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ASSESSMENT OF THE EFFECTS OF EDIBLE MICROALGAE IN A CANINE GUT MODEL

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Abstract: Gut microbiota plays a fundamental role in maintaining host health and metabolism and is considered a potential target of novel therapeutics. Microalgae represent an interesting source of bioactive compounds such as protein, fatty acids, fibre, and minerals for nutritional supplementation in humans and animals. Nevertheless, there is a lack of information on the effect of microalgae on canine gut microbiota. The aim of the study was to evaluate in a *in vitro* canine gut model the effects of four microalgae *Arthrospira platensis* (AP), *Haematococcus pluvialis* (HP), *Phaeodactylum tricornutum* (PT), *Chlorella vulgaris* (CV), on some faecal microbial populations and metabolites. Following the *in vitro* fermentation, chemical and microbiological analysis displayed significant differences between the control and microalgae groups. In particular, after 6h of incubation, microalgae increased propionate (+36% for CV; *p=0.001*) and butyrate (+24% for CV *p=0,013*), and decreased total BCFA (-47% for both PT and CV; *p=0.006*), isobutyrate (-52% for CV; *p=0.022*) and isovalerate (-43% for AP, CV, PT; *p=0.009*) and *C. hiranonis* (-0.46 log10 copies/75 ng DNA for CV; *p*=0.052); after 24h microphytes increased propionate (+21% for CV; *p=0.001*) and isovalerate (+10% for CV; *p=0.041*), and decreased the abundance of *Turicibacter* spp. (7.18 vs. 6.69 and 6.56 log10 copies/75 ng DNA for CTRL vs HP and CV, respectively; *p*=0.018), *C. leptum* (-1.12 log10 copies/75 ng DNA for PT; *p*=0.008) and *Enterococcus spp.* (-0.37 log10 copies/75 ng DNA for PT; *p*=0.044). These findings suggest a potential modulatory effect of microalgae on metabolism of canine faecal microbiota.

Key words: dog intestinal microbiota, microalgae, canine nutrition, in vitro fermentation

Introduction

The gastrointestinal tract of mammals harbours numerous bacterial species known to be, along with fungi, protozoa and viruses, one of the largest and most complex ecosystems known. Recently, there has been a growing understanding that the intestinal microbiota plays a crucial role in supporting host health (*Tuddenham and Sears, 2015*). In this vein, modulation of intestinal microbiota should be taken into account as a potential novel therapeutic (*Ercolini and Fogliano, 2018*). Among the bacterial metabolites straight short-chain fatty acids (SCFA) acetate, propionate, and butyrate and branched chain fatty acids (BCFA) isobutyrate and isovalerate (*Mondo et al., 2019*) play a very different but important role in the host body (*Rowland et al., 2018*).

The diet plays a fundamental role in shaping the composition of gut microbiota and its relation with the host (*Ercolini and Fogliano, 2018*). In last decades, scientific research has widely investigated different nutritional strategies aimed to positively influence the microbial ecosystem of human gastrointestinal tract (*Conlon and Bird, 2014*). Among the several dietary components investigated in this context, edible microalgae, also called microphytes, represent an interesting source of bioactive compounds including protein, polyunsaturated fatty acids, polysaccharides, pigments, vitamins, minerals, phenolic compounds, volatile compounds, and sterols, hence offering several possible health benefits (*Camacho et al., 2019*). Microalgae are ancestral living organisms belonging to a phylogenetically diverse group, encompassing a number of different phyla and classes of organisms; in some cases, cyanobacteria are also included (*Kay and Barton, 1991*). For their characteristic, microphytes have been proposed as encouraging sustainable alternatives to conventional animal feed resources and for their possible application as health-promoting ingredients both in human diet (*Guerin et al., 2003; Vaz et al., 2016*) and animal feeds (*de Medeiros et al., 2021*), particularly, in the aquaculture sector (*Charoonnart et al., 2018*). However, successful inclusion of microalgae and microalgae-based products in feed requires a clear understanding of their effects on the intestinal microbiota and bacterial metabolome of the host (*Sagaram et al., 2021*).

Over the last years, studies have identified many positive benefits of microalgae, including immunomodulatory (*Manzo et al., 2017; Satyaraj et al., 2021*), antioxidant (*Guzmán-Gómez et al., 2018*), anti-inflammatory (*Montero-Lobato et al., 2018; Rodríguez-Luna et al., 2018*), and anti-bacterial effects (*Martínez et al., 2019*). In addition, some microalgae are also known to have prebiotic properties (*Patel et al., 2021*), thus modulating the gastrointestinal microbiota. For example, colonic microbiota composition of rats changed after diet supplementation with some edible blue-green algae (cyanobacteria) including

Spirulina (*Rasmussen et al., 2009*). Recently, *Jin et al. (2020)* have demonstrated that supplementation with microalgae, including *Chlorella vulgaris*, increased propionate-producing bacteria in an *in vitro* human gut fermentation model (*Jin et al., 2020*). However, there is a paucity of studies investigating the effects of microphytes on canine gut microbiota and intestinal concentrations of metabolites deriving from the bacterial metabolism such as SCFA, BCFA, ammonia and biogenic amines, which are known to be of crucial relevance in host–microbial interactions (*Mccarville et al., 2020*). Moreover, some of aforementioned metabolites (SCFA in particular) represent important indices of gut health (*Alexander et al., 2019*).

The aim of the present study was to evaluate in an *in vitro* canine gut model the effects of four microalgae *Arthrospira platensis* (AP), *Haematococcus pluvialis* (HP), *Phaeodactylum tricornutum* (PT), and *Chlorella vulgaris* (CV) on some fecal microbial populations and metabolites. We supposed that composition and metabolism of canine faecal microbiota would have been positively influenced by microalgae supplementation.

Materials and Methods

The present study was conducted at the Laboratory of Animal Production of the Department of Veterinary Medical Sciences, University of Bologna, Italy.

Experimental Set Up

In order to simulate the digestion processes that take place in the stomach and small intestine of dogs, the microalgae were preliminarily subjected to *in vitro* digestion, according to the method proposed by *Biagi et al. (2016)*. The undigested fraction was subjected to analysis (Table 1) and subsequently tested as a fermentation substrate.

Table 1. Proximate analysis of the undigested fraction of four microalgae.

	Crude protein%	Crude fat, %	Crude ash, %
Arthrospira platensis	55.7	5.81	4.04
Haematococcus pluvialis	10.2	16.6	1.53
Phaeodactylum tricornutum	18.0	10.7	10.7
Chlorella vulgaris	18.6	3.28	27.0

Five healthy adult dogs (mixed breed; average body weight of 21 kg; age 3.6 years), house hold, were fed the same commercial dry diet for adult dogs (Stuzzy New Zealand & Australia Dry Line with venison, Agras Delic Spa, Italy) for 4 weeks. The diet contained the following ingredients: corn, barley, dehydrated venison, potato protein, purified pork fat, dried beet pulp, sunflower oil, brewer's yeast, dried chicory pulp, FOS, cod liver oil, dicalcium phosphate, potassium chloride, sodium chloride, herbs (dog rose, bearberry, blackcurrant, taraxacum, and thistle), and *Yucca schidigera*. The macronutrient composition of the diet (per kg on dry matter basis) was the following: crude protein (CP) 236 g, ether extract (EE) 125 g, crude ash (ash) 57.1 g, starch 389 g, and crude fibre (CF) 20.8 g.

The same dry food that was fed to the dogs used as faecal donors was subjected to *in vitro* digestion using the two-step procedure proposed by *Biagi et al. (2016)*. After *in vitro* digestion, the undigested fraction was dried at 65°C until a constant dry weight was obtained (18.5 g of undigested residue were obtained from 100 g of food dry matter [DM]) and its chemical composition per kg was the following: CP 173 g, EE 24.3 g, starch 38.7 g, ash 146 g, and CF 99.4 g.

After the 4-week feeding period, a sample of fresh faeces was collected from each dog immediately after excretion; faeces were pooled and suspended at 10 g/L in prereduced Wilkins Chalgren anaerobe broth. The faecal suspension was used to inoculate (100 mL/L) a previously warmed (39°C) and prereduced medium prepared according to *(Sunvold et al., 1995)*. Five 30 mL bottles (each bottle containing 21 mL of faecal culture) were set up per treatment.

Five treatments were carried out: (1) control diet with no addition of experimental substrates and control diet with (2) *Arthrospira platensis* (AP), (3) *Haematococcus pluvialis* (HP), (4) *Phaeodactylum tricornutum* (PT), or (5) *Chlorella vulgaris* (CV). All bottles contained the undigested residue of the commercial dry food for dogs at 10 g/L. The amount of microalgae that was added to the inocula is reported in table 2. Amounts were calculated based on the different *in vitro* digestibility coefficients of microalgae (Table 2). The dose that was used should reflect the amount of microalgae that reach the hindgut when they are included in a commercial extruded food for dogs (with a digestibility of approximately 90%) at a concentration of 40 g/kg.

Table 2. Amount of undigested fraction of the commercial dry food and microalgae that were added to bottles and digestibility coefficients of microalgae subjected to *in vitro* digestion.

Treatment	Commercial dry food, undigested fraction (mg)	Algae, total digestibility (%)	Algae, undigested fraction (mg)
CTRL	210	-	-
AP	210	86.2	11.6
HP	210	7.87	77.4
PT	210	67.5	27.3
CV	210	55.3	37.5

CTRL, control; AP, *Arthrospira platensis;* HP, *Haematococcus pluvialis;* PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris.*

The pH of faecal cultures was adjusted to 6.7; bottles were sealed and incubated for 24 h at 39°C in an anaerobic cabinet (Anaerobic System; Forma Scientific Co., Marietta, OH; under an 85% N2, 10% CO2 and 5% H2 atmosphere). Samples of fermentation fluid were collected from each bottle at 6 and 24 h for the determination of pH, ammonia, biogenic amines, SCFA, and for microbial analysis.

Chemical Analyses

The commercial dry food and its undigested residue were analysed according to the AOAC International standard methods (method 950.46 for water, method 954.01 for CP, method 920.39 for EE, method 920.40 for starch, method 942.05 for ash and method 962.09 for CF). Ammonia was measured using a commercial kit (Urea/BUN—Color; BioSystems S.A., Spain). The SCFA and BCFA were separated on a 2-m glass column (inner diameter, 3 mm) of 10% SP-1000 + 1% H_3PO_4 on 100/120 Chromosorb W AW with nitrogen as the carrier. The chromatograph was a Fisons HRGC MEGA 2 series 8560 with a flame ionization detector. The temperatures of the injector and detector were 200 °C, and the oven temperature was 155 °C. 2-ethylbutyric acid was used as the internal standard. For the determination of biogenic amines, samples were diluted 1:5 with perchloric acid (0.3 M); biogenic amines were later separated by HPLC and quantified through fluorimetry (*Stefanelli et al., 1986*).

Microbial Analysis

At each sampling time, a 1 mL portion of fermentation fluid was collected from each vessel and centrifuged at 4°C for 5 min, at 18,000 X g. The supernatant

was removed and immediately frozen at -80°C for further analysis. Bacterial genomic DNA was extracted from remaining pellet using the Stool DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada). Isolated DNA concentration (ng/µL) and purity were measured using a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Template DNA was diluted to 50 ng/µL and stored at -20°C until further analysis. Turicibacter, Ruminococcaceae, Blautia, *Escherichia coli*, *Bifidobacterium* spp., *Lactobacillus* spp., *Enterococcus* spp., C*lostridium* cluster XIV, *Clostridium coccoides*, *Clostridium leptum* e *Clostridium hiranonis* were quantified via quantitative polymerase chain reaction (qPCR) using specific primers. The qPCR assay was performed using a CFX96 Touch thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification was performed in duplicate for each bacterial group within each sample, while standard curves were run in triplicate.

Briefly, the PCR reaction contained 7.5 µL 2XSensiFAST No-ROX PCR MasterMix (Meridian Bioscience Inc, Cincinnati, OH, USA), 4.8 µL of nuclease-free water, 0.6 µL of each 10 pmol primer and 1.5 µL of template DNA for a final reaction volume of 15 µL. The amplification cycle was as follows: initial denaturation at 95°C for 2 min, 95°C for 5 s, primer annealing at 56-64°C for 10 s and 72°C for 8 s. The cycle was repeated 40 times. A negative control (without the DNA template) was also run for each primer pair. Standard curves were constructed from eight tenfold dilutions for each target. Cycle threshold values were plotted against standard curves for the quantification of the target bacterial DNA from faecal inoculum. Melting curves were checked after amplification to ensure the single product amplification of a consistent melting temperature.

Statistical Analyses

Kruskal-Wallis One-ANOVA with Dunn's multiple comparisons were performed for data with unequal variances, while normally distributed data were compared using one-way ANOVA with Dunnett's multiple comparison test. Differences between groups were considered significant for $p<0.05$. Each vial represented a single experimental unit. Significance and tendency for statistical tests were set at $p<0.05$ and $0.05<p<0.1$, respectively. Statistical analyses were performed using Statistica 10.0 software (Stat Soft Italia, Padua, Italy).

Results

The chemical parameters evaluated on samples of fermentation fluid collected after 6 and 24 h of incubation are shown in Tables 3 and 4, respectively. After 6 h of incubation, pH was decreased by HP, PT and CV compared to CTRL (6.58, 6.56, 6.63 vs. 6.71 respectively; p=0.005). Conversely, after 24 h of incubation, the pH was not statistically different between CTRL and microphyte groups (p>0.05). Moreover, the concentration of ammonia did not change after 6 and 24 h of incubation. Total concentrations of SCFA were not influenced by treatments after 6 and 24 h. On the contrary total BCFA were decreased in flasks containing PT and CV (-46% for both; p=0.006) at 6 h, however, this effect was no longer present after 24 h. At 6 h, flasks with CV contained higher concentration of propionate (+36%; p=0.001) and butyrate (+24%; p=0.013). Moreover, after 6 h of incubation, isobutyrate was reduced by CV (-52%, p=0.022) and isovalerate was decreased by all treatments, except HP (-43% for AP, CV, PT; p=0.009). At 24 h, propionate was still higher in vessels containing CV (+21%; p = 0.001) while BCFA were not affected by microalgae with the exception of isovalerate concentration that was higher in CV (+10%; p=0.041). In addition, no significant effects were observed in regard to biogenic amines both at 6 and 24 h, as reported in Table 5.

Table 3. pH values, ammonia and short-chain fatty acids concentrations after 6 h of an *in vitro* incubation of canine faecal inoculum supplemented with microalgae.[1]

Item	CTRL	AP	HP	PT	CV	pooled SEM	anova p-value
pH	6.71	6.63	6.58*	6.56*	6.63*	0.03	0.005
Ammonia, mmol/L	30.2	32.2	31.4	31.9	29.6	1.62	0.586
Straight-chain SCFA, mmol/L							
Acetate	8.62	8.66	8.97	8.57	8.85	0.42	0.954
Propionate	4.54	4.92	5.13	5.14	6.19*	0.23	0.001
Butyrate	2.55	2.58	2.62	2.69	3.16*	0.12	0.013
Total SCFA	15.7	15.7	16.7	16.4	18.2	0.78	0.232
BCFA, mmol/L							
Isobutyrate	0.27	0.15	0.15	0.13	0.13*	0.03	0.022
Isovalerate	0.46	0.26*	0.30	0.26*	0.26*	0.03	0.009
Total BCFA	0.73	0.41	0.45	0.39*	0.39*	0.08	0.006

[1] Values are the means of five bottles per treatment.
* Significantly different from CTRL, $p<0.05$
CTRL, control; AP, *Arthrospira platensis;* HP, *Haematococcus pluvialis;* PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris;* SCFA, short chain fatty acid; BCFA, branched chain fatty acid.

Table 4. pH values, ammonia and short-chain fatty acids concentrations 24 h of an in vitro incubation of canine faecal inoculum with a control diet supplemented with microalgae.[1]

Item	CTRL	AP	HP	PT	CV	pooled SEM	anova p-value
pH	5.84	5.84	5.81	5.81	5.95	0.01	0.004
Ammonia, mmol/L	39.6	39.9	36.0	35.7	38.0	1.29	0.065
Straight-chain SCFA, mmol/L							
Acetate	16.7	16.9	16.6	16.5	16.5	0.48	0.960
Propionate	9.68	10.5	10.3	10.7	11.7*	0.28	0.001
Butyrate	5.43	5.73	5.31	5.61	5.65	0.14	0.271
Total SCFA	31.81	33.13	32.21	32.81	33.85	0.89	0.536
BCFA, mmol/L							
Isobutyrate	0.60	0.64	0.60	0.62	0.64	0.02	0.289
Isovalerate	0.92	0.95	0.90	0.94	1.01*	0.02	0.041
Total BCFA	1.52	1.59	1.50	1.56	1.65	0.04	0.086

[1] Values are the means of five bottles per treatment.
* Significantly different from CTRL, $p<0.05$
CTRL, control; AP, *Arthrospira platensis;* HP, *Haematococcus pluvialis;* PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris;* SCFA, short chain fatty acid; BCFA, branched chain fatty acid.

Table 5. Biogenic amines concentrations (nmol/mL) 6 h and 24 h of an in vitro incubation of canine faecal inoculum with a control diet supplemented with microalgae.[1]

Item	CTRL	AP	HP	PT	CV	pooled SEM	anova p-value
6 h							
Putrescine	177.4	186.6	175.6	179.0	169.2	4.87	0.241
Cadaverine	101.0	124.6	132.4	96.4	87.4	15.1	0.371
Spermidine	24.4	68.8	36.4	23.6	21.6	9.55	0.043
Spermine	3.80	3.70	5.02	1.28	0.98	6.85	0.041
24 h							
Putrescine	166.4	174.0	111.4	107.8	140.4	10.2	0.007
Cadaverine	129.8	154.4	72.4	113.8	97.4	25.6	0.223
Spermidine	22.0	21.8	18.2	24.6	22.6	3.00	0.669
Spermine	1.32	1.16	1.04	2.12	0.58	0.53	0.414

[1] Values are the means of five bottles per treatment.
CTRL, control; AP, *Arthrospira platensis;* HP, *Haematococcus pluvialis;* PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgari.*

The data relating to the composition of the faecal microbiota evaluated at 6 and 24 h of incubation are presented in Table 6 and 7, respectively. After 6 h, treatments containing CV tended to decrease the abundance of *C. hiranonis* (6.88 vs. 7.34 log10 copies /75 ng DNA; p=0.052). Microphyte treatments decreased the presence of some bacterial population after 24 h. In particular, the abundance of *Turicibacter* spp. was reduced by HP and CV (6.69 and 6.56 vs.7.18 log10 copies /75 ng DNA, respectively; p=0.018). Finally, *C. leptum* (8.26 vs. 9.38 log10 copies /75 ng DNA p=0.008) and *Enterococcus* spp. (6.99 vs. 7.36 log10 copies/75 ng DNA p=0.044) were less abundant in flasks containing PT.

Table 6. Microbial analysis after 6 h of an in vitro incubation of canine faecal inoculum with a control diet supplemented with microalgae.[1]

Target	CTRL	AP	HP	PT	CV	pooled SEM	anova p-value
Bifidobacterium spp.	7.10	7.21	6.91	7.11	6.63	0.23	0.415
Blautia	6.51	6.60	6.42	6.37	6.16	0.19	0.571
Clostridium cluster XIV	8.14	8.21	7.87	8.07	7.97	0.19	0.639
Clostridium coccoides	7.51	7.50	7.26	7.43	7.31	0.25	0.929
Clostridium hiranonis	7.34	7.16	7.20	6.96	6.88*	0.11	0.052
Clostridium leptum	7.42	7.34	7.35	7.20	6.99	0.13	0.183
Escherichia coli	7.01	7.30	7.23	6.93	7.00	0.12	0.346
Enterococcus spp.	7.16	7.27	6.70	7.19	7.32	0.24	0.409
Lactobacillus spp.	5.84	5.77	5.52	5.62	4.87	0.32	0.283
Ruminococcaceae	8.49	8.50	8.32	8.34	8.11	0.19	0.595
Turicibacter spp.	6.30	6.36	6.13	6.10	6.07	0.16	0.631

[1] Values are the means of five bottles per treatment.
The qPCR data was expressed as log10 copies of DNA for each particular bacterial target per 75 ng of isolated total DNA.
* Significantly different from CTRL, $p<0.05$
CTRL, control; AP, *Arthrospira platensis;* HP, *Haematococcus pluvialis;* PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris*

Table 7. Microbial analysis after 24 h of an in vitro incubation of canine faecal inoculum with a control diet supplemented with microalgae.[1]

Target	CTRL	AP	HP	PT	CV	pooled SEM	anova p-value
Bifidobacterium spp.	8.23	7.92	8.11	8.32	7.91	0.11	0.066
Blautia	7.63	7.38	7.60	7.46	7.46	0.09	0.313
Clostridium cluster XIV	8.33	8.07	8.38	8.26	8.31	0.08	0.134
Clostridium coccoides	9.38	8.97	8.88	8.26*	9.09	0.19	0.008
Clostridium hiranonis	8.14	8.06	8.03	8.07	7.95	0.10	0.754
Clostridium leptum	8.41	8.37	8.31	8.15	8.27	0.09	0.310
Escherichia coli	8.45	8.41	8.37	8.16	8.54	0.11	0.206
Enterococcus spp.	7.36	7.19	7.30	6.99*	7.40	0.10	0.044
Lactobacillus spp.	7.29	7.41	7.16	7.19	6.80	0.19	0.256
Ruminococcaceae	9.65	9.47	9.60	9.42	9.48	0.08	0.276
Turicibacter spp.	7.18	6.92	6.69*	6.74	6.56*	0.12	0.018

[1] Values are the means of five bottles per treatment.
The qPCR data was expressed as log10 copies of DNA for each particular bacterial target per 75 ng of isolated total DNA.
* Significantly different from CTRL, $p<0.05$
CTRL, control; AP, *Arthrospira platensis;* HP, *Haematococcus pluvialis;* PT, *Phaeodactylum tricornutum*; CV, *Chlorella vulgaris*

Discussion

The purpose of this investigation was to evaluate the *in vitro* effects of four microalgae on some canine faecal microbial populations and metabolites. It must be emphasized that very few studies have investigated the use of microphytes in dogs and they were mainly focused on anti-inflammatory and immunomodulating activities of microalgae (*Satyaraj et al., 2021*). In this study, supplementation with microalgae partially affected the gut ecology.

Particularly, pH was decreased after 6 h in three of the four microalgae groups (HP, CV, PT). The reduction of intestinal pH could be a desirable effect, as the acidification of the environment has a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria. It is known how the colonic pH is influenced by fermentation processes of bacterial populations, in particular in the proximal colon, where the pH is lower due to the production of SCFA that mainly derive from the fermentation of carbohydrates (*Hamer et al., 2012*). However, in the present investigation, total concentration of SCFA was not affected by treatments.

After 6 h of incubation, the concentration of propionate and butyrate was increased by CV. A previous study conducted in an *in vitro* human gut model demonstrated that supplementation with microphytes, including CV, could affect both intestinal microbiota composition and metabolites. Particularly, *Jin et al. (2020)* investigated the effects of three edible microalgae (*Chlorella vulgaris, Chlorella protothecoides*, and *Schizochytrium* sp.) on gut microbiota showing that microalgae supplementation increased the proportion of propionate in the colonic culture together with the relative abundance of some bacterial populations involved in propionate metabolism (genera *Bacteroides* spp. and *Dialister* spp.). Moreover, total SCFA were significantly increased by *C. vulgaris*. Similar effects were observed in the present study regarding the higher concentration of propionate in CV group, both after 6 and 24 h. Intestinal SCFA are linked with some health-promoting effects, such as anti-inflammatory, anticarcinogenic, and immune-regulatory functions (*O'Keefe, 2016*). Specifically, propionate is metabolized in the liver and plays a role in reducing the concentration of blood sugar and serum cholesterol, while butyrate is an important source of energy for colonocytes (*Guarner and Malagelada, 2003*). In addition, butyrate is known to be effective in preventing colon cancer (*McNabney and Henagan, 2017*).

Nevertheless, in the present trial higher concentration of SCFA did not reflect a change in microbial populations known as SCFA producers. Moreover, after 24 h of fermentation, lower presence of genera *Enterococcus* spp. (PT), *Turicibacter* spp. (CV and HP) and *C. coccoides* subgroup (PT) were detected in three of the groups to which microalgae were added. These last outcomes are in contrast with the results recently obtained by *Wan et al. (2019)* who studied the effect of a bioactive polysaccharide from microalga *Chlorella pyrenoidosa* (CPP) at the dosage of 150 and 300 mg/kg, on gut microbiota of mice fed a high-fat diet. The authors pointed out that the growth of some bacterial genera, including *Turicibacter*, and the concentrations of acetate, propionate, and butyrate were drastically increased in both CPP treatments. *Turicibacter* spp., belonging to the *Firmicutes phylum*, was considered an important producer of SCFA *(Sivaprakasam et al., 2016)*, suggesting an important role of *Turicibacter* spp. in promoting gut health.

For what concerns the decrease of *C. coccoides* that was observed in the present study, existing literature appears to report controversial findings regarding the abundance of this bacterial group in host physiology. *C. coccoides*, belongs to the *Firmicutes phylum*, one of the most predominant groups in the human gut, and many species in this class, such as *Eubacterium* spp., *Roseburia* spp., *Subdoligranulum variabile*, and *Faecalibacterium prausnitzii* directly produce butyrate from dietary polysaccharides and other substrates (*Jamar et al., 2018*); moreover, its presence is also correlated with an increased capacity to harvest

energy from diet (*Turnbaugh et al., 2006*). Microbial analysis showed that, at 24 h, PT treatment decreased enterococci and *C. leptum* subgroup. The last is known as a butyrate-producing bacterium previously reported to be less abundant in faecal samples of human patients suffering from gastrointestinal disorders like inflammatory bowel disease (*Wang et al., 2014*).

After 6 h of incubation, CV resulted in decreased abundance of *C. hiranonis*. *C. hiranonis* is a bacterial species of interest, as it shows bile acid 7 alpha-dehydroxylating activity, and a decrease in *C. hiranonis* may suggest bile acid dysmetabolism (*AlShawaqfeh et al., 2017*). These findings are apparently in contrast with previously mentioned studies (*Jin et al.,2020; Wan et al., 2019*), in which microphytes seemed to improve intestinal health by promoting the growth of positive bacterial population, such as SCFA-producing bacteria. Certainly, it must be underlined that, in the present study, only few of the main populations of canine microbiota have been evaluated. This fact represents a limitation as we cannot exclude that changes regarding other bacteria could not have been detected.

One of the main reasons for considering microalgae as an interesting source of food is their high protein content (e.g., 55% -70% for *S. platensis* and 42%-55% for *C. vulgaris* on a dry matter basis; *Matos, 2019*). In this study, microalgae were preliminarily subjected to *in vitro* digestion and the undigested fraction was used as the fermentation substrate. Proteins were highly represented in the undigested fraction of AP (55.7%), CV (18.6%), PT (18.0%). Interestingly, the presence of microalgae, despite the increased presence of protein, decreased BCFA after 6 h and did not result in higher concentrations of ammonia and biogenic amines, all metabolites deriving from bacterial proteolysis (*Blachier et al., 2006; Mccarville et al., 2020*). In particular, CV seemed to have the greatest effect on BCFA by decreasing both the concentration of isobutyrate and isovalerate. The biological significance of BCFA and biogenic amines is still poorly understood. The former originate from branched chain amino acids in the colon and it has been hypothesized that BCFA may have a role in the regulation of ionic exchanges in colonic mucosa (*Musch et al., 2001*) and that isobutyrate may act as a potential source of energy for colonocytes after exhaustion of butyrate (*Jaskiewicz et al., 1996*). Similarly, biogenic amines seem to have a beneficial influence on the intestinal mucosa (*Heby, 1981*) but, on the other hand, they could act as precursors in the formation of nitrosamines, known as carcinogens in humans (*Smith and Macfarlane, 1996*). The decrease of BCFA that we observed could indicate a reduction of proteolytic activities operated by some bacterial populations. However, other parameters, including concentration of ammonia and biogenic amines, did not reflect this trend. In this regard, the effects of microalgae supplementation on metabolites deriving from bacterial proteolysis are still poorly investigated, hence it could represent an interesting aspect to be explored.

Conclusion

During the present *in vitro* study microalgae partially affected canine faecal microbiota. Among the four microphytes, CV showed the major effect on microbial metabolites after 6 h of incubation by increasing propionate, butyrate and decreasing BCFA. These outcomes suggest that microalgae, especially CV, could have a potential modulatory effect on the metabolic activities of canine faecal microorganisms. However, CV led to a reduction of *C. hiranonis* at 6 h, while after 24 h HP, PT and CV resulted in a decrease of some beneficial bacterial populations belonging to *Firmicutes*, known to be butyrate-producing bacteria.

The present study regarding the influence of microalgae on the intestinal microbiota of dogs has led to controversial results and should be considered as a preliminary study for future investigations.

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