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Dynamic Multi-Stage Gastrointestinal Digestion Model Assessment of Microbial Fermentation Products of Collagen Hydrolysates †

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Abstract: Proteins, peptides and amino acids (AA) that bypass upper gastrointestinal (GI) digestion can be fermented in the colonic regions. This could lead to microbial production of health promoting short-chain fatty acid (SCFAs). Nitrogenous compounds can also be fermented to generate potentially harmful branched chain fatty acid (BCFAs). As collagen hydrolysate (CH) supplements contain a high peptide content, we evaluated whether peptides that undergo intestinal CH digestion and microbial fermentation can generate SCFAs and BCFAs. Two bovine-sourced CH formulations (CH-GL and CH-OPT) underwent digestive processes and microbial fermentation for 24 h in a dynamic GI digestion model containing human fecal matter. After 24 h, CH-OPT showed a significant ($p < 0.05$) increase in SCFAs (propionic, butyric and valeric acids) in the ascending colonic vessel with no changes observed with CH-GL. Only CH-OPT showed a significant ($p < 0.05$) increase in BCFAs, also noted in the ascending colon. No significant ($p < 0.05$) changes to SCFAs and BCFAs were observed in the transverse and descending colons for both CHs. These findings demonstrate that CHs can induce microbial production of SCFAs and BCFAs although this appears to depend on the CH tested. More studies are needed to determine the physiological significance of these microbial metabolites from intake of CH supplements.

Keywords: nutraceutical; in vitro digestion; collagen hydrolysate; prebiotic; SCFA; BCFA; osteoarthritis; ammonia; hydrogen sulfide; antioxidant

1. Introduction

Osteoarthritis (OA) is a debilitating condition, affecting 50% of people over 75 years old, and accounting for 25% of visits to family doctors [1–3]. OA results in pain, mobility limitations and significant swelling in joint areas most often in the knees and hips. Risk factors include aging, genetic predisposition, previous injuries, sex, but is also highly associated with metabolic diseases and conditions such as obesity, diabetes, hypertension and dyslipidemia [4–8]. Although in a classical sense, OA is not often regarded as a metabolic disorder, there have been some insights showing the relationship between OA and increased risk of metabolic syndrome [9,10]. Treatment options are limited, although a number of clinical trials have shown that ingestion of collagen hydrolysates (CHs) allows for decreased pain and increased mobility [11–17]. CH products contain a cocktail of peptides and amino acids (AAs), whereby the peptides can be further broken down into bioactive peptides (BAPs) in the stomach and small intestine [18–21]. It is conceivable that the rich content of peptides and AAs present in CHs can lead to the generation of microbial nitrogenous fermentation products in the colon, and affect the composition and function of the human gut microbiota community. Such fermentation processes could be important for OA as gut health has been linked to joint health [7,22].

A recent study on obesity showed a direct link between OA and the gut microbiome, and its effects on systemic inflammation [7]. Therefore, further insights as to how CH products impact on gut microbial fermentation is warranted, particularly as patients are increasingly utilizing nutraceuticals to mitigate the symptoms of OA [23,24].

Prebiotics are dietary components that can induce beneficial changes in the growth, activity or composition of microorganisms found in the gastrointestinal (GI) tract, otherwise known as the microbiota. Microbial fermentation products of prebiotics have been implicated to provide several health benefits upon the host [25]. Prebiotics have been shown to regulate inflammation, exhibit antioxidant activity as well as reduce symptoms associated with metabolic disorders such as arthritis [7,22,26–28]. As the definition of a prebiotic now includes fermented proteins, peptides and AAs [29], the investigation into the effects of colonic fermentation of CH products is merited as this could provide additional health benefits.

Short chain fatty acids (SCFAs) are well established products of fermentation of prebiotics and their production is an indicator of a healthy microbial community [30]. SCFA assessment includes acetic, propionic and butyric acids, which are normally present in ratios ranging from 3:1:1 to 10:2:1 [27]. SCFA production is considered as one of the major benefits associated with prebiotics, and the relative abundance of fecal SCFAs has been used as a biomarker of gut health as well as overall systemic health [31,32]. Although only a small fraction of SCFAs are absorbed, there are numerous biological functions attributed to SCFAs that are under active investigation. For example, butyric acid has been implicated in the control of inflammation [33], appetite [34] and liver mitochondrial function [35]. Although less is known about minor SCFAs such as valeric and caproic acids, they also have the potential to affect human health [36,37].

CH supplementation could also lead to increased microbial production of branched chain fatty acids (BCFAs; isobutyric, isopropionic, isovaleric, isocaproic acid), which are products derived from colonic microbial fermentation of branched-chain amino acids. The health impact of BCFAs is still under debate. Increased production of BCFAs has been associated with prevention of irritable bowel syndrome [38] whereas other studies have increasingly linked exposure to BCFAs with insulin resistance and obesity [39]. To determine the possible production of SCFAs and BCFAs associated with CH intake, a dynamic multistage computer-controlled GI model was used to observe the production of colonic microbial metabolites from two commercially available CH products

2. Materials and Methods

2.1. Dynamic In Vitro Gastrointestinal Digestion of Collagen Hydrolysates

An established dynamic computer-controlled GI model was used to digest the CHs products, which has been previously validated [40,41]. The model consists of five bioreactor vessels: stomach, small intestine, ascending colon (ASNC), transverse colon (TRSC, Bangkok, Thailand) and descending colon (DESC). For each vessel, the pH is continuously measured and adjusted by a computer system, with either the addition of 0.2 M NaOH or 0.5 M HCL. The temperature of the GI model is kept at 37 °C and is monitored and controlled by flowing water through double- jacketed reactor vessels in which the GI bioreactors components are found. The model components are attached by plastic tubing and the contents of the reactor vessels are moved by peristaltic pumps. The vessel contents are continuously agitated by stir plates. The colonic vessels were inoculated with fecal matter and allowed to stabilize over a two-week period to allow for optimal bacterial growth. The two bovine-sourced CH products used for this study were Original Formula® (Genacol, Blainville, QC, Canada) (CH-GL) and Selection (Uniprix, QC, Canada) (CH-OPT). The CH treatment dose of 1200 mg was added to a gastrointestinal food mixture, as previously described by Sadeghi Ekbatan et al., (2016) and Gaisawat et al., (2019), and which was slowly pumped into the stomach vessel. The treatment dose was based on the daily dose of the Genacol Original Formula® that was shown to reduce joint pain in clinical trials [11,12,17]. To our knowledge, no information is available regarding the clinical efficacy of the Selection CH product. An enzyme solution of α-amylase (Sigma A3176, St. Louis, MO, USA) prepared in sterile deionized water was added to the GI food mixture to replicate

salivary digestion. Pepsin (Sigma P7125) prepared in 0.1 M HCL was added to the stomach vessel and 35 mL of a bile solution composed of pancreatin (Sigma P7545), bile extract (Sigma B8631) and sodium bicarbonate were added to the small intestine, as described by Sadeghi Ekbatan et al., (2016) and Gumienna et al., (2011) [41,42]. Sub-samples from each vessel were obtained at times 0, 8, 16 and 24 h and filtered using a 0.45 μm Millipore syringe-driven filter. Two separate digestion runs were completed for each treatment, with a washout/re-stabilization period of 3 days between treatments. Previous in vitro fermentation experiments have also utilized two separate digestion runs [43].

2.2. Short and Branched Chain Fatty Acids

The SCFA and BCFA content was measured using gas chromatograph system equipped with a flame ionization detector (GC-FID) (6890A series, Agilent Technologies, Santa Clara, CA, USA) using an adapted method from Sadeghi Ekbatan et al., (2016) and Gaisawat et al., (2019) [41,44]. Pre-filtered subsamples from the GI model for each colonic vessel were obtained, and then diluted 1:1 with methanol. A 1 μL volume was injected into the GI-FID system. An HP-INNOWAS 30 m fused capillary column (Agilent Technologies, Santa Clara, CA, USA) with a 250 μm ID and a film thickness of 0.25 μm was used to separate the SCFAs and BCFAs from both the standard mixes and samples. A flow rate of 1 mL/min of helium gas was used. The inlet and detector temperatures were set at 220 °C and 230 °C, respectively. The oven temperature was originally set at 150 °C and held for 10 min and then increased by 10 °C/min to 180 °C and held for 5 min. SCFAs were identified using a standard mix (46975-U, Sigma Aldrich, St. Louis, MO, USA) and quantified (mM) using an external calibration curve.

2.3. Statistical Analysis

Data is reported as mean ± standard error of the mean (SEM). For each treatment, differences between timepoints was assessed using a one-way ANOVA, followed by Dunnett's post hoc test, using time 0 h as control. All analysis was completed using JMP (JMP®, Version 13.2.1. SAS Institute Inc., Cary, NC, USA) and were considered statistically significant if $p < 0.05$.

3. Results

SCFAs and BCFAs

In the ascending colon, no changes in SCFAs were observed after CH-GL digestion (Table 1) whereas individual SCFA profiles showed variability between baseline control (time 0 h) after the digestion of CH-OPT (Table 2). Specifically, propionic acid (mM) content increased from a baseline value of 0.50 ± 0.47 to 7.59 ± 0.59 and 6.53 ± 1.71 after 16 h and 24 h, respectively. Similarly, a significant ($p < 0.05$) increase in butyric acid (mM) was also observed after 16 h and 24 h (6.97 ± 0.20 and 5.78 ± 1.21, respectively) from time 0 (2.92 ± 0.21). No significant changes in acetic acid was reported for CH-OPT, for any timepoint. Valeric acid also increased after 8, 16 and 24 h. No changes in caproic acid or heptanoic acid were observed. For both CHs, no changes in SCFA or BCFA content were observed in the transverse and descending colon compared to baseline (time 0 h).

An increase in BCFAs in the ascending colonic reactors was observed only with CH-OPT supplementation (Table 3). Specifically, isovaleric acid (mM) increased from 2.20 ± 0.09 at baseline to 3.69 ± 0.34 after 16 h ($p < 0.05$). Although not significant, a trend for an increase in isovaleric acid was observed after 24 h ($p = 0.0588$).

No increase in BCFA content was observed after digestion of CH-GL, although a significant decrease in isobutyric acid (mM) was detected (Table 4). Isobutyric acid (mM) decreased from 0.40 ± 0.05 at baseline to 0.19 ± 0.01 after 24 h ($p < 0.05$). A trend for isobutyric acid to decrease relative to control time 0 h was also observed at 8 h (0.23 ± 0.04) ($p = 0.0531$).

Similar to SCFAs, there were no changes in BCFAs in the transverse and descending colon vessels for either CH treatment.

Table 1. SCFA for CH-GL at times 0, 8, 16 and 24 h for each colonic region.

Time (h)	Acetic Acid (mM)	Propionic Acid (mM)	Butyric Acid (mM)	Valeric Acid (mM)	Caproic Acid (mM)	Heptanoic Acid (mM)
Ascending colon						
0	12.43 ± 4.43	0.08 ± 0.07	0.05 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
8	24.83 ± 1.84	0.16 ± 0.05	0.05 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16	25.17 ± 1.95	0.18 ± 0.04	0.05 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
24	21.48 ± 0.20	0.22 ± 0.06	0.05 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
Transverse colon						
0	3.10 ± 0.67	1.34 ± 1.27	4.67 ± 3.21	1.56 ± 1.24	0.43 ± 0.43	0.00 ± 0.00
8	6.87 ± 2.68	1.52 ± 1.49	5.26 ± 2.65	1.74 ± 1.12	0.41 ± 0.38	0.00 ± 0.00
16	10.56 ± 6.86	1.74 ± 1.72	3.85 ± 2.35	1.42 ± 1.09	0.29 ± 0.03	0.00 ± 0.00
24	12.2 ± 10.56	1.29 ± 0.51	2.16 ± 1.14	0.85 ± 0.54	0.14 ± 0.12	0.00 ± 0.00
Descending colon						
0	4.50 ± 1.73	2.08 ± 1.90	4.56 ± 3.54	1.09 ± 1.60	0.46 ± 0.42	0.00 ± 0.00
8	5.56 ± 1.12	1.75 ± 1.51	4.70 ± 1.98	1.80 ± 0.99	0.41 ± 0.32	0.00 ± 0.00
16	3.83 ± 0.13	1.49 ± 1.44	3.83 ± 2.04	1.49 ± 0.98	0.40 ± 0.28	0.00 ± 0.00
24	6.80 ± 2.98	1.51 ± 1.43	4.63 ± 0.92	1.68 ± 0.69	0.38 ± 0.26	0.00 ± 0.00

Values are expressed as mean ± SEM in mM. Within a column.

Table 2. SCFA for CH-OPT at times 0, 8, 16 and 24 h for each colonic region.

Time (h)	Acetic Acid (mM)	Propionic Acid (mM)	Butyric Acid (mM)	Valeric Acid (mM)	Caproic Acid (mM)	Heptanoic Acid (mM)
Ascending colon						
0	3.96 ± 2.18	0.50 ± 0.47	2.92 ± 0.21	2.12 ± 0.05	1.80 ± 0.22	1.50 ± 0.08
8	8.55 ± 3.71	5.05 ± 0.43	4.90 ± 0.26	**4.42 ± 0.21** *	3.65 ± 0.10	2.87 ± 0.54
16	14.12 ± 2.73	7.59 ± 0.59 *	**6.97 ± 0.20** *	**5.91 ± 0.37** *	4.44 ± 1.13	3.10 ± 0.36
24	14.20 ± 7.02	6.53 ± 1.71 *	**5.78 ± 1.21** *	**5.07 ± 0.70** *	3.83 ± 0.08	3.12 ± 0.49
Transverse colon						
0	3.27 ± 2.13	0.16 ± 0.03	1.68 ± 0.38	1.29 ± 0.21	0.89 ± 0.03	0.78 ± 0.16
8	2.75 ± 0.41	0.82 ± 0.72	1.77 ± 0.50	1.52 ± 0.26	1.11 ± 0.47	0.76 ± 0.13
16	2.94 ± 1.46	1.06 ± 1.00	1.37 ± 0.18	1.09 ± 0.03	0.82 ± 0.23	0.57 ± 0.08
24	6.63 ± 2.54	1.52 ± 1.46	2.17 ± 0.18	1.84 ± 0.12	1.20 ± 0.37	0.91 ± 0.05
Descending colon						
0	2.43 ± 0.37	0.85 ± 0.68	2.23 ± 0.39	1.35 ± 0.17	0.83 ± 0.23	0.46 ± 0.10
8	4.34 ± 1.07	1.21 ± 0.92	3.70 ± 0.60	2.26 ± 0.40	1.4 ± 0.0.41	0.61 ± 0.21
16	3.84 ± 1.16	0.37 ± 0.08	3.72 ± 1.27	2.01 ± 0.05	1.34 ± 0.06	0.54 ± 0.11
24	3.39 ± 3.39	0.43 ± 0.12	3.95 ± 1.45	2.12 ± 0.12	1.32 ± 0.01	0.54 ± 0.11

Values are expressed as mean ± SEM in mM. Within a column, * symbol indicates differences from control (Time 0 h) ($p < 0.05$).

Table 3. BCFA for CH-OPT at times 0, 8, 16 and 24 h for each colonic region.

Time (h)	Isobutyric Acid (mM)	Isovaleric Acid (mM)	Isocaproic Acid (mM)
Ascending colon			
0	4.27 ± 1.39	2.20 ± 0.09	1.56 ± 0.05
8	3.80 ± 0.22	3.20 ± 0.13	2.66 ± 0.55
16	5.19 ± 0.13	**3.69 ± 0.34 ***	2.80 ± 0.18
24	4.65 ± 0.61	**2.81 ± 0.17** +	2.55 ± 0.47
Transverse colon			
0	2.67 ± 1.26	0.86 ± 0.04	0.64 ± 0.19
8	2.20 ± 0.10	0.98 ± 0.36	0.59 ± 0.10
16	2.20 ± 0.07	0.82 ± 0.10	0.52 ± 0.05
24	5.64 ± 0.87	1.09 ± 0.17	0.73 ± 0.02
Descending colon			
0	1.97 ± 0.09	1.09 ± 0.07	0.40 ± 0.13
8	2.35 ± 0.11	1.76 ± 0.17	0.45 ± 0.18
16	3.38 ± 0.73	1.73 ± 0.15	0.52 ± 0.17
24	4.17 ± 0.95	1.76 ± 0.24	0.46 ± 0.13

Values are expressed as mean ± SEM in mM. Within a column, * symbol indicates differences from control (Time 0 h) ($p < 0.05$). The symbol + indicates a possible trend ($p = 0.0588$).

Table 4. BCFA for CH-GL at times 0, 8, 16 and 24 h for each colonic region.

Time (h)	Isobutyric Acid (mM)	Isovaleric Acid (mM)	Isocaproic Acid (mM)
Ascending colon			
0	0.40 ± 0.05	0.04 ± 0.01	0.00 ± 0.00
8	**0.23 ± 0.04** +	0.04 ± 0.00	0.00 ± 0.00
16	0.26 ± 0.01	0.04 ± 0.00	0.01 ± 0.01
24	**0.19 ± 0.01 ***	0.03 ± 0.01	0.01 ± 0.00
Transverse colon			
0	0.54 ± 0.17	0.35 ± 0.18	0.01 ± 0.01
8	0.55 ± 0.09	0.43 ± 0.22	0.01 ± 0.01
16	0.40 ± 0.23	0.35 ± 0.27	0.02 ± 0.01
24	0.32 ± 0.10	0.26 ± 0.16	0.02 ± 0.01
Descending colon			
0	0.79 ± 0.33	0.50 ± 0.26	0.01 ± 0.01
8	1.00 ± 0.53	0.60 ± 0.11	0.01 ± 0.01
16	0.60 ± 0.02	0.50 ± 0.17	0.02 ± 0.02
24	0.76 ± 0.23	0.60 ± 0.07	0.02 ± 0.02

Values are expressed as mean ± SEM in mM. Within a column, * symbol indicates differences from control (Time 0 h) ($p < 0.05$). The symbol + indicates a possible trend ($p = 0.0531$).

4. Discussion

To our knowledge, this simulated dynamic GI digestion model study provides the first assessment of the production of SCFAs and BCFAs following fermentation of hydrolyzed collagen by human gut microbiota. Although they were both derived from bovine collagen, only the CH-OPT treatment was associated with an increase in colonic SCFA and BCFA content. This finding is most likely due to differences in the peptide profiles in the two CH products that can result from differing collagen hydrolysate preparation or purification methods [45,46]. In that regard, CH-GL is a formulation that consists of a low molecular weight peptide complex [47]. Thus, the contrasting findings between the two CH supplements might be due to the putative lower molecular weight peptide complex of CH-GL being less amenable to microbial metabolism. Conversely, it appears that

sufficient amounts of specific peptide sequences in CH-OPT remained intact following upper GI digestive processes to induce microbial generation of SCFAs in terms of butyric, propionic and valeric acids and the BCFA, isovaleric acid. As no changes in SCFAs and BCFAs were observed in the transverse or descending colonic vessels, it is likely that insufficient amounts of peptides reached those vessels to support further microbial fermentation. Although there are no analogous studies involving CH fermentation, an increase in butyrate and propionate content was observed from fermentation of casein hydrolysates using single stage, anaerobic fermentation chambers inoculated with human fecal matter [48]. In contrast to the present work, the latter study did not include stomach and small intestine digestive processes that can modify peptide profiles prior to their exposure to microbial metabolism. Other reports have shown that wheat arabinogalactan peptides were associated with an increase in SCFAs after 24 h, although this was assessed using in vitro batch fermentation rather than a dynamic GI model system [43]. The multistage dynamic GI model can provide a more accurate representation of human digestive and microbiota metabolism of dietary components as compared to either static digestion models or animal models [49,50].

There are possible metabolic health benefits that might accrue from increased colonic generation of propionic and butyric acid that was associated with CH-OPT supplementation [31,32]. On the other hand, these benefits could be partially offset by the corresponding increase in isovaleric acid seen with the CH-OPT treatment since enhanced gut exposure to BCFAs has been linked to an increased risk for diabetes and obesity [39]. Although not much information is currently known about the health modulatory properties of minor SCFAs, recent research has suggested that fecal valeric acid may serve as an indicator of gut microbial dysbiosis [36]. Hence, the increase in valeric acid concentrations observed with CH-OPT could be indicative of adverse changes in gut microbial composition. An additional potential concern in relation to OA are reports that fecal valeric acid is positively correlated with the pro-inflammatory C-reactive protein in patients with ischemic stroke [51]. Conversely, the conjugated base of valeric acid has been associated with enhancing interleukin-10 production and suppressing Th17 cells, which could provide anti-inflammatory benefits [52]. The immunomodulatory effects of valeric acid needs further investigation, particularly in relation to OA and rheumatoid arthritis as these are conditions associated with an increase in joint and whole body proinflammatory processes [53]. Interestingly, the lack of effect of the CH-GL on the SCFA and BCFA production indicates that this supplement does not have either prebiotic or dysbiotic properties in contrast to CH-OPT.

5. Conclusions

To date, there is limited information regarding the effects of food-derived peptides on the gut microbiome and microbial fermentation products such as SCFAs and BCFAs. The present study provides the first evidence that CHs can lead to the generation of SCFAs and BCFAs but that this microbial metabolic activity appears to be dependent on the nature of the CH tested. The application of peptidomics to characterize the heterogeneity of peptides generated via digestive and fermentative processes could provide more insight regarding the peptide sequences involved in the colonic microbial fermentation. A recent review has highlighted that long-term dietary choices, such as greater protein content, could exert effects on GI microbial populations, which has implications towards development of metabolic diseases such as obesity and diabetes [29]. This review emphasized that important knowledge gaps exist concerning dietary protein-mediated generation of colonic microbial molecules that could exert bioactivities towards gut inflammation and permeability. Accordingly, it is possible that CH supplements, which contain a rich content of peptides, can impact the structure and function of gut microbial communities. The dynamic GI model platforms, such the one utilized in the present study, can be a useful tool to further investigate the impact of CH supplementation on the gut microbiome and other biomarkers of gut health in order to more fully understand the impact these nutraceuticals on GI and systemic health.

Author Contributions: Conceptualization, methodology was completed by C.E.L., M.I. and S.K. Data curation, formal analysis, writing—original draft preparation was done by C.E.L. Validation, project administration, writing—review and editing was completed by M.I. and S.K. Resources, supervision, funding acquisition was done by S.K. All authors have read and agreed to the published version of the manuscript.

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