpm with a ZM-1000 grinder (Retsch, Haan, DE) equipped with a 12-tooth rotor and without a screen. A 3 kg batch consisting of 300 g/kg sodium butyrate (Nutri-Ad International NV, Dendermonde, BE) and 700 g/kg ground wax was mixed with a pedal mixer model 305 (Dinissen, Sevenum, NL) at 50 % of the maximum speed for 5 min. The wax-butyrate mix was subsequently extruded with a twin screw extruder (M.P.F.50, Baker Perkins Ltd., Peterborough, UK). Extrudates were cooled to room temperature overnight and subsequently ground at 10,000 rpm with a ZM-1000 grinder (Retsch) equipped with a 12-tooth rotor without a screen. Ground extrudates were sieved manually to obtain the 0.8-1.4 mm fraction. The CAB additive was purchased from Fisher-Scientific (10549813; butyryl content 50-54%; Waltham, USA). Basal starter, grower and finisher diets were formulated to meet or exceed the nutritional requirements of broiler chickens (CVB, 2007, Table 1).

Table 1. Ingredients and calculated nutrient composition of the basal diets

	Starter	Grower	Finisher
Ingredients (g/kg as is basis)			
Corn	467.27	412.06	371.86
Wheat	147.90	197.29	246.67
Soybean meal	266.67	271.61	271.61
Fish meal	24.69	14.82	0.00
Potato protein	19.75	9.88	9.88
Soybean oil	21.72	49.66	56.28
Vitamin and mineral premix[1]	4.94	4.94	4.94
Limestone	14.82	12.35	11.85
Monocalcium phosphate	10.86	6.42	5.43
Salt	1.58	1.78	1.98
Sodium bicarbonate	2.57	2.17	1.98
L-Lysine	1.48	1.58	1.88
DL-Methionine	2.57	2.47	2.37
L-Threonine	0.40	0.49	0.49
L-Valine	0.15	0.15	0.15
Natuphos 10000G	0.05	0.05	0.05
Chromic sesquioxide	0.25	0.25	0.25
Butyrate containing additives[2]	12.33	12.33	12.33
Calculated nutrient composition (as is basis)			
Crude protein (g/kg)	221.70	209.70	201.00
Metabolisable energy (MJ/kg)	11.91	12.60	12.76
Calcium (g/kg)	9.20	7.20	6.50
Available phosphorus (g/kg)	4.40	3.10	2.60
Methionine (g/kg)	6.31	5.85	5.46
Lysine (g/kg)	13.42	12.50	11.97

[1]Provided per kg of diet: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 50 mg; vitamin B_2, 7.5 mg; vitamin B_6, 3.5 mg; vitamin B_1, 2.0 mg; vitamin K_3, 1.5 mg; vitamin B_{12}, 20 µg; choline chloride, 460 mg; antioxidant (oxytrap PXN), 125 mg; niacin, 35 mg; pantothenic acid, 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg; Fe, 80 mg; Zn, 60 mg; Cu, 12 mg; I, 0.8 mg; Se, 0.15 mg.
[2]See Table 2.

Experimental diets were derived from the basal diets by adding the ingredients presented in Table 2. Butyrate-supplemented diets contained 1 g/kg of butyrate anion ($C_4H_7O_2^-$; as-is basis). Ingredient constituents used in the production of butyrate additives such as palm fat, cellulose or silica were present in similar amounts across diets. Sodium bicarbonate was used to maintain equal sodium supply across diets. Analysed nutrient values of the experimental diets are presented in Table 2. Birds were allowed *ad libitum* access to feed and water. Starter diets were fed during the 0-10 d period, grower diets during the 10-24 d and finisher diets during the reminder of the experiment.

Table 2. Ingredients added to the basal diets to derive the experimental diets and their analysed nutrient composition

	Dietary treatment					
	Control	UP	TB	FCB	WAX	CAB
Ingredients added (g/kg as is basis)						
Soybean oil	1.16	0.86	0.00	0.86	0.86	0.16
Fat-coated butyrate[2]	0.00	0.00	0.00	4.21	0.00	0.00
Tributyrin[3]	0.00	0.00	1.78	0.00	0.00	0.00
Sodium butyrate[4]	0.00	1.40	0.00	0.00	0.00	0.00
Wax protected butyrate[5]	0.00	0.00	0.00	0.00	6.76	0.00
Cellulose acetate butyrate[6]	0.00	0.00	0.00	0.00	0.00	2.00
Cellulose	1.00	1.00	1.00	1.00	1.00	0.00
Palm fat	2.95	2.95	2.95	0.00	2.95	2.95
Lunacera M wax	5.50	5.50	5.50	5.50	0.00	5.50
Silica	0.62	0.62	0.00	0.62	0.62	0.62
Dextrin	0.14	0.00	0.14	0.14	0.14	0.14
Sodium bicarbonate	0.96	0.00	0.96	0.00	0.00	0.96
Total	**12.33**	**12.33**	**12.33**	**12.33**	**12.33**	**12.33**
Analysed nutrient composition of the experimental diets (as is basis)						
Starter						
Dry matter (g/kg)	916.40	928.10	942.50	930.10	935.90	937.40
Fat (g/kg)	55.30	55.10	56.00	54.40	54.10	55.60
Crude protein (g/kg)	224.44	230.19	233.19	228.75	231.06	232.06
Crude fibre (g/kg)	27.70	29.50	31.50	29.30	27.60	28.40
Gross energy (MJ/kg)	17.49	17.74	17.94	17.69	17.77	17.87
Grower						
Dry matter (g/kg)	876.30	877.90	877.70	879.30	879.30	879.10
Fat (g/kg)	77.30	76.80	76.90	76.40	78.70	76.70
Crude protein(g/kg)	204.13	207.38	205.81	201.44	203.81	207.06
Crude fibre (g/kg)	28.50	26.60	27.80	26.50	27.20	27.00
Gross energy (MJ/kg)	17.41	17.52	17.46	17.53	17.53	17.54
Finisher						
Dry matter (g/kg)	875.30	879.00	878.90	879.40	868.40	877.80
Fat (g/kg)	79.80	81.00	84.80	80.70	78.50	81.30
Crude protein (g/kg)	196.81	193.63	194.19	194.88	194.38	195.94
Crude fibre (g/kg)	26.40	26.00	26.40	26.50	25.10	26.10
Gross energy (MJ/kg)	17.61	17.64	17.74	17.65	17.45	17.60

[1]UP: Unprotected butyrate, TB: Tributyrin, FCB: Fat-coated butyrate, WAX: wax-coated butyrate, CAB: Cellulose acetate butyrate.
[2]Adimix Precision (Nutriad International, Dendermonde, BE). Contains 700 g/kg palm fat; 237 g/kg $C_4H_7O_2^-$; 63 g/kg Na^+.
[3]Contains 650 g/kg tributyrin (Sigma-Aldrich, Saint-Louis, MO, USA); 350 g/kg silica.
[4]Adimix Easy (Nutriad International, Dendermonde, BE); contains 712 g/kg $C_4H_7O_2^-$; 188 g/kg Na^+, 100 g/kg dextrin.
[5]Contains 813 g/kg Lunacera M wax (Füller GmbH, Lüneburg, DE); 147.73 g/kg $C_4H_7O_2^-$, 39.27 g/kg Na^+.
[6]Fisher-Scientific (Waltham, USA). Contains 500-540 g/kg $C_4H_7O_2^-$.

Experimental procedures

Birds and housing conditions. The experiment was conducted at the research farm Carus of Wageningen University (Wageningen, NL). All the experimental procedures were approved by the Animal Care and Use Committee of Wageningen University. A total of 240 one-day-old male broilers (initial BW 43.4 ± 3.4 g; Ross 308, Aviagen Group, Newbridge, UK) were obtained from a commercial hatchery (Kuikenbroederij Morren B.V., Lunteren, NL). Day-old chicks originated from a single breeder flock, were hatched in the same incubator and were transported in the same truck to minimize initial variation in microbiota composition. Upon arrival, birds were individually weighed,

neck-tagged and randomly assigned to one of the replicate pens. Each pen housed 8 birds, had a dimension of 1.85 × 1 m (L × W) and was enriched with a perch. Birds were kept 15 cm above the ground floor on a slatted floor covered with cardboard and pelleted lignocellulose (Softcell; Agromed Austria GmbH, Kremsmünster, AT) as bedding material. Ambient temperature was maintained at 32°C until d 3 and thereafter gradually reduced to 22°C at d 23. A 23L:1D photoperiod was applied until d 3 and was changed thereafter to 16L:8D. Individual and pen BW as well as pen feed intake (FI) were recorded every week. Each 20 kg bag of feed used in the experiment was sampled at the top, middle and bottom. Feed samples were pooled by diet type and kept at 4°C.

Human serum albumin immunization. Two birds per pen having an individual BW closest to the pen average at d 14 were selected and marked with aerosol paint. At d 21 and 22 post-hatch, these birds received an immunological challenge consisting of intratracheal administration of HuSA (0.5 mg/d in 0.5 ml physiological saline solution on two consecutive days). Marked birds remained with the unchallenged birds in their respective pens after HuSA administration. The HuSA was obtained from Sigma-Aldrich (A8763) and dissolved in physiological saline at the concentration of 1 mg/ml. Blood was collected from the wing vein before initial HuSA administration (d 0) and at d 3, 7 and 18 post initial challenge. On d 39 post-hatch (i.e. d 18 post initial challenge), HuSA-challenged birds were euthanized by intravenous injection of sodium pentobarbital. Slaughter weights were recorded, body cavities were opened and different GIT segments were carefully separated using tie-raps: crop, proventriculus + gizzard, duodenum + jejunum (from the pyloric junction to Meckel's diverticulum), ileum (from Meckel's diverticulum to the ileocecal valve), colon, and ceca. Contents of each GIT segment were collected by gentle finger stripping and frozen at -20°C pending further analysis.

LPS challenge and nutrient balance. Immediately after the blood sampling at 42 d, all birds received an i.p. injection of *E. coli* LPS (0.5 mg/kg BW, E. coli serotype 055:B5; L2880, Sigma-Aldrich) dissolved in a phosphate-buffered saline solution at the concentration of 0.5 mg/ml. The LPS dose injected was, therefore, adjusted to the BW of each bird. Bedding and cardboard were concomitantly removed from the slatted floor in order to transform the pens into metabolic cages. A balance period was conducted during the 24 h following i.p. LPS injection wherein FI, BW gain and mortality were recorded. Droppings were collected at 12 and 24 h following i.p. LPS injection, pooled per pen and stored at -20°C.

Analytical methods

Short chain fatty acids. A representative fraction of the digesta content taken from birds dissected at 39 d post-hatch (i.e. 18 d post initial HuSA challenge, but 3 d prior to LPS challenge) was analysed for DM and SCFA contents (15 birds per treatment group, 6 GIT segments per bird). Dry matter was measured according to standard methods (ISO 6496; ISO, 1999) and SCFA contents were determined according to the method described by Qaisrani and coworkers (2014). Blood samples taken from these birds were analysed according to the aforementioned procedure.

Immunoglobulins binding HuSA or KLH. Blood samples taken before initial HuSA administration (d 0) and at d 3, 7 and 18 relative to the initial HuSA challenge were analysed for IgM and IgY binding HuSA and keyhole limpet hemocyanin (KLH). Immunoglobulin titers were determined by ELISA, as described previously (Simon *et al.*, 2014).

Nitrogen and energy balance during the LPS challenge. Finisher feed samples and freeze-dried droppings collected during the 24 h post i.p. LPS injection were ground to pass a 1 mm sieve and analysed for DM (ISO 6496; ISO, 1999), nitrogen (method ISO 5983-1; ISO, 2005) and gross energy (method ISO 9831; ISO, 1998).

Calculations and statistical analysis

Short chain fatty acids. The SCFA concentration in the digesta of different GIT segments was analysed using the PROC GLM of SAS (version 9.3, SAS Institute Inc., Cary, NC). Diet was used as main effect. Means were separated using the Tukey-Kramer post-hoc test when a significant diet effect was detected.

Growth performance. Growth performance was analysed during the 0-35 d period to avoid the confounding influence of dissections (d 39) and LPS challenge (d 42). Average FI, BW gain and feed conversion ratio (**FCR**) were analysed using the PROC MIXED of SAS (version 9.3, SAS Institute Inc., Cary, NC) using the following model:

$$Y_{ijkm} = \mu + D_i + W_j + DW_{ij} + B_k + \varepsilon_{ijkm}$$

where Y_{ijkm} is the performance response of the m^{th} pen (m = 1 to 30) fed the i^{th} diet (i = CTR, UP, TB, FCB, WAX, CAB) during the j^{th} measurement week (j = 1 to 5) in the k^{th} block (k = 1 to 5). Diet (D_i), measurement week (W_j) and the interaction between diet and measurement week (DW_{ij}) where included as fixed effects while block (B_k) was included as a random factor. Means related to significant fixed effects were separated using the Tukey-Kramer post-hoc test. The effect of butyrate presence in the diet was tested using a contrast (Butyrate: CTR vs UP+TB+FCB+WAX+CAB).

Immunoglobulins binding HuSA or KLH. Immunoglobulin titers were analysed with the PROC MIXED procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC) using the following model:

$$Y_{ijkm} = \mu + D_i + S_j + DS_{ij} + B_k + \varepsilon_{ijkm}$$

where Y_{ijkm} is the Ig titer of m^{th} bird (m = 1, 2) fed the i^{th} diet (i = CTR, UP, TB, FCB, WAX, CAB) in the k^{th} block (k= 1 to 5) and sampled on the j^{th} day post initial i.p. HuSA administration (j = 0, 3, 7, 18). Diet (D_i), sampling day (S_j) and the interaction between diet and sampling day (DS_{ij}) where included as fixed effects while block (B_k) was included as a random factor. Means related to significant fixed effects were separated using the Tukey-Kramer post-hoc test. The effect of butyrate presence in the diet was tested using a contrast (Butyrate: CTR vs UP+TB+FCB+WAX+CAB).

Performance, energy and nitrogen retention during the LPS challenge. Nitrogen retention (NR; g/kg diet consumed) was calculated at a pen level during the balance period (0-24 h post i.p. LPS injection) as follows:

$$NR = \frac{(I \times N_{feed}) - (D \times N_{droppings})}{(I \times N_{feed})} \times 100$$

where I is the pen FI in g; N_{feed} and $N_{droppings}$ are as-is basis nitrogen contents of the feed and of the droppings, respectively; D is the pen dropping mass in g.

Total nitrogen corrected apparent metabolizable energy (AME_n) intake (MJ) was calculated at a pen level during the balance period using the equation of Meloche and coworkers (2013). A nitrogen correction factor of 8.73 kcal/g was used (Titus, 1956) and negative nitrogen retention values were set to zero. Apparent ME_n (MJ/kg) was calculated as follows:

$$AME_n = \frac{Total\ AME_n\ intake}{I}$$

Bodyweight gain, FI, NR and AME_n during the balance period were analysed using the PROC GLM procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC). Diet was considered as a main effect. Mortality during the balance period was analysed using the Chi-square test of the PROC FREQ procedure of SAS.

RESULTS

Short chain fatty acids

Table 3 shows the effect of the dietary treatments on SCFA concentrations in the digesta of different GIT segments of broilers at 39 d of age. Significant dietary effects were observed for butyrate concentrations in the contents of the crop (P<0.001), pooled proventriculus + gizzard (P<0.001) and pooled duodenum + jejunum (P<0.001). Post-hoc analysis showed that birds fed the WAX additive resulted in significantly higher butyrate concentration in the crop and pooled proventriculus + gizzard contents than birds fed other diets (P<0.05). The UP, TB, and FCB additives also significantly increased butyrate concentration in the crop and pooled proventriculus + gizzard contents in comparison to the CTR, albeit to a lesser extent than the WAX additive (P<0.05). In addition, post-hoc analysis revealed that the TB diet significantly increased butyrate concentration in pooled duodenum and jejunum contents compared to the control diet (P<0.05). Acetate and propionate concentrations in the digesta of the different GIT segments were not influenced (P>0.05) by dietary intervention.

Table 3. Mean acetate, propionate and butyrate concentration in the digesta of distinct gastrointestinal tract segments of 39 day old broilers fed a diet without or with various butyrate additives

Fatty acid	Dietary treatment groups[1]						SEM	Treatment
Location[2]	CTR	UP	TB	FCB	WAX	CAB		*P*-value
Acetate (µmol/ g DM)								
Crop	33.3	20.6	41.7	21.2	23.4	22.9	7.9	0.355
P + G	12.4	8.7	15.0	9.8	10.3	11.4	2.5	0.563
D + J	11.0	23.8	21.5	22.2	21.7	21.7	3.3	0.113
Ileum	43.4	42.9	48.7	40.6	36.8	42.3	4.2	0.533
Colon	175.4	227.0	107.8	82.8	87.1	135.6	47.6	0.225
Ceca	455.9	500.8	552.5	448.5	422.5	426.9	50.2	0.450
Propionate (µmol/ g DM)								
Crop	0.7	0.7	1.1	0.6	1.1	0.7	0.2	0.322
P + G	1.1	0.7	0.8	0.6	1.0	0.9	0.2	0.457
D + J	1.1	2.4	2.0	1.9	2.0	2.0	0.4	0.439
Ileum	2.7	2.6	2.7	2.5	2.2	2.6	0.3	0.733
Colon	7.6	10.9	6.5	4.7	4.9	7.3	2.6	0.531
Ceca	41.0	30.9	53.5	35.7	33.0	30.5	6.4	0.135
Butyrate (µmol/ g DM)								
Crop	0.1^{c}	6.5^{b}	6.1^{b}	6.2^{b}	13.1^{a}	0.1^{c}	0.9	**<0.001**
P + G	0.1^{c}	2.6^{b}	1.9^{b}	2.2^{b}	3.7^{a}	0.1^{c}	0.4	**<0.001**
D + J	0.1^{c}	0.4^{b}	1.6^{a}	0.5^{bc}	0.8^{ab}	0.1^{c}	0.2	**<0.001**
Ileum	0.1	0.3	0.3	0.1	0.1	0.1	0.1	0.120
Colon	25.9	20.1	9.7	2.9	2.8	11.0	6.9	0.145
Ceca	104.0	84.8	138.8	86.0	99.7	95.6	14.5	0.136

[1]Dietary treatments groups: CTR (no butyrate supplementation), UP (butyrate anion provided as unprotected salt), TB (butyrate anion provided as tributyrin), FCB (butyrate anion provided as fat-coated butyrate), WAX (butyrate anion provided as wax-coated butyrate), CAB (butyrate anion provided as cellulose-acetate-butyrate). Butyrate was provided at 1 g/kg as is basis. Values are means of 10 birds per treatment group.

[2]P + G: Proventriculus-gizzard; D + J: Duodenum-jejunum.

Growth performance

There was no significant effect of dietary treatment on the growth performance of HuSA-challenged and non-challenged birds during the 1-35 d period, as indicated in Table 4. Dietary treatments tended (P=0.085) to influence FI of birds but did not (P>0.05) influence FCR.

Table 4. Growth performance (d 1 to 35) of broiler chickens fed a diet without or with various butyrate additives

Parameter[1]	Dietary treatment groups[2]						SEM	*P*-values[3]			
	CTR	UP	TB	FCB	WAX	CAB		D	W	D x W	B[4]
ADG (g)	76	77	77	75	75	77	8.4	0.704	**<0.001**	0.915	0.787
FI (g)	108	109	110	107	105	109	9.5	***0.085***	**<0.001**	0.819	0.755
FCR	1.42	1.41	1.43	1.42	1.41	1.43	0	0.759	**<0.001**	0.271	0.365

[1]ADG: Average daily gain; FI: Feed intake; FCR: Feed conversion ratio.

[2]Dietary treatment groups: No butyrate supplementation (CTR) or 1 g/kg butyrate anion provided in the diet in the form of unprotected butyrate (UP), tributyrin (TB), fat coated butyrate (FCB), wax coated butyrate (WAX) or cellulose acetate butyrate (CAB). Means represent average values of 5 replicate pens per group

[3]D: Diet; W: Week; B: Butyrate.

[4]Statistical contrast: CTR vs UP+TB+FCB+WAX+CAB.

Immunoglobulins binding HuSA or KLH

Dietary intervention did not influence titers of IgM (P=0.636) and IgY (P=0.849) binding HuSA. There was no significant interaction between number of days following the initial i.t. HuSA administration and dietary intervention for both IgM (P=0.867) and IgY (P=0.984) binding HuSA. Titers of IgM and IgY binding HuSA did increase significantly from 0 to 18 d following the initial i.t. HuSA administration (P<0.001; Figure 1). Birds subjected to i.t. HuSA administration tended (P=0.079) to have a lower ADG than their non-challenged counterparts during the 21-35 d period.

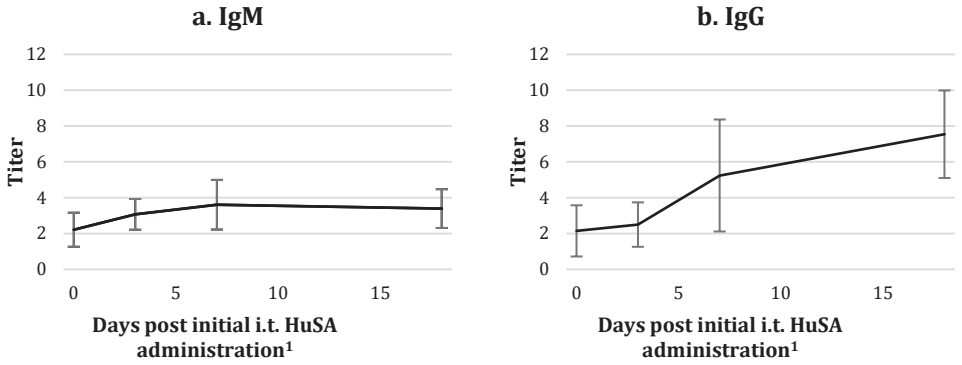

Figure 1. Anti-human serum albumin (HuSA) IgM (panel a) and IgG (panel b) antibodies at d 0, 3, 7 and 18 post initial intratracheal administration of HuSA. HuSA administered at d 21 and 22 post-hatch at a dose of 0.5 mg/day to 10 birds per treatment group.

Titers of IgM and IgY binding KLH increased significantly from 0 to 18 d following the initial i.t. HuSA administration (P<0.001; Table 5). A significant diet effect was found for the titers of IgY binding KLH (P=0.002). Contrast analysis indicated that butyrate supplementation significantly increased titers of IgY binding KLH (P=0.002). Post-hoc analysis revealed that birds fed the TB diet had significantly higher titers of IgY binding KLH than birds fed the other diets (P<0.05). A linear relationship between IgY NAbs and BW was observed at 39 d of age (P=0.007, R^2=0.151; Figure 2). For every additional unit of IgY titers, birds grew, on average, 3.5 g/d more over the 0-39 d period. Dietary intervention did not influence titers of IgM binding KLH (P=0.413). There was no significant interaction

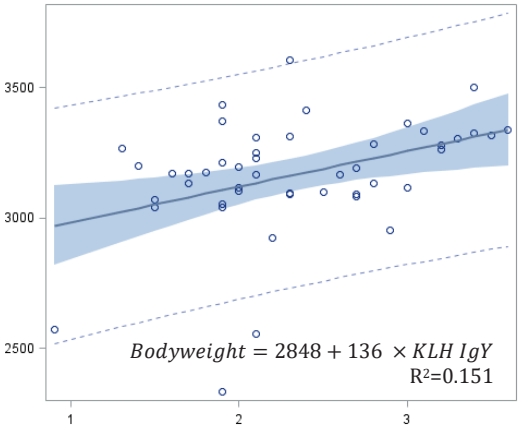

Figure 2. Effect of anti-KLH IgY natural antibody titers on bodyweight of broilers at 39 days of age. Human serum albumin administered at d 21 and 22 post-hatch at the dose of 0.5 mg/day. Outliers (n=3) were identified as being more than two standard deviations away from the calculated mean and subsequently removed from the regression analysis

between number of days following the initial i.t. HuSA administration and dietary intervention for both IgM (P=0.637) and IgY (P=0.516) binding KLH.

Table 5. Effect of dietary treatments on titers of Ig binding keyhole lympet hemocyanin (KLH) proteins at d 0, 3, 7 and 8 after initial intratracheal administration of HuSA[1]

IgM	Dietary treatment groups[2]							*P*-values			
	CTR	UP	TB	FCB	WAX	CAB	**Mean**	Diet	Day	Diet*Day	B[3]
d 0	1.7	1.8	1.9	1.4	1.4	1.6	**1.6**^{b}	0.413	**0.001**	0.637	0.195
d 3	2.2	2.7	2.8	2.3	2.4	2.6	**2.5**^{ab}				
d 7	2.3	2.3	2.7	2.6	2.1	2.3	**2.4**^{ab}				
d 18	2.9	3.3	3.3	3.1	3.8	3.3	**3.3**^{a}				
Mean	**2.3**	**2.5**	**2.6**	**2.3**	**2.4**	**2.5**	**-**				
SEM	0.14	0.14	0.14	0.14	0.14	0.14					
IgY	Dietary treatment groups[2]							*P*-values			
	CTR	UP	TB	FCB	WAX	CAB	**Mean**	Diet	Day	Diet*Day	B[3]
d 0	1.0	1.0	1.0	1.0	1.4	1.3	**1.1**^{b}	**0.002**	**0.001**	0.516	**0.002**
d 3	0.9	1.2	1.6	0.9	1.5	1.3	**1.2**^{b}				
d 7	1.1	1.3	1.3	1.2	1.2	1.4	**1.3**^{b}				
d 18	1.8	2.3	3.2	2.3	2.9	2.9	**2.5**^{a}				
Mean	**1.2**^{B}	**1.5**^{B}	**1.8**^{A}	**1.3**^{B}	**1.7**^{B}	**1.7**^{B}	**-**				
SEM	0.15	0.15	0.15	0.14	0.15	0.15					

[1]Human serum albumin administered at d 21 and 22 post-hatch at the dose of 0.5 mg/day to 10 birds per group.
[2]Dietary treatment groups: No butyrate supplementation (CTR) or 1 g/kg butyrate anion provided in the diet in the form of unprotected butyrate (UP), tributyrin (TB), fat coated butyrate (FCB), wax coated butyrate (WAX) or cellulose acetate butyrate (CAB).
[3]Butyrate; Statistical contrast: CTR vs UP+TB+FCB+WAX+CAB.
[a-c]Means within a column lacking a common superscript differ (P< 0.05).
[A-B]Means within a row lacking a common superscript differ (P< 0.05).

Performance, energy and nitrogen retention during the LPS challenge

Growth, FI, mortality, nitrogen retention and AME_n data during the 24 h following i.p. LPS injection are presented in Table 6. There were no significant effects of dietary treatment on any of the measured parameters.

Table 6. Body weight gain (BWG), feed intake (FI), mortality, nitrogen retention (NR) and nitrogen-corrected apparent metabolizable energy (AME_n) of broiler chickens 0-24 h post injection of lipopolysaccharide (0.5 mg/kg BW) fed a diet without or with various butyrate additives

Parameter	Dietary treatments groups[1]						SEM	*P*-value
	CTR	UP	TB	FCB	WAX	CAB		
BWG (g/d)	-85.0	-41.1	-69.8	-89.1	-63.6	-66.2	21.0	0.641
FI (g/d)	112.5	89.4	110.4	127.3	114.2	86.3	15.3	0.332
Mortality (%)	6.9	0.0	7.7	3.4	11.5	7.1	4.0	0.585
NR (% N intake)	30.6	27.4	34.5	33.7	41.2	24.6	7.9	0.677
AME_n (MJ/kg)	-71.7	-33.6	-80.4	-78.8	-98.4	-42.9	4575	0.186

[1]Dietary treatment groups: No butyrate supplementation (CTR) or 1 g/kg butyrate anion provided in the diet in the form of unprotected butyrate (UP), tributyrin (TB), fat coated butyrate (FCB), wax coated butyrate (WAX) or cellulose acetate butyrate (CAB).

Birds showed strong sickness behaviour after i.p. LPS injection. Animals displayed within an hour lethargic and anorectic behaviour which lasted for approximatively 15 hours. Birds had difficulty breathing during the first 12 h following LPS injection as their respiratory tract seemed to contain fluids. A total of 6.0 % (n= 10) of the animals died during the 24 hours that followed the injection. Mortality was not affected by dietary intervention (P=0.585).

DISCUSSION

Butyrate concentration in the digesta of distinct GIT segments

The current study aimed to assess the effect of butyrate presence in distinct GIT segments on immune responses of broiler chickens subjected to non-infectious immune challenges. Differences in luminal butyrate concentration across GIT segments were achieved using five different butyrate additives (UP, TB, FCB, WAX, CAB). Birds fed the WAX diet had a significantly higher butyrate concentration in the crop, and proventriculus + gizzard contents compared to the birds fed the other experimental diets. Such findings contrasts with preliminary *in vitro* work showing that WAX prevented butyrate release in the proventriculus and small intestine more effectively than FCB. Damages to the WAX matrix during feed production, e.g. by steam conditioning and pelleting, may explain the difference between this *in vitro* and *in vivo* observation.

The UP, TB, and FCB additives also significantly increased butyrate concentration in the crop and proventriculus + gizzard contents, albeit to a lesser extent than the WAX additive. Increased butyrate concentration in the proximal GIT of birds fed UP, TB or FCB additives have already been reported elsewhere (Moquet *et al.*, 2018). The release profile of UP and FCB are consistent with the intrinsic properties of these products: UP is quickly absorbed in the proventriculus while FCB provides only a partial protection against gastric absorption (Moquet *et al.*, 2016b). Tributyrin is, however, supposed to only be released in the proximal small intestine under the action of pancreatic lipase (Moquet *et al.*, 2016b). Pancreatic lipase can, however, end up in the gizzard of chickens due to retrograde peristalsis (Sklan *et al.*, 1978). Such gastro-duodenal reflux may explain why TB-fed birds had significantly more butyrate in the crop, proventriculus + gizzard contents than to their CTR-fed counterparts.

Previous reports indicated that exogenous butyrate can be delivered to the colon and ceca of broilers by feeding FCB (Smith *et al.*, 2012; van den Borne *et al.*, 2015; Moquet *et al.*, 2018) or butyrylated polysaccharides such as CAB (M'Sadeq *et al.*, 2015). Supplementing broiler diets with FCB or CAB failed, however, to significantly increase butyrate concentration beyond the jejunum in the present experiment. The investigation of the effects of butyrate on immune responses along *all* segments of the birds' GIT tract was, therefore, incomplete. Discrepancies between our study and data in the literature in the effects of feeding FCB or CAB on butyrate concentration in the distal GIT may be attributable to diet formulation. Rapeseed meal (RSM) based diets were used in the study of Moquet and coworkers (2018) while soybean meal (SBM) based diets were used in the present work. Soybean meal contains 3.3 times more oligosaccharides than RSM (Knudsen and Li, 1991). Raffinose and stachyose make up

half of SBM oligosaccharides (Choct *et al.*, 2010). Such SBM oligosaccharides have a very low pre-cecal digestibility and are extensively fermented in the colon and ceca of birds (Coon *et al.*, 1990). Hence, cecal butyrate production is increased when RSM is replaced by SBM as main protein source in broiler diets (Qaisrani *et al.*, 2014).

The final step of butyrate production by the cecal microbiota is achieved by the butyryl-CoA: acetate CoA enzyme (Onrust *et al.*, 2015). The gene coding for butyryl-CoA: acetate CoA is more abundantly expressed by the cecal microbiota of broilers when diets are diluted with purified cellulose (Bortoluzzi *et al.*, 2017). This increase was, however, only observed in diets without supplemented butyrate. Using cecal contents of 28 d old broilers in an *in vitro* fermentation model, Onrust (Department of Pathology, Bacteriology and Avian Diseases, Ghent, BE, personal communication) demonstrated that the butyrogenic effect of purified cellulose as fermentation substrate is surpassed by the one of CAB only after 6 hours of fermentation. Mean retention time of solid particles in the ceca of broilers varies greatly across studies, with values ranging from 13-50 min (Rougière and Carré, 2010) to 119 min (Denbow and Scanes, 2015) or longer (Warriss *et al.*, 2004; Liu *et al.*, 2017). Diet structure, fibre composition as well as age of the bird are thought to influence cecal functions (Svihus, 2014). Hence, it is hitherto difficult to conclude if CAB has a superior butyrogenic effect than purified cellulose *in vivo*. In the present experiment, purified cellulose was added to the CTR, UP, TB, FCB and WAX diets to correct for the cellulose present in the CAB diet. In addition to SBM oligosaccharides, purified cellulose may have modified the endogenous (microbial) butyrate production in the ceca, thereby, masking the effect of exogenous (dietary) butyrate supplementation. Onrust and coworkers (2015) listed a series of strategies that could stimulate microbial butyrate production in the hindgut of broilers. It may be sound to use such strategies rather than protected butyrate additives in future studies aimed at increasing butyrate concentration in the hindgut.

Growth performance

Immunologic stress depresses growth performance of broilers by reducing FI, shifting nutrient partitioning away from skeletal muscle deposition and increasing basal metabolism rate (Klasing and Johnstone, 1991). Antibiotic growth promoters (AGP) are known to prevent immunologic stress in chicks (Roura *et al.*, 1992). Hence, sub-optimal rearing conditions are required to observe the growth-promoting effect of AGP. This has led Niewold (2007) to refer preferably to AGP as growth-permitting rather than growth-promoting molecules. Butyrate has been proposed as an alternative to the use of AGP in broiler diets (Huyghebaert *et al.*, 2011). In the present experiment, dietary intervention did not change the growth performance of unchallenged birds during the 1-35 d period. In contrast, feeding butyrate was reported to influence growth performance of broilers subjected to i.p. LPS injections (Zhang *et al.*, 2011), *Salmonella* infection (Liu *et al.*, 2017) or feed challenge (Moquet *et al.*, 2018). Overall, these observations indicate that growth-modulating effects of both butyrate and AGP are more prominent under sub-optimal rearing conditions.

Specific and natural Ig titers

In the present study, i.t. HuSA administration was used at 21 and 22 d post-hatch to elicit a SpAb response. We did not observe any significant effect of dietary butyrate supplementation on HuSA-SpAb response. In contrast, Sikandar and coworkers (2017) reported that sodium butyrate enhanced the SpAb response of broilers after immunization against Newcastle disease virus via the ocular route at 1 d old and against SRBC via the intravenous route at 14 d. Newcastle disease vaccines contain pathogen-associated molecular patterns (**PAMP**) that can trigger an innate immune response (Schirrmacher, 2017). In contrast, purified model antigens such as HuSA allow to specifically stimulate the adaptive immune system without triggering a robust innate immune response (Klasing, 2004). The effect of butyrate on SpAb response may, therefore, be more prominent with PAMP-containing antigens. Discrepancies may be attributable, in addition, to differences in age and administration route.

Natural antibodies are defined as the Ig produced in absence of prior (known) external antigen stimulation (Schwartz-Albiez *et al.*, 2009). Parmentier and colleagues (2004) reported that the serum of chickens contains Ig that can bind antigens to which chickens were never voluntarily exposed, e.g. KLH. Such Ig are considered to be avian NAb in the present study. There are two subpopulation of NAb: overt NAb are readily active in the serum while cryptic NAb only become active after cell damage (Berghof *et al.*, 2015). In this study, overt NAb titers against KLH were measured. In agreement with earlier reports, both IgM and IgY NAb titers were found to increase significantly with age from 21 d post-hatch onwards (Simon *et al.*, 2014). This increase is likely to be related to the maturation of the immune system and not to the HuSA challenge (Simon *et al.*, 2014). Interestingly, dietary butyrate supplementation was found to significantly increase IgY NAb titers in the present study.

Butyrate has been shown to exert immuno-modulatory effects on mammalian gut cells through distinct mechanisms. Firstly, presence of butyrate in the cytoplasm of colonocytes and intestinal macrophages modifies IKK proteins, thereby down regulating NF-κB pathway activation (Luhrs *et al.*, 2001). Secondly, the presence of butyrate in the nucleus of intestinal macrophages is thought to inhibit histone deacetylase enzymes in a manner where it can reduce production of pro-inflammatory cytokines (Chang *et al.*, 2014). Finally, butyrate can be used as an energy substrate by regulatory T cells (**Treg**) and alternatively activated (**M2**) macrophages, but not by other immune cells having a more limited capacity to perform fatty acid oxidation and oxidative phosphorylation (Pearce and Pearce, 2013; O'Neill *et al.*, 2016). Collectively, immuno-modulatory properties of butyrate are known to promote IL-10 production (Corrêa-Oliveira *et al.*, 2016; Kim *et al.*, 2014). Interleukin 10 influences the renewal and migration of NAb-producing B cells to their secretory sites in mice (Baumgarth, 2011). Similar mechanisms could underlie the observed increase in IgY NAb in the present experiment.

Interestingly, the increase in IgY NAb was more pronounced when butyrate was delivered in the duodenum and jejunum in comparison to other segments. Tissue-specific differences in butyrate uptake and metabolism may explain this observation. Butyrate may have limited effects on immunity when delivered in the crop,

proventriculus and gizzard, because these locations are sparsely populated with immune cells (Smith *et al.*, 2014). When present in these proximal GIT segments, butyrate cannot reach distally located immune cells due to simple, non-ionic diffusion in the proventriculus (Moquet *et al.*, 2016b). Hence, butyrate has to be delivered beyond the proventriculus to reach the dense immune cells populations harboured by the small and large intestine. Once absorbed in the small intestine, butyrate escapes oxidation in the epithelium since avian enterocytes rely preferentially on glucose, glutamate and glutamine for energy production (Watford *et al.*, 1979). Enteric butyrate is, therefore, widely available to immune cells.

There were no significant differences among diets concerning digesta butyrate concentration in the colon/ceca in the present experiment. It is therefore difficult to compare the effect of an increasing digesta butyrate concentration in the small intestine or in the colon/ceca on immune responses of birds. One may argue that colonic butyrate is less likely to reach immune cells than enteric butyrate due to the extensive butyrate oxidation occurring in avian colonocytes (Sunkara *et al.*, 2011). On the other hand, Moquet and coworkers (2018) reported that butyrate presence in the hindgut can modify the microbiota in a way that stimulates propionate production. In mammals, propionate regulates the number and function of the colonic Treg (Smith *et al.*, 2013). This suggests that immune system homeostatis may be modulated in the colon by butyrate-induced changes in microbiota composition.

A significant linear relationship was found between IgY NAb titers and BW at 39 d of age. Avian IgY is structurally and functionally homologous to mammalian IgG (Härtle *et al.*, 2014). Physiological roles of IgY/IgG NAb are less clearly established than those of IgM NAb (Panda and Ding, 2015). Mammalian IgG NAb appear to collaborate with soluble pattern recognition receptors such as mannan-binding lectin or ficolin to facilitate the phagocytosis of bacteria under inflammatory conditions (Panda *et al.*, 2013). Other roles of IgG NAb in inflammation, immune regulation and tissue homeostasis remain unclear (Panda and Ding, 2015). It is hitherto difficult to speculate on the exact mechanisms underlying the relationship between IgY NAb titers and BWG in the present experiment.

Nutrient retention during the LPS Challenge

Zhang and coworkers (2011) reported that feeding butyrate alleviated the negative effects of i.p. LPS injection on BWG of 21 d old broilers. We could not replicate such results with 42 d old broilers despite using the identical LPS strain, injection route and dose. The intestinal immune system of broilers undergoes dynamic changes in the 1-42 d period (Schokker *et al.*, 2009; Lammers *et al.*, 2010; Simon *et al.*, 2014). Age-related differences in intestinal immune development may explain why butyrate failed to alleviate the negative effects of i.p. LPS injection on growth performance of broilers in the present study.

The fact that LPS dose was injected in the body cavity after an adjustment for BW may also explain partly such discrepancy. In fast growing broilers, the weight of the intestines relative to total BW increases during the 1-4 d post-hatch, reaches a peak at 4 d, and then decreases continuously till 42 d (Sørensen *et al.*, 2012). In other words, the size of the body cavity increases at a lower rate than the BW of the bird after 4 d

post hatch. According to our calculations, broilers subjected to the LPS challenge in the present experiment received a dose identical to those of Zhang and coworkers (2011) when expressed as mg LPS/kg BW. The dose used in our study was, however, 1.9 fold higher than the one of Zhang and coworkers (2011) when expressed as mg LPS/kg empty intestine weight. Ji and coworkers (2015) studied in 22 d old broilers the effect of different i.p. LPS injection doses on intestinal inflammation using mucosal IL-1β concentration as biomarker. A dose of 0.5 mg LPS/kg BW was found to partly reduce FI compared to an unchallenged control while a dose of 1 mg/kg BW was found to supress completely FI while triggering significantly more inflammation than lower LPS doses. Hence, inflammation induced by i.p. LPS injection may have been greater in the present study compared to the one of Zhang and coworkers (2011) due to age-related differences in GIT growth allometry. When considering i.p. LPS injection in future studies, it may be sound to adjust the LPS dose based on the predicted empty GIT weight instead of the measured BW.

In the present experiment, birds realised only half of their predicted *ad libitum* FI during the 24 h following the i.p. LPS injection (Aviagen, 2014). This observation is in line with reported anorexigenic effects of pro-inflammatory cytokines in poultry (Klasing and Johnstone, 1991). In chickens, inflammatory challenges result in increased skeletal muscle catabolism (Klasing and Austic, 1984a; Klasing *et al.*, 1987), reduced skeletal muscle synthesis as well as increased protein accretion in primary and secondary lymphoid organs (Klasing and Austic, 1984b). Such changes in protein metabolism result in a net protein loss from skeletal muscles that could explain the body weight loss and low nitrogen retention reported in the present study.

CONCLUSION

In agreement with previous broiler studies, the present work demonstrates that different butyrate additives can be used to create a contrast in luminal butyrate concentrations across GIT segments until the end of the jejunum. It is, however, more difficult to achieve consistent contrasts in butyrate concentration beyond the small intestine across studies using such additives. This may be due to the confounding influence of diet composition on endogenous microbial butyrate production. Dietary butyrate supplementation increased IgY NAb titers of birds in the present experiment. The positive effect of butyrate on IgY NAb was more pronounced when the molecule was present in the duodenum and jejunum. This indicates that tissue-specific differences in butyrate uptake and metabolism modulate the immuno-modulatory effects of the molecule. We could not replicate the findings of other researchers concerning the positive effect of dietary butyrate on specific humoral responses of birds and on their growth performance during i.p. LPS challenge. Various factors may explain such discrepancies and additional research is required to better understand the effect of butyrate on inflammatory and specific immune responses of birds.

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LITERATURE CITED

Aviagen. 2014. Ross 308 broiler perfomance objectives. Accessed Dec. 2017. http://eu.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-308-Broiler-PO-2014-EN.pdf.

Baumgarth, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat. Rev. Immunol. 11:34-46.

Berghof, T. V. L., S. A. S. van der Klein, J. A. J. Arts, H. K. Parmentier, J. J. van der Poel, and H. Bovenhuis. 2015. Genetic and non-genetic inheritance of natural antibodies binding keyhole limpet hemocyanin in a purebred layer chicken line. PLOS ONE 10:e0131088.

Boes, M. 2000. Role of natural and immune IgM antibodies in immune responses. Mol. Immunol. 37:1141-1149.

Bortoluzzi, C., A. A. Pedroso, J. J. Mallo, M. Puyalto, W. K. Kim, and T. J. Applegate. 2017. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. Poult. Sci. 96(11):3981-3993.

Chang, P. V., L. Hao, S. Offermanns, and R. Medzhitov. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc. Natl. Acad. Sci. USA 111:2247-2252.

Choct, M., Y. Dersjant-Li, J. McLeish, and M. Peisker. 2010. Soy oligosaccharides and ssoluble non-starch polysaccharides: a review of digestion, nutritive and anti-nutritive effects in pigs and poultry. Asian-Australas. J. Anim. Sci. 23:1386-1398.

Coon, C. N., K. L. Leske, O. Akavanichan, and T. K. Cheng. 1990. Effect of oligosaccharide-free soybean meal on true metabolizable energy and fiber digestion in adult roosters. Poult. Sci. 69:787-793.

Corrêa-Oliveira, R., J. L. Fachi, A. Vieira, F. T. Sato, and M. A. R. Vinolo. 2016. Regulation of immune cell function by short-chain fatty acids. Clin. Transl. Immunol. 5(4):e73.

CVB. 2007. Tabellenboek veevoeding 2007. Voedernormen landbouwhuisdieren en voederwaarde veevoeders. Centraal Veevoederbureau, Lelystad, NL.

Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Härtle, S., K. E. Magor, T. W. Göbel, F. Davison, and B. Kaspers. 2014. Chapter 6 - Structure and evolution of avian immunoglobulins. Pages 103-120 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Hu, Z., and Y. Guo. 2007. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. Anim. Feed Sci. Technol. 132:240-249.

Huyghebaert, G., R. Ducatelle, and F. V. Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:182-188.

International organization for standardization (ISO). 1998. ISO 9831:1998. Animal feeding stuffs, animal products, and faeces or urine — Determination of gross calorific value — Bomb calorimeter method. ISO, Geneva, CH.

International organization for standardization (ISO). 1999. ISO 6496:1999. Animal feeding stuffs - Determination of moisutre and other volatile matter content. ISO, Geneva, CH.

International organization for standardization (ISO). 2005. ISO 5983-1:2005. Animal feeding stuffs - Determination of crude protein content - Part 1: Kjeldahl method. ISO, Geneva, CH.

Kaiser, P., and P. Stäheli. 2014. Chapter 10 - Avian Cytokines and Chemokines. Pages 189-204 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Kim, C. H., J. Park, and M. Kim. 2014. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. Immune Netw. 14:277-288.

Klasing, K. C. 2004. The costs of immunity. Acta Zool. Sin. 50:961-969.

Klasing, K. C., and R. E. Austic. 1984a. Changes in protein degradation in chickens due to an inflammatory challenge. Proc. Soc. Exp. Biol. Med. 176:292-296.

Klasing, K. C., and R. E. Austic. 1984b. Changes in protein synthesis due to an inflammatory challenge. Proc. Soc. Exp. Biol. Med. 176:285-291.

Klasing, K. C., and B. J. Johnstone. 1991. Monokines in growth and development. Poult. Sci. 70:1781-1789.

Klasing, K. C., D. E. Laurin, R. K. Peng, and D. M. Fry. 1987. Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. J. Nutr. 117:1629-1637.

Knudsen, K. E. B., and B. W. Li. 1991. Determination of oligosaccharides in protein-rich feedstuffs by gas-liquid chromatography and high-performance liquid chromatography. J. Agric. Food Chem. 39:689-694.

Koutsos, E. A., K. C. Klasing, B. Kaspers, and P. Kaiser. 2014. Chapter 17 - Factors modulating the avian immune system. Pages 299-313 in Avian immunology (Second edition) K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Lammers, A., W. H. Wieland, L. Kruijt, A. Jansma, T. Straetemans, A. Schots, G. den Hartog, and H. K. Parmentier. 2010. Successive immunoglobulin and cytokine expression in the small intestine of juvenile chicken. Dev. Comp. Immunol. 34:1254-1262.

Li, J., Y. Hou, D. Yi, J. Zhang, L. Wang, H. Qiu, B. Ding, and J. Gong. 2015. Effects of tributyrin on intestinal energy status, antioxidative capacity and immune response to lipopolysaccharide challenge in broilers. Dev. Comp. Immunol. 28:1784-1793.

Liu, J. D., H. O. Bayir, D. E. Cosby, N. A. Cox, S. M. Williams, and J. Fowler. 2017. Evaluation of encapsulated sodium butyrate on growth performance, energy digestibility, gut development, and Salmonella colonization in broilers. Poult. Sci. 96:3638-3644.

Lochmiller, R. L., and C. Deerenberg. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87-98.

Luhrs, H., T. Gerke, J. G. Muller, R. Melcher, J. Schauber, F. Boxberge, W. Scheppach, and T. Menzel. 2002. Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. Scand. J. Gastroenterol. 37:458-466.

M'Sadeq, S. A., S. B. Wu, R. A. Swick, and M. Choct. 2015. Dietary acylated starch improves performance and gut health in necrotic enteritis challenged broilers. Poult. Sci. 94:2434-2444.

Meloche, K. J., B. J. Kerr, G. C. Shurson, and I. W. A. Dozier. 2013. Apparent metabolizable energy and prediction equations for reduced-oil corn distillers dried grains with solubles in broiler chicks from 10 to 18 days of age. Poult. Sci. 92:3176-3183.

Moore, G. E., R. E. Gerner, and H. A. Franklin. 1967. Culture of normal human leukocytes. JAMA. 199:519-524.

Moquet, P. C. A., L. Onrust, R. Van Emous, and R. P. Kwakkel. 2016a. Development and *in vitro* evaluation of protected butyrate formulations for broiler chickens. Proc. Poult. Sci. Ann. Meet. 436P (Abstr.)

Moquet, P. C. A., L. Onrust, F. Van Immerseel, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2016b. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72:61-80.

Moquet, P. C. A., S. A. Salami, L. Onrust, W. H. Hendriks, and R. P. Kwakkel. 2018. Butyrate presence in distinct gastrointestinal tract segments modifies differentially digestive processes and amino acid bioavailability in young broiler chickens. Poult. Sci. 97:167-176.

Niewold, T. A. 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poult. Sci. 86:605-609.

O'Neill, L. A., R. J. Kishton, and J. Rathmell. 2016. A guide to immunometabolism for immunologists. Nat. Rev. Immunol. 16:553-565.

Onrust, L., R. Ducatelle, K. Van Driessche, C. De Maesschalck, K. Vermeulen, F. Haesebrouck, V. Eeckhaut, and F. Van Immerseel. 2015. Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. Front. Vet. Sci. 2:75. doi: 10.3389/fvets.2015.00075.

Panda, S., and J. L. Ding. 2015. Natural Antibodies Bridge Innate and Adaptive Immunity. J. Immunol. 194:13-20.

Panda, S., J. Zhang, N. S. Tan, B. Ho, and J. L. Ding. 2013. Natural IgG antibodies provide innate protection against ficolin-opsonized bacteria. EMBO J. 32:2905-2919.

Parmentier, H. K., A. Lammers, J. J. Hoekman, G. D. V. Reilingh, I. T. A. Zaanen, and H. F. J. Savelkoul. 2004. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. Dev. Comp. Immunol. 28:39-49.

Pearce, E. L., and E. J. Pearce. 2013. Metabolic pathways in immune cell activation and quiescence. Immun. 38:633-643.

Qaisrani, S. N., P. C. Moquet, M. M. van Krimpen, R. P. Kwakkel, M. W. Verstegen, and W. H. Hendriks. 2014a. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93:3053-3064.

Rougière, N., and B. Carré. 2010. Comparison of gastrointestinal transit times between chickens from D+ and D– genetic lines selected for divergent digestion efficiency. Anim. 4:1861-1872.

Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. J. Nutr. 122:2383-2390.

Schirrmacher, V. 2017. Immunobiology of newcastle disease virus and its use for prophylactic vaccination in poultry and as adjuvant for therapeutic vaccination in cancer patients. Int. J. Mol. Sci. 18:1103.

Schokker, D., A. J. W. Hoekman, M. A. Smits, and J. M. J. Rebel. 2009. Gene expression patterns associated with chicken jejunal development. Dev. Comp. Immunol. 33:1156-1164.

Schwartz-Albiez, R., R. C. Monteiro, M. Rodriguez, C. J. Binder, and Y. Shoenfeld. 2009. Natural antibodies, intravenous immunoglobulin and their role in autoimmunity, cancer and inflammation. Clin. Exp. Immunol. 158(1):43-50.

Sikandar, A., H. Zaneb, M. Younus, S. Masood, A. Aslam, F. Khattak, S. Ashraf, M. S. Yousaf, and H. Rehman. 2017. Effect of sodium butyrate on performance, immune status, microarchitecture of small intestinal mucosa and lymphoid organs in broiler chickens. Asian-Australas. J. Anim. Sci. 30:690-699.

Simon, K., G. de Vries Reilingh, B. Kemp, and A. Lammers. 2014. Development of ileal cytokine and immunoglobulin expression levels in response to early feeding in broilers and layers. Poult. Sci. 93:3017-3027.

Sklan, D., B. Shachaf, J. Baron, and S. Hurwitz. 1978. Retrograde movement of digesta in the duodenum of the chick: extent, frequency, and nutritional implications. J. Nutr. 108:1485-1490.

Smith, A. L., C. Powers, R. K. Beal, B. Kaspers, and P. Kaiser. 2014. Chapter 13 - The avian enteric immune system in health and disease. Pages 227-250 in Avian immunology (Second edition) K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Smith, D. J., A. Barri, G. Herges, J. Hahn, A. G. Yersin, and A. Jourdan. 2012. In vitro dissolution and in vivo absorption of calcium [1-^{14}C]butyrate in free or protected forms. J. Agric. Food Chem. 60:3151-3157.

Smith, P. M., M. R. Howitt, N. Panikov, M. Michaud, C. A. Gallini, Y. M. Bohlooly, J. N. Glickman, and W. S. Garrett. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Sci. 341:569-573.

Sørensen, P., R. Labouriau, and S. Steenfeld. 2012. Allometric growth of intestine in fast and moderate growing broilers. Proc. Worlds Poult. Sci. Conf. 24:43-46.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R. G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLOS ONE 6:e27225.

Svihus, B. 2014. Function of the digestive system. J. Appl. Poutl. Res. 23:1-9.

Titus, H. W. 1956. Energy values of feedstuffs for poultry. Pages 10–14 in Proc. Semi-Annu. Mtg., Nutr. Council. Am. Feed Manufact. Assoc., St. Louis, MO.

van den Borne, J. J. G. C., M. J. W. Heetkamp, J. Buyse, and T. A. Niewold. 2015. Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. Livest. Sci. 175:96-100.

Warriss, P. D., L. J. Wilkins, S. N. Brown, A. J. Phillips, and V. Allen. 2004. Defaecation and weight of the gastrointestinal tract contents after feed and water withdrawal in broilers. Br. Poult. Sci. 45:61-66.

Watford, M., P. Lund, and H. A. Krebs. 1979. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589-596.

Yin, L., G. Laevsky, and C. Giardina. 2001. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. J. Biol. Chem. 276:44641-44646.

Zhang, W. H., Y. Jiang, Q. F. Zhu, F. Gao, S. F. Dai, J. Chen, and G. H. Zhou. 2011. Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. Br. Poult. Sci. 52:292-301.

Zhou, F., C. Vervaet, and J. P. Remon. 1996. Matrix pellets based on the combination of waxes, starches and maltodextrins. Int. J. Pharm. 133:155-160.

Zhou, Z. Y., B. Packialakshmi, S. K. Makkar, S. Dridi, and N. C. Rath. 2014. Effect of butyrate on immune response of a chicken macrophage cell line. Vet. Immunol. Immunopathol. 162:24-32.

Chapter 8

General discussion

INTRODUCTION

There is an apparent consensus among current-day scientists to describe butyrate as a health-promoting molecule for humans (Canani *et al.*, 2011), poultry (Dethlefsen *et al.*, 2007; Moquet *et al.*, 2016), pigs and other monogastric farm animals (Guilloteau *et al.*, 2010a; Kaiser and Stäheli, 2014). Butyrate is portrayed in both the scientific and non-scientific literature as a molecule that elicits **unique and beneficial effects in the gut** (Textbox 1). Butyrate was discovered in 1815 and characterized in 1817 by the chemist E.M. Chevreul (Chevreul, 1823). Until the late 1960s, the physiological effects of butyrate remained largely unknown and butyrate was described as a potentially toxic pollutant present in the smoke of cigarettes (Gold *et al.*, 1946).

Textbox 1. BUTYRATE IN THE SCIENTIFIC AND NON-SCIENTIFIC LITERATURE

Scientific journals

When considering signals derived from bacterial fermentation, butyric acid has been shown to be the main driving force toward an optimal gut health. Onrust *et al.*, 2015 in Frontiers in Veterinary Sciences

Among these [short chain fatty acids], *butyric acid possesses interesting characteristic features.* (Ahsan *et al.*, 2016) in World's Poultry Science Journal

Online animal nutrition magazines

SCFA, butyrate in particular, have a strong capacity to enhance [...] *critical components of the animal's innate immunity.* Byrne, 2017 on Feed Navigator

Multifarious effect of butyrate on intestinal health: [...] *Butyrate is a short chain fatty acid naturally produced in the digestive tract by the fermentation of fibres.* Ahsan, 2016 on the Poultry Site

Feed additives companies communications

Butyrate is somewhat unique among its biological functions when compared with other short chain fatty acids. Nutriad

Butyrate is [a] *very important energy source for the villi, and plays a vital role in balancing a healthy intestinal flora.* Palital

Newspaper articles

[Goat butter] *contains a short-chain fatty acid called butyrate which may also enhance intestinal barrier function - i.e. gut health.* Chalmers, 2017 in the Daily Mail

Ghee also has butyric acid, shown to support healthy digestion. Seidenberg, 2017 in the Washington Post

A renewal for the interest in butyrate research was sparked in the late 1970s when butyrate was identified as a potential chemo-therapeutic agent in humans (for review, see Prasad and Sinha, 1976). The physiological effects of butyrate have been more thoroughly investigated than those of other short chain fatty acids (**SCFA**) as a consequence of the prime discovery of its potent anti-tumorigenic properties in humans and associated animal models (Fung *et al.*, 2012). The initial findings summarized by Prasad and Sinha (1976) initiated an era of unprecedented activity in the history of butyrate research that is, to date, ongoing (Figure 1). There were nearly three times more scientific articles published on butyrate in 2017 than in the first 150 years that followed its discovery. Numerous research teams studied the regulatory effect of butyrate on digestive functions, immune responses, cell proliferation and gut integrity using animal models and human cell cultures (for review, see Guilloteau *et al.*, 2010a).

In the wake of these intense scientific developments, Mroz and colleagues (1997) started to evaluate the effect of dietary butyrate supplementation on the growth performance of swine in the late

1990s. At that time, there were only a few studies investigating the effect of organic acids on the growth performance of poultry (Dibner and Buttin, 2002). Such studies were mostly focused on fumaric acid and the growth-promoting potential of butyrate was unexplored (Dibner and Buttin, 2002). As a matter of fact, butyrate was merely considered for its influence on voluntary feed intake of chickens (Pinchasov and Jensen, 1989). The results of Mroz and colleagues (1997) in swine prompted poultry nutritionists to investigate the effect of dietary butyrate supplementation on the growth performance of poultry. To the best of my knowledge, 2005 was the year when the first *in vivo* studies investigating the effect of butyrate on the health and growth performance of broiler chickens were published (Leeson *et al.*, 2005; Van Immerseel *et al.*, 2005a). Such pioneering works did not go unnoticed and, in the following decade, no less than 90 *in vivo* experiments investigated the effect of dietary butyrate supplementation on the growth performance of chickens (Polycarpo *et al.*, 2017). This body of literature has established butyrate as a relatively unique and beneficial molecule in the eyes of poultry nutritionists (Onrust *et al.*, 2015; Ahsan *et al.*, 2016; Moquet *et al.*, 2016).

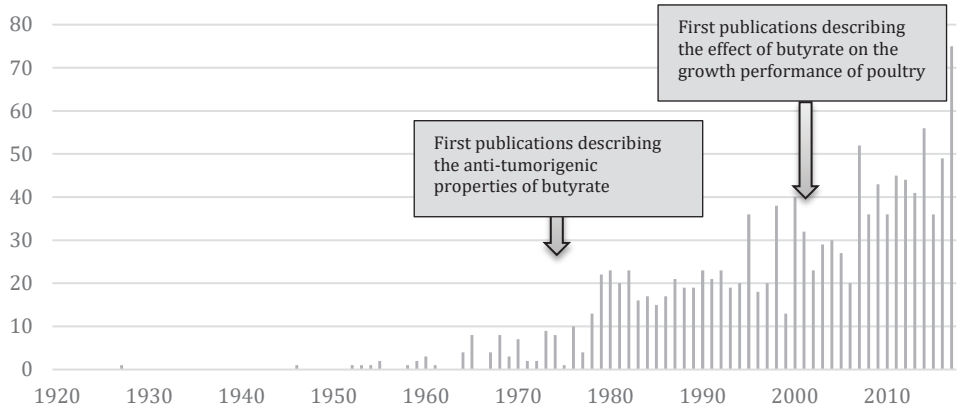


Figure 1. Number of scientific publications having a title containing 'butyrate' between 1823 and 2017. No data are shown between 1823 and 1920 because there were no results. Data generated using the search engine of Sciencedirect (https://www.sciencedirect.com/science/search)

Nowadays, the positive image of butyrate has overstepped the bounds of the scientific community. Increasing butyrate presence in the intestines by eating specific fibres or butyrate-containing food items is widely perceived by food manufacturers and consumers as a good strategy to correct health issues that may arise from the consumption of heavily processed western diets (Textbox 1; Fung *et al.*, 2012).

The perception of butyrate by scientists has swung from neutral to bad to good over time, like a pendulum (Figure 2). The very first reported research endeavours mentioning butyrate were non-judgmental as they only considered the chemical structure of the molecule. Initial research into the physiological effects of butyrate portrayed the molecule negatively as a toxic pollutant present in the smoke of cigarettes. This view changed gradually as several researchers proposed to use butyrate as a chemotherapeutic agent. In other words, butyrate became a toxic substance that could have some usefulness in human medicine. This change in paradigm opened an era of intense investigations on the physiological effects of butyrate. As a result of such

investigations, the perception of butyrate evolved further and, nowadays, butyrate is seen as a molecule having unique and beneficial effects for intestinal health in both humans and non-ruminant farm animals (Textbox 1).

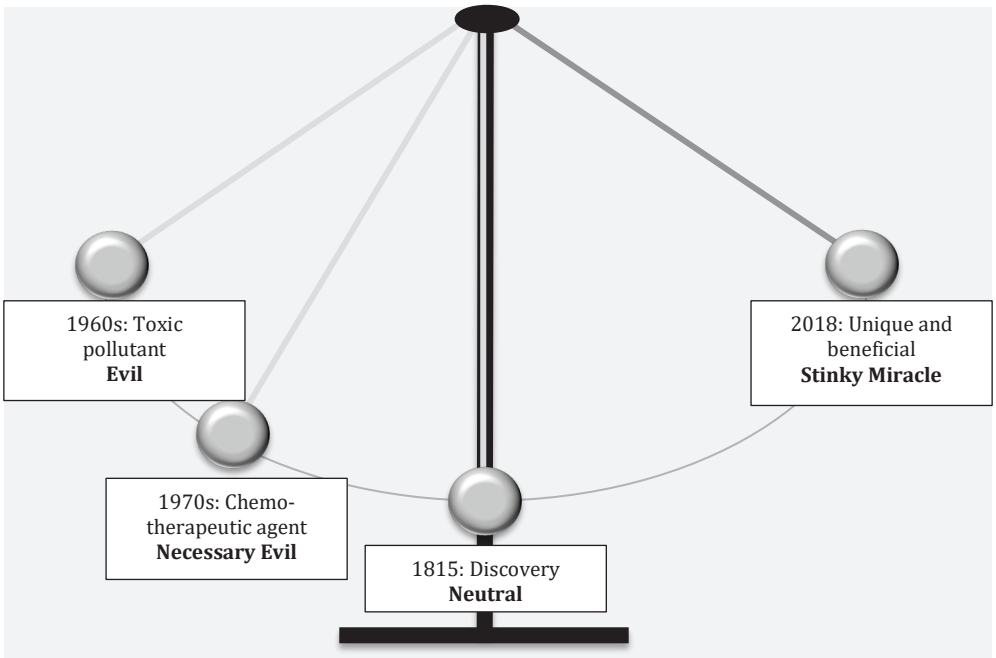


Figure 2. Depiction of the perception of butyrate by scientists from its discovery to the present

Yet, butyrate has not always achieved all the multifarious, positive effects in a consistent manner in mammals and poultry (Moquet *et al.*, 2016). As a result, reviews related to this molecule sometimes contain interrogative titles, e.g. '*The butyrate story: old wine in new bottles?*' (Scheppach and Weiler, 2004) and '*Butyrate: what is the future for this old substance?*' (Sossai, 2012).

The aim of this thesis was to ascertain the effect of dietary butyrate supplementation on the digestive function, microbiota composition and immune responses of broilers. In addition, the aim of this thesis was to verify whether such effects were conditioned by the gastrointestinal tract (**GIT**) segment wherein butyrate is present.

Proven and putative mechanisms underlying the effects of butyrate in the avian GIT were identified through a literature review (Chapter 2). This review highlighted a potential role of butyrate presence in the digesta of distinct GIT segments in triggering specific effects. An *in vitro* model was developed to compare the release profile of commercially available and experimental butyrate additives (Chapter 3). Based on the outcome of this *in vitro* screening, several additives were included in two *in vivo* trials (Chapters 4 and 7). Samples collected during these two trials allowed detailed investigation of the effect of butyrate on digestive processes (Chapter 4), energy metabolism and endocrine regulation of the gut (Chapter 5), microbiota composition (Chapter 6) and immune responses (Chapter 6 and 7) of broiler chickens. In this final Chapter, the results of the *in vitro* and *in vivo* studies are integrated and discussed.

From the literature review (Chapter 2), a popular view among scientists emerged. Several observations reported in the present Thesis, however, do not agree with this popular view that depicts butyrate as a molecule that exerts systematically beneficial effects on growth performance (e.g. observed reduction in amino acid digestibility in Chapter 4) and the health status of birds (e.g. observed dysbiosis that resulted in inflammation in Chapter 6). Hence, in this final Chapter I will revisit the popular view that portrays butyrate as a molecule having unique and beneficial properties in the gut of poultry. In the first part of the discussion, I have assessed the uniqueness of the multifarious effect of butyrate by comparing its physiological effects to those elicited by other SCFA and by describing the microbial fermentation pathways that contribute to the production of SCFA including butyrate. In the second part, I have discussed whether the effects elicited by butyrate are beneficial for the health and growth performance of broilers.

PART I:
EXPLAINING THE MULTIFARIOUS EFFECTS OF BUTYRATE

Main messages:

- Acetate and propionate may replicate the physiological effects of butyrate with various degrees of efficacy
- Conditions that contribute to butyrate production can be either positive or negative to the host, depending on the location and substrate considered
- Host-microbiota crosstalk is more likely to be driven by the integration of various signals (e.g. short chain fatty acids, pathogen associated molecular patterns, biogenic amines, hydrogen sulphide) than being governed uniquely by microbial butyrate production

INTRODUCTION

At the beginning of this millennium, Gibson (2000) stated "*A vexing question has been how this small molecule exerts such a wide array of effects*". Concerted efforts of different research teams addressed over the subsequent years this question and have unravelled many mechanisms underlying the multifarious effects of butyrate on prokaryotic and eukaryotic cells (For review in poultry see Ahsan *et al.*, 2016; Moquet *et al.*, 2016 and in mammals Guilloteau *et al.*, 2010a; Meijer *et al.*, 2010; Astbury and Corfe, 2012). A logical follow-up question would be: "*Why does this small molecule exerts such widespread effects?*"

Intricate relationships exist between the host and the microbial meta-communities residing in distinct GIT segments. Such relationships can be characterized by the classical ecological concepts, e.g. parasitism, commensalism or mutualism (Ley *et al.*, 2006). The diversity of relationships that may occur between the host and the microorganisms populating distinct segments of the GIT bears significant implications for poultry health and energy harvest (Gabriel *et al.*, 2006). Positive and negative feedback loops exist between the host and the microbial meta-communities populating distinct GIT segments. The succession of feedback loops constitutes a crosstalk that favours the onset of a healthy steady state (Lozupone *et al.*, 2012). At a mucosal level, host-microbiota crosstalk is mediated by pathogen-associated molecular patterns (PAMP) and by microbial metabolites (e.g. SCFA; Wells *et al.*, 2011).

In nature, butyrate is produced in the GIT of vertebrates as a metabolite by the microbiota (Guilloteau *et al.*, 2010a) One could argue that the multifarious effects exerted by the presence of butyrate in the GIT indicates that this molecule has been selected through evolutionary processes as a key mediator of host-microbiota crosstalk. This theory would require three pieces of empirical evidence:

(1) Proposed mechanisms mediating host responses to butyrate presence have to be butyrate-specific or, at least, butyrate should be the most potent trigger when compared to other SCFA
(2) Microbial butyrate production needs to reflect the presence and activities of specific microbial meta-communities that are of evolutionary interest for the host (i.e. influence reproductive fitness or survival)
(3) Host's responses to butyrate presence have to be coherent with the microbial meta-community that contributed to butyrate production (i.e. host's responses to butyrate-feedback should be meaningful)

Due to a dearth of avian research covering such fundamental issues, this section will focus on work conducted on more thoroughly investigated mammal species such as pigs, mice and humans. As a consequence, the validity of argument extrapolation from mammals to avian species has also to be addressed.

ASSESSING THE SPECIFICITY OF BUTYRATE-INDUCED HOST RESPONSES

Epigenetic effects

Epigenetic effects of butyrate are mediated by its histone deacetylase (**HDAC**) inhibitory properties, as demonstrated in human cell cultures (Riggs *et al.*, 1977; Sealy and Chalkley, 1978). Butyrate is the most potent HDAC inhibitor among SCFA, followed by propionate and then acetate as assessed with human cell cultures (Cousens *et al.*, 1978; Sealy and Chalkley, 1978; Waldecker *et al.*, 2008). Little research has been conducted to compare the respective merits of each SCFA in modulating gene expression. HDAC classes that are inhibited by acetate and propionate are, to the best of my knowledge, unknown. Butyrate starts to act as an HDAC inhibitor when present at the 0.01 mM concentration (Cousens *et al.*, 1978). This concentration is in the range of those observed for intracellular butyrate across GIT segments of broilers (Chapter 5).

Anti-inflammatory properties

The anti-inflammatory effect of butyrate has been extensively studied over the past decades in a variety of animal species including humans (Segain *et al.*, 2000), pigs (Grilli *et al.*, 2016), mice (Jiminez *et al.*, 2017) and chickens (Li *et al.*, 2015; Bortoluzzi *et al.*, 2017). Much less is known, however, about the anti-inflammatory properties of acetate and propionate. Butyrate's anti-inflammatory properties are, among others, mediated by its inhibitory effect on the cytoplasmic IκB kinase enzyme that results in a reduced nuclear factor κB (**NF-κB**) pathway activity (Segain *et al.*, 2000; Yin *et al.*, 2001; Luhrs *et al.*, 2002). Propionate has also been reported to exert similar effects (Zapolska-Downar and Naruszewicz, 2009). The potency of SCFA to inhibit NF-κB pathway of murine colon cells *in vitro* is as follows: butyrate > propionate > acetate (Tedelind *et al.*, 2007). The half maximal effective concentration of butyrate to inhibit NF-κB activation is 0.06 mM (Tedelind *et al.*, 2007). This concentration is in the range of those observed for intracellular butyrate across GIT segments of broilers (Chapter 5).

Free fatty acid receptors activation

Free fatty acid receptors (**FFAR**) 2 and 3, respectively, were referred to as G-protein coupled receptor (**GPR**) 43 and 41 up until 2003 (Kaji *et al.*, 2014). Several attempts have been made to compare, on a molar basis, the capacity of SCFA to activate FFAR2 and FFAR3. It was concluded that for FFAR2 the order of activation is: acetate = propionate > butyrate while that of FFAR3 it is: propionate > butyrate > acetate (Brown *et al.*, 2003; Le Poul *et al.*, 2003). However, Kaji and coworkers (2014) also suggested that ligand specificity may vary across species for both FFAR2 and FFAR3. The G-protein coupled receptor GPR109a is known to be activated by butyrate (Singh *et al.*, 2014) but, to the best of my knowledge, it is unknown whether GPR109a can also be activated by other SCFA. Only FFAR2 has been identified in the chicken genome and its expression seems to be reduced by the presence of butyrate in gastric and enteric digesta (Chapter 6).

G-cells secretory activities

Gastrin is a peptide hormone produced by G-cells. In chickens, these cells are mostly located in the pyloric region and to a lower extent in the duodenum (Rawdon and Andrew, 1999). Gastrin release is triggered by dietary compounds such as calcium, amino acids or amines (Dockray *et al.*, 2001) but not by SCFA despite the presence of FFAR3 in 90% of the G-cells of mice (Nohr *et al.*, 2013). This confirms that expression of FFAR2 and FFAR3 does not necessarily indicate a cellular response to SCFA sensing (Liou, 2013). Nonetheless, dietary supplementation with butyrate (3 g/kg DM) has been reported to increase the expression of gastrin mRNA in the pyloric mucosa of weaned piglets (Mazzoni *et al.*, 2008). It has been postulated that the autonomic nervous system can transmit a butyrate-induced nervous signal from the hindgut to the central nervous system, resulting in the release of trophic signals such as gastrin (Sakata, 1989; Reilly *et al.*, 1995). This mechanism has been scarcely investigated. It is hitherto impossible to state if it is butyrate-specific or not. In the present thesis, there was no evidence at the transcriptional level that butyrate presence in the gastric region affected the secretory activity of G-cells (Chapter 5)

I-cells secretory activities

Cholecystokinin (**CCK**) is a peptide hormone produced by I-cells. In chickens, I-cells are located in the proximal half of the small intestine (Rawdon and Andrew, 1999). Up until the end of the second millennium, SCFA were thought to be unable to trigger CCK release (Liddle, 1997; McLaughlin *et al.*, 1998). This view was challenged by reports indicating that supplementing diets with unprotected butyrate (3 g/kg, as fed basis) increased circulatory CCK levels in piglets (Kotunia *et al.*, 2004) and calves (Guilloteau *et al.*, 2010b). Pancreatic enzyme outflow can be considered as indicative of circulatory CCK level. Such outflow has been shown to be positively influenced by ileal infusion of butyrate and propionate (5 mM and 7.5 mM, respectively) in growing pigs (Sileikiene *et al.*, 2005). Interestingly, the outflow was unchanged compared to a control when butyrate concentration was increased to 10 mM or even significantly reduced when priopionate was infused at 15 mM. Finally, pancreatic enzyme outflow was decreased by acetate (85 and 170 mM). Such effects could be attributed to the presence of FFAR3 in I-cells (Nohr *et al.*, 2013). Collectively, these results indicate that butyrate is not the sole SCFA having effect on CCK release and that release location, dose and species should be taken into consideration when discussing this effect. We observed that, in broilers, the presence of butyrate in the digesta of the small intestine increased the expression of the CCK prohormone (Chapter 5)

L-cells secretory activities

Localization of L-cells. Peptide YY (**PYY**) and glucagon-like peptides (**GLP**) are peptide hormones produced by L-cells. In mammals, L-cells are localized in the distal ileum and in the colon (Liou, 2013; Kaji *et al.*, 2014). The distribution of L-cells seems to be different in chickens. In birds, L-cells are equally present in the duodenum and ileum, as indicated by PYY positive cell counts (Rawdon and Andrew, 1999). Both FFAR2 and FFAR3 are expressed in L-cells (Liou, 2013). Recent evidence supports a role of FFAR2 sensing for GLP-1 release (Lin *et al.*, 2012; Psichas *et al.*, 2015) whilst acetate did not significantly increase PYY. Similarly, ileal SCFA infusion did not change

PYY levels in portal blood whereas colonic infusion resulted in a significant increase in PYY release (Fu-Cheng *et al.*, 1995). Collectively, these results indicate that the small intestine reacts indistinctly to abnormal elevated concentrations of propionate or butyrate in rats. In contrast, physiological concentrations of these SCFAs in the colon can induce PYY release, with a markedly higher release observed for butyrate.

GLP-1 release. Concerning GLP-1, a release was observed in mice after oral dosing with butyrate (400 mg/kg BW) but an identical dose of propionate did not elicit any endocrine change (Lin *et al.*, 2012). Colonic administration of propionate (180 mmol/min with a total of 0.45 mmol) resulted in a significant release of GLP-1 in rats. In contrast with these results, acetate, propionate and butyrate infusion in an isolated rat colon at physiological (5 mM) and supraphysiological concentrations (20 and 100 mM), did not increase GLP-1 in the portal blood (Plaisancie *et al.*, 1995). Factors such as dose, administration route and specie-specific ligand affinity may, therefore, have a confounding influence on the observed effects of SCFA presence in the hindgut on GLP-1 release. In the present thesis, we did not find any evidence at the transcriptional level that butyrate presence in the small intestine and colon affected the secretion of GLP-1 (Chapter 5)

FFAR3 sensing mediates PYY release (Liou, 2013). It is, therefore, likely that butyrate can trigger PYY and GLP-1 release. Ligand affinity of FFAR also indicates that this capacity is not butyrate-specific, with other SCFA being more potent triggers (e.g. propionate for PYY release through FFAR3) (Brown *et al.*, 2003). The body of published evidences illustrates the difficulty to generalize the effect of butyrate sensing on GLP-1 and PYY release.

PYY release. When considered individually, the SCFA concentration seldom reaches the 1-5 mM range in the in the digesta of the small intestine. When infused in this range in the ileum of rats, SCFA failed to induce an increase in PYY release. Higher concentrations of propionate and butyrate (20 mM) induce a transient release of PYY in portal blood (Dumoulin *et al.*, 1998). Provision of butyrate (0.5 to 5 mM) to the isolated colon of rats and rabbits increases the release of PYY in the portal blood in a dose-dependent manner (Longo *et al.*, 1991; Plaisancie *et al.*, 1996). Propionate was reported to be less potent in eliciting a PYY release than butyrate whilst acetate did not significantly increase PYY. Similarly, ileal SCFA infusion did not change PYY levels in portal blood whereas colonic infusion resulted in a significant increase in PYY release (Fu-Cheng *et al.*, 1995). Collectively, these results indicate that the small intestine reacts indistinctly to abnormal elevated concentrations of propionate or butyrate in rats. In contrast, physiological concentrations of these SCFA in the colon can induce PYY release, with a markedly higher release observed for butyrate.

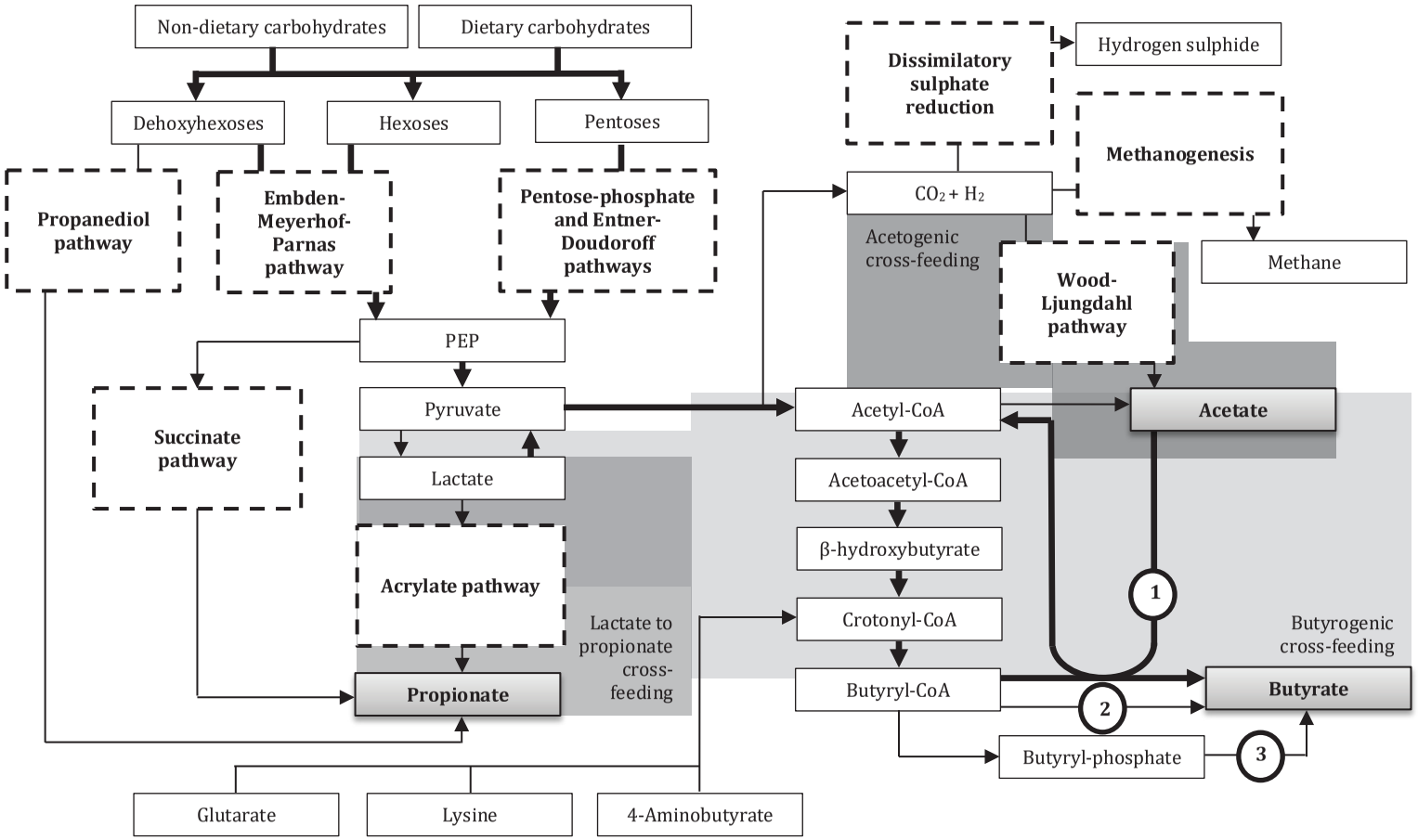
Conclusion: Acetate and propionate may replicate the effect of butyrate with various degrees of efficacy

Table 1. Potency of different short chain fatty acids to elicit effects observed when butyrate is present in the digestive tract of mammals

Butyrate-induced effect	Proposed mechanism[1]	Butyrate specificity	SCFA potency ranking[2]
Changes in gene expression	HDAC inhibition	No	B>P>A
Reduced inflammation	NF-κB inhibition	No	B>P>A
G-cells activity	Gastrin secretion: Hindgut derived mechanisms mediated by FFAR-sensing and nervous system	Unclear	Unknown
I-cells activity	CCK secretion: Stomach-derived mechanism mediated by nervous or peptides intermediaries	Unclear	Unknown
L-cells activity	PYY secretion: FFAR3 sensing	No	P>B>A; possibly specie-dependent
	GLP-1 secretion: FFAR2 sensing	No	A=P>B; possibly specie-dependent

[1]HDAC: Histone deacetylase, NF-κB: Nuclear factor κB, FFAR: Free fatty acid receptor, CCK: Cholecystokinin, PYY: Peptide YY, GLP-1: Glucagon like peptide 1.
[2]B: Butyrate, P: Propionate, A: Acetate.

It can be concluded that none of the butyrate-induced effects are proven to be a unique feature of butyrate (Table 1). Changes in gene expression and anti-inflammatory effects, however, do seem to be more marked with butyrate than with propionate and acetate. Underlying mechanisms mediating the release of gastrin and CCK are unclear. It is, therefore, impossible to determine if such effects are butyrate-specific. On the other hand, release of GLP-1 and PYY hormones is mediated by FFAR2 and 3 triggering, respectively. Such receptors can be activated by other SCFA, with a SCFA potency ranking that could be specie-specific. This indicates that GLP-1 and PYY responses can be triggered by other SCFA than butyrate. As a consequence, most of the effects of butyrate on eukaryotic cells can be replicated with other SCFA as well, with various degrees of efficacy.



Solid line boxes indicate chemical compounds. Heavy dashed boxes indicate pathways. Thick arrows indicate the main butyrate production pathway. Final conversion of butyryl-CoA to butyrate can be achieved through butyrogenic cross-feeding (1), classical pathway (2) or by the butyryl-phosphate pathway (3). PEP: Phosphoenol pyruvate.

Figure 3. Fermentation pathways leading to the production of acetate, propionate and butyrate

MICROBIAL PATHWAYS LEADING TO THE PRODUCTION OF BUTYRATE

Fermentation is the metabolic processing of different substrates, e.g. fibres or proteins, by microbial enzymes. Fermentation results in the production of various metabolites, including butyrate. If butyrate has been selected by evolutionary processes as a key mediator of intestinal host-microbiota crosstalk, then butyrate production must reflect microbiota conditions that are of evolutionary interest to the host.

Carbohydrate fermentation

Carbohydrates of dietary and non-dietary origin can be fermented in the GIT (Figure 3). During carbohydrate fermentation, primary reactions consume oxidized pyridine nucleotides (i.e. soluble coenzymes: NAD^+, $NADP^+$) and produce substantial amounts of their reduced equivalents (i.e. NADH, NADPH). The progression of carbohydrate fermentation is, therefore, partly conditioned by the replenishment of oxidized pyridine nucleotides pools (Cummings and Branch, 1986), which is predominantly achieved by hydrogen (**H_2**) formation (Macfarlane and Macfarlane, 2003). Due to thermodynamic constrains, H_2 formation is facilitated by low hydrogen partial pressure (den Besten *et al.*, 2013). Hence, H_2 removal is critical for the maintenance of the redox state of the pyridine nucleotides pool and, consequently, for the progression of carbohydrate fermentation processes.

H_2 removal can be achieved by hydrogenotrophic acetogenesis, methanogenesis or dissimilatory sulphate reduction (Gibson *et al.*, 1993;; Sahakian *et al.*, 2010; Louis *et al.*, 2014; Figure 3). The relative importance of these pathways is influenced by various factors, including thermodynamic properties (Sahakian *et al.*, 2010), pH (Gibson *et al.*, 1990), passage rate (Sahakian *et al.*, 2010) or the presence of chemical compounds other than H_2 such as sulphur amino acids (Takahashi *et al.*, 1997) or lactate (Dar *et al.*, 2008). In birds, the relative importance of dissimilatory sulphate reduction and acetogenesis in cecal hydrogen removal seems to be increased when butyrate is present in the gastric region (Chapter 6).

When not restrained by H_2 partial pressure, primary fibre fermentation results in the formation of large amounts of phosphoenol pyruvate, which is converted to pyruvate (Figure 3). A large proportion of pyruvate is converted to acetyl-CoA in a reaction that also generates H_2 and CO_2 (den Besten *et al.*, 2013). Acetyl-CoA is a precursor used in butyrate synthesis, leading to the production of butyryl-CoA in a four step reaction that is remarkably similar to the β-oxidation of fatty acids observed in eukaryotes (Bennett and Rudolph, 1995). This pathway, referred to as acetyl-CoA pathway, is the most prevalent in the human hindgut (Vital *et al.*, 2014). Final conversion of butyryl-CoA to butyrate can be achieved through three distinct pathways. In the classical pathway, butyryl-CoA is converted to CoASH, butyrate and ATP (den Besten *et al.*, 2013). Alternatively, butyryl-CoA can be converted to butyryl-phosphate and then to butyrate in a pathway that seems restricted to a few bacterial species (Louis *et al.*, 2014). Finally, the butyrogenic cross-feeding pathway involves the condensation of butyryl-CoA with exogenous acetate to form butyrate and acetyl-CoA. The butyrogenic cross-feeding pathway seems to predominate over other pathways in the conversion of butyryl-CoA to butyrate in the human hindgut (Duncan *et al.*, 2002).

Summarizing, butyrate originates mostly from the fermentation of carbohydrates by commensal gut bacteria present in the colon and ceca of non-ruminant species (e.g. *Faecalibacterium, Roseburia, Butyrivibrio*; Anand *et al.*, 2016). This production involves bacterial cross-feeding and requires, therefore, a diverse and stable microbiota (Onrust *et al.*, 2015). In poultry, bacterial diversity is lower in the crop, gastric region and small intestine than in more distal GIT segments (Pedroso and Lee, 2014). As a consequence, digesta butyrate concentration is very low in the proximal GIT of broilers that are not supplemented with dietary butyrate (Chapters 4 and 7). In addition to butyrate, carbohydrate fermentation can also generate other SCFA such as acetate and propionate (Figure 3).

Amino acid fermentation

Next to carbohydrate fermentation, amino acid (**AA**) fermentation can also result in the production of butyrate. The production of butyrate from AA fermentation requires additional pathways that are coined after their respective precursors, namely glutarate, lysine and succinate/4-aminobutyrate (Herrmann *et al.*, 2008). Each pathway consists in a specific set of enzymes that catalyse the transformation of their respective substrate to crotonyl-CoA. Crotonyl-CoA is then converted to butyryl-CoA (Figure 3). Glutarate is produced during the catabolism of several AA including lysine and tryptophan while succinate originates from the fermentation other AA such as arginine (Anand *et al.*, 2016). When originating from AA fermentation, the production of butyrate is accompanied by the release of potentially harmful metabolites such as ammonia or biogenic amines (Anand *et al.*, 2016). Hence, butyrate may occasionally originate from the fermentation of essential amino acids by butyrogenic gut pathogens (e.g. *Fusobacterium*; Anand *et al.*, 2016). Butyrate production is, therefore, not an absolute indicator of gut health. Ratios between different microbial metabolites may provide more relevant information on the potential effect of microbiota activities on host health. A beneficial microbiota may, for instance, be indicated by a high butyrate production and, concomitantly, low ammonia and biogenic amines production.

COHERENCE OF HOST RESPONSES TO GUT CONDITIONS THAT CONTRIBUTE TO BUTYRATE PRODUCTION

Butyrate signalling in the *proximal* GIT of broilers

Two separate situations should be considered when discussing the relevance of butyrate production as an indicator of microbiota activities to the host. Butyrate is present at very low concentrations in the digesta of the crop, gastric region and small intestine of birds that are not supplemented with dietary butyrate (Chapters 4 and 7). Diverse and stable microbiota activities are required to produce butyrate (Onrust *et al.*, 2015; Figure 3). Hence, butyrate production in proximal GIT segments such as the crop, gastric region or small intestine of birds may indicate a complex microbiota and, consequently, a competition between the host and local meta-communities of microbiota for the use of digestible carbohydrates and AA, as described in pigs (Gaskins, 2008). The presence of butyrate in such proximal GIT segments signals, therefore, a potential detrimental microbiota overgrowth. The host increases, in response to such signalling, intestinal retention time (Chapter 4), pepsin output (Chapter 4; Qaisrani *et al.*, 2014)), host defence peptides gene expression (Chapter 6; Sunkara *et al.*, 2011; Sunkara *et al.*, 2014), mucus gene expression (Chapter 6) and, sometimes villus length (for review, see Chapter 2). The host reduces, at the same time feed intake (Chapters 4 and 7) and experiences inflammation (Chapter 6). Such physiological changes evoke an attempt to improve digestive efficiency (retention time, gut morphology and pepsin secretion) while trying to deal with microbial overgrowth using local and systemic mechanisms (local mechanisms: increased mucus and host defence peptides; systemic mechanisms: reduced feed intake, recruitment of leukocytes as a result of inflammatory chemotaxis). Overall, physiological changes induced by the presence of butyrate in the proximal GIT may be positive for growth performance of poultry (Chapter 2) but they originate, nonetheless, from the perception of a negative signal by the host. The dietary interventions reported in Chapter 4 resulted in changes in digesta butyrate concentration in the gastric region, duodenum and ileum that were sufficient to reduce the expression of levels of FFAR2 (Chapter 5). This may indicate that the signalling was so strong that expression of FFAR2 receptor in gut cells was reduced, as is seen for instance with insulin resistance in the context of type II diabetes (Krupp and Lane, 1981). The tolerance threshold to increases in digesta butyrate concentration may, therefore, be low in the crop, gastric region and small intestine of poultry. Like butyrate, propionate is scarcely present in the digesta of the proximal GIT of poultry (Chapters 4 and 7) and requires a diversified and stable microbiota to be produced (Figure 5). In rats, L-cells seems to react indistinctly to the presence of butyrate and propionate in the digesta of the small intestine (Dumoulin *et al.*, 1998). This may indicate that, in the proximal GIT of poultry, propionate could induce similar responses as butyrate.

Butyrate signalling in the *distal* GIT of broilers

In birds, the ceca and colon are major fermentation sites (Józefiak *et al.*, 2004) Hence, butyrate concentration is much higher in the colon and ceca than in more proximal GIT segments (Control groups in Chapters 4 and 7). Cecal and colonic butyrate production results mostly from the fermentation of undigested carbohydrates by commensal gut bacteria (Onrust *et al.*, 2015; Anand *et al.*, 2016). Thus, butyrate can be considered to be a double messenger molecule. Not only is it indicative for microbial

activities that can be detrimental to the host when present in the crop, gastric region and small intestine of birds, but when present in the colon and ceca, butyrate can be a signalling molecule for a cooperative relationship between the host and the microbiota. Butyrate may, occasionally, also originate from the fermentation in the distal GIT of nutrients that are critical for growth of the host (e.g. essential AA or starch; Anand *et al.*, 2016). Butyrate signalling in the distal GIT of poultry is, therefore, nonspecific. Such signalling results in increased intestinal retention time (Chapter 4), possibly due to changes in L-cell secretory activities (Onrust *et al.*, 2015). Such response does not seem to induce any inflammation (Chapter 6). Hence, the distal GIT seems to tolerate higher butyrate levels than the proximal GIT. It is also likely that, in the distal GIT, detrimental shifts in microbiota activity are not only signalled by changes in SCFA levels, but also by concomitant increases in other metabolites such as biogenic amines or ammonia.

Textbox 2. SPECIFICITIES OF CHICKENS COMPARED TO PIGS
Chickens are not mice with feathers
J. Kaufman

Digestive physiology

Existence of reverse peristalsis (reflux)

Presence of a crop and muscular stomach (gizzard)

Transport of lipids to the liver by portomicrons

Short transit time

Microbiota

Paired ceca as primary fermentation organs

Very short colon

Lactic fermentation in the crop

Immune responses

Higher resistance to the toxic effects of LPS compared to mammals

In ovo transfer of maternal IgG, IgM and IgA

B cells maturation occurs in the bursa of Fabricius

Existence of several cytokines still controversial

No neutrophils but presence of heterophils

Chickens lack encapsulated lymph nodes but have lymphoid nodules along the course of lymphatics

Very confined period of developmental oral tolerance (5-10 d post-hatch)

Butyrate is not the only metabolite mediating host-microbiota crosstalk

Our current knowledge indicates that, in mammals, acetate and propionate may replicate the effect of butyrate with various degrees of efficacy. In other words, butyrate does not seem to trigger unique responses in the gut of mammals. The conditions that contribute to butyrate production in the hindgut can be either positive or negative for the host, depending on the location and substrate considered. It is, therefore, unlikely that butyrate is a unique compound which has been selected through evolutionary processes to be a central molecule for host-microbiota crosstalk. Such crosstalk is more likely to be driven by the integration of various signals (e.g. SCFA, PAMP, biogenic amines, hydrogen sulphide, etc). It is known that birds differ from other monogastric farm animals such as pigs in many aspects of their physiology (Textbox 2). The absence of a clear host-microbiota crosstalk based solely on the existence butyrate in mammals does not incline me to think that such relationship exists in poultry.

PART II:
ASSESSING THE BENEFITS OF BUTYRATE

Main messages:

- Presence of butyrate in the gastric region and small intestine can induce dysbiosis and inflammation
- Immunomodulatory effects of butyrate are inconsistent and their relevance in improving the health performance of broilers is conditioned by the type of challenge encountered
- Butyrate sensing induces hormonal changes that can increase the digestibility of amino acids, fat and energy while reducing feed intake
- Butyrate may affect calcium and phosphorus metabolism in broilers
- Intracellular butyrate concentration is tightly regulated by butyrate transport and oxidation, possibly to avoid cytotoxic effects
- Endogenous production of butyrate is a major confounding factor in studying the effects of exogenous butyrate supplementation
- The industry could consider supplementing broiler diets with fat coated butyrate in the starter phase and with butyrogenic fibres in the grower/finisher phases

INVESTIGATING HOST RESPONSES TO BUTYRATE PRESENCE IN DIFFERENT GIT SEGMENTS

How to discriminate between endogenous and exogenous butyrate?

The signalling effect of butyrate may vary depending on the GIT segment considered (Chapter 2). Feeding butyrate additives with markedly different release kinetics may allow the study of such location effects in poultry. Release profiles of five experimental butyrate formulations and of five commercially available butyrate additives were evaluated in a novel *in vitro* method in Chapter 3. Changes in digesta butyrate concentration along the GIT of broilers reported in Chapter 4 were consistent with the *in vitro* release patterns of the butyrate additives used. Changes in digesta butyrate concentration reported in Chapter 7, however, were in constrat with the *in vitro* release patterns of the additives butyrate used. Such differences may be attributable to differences in ingredient composition between the basal diets used in the first and second *in vivo* experiment and in particular the fibre composition and levels (Chapter 7). Such differences may have resulted in distinct endogenous butyrate production patterns across studies . The method used to determine butyrate release patterns *in vivo* did not allow discrimination among exogenous (dietary) and endogenous (microbial) butyrate. Such limitation may be overcome by labelling exogenous butyrate.

Due to the approach chosen, it is unclear if the responses described in Chapter 4 to 7 are triggered by exogenous or endogenous butyrate. Hence, results described in the present Thesis are the results of an overall "butyrate location effect" and not a precise "dieterary butyrate release location effect" as initially intended (Chapters 1 and 2). Although labelling butyrate with stable isotopes has been used by others (Smith *et al.*, 2012; van den Borne *et al.*, 2015) in order to characterize the release pattern of butyrate additives, two assumptions are made when the appearance of stable isotopes in the breath is used as a proxy of butyrate release time:

(1) Butyrate is absorbed by the gut immediately upon release
(2) Butyrate is oxidized immediately upon absorption

Changes in digesta butyrate concentration reported in Chapters 4 and 7 indicate that a delay may exist between butyrate release and absorption. Results reported in Chapter 5 indicate that assumption 2 may be invalid. As such, the use of stable isotopes in the breath to study dietary butyrate release location in the avian GIT has inherent limitations.

Butyrate concentration and butyrate fluxes

Figure 4 illustrates the fluxes of butyrate occurring in the digesta, gut wall and liver of broilers. Digesta butyrate concentration is the net result of the production, release and absorption of butyrate in the lumen of the gut. Digesta butyrate concentration is, therefore, an incorrect estimate of endogenous (microbial) production (Montoya *et al.*, 2017) and, by extension, of exogenous (dietary) butyrate release.

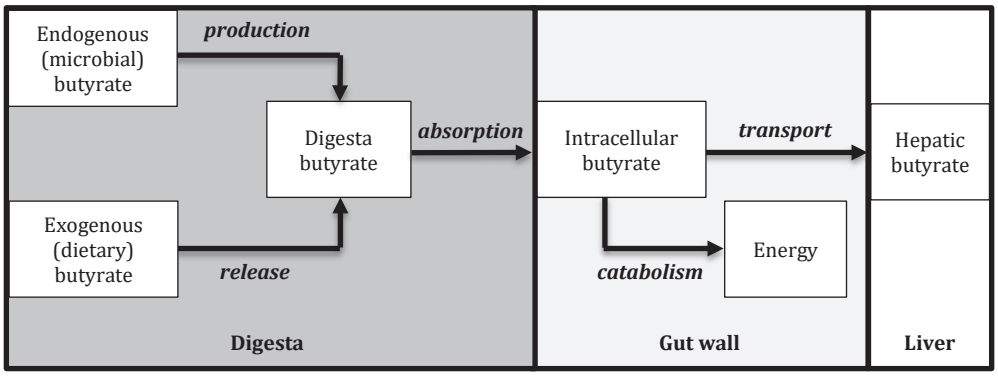


Figure 4. Schematic representation of butyrate fluxes occurring in broilers from the digesta to the liver

It is likely that the pleiotropic effects of butyrate on digestive functions, microbiota composition and immune responses of broilers are more influenced by butyrate fluxes in the digesta (i.e. production, release and absorption) than by the absolute digesta butyrate concentration. Digesta butyrate concentration was, nonetheless, used in Chapters 4 to 7 as an explanatory variable to interpret the observed effects of butyrate in poultry. The validity of the latter can be questioned.

REGULATION OF DIGESTIVE PROCESSES BY BUTYRATE

Hormones involved in the regulatory effect of butyrate on digestive functions

Presence of butyrate in various GIT segments is thought to modulate the secretion of numerous gut hormones, e.g. gastrin, CCK, PYY and GLP (Chapter 2). The results in Chapter 4 indicate that the presence of butyrate in the colon and ileum of broilers increases intestinal digesta retention time. Qaisrani and coworkers (2014) reported that fat coated butyrate increased the proteolytic activity of the proventriculus. Here similar effects were observed when butyrate was present in the gastric region (Chapter 4). Such changes evoke shifts in the secretory activities of G and L cells, which could not be demonstrated at the transcriptional level in the present Thesis (Chapter 6). It is known in mammals that butyrate presence in the colon increases gastrin secretion (Sakata, 1989). Such indirect effect was not evaluated in the present Thesis and may explain the results reported by Qaisrani and coworkers (2014). Finally, the presence of butyrate in the duodenum increased the expression of the CCK prohormone (Chapter 5).

Cellular mechanisms that may explain the effect of butyrate on the secretion of regulatory peptides in the gut have been addressed above in this Chapter. These mechanisms involve the sensing of butyrate by FFAR by enteric nerves or by enteroendocrine cells. Butyrate was found to decrease the expression of FFAR2 all measured GIT segments except the colon (Chapter 6). As mentioned earlier, this may indicate that butyrate signalling was too strong due to high concentrations. Hence, further studies should inverstigate different butyrate inclusion rates to gain insight into possible dose-response effects of butyrate on regulatory peptides of the poultry gut.

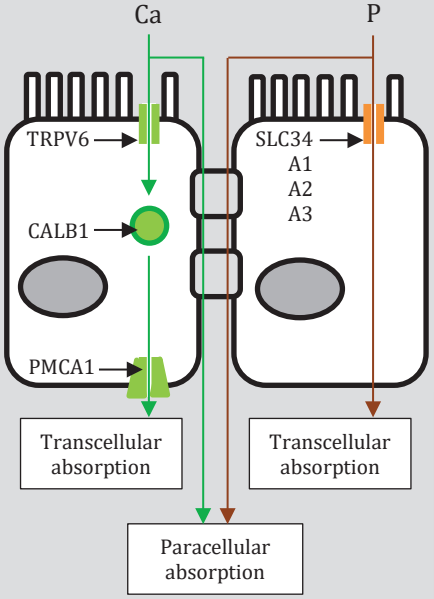
Effects on the digestibility of AA, fat and energy

The apparent ileal digestibility of methionine and histidine tended to be improved when butyrate was present in the colon and ceca, possibly as a results of the putative activation of L-cells (Chapter 4). The influence of butyrate on CCK prohormone secretion indicates a change in I cell activity (Chapter 5). Increased CCK secretion may result in increased bile salt and pancreatic enzymes outputs (Denbow, 2015). This may explain why fat coated butyrate and tributyrin, two butyrate additives that extend the activity of butyrate beyond the gastric region, have been reported to increase the apparent metabolisable energy of broiler diets as well as fat digestibility (Smilukowska *et al.*, 2007; Kaczmarek *et al.*, 2016; Liu *et al.*, 2017). Such effects can be considered as positive for the growth performance of broilers. Butyrate may, in addition, influence the digestibility and metabolism of calcium (**Ca**) and phosphorus (**P**), as discussed below.

Effects on Ca and P metabolism

Evidence describing the influence of intestinal microbiota activity, including butyrate production on Ca and P mammalian metabolism have been reviewed to more fully understand the origin of postmenopausal osteoporosis (Xu *et al.*, 2017). The intestinal microbiota is thought to have a threefold effect on Ca/P metabolism in mammals. Firstly, SCFA have been shown to induce the expression of different Ca transporters, e.g. TRPV6 or CALB1 (Fukushima *et al.*, 2009; Fukushima *et al.*, 2012) *in vitro* and *in vivo*. This may explain why feeding various fermentable fibre sources influences Ca absorption in rats (Coudray *et al.*, 2003; Kruger *et al.*, 2003; Demigne *et al.*, 2008) and humans (Teramoto *et al.*, 2006; Whisner *et al.*, 2013). Secondly, butyrate is known to influence the expression of tight junctions proteins (Peng *et al.*, 2009). This may influence the paracellular absorption of Ca and P (Xu *et al.*, 2017). Finally, intestinal butyrate inhibits the release of INF-γ and TNF-α by leukocytes after *Staphylococcus aureus* (Saemann *et al.*, 2000) and lipopolysaccharide (**LPS**) stimulation (Nancey *et al.*, 2002). Both INF-γ and TNF-α are known to modulate the activity of osteoblasts and osteoclasts, and consequently, bone mass and micro-architecture (Kobayashi *et al.*, 2000; Lam *et al.*, 2000; Duque *et al.*, 2011). Microbiota activity can, therefore, affect Ca/P metabolism in mammals (Xu *et al.*, 2017).

Textbox 3. CALCIUM (Ca) AND PHOSPHORUS (P) ABSORPTION IN THE GUT OF BROILERS



Model depicting Ca and P absorption in broiler chickens.
Transcellular Ca absorption: Uptake is mediated by TRPV6, intracellular transport by CALB1 and transport across the basolateral membrane by PMCA1. Transcellular P absorption: uptake is mediated by SLC34 A1, A2 and A3 family members. Paracellular absorption may occur depending on the integrity of the gut.

Phosphorus utilisation is thought to be important for the sustainability of poultry production because excessive P excretion leads to eutrophication (Valable *et al.*, 2017). In addition, impairments of Ca/P metabolism increases the occurrence of skeletal abnormalities (Proszkowiec-Weglarz and Angel, 2013). In broilers, Ca and P absorption is the result of transcellular and paracellular mechanisms (Textbox 3). The expression of Ca and P transporters in the GIT of poultry is modulated by the dietary levels of digestible Ca and P (e.g. Centeno *et al.*, 2004; Ashwell and Angel, 2010). Such mechanisms have been used to improve Ca/P efficiency of broilers through depletion/repletion strategies (Yan *et al.*, 2005). The results of such strategies, are however, difficult to replicate (van Krimpen *et al.*, 2017).

Levels of SCFA in the gut may explain partly such inconsistencies. As seen in chapter 6, levels of butyrate can affect the expression of TJ proteins in the gut of poultry. This, in turn, may affect the paracellular absorption of Ca and P. Butyrate modifies, in addition, the cytokine response of avian macrophages after LPS challenge and, in particular, INF-γ release (Zhou *et al.*, 2014). This may influence the activity of osteoclasts and osteoblasts and, consequently, bone mass and microarchitecture. Finally, the presence of butyrate in the GIT of poultry seems to influence on the expression of Ca and P transporters in a location-dependent manner (Table 2, using the same approach as Table 3 in Chapter 5).

Table 2. Effect of dietary treatments[1] on the genetic expression of calcium and phosphorus transporters in different intestinal tract segments of broilers at 21-22 d of age

	Average gene expression[2]					
Location	**TRPV6**	**CALB1**	**PMCA1**	**SLC34A1**	**SLC34A2**	**SLC34A3**
Duodenum						
CTR	71.1	73.6	95.8	100.0	89.1	80.0
TB	37.4	100.0	100.0	69.8	100.0	77.9
P-value	0.27	**0.033**	0.318	0.114	0.215	0.386
Ileum						
CTR	11.80	18.70	36.40	45.80	10.60	68.60
FCB	24.40	14.50	31.10	45.30	8.90	53.00
P-value	***0.072***	0.135	**0.026**	0.318	0.186	0.159
Colon						
CTR	27.20	0.40	18.60	52.70	0.60	82.10
FCB	16.20	0.20	20.50	61.80	0.50	100.00
P-value	0.181	0.500	0.343	***0.095***	0.282	0.112

[1]Dietary treatments groups: CTR (no dietary butyrate); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg as in basis. Values are means of 8 birds per treatment.

[2]Arbitrary units. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. For each gene, the highest average gene expression across location and treatments was set to 100.

The link between Ca/P and microbiota has already been studied in broilers, but, as far as I know, it was always approached on the direction of the effect of Ca, P and phytase on microbiota activity (e.g. Ptak *et al.*, 2015; Witzig *et al.*, 2015; Borda-Molina *et al.*, 2016). I suggest that the relationship is actually bidirectional. In other words, luminal butyrate, and, more generally, SCFA may influence Ca/P metabolism in broilers and such studies should be conducted to further improve the utilisation of these minerals by poultry.

IS IT A TOXIN?: BUTYRATE METABOLISM IN THE GIT OF POULTRY

Unlike other SCFA, butyrate does not reach peripheral tissues

One unexpected result came from the intracellular butyrate data in Chapter 5. Some treatments led to a nearly 10 fold increase in digesta butyrate concentration compared to the control, but intracellular butyrate concentration was not affected and remained low. Iso-valeric and valeric acid are present in much lower concentrations than butyrate in the digesta of poultry. Yet, butyrate was found in the same concentration range as these SCFA in the tissue of the different GIT segment. It was also very clear from observed changes in the transcription of genes involved in SCFA transportation and energy metabolism that butyrate had been absorbed as number were upregulated. In mammals, it is known that butyrate is metabolized in the GIT and liver in such a way that very little of it passes to the peripheral circulation (Astbury and Corfe, 2012). This is in contrast to other SCFA such as acetate and propionate, which may reach the peripheral circulation (Astbury and Corfe, 2012). As shown in Table 3, similar observations can be obtained in broilers (Moquet, unpublished).

Table 3. Effects of dietary treatments on short chain fatty (SCFA) concentrations in the plasma of the wing vein (peripheral blood) of broilers[1] at 39 d of age

	Dietary treatments[2]						Diet *P*-value[3]
SCFA in mM	CTR	UP	TB	FCB	WAX	CAB	
Acetate	2.12	2.00	1.89	1.57	2.10	2.05	0.588
Propionate	0.35	0.29	0.26	0.22	0.32	0.34	0.281
Butyrate	**0.01**	**0.00**	**0.01**	**0.01**	**0.01**	**0.01**	**0.717**
Iso-butyrate	0.01	0.01	0.01	0.01	0.02	0.01	0.484
Valerate	0.01	0.01	0.01	0.01	0.01	0.01	0.568
Iso-valerate	0.04	0.03	0.03	0.03	0.04	0.03	0.568

[1]Birds received at d 21 and 22 post-hatch intratracheal administrations of human serum albumin at the dose of 0.5 mg/day.
[2]Dietary treatment groups: No butyrate supplementation (CTR) or 1 g butyrate radical per kg diet in the form of unprotected butyrate (UP), tributyrin (TB), fat coated butyrate (FCB), wax coated butyrate (WAX) or cellulose acetate butyrate (CAB). Values are the average of 10 birds per treatment.
[3]Data were analysed using PROC GLM of SAS 9.4.

Two theories can explain these observations:

(1) Butyrate is a very valuable energy source that is oxidized in priority over other energy sources
(2) Butyrate is a toxin whose concentration is tightly regulated in the body to avoid detrimental effects

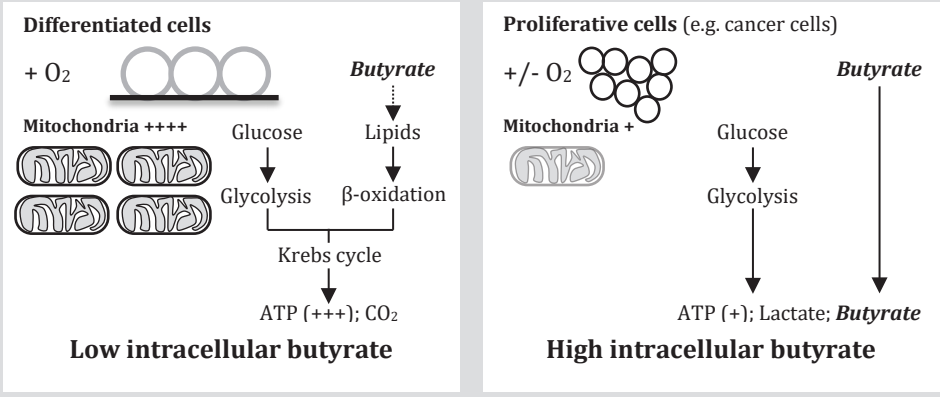
The first theory may be meaningful in the context of colonocytes. In poultry, dietary lipid, protein and starch is mostly absorbed before the colon (Denbow, 2015). Hence, colonocytes rely to a large extent on SCFA to cover their energy requirements (Roediger, 1982). Cellular energy is derived from the oxidation of SCFA in the mitochondria (Bender, 2014). Mitochondria biogenesis involves the synthesis of hundreds of proteins and is, therefore, an energy-demanding process (Cherry and Piantadosi, 2015). Energy yield increases with the chain length of SCFA (Bender, 2014). Butyrate, being a longer (C4) SCFA than acetate (C2) and propionate (C3), may be oxidized in priority to offer a quicker 'return on investment' in the energy homeostasis of colonocytes.

This theory does not hold, however, for enterocytes located in the proximal part of the small intestine. These cells have access to other preferred energy sources such as glucose, glutamine or glutamate (Watford *et al.*, 1979). The presence of butyrate in the digesta of the proximal part of the small intestine results in an upregulation of the genes involved in glycolysis, indicating that the β-oxidation of lipids, including butyrate, was reduced (Chapter 5). Hence, butyrate cannot be considered a valuable energy source for the enterocytes located in the proximal part of the small intestine. Yet, intracellular butyrate also remains low in such cells.

Cytotoxic effects of butyrate

As stated in Chapter 5, the influence of intracellular butyrate regulation on the survival of healthy and cancerous colonocytes is well established. Butyrate may act as a survival factor in healthy colonocytes or as an apoptotic agent in cancerous ones (Luciano *et al.*, 2002). This paradox is explained by differences in energy metabolism existing between differentiated and proliferative cells, e.g. subtypes of cancerous cells and activated immune cells. Because of a limited number of mitochondria, proliferative cells rely on aerobic glycolysis (Textbox 4, Vander Heiden *et al.*, 2009). In addition, proliferative cells use lipids as structural components to build novel cell membranes. Hence, such cells show enhanced lipid biosynthesis (Mashima *et al.*, 2009). Cytosolic lipid biosynthesis inhibits mitochondrial β-oxidation to avoid the occurrence of a futile cycle (Bender, 2014).

Textbox 4. THE WARBURG EFFECT. This mechanism influences cellular energy metabolism in proliferative and differentiated cells and, consequently, intracellular butyrate concentration.



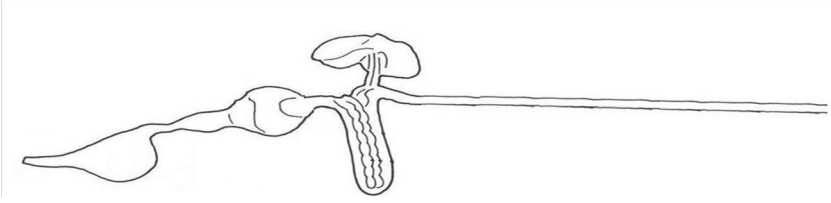
Intracellular butyrate concentration is thought to modulate the inhibitory effect of butyrate on histone deacetylase (HDAC) enzyme activity (Astbury and Corfe, 2012). Butyrate seems to exert stronger inhibition of total HDAC activity than other SCFA (Waldecker *et al.*, 2008). Butyrate and its pharmaceutical analogues inhibit specifically class I HDAC enzymes, i.e. HDAC 1-2-3-8 (Fass *et al.*, 2011). Such enzymes play a major role in the regulation of cell survival and proliferation (Licciardi *et al.*, 2011). The inhibitory effect of butyrate (mM range) on class I HDAC enzyme results in cell death in peripheral blood monocytes (Licciardi *et al.*, 2011) and cancer cells (Waldecker *et al.*, 2008).

Hence, it can be hypothesised that butyrate concentration is tightly regulated in peripheral blood and tissues to avoid cytotoxic effects.

The two theories presented above are not mutually exclusive. Butyrate could be a potent toxic compound for different organs due to its HDAC activity while remaining a valuable energy source for the cells populating the distal GIT of poultry. I think that, in poultry, intracellular butyrate concentration is tightly regulated to prevent adverse effects on host cell liveability.

Intracellular butyrate concentration is controlled by oxidation in the gut and liver

The extent to which butyrate is oxidized in the gut of poultry appears to vary depending on the GIT segment considered (Chapter 5). In addition, the expression of putative butyrate transporters is induced in the proximal GIT while being constitutive in the more distal GIT (Figure 5). Such differences may reflect an evolutionary adaptation. Colonocytes of wild birds are, for instance, more likely to encounter butyrate than duodenal enterocytes due to the fermentation patterns observed in the gut (Pedroso and Lee, 2014). The classical work of Imondi and Bird (1966) indicated, in addition, that cell turnover rate is much lower in the ileum than in the duodenum of chicks. As shown in Textbox 3, high cell proliferation rates hamper butyrate catabolism. Hence, differences in cell turnover rates may also explain partly the differences in butyrate metabolism observed across GIT segments. As indicated in Table 3, butyrate does not pass into the peripheral circulation. The work of Yin and coworkers (2016) indicates that butyrate is oxidized in the liver. As shown in Figure 5, a variable fraction of the absorbed butyrate may escape oxidation in the gut to reach the liver. This transport of butyrate to the liver have significant implications for the carcass quality of broilers.



	Gastric region	Duodenum	Jejunum	Ileum	Colon
Uptake	Passive	Active & induced	?	Active & constitutive	Active & constitutive
Butyrate metabolism	Clues of oxidations	Not oxidized	Clues of oxidation (Yin *et al.*, 2016)	Clues of oxidations	Clues of oxidations
Transport to the liver	++ (?)	++++ (?)	?	++ (?)	+ (?)

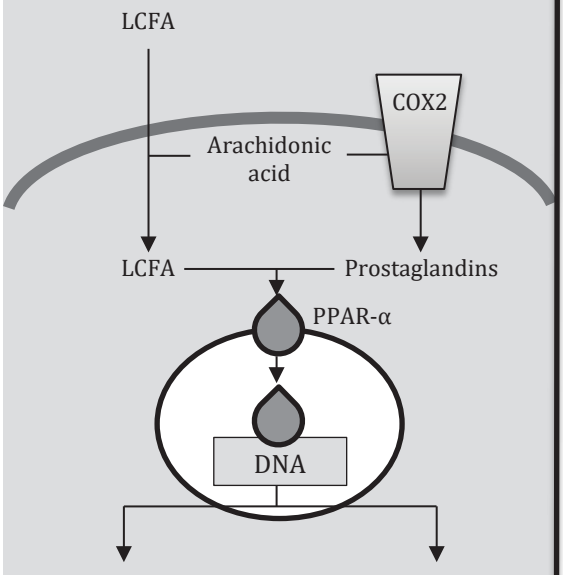
Figure 5. Summary of the sensing, uptake and metabolism. Based on the data presented in Chapter 5 and in Yin *et al.*, (2016)

BUTYRATE MAY IMPROVE CARCASS QUALITY BY INDUCING PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-A EXPRESSION IN THE LIVER

Butyrate is known to induce the expression of the peroxisome proliferator-activated receptor-α (PPAR-α) in the murine liver (Weng *et al.*, 2015). Yin and coworkers (2016) supplemented broiler diets with TB, a butyrate glyceride that increases digesta butyrate concentration in the duodenum and jejunum, and reported significant changes in jejunal and hepatic transcriptomes that evoked an increase in PPAR-α activity. Such signalling pathway is known to influence energy homeostasis (Textbox 5). This may explain why butyrate has been reported to improve carcass leanness of broilers fed butyrate glycerides (Bedford *et al.*, 2017a,b) but not an when fed as unprotected salt (Panda *et al.*, 2009).

Underlying mechanisms mediating the positive effect of butyrate on PPAR-α signalling are unclear. It is known that, in mammals, PPAR-α is activated by long chain fatty acids (e.g. palmitic, oleic or linoleic acids; Banner *et al.*, 1993) and by eicosanoids (e.g. prostaglandins; Bishop-Bailey and Wray, 2003). In Chapter 6 it is reported that the presence of butyrate in the duodenum increased the expression of COX2, a prostaglandin forming enzyme. A similar trend was observed in the ileum. In addition and as previously explained, butyrate may enhance fat digestibilty by modulating CCK expression. Such changes may have influenced hepatic PPAR-α activity.

Textbox 5. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ALPHA SIGNALLING IN THE LIVER



PPAR-α is a nuclear receptor and upon activation, acts as a transcription factor regulating inflammatory responses and energy homeostasis. There are other PPAR family members, namely PPAR-γ and PPAR-β/δ which are characterized by their tissue expression, ligands and target genes (Grygiel-Górniak, 2014).

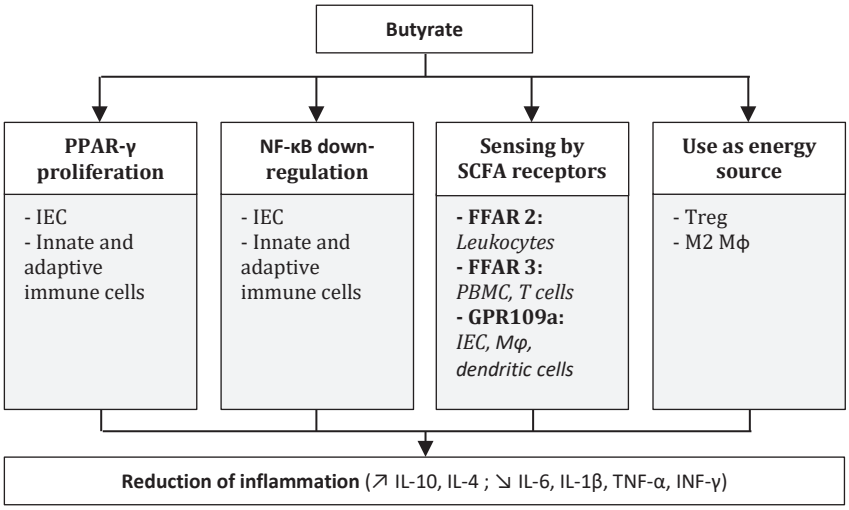
LCFA: long chain fatty acid; COX2: Cyclooxygenase 2

Finally, butyrate is known to activate PPAR-α in the liver of mice as a results of its inhibitory effect on HDAC 3 activity (Li *et al.*, 2012). Hence, the increased carcass leanness observed when birds are fed butyrate glycerides (Bedford *et al.*, 2017a,b) may also reflect a direct effect of butyrate on hepatocytes. Physiological effects of PPAR-α signalling go beyond the regulation of energy homeostasis and affect also immune responses (Textbox 5). Hence, this mechanism may also explain partly the immuno-modulatory properties of butyrate in poultry.

IMMUNO-MODULATORY PROPERTIES OF BUTYRATE

Modes of action described *in mammals*

In mammals, butyrate is thought to influence immune responses in different ways (Figure 6). Firstly, the presence of butyrate in the mM range in the culture medium of Caco 2 cells upregulates PPAR-γ expression (Wächtershäuser *et al.*, 2000). It is, however, unclear if the observations made by the latter authors on cancer cell lines can be extrapolated to healthy enterocytes given the metabolic differences between cancerous and healthy cells (Textbox 4).



IEC: intestinal epithelial cell; NF-κB: nuclear factor kappa B; SCFA: short chain fatty acid; FFAR: free fatty acid receptor; PBMC: peripheral blood monocytes; GPR: G-protein coupled receptor; Mφ: macrophage; Treg: Regulatory T-cell; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon

Figure 6. Immunomodulatory effects of butyrate in the mammalian GIT

Secondly, butyrate down regulates the NF-κB pathway by inhibiting cytoplasmic IκB kinase enzymes activity (Luhrs *et al.*, 2001). Thirdly, the sensing of butyrate and other SCFA by FFAR affects inflammatory responses in the gut (Maslowski *et al.*, 2009). Finally, butyrate can be used as an energy substrate by regulatory T cells (**Treg**) and alternatively activated (**M2**) macrophages, but not by other immune cells having a more limited capacity to perform fatty acid oxidation and oxidative phosphorylation (O'Neill *et al.*, 2016; Pearce and Pearce, 2013). It is generally accepted that the combined action of butyrate on different gut cells leads to a reduction of gut inflammation (Figure 6; For review, see Meijer *et al.*, 2010; Kim *et al.*, 2014; Corrêa-Oliveira *et al.*, 2016).

Suggested modes of action *in poultry*

Butyrate has been shown to modulate the expression of pro-inflammatory cytokines IL-1β and IL-6 in chickens in both *in vitro* (Zhou *et al.*, 2014) and *in vivo* (Li *et al.*, 2015; Bortoluzzi *et al.*, 2017) experiments. Dietary butyrate supplementation has also been shown to alleviate the negative effects of intraperitoneal injections of the potent NF-κB inducer *E. coli* LPS on growth of broilers (Zhang *et al.*, 2011). Immunologic stress depresses growth performance of broilers by reducing feed intake, shifting nutrient

partitioning away from skeletal muscle deposition and increasing basal metabolic rate (Klasing and Johnstone, 1991). Reduction of immunological stress and, more precisely inflammation, is thought to be one of the mechanisms mediating the positive effect of antibiotic growth promoters on the growth performance of poultry (Roura *et al.*, 1992; Niewold, 2007). In addition, it is thought that a decrease in NF-κB activity shifts adaptive immunity from cell-mediated to humoral responses in birds (Koutsos *et al.*, 2014). This was confirmed by Sikandar (2017) who reported that butyrate improved the specific antibody (**SpAb**) response of broilers. Hence, published evidence indicates that the immune-modulatory properties of butyrate in mammals can be extrapolated to poultry species.

The data reported in Chapter 6 and 7 indicate that the immunomodulatory effects of butyrate are inconsistent in poultry. In contrast data in the literature, in these Chapters it is reported that butyrate may induce bacterial dysbiosis and, hence, increase the intestinal expression of IL-6 (Chapter 6, in agreement with Bortoluzzi *et al.*, 2017 and in contrast with Li *et al.* 2015). In addition, no improvement in specific antibody (**SpAb**) response was observed when broiler diets were supplemented with butyrate (Chapter 7 in contrast with Sikandar *et al.*, 2017). Finally, dietary butyrate did not alleviate the negative effect of i.p. LPS injection on the growth performance of broilers (Chapter 7, in contrast with Zhang *et al.*, 2011). Possible reasons explaining the discrepancies have been discussed in Chapter 7 and may relate to differences in age and types of challenges used.

It must be stressed that resolving such discrepancies will be a real challenge given the scarcity of resources available to study avian immunology at the protein expression level (Kaiser, 2014), and the ongoing debate concerning the existence in poultry of several genes critical to the understanding of the immuno-modulatory effect of butyrate (Textbox 6). An illustrative example is the paper from Zhang and coworkers (2011), wherein the effect of dietary butyrate supplementation on serum cytokines was evaluated in broilers using commercially available ELISA kits. It appears that such kits are not existent for poultry (Kaiser, 2014; Kaiser and Stäheli, 2014). ELISA kits referenced in the aforementioned paper have been originally designed for use in human and were not referenced by the supplier for chickens (J.F. Bellec, Bio-Techne, Minneapolis, US, personal communication). In addition, one of the cytokines measured by Zhang and workers (2011) was TNF-α, a cytokine whose existence is still controversial in poultry (Kaiser and Stäheli, 2014; Textbox 6).

Textbox 6. THE MISSING GENES OF CHICKENS

About 274 genes are thought to be missing in chickens while still being present in most of the other vertebrate lineages (Lovell *et al.*, 2014). Such missing genes are associated with gastrointestinal diseases (n=40), inflammatory responses (n=27), immune cell trafficking (n=23), G-protein coupled receptor signalling (n=9) and humoral immune responses (n=6) (Lovell *et al.*, 2014). This may explain why several genes that are critical in understanding the effect of butyrate in mammals are missing in the chicken genome (e.g. TNF-α, FFAR3, GPR109a; Kaiser, 2014; Meslin *et al.*, 2015).

Recent evidences indicates, however, that several genes that have been described as missing in birds by Lovell and colleagues (2014) may have simply escaped sequencing due to their high GC content and long GC-rich stretches (Hron *et al.*, 2015). Updated versions of the avian genome may help to design primers and monoclonal Ab in the near future to study several aspects of avian immunity that could not be captured so far (e.g. FOXP3 and TNF-α) as well of missing butyrate-sensing receptors (e.g. FFAR3 and GPR109a).

Focus on butyrate and humoral immunity in broilers

In humans, butyrate has been reported to prevent the polarization of $CD4^+$ T cells into T helper (**T_H**) 1 while promoting T_H2 polarization (Saemann *et al.*, 2000). This may be due to the changes in cytokine milieu induced by butyrate presence in the mammalian gut (↗IL-4, ↘INF-γ; Figure 6). Such findings are partly in agreement with those of Nancey and coworkers, who reported that butyrate prevented T_H1 polarization without resulting in a clear T_H2 profile (Nancey *et al.*, 2002). The T_H1/T_H2 balance is, therefore, affected by butyrate in mammals. Such balance is known to influence the antibody (**Ab**) response (Smith *et al.*, 2000).

In poultry, the existence of a T_H1-T_H2 paradigm has not been clearly demonstrated yet, but a mounting body of evidence corroborates its existence (For review, see Kaiser and Stäheli, 2014). This may explain why, in birds, a decrease in NF-κB activity shifts adaptive immunity from cell-mediated to humoral responses in birds (Koutsos *et al.*, 2014). The presence of butyrate along the intestine of broilers has an influence on IgM expression, but not on IgG expression nor IgG:IgM ratios (Table 4, using the same approach as Table 3 in Chapter 5).

Table 4. Effect of dietary treatments[1] on the genetic expression of immunoglobulins (Ig) M and G and the IgG:IgM ratio in different intestinal tract segments of broilers at 21-22 d of age

Intestinal segment	Average gene expression[2]		IgG:IgM ratio
Diet	IgM	IgG	
Duodenum			
CTR	100	8	0.08
TB	65	9	0.15
P-value	***0.059***	0.396	0.477
Ileum			
CTR	42	57	1.36
FCB	81	100	1.24
P-value	**0.014**	0.282	0.128
Colon			
CTR	60	44	0.73
FCB	76	38	0.50
P-value	***0.078***	0.215	0.159

[1]Dietary treatments groups: CTR (no dietary butyrate); TB (butyrate radical provided as tributyrin); FCB (butyrate radical provided as fat-coated butyrate). Butyrate was provided at 1 g/kg diet as is basis. Values are means of 8 birds per treatment.

[2]Arbitrary units. Quantitative mRNA measurement was performed by establishing a linear calibration using 10-fold serial dilutions of cDNA templates. Data were standardized using IPO8 as housekeeping gene. For each gene, the highest average gene expression across location and treatments was set to 100.

IgM exists as a membrane-bound Ig (i.e. B cell receptor) or as a secretory Ig (i.e. Ab). Assuming that IgM mRNA represents mostly naïve B cells and only a few activated B cells undergoing class switching, the results presented in this Thesis may indicate that butyrate has a location-dependent modulatory effect on B cell trafficking to the gut. In mammals, such trafficking is directed by chemokines such as CXCL13 (also known as BLC, B lymphocyte chemoattractant). It is known that, in mammals, butyrate induces CXCL13 expression in the small intestine (Lucero *et al.*, 2013). Compared to mammals, birds have a somewhat more complex B cell development pathway that involves different locations, i.e. the bone marrow, Bursa of Fabricius and spleen. A range of three homeostatic B cell chemoattractant has, therefore, evolved in chickens from the expansion of an ancestral CXCL13 gene (Kaiser and Stäheli, 2014). The effect of butyrate on the expression of CXC chemokines is undocumented in poultry. If existing, such effect may bear implications for the production of secretory IgA (**sIgA**), a central Ig in mucosal immunity (Härtle *et al.*, 2014). Unfortunately, IgA mRNA was not measured in the present work.

Other evidence pointing to an effect of butyrate on the humoral immunity of chickens came from the positive effect of dietary butyrate supplementation on keyhole limpet hemocyanin (**KLH**) binding IgY (Chapter 7). Such titers were positively correlated with the bodyweight of broilers. Titers of overt Ab binding KLH were used as proxy to evaluate natural antibodies (**NAb**) in birds that were not previously immunized against KLH. Keyhole limpet hemocyanin is a large antigen of approximately 390 kDa which shares epitopes with viruses, bacteria and protists (e.g. Hepatitis C, *Escherichia coli* and *Trypanosoma*; Sundsmo *et al.*, 2012; Geyer *et al.*, 2004). Hence, it is unclear whether KLH Ab titers reflect truly NAb levels or cross-reactive SpAb. In mammals, natural Ab are produced by B1 cells, a subpopulation of B cells expressing a clearly defined set of cellular markers (Baumgarth, 2004). In contrast to mammals, cellular markers specific to B1 cells have yet to be identified in birds. Surface markers that are specific to B1a cells in mammals such as $CD5^+$ are common to all avian B cells (Ratcliffe *et al.*, 2014). The existence of true NAb-producing B cells is, therefore, questionable in poultry. This has led Berghof (2018) to speculate that IgM NAb represent, in poultry, humoral adaptive baseline immunity rather than a pool of specialized NAb-producing B cells. The change in overt IgY binding KLH reported in Chapter 7 may, therefore, reflect secretory NAb levels or, alternatively, cross reactive Ab produced by B cells after class switching.

Overall, the results in this thesis and those of Sikandar and coworkers (2017) indicate that butyrate has an effect on the humoral immunity of broilers. Such effect, however, requires additional research to refine our understanding of the responses elicited by butyrate (change in SpAb, NAb, sIgA) and their underlying mechanisms (T_H1/T_H2 balance, CXCL chemokines).

Immuno-modulation does not always rhyme with improved health

Two factors hamper my capacity to discuss the relevance of dietary butyrate supplementation strategies to improve the health and growth performance of broilers. First, there are numerous discrepancies between the literature and the results in this Thesis concerning the immunomodulatory effect of butyrate in broilers diets. Exact reasons for such discrepancies remain unknown, partly because of a lack of experimental evidence, but also because reagents available to study immunology in birds are scarce. Secondly, it is known that, in poultry, the concept of adding one particular ingredient to the diet to prevent the adverse effect of a specific challenge may, at the same time, increase susceptibility to a different challenge (Klasing, 2007). This means that, even if the effect of butyrate on immune competence would have been consistent, the overall benefit to the health and growth performance would have been context-dependent. It is, therefore difficult to speculate on the relevance of immunomodulatory interventions in poultry production (Textbox 7).

Textbox 7. A WORD OF WARNING ON THE INTERPRETATION OF IMMUNOCOMPETENCE RESULTS IN POULTRY STUDIES

Because the immune system is a complex network of many cell types and accessory proteins, assessing its state of competence is difficult. Many nutritionists are not trained in immunology and lack the necessary background to design informative studies on immunocompetence or to interpret their results. This has resulted in a confusing and ugly literature rife with poorly thought-through experiments and misinterpreted results. Simply measuring every immunological end point possible is sure to find differences due to diet, but interpreting these differences and establishing whether a change is beneficial or detrimental is often impossible.

K.C. Klasing
Speech to the UK branch of the World's Poultry Science Association in 2007

Salmonella entrica enteritidis and *Eimeria* infections have a very large impact on poultry production when taking into consideration economic impact, societal concern, zoonotic potential and, last but not least, animal welfare (Davies *et al.*, 2009). Butyrate has been shown to reduce the colonization of the broiler GIT by *Salmonella enteritidis* under experimental conditions (Van Immerseel *et al.*, 2005b; Sunkara *et al.*, 2011) due to direct effects elicited on *Salmonella* (Gantois *et al.*, 2006) and on the host (Sunkara *et al.*, 2011; Sunkara *et al.*, 2014).

Dietary strategies that have proven useful against *Eimeria* infections in poultry increase oxidative stress (e.g. flaxseed, fish oil or *Artemisia annua* supplementation) or IFN-γ levels in the gut (e.g. plum supplementation Allen *et al.*, 1997a; Allen *et al.*, 1997b; Danforth *et al.*, 1997; Lee *et al.*, 2008). Such effects are diametrically opposed to those elicited by dietary butyrate supplementation (Figure 6). Hence, the relevance of dietary butyrate supplementation in improving the health of broilers is conditioned by the type of challenge encountered.

EFFECTS OF DIETARY BUTYRATE SUPPLEMENTATION ON MICROBIOTA COMPOSITION AND ACTIVITY

Direct and indirect modes of action

Several modes of action may explain the effect of butyrate presence in the GIT on the microbiota composition of the gut (Chapter 2). Briefly, such modes of action can be divided into two categories, depending on whether butyrate acts on the microbiota or on the host.

Direct effects of butyrate on the microbiota include epigenetic effects, such as those reported by Gantois and coworkers (2006) concerning the expression of genes regulating the invasiveness of *Salmonella*. Quorum sensing is an emerging concept in microbiology. This concept describes a cell to cell communication process that allows bacteria to adjust their activity based on the environmental clues provided by extracellular molecules (Papenfort and Bassler, 2016). It is hitherto unclear whether SCFA, including butyrate, can mediate a 'quorum sensing' type of effect. It has been reported in the literature that, in the oral cavity, metabolic end products of bacterial fermentation such as SCFA do not affect the growth of the producing species but inhibit the growth of other microorganisms with various degrees of efficacy (Huang *et al.*, 2011). It remains, however, unclear whether the supply or exogenous (dietary) butyrate stimulates the (endogenous) microbial production of butyrate in the GIT of poultry while inhibiting the growth of other bacteria. Bortoluzzi and coworkers (2017) reported that dietary feeding of fat-coated butyrate did not affect the butyrogenic capacity of the cecal microbiota of broilers, as measured by the abundancy of the butyryl-CoA:acetate CoA transferase mRNA abundancy. The use of stable isotopes, e.g. ^{13}C-labelled butyrate, may help to address this question.

Undissociated butyrate may, like many other organic acids, exert direct bacteriostatic and bactericidal effects (Van Immerseel *et al.*, 2004; Ahsan *et al.*, 2016). Butyrate is a weak organic acid (pKa of 4.82) that exists mostly in a undissociated form under acidic conditions (Ahsan *et al.*, 2016). Such conditions are encountered in the crop, proventriculus and gizzard of chickens (Denbow, 2015). Based on the initial observations of Cherrington and coworkers (1991), poultry researchers speculated that undissociated butyrate diffuses through bacterial membranes (Ahsan *et al.*, 2016). Subsequent dissociation in the circumneutral bacterial cytoplasm is thought to induce an acid stress that can impair bacterial fitness in a specie-dependent manner (Sun *et al.*, 2013). This putative mode of action, although commonly relayed in recent reviews (Ahsan *et al.*, 2016; Moquet *et al.*, 2016), has been questioned as it fails to explain differences in susceptibility of *Salmonella* to organic acids of different carbon chain length (Van Immerseel and Atterbury, 2013). Accumulation of acid anions has been proposed as an alternative mechanism explaining the bacteriostatic effects of organic acids (Russell and Diez-Gonzalez, 1998).

Butyrate exerts not only direct effects on bacteria, but also elicits effects on the host that may affect indirectly microbiota composition. As described in the literature and in the present Thesis, butyrate modulates the expression of various mucus and host defence peptides (**HDP**) genes across the GIT (Sunkara *et al.*, 2011; Sunkara *et al.*, 2014; Chapter 6). Butyrate seems, in addition, to have a marked influence on humoral immunity at the systemic (SpAb and NAb: Sikandar *et al.* 2017; Chapter 7) and gut level (intestinal IgM mRNA; Chapter 8). Such influence may also modify sIgA production in the gut, which is a major mechanism by which the host influences microbiota composition. Finally, butyrate modulates digestive processes and nutrient digestibility (Chapter 4). Such effects may, theoretically, influence microbiota composition by changing the substrate left for growth in the hindgut. My results indicate, however, that butyrate-induced changes in digestive functions had little to no relevance in explaining the changes in cecal microbiota reported in Chapter 6.

Relevance for the health and growth performance of broilers

High-throughput sequencing of 16S rRNA does not allow a phylogenetic identification that goes beyond the genus level in most cases, as shown in Chapter 6 (Zoetendal and Smidt, 2018). In addition, strains dominating the cecal microbiota of chickens are poorly characterized (Pedroso and Lee, 2014) and there is still a certain controversy regarding the specificity of the roles exerted by the bacterial species populating the GIT (Textbox 8). Yet, literature indicates that supplementing broiler diets with butyrate influences cecal microbiota composition in a manner that is positive for the health and growth performance of broilers (e.g. reduced occurrence of potentially pathogenic bacteria, improved energy yield; Qaisrani, 2014; Bortoluzzi *et al.*, 2017). In contrast, the results presented in this Thesis indicate that the presence of butyrate in the gastric region of broilers induces cecal microbiota dysbiosis and inflammation in the distal GIT (Chapter 6) as well as intestinal *Lactobacilli* overgrowth and impaired AA digestibility (Chapter 4). My results indicate, in addition, that the presence of butyrate in the proximal part of the small intestine may result in fungal dysbiosis (Chapter 6). The presence of butyrate in the gastric region and in the proximal part of the small intestine seemed, therefore, to reduce gut health of broilers under the experimental conditions employed. The presence of butyrate in the more distal GIT did not result, in contrast, in measurable effects on microbiota composition and inflammation indicators.

Textbox 8. REDUNDANT FUNCTIONS OR KEYSTONE SPECIES?

Phylogenetic dendograms of microbial communities inhabiting the hindgut of avian species are characterized by palm tree-like architectures (Zhu *et al.*, 2002). Such architectures indicate a paucity of diversity at the phyla, genus and family levels and contrastingly high levels of species and strain variations (Lagerström *et al.*, 2006; Ley *et al.*, 2006; Onrust *et al.*, 2015). In addition, hierarchy theory indicates that the higher level of complexity imposes selective constraints over lower, less complex levels (Koestler, 1967). This top-down selection process favours the settlement of stable microbial communities characterized by large degree of overlaps in functionalities (Benson *et al.*, 2010). As a consequence, cecal microbiota is characterized by a paucity in diversity at intermediate levels of the phylogenetic dendogram, high levels of strain variation and overlaps in functions across strains.

At the same time, an opposing theory states that microbiota harbours bacterial species and strains having very unique and central functions that are referred to keystone species. Such species are thought to be the source of major interindividual differences in disease susceptibility and could be targeted by dietary interventions (Ze *et al.*, 2013).

RECOMMENDATIONS TO THE INDUSTRY

Increasing butyrate concentration in the colon and ceca seemed to be a way to positively influence digestive processes and performance (Chapter 4) without degrading the inflammatory status of the gut and the microbiota composition of broilers (Chapter 6). Feeding fat-coated butyrate to broilers is thought to increase the availability of butyrate beyond the ileum (van den Borne *et al.*, 2015). A practical recommendation for the industry may be, therefore, to include fat-coated butyrate in the starter diets of broilers (0-10 d of age) to ensure that this location is provided with butyrate until the cecal microbiota are fully developed. When switching to grower and finisher diets, fat-coated butyrate may be replaced by a butyrogenic fibre source, e.g. inulin. Such a strategy may allow maximization of the positive effects of luminal butyrate on growth performance of broilers, especially when basal diets are devoid of butyrogenic fibres. Onrust and coworkers (2015) have published a list of dietary interventions that may stimulate endogenous butyrate production.

CONCLUSION

CONCLUSION

The release profile of experimental and commercially available butyrate feed additives was measured in an *in vitro* study. Butyrate derivatives having markedly different release profiles were used in two *in vivo* experiments to inverstigate the effect of butyrate presence in distinct GIT segments on digestive processes, microbiota composition and immune responses of broilers. These studies have led to the following conclusions:

- Unprotected butyrate is active in the gastric region of broilers. Tributyrin is active in the small intestine. There is an important variability of release profiles among fat-coated butyrate additives that have similar macroscopic shapes, colours and particle sizes. Some fat coated butyrate additives can extend the effect of butyrate to the colon and ceca.
- Butyrate when present in the gastric region of poultry induces inflammation and dysbiosis in the colon and ceca.
- Butyrate when present in the small intestine increases IgY NAb titers in peripheral blood and induces inflammation in the colon and ceca.
- Butyrate when present in the colon and ceca of broilers increases intestinal retention time in a manner that can improve the digestibility of nutrients.
- Transport of butyrate across the epithelium is a passive process in the gastric region and an active process in the small intestine and colon. The extent to which butyrate is oxidized in the gut of poultry appears to vary depending on the GIT segment considered. Intracellular butyrate concentration is low in the GIT and butyrate does not pass into the peripheral circulation.

Butyrate does not always elicit positive effects for the gut health of broilers. Such findings support a shift in paradigm, from butyrate being a beneficial molecule to butyrate having effects that may be positive or negative depending on the GIT location considered and the concentration present. Broilers appear to react strongly to increases in digesta butyrate concentration in GIT segments where butyrate production is normally limited (crop, proventriculus, small intestine). Tolerance for the presence of butyrate in the digesta in the gastric region and small intestine may be low. The detrimental effects reported in the present thesis may be related to a strong response of broilers to butyrate signaling in such GIT segments. In contrast, GIT segments where normally butyrate production is substantial (colon and ceca) appear to have a higher tolerance for the presence of butyrate in digesta. And so the pendulum depicted in Figure 2 appears to swing back.

LITERATURE CITED

Ahsan, U., Ö. Cengíz, I. Raza, M. F. A. Cacher, Z. Iqbal, S. Umar, and S. Çakir. 2016. Sodium butyrate in chicken nutrition: the dynamics of performance, gut microbiota, gut morphology, and immunity. Worlds Poult. Sci. J. 72:265-275.

Allen, P. C., H. Danforth, and O. A. Levander. 1997a. Interaction of dietary flaxseed with *coccidia* infections in chickens. Poult. Sci. 76:822-827.

Allen, P. C., J. Lydon, and H. D. Danforth. 1997b. Effects of components of *Artemisia annua* on *coccidia* infections in chickens. Poult. Sci. 76:1156-1163.

Anand, S., H. Kaur, and S. S. Mande. 2016. Comparative in silico analysis of butyrate production pathways in gut commensals and pathogens. Front. Microbiol. 7:1945. doi 10.3389/fmicb.2016.01945

Ashwell, C. M., and R. Angel. 2010. Nutritional genomics: a practical approach by early life conditioning with dietary phosphorus. R. Bras. Zootec. 39:268-278.

Astbury, S. M., and B. M. Corfe. 2012. Uptake and metabolism of the short-chain fatty acid butyrate, a critical review of the literature. Curr. Drug. Metab. 13:815-821.

Banner, C. D., M. Gottlicher, E. Widmark, J. Sjovall, J. J. Rafter, and J. A. Gustafsson. 1993. A systematic analytical chemistry/cell assay approach to isolate activators of orphan nuclear receptors from biological extracts: characterization of peroxisome proliferator-activated receptor activators in plasma. J. Lipid Res. 34:1583-1591.

Baumgarth, N. 2004. B-Cell Immunophenotyping. Pages 643-662 in Methods in cell biology. R. P. Mecham, ed. Academic Press, San Diego, CA.

Bedford, A., H. Yu, M. Hernandez, J. Squires, S. Leeson, Y. Hou, and J. Gong. 2017a. Response of Ross 308 and 708 broiler strains in growth performance and lipid metabolism to diets containing tributyrate glycerides. Canad. J. Anim. Sci. 98:98-108.

Bedford, A., H. Yu, E. J. Squires, S. Leeson, and J. Gong. 2017b. Effects of supplementation level and feeding schedule of butyrate glycerides on the growth performance and carcass composition of broiler chickens. Poult. Sci. 96:3221-3228.

Bender, D. A. 2014. Energy nutrition – The metabolism of carbohydrates and fat. Pages 117-167 in Introduction to nutrition and metabolism (Fifth edition). D. A. Bender, ed. CRC Press/Taylor & Francis Group, Boca Raton, FL.

Bennett, G. N., and F. B. Rudolph. 1995. The central metabolic pathway from acetyl-CoA to butyryl-CoA in Clostridium acetobutylicum. FEMS Microbiol. Rev. 17:241-249.

Benson, A. K., S. A. Kelly, R. Legge, F. Ma, S. J. Low, J. Kim, M. Zhang, P. L. Oh, D. Nehrenberg, K. Hua, S. D. Kachman, E. N. Moriyama, J. Walter, D. A. Peterson, and D. Pomp. 2010. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. USA 107:18933-18938.

Berghof, T. V. L. 2018. Selective breeding on natural antibodies in chickens. PhD dissertation. Wageningen University, Wageningen, NL.

Bishop-Bailey, D., and J. Wray. 2003. Peroxisome proliferator-activated receptors: a critical review on endogenous pathways for ligand generation. Prostaglandins Other Lipid. Mediat. 71:1-22.

Borda-Molina, D., M. Vital, V. Sommerfeld, M. Rodehutscord, and A. Camarinha-Silva. 2016. Insights into broilers' gut microbiota fed with phosphorus, calcium, and phytase supplemented diets. Front. Microbiol. 7:2033. doi 10.3389/fmicb.2016.02033.

Bortoluzzi, C., A. A. Pedroso, J. J. Mallo, M. Puyalto, W. K. Kim, and T. J. Applegate. 2017. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. Poult. Sci. 96(11):3981-3993.

Brown, A. J., S. M. Goldsworthy, A. A. Barnes, M. M. Eilert, L. Tcheang, D. Daniels, A. I. Muir, M. J. Wigglesworth, I. Kinghorn, N. J. Fraser, N. B. Pike, J. C. Strum, K. M. Steplewski, P. R. Murdock, J. C. Holder, F. H. Marshall, P. G. Szekeres, S. Wilson, D. M. Ignar, S. M. Foord, A. Wise, and S. J. Dowell. 2003. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J. Nutr. Biochem. 278:11312-11319.

Byrne, J. 2017. Sodium butyrate has benefits for birds with necrotic enteritidis. Feed Navigator. https://www.feednavigator.com/Article/2017/09/04/Sodium-butyrate-has-benefits-for-birds-with-necrotic-enteritis-study Accessed Jan. 2018.

Canani, R. B., M. D. Costanzo, L. Leone, M. Pedata, R. Meli, and A. Calignano. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J. Gastroenterol. 17:1519-1528.

Centeno, V. A., G. E. Diaz de Barboza, A. M. Marchionatti, A. E. Alisio, M. E. Dallorso, R. Nasif, and N. G. Tolosa de Talamoni. 2004. Dietary calcium deficiency increases Ca_2^+ uptake and Ca_2^+ extrusion mechanisms in chick enterocytes. Comp. Biochem. Physiol. A Physiol. 139:133-141.

Chalmers, V. 2017. I treated my GUT to cure my eczema: Woman who swapped milk for kefir and bread for brown rice says the diet transformed her skin in just 7 days. Daily Mail. http://www.dailymail.co.uk/health/article-4899194/Seven-steps-rid-eczema.html Accessed Jan. 2018.

Cherrington, C. A., M. Hinton, G. R. Pearson, and I. Chopra. 1991. Short-chain organic acids at ph 5.0 kill *Escherichia coli* and *Salmonella* spp. without causing membrane perturbation. J. Appl. Microbiol. 70:161-165.

Cherry, A. D., and C. A. Piantadosi. 2015. Regulation of mitochondrial biogenesis and its intersection with inflammatory responses. Antiox. Redox Signal. 22:965-976.

Chevreul, M. E. 1823. Recherches chimiques sur les corps gras d'origine animale. Levrault, Paris, FR.

Corrêa-Oliveira, R., J. L. Fachi, A. Vieira, F. T. Sato, and M. A. R. Vinolo. 2016. Regulation of immune cell function by short-chain fatty acids. Clin. Transl. Immunol. 5:e73. doi 10.1038/cti.2016.17.

Coudray, C., J. C. Tressol, E. Gueux, and Y. Rayssiguier. 2003. Effects of inulin-type fructans of different chain length and type of branching on intestinal absorption and balance of calcium and magnesium in rats. Europ. J. Nutr. 42:91-98.

Cousens, L. S., D. Gallwitz, and B. M. Albert. 1978. Different accessibilities in chromatin to histone acetylase. J. Bio. Chem. 254:1716-1723.

Cummings, J. H., and W. J. Branch. 1986. Fermentation and the production of short-chain fatty acids in the human large intestine. Pages 131-149 in Dietary fiber: Basic and clinical aspects. G. V. Vahouny and D. Kritchevsky, eds. Springer US, Boston, MA.

Danforth, H. D., P. C. Allen, and O. A. Levander. 1997. The effect of high n-3 fatty acids diets on the ultrastructural development of Eimeria tenella. Parasitol. Res. 83:440-444.

Dar, S. A., R. Kleerebezem, A. J. M. Stams, J. G. Kuenen, and G. Muyzer. 2008. Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. Appl. Microbiol. Biotech. 78:1045-1055.

Davies, G., S. Genini, S. C. Bishop, and E. Giuffra. 2009. An assessment of opportunities to dissect host genetic variation in resistance to infectious diseases in livestock. Animal 3:415-436.

Demigne, C., H. Jacobs, C. Moundras, M. J. Davicco, M. N. Horcajada, A. Bernalier, and V. Coxam. 2008. Comparison of native or reformulated chicory fructans, or non-purified chicory, on rat cecal fermentation and mineral metabolism. Europ. J. Nutr. 47:366-374.

den Besten, G., K. van Eunen, A. K. Groen, K. Venema, D.-J. Reijngoud, and B. M. Bakker. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J. Lipid Res. 54:2325-2340.

Denbow, D. M. 2015. Chapter 14 - Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's avian physiology (Sixth edition). C. G. Scanes, ed. Academic Press, San Diego, CA.

Dethlefsen, L., M. McFall-Ngai, and D. A. Relman. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449:811-818.

Dibner, J. J., and P. Buttin. 2002. Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. J. App. Poult. Res. 11:453-463.

Dockray, G. J., A. Varro, R. Dimaline, and T. Wang. 2001. The gastrins: their production and biological activities. Ann. Rev. Physiol. 63:119-139.

Dumoulin, V., F. Moro, A. Barcelo, T. Dakka, and J. C. Cuber. 1998. Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. Endocrinol. 139:3780-3786.

Duncan, S. H., A. Barcenilla, C. S. Stewart, S. E. Pryde, and H. J. Flint. 2002. Acetate utilization and butyryl Coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. Appl. Environ. Microbiol. 68:5186-5190.

Duque, G., D. C. Huang, N. Dion, M. Macoritto, D. Rivas, W. Li, X. F. Yang, J. Li, J. Lian, F. T. Marino, J. Barralet, V. Lascau, C. Deschenes, L. G. Ste-Marie, and R. Kremer. 2011. Interferon-gamma plays a role in bone formation in vivo and rescues osteoporosis in ovariectomized mice. J. Bone Miner. Res. 26:1472-1483.

Fass, D. M., R. Shah, B. Ghosh, K. Hennig, S. Norton, W.-N. Zhao, S. A. Reis, P. S. Klein, R. Mazitschek, R. L. Maglathlin, T. A. Lewis, and S. J. Haggarty. 2011. Short-chain HDAC inhibitors differentially affect vertebrate development and neuronal chromatin. ACS Med. Chem. Lett. 2:39-42.

Fu-Cheng, X., Y. Anini, J. Chariot, T. Voisin, J. P. Galmiche, and C. Roze. 1995. Peptide YY release after intraduodenal, intraileal, and intracolonic administration of nutrients in rats. Pflugers Archiv. 431:66-75.

Fukushima, A., Y. Aizaki, and K. Sakuma. 2009. Short-chain fatty acids induce intestinal transient receptor potential vanilloid type 6 expression in rats and Caco-2 cells. J. Nutr. 139:20-25.

Fukushima, A., Y. Aizaki, and K. Sakuma. 2012. Short-chain fatty acids increase the level of calbindin-D9k messenger RNA in Caco-2 cells. J. Nutr. Sci. Vitaminol. 58:287-291.

Fung, K. Y. C., L. Cosgrove, T. Lockett, R. Head, and D. L. Topping. 2012. A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate. Br. J. Nutr. 108:820-831.

Gabriel, I., M. Lessire, S. Mallet, and J. F. Guillot. 2006. Microflora of the digestive tract: critical factors and consequences for poultry. Worlds Poult. Sci. J. 62:499-511.

Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, I. Hautefort, A. Thompson, J. C. Hinton, and F. Van Immerseel. 2006. Butyrate specifically down-regulates *Salmonella* pathogenicity island 1 gene expression. Appl. Environ. Microbiol. 72:946-949.

Gaskins, H. R. 2008. Host and intestinal microbiota negotiations in the context of animal growth efficiency. Pages 29-35 in Gut efficiency; the key ingredient in pig and poultry production: elevating animal performance and health. J. A. Taylor-Pickard, and P. Spring, eds. Wageningen Academic Publishers, Wageningen, NL.

Geyer, H., M. Wuhrer, T. Kurokawa, and R. Geyer. 2004. Characterization of keyhole limpet hemocyanin (KLH) glycans sharing a carbohydrate epitope with *Schistosoma mansoni glycoconjugates*. Micron 35:105-106.

Gibson, G. R., J. H. Cummings, G. T. Macfarlane, C. Allison, I. Segal, H. H. Vorster, and A. R. Walker. 1990. Alternative pathways for hydrogen disposal during fermentation in the human colon. Gut 31:679-683.

Gibson, G. R., G. T. Macfarlane, and J. H. Cummings. 1993. Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34:437-439.

Gibson, P. R. 2000. The intracellular target of butyrate's actions: HDAC or HDON'T? Gut 46:447.

Gold, H., H. L. Otto, W. Model, and S. L. Halpern. 1946. Behavior of synthetic esters of strophanthidin, the acetate, propionate, butyrate, and benzoate, in man. J. Pharmacol. Exper. Therap. 86: 301-309.

Grilli, E., B. Tugnoli, C. J. Foerster, and A. Piva. 2016. Butyrate modulates inflammatory cytokines and tight junctions components along the gut of weaned pigs. J. Anim. Sci. 94:433-436.

Grygiel-Górniak, B. 2014. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications - a review. J. Nutr. 13:17-27.

Guilloteau, P., L. Martin, V. Eeckhaut, R. Ducatelle, R. Zabielski, and F. Van Immerseel. 2010a. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23:366-384.

Guilloteau, P., G. Savary, Y. Jaguelin-Peyrault, V. Rome, L. Le Normand, and R. Zabielski. 2010b. Dietary sodium butyrate supplementation increases digestibility and pancreatic secretion in young milk-fed calves. J. Dairy Sci. 93:5842-5850.

Härtle, S., K. E. Magor, T. W. Göbel, F. Davison, and B. Kaspers. 2014. Chapter 6 - Structure and Evolution of Avian Immunoglobulins. Pages 103-120 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Herrmann, G., E. Jayamani, G. Mai, and W. Buckel. 2008. Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. J. Bacteriol. 190:784-791.

Hron, T., P. Pajer, J. Pačes, P. Bartůněk, and D. Elleder. 2015. Hidden genes in birds. Genome Biol. 16:164-168.

Huang, C. B., Y. Altimova, T. M. Myers, and J. L. Ebersole. 2011. Short- and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms. Arch. Oral. Biol. 56:650-654.

Imondi, A. R., and F. H. Bird. 1966. The turnover of intestinal epithelium in the chick. Poult. Sci. 45:142-147.

Jiminez, J. A., T. C. Uwiera, D. W. Abbott, R. R. E. Uwiera, and G. D. Inglis. 2017. Butyrate supplementation at high concentrations alters enteric bacterial communities and reduces intestinal inflammation in mice infected with *Citrobacter rodentium*. mSphere 2. doi 10.1128/mSphere.00243-17

Józefiak, D., A. Rutkowski, and S. A. Martin. 2004. Carbohydrate fermentation in the avian ceca: a review. Anim. Feed Sci. Technol. 113:1-15

Kaczmarek, S. A., A. Barri, M. Hejdysz, and A. Rutkowski. 2016. Effect of different doses of coated butyric acid on growth performance and energy utilization in broilers. Poult. Sci. 95:851-859.

Kaiser, P. 2014. Appendix B - Resources for studying avian immunology. Pages 425-428 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Kaiser, P., and P. Stäheli. 2014. Chapter 10 - Avian cytokines and chemokines. Pages 189-204 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Kaji, I., S. Karaki, and A. Kuwahara. 2014. Short-chain fatty acid receptor and its contribution to glucagon-like peptide-1 release. Digest. 89:31-36.

Kim, C. H., J. Park, and M. Kim. 2014. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. Immune Netw. 14:277-288.

Klasing, K. C. 2007. Nutrition and the immune system. Br. Poult. Sci. 48:525-537.

Klasing, K. C., and B. J. Johnstone. 1991. Monokines in growth and development. Poult. Sci. 70:1781-1789.

Kobayashi, K., N. Takahashi, E. Jimi, N. Udagawa, M. Takami, S. Kotake, N. Nakagawa, M. Kinosaki, K. Yamaguchi, N. Shima, H. Yasuda, T. Morinaga, K. Higashio, T. J. Martin, and T. Suda. 2000. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J. Exp. Med. 191:275-286.

Koestler, A. 1967. The ghost in the machine. Hutchinson, London, UK.

Kotunia, A., J. Wolinski, D. Laubitz, M. Jurkowska, V. Rome, P. Guilloteau, and R. Zabielski. 2004. Effect of sodium butyrate on the small intestine development in neonatal piglets fed [correction of feed] by artificial sow. J. Physiol. Pharmacol. 55 Suppl 2:59-68.

Koutsos, E. A., K. C. Klasing, B. Kaspers, and P. Kaiser. 2014. Chapter 17 - Factors modulating the avian immune system. Pages 299-313 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Krimpen, M. M., E. Willems, and J. van Harn. Year. Early life adaptation of broilers to low dietary phosphorus. Proc. 21st European Symposium on Poultry Nutrition, Salou, SP.

Kruger, M. C., K. E. Brown, G. Collett, L. Layton, and L. M. Schollum. 2003. The effect of fructooligosaccharides with various degrees of polymerization on calcium bioavailability in the growing rat. Exp. Biol. Med. 228:683-688.

Krupp, M., and M. D. Lane. 1981. On the mechanism of ligand-induced down-regulation of insulin receptor level in the liver cell. J. Biol. Chem. 256(4):1689-1694.

Lagerström, M. C., A. R. Hellström, D. E. Gloriam, T. P. Larsson, H. B. Schiöth, and R. Fredriksson. 2006. The G protein–coupled receptor subset of the chicken genome. PLOS Comput. Biol. 2:e54.

Lam, J., S. Takeshita, J. E. Barker, O. Kanagawa, F. P. Ross, and S. L. Teitelbaum. 2000. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J. Clin. Investig. 106:1481-1488.

Le Poul, E., C. Loison, S. Struyf, J. Y. Springael, V. Lannoy, M. E. Decobecq, S. Brezillon, V. Dupriez, G. Vassart, J. Van Damme, M. Parmentier, and M. Detheux. 2003. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J. Bio. Chem. 278:25481-25489.

Lee, S. H., H. S. Lillehoj, E. P. Lillehoj, S. M. Cho, D. W. Park, Y. H. Hong, H. K. Chun, and H. J. Park. 2008. Immunomodulatory properties of dietary plum on coccidiosis. Comp. Immunol. Microbiol. Infect. Dis. 31(5):389-402.

Leeson, S., H. Namkung, M. Antongiovanni, and E. H. Lee. 2005. Effect of butyric acid on the performance and carcass yield of broiler chickens. Poult. Sci. 84:1418-1422.

Ley, R. E., D. A. Peterson, and J. I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124:837-848.

Li, H., Z. Gao, J. Zhang, X. Ye, A. Xu, J. Ye, and W. Jia. 2012. sodium butyrate stimulates expression of fibroblast growth factor 21 in liver by inhibition of histone deacetylase 3. Diabetes 61:797-806.

Li, J., Y. Hou, D. Yi, J. Zhang, L. Wang, H. Qiu, B. Ding, and J. Gong. 2015. Effects of tributyrin on intestinal energy status, antioxidative capacity and immune response to lipopolysaccharide challenge in broilers. Asian Australas. J. Anim. Sci 28:1784-1793.

Licciardi, P. V., K. Ververis, and T. C. Karagiannis. 2011. Histone deacetylase inhibition and dietary short-chain fatty acids. ISRN Allergy 2011:869647.

Liddle, R. A. 1997. Cholecystokinin cells. Ann. Rev. Physiol. 59:221-242.

Lin, H. V., A. Frassetto, E. J. Kowalik Jr, A. R. Nawrocki, M. M. Lu, J. R. Kosinski, J. A. Hubert, D. Szeto, X. Yao, G. Forrest, and D. J. Marsh. 2012. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLOS ONE 7:e35240.

Liou, A. P. 2013. Digestive physiology of the pig symposium: G protein-coupled receptors in nutrient chemosensation and gastrointestinal hormone secretion. J. Anim. Sci. 91:1946-1956.

Liu, J. D., H. O. Bayir, D. E. Cosby, N. A. Cox, S. M. Williams, and J. Fowler. 2017. Evaluation of encapsulated sodium butyrate on growth performance, energy digestibility, gut development, and Salmonella colonization in broilers. Poult. Sci. 96:3638-3644.

Longo, W. E., G. H. Ballantyne, P. E. Savoca, T. E. Adrian, A. J. Bilchik, and I. M. Modlin. 1991. Short-chain fatty acid release of peptide YY in the isolated rabbit distal colon. Scand. J. Gastroenterol. 26:442-448.

Louis, P., G. L. Hold, and H. J. Flint. 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat. Rev. Microbiol. 12:661-672.

Lovell, P. V., M. Wirthlin, L. Wilhelm, P. Minx, N. H. Lazar, L. Carbone, W. C. Warren, and C. V. Mello. 2014. Conserved syntenic clusters of protein coding genes are missing in birds. Genome Biol. 15:565.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220-230.

Lucero, C. M., B. Fallert Junecko, C. R. Klamar, L. A. Sciullo, S. J. Berendam, A. R. Cillo, S. Qin, Y. Sui, S. Sanghavi, M. A. Murphey-Corb, and T. A. Reinhart. 2013. Macaque paneth cells express lymphoid chemokine CXCL13

and other antimicrobial peptides not previously described as expressed in intestinal crypts. Clinic. Vaccine Immunol. 20:1320-1328.

Luciano, L., S. Groos, R. Busche, W. Von Engelhardt, and E. Reale. 2002. Massive apoptosis of colonocytes induced by butyrate deprivation overloads resident macrophages and promotes the recruitment of circulating monocytes. Cell Tissue Res. 309:393-407.

Luhrs, H., T. Gerke, J. G. Muller, R. Melcher, J. Schauber, F. Boxberge, W. Scheppach, and T. Menzel. 2002. Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. Scand. J. Gastroenterol. 37:458-466.

Macfarlane, S., and G. T. Macfarlane. 2003. Regulation of short-chain fatty acid production. Proc. Nutr. Sci. 62:67-72.

Mashima, T., H. Seimiya, and T. Tsuruo. 2009. De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy. Br. J. Cancer 100:1369-1372.

Maslowski, K. M., A. T. Vieira, A. Ng, J. Kranich, F. Sierro, D. Yu, H. C. Schilter, M. S. Rolph, F. Mackay, D. Artis, R. J. Xavier, M. M. Teixeira, and C. R. Mackay. 2009. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461:1282-1286.

Mazzoni, M., M. Le Gall, S. De Filippi, L. Minieri, P. Trevisi, J. Wolinski, G. Lalatta-Costerbosa, J. P. Lalles, P. Guilloteau, and P. Bosi. 2008. Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. J. Nutr. 138:1426-1431.

McLaughlin, J. T., R. B. Lomax, L. Hall, G. J. Dockray, D. G. Thompson, and G. Warhurst. 1998. Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca(2+)-dependent mechanism in the enteroendocrine cell line STC-1. J. Physiol. 513:11-18.

Meijer, K., P. de Vos, and M. G. Priebe. 2010. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? Curr. Opin. Clin. Nutr. Metab. Care. 13:715-721.

Meslin, C., C. Desert, I. Callebaut, A. Djari, C. Klopp, F. Pitel, S. Leroux, P. Martin, P. Froment, E. Guilbert, F. Gondret, S. Lagarrigue, and P. Monget. 2015. Expanding duplication of free fatty acid receptor-2 (GPR43) genes in the chicken genome. Genome Biol. Evol. 7:1332-1348.

Montoya, C. A., S. M. Rutherfurd, and P. J. Moughan. 2017. Ileal digesta nondietary substrates from cannulated pigs are major contributors to in vitro human hindgut short-chain fatty acid production. J. Nutr. 147:264-271.

Moquet, P. C. A., L. Onrust, F. Van Immerseel, R. Ducatelle, W. H. Hendriks, and R. P. Kwakkel. 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72:61-80.

Mroz, Z., A. Jongbloed, K. Partanen, J. van Diepen, P. Kemme, and J. Kogut. 1997. Apparent digestibility of amino acids and balance of nitrogen and minerals as in¯uenced by buffering capacity and organic acids in diets for growing swine. J. Anim. Sci. 75:Abstr. 177.

Nancey, S., J. Bienvenu, B. Coffin, F. Andre, L. Descos, and B. Flourie. 2002. Butyrate strongly inhibits in vitro stimulated release of cytokines in blood. Dig. Dis. Sci. 47:921-928.

Niewold, T. A. 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poult. Sci. 86:605-609.

Nohr, M. K., M. H. Pedersen, A. Gille, K. L. Egerod, M. S. Engelstoft, A. S. Husted, R. M. Sichlau, K. V. Grunddal, S. S. Poulsen, S. Han, R. M. Jones, S. Offermanns, and T. W. Schwartz. 2013. GPR41/FFAR3 and GPR43/FFAR2

as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. Endocrinol. 154:3552-3564.

Nutriad. 2018. Butyrate: feeding the gut and beyond for animal health. https://nutriad.com/2015/01/butyrate-feeding-the-gut-and-beyond-for-animal-health-2/. Accessed Jan. 2018.

O'Neill, L. A., R. J. Kishton, and J. Rathmell. 2016. A guide to immunometabolism for immunologists. Nat. Rev. Immunol. 16:553-565.

Onrust, L., R. Ducatelle, K. Van Driessche, C. De Maesschalck, K. Vermeulen, F. Haesebrouck, V. Eeckhaut, and F. Van Immerseel. 2015. Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. Fronti. Vet. Sci. 2:75. doi 10.3389/fvets.2015.00075

Paap, P. 2016. Necrotic enteritis, coccidiosis - multifactorial role of butyrate on intestinal health. The Poultry Site. http://www.thepoultrysite.com/articles/3615/necrotic-enteritis-coccidiosis-multifactorial-role-of-butyrate-on-intestinal-health/. Accessed Jan. 2018.

Pace, D. M., B. T. Aftonomos, A. Elliott, and S. Sommer. 1967. Observations on some effects of the sodium salts of certain monocarboxylic acids on established cell lines. Canad. J. Biochem. 45:81-88.

Palital. 2018. Butyrate concepts - For pigs, poultry, veal- and rearing calves, fish and pets. http://www.palital.com/en/products/intest-plus-en/. Accessed Jan. 2018.

Panda, A. K., S. V. R. Rao, M. V. L. N. Raju, and G. S. Sunder. 2009. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. Asian-Australas. J. Anim. Sci. 22:1026-1031

Papenfort, K., and B. L. Bassler. 2016. Quorum sensing signal–response systems in Gram-negative bacteria. Nat. Rev. Microbiol. 14:576-588.

Pearce, E. L., and E. J. Pearce. 2013. Metabolic pathways in immune cell activation and quiescence. Immunity 38:633-643.

Pedroso, A. A., and M. D. Lee. 2014. Chapter 2: The composition and role of the microbiota in chickens. Pages 21-50 in Intestinal health. T. Niewold, ed. Wageningen Academic Publishers, Wageningen, NL.

Peng, L., Z. R. Li, R. S. Green, I. R. Holzman, and J. Lin. 2009. Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. J. Nutr. 139:1619-1625.

Pinchasov, Y., and L. S. Jensen. 1989. Effect of short-chain fatty acids on voluntary feed of broiler chicks1. Poult. Sci. 68:1612-1618.

Plaisancie, P., V. Dumoulin, J. A. Chayvialle, and J. C. Cuber. 1995. Luminal glucagon-like peptide-1(7-36) amide-releasing factors in the isolated vascularly perfused rat colon. J. Endocrinol. 145:521-526.

Polycarpo, G. V., I. Andretta, M. Kipper, V. C. Cruz-Polycarpo, J. C. Dadalt, P. H. M. Rodrigues, and R. Albuquerque. 2017. Meta-analytic study of organic acids as an alternative performance-enhancing feed additive to antibiotics for broiler chickens. Poult. Sci. 96:3645-3653.

Prasad, K. N., and P. K. Sinha. 1976. Effect of sodium butyrate on mammalian cells in culture: A review. In Vitro 12:125-132.

Proszkowiec-Weglarz, M., and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility1. J. App. Poult. Res. 22:609-627.

Psichas, A., M. L. Sleeth, K. G. Murphy, L. Brooks, G. A. Bewick, A. C. Hanyaloglu, M. A. Ghatei, S. R. Bloom, and G. Frost. 2015. The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. Int. J. Obesity 39:424-429.

Ptak, A., M. R. Bedford, S. Świątkiewicz, K. Żyła, and D. Józefiak. 2015. Phytase modulates ileal microbiota and enhances growth performance of the broiler chickens. PLOS ONE 10:e0119770

Qaisrani, S. N. 2014. Improving performance of broilers fed lower digestible protein diets. PhD dissertation. Wageningen University, Wageningen, NL.

Qaisrani, S. N., P. C. Moquet, M. M. van Krimpen, R. P. Kwakkel, M. W. Verstegen, and W. H. Hendriks. 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93:3053-3064.

Ratcliffe, M. J. H., S. Härtle, B. Kaspers, and P. Kaiser. 2014. Chapter 4 - B cells, the bursa of fabricius and the generation of antibody repertoires. Pages 65-89 in Avian immunology (Second edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Academic Press, San Diego, CA.

Rawdon, B. B., and A. Andrew. 1999. Gut endocrine cells in birds: an overview, with particular reference to the chemistry of gut peptides and the distribution, ontogeny, embryonic origin and differentiation of the endocrine cells. Prog. Histochem. Cytochem. 34:3-82.

Reilly, K. J., W. L. Frankel, A. M. Bain, and J. L. Rombeau. 1995. Colonic short chain fatty acids mediate jejunal growth by increasing gastrin. Gut 37:81-86.

Riggs, M. G., R. G. Whittaker, J. R. Neumann, and V. M. Ingram. 1977. n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature 268:462-464.

Roediger, W. E. 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterol. 83:424-429.

Roura, E., J. Homedes, and K. C. Klasing. 1992. Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. J. Nutr. 122:2383-2390.

Russell, J. B., and F. Diez-Gonzalez. 1998. The effects of fermentation acids on bacterial growth. Adv. Microb. Physiol. 39:205-234.

Saemann, M. D., G. A. Bohmig, C. H. Osterreicher, H. Burtscher, O. Parolini, C. Diakos, J. Stockl, W. H. Horl, and G. J. Zlabinger. 2000. Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. FASEB 14:2380-2382.

Sahakian, A. B., S.-R. Jee, and M. Pimentel. 2010. Methane and the gastrointestinal tract. Dig. Dis. Sci. 55:2135-2143.

Sakata, T. 1989. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation of isolated and denervated jejunal segment of the rat. Scand. J. Gastroenterol. 24:886-890.

Scheppach, W., and F. Weiler. 2004. The butyrate story: old wine in new bottles? Curr. Opin. Clin. Nutr. Metab. Care. 7:563-567.

Sealy, L., and R. Chalkley. 1978. The effect of sodium butyrate on histone modification. Cell 14:115-121.

Segain, J. P., D. Raingeard de la Bletiere, A. Bourreille, V. Leray, N. Gervois, C. Rosales, L. Ferrier, C. Bonnet, H. M. Blottiere, and J. P. Galmiche. 2000. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut 47:397-403.

Seidenberg, C. 2017. Why cooks (and bakers) should give ghee a try. Washinghton Post. https://www.washingtonpost.com/lifestyle/wellness/why-cooks-and-bakers-should-give-ghee-a-try/2017/10/30/10f226ca-b8e2-11e7-9e58-e6288544af98_story.html?utm_term=.7b2181fb7880. Accessed Jan. 2018.

Sikandar, A., H. Zaneb, M. Younus, S. Masood, A. Aslam, F. Khattak, S. Ashraf, M. S. Yousaf, and H. Rehman. 2017. Effect of sodium butyrate on performance, immune status, microarchitecture of small intestinal mucosa and lymphoid organs in broiler chickens. Asian-Australas. J. Anim. Sci. 30:690-699.

Sileikiene, V., R. Mosenthin, M. Tafaj, D. Kruszewska, B. Westrom, I. Mattsson, and S. G. Pierzynowski. 2005. Effect of short chain fatty acids infused intraileally on interdigestive exocrine pancreatic secretions in growing pigs. J. Anim. Physiol. Anim. Nutr. 89:253-259.

Singh, N., A. Gurav, S. Sivaprakasam, E. Brady, R. Padia, H. Shi, M. Thangaraju, P. D. Prasad, S. Manicassamy, D. H. Munn, J. R. Lee, S. Offermanns, and V. Ganapathy. 2014. Activation of GPR109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 40:128-139.

Smith, D. J., A. Barri, G. Herges, J. Hahn, A. G. Yersin, and A. Jourdan. 2012. In vitro dissolution and in vivo absorption of calcium [1-$^{(14)}$C]butyrate in free or protected forms. J. Agric. Food Chem. 60:3151-3157.

Smith, K. M., L. Pottage, E. R. Thomas, A. J. Leishman, T. N. Doig, D. Xu, F. Y. Liew, and P. Garside. 2000. Th1 and Th2 CD4^{+} T Cells provide help for b cell clonal expansion and antibody synthesis in a similar manner *in vivo*. J. Immunol. 165:3136.

Sossai, P. 2012. Butyric acid: what is the future for this old substance? Swiss Med. Wkly. 142:w13596.

Sun, Y., M. X. D. O'Riordan, S. Sima, and M. G. Geoffrey. 2013. Chapter three - regulation of bacterial pathogenesis by intestinal short-chain fatty acids. Adv. Appl. Microbiol. 85:93-118.

Sundsmo, J. S., H. Chow, and M. Sagermann. 2012. Keyhole limpet hemocyanin (KLH) N-glycans share similar structural features with saccharides of *Schistosoma, Trypanosoma, Leishmania, Cryptococcus, Aspergillis, candida, E. coli, Salmonella, Costridium* and hepatitis c virus (HCV). Proc. 52nd ICAAC, San Francisco, CA.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R. G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLOS ONE 6:e27225.

Sunkara, L. T., X. Zeng, A. R. Curtis, and G. Zhang. 2014. Cyclic AMP synergizes with butyrate in promoting beta-defensin 9 expression in chickens. Mol. Immunol. 57:171-180. d

Takahashi, J., A. S. Chaudhry, R. G. Beneke, Suhubdy, and B. A. Young. 1997. Modification of methane emission in sheep by cysteine and a microbial preparation. Sci. Total Environn. 204:117-123.

Tedelind, S., F. Westberg, M. Kjerrulf, and A. Vidal. 2007. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World J. Gastroenterol. 13:2826-2832.

Teramoto, F., K. Rokutan, Y. Sugano, K. Oku, E. Kishino, K. Fujita, K. Hara, K. Kishi, M. Fukunaga, and T. Morita. 2006. Long-term administration of 4G-beta-D-galactosylsucrose (lactosucrose) enhances intestinal calcium absorption in young women: a randomized, placebo-controlled 96-wk study. J. Nutr. Sci. Vitaminol. 52:337-346.

Valable, A. S., A. Narcy, M. J. Duclos, C. Pomar, G. Page, Z. Nasir, M. Magnin, and M. P. Letourneau-Montminy. 2017. Effects of dietary calcium and phosphorus deficiency and subsequent recovery on broiler chicken growth performance and bone characteristics. Animal. doi 10.1017/s1751731117003093.

van den Borne, J. J. G. C., M. J. W. Heetkamp, J. Buyse, and T. A. Niewold. 2015. Fat coating of Ca butyrate results in extended butyrate release in the gastrointestinal tract of broilers. Livest. Sci. 175:96-100.

Van Immerseel, F., and R. J. Atterbury. 2013. Other approaches to infection control. Pages 518-535 in Salmonella in domestic animals. P. A. Barrow and U. Methner, eds. CAB International, Oxon, UK.

Van Immerseel, F., F. Boyen, I. Gantois, L. Timbermont, L. Bohez, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2005a. Supplementation of coated butyric acid in the feed reduces colonization and shedding of Salmonella in poultry. Poult. Sci. 84:1851-1856.

Van Immerseel, F., F. Boyen, I. Gantois, L. Timbermont, L. Bohez, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2005b. Supplementation of coated butyric acid in the feed reduces colonization and shedding of Salmonella in poultry. Poult. Sci. 84:1851-1856.

Van Immerseel, F., V. Fievez, J. de Buck, F. Pasmans, A. Martel, F. Haesebrouck, and R. Ducatelle. 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with *Salmonella enteritidis* in young chickens. Poult. Sci. 83:69-74.

Vander Heiden, M. G., L. C. Cantley, and C. B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324:1029-1033.

Vital, M., A. C. Howe, and J. M. Tiedje. 2014. Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. mBio 5:e00889-00814.

Wächtershäuser, A., S. M. Loitsch, and J. Stein. 2000. PPAR-γ is selectively upregulated in Caco-2 cells by butyrate. Biochem. Biophys. Res. Comm. 272:380-385.

Waldecker, M., T. Kautenburger, H. Daumann, C. Busch, and D. Schrenk. 2008. Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. J. Nutr. Biochem. 19:587-593.

Watford, M., P. Lund, and H. A. Krebs. 1979. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochemi. J. 178:589-596.

Wells, J. M., O. Rossi, M. Meijerink, and P. van Baarlen. 2011. Epithelial crosstalk at the microbiota–mucosal interface. Proc. Natl. Acad. Sci. USA 108:4607-4614.

Weng, H., K. Endo, J. Li, N. Kito, and N. Iwai. 2015. Induction of peroxisomes by butyrate-producing probiotics. PLOS ONE 10:e0117851.

Whisner, C. M., B. R. Martin, M. H. Schoterman, C. H. Nakatsu, L. D. McCabe, G. P. McCabe, M. E. Wastney, E. G. van den Heuvel, and C. M. Weaver. 2013. Galacto-oligosaccharides increase calcium absorption and gut bifidobacteria in young girls: a double-blind cross-over trial. Br. J. Nutr. 110:1292-1303.

Witzig, M., A. Camarinha da Silva, R. Green-Engert, K. Hoelzle, E. Zeller, J. Seifert, L. E. Hoelzle, and M. Rodehutscord. 2015. Spatial variation of the gut microbiota in broiler chickens as affected by dietary available phosphorus and assessed by T-RFLP analysis and 454 pyrosequencing. PLOS ONE 10:e0143442.

Xu, X., X. Jia, L. Mo, C. Liu, L. Zheng, Q. Yuan, and X. Zhou. 2017. Intestinal microbiota: a potential target for the treatment of postmenopausal osteoporosis. Bone Res. 5:17046.

Yan, F., R. Angel, C. Ashwell, A. Mitchell, and M. Christman. 2005. Evaluation of the broiler's ability to adapt to an early moderate deficiency of phosphorus and calcium. Poult. Sci. 84:1232-1241.

Yin, F., H. Yu, D. Lepp, X. Shi, X. Yang, J. Hu, S. Leeson, C. Yang, S. Nie, Y. Hou, and J. Gong. 2016. Transcriptome analysis reveals regulation of gene expression for lipid catabolism in young broilers by butyrate glycerides. PLOS ONE 11:e0160751.

Yin, L., G. Laevsky, and C. Giardina. 2001. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. J. Biol. Chem. 276:44641-44646.

Zapolska-Downar, D., and M. Naruszewicz. 2009. Propionate reduces the cytokine-induced VCAM-1 and ICAM-1 expression by inhibiting nuclear factor-kappa B (NF-kappaB) activation. J. Physiol. Pharmacol. 60:123-131.

Ze, X., F. Le Mougen, S. H. Duncan, P. Louis, and H. J. Flint. 2013. Some are more equal than others: The role of "keystone" species in the degradation of recalcitrant substrates. Gut Microbes 4:236-240.

Zhang, W. H., Y. Jiang, Q. F. Zhu, F. Gao, S. F. Dai, J. Chen, and G. H. Zhou. 2011. Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. Br. Poult. Sci. 52:292-301.

Zhou, Z. Y., B. Packialakshmi, S. K. Makkar, S. Dridi, and N. C. Rath. 2014. Effect of butyrate on immune response of a chicken macrophage cell line. Vet. Immunol. Immunopathol. 162:24-32.

Zhu, X. Y., T. Zhong, Y. Pandya, and R. D. Joerger. 2002. 16S rRNA-Based Analysis of Microbiota from the Cecum of Broiler Chickens. Appl. Environ. Microbiol. 68:124-137.

Zoetendal, E. G., and H. Smidt. 2018. Endothelial dysfunction: what is the role of the microbiota? Gut 67:201-202.

SUMMARY (EN)

The broiler production sector is prompted to find strategies that can ensure improved growth performance, health and welfare of birds while using new feed ingredients that decrease cereal use and soybean meal dependence as well as the use of antibiotics. Among feed additives, butyrate has received much attention and has been proposed as a potential solution to assist the poultry sector to address the aforementioned challenges. Dietary butyrate supplementation has, however, an inconsistent effect on growth performance of broilers. Several mechanisms underlying the observed effects of butyrate on growth of livestock have been hypothesised, but, at present, a limited number of studies have investigated the existence of such mechanisms in poultry. It remains, in addition, uncertain whether the effects elicited by butyrate are conditioned by the gastrointestinal tract (**GIT**) segment wherein the molecule is present.

The aim of this thesis was to evaluate the effect of dietary butyrate supplementation on digestive functions, microbiota composition and immune responses of broilers and to verify whether such effects were conditioned by the GIT segment wherein butyrate is present. This research objective was addressed by supplementing broiler diets with butyrate derivatives having markedly different release profiles in the GIT. This thesis describes the results of an *in vitro* experiment and two *in vivo* trials. Chapter 2 consists of a review of the literature concerning potential modes of action of butyrate in the avian GIT, highlighting a potential role of butyrate presence in the digesta of distinct GIT segments in triggering specific effects. In Chapter 3, an *in vitro* model was used to compare the release profiles of experimental and commercially available butyrate derivatives along a simulated poultry GIT. Based on the outcome of this *in vitro* screening, several derivatives were included in the trial described in Chapter 4, in order to investigate the effects of butyrate presence in the digesta of distinct GIT segments on digestive processes and amino acid digestibility. In Chapter 5, a gene expression approach was applied in an effort to associate observed changes in digestive processes to changes in the secretory activities of different enteroendocrine cell subsets. In addition, the relationship between mucosal butyrate concentration and the expression of genes related to butyrate metabolism were investigated. In Chapter 6, the intricate relationship between cecal microbiota composition, pre-cecal digestive processes, butyrate presence along the GIT and expression of immune effectors were explored. In Chapter 7, a study is described where the effect of butyrate presence in the digesta of distinct GIT segments investigated on immune responses after *in vivo* stimulations. Finally, Chapter 8 summarizes and discusses the results of the previous chapters.

Butyrate signalling in the proximal GIT

Microbial butyrate production is naturally low in the proximal GIT segments of the avian GIT such as the gastric region and the small intestine. Butyrate signalling in the gastric region increased the proteolytic activity of the proventriculus, prolonged intestinal digesta retention time and modulated the expression of host defence peptides and mucus genes in the proventriculus and pylorus (Chapters 4 and 6). Such changes were associated with changes in cecal microbiota composition that evoked dysbiosis

and inflammation (Chapter 6). Butyrate signalling in the small intestine increased the expression of cholecystokinin prohormone (Chapter 5), modulated the expression of host defence peptides and mucus genes (Chapter 6) and increased the titers of natural IgY antibodies in the peripheral blood (Chapter 7). Such changes were associated with intestinal inflammation (Chapter 6) and signs of intestinal microbiota overgrowth (Chapter 4). The presence of butyrate in the gastric region and small intestine reduced the expression of the free fatty acid receptor 2 (Chapter 5). The changes observed appear to be alterations in physiology and metabolism to improve digestive efficiency (retention time, gut morphology and pepsin secretion) while trying to deal with microbial overgrowth using local and systemic mechanisms (local mechanisms: increased mucus and host defence peptides; systemic mechanisms: reduced feed intake, recruitment of leukocytes as a result of inflammatory chemotaxis). Butyrate in this part of the GIT of poultry appears to be a negative signal to the broiler.

Butyrate signalling in the distal GIT

In broilers, the ceca and colon are the major fermentation sites. Hence, butyrate concentration is much higher in the colon and ceca in comparison to more proximal GIT segments (no butyrate supplementation; Chapter 4 and 7). Butyrate signalling in the colon and ceca increased intestinal retention time and tended to improve apparent pre-cecal digestibility of methionine and feed efficiency of broilers (Chapter 4). Such changes may be due to changes in L-cell secretory activities, although this mechanism could not be demonstrated at the transcriptional level (Chapter 5). Butyrate signalling in the distal GIT did not seem to induce gut inflammation (Chapter 6). In Chapter 8, a view emerged wherein broilers seem to react strongly to increases in digesta butyrate concentration in the GIT segment where naturally, butyrate production is limited. The tolerance threshold of the gastric region and small intestine to the presence of butyrate in the digesta may, therefore, be low. The detrimental effects reported in the present thesis may be related to a vigorous response of broilers to butyrate signaling in such GIT segments. In contrast, the GIT segments where naturally butyrate production is substantial, such as the colon and ceca, may have a higher tolerance threshold.

Butyrate metabolism in the avian GIT

Butyrate did not accumulate in gut tissues (Chapter 5) and did not pass into the peripheral circulation (Chapter 8), irrespective of the GIT segment considered. This indicates that, in the poultry host, intracellular butyrate concentration is tightly regulated. This may be related to possible cytotoxic effects of butyrate as a microbial metabolite (Chapter 8). Yet, an active uptake of butyrate was shown by changes in the expression of potential SCFA transporters along the GIT of broilers (Chapter 5). Transcriptional changes observed in the gastric region, ileum and colon indicated that butyrate may be oxidized in such tissues (Chapter 5). This may show that, from an evolutionary perspective, the benefits in term of energy harvest of a rapid butyrate oxidation outweighs possible detrimental effects incurred by intracellular butyrate accumulation in host tissues.

Recommendations

The results of this thesis indicate, therefore, that butyrate does not always elicit positive effects on gut health of broilers. Such findings support a paradigm shift, from

butyrate being a beneficial molecule to butyrate having effects that may be positive or negative depending on the GIT location considered. Unprotected butyrate was mostly active in the crop and gastric region of birds (Chapter 4). Results of the *in vitro* and *in vivo* studies presented in this thesis indicated that tributyrin, the triglyceride analogue of butyrate, had a release profile targeting enteric segments (Chapters 3, 4, and 7). Wax coated butyrate, an experimental derivative, had a sustained release profile *in vitro* (Chapter 3) but did not result in a protection of butyrate *in vivo* (Chapter 7). Commercial fat-coated products showed heterogeneous release profiles in the *in vitro* model, some products being non-protective whereas others displayed a sustained release profile (Chapter 4). Differences between the two *in vivo* studies concerning the patterns of digesta butyrate concentration along the GIT of birds fed the same fat-coated butyrate derivative (Chapters 4 and 7) were observed. This difference may have been caused by the composition of the basal diet (e.g. fibre composition and level) which can affect endogenous butyrate production in a way that may confound the effects induced by exogenous butyrate supplementation. Hence, it is difficult to propose, on the basis of the data presented in this thesis, practical recommendations for poultry nutritionists. It is recommended, nonetheless, to use sustained release butyrate formulations such as fat-coated butyrate in starter diets and to rely on endogenous butyrate production in later production stages by supplementing grower and finisher diets with butyrogenic fibres such as fructooligosaccharides or inulin. Such a strategy may allow to maximize the positive effects of luminal butyrate on growth performance of broilers, especially when basal diets are devoid of butyrogenic fibres.

RESUME (FR)

Le secteur avicole doit trouver des stratégies permettant d'améliorer les performances zootechniques, la santé et le bien être des volailles tout en réduisant l'utilisation d'antibiotiques et l'importation de matières premières de contrées éloignées. Parmi les additifs alimentaires, le butyrate a reçu beaucoup d'attention de la part des nutritionnistes aviaires et a été proposé comme une solution possible pouvant aider le secteur avicole à relever les défis précédemment cités. La supplémentation alimentaire en butyrate a, cependant, des effets inconsistants sur les performances zootechniques des poulets de chair. Différents mécanismes ont été proposés pour expliquer les effets bénéfiques du butyrate sur les performances zootechniques des animaux de rentes mais, à l'heure actuelle, peu d'études ont été conduites pour vérifier l'existence de ces mécanismes chez le poulet de chair. Il est également incertain que les effets produits par le butyrate sont conditionnés par la partie du tractus digestif (**TD**) dans laquelle la molécule est présente.

Le but de cette thèse est d'évaluer les effets d'une supplémentation alimentaire en butyrate sur les fonctions digestives, la composition du microbiote et les réponses immunitaires du poulet de chair et de vérifier si ces effets sont conditionnés par la partie du TD dans laquelle la molécule est présente. Cette thématique de recherche a été traitée en utilisant des dérivatifs de butyrate ayant des profils de libération nettement différents. Cette thèse décrit les résultats d'une étude *in vitro* et de deux essais *in vivo*. Le chapitre 2 est une revue de la littérature portant sur les modes d'action du butyrate dans le TD de la volaille. Ce travail a mis en lumière le fait que la présence du butyrate dans des parties distinctes du TD peut potentiellement provoquer des réponses spécifiques. Un modèle *in vitro* a été utilisé dans le chapitre 3 pour comparer les cinétiques de libération de dérivatifs commerciaux et expérimentaux à base de butyrate. Sur la base de ce travail *in vitro*, différents dérivatifs ont été sélectionnés et utilisés dans l'essai décrit dans le Chapitre 4. Cet essai avait pour but d'évaluer les effets de la présence de butyrate dans différentes parties du TD du poulet de chair sur la physiologie digestive et sur la digestibilité des acides aminés. Une approche transcriptomique a été utilisée dans le Chapitre 5 pour tenter d'associer les changements observés en terme de physiologie digestive à des changements de l'activité sécrétrice des cellules entéroendocrines. Ce travail a permis, de plus, d'évaluer la relation existant entre le concentration en butyrate de la muqueuse digestive d'une part et l'expression de gènes liés au métabolisme du butyrate d'autre part. Dans le chapitre 6, les relations complexes existant entre la composition du microbiote caecal, les processus digestifs pré-caecaux, la présence de butyrate dans le TD et l'expression de gênes liés à l'immunité ont été explorés. Finalement, les effets de la présence de butyrate dans différentes parties du TD sur les réponses immunitaires du poulet ont été évalués dans le chapitre 7.

Signalisation du butyrate dans la partie *proximale* du TD

La production microbienne de butyrate est basse à l'état naturel dans la partie proximale du TD de la volaille, comme par exemple dans la région gastrique ou l'intestin grêle. La signalisation cellulaire du butyrate dans la région gastrique du poulet de chair a augmenté l'activité protéolytique du proventricule, la rétention intestinale du digesta et a modulé l'expression des peptides antimicrobiens et des mucines dans le

proventricule et le pylore (Chapitres 4 et 6). Ces changements ont été associés à une inflammation intestinale et à des modifications de la composition du microbiote caecal évoquant une dysbiose (Chapitre 6). La signalisation cellulaire du butyrate dans l'intestin grêle a augmenté l'expression du précurseur de la cholécystokinine (Chapitre 5), a modulé l'expression des peptides antimicrobiens et des mucines (Chapitre 6) et a augmenté le titre des anticorps naturels IgY dans la circulation périphérique (Chapitre 7). Ces changements ont été associés à une inflammation de l'intestin grêle et à des signes suggérant une croissance excessive du microbiote intestinal. La présence du butyrate dans la région gastrique et dans l'intestin grêle ont réduit l'expression du récepteur sensible aux acides gras volatils FFAR2 (Chapitre 5). Ces changements physiologiques évoquent une réponse de l'hôte visant à améliorer l'efficacité digestive (temps de rétention du digesta, sécrétions enzymatiques) et à contenir une croissance excessive du microbiote (localement avec les peptides antimicrobiens et les mucines, de manière systémique en réduisant l'ingéré et en recrutant des leucocytes par l'inflammation). Dans l'ensemble, l'adaptation de l'hôte à la présence de butyrate dans la partie proximale du TD peut avoir des effets variables sur les performances zootechniques (Chapitre 2). Cette adaptation résulte de l'intégration d'un signal négatif par l'hôte.

Signalisation du butyrate dans la partie *distale* du TD

Chez les oiseaux, les caeca et le colon sont des sites majeurs de fermentation. La concentration du butyrate dans le digesta du colon et des caeca est, en conséquence, supérieure à celle observée dans les parties du TD qui sont situées en amont de ces organes (Chapitres 4 et 7). La signalisation cellulaire du butyrate dans les caeca et le colon a augmenté le temps de rétention du digesta dans l'intestin grêle et a eu tendance à améliorer la digestibilité apparente iléale de la méthionine. Ces changements sont peut-être dus à une modification de l'activité sécrétrice des cellules entéroendocrines L, bien que ce mécanisme n'a pas pu être démontré au niveau transcriptionnel (Chapitre 5). La signalisation cellulaire du butyrate dans la partie distale du TD n'a pas semblé induire d'inflammation intestinale (Chapitre 6). Il est apparu dans le chapitre 8 que les poulets de chair semblent réagir vigoureusement à l'augmentation de la concentration en butyrate dans le digesta de parties du TD où, à l'état naturel, la production naturelle en butyrate est basse. Le niveau de tolérance de la région gastrique et de l'intestin grêle est donc probablement basse. Les effets négatifs de la signalisation cellulaire du butyrate dans ces parties du TD pourraient être attribuables à une réponse excessive de l'hôte. Les parties distales du TD qui, par nature, ont une production de butyrate supérieure, pourraient avoir un niveau de tolérance supérieur.

Métabolisme du butyrate dans le TD de la volaille

Il n'y a eu aucune accumulation de butyrate dans les tissus du TD (Chapitre 5) et le butyrate ne passe pas dans la circulation sanguine périphérique (Chapitre 8), quelle que soit la partie du TD où la molécule est présente. Cela suggère que, chez la volaille, la concentration intracellulaire en butyrate est finement régulée, possiblement pour éviter des effets cytotoxiques. Le transport actif de cette molécule a été néanmoins suggéré par des changements de l'expression de canaux transportant potentiellement les acides gras volatils à travers l'épithélium (Chapitre 5). Des changements au niveau transcriptionnel laissent également à penser que le butyrate pourrait être oxydé dans

la région gastrique, l'iléon et le colon. Cela semble indiquer que, d'un point de vue évolutif, les bénéfices en terme de couverture énergétique d'une oxydation rapide du butyrate sont supérieurs aux effets potentiellement délétères d'une accumulation de cette molécule dans le cytoplasme des cellules de l'hôte.

Recommandations

Les résultats de cette thèse indiquent donc que le butyrate n'exerce pas systématiquement des effets bénéfiques pour la santé des poulets de chair. Ces observations plaident pour un changement de paradigme : le butyrate n'est pas systématiquement associé à la santé intestinale. Cette molécule peut avoir des effets négatifs ou positifs en fonction de la partie du TD considérée. Le butyrate non protégé est principalement actif dans le jabot et la région gastrique des oiseaux (Chapitre 4). Les résultats de nos études *in vitro* et *in vivo* indiquent que la tributyrine, un triglycéride du butyrate, a un profil de libération ciblant l'intestin grêle (Chapitres 3, 4 and 7). Une encapsulation du butyrate dans de la cire permet un profil de libération graduel *in vitro* (Chapitre 3) mais n'offre que peu de protection *in vivo* (Chapitre 7). Les butyrates encapsulés avec de la matière grasse végétale qui sont disponibles commercialement ont des profils de libération hétérogènes; certains n'offrant aucune protection tandis que d'autres permettent une libération graduelle (Chapitre 4). Nous avons observé des différences du profil de concentration du butyrate dans le digesta du TD des volailles entre nos deux essais *in vivo* en dépit de l'utilisation du même produit commercial (Chapitres 4 et 7). Cela suggère que la composition de l'aliment (e.g. composition et niveau des fibres alimentaires) peut avoir un effet sur la production endogène de butyrate par le microbiote, ce qui rend plus difficile de distinguer les effets de la supplémentation alimentaire en butyrate. Il est donc difficile, à partir de cette thèse, de tirer des conclusions générales et pratiques à destination des nutritionnistes avicoles. Il est néanmoins recommandé d'utiliser un dérivatif du butyrate ayant un profil de libération graduel dans les aliments de démarrage. Certains produits commerciaux utilisant une encapsulation à base de matière grasse végétale pourraient être utilisés à cet effet. Il est ensuite recommandé de s'appuyer sur la production endogène de butyrate par le microbiote en supplémentant les aliments croissance et finition avec des fibres butyrogéniques tel que l'inuline ou les fructooligosaccharides. Cette stratégie alimentaire peut permettre maximiser les effets positifs du butyrate sur les performances zootechniques du poulet de chair, en particulier dans lorsque l'aliment est pauvre en fibres butyrogéniques.

SAMENVATTING (NL)

Kippenvleesproductie in de toekomst zal worden gekenmerkt door een efficiënte groei van gezonde kuikens in een diervriendelijke omgeving die minder milieubelastend zal zijn. Daartoe wordt gebruik gemaakt van voeradditieven om de afhankelijkheid van (ook voor de mens geschikte) granen en soja te verminderen, alsook het gebruik van antibiotica. Een voorbeeld van zo'n voeradditief is boterzuur (butyraat). Boterzuur heeft in de wetenschappelijke en technische literatuur veel aandacht gekregen als mogelijke oplossing voor bovengenoemde uitdagingen voor de sector. Boterzuurtoevoeging aan het voer laat echter tegengestelde effecten zien op groeiprestaties van vleeskuikens. Verschillende hypotheses zijn geformuleerd omtrent de werkingsmechanismen van boterzuur, maar slechts enkele studies bestudeerden daadwerkelijk dit soort mechanismen bij pluimvee. Het blijft daardoor onduidelijk in hoeverre algemene effecten toegeschreven aan boterzuur wellicht meer specifiek zijn toe te schrijven aan het maagdarmsegment waarin dit molecuul vrijkomt.

Het doel van dit proefschrift was de effecten van het toevoegen van boterzuur aan het voer voor vleeskuikens te bestuderen op verteringskinetiek, microbiota-compositie en immunologische responsen. En daarbij te bepalen of de gemeten effecten afhankelijk waren van het maagdarmsegment waar boterzuur aanwezig was. In de proeven werd dit gerealiseerd door boterzuurformuleringen te gebruiken met een verschillende kinetiek in het vrijkomen van boterzuur in het maagdarmkannal (MDK). Dit proefschrift beschrijft de resultaten van een in vitro en twee in vivo experimenten. Hoofdstuk 2 bestaat uit een literatuuroverzicht waarin de werkingsmechanismen van boterzuur in de darm(inhoud) worden belicht. In Hoofdstuk 3 wordt een in vitro experiment beschreven, waarbij de kinetiek van verschillende experimentele en commerciële boterzuurformuleringen in het MDK op laboratoriumschaal wordt gesimuleerd. Gebaseerd op de uitkomsten van deze in vitro studie is een experiment uitgevoerd met verschillende boterzuurformuleringen om de effecten op vertering van nutriënten en specifiek aminozuren te bestuderen (Hoofdstuk 4). In Hoofdstuk 5 is de secretoire activiteit van verschillende entero-endocriene cellen in de darm door middel van genexpressie bestudeerd om deze activiteit te relateren aan verteringsprocessen. Daarnaast is bestudeerd of de boterzuur concentratie in de darmmucosa gerelateerd was aan de expressie van genen die coderen voor boterzuurmetabolisme. De complexe relatie tussen microbiota in de ceca, verteringsprocessen, de beschikbaarheid van boterzuur in bepaalde maagdarmsegmenten en de expressie van immunologische cellen werd bestudeerd in Hoofdstuk 6. Het effect van de aanwezigheid van boterzuur in specifieke maagdarmsegmenten op de immuunrespons na immunisatie was onderwerp van studie in Hoofdstuk 7. In het laatste hoofdstuk (Hoofdstuk 8) worden de bevindingen uit de eerdere hoofdstukken bediscussieerd.

Signaalfunctie van boterzuur in het begin van het MDK

Van nature vindt er weinig boterzuurproductie door microben plaats in het begin van het MDK (magen en dunne darm) bij pluimvee. Uit deze studie bleek dat de boterzuuractiviteit in de magen de proteolytische activiteit van de kliermaag verhoogt, alsook een langere retentietijd van de digesta in de darm veroorzaakt. Daarnaast verandert de genexpressie van eiwitten die de gastheer beschermen en die van mucus in kliermaag en pylorus (Hoofdstukken 4 en 6). Deze veranderingen gingen gepaard

met veranderingen in microbiota-compositie, die dysbiose en ontsteking veroorzaken (Hoofdstuk 6). Boterzuuractiviteit in de dunne darm verhoogde de expressie van het cholecystokine pro-hormoon (Hoofdstuk 5), en veranderde de genexpressie van host defense peptides en die van mucus (Hoofdstuk 6), en het verhoogde de titers van natuurlijke IgY antilichamen in de bloedbaan (Hoofdstuk 7). Deze veranderingen gingen gepaard met darmontstekingen (Hoofdstuk 6) en tekenen van overmatige darmbacteriegroei (Hoofdstuk 4). De aanwezigheid van boterzuur in magen en dunne darm verlaagde de expressie van de vluchtige vetzuurreceptor FFAR2 (Hoofdstuk 5). Deze fysiologische veranderingen suggereren een poging van het metabolisme om de voerefficiëntie te verhogen (retentietijd, darmmorfologie en pepsine secretie), terwijl tegelijkertijd getracht wordt de overmatige groei van microben te beperken, gebruikmakend van lokale (meer mucus en host defense peptides) en systemische mechanismen (verlaagde voeropname en toename van leukocyten ten gevolge van een ontstekingsreactie). Samenvattend, fysiologische veranderingen ten gevolge van de aanwezigheid van boterzuur in het begin van het MDK hebben een wisselend effect op groei bij vleeskuikens (Hoofdstuk 2). Dit komt door het negatieve signaal dat wordt afgegeven door het kuiken zelf.

Signaalfunctie van boterzuur aan het einde van het MDK

Bij vogels zijn de blinde darmzakken en de dikke darm de belangrijkste plaatsen van fermentatie. Vandaar dat de boterzuurconcentratie in die delen van de darm veel hoger ligt dan in de meer proximale delen van het MDK. Dit bleek uit de studies waarin dieren geen boterzuurtoevoeging aan het voer kregen (Hoofdstukken 4 en 7). Boterzuur activiteit in de blinde darmzakken en dikke darm veroorzaakte een langere retentietijd van de digesta in de darm en een tendens was zichtbaar van een verbeterde schijnbare darmverteerbaarheid van methionine en een verbeterde voerefficiëntie (Hoofdstuk 4). Dit type veranderingen kan veroorzaakt zijn door een gewijzigd afgiftepatroon van L-cellen, hoewel dit niet op transcriptieniveau kon worden aangetoond (Hoofdstuk 5). Boterzuur activiteit in de voorste delen van het MDK induceerden geen ontstekingsreacties (Hoofdstuk 6). Uit de resultaten in Hoofdstuk 8 blijkt echter dat kuikens sterk reageerden op een verhoogde boterzuurconcentratie in die segmenten van het MDK waar van nature slechts weinig boterzuur wordt geproduceerd. Het tolerantieniveau in het voorste deel van het MDK lijkt dus laag. De nadelige effecten, zoals beschreven in deze thesis, kunnen gerelateerd zijn aan een krachtige respons van vleeskuikens op de activiteit van boterzuur in deze maagdarmsegmenten. Delen van het MDK waar een hogere boterzuurproductie plaatsvindt, zoals in blinde en dikke darm, hebben wellicht een hogere drempelwaarde.

Boterzuurmetabolisme in het maagdarmkanaal bij pluimvee

Boterzuur hoopte zich niet op in het maagdarmepitheel (Hoofdstuk 5) en evenmin in de perifere circulatie (Hoofdstuk 8), ongeacht het bestudeerde maagdarmsegment. Dit suggereert dat bij pluimvee de intracellulaire boterzuurconcentratie binnen nauwe grenzen varieert. Dit kan te maken hebben met mogelijk cytotoxische effecten van boterzuur als metaboliet (Hoofdstuk 8) en dat zou kunnen betekenen dat boterzuur actief wordt opgenomen ten gevolge van een verandering in expressie van bepaalde korte keten vrije vetzuren in het MDK bij pluimvee (Hoofdstuk 5). Vanuit evolutionair oogpunt kan dit een manier zijn om de energie uit een snelle oxidatie van boterzuur te

benutten om daarmee mogelijk nadelige effecten van een intracellulaire ophoping in darmweefsel te voorkomen.

Aanbevelingen

Uit de resultaten van dit onderzoek blijkt dat boterzuur de maagdarmgezondheid niet uitsluitend positief beïnvloedt. De resultaten ondersteunen een veranderend paradigma rondom de effecten van boterzuur, waarbij het molecuul niet slechts positief werkt maar een werking (positief of negatief) laat zien die afhankelijk is van het segment van de darm waar het molecuul aanwezig is. Vrij boterzuur was het meest actief in de krop en magen (Hoofdstuk 4). Daarentegen bleek uit de in vitro en in vivo studies dat tributerine, het triglyceride analoog van boterzuur, vooral vrijkwam in de dunne darmsegmenten (Hoofdstukken 3, 4 en 7). Boterzuur gecoat met wax, een experimentele formulering, kwam in vitro heel geleidelijk vrij in het MDK (Hoofdstuk 3), maar deze coating gaf in vivo geen enkele bescherming (Hoofdstuk 7). Commerciële vet-gecoate formuleringen lieten in vitro een grote variatie zien in het vrijkomen van boterzuur; sommige formuleringen beschermden totaal niet, waar andere formuleringen een geleidelijk vrijkomen van boterzuur lieten zien (Hoofdstuk 4). Tussen de twee in vivo experimenten zagen we verschillen in boterzuurconcentratie in hetzelfde darmsegment van de vet-gecoate formulering (Hoofdstukken 4 en 7). Dit zou kunnen betekenen dat het voer van de kuikens (bv de vezelfractie, die varieerde tussen beide experimenten) de endogene boterzuur productie kan hebben beïnvloed, waardoor de effecten van de exogene toediening vertroebeld werden. Vandaar dat het lastig is om op basis van de resultaten uit dit proefschrift een algemeen geldende praktische aanbeveling voor pluimveenutritionisten te doen. Desalniettemin zou ik, op basis van deze resultaten, aanbevelen om boterzuur geleidelijk te laten vrijkomen in het MDK (door bv vet-gecoat boterzuur te gebruiken) tijdens de startfase bij vleeskuikens en in latere fasen van de groei te vertrouwen op de endogene boterzuurafgifte ondersteund door een voer met boterzuurstimulerende vezels zoals fructo-oligosacchariden of inuline. Een dergelijke voerstrategie ondersteunt de groei en gezondheid van vleeskuikens, met name in de voeders die geen vezels bevatten.

About the author

My my, hey hey

Out of the blue and into the black

Neil Young

Curriculum Vitae
List of publications
Training and supervision

CURRICULUM VITAE

Pierre Moquet was born on April 3rd, 1989 in Mayenne, France. In 2007 he graduated from high school 'Lycée Lavoisier' in Mayenne after which he started his studies as 'Ingénieur en Agriculture' at Ecole Supérieure d'Agriculture (ESA; Angers, France). During his BSc education at ESA, Pierre followed internships on a dairy farm (GAEC de la Voie Lactée, Mars, France) and in a feed factory (Aliments Genouel, Juvigné, France). Following his BSc, Pierre joined the MSc program EURAMA (EURopean Animal MAnagement) in 2010. This program is a collaboration between Wageningen University (WUR; Wageningen, The Netherlands) and EI Purpan (Toulouse, France) and ESA. Through this program, Pierre followed MSc courses at the Animal Nutrition Group (WUR) and the 'D.A. Lait-Viande' specialization at ESA. For his MSc thesis, Pierre investigated the effect of particle size on hindgut protein fermentation and intestinal health in broilers under the supervision of Dr S.N. Qaisrani and Dr R.P. Kwakkel. During his MSc internship, Pierre worked on nutritional and technical solutions to improve the productivity of lactating sows. This work was conducted under the supervision of Dr Y. Lautrou and Ir N. Cottais at MG2Mix (Chateaubourg, France). Pierre graduated in 2013 from WUR (MSc in Animal Science), E.I. Purpan (MSc Eurama) and ESA (Ingénieur en Agriculture, mention exceptionnelle et félicitations du jury). In 2013, Pierre started his PhD research focussing on the various effects of dietary butyrate supplementation in broilers at the Animal Nutrition Group (WUR) within the framework of the BUTGIT project. The project was co-financed by Nutriad International (Dendermonde, Belgium) and the Agency for Innovation by Science and Technology (VLAIO -formerly IWT-, Brussels, Belgium). From September 2016, Pierre did a half-year sabbatical at ESA where he gave lectures to MSc students on animal nutrition and immunology. He returned to Wageningen in February 2017 to complete the final year of his PhD program. Pierre was employed part-time (25%) from September 2017 as poultry nutritionist at the technical poultry department of MiXscience (Bruz, France) while continuing his PhD at WUR (75%). Pierre will continue working at MiXscience after his graduation.

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Kasper, thank you for the many practical advices that helped me a lot in organizing my work efficiently. Thanks also for the long discussions while cycling, you kept me convinced that I could achieve this journey. My gratitude is extended to Amanda, with whom we shared good moments in NL and FR. You and your family are welcome to visit me anytime in France.

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LIST OF PUBLICATIONS

Peer reviewed scientific publications

Moquet, P.C.A., S.A. Salami, L. Onrust, W.H. Hendriks and R.P. Kwakkel. 2018. Butyrate presence in distinct gastrointestinal tract segments modifies differentially digestive processes and amino acid bioavailability in young broiler chickens. Poult. Sci. 97(1):167-176.

Moquet, P.C.A., L. Onrust, F. van Immerseel, R. Ducatelle, W.H. Hendriks and R.P. Kwakkel. 2016. Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. Worlds Poult. Sci. J. 72: 61-80.

Qaisrani, S.N., **P.C.A. Moquet**., M.M. van Krimpen, R.P. Kwakkel., M.W.A. Verstegen and W.H. Hendriks. 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93(12):3053-3064.

Patents

Goossens, T., R.P. Kwakkel, **P.C.A. Moquet**, F. van Immerseel, B.G. de Geest, J.P. Remon. 2015. Voedingsadditief voor dieren. Nutri Ad International N.V., as -signee. BE Pat. No. 2015/5629.

Goossens, T., R.P. Kwakkel, **P.C.A. Moquet**, F. van Immerseel, R. Ducatelle, L. Onrust. 2017. Animal feed material. Nutri Ad International N.V., as -signee. BE Pat. No. 2017/5984.

Conference and symposia proceedings

Kwakkel, R.P. and **Moquet, P.C.A.** 2013. Coarse diets for poultry: Effects on N-efficiency and gut health. Proc. Curso de especialización FEDNA. Madrid, SP. pp. 103-110.

Moquet, P.C.A., L. Onrust, R. van Emous and R.P. Kwakkel. 2015. A novel *in vitro* poultry model to compare release kinetics of (un)protected butyrate. Proc. European Symposium on Poultry Nutrition. Prague, CZ. p. 159 (Abstr.).

Moquet, P.C.A., L. Onrust, R. van Emous and R.P. Kwakkel. 2016. Development and *in vitro* evaluation of protected butyrate formulations for broiler chickens. Proc. Poultry Science Annual Meeting. New Orleans, US. (Abstr. #439P).

Moquet P.C.A., S.A. Salami, L. Onrust, W.H. Hendriks and R.P. Kwakkel. 2016. Effect of dietary butyrate release location on gut functions and protein digestibility of broilers in a feed challenge model. Proc. World Poultry's Conference. Beijing, CN. p. 84 (Abstr.).

L. Onrust, **P.C.A. Moquet**, R.P. Kwakkel., R. Ducatelle and F. van Immerseel. 2016. A wax matrix is an efficient butyrate carrier that can be used as a feed additive to reduce Salmonella colonization. Proc. World Poultry's Conference. Beijing, CN. p. 390 (Abstr.).

Moquet, P.C.A., L. Onrust and R.P. Kwakkel. 2016. Butyrate in broiler diets: New insights on release location effects in the GIT. Proc. International Animal Nutrition Congress. Antalya, TK. pp. 15-17.

Moquet P.C.A., S.A. Salami, L. Onrust, W.H. Hendriks and R.P. Kwakkel. 2017. Luminal butyrate concentration influences digestive functions, cecal microbiota and amino acid digestibility in a feed challenge model. Proc. Animal Nutrition Research Forum. Gent, BE. pp. 59-60 (Abstr.).

Moquet, P.C.A., G.D.P. Konnert, A. Lammers., L. Onrust and R.P. Kwakkel. 2017. Effect of butyrate concentration in the GIT on innate and adaptive immune responses of broilers. Proc. European Symposium on Poultry Nutrition. Salou, ES. (Abstr. #25757).

TRAINING AND SUPERVISION PLAN

EDUCATION AND TRAINING		
The Basic Package	Year	Credits
WIAS Introduction Course	1	1.5
Course on philosophy of science and/or ethics	1	1.5
Introduction interview with WIAS scientific director and secretary	1	-
Introduction interview with WIAS education coordinator	1	-
Introduction interview with WIAS PhD students confidant	1	-
Subtotal Basic Package		**3**
Scientific Exposure	Year	Credits
International conferences		
European Symposium on Poultry Nutrition (Prague, CZ; 2015)	2	1.2
Poultry Science Association meeting (New Orleans, US; 2016)	3	1.2
World Poultry Conference 2016 (Beijing, CN; 2016)	3	1.2
International Animal Nutrition Congress (Antalya, TR; 2016)	3	0.9
European Symposium on Poultry Nutrition (Salou, ES; 2017)	4	1.2
Poultry Science Association meeting (Orlando, US; 2017)	4	1.2
Seminars and workshops		
Animal Nutrition Research Forum (Gent, BE; 2017)	4	0.3
Intestinal Health Workshop (Gent, BE; 2017)	4	0.9
Presentations		
Poster: European Symposium on Poultry Nutrition (Prague, CZ; 2015)	2	1.0
Poster: Poultry Science Association meeting (New Orleans, US; 2016)	3	1.0
Oral: World Poultry Conference 2016 (Beijing, CN; 2016)	3	1.0
Invited Speaker: International Animal Nutrition Congress (Antalya, TK; 2016)	3	1.0
Oral: Animal Nutrition Research Forum (Gent, BE; 2017)	4	1.0
Oral: European Symposium on Poultry Nutrition 2017 (Salou, BE; 2017)	4	1.0
Oral: Poultry Science Association meeting (Orlando, US; 2017)	4	1.0
Subtotal Scientific Exposure		**15**
In-Depth Studies	Year	Credits
Disciplinary and interdisciplinary courses		
Poultry Production and Health (Aarhus, DK)	2	5.0
Gut health in pigs and poultry (Wageningen, NL)	2	0.5
Energy metabolism and body composition (Wageningen, NL)	3	1.0
Advanced statistics courses		
Design of experiments (Wageningen, NL)	2	1.0
Statistics for life sciences (Wageningen, NL)	1	2.0
PhD students' discussion groups		
Animal Health and Immunology (Wageningen, NL)	2;3;4	2.0
Subtotal In-Depth Studies		**12**

EDUCATION AND TRAINING (continued)			
Statutory Courses		Year	Credits
Use of Laboratory Animals		1	3
Subtotal Statutory Courses			**3**
Professional Skills Support Courses		Year	Credits
Project and time management		3	1.5
Scientific writing		4	1.8
Subtotal Professional Skills Support Courses			**3**
Research Skills Training		Year	Credits
Training at the Pharmaceutical Technology Lab of UGent (Gent, BE)		1	2.0
Training at the Animal Science Department of ESA Angers-Loire (Angers, France)		4	-
Subtotal Research Skills Training			**2**
Didactic Skills Training		Year	Credits
Lecturing			
Designing and teaching a MSc Course on Immunology		4	3.0
Designing and teaching a MSc Course on Nutrition		4	3.0
Supervising practicals and excursions			
Review RMC proposal		1;2;4	1.5
PAN practicals		1;2	2.0
Supervising theses			
Joice san Andres	(MSc; Major thesis)	1	2.0
Rosalie van Emous	(MSc; Major thesis)	2	2.0
Saheed Salami	(MSc; Major thesis)	2	2.0
Chuanlan Tang	(MSc; Major thesis)	3	2.0
Gauthier Konnert	(MSc; Major thesis)	3	2.0
Subtotal Didactic Skills Training			**20**
Management Skills Training		Year	Credits
Organisation of seminars and courses			
Organisation of Animal Health and Immunology masterclasses		3	1.0
Membership of boards and committees			
WIAS science day board member		3	1.0
Chairman of the Animal Health and Immunology discussion group		3	1.0
Subtotal Management Skills Training			**3**
Education and Training Total			**60**

COLOPHON

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