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Development and validation of the Simulator of the Canine Intestinal Microbial Ecosystem (SCIME™)

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ABSTRACT

Whereas a wide variety of *in vitro* models have been developed and validated to assess the effect of specific food ingredients on the human gut microbiome, such models have only been developed and applied to a limited extent for companion animals. Since the use of pre- and probiotics to improve gut health **is** an emerging research topic in the field of companion animals and as dogs are often used as laboratory animals in developing and testing of pharmaceuticals, the current study aimed to establish an adequate canine *in vitro* model. This consisted of a four-stage reactor composed of a stomach and small intestinal compartment followed by a proximal and distal colon. This semi-continuous gastrointestinal tract model allowed a long-term, region-dependent and pH-controlled simulation of the colon-associated microbial community of dogs. Upon reaching a functional steady state, the simulated canine microbial community composition proved to be representative of the *in vivo* situation. Indeed, the predominant bacterial phyla present in the *in vitro* proximal and distal colon corresponded with the main bacterial phyla detected in the fecal material of the dogs, resulting in an average community composition along the simulated canine gastrointestinal tract of 50.5% Firmicutes, 34.5% Bacteroidetes, 7.4% Fusobacteria, 4.9% Actinobacteria and 2.7% Proteobacteria. A parallel *in vivo-in vitro* comparison assessing the effects of fructo-oligosaccharides on the canine microbial community composition showed a consistent stimulation of *Lactobacillus* concentrations in the *in vivo* fecal samples as well as in the *in vitro* canine gut model. Furthermore, the *in vitro* platform provided additional insights about the prebiotic effect of fructo-oligosaccharides supplementation of dogs, such as a reduced abundance of *Megamonas* spp. which are only present in very low abundance in *in vivo* fecal samples, indicating an interesting application potential of the developed canine *in vitro* model in research related to gastrointestinal health of dogs.

Key words: dog; validation; SCIME; FOS; *in vitro*.

INTRODUCTION

The canine gastrointestinal tract harbors a complex community of bacteria, fungi, archaea and viruses, in the same order of magnitude as the host cells (Sender et al., 2016). With 10^9 – 10^{10} CFU/g, the amount of bacterial cells present in the large intestine of dogs outnumbers the other parts of the canine gastrointestinal tract (Honneffer et al., 2014). Similar to humans, the composition of the intestinal microbial community of dogs mainly consists of the bacterial phyla Firmicutes, Bacteroides, Proteobacteria, Fusobacteria and Actinobacteria in a similar distribution, thereby covering 99% of all bacteria in the canine intestine (Middelbos et al., 2007; Turnbaugh et al., 2009; Suchodolski, 2011; Schmitz and Suchodolski, 2016). It has been shown that the colon-associated canine microbiome plays a crucial role in food digestion, prevention from pathogenic infection, bioconversion of endogenous and exogenous compounds and immunomodulation (Garcia-Mazcorro et al., 2012). Therefore, much attention has recently been given to strategies that modulate the composition and metabolism of the canine intestinal microbial population in order to improve canine health (Pinna and Biagi, 2014). The most extensively studied approach for microbial community modulation in dogs is the use of prebiotics. Prebiotic compounds are classified as non-digestible substrates that are selectively used by the gut microbial community, thereby conferring a health benefit for the host (Gibson et al., 2017). Although the use of novel next-generation sequencing techniques gave rise to new insights regarding effects of prebiotics on microbial structure and function, final conclusions remain conflicting (Sunvold et al., 1995; Swanson et al., 2002a; Swanson et al., 2002b; Flickinger et al., 2003; Hesta et al., 2003; Suchodolski, 2011; Beloshapka et al., 2013; Pinna and Biagi, 2014), emphasizing the need of additional studies to unravel the effect of prebiotics on the canine gastrointestinal community.

As *in vivo* studies of dietary effects on the intestinal microbiota frequently encounter technical and ethical constraints, much attention has been given to the development of *in vitro* models. Whereas a wide variety of *in vitro* gut models to study the effect of test products, including prebiotics, on the gut microbiome have been developed and validated for human applications (Van den Abbeele et al., 2010), the use of such models for companion animals such as dogs remains

limited. Moreover, since dogs are often used as laboratory animals in the development of pharmaceutical compounds, the establishment of an adequate canine *in vitro* gut model is of high interest.

As reviewed by Suchodolski (Suchodolski, 2011), prebiotic *in vitro* studies in the field of companion animals are restricted to short term batch experiments (Sunvold et al., 1995; Barry et al., 2011). Such experiments are often not representative of the complex colonic environment as they lack pH control, repeated feeding cycles and refreshment of the media, and therefore do not simulate accurately the *in vivo* situation. This indicates a clear need for better designed *in vitro* gut models simulating the gastrointestinal tract of companion animals.

In the current study, a dynamic *in vitro* gut model simulating the canine gastrointestinal tract was developed with focus on the colon-associated microbial community. To validate the developed canine gut model, a simultaneous *in vivo-in vitro* comparison was performed in which beagle dogs were subjected to common prebiotic treatment *in vivo*, whereas the fecal microbiota of the dogs was used as inoculum for a parallel *in vitro* prebiotic supplementation study.

MATERIALS AND METHODS

Simulator of the canine intestinal microbial ecosystem

Considering the high similarity at the compositional and functional levels between the dog and human gut microbiota, the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) as described by Molly *et al.* (Molly et al., 1993) was adapted to simulate the canine gastrointestinal tract. The Simulator of the Canine Intestinal Microbial Ecosystem (SCIME™) set-up included four temperature-controlled reactors representing the different parts of the canine gastrointestinal tract, i.e. stomach, small intestine, proximal (PC) and distal colon (DC), as shown in Figure 1. The system was operated at 39°C and kept anaerobic by daily flushing with N_2-gas. The first two reactors were of the fill-and-draw principle to simulate food uptake and digestion. In the stomach compartment a defined amount of simulated nutritional medium, composed of 9 g/L dog food (Hill's Science plan adult advance Fitness Lamb and Rice), 4 g/L mucin, 0.5 g/L cystein (Sigma-Aldrich, Bornem, Belgium), 4 g/L special peptone and 1.5 g/L yeast extract (Oxoid, Aalst, Belgium), was added twice a day. Similarly, simulated pancreatic juice, composed of 2 g/L bile extract (Oxgall, Difco, Bierbeek, Belgium), 0.9 g/L pancreatin (Applichem, Zedelgem, Belgium) and 12.5 g/L $NaHCO_3$ (VWR, Heverlee, Belgium) was added to the small intestinal compartment upon entering of the gastric suspension into this reactor. The last two reactor vessels, representing the canine large intestine, were continuously stirred with constant volume and pH control, and received the digested suspension from the small intestinal compartment at pre-defined intervals, **with 12h in-between each feeding cycle**. The pH controllers, peristaltic pumps for liquid transfer and flushing equipment were incorporated in an automated setup controlled by LabVIEW software (TWINSHIME®, ProDigest, Zwijnaarde, Belgium). Residence times and pH were controlled to resemble *in vivo* conditions in the different parts of the canine gastrointestinal tract. Table 1 gives an overview of the parameters implemented in the SCIME™ model as compared to the SHIME® setup.

In vivo validation study

The animals were housed at the Faculty of Veterinary Medicine (Ghent, Belgium) according to the European animal welfare conditions. Prior to the study, dogs were adapted to the standard diet (Hill's Science plan adult advance Fitness Lamb and Rice) during 14 days. During the *in vivo* trial, 10 beagle dogs (5 male, 5 female) were fed the standard diet during a 10-day control period. This was followed by a 20-day treatment period, during which the standard diet was supplemented with 3% (w:w) fructo-oligosaccharides (FOS) from chicory root (Fibrulose®, Cosucra Groupe Warcoing S.A., Belgium). Four out of the ten dogs were randomly selected for validation of the *in vitro* SCIME™ model. During the control period, fresh fecal material from these four dogs was collected in an anaerobic container using an anaerobic bag (Anaerogen, Oxoid, Aalst, Belgium) for transport. Fecal material was processed within the hour after defecation as previously reported by De Boever et al. (De Boever et al., 2000) and used for inoculation of four parallel *in vitro* SCIME™ platforms. After inoculation, the canine microbial community was allowed to stabilize during 14 days to the *in vitro* reactor conditions. Following this stabilization period, the *in vitro* experiment consisted of a two-week control period in which the standard nutritional medium was administered, followed by a two-week treatment period

where the standard nutritional medium was supplemented with 3% FOS (Fibrulose®, Cosucra Groupe Warcoing S.A., Belgium), corresponding to a daily dose of 7.5 g/day.

Sample collection

Sampling of each reactor vessel of the SCIME™ was performed every two days during the stabilization, control and treatment periods. Liquid samples for subsequent analysis of microbial metabolic activity were immediately frozen at -20°C, while pelleted cells (5 min, 9000*g*) originating from 1 mL liquid sample were frozen at -20°C for subsequent molecular analysis.

During the *in vivo* trial, fecal material was collected 30 min after defecation every two days during the control and treatment periods and stored at -20°C prior to analysis. Fecal material for metabolic analysis was diluted in distilled water 12.5% (w:v) and analyzed immediately. DNA extraction was performed directly on 0.5g fecal material.

Microbial metabolic activity

Short chain C2-C6 fatty acids (SCFA), including isoforms C4-C6, were measured by gas chromatography as described by Andersen *et al.* (Andersen et al., 2014). Ammonium concentrations were determined by steam distillation as described by Possemiers *et al.* (Possemiers et al., 2004).

Microbial community composition

Total DNA was extracted using the method described previously by Van den Abbeele *et al.* (Van den Abbeele et al., 2018). The extracted DNA was dissolved in DNAse free water and stored at -20°C for subsequent analysis. Firstly, the microbial community composition was determined through Illumina sequencing. The V1-2 region of the 16S rRNA gene was amplified as previously described (Camarinha-Silva et al., 2014) with some minor modifications. In the first 20 cycles of the polymerase chain reaction (PCR) the 16S rDNA target was enriched using the well-documented 27F and 338R primers (Lane, 1991; Etchebehere and Tiedje, 2005) as specified by Chaves-Moreno *et al.* (Chaves-Moreno et al., 2015). Libraries were sequenced on an Illumina MiSeq platform. Furthermore, quantitative PCR (qPCR) for *Lactobacillus* spp. was performed as previously reported by Furet *et al.* (Furet et al., 2009).

Statistical analysis

Statistical data analyses for the *in vivo* - *in vitro* validation study was performed with the statistical software R, version 3.0.2. for Windows (http://www.r-project.org). Linear mixed model analysis was performed using the linear mixed-effects models (lme) package (Laird and Ware, 1982; Lindstrom and Bates, 1988). The restricted maximum likelihood (REML) approach was used to fit the mixed model (Pinheiro and Bates, 1996). The residuals were plotted against the fitted data. The distribution of the residuals and the normality was visually checked. Additionally, normality of the residuals was tested using the Shapiro-Wilk Normality Test. In case of non-normality, the data were transformed to reach normality. The optimal transformation was determined using the box-cox transformation function (Box and Cox, 1964) in R. P-values to examine the effect of the prebiotic treatment were obtained using ANOVA of the mixed models. P-values below 0.05 were considered statistically significant.

For the *in vivo* and *in vitro* experiment, normality of the data and equality of the variances were assessed using the Kolgomorov-Smirnov test and Levene's test. Comparison of normally distributed data was performed with Student's test for pairwise comparisons. Comparison of means of non-normally distributed data was evaluated with nonparametric Kruskal-Wallis test. P-values below 0.05 were considered statistically significant.

Bioinformatic processing of the Illumina sequencing data was conducted as previously described (Camarinha-Silva et al., 2014) with some modifications. Pair-end raw sequences were assembled according to Cole *et al.* (Cole et al., 2014) and subsequently aligned (gotoh algorithm with the SILVA reference database) and pre-clustered (diff=2) using MOTHUR (Schloss et al., 2009). Obtained phylotypes were filtered to include only those present in an average abundance of $\geq$ 0.001% in all samples. Rarefaction curves and statistics were generated using the vegan package in R. All phylotypes were assigned a taxonomic affiliation based on RDPs Naïve Bayesian Classifier (RDP classifier) (Wang et al., 2007) applying an 80% of threshold. Overall, a total of 1,113,350 reads were obtained (with an average of 34,792 reads per sample) that clustered into 1,942 phylotypes.

RESULTS

Stability of canine microbial community in SCIME™

Functional stability of the SCIME™ reactors was determined by plotting the correlation coefficients of microbial metabolic parameters between a sampling point and its preceding sampling point as a function of time during the stabilization and control periods (Figure 2). It was observed that the calculated correlation coefficients for the measured metabolic parameters, i.e. acetate, propionate, butyrate, total SCFA (sum of acetate, propionate, butyrate and branched SCFA) and ammonium, exceeded the minimal threshold of 80% within 14 days of reactor operation. In the DC, acetate, propionate and total SCFA concentrations reached the 80% threshold after 7 days of operation, while a stabilization period of 14 days was needed in the PC. For butyrate, a stabilization period of 14 days was needed in both colon regions in order to reach the stability threshold of 80%. The fastest stability was reached for ammonium concentrations, as the 80% correlation was reached after 4 and 7 days for the PC and DC respectively.

Microbial community composition in SCIME™

Predominant bacterial phyla present in the PC and DC of the SCIME™ consisted of Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria, which corresponded with the main bacterial phyla detected *in vivo* (Table 2). The Firmicutes phylum dominated the fecal material of the dogs with a relative abundance of 94.4% (ranging from 98.7% to 91.1%), followed by Actinobacteria with relative abundances ranging from 7.0% to 0.9%. Similarly, Firmicutes were the most abundant phylum in the simulated canine microbial community of the SCIME™, comprising 52.4% and 48.6% in the PC and DC respectively. Actinobacteria were equally enriched in the PC and DC of the SCIME™, resulting in similar relative abundances as observed in the fecal microbial community of the dogs. Further, as compared with the *in vivo* samples, the simulated canine microbial community of the SCIME™ was enriched in Bacteroidetes (40.1% in PC and 28.8% in DC as compared to 1.2% *in vivo*) and Proteobacteria (2.3% in PC and 3.2% in DC as compared to <0.1% *in vivo*) at the expense of Firmicutes levels. Furthermore, in the DC an enrichment of Fusobacteria was observed, as seen by an overall relative abundance of 14.5%.

Within the Firmicutes phylum, the predominant bacterial phylotypes observed in the *in vivo* samples belonged to the genus *Lactobacillus*. In the simulated canine microbial community of the SCIME™, the *Lactobacillus* genus was also highly abundant, especially in the PC, highly similar to the *in vivo* microbiota (17.2% and 6.3% in PC and DC, respectively, as compared to 26.7% *in vivo*). Similarly, the bacterial phylotypes belonging to the *Clostridium* cluster XI group were enriched *in vivo*, while they were detected in lower concentrations in the PC and DC compartments of the SCIME™ model. The genera *Clostridium* cluster XIVa and *Megamonas* on the other hand were enriched in the *in vitro* simulation model as compared to the fecal material of the dogs.

Effect of FOS supplementation on microbial community composition

In vivo, the addition of FOS resulted in a strong and significant increase in *Lactobacillus* concentrations. This key effect of FOS *in vivo* was also observed in both the PC and DC of the *in vitro* SCIME™ model (Table 3). Furthermore, FOS supplementation resulted in a significant decrease in two bacterial phylotypes belonging to *Clostridium* cluster XI *in vivo*, while this was only observed in the PC for one of these phylotypes *in vitro*. Finally, slightly enhanced levels of the genus *Collinsella*, belonging the Actinobacteria phylum, and some bacterial phylotypes within the Bacteroidetes phylum were significantly enriched upon FOS administration *in vivo*, with similar effects being observed *in vitro*, though not reaching statistical significance. On the contrary, the simulated canine microbial community of the SCIME™ was characterized by some minor decreases in *Bacteroides* genera and in a significant decrease in *Megamonas* upon FOS supplementation, which were not observed *in vivo*. In the DC compartment of the SCIME™, a remarkable increase in several *Allobaculum* phylotypes was observed, which was not observed in the *in vivo* fecal samples.

To confirm the results obtained through 16S-targeted Illumina sequencing, the most abundant bacterial genus, i.e. *Lactobacillus* spp., was quantified by qPCR (Figure 3). *Lactobacillus* concentrations significantly increased upon FOS supplementation in the PC of the SCIME™ model ($p=0.040$) as well as in the *in vivo* fecal samples ($p=0.021$). Also in the DC of the SCIME™ model an increase in *Lactobacillus* concentrations was observed, though this did not reach statistical significance.

Microbial community activity in SCIME™

SCFA profiles of the four SCIME™ platforms during the control period revealed that the PC was characterized by a SCFA profile consisting of 41% acetate, 41% propionate, 15% butyrate and 2% branched SCFA, while in the DC a proportional ratio of 40% acetate, 37% propionate, 20% butyrate and 3% branched SCFA was observed (Table 4). In the DC, a shift towards increased butyrate concentrations was observed ($p<0.001$), mainly at the expense of propionate concentrations ($p=0.083$). Furthermore, absolute branched SCFA concentrations were significantly higher in the DC as compared to the PC ($p<0.001$). Similarly, ammonium concentrations were significantly enriched in the DC compartments as compared to the PC ($p<0.001$).

FOS supplementation resulted in a significant increase in acetate, propionate and butyrate concentrations in both the PC and DC compartments of the SCIME™ model, with the strongest effects being observed for propionate concentrations (Table 4). *In vivo*, increases in the aforementioned SCFA were not detected and even a significant decrease in butyrate concentrations was observed upon FOS administration. In contrast to the non-branched SCFA, the branched SCFA significantly decreased upon FOS supplementation in both colon compartments of the SCIME™, thereby confirming the effects that were observed *in vivo*. Finally, ammonium production decreased significantly in PC as well as in DC, which was also observed in the fecal samples of *in vivo* experiment.

DISCUSSION

The aim of this research was to establish a continuous *in vitro* model simulating the canine gastrointestinal tract with focus on the colon-associated microbial community. Due to high similarities in microbial function and composition between the gastrointestinal tract of humans and dogs, the SHIME®, a validated *in vitro* model simulating the human gastrointestinal tract (Molly et al., 1993), was adapted in order to meet the canine physiology in terms of feed composition and regimen, gastrointestinal regions, digestive enzymes, pH control, temperature, residence time and fecal inoculum applied.

With respect to the simulated canine microbial community composition, it was observed that the PC and DC of the SCIME™ harbored distinct microbial communities. For the human *in vitro* simulation platform this region-dependency of the colon-associated microbial community in terms of functionality and composition has been extensively validated using colonic samples from sudden death victims and proved to be mainly attributed to substrate availability, pH, residence time and redox potential (Macfarlane et al., 1998). Although such an extensive validation for the dog has not been performed yet, the current study indicates that two distinct bacterial communities were sustained in the proximal and distal compartments of the SCIME™. While the PC was characterized by high abundance of members from the *Prevotellaceae* and *Lactobacillaceae* families, the DC was enriched in *Fusobacteriaceae* as well as species belonging to *Allobaculum* genus. It has been reported that *Lactobacillaceae* are more enriched in the proximal part of the canine intestine due to their resilience towards lower pH and higher bile salt concentrations, and due to the shorter residence times in the upper parts of the gastrointestinal tract (Perelmuter et al., 2008), thereby confirming data obtained in the current study. Furthermore, it is well described that the proximal microbial community is adapted towards saccharolytic fermentation due to influx of undigestible carbohydrates in this part of the large intestine (Macfarlane et al., 1992). For instance, several members of the Bacteroidetes phylum, including *Prevotellaceae*, have been associated with high-fiber degradation potential (De Filippo et al., 2010), and therefore specifically colonize the proximal colon areas (Van den Abbeele et al., 2010). The distal colon on the other hand is generally characterized by a microbial community rich in species with specific metabolic functions, such as mucin (Van Herreweghen et al., 2017) and protein degradation (Macfarlane et al., 1992). In the present study, the DC of the SCIME™ showed increased concentrations of branched SCFA and ammonium, which have been linked with proteolytic fermentation. However, also increased production of butyrate was observed in the DC of the simulated canine gastrointestinal tract, which could be linked with the specific enrichment of *Allobaculum* in this colonic area. Indeed, members of the *Allobaculum* genus have been reported as potent butyrate producers in the canine gastrointestinal tract (Greetham et al., 2004). The same butyrate producing functionality has been described in many members of *Clostridium* cluster XIVa in humans (Louis and Flint, 2009). *Clostridium* cluster XIVa was also enriched in the DC compartment

of the SCIME™ model during the current study, though to a much lower extent as compared to the canine-specific *Allobaculum* genus.

Recent advances in molecular tools have greatly improved our knowledge of the canine colon-associated microbial community. Kim *et al*. (Kim et al., 2017) showed by using the Illumina Miseq platform that the canine microbial community composition consists on average of Firmicutes (64.2–73.3%), Bacteroidetes (17.3–19.9%), Proteobacteria (0.9–8.7%), Fusobacteria (0–13.6%) and Actinobacteria (0.6–1.5%). However, Garcia-Mazcorro *et al.* showed a more dominant presence of Firmicutes (75–98%) in the fecal community of dogs using 454-pyrosequencing (Garcia-Mazcorro et al., 2012). These observed discrepancies might be explained by the molecular method used, which should be considered when abundances of gut microbiota are compared among different studies (Garcia-Mazcorro and Minamoto, 2013). While the Firmicutes phylum strongly dominated the canine feces in the current study, with a relative abundance of 94.4%, the predominant bacterial phyla present in the PC and DC of the SCIME™ corresponded with the main bacterial phyla detected in the fecal material of the dogs, resulting in an average community composition along the simulated canine gastrointestinal tract of 50.5% Firmicutes, 34.5% Bacteroidetes, 7.4% Fusobacteria, 4.9% Actinobacteria and 2.7% Proteobacteria. Overall, all bacterial groups that were present in the fecal material of the dogs in the current study proliferated well in the *in vitro* environment, resulting in highly representative microbial communities along the colonic regions of the SCIME™ model. However, when comparing the fecal microbial composition in the current study with the *in vitro* results, the microbial community in the SCIME™ model was significantly enriched in Bacteroidetes and Proteobacteria at the expense of Firmicutes. A similar enrichment has been previously observed in human *in vitro* gut models (Rajilic-Stojanovic et al., 2010; Van den Abbeele et al., 2010) and could be partly associated with the absence of mucosal adhesion sites (Van den Abbeele et al., 2013). Indeed, it has been shown that inclusion of a mucosal environment in the SHIME® model prevented the wash out of typically mucin associated butyrate producers (Van den Abbeele et al., 2013) and could therefore also be an interesting extension of the SCIME™ platform. Finally, the microbial community in the SCIME™ model showed a specific enrichment of *Megamonas* species in both the PC and DC. As predominant members of the *Veillonellaceae* family, *Megamonas* species are known to produce propionate and butyrate from lactate (Lin et al., 2011). Furthermore, in dogs, *Megamonas* spp. have been described to be enhanced upon supplementation of the prebiotic FOS, indicating their potential beneficial effect on the canine gastrointestinal health (Beloshapka et al., 2013). The fact the *in vitro* model sustained the growth of *Megamonas* could potentially allow a better understanding of the functionality of this genus in the microbial community of the canine associated gastrointestinal tract.

To assess the effect of a test product on intestinal microbial composition and functioning *in vitro*, the establishment of steady-state bacterial community prior to the actual start of the treatment is of utmost importance as stability of the microbial community guarantees that any effects observed during a specific treatment truly result from the administered test product (Van den Abbeele et al., 2010). Results showed that functional stability of the microbial community in the SCIME™ model was reached after 14 days following inoculation, which nicely corresponded with the results obtaining with the SHIME® model for human applications (Possemiers et al., 2004; Van den Abbeele et al., 2010). Upon reaching a stable microbial community in the current study, treatment with FOS from chicory root was initiated for a duration of two weeks in a parallel *in vitro*-*in vivo* experiment. This non-digestible fiber has been well described for its prebiotic potential in both dogs (Swanson et al., 2002b; Pinna and Biagi, 2014) and humans (Van de Wiele et al., 2004; Grootaert et al., 2009). Although conflicting findings on the effect of prebiotic administration in dogs on markers of proteolytic fermentation have been described (Beynen et al., 2002; Flickinger et al., 2003; Propst et al., 2003), our study revealed a consistent reduction of ammonium and branched SCFA concentrations both *in vivo* as well as *in vitro*. Furthermore, a consistent stimulation of *Lactobacillus* concentrations was observed upon FOS supplementation in the *in vivo* fecal samples as well as in the PC and DC of the SCIME™ model. On the other hand, FOS supplementation resulted in increased acetate, propionate and butyrate concentrations in the simulated canine gastrointestinal tract, while fecal levels of the aforementioned SCFA were not affected and even slightly reduced. Though, it is well known that fecal SCFA are not fully representative for the *in vivo* situation, as they are absorbed along the colon by the host (Von Engelhardt et al., 1989). Furthermore, large inter-individual variations often result in the absence of significant effects. Similarly,

some conflicting effects on microbial community composition were observed between the parallel *in vitro* and *in vivo* study. For instance, reduced levels of *Megamonas* spp. were observed in the SCIME™ model upon treatment with FOS, while fecal concentrations remained unaffected. However, the low abundance of this genus in the *in vivo* samples made significant observations difficult. Several studies reported increased fecal abundance of *Megamonas* spp. upon treatment with FOS (Beloshapka et al., 2013). However, fecal community composition does not reflect the *in vivo* situation, where specific microbes can thrive in specific areas of the gastrointestinal tract. *In vitro* studies could therefore help to unravel the health-related effects of specific bacterial genera on the host and to measure prebiotic effects at the site of fermentation.

In conclusion, a dynamic *in vitro* gut model simulating the canine gastro-intestinal tract was developed, with focus on the colon-associated microbial community allowing to culture the complex gut microbiota over a longer period under representative conditions of the different intestinal regions. Advanced molecular analysis demonstrated the colon-region specificity of the colonization process, with the PC being characterized by a saccharolytic microbial community, while the DC was enriched in species with specific metabolic functions such as butyrate production and protein degradation. Upon reaching a functional steady state, the simulated canine microbial community composition proved to be representative for the *in vivo* condition, though the absence of mucosal adhesion sites resulted in a specific enrichment of Bacteroidetes and Proteobacteria at the expense of Firmicutes. **Furthermore, as the current study mainly focused on validation of the activity and composition of the colon-associated microbial community in the SCIME™ model, chemical assessment of the digesta at each step of the digestion process might be an interesting approach for further validation of the *in vitro* canine gut model. Overall,** the *in vitro* platform provided additional insights in the prebiotic effect of repeated FOS supplementation in dogs, indicating an interesting application potential of the SCIME™ model in research related to gastrointestinal health in dogs.

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FIGURE LEGENDS

Figure 1. SCIME™ reactor setup. Schematic overview of the SCIME™ (Simulator of the Canine Intestinal Microbial Ecosystem) simulating the full canine gastrointestinal tract.

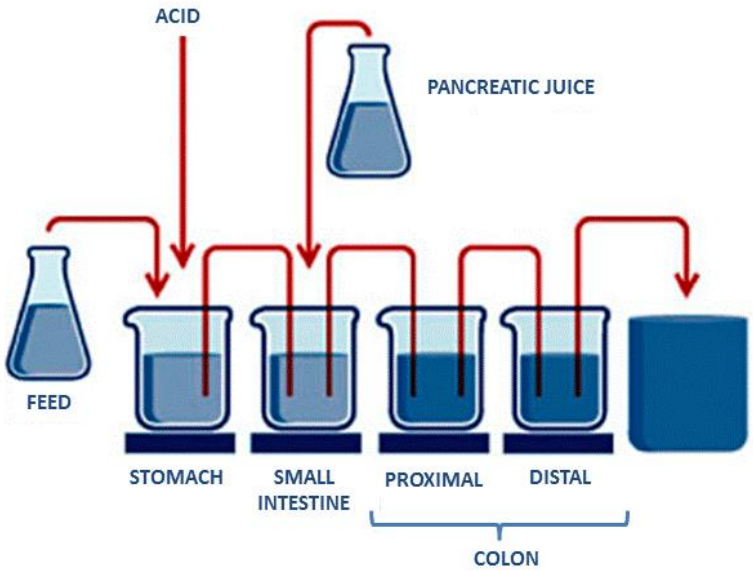


Figure 2. Stability of the Canine Microbial Community in SCIME™. Variability of microbial metabolic activity obtained in the SCIME™ during the stabilization (day 1-14) and control period (day 15-30) expressed as correlation between a sampling point and its preceding sampling point (%) for acetate, propionate, butyrate, total SCFA and ammonium concentrations in the proximal and distal colon (n=1). The threshold for stability was put at 80% (dashed line) according to Van den Abbeele *et al.* (Van den Abbeele et al., 2010).

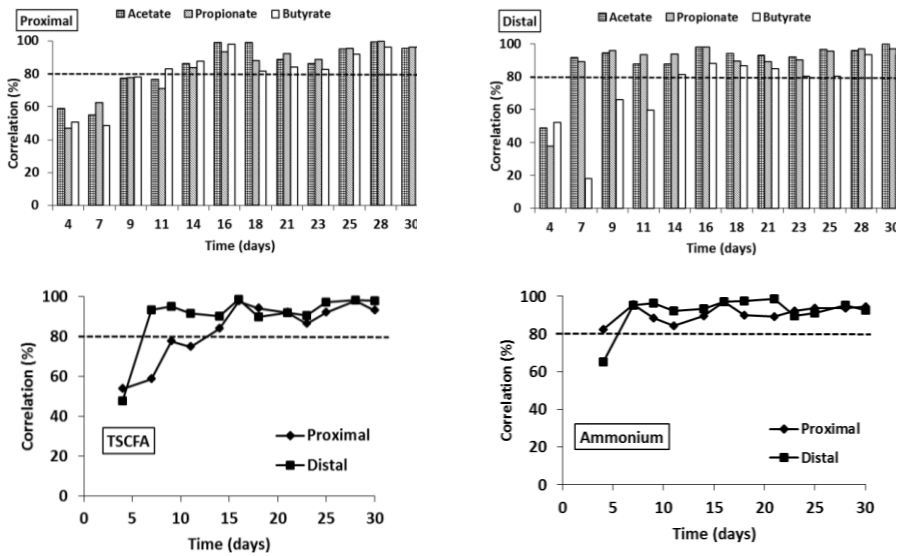
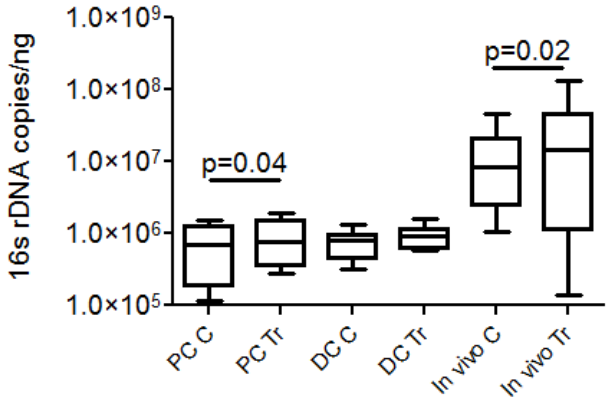


Figure 3. Microbial community composition as assessed via qPCR. ***Lactobacillus*** **concentrations** (16S rDNA copies/ng DNA) as assessed via qPCR at the end of the control (C) and treatment (Tr) period with FOS in both the proximal (PC) and distal colon (DC) of the SCIME™ model, as well as in the fecal material of the dogs (*in vivo*). Statistically significant differences are indicated by their p-value.



TABLES

Table 1. SCIME™ parameters. Reactor setup of the Simulator of the Canine Intestinal Microbial Ecosystem (SCIME™) including reactor volumes (mL), residence times (h) and pH compared to the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) (Possemiers et al., 2004).

	SCIME™			**SHIME®**		
	Volume (mL)	**Residence time (h)**	**pH**	**Volume (mL)**	**Residence time (h)**	**pH**
Stomach	140	1	2.00	200	2	2.00
Small intestine	200	4	6.80	200	4	6.80
Proximal colon	100	6	5.60-5.90	500	20	5.60-5.90
Transverse colon	-	-	-	800	32	6.10-6.40
Distal colon	167	10	6.65-6.90	600	24	6.60-6.40
Feeding regimen	2x/day			3x/day		

Table 2. Canine microbial community composition. Abundances of the dominant bacterial phylotypes belonging to the Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria phylum (% ± SEM) as assessed via 16S-targeted Illumina sequencing in samples collected from the SCIME™ during the control period in both the proximal (PC) and distal colon (DC), as well as in the fecal material of the dogs during the control period (n=4). Statistically significant differences from the samples collected from the SCIME™ as compared to the *in vivo* samples are indicated in bold, **while different letters indicate statistical differences between each of the tested samples (p<0.05).**

	In vitro				***In vivo***	
	PC		**DC**			
	%	**SEM**	**%**	**SEM**	**%**	**SEM**
Actinobacteria	4.9	1.0	4.9	1.0	3.8	0.6
Bifidobacterium	1.0	0.3	1.0	0.2	0.5	0.1
Coriobacteriaceae	3.8	0.7	4.0	0.7	3.6	0.7
Bacteroidetes	**40.1** [b]	0.8	**28.8** [c]	1.9	1.2 [a]	0.2
Bacteroides	**10.5** [b]	0.6	**10.5** [b]	1.8	0.3 [a]	0.1
Prevotellaceae	**29.5** [b]	1.3	**18.2** [c]	1.5	0.8 [a]	0.2
Uncultured Bacteroidetes	0.1	0.1	0.1	0.0	0.1	0.1
Firmicutes	**52.4** [b]	0.5	**48.6** [b]	0.7	94.4 [a]	0.1
Lactobacillus	17.2 [a,b]	1.5	**6.3** [b]	0.4	26.7 [a]	4.1
Streptococcus	0.1	0.1	<0.1	<0.1	5.0	2.1
Blautia	0.7	0.2	1.0	0.4	1.5	0.3
***Clostridium* cluster XIVa**	0.6	0.1	1.1	0.2	<0.1	<0.1
***Clostridium* cluster XI**	**0.9** [b]	0.1	**1.1** [b]	0.1	17.3 [a]	2.4
Uncultured Clostridiales	0.1	0.1	0.8	0.1	0.1	0.3
Allobaculum	7.2	0.6	13.5	0.4	13.3	4.0
Catenibacterium	0.1	<0.1	<0.1	<0.1	2.4	0.7
Erysipelotrichaceae	0.1	<0.1	0.0	0.1	2.3	0.6
Turicibacter	**0.1** [b]	<0.1	**<0.1** [b]	<0.1	14.8 [a]	3.3
Uncultured *Acidaminococcaceae*	0.5	0.1	0.8	0.1	<0.1	<0.1

Megamonas	**19.1** [b]	1.0	**20.5** [b]	1.1	0.1 [a]	0.1
Uncultured Firmicutes	5.8 [a,b]	0.4	**3.3** [b]	0.3	11.1 [a]	1.9
Fusobacteria	0.3 [a]	0.1	**14.5** [b]	0.8	0.3 [a]	0.1
Uncultured *Fusobacteriaceae*	0.3 [a]	0.1	**14.5** [b]	0.8	0.3 [a]	0.1
Proteobacteria	**2.3** [b]	0.2	**3.2** [b]	0.2	<0.1 [a]	<0.1
Sutterella	**1.2** [b]	0.1	**1.8** [b]	0.2	<0.1 [a]	<0.1
Anaerobiospirillum	**1.0** [b]	0.2	0.3 [a,b]	0.1	<0.1 [a]	<0.1
Pseudomonas	0.1 [a]	0.1	**1.1** [b]	0.2	<0.1 [a]	<0.1

Table 3. Microbial Community Shift upon FOS Supplementation. Increase or decrease in relative abundances of the dominant bacterial phylotypes belonging to the Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria phylum (%) as assessed via 16S-targeted Illumina sequencing at the end of the treatment period with FOS as compared to the control period in samples collected from the SCIME™ in both the proximal (PC) and distal colon (DC), as well as in the fecal material of the dogs (n=4). Statistically significant increases or decreases as compared to the control period are indicated with their p-value and are shown in bold when p<0.05.

	In vitro				***In vivo***	
	PC (%)	**p-value**	**DC (%)**	**p-value**	**%**	**p-value**
Actinobacteria						
14_*Collinsella*	+0.5	0.556	-0.4	0.750	**+0.4**	**0.036**
Bacteroidetes						
38_*Bacteroides*	**-1.2**	**0.035**	-0.1	0.819	<0.1	0.873
65_*Bacteroides*	-0.4	0.150	**-0.1**	**0.028**	<0.1	0.333
123_*Bacteroides*	<0.1	0.483	+0.1	0.335	**+0.6**	**0.005**
95_Bacteroidetes	<0.1	0.566	+0.2	0.381	**+0.7**	**0.020**
Firmicutes						
1_*Lactobacillus*	**+7.9**	**0.042**	**+2.9**	**0.014**	**+18.3**	**0.002**
8_*Clostridium* XI	**-0.2**	**0.010**	<0.1	0.744	**-7.7**	**0.024**
15_*Clostridium* XI	-0.2	0.193	-0.5	0.104	**-3.2**	**0.014**
10_*Allobaculum*	-1.3	0.104	**+4.4**	**0.023**	-0.3	0.778
21_*Allobaculum*	-0.5	0.099	**+1.8**	**0.003**	-0.1	0.897
28_*Allobaculum*	-0.3	0.104	**+1.3**	**0.021**	<0.1	0.977
33_*Allobaculum*	-0.2	0.191	**+1.8**	**0.047**	-0.1	0.522
63_*Allobaculum*	-0.1	0.208	**+0.7**	**0.024**	<0.1	0.946
25_*Megamonas*	**-1.7**	**0.013**	**-2.1**	**0.030**	+0.1	0.258
Fusobacteria						
27_*Fusobacteriaceae*	-0.1	0.601	**-6.9**	**0.008**	-0.3	0.069

Table 4. Microbial Metabolic Activity. Average acetate (mM), propionate (mM), butyrate (mM), branched SCFA (mM) and ammonium (mg/L) concentrations over the control (C) and the treatment (TR) period in the proximal (PC) and distal colon (DC) of the SCIME™ model as well as in fecal samples from the *in vivo* experiment upon treatment with FOS. Data are presented as mean ± SEM. Statistically significant differences relative to the control period are indicated by their p-value ($p<0.05$).

	***In vitro* PC**			***In vitro* DC**			***In vivo***		
	C	**TR**	**p value**	**C**	**TR**	**p value**	**C**	**TR**	**p value**
Acetate (mM)	39 ± 4	45 ± 5	**0.018**	39 ± 3	43 ± 4	**0.040**	55 ± 7	49 ± 12	0.066
Propionate (mM)	39 ± 3	60 ± 5	**<0.001**	36 ± 3	46 ± 3	**<0.001**	42 ± 7	41 ±10	0.526
Butyrate (mM)	14 ± 2	18 ± 4	**0.011**	19 ± 2	28 ± 2	**<0.001**	22 ± 9	15 ± 8	**0.003**
Branched SCFA (mM)	2.7 ± 0.1	2.1 ± 0.1	**<0.001**	3.1 ± 0.2	2.7 ± 0.1	**<0.001**	1.0 ± 0.3	0.5 ± 0.3	**<0.001**
Ammonium (mg/L)	427 ± 13	358 ± 9	**<0.001**	527 ± 17	449 ± 12	**<0.001**	292 ± 61	222 ± 63	**0.002**