CATÓLICA
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PORTO

EFFECTS OF PROLONGED FOOD ADDITIVE INTAKE ON THE HUMAN GUT MICROBIOTA

by
Ana Cristina Lopes Pinto Fernandes

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Applied Microbiology

by
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"Do not go where the path may lead, go instead where there is no path and leave a trail."

- Ralph Waldo Emerson

Resumo

Aditivos alimentares, tipicamente incorporados em alimentos e bebidas, são amplamente considerados seguros pelos meios reguladores. No entanto, pesquisas emergentes sugerem que certos aditivos podem alterar a composição e funcionalidade da microbiota intestinal, levantando preocupações sobre os seus potenciais impactos na saúde. Embora muitos estudos tenham focado em como os principais componentes da dieta influenciam a microbiota intestinal, os efeitos do consumo prolongado de aditivos alimentares nessas comunidades microbianas permanecem por explorar.

Este estudo investiga o impacto de três aditivos alimentares – carboximetilcelulose (CMC) e *iota* e *kappa* carragenanos ι-CGN e κ-CGN – na microbiota intestinal, utilizando um modelo *in vitro* de digestão gastrointestinal. Após caracterizar o processo de digestão para cada aditivo, os seus efeitos foram avaliados por meio de fermentação *in vitro* durante 192 horas, com amostras de fezes humanas frescas de doadores voluntários. Esta abordagem permitiu a análise das respostas metabólicas e das dinâmicas populacionais microbianas na microbiota intestinal.

O estudo demonstra que a CMC é imediatamente fermentada por microrganismos intestinais, resultando num aumento inicial na produção de ácido acético (≈ 5000 μM), mas com baixos rendimentos de ácidos propiônico e butírico (< 1000 μM). Em contraste, ι-CGN e κ-CGN passam por um processo de fermentação mais lento e contínuo, dando prioridade à produção de ácido butírico (≈ 5000 μM). No geral, a produção total de ácidos gordos de cadeia curta (SCFAs) nas amostras digeridas permaneceu baixa (< 6000 μM). A análise de previsão funcional indicou que as amostras tratadas com ι-CGN, κ-CGN e CMC apresentaram menor atividade metabólica microbiana ($p < 0,05$), sugerindo que estes aditivos podem promover disbiose durante a fermentação, resultando numa comunidade microbiana menos versátil metabolicamente. As mudanças microbianas observadas nas amostras tratadas com aditivos incluíram alterações no ratio *Firmicutes-Bacteroidota* para 2:1 e aumentos relativos nos filos *Proteobacteria* e *Synergistota*.

Estes resultados sugerem que o consumo crônico destes aditivos alimentares pode alterar a dinâmica microbiana e a síntese de SCFAs, com potenciais implicações adversas para a saúde intestinal.

Palavras-chave: Aditivos Alimentares, Microbiota Intestinal, Carragenina, Carboximetilcelulose, Inflamação.

Abstract

Food additives, commonly incorporated into food and beverages are widely regarded as safe by regulatory bodies. However, emerging research suggests that certain additives may alter gut microbiota composition and functionality, raising concerns about their potential health impacts. While much research has focused on how major dietary components influence gut microbiota, the effects of long-term intake of food additives on these microbial communities remain underexplored.

This study investigates the impact of three dietary additives—carboxymethyl cellulose (CMC) and the carrageenans ι-CGN and κ-CGN—on gut microbiota using an *in vitro* model of gastrointestinal digestion. After characterizing the digestion process for each additive, their effects were further evaluated through *in vitro* fermentation for 192h, with fresh human fecal samples from volunteer donors. This approach allowed for the analysis of metabolic responses and microbial population dynamics within the intestinal microbiota.

This study demonstrates that CMC, is readily fermented by gut microbes, leading to an initial increase in acetic acid production (≈ 5000 μM) but minimal yields of propionic and butyric acids (< 1000 μM). In contrast, the more complex ι-CGN and κ-CGN undergo a slower, sustained fermentation process, prioritizing butyric acid production (≈ 5000 μM). Overall, total short-chain fatty acid (SCFAs) production from digested samples remained low (< 6000 μM). Additionally, functional prediction analysis indicated that samples treated with ι-CGN, κ-CGN, and CMC had lower microbial metabolic activity ($p < 0.05$), suggesting that these additives may promote dysbiosis during fermentation, resulting in a less metabolically versatile microbial community. The significant microbial shifts observed in additive-treated samples include changes in the *Firmicutes*-to-*Bacteroidota* ratio to 2:1 and relative increases in the *Proteobacteria* and *Synergistota* phyla.

These findings suggest that chronic consumption of these food additives may substantially alter microbial dynamics and SCFAs synthesis, with potential adverse implications for gut health.

Keywords: Food Additives, Gut Microbiota, Carrageenan, Carboxymethyl Cellulose, Inflammation.

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CHAPTER I

1. Introduction

1.1. The Human Gut Microbiota Composition and Interaction

The gut microbioma is a thriving ecosystem of microorganisms that carries out an intricate process that profoundly influences our health and well-being. This ecosystem evolves alongside its host, leading to a vast diversity of species (Adak & Khan, 2019). According to numerous studies, the human gut microbiota comprises over 1000 microbial species (Cao et al., 2020). The composition of an individual's gut microbiota can vary significantly from person to person, influenced by factors such as genetics, diet, age, and environment. Additionally, microbiota composition differs across various regions of the gastrointestinal tract, which leads to the assembly of specific communities (Cao et al., 2020). The main phyla encountered in gut microbiota are *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Cyanobacteria*, *Verrucomicrobia*, and *Actinobacteria* (Table 1 in Appendixes)
(X. Zhou et al., 2023). The Human Microbiome Project Consortium also identified the pathogenic species that can be observed in healthy individuals (see Table 2 in Appendixes) through shotgun metagenomic data, using unique marker sequences. This analysis, encompassing the the largest cohort and set of distinct, clinically relevant body habitats offers valuable insights (Huttenhower et al., 2012).
The existence of several "pathogenic" species in healthy individuals, when studying changes in the gut microbiota, can explain or support the development of several serious diseases, particularly inflammatory conditions. It is essential to discriminate the augmentation or reduction of these same species, bearing in mind that their presence specific at a specific abundance does not necessarily imply risk.. So, it is relevant to understand the ratio between species, how these interact, and the final impact on the microbiota microenvironment to understand the gut microbiome. Since *Firmicutes* and *Bacteroidetes* are in the majority, studying them, their ratios, and the balance between the two phyla is essential.

i. Influence of *Firmicutes*-to-*Bacteroidetes* Ratio

Firmicutes phyla has more than 200 different genera identified, in which *Clostridium* represents 95% of its composition, with the predominant genera of *Bacillus, Clostridium, Enterococcus, Lactobacillus, and Ruminicoccus* (Stojanov et al., 2020), while for *Bacteroidetes*, its predominant genera consist of *Prevotella* and *Bacteroides*. Still, it can be found that the abundance of *Firmicutes* in the gut microbiota of healthy individuals can vary between 11-95% and that of *Bacteroidetes* between 0.6-86.6%, demonstrating significant variability even among

individuals in the same population (Magne et al., 2020; Rinninella et al., 2019). Furthermore, according to Mariat et al., (2009), *Clostridium leptum, Clostridium coccoides, Bacteroides*, and *Bifidobacterium* characterize the leading groups of the adult fecal microbiota, being the sub-dominant portrayed by *Lactobacilli, Enterobacteriaceae, Desulfovibrio, Sporomusa, Atopobium*, among others.

Both these phyla are of great importance for gut health; *Bacteroidetes* can protect from pathogens and supply nutrients to other microbial residents by altering the nutritional landscape to produce certain food sources of the gut, assisting in the stability of the immune system (Wexler & Goodman, 2017). They are also key producers of short-chain fatty acids (SCFAs), mainly acetate and propionate, and can grow in a wide pH range. *Firmicutes*, on the other hand thrive in neutral or slightly acidic pH conditions and adapt to the gastrointestinal tract (GIT) zone they inhabit. They are fundamental for various metabolic pathways within the gut, including the metabolism of dietary nutrients, bile acids, and host-derived compounds, being able to metabolize a wide range of substrates, influencing nutrient absorption, energy balance, and the production of bioactive molecules (Bäckhed et al., 2004).

The ratio of *Firmicutes* to *Bacteroidetes* is crucial for maintaining guthomeostasis, leading to possible pathologies if stability is not achieved, which can happen when there is an increase in the *Bifidobacterium*, *Bacteroides*, *Faecalibacterium, Akkermansia, Enterococcus, Bacillales, Enterobacteriaceae*, *Fusobacterium, Parvimonas, Gemella, Leptotrichia and Roseburia* genera and (or), by the decrease in the *Lachnospiraceae* family or the *Clostridium, Bifidobacterium, Faecalibacterium, Blautia* genera. (Stojanov et al., 2020). Tables 1.1 and 1.2 show that dysbiosis—an imbalance in microbial communities—can lead to diseases through either the growth or reduction of specific species.

The effects of dysbiosis on gut health depend on which bacteria are involved.*Actinobacteria, Firmicutes,* and *Proteobacteria* will support the production of SCFAs by degrading complex carbohydrates. One of the most interesting gut microorganisms to consider is *Clostridium leptum*, can reduce inflammation due to its SCFA production, such as butyric acid. While the presence of *E. coli* can induce inflammation, and *Ruminococcus gnavus* can produce toxins (Abiega-Franyutti & Freyre-Fonseca, 2021).

Table 1.1 – Bacterial Species Whose Overgrowth Leads to Pathogenicity.

Phylum	Class	Order	Family	Genus	Species
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	*Enterotoxigenic Bacteroides fragilis*
					Bacteroides vulgatus
Bacillota	*Erysipelotrichia*	*Erysipelotrichales*	*Coprobacillaceae*	*Coprobacillus*	*Coprobacillus catenaformis*
	Negativicutes	*Selenomonadales*	*Veillonellaceae*	*Veillonella*	*Veillonella denticariosi*
	Clostridia	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*Ruminococcus bromii*
					Ruminococcus torques
Firmicutes			*Clostridiaceae*	*Clostridium*	*Clostridium cocleatum*
					Clostridium hatheway
					Clostridium symbiosum
					Clostridium thermosuccinogenes
	Bacilli	*Lactobacillales*	*Streptococcaae*	*Streptococcus*	*Streptococcus bovis*
Fusobacteria	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*Fusobacterium*	*Fusobacterium nucletum*
Proteobacteria	*Myxococcota*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*	*Desulfovibrio*
	Campylobacteria	*Campylobacteriales*	*Helicobacteraceae*	*Helicobacter*	*Helicobacter pylori*

Table 1.2 - Bacterial Species Whose Decrease Leads to Pathogenicity.

Phylum	Class	Order	Family	Genus	Species
Bacillota	*Clostridia*	*Oscillpirales*	*Ruminococcaceae*	*Faecalibacterium*	*F. prausnitzii*
		Eubacteriales	*Lachnospiraceae*	*Faecis*	*Blautia faecis*
Firmicutes		*Clostridiales*	*Clostridiaceae*	*Clostridium*	*Clostridium coccoides*
					Clostridium lavalense
					Clostridium leptum
				Roseburia	*Roseburia inulinivorans*
Verrucomicrobiota	*Verrucomicrobiae*	*Verrucoicrobiales*	*Akkermansiaceae*	*Akkermansia*	*Akkermansia muciniphila*

SOURCE TABLE 3 & 4: Stojanov, S., Berlec, A., & Štrukelj, B. (2020). The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. In *Microorganisms* (Vol. 8, Issue 11, pp. 1–16). MDPI AG.

1.2. Gut Microbiota and its Impact on Human Health

One of the gut microbiota's essential purposes is to participate in human immune function since it affects host cells' gene expression and physiology (Belkaid & Hand, 2014). Several research articles have demonstrated the in-depth connection of the gut microbiome to the physiological development and regulation of innate and adaptive immune functions, which aid the human host's health. In some cases, dysbiosis in GIT microbiota can be the cause of diseases, playing an essential role in conditions such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), diabetes, obesity, cancer, cardiovascular problems, and central nervous system disorders, being of relevance to understanding in which way it can lead to its development or activation.

i. Inflammatory Bowel Disease and Irritable Bowel Syndrome

IBD and IBS are a group of chronic inflammatory conditions that affect the GIT. In the case of IBD, it includes Crohn's disease and ulcerative colitis. Individuals with IBD often exhibit dysbiosis, showing changes in the abundance and diversity of specific microbial species in the gut (Frank et al., 2007). IBS is a common gastrointestinal disorder that affects the large intestine (colon), leading to abnormal motility and inflammation. Several studies have linked these syndromes to gut microbiota dysbiosis, which may contribute to the inflammation observed in IBD and IBS. So it is essential to understand in depth which alterations can lead to them. In both diseases, the microbiota is dominated by *Firmicutes* and by *Bacteroidetes*; in the case of IBS, more specifically, by *Enterococcus* and *Bacillales* (Krogius-Kurikka et al., 2009; T. Zuo & Ng, 2018), presenting an increase in *Bacteroidetes*, with a high decrease in *Firmicutes*, which includes *Faecalibacterium prausnitzii*, which according to Jacob (2023), can promote anti-inflammatory effect due to the production of butyrate. This sequentially leads to decreased patient SCFAs concentration (Frank et al., 2007; Stojanov et al., 2020).

Nevertheless, it is characteristic of IBD patients that the impact of the dysbiosis depends on the location in the GIT, presenting a higher phylogenetic diversity in the large intestine when compared with the small intestine. In the case of the small intestine, there is a higher abundance of *Lactobacillus*, with a variety of members of the family *Lachnospiraceae* being less abundant than the colon (Frank et al., 2007). Throughout the whole GIT, IBD patients present a decrease of *Blautia faecis, Roseburia inulinivorans, Ruminococcus torques,* and *Clostridium lavalense* (Jacob, 2023). On the other side, they show a high amount of *Desulfovibrio,* a sulfate-reducing bacteria, which damages intestinal epithelial cells and induces mucosal inflammation (Jacob, 2023) and a subtle increase in *Proteobacteria*, such as *E. coli* with adhesion-invasive capacities

(AIEC), which can be related with increased pro-inflammation of intestinal mucosa (Quaglio et al., 2022).

A significant decrease in the abundance of *Faecalibacterium* and *Bifidobacterium* has been exclusively observed in IBS, with the microbiota predominantly dominated by *Enterobacteriaceae* (Krogius-Kurikka et al., 2009; Stojanov et al., 2020), presenting prevalence of pro-inflammatory phylotypes, such as *Clostridium cocleatum* 88%, *Clostridium thermosuccinogenes* 85%, *Coprobacillus catenaformis* 91%, *Ruminococcus bromii-like* 91%, and *R. torques* 93% (Krogius-Kurikka et al., 2009).

ii. Colorectal Cancer

Colorectal cancer (CRC) occurs when there is a growth of cells that begins in the large intestine being one of the most common cancers worldwide (Center et al., 2009; Karsa et al., 2010). There is reason to believe that gut dysbiosis is related to the progression of cancer in the light of several studies on CRC being associated with the pathogenetic bacterium, various being identified as carcinogens (Gao et al., 2017).

According to tissue and stool assays, it is possible to identify that *Fusobacterium, Parvimonas, Gemella, Leptotrichia, Enterococcus, Escherichia/ Shigella, Klebsiella, Streptococcus*, and *Peptostreptococcus* are enriched, and anti-inflammatory *F. prausnitzii, Bifidobacterium, Faecalibacterium,* and *Blautia* have a lower abundance in CRC patients (Gao et al., 2017; Quaglio et al., 2022). Furthermore, enterotoxigenic *Bacteroides fragilis* (ETBF), which encoded *B. fragilis* metalloprotease toxin (BFT) to induce diarrhea in most reports, and *Fusobacterium nucletum,* which has proven to be invasive, are highly expressed in CRC tissue when compared with healthy tissue (Gao et al., 2017). On a separate issue, CRC patients also present a significantly lower abundance of SCFAs and SCFAs-producing bacteria, which can be instigated by the reduction of *Firmicutes* and an increase in *Bacteroides*, leading to the development and progression of CRC (Hou et al., 2022).

iii. Diabetes

Research suggests that the composition and function of the gut microbiota may play a role in the development and progression of Type 2 diabetes (T2DM). While healthy patients have a high quantity of butyrate-producing bacteria, T2DM patients have a reduction, mainly of *Clostridium coccoides, Clostridium leptum, Akkermansia muciniphila*, and *Faecalibacterium prausitizii. On the other side*, they show an increase in multiple pathogenic bacteria, such as *Clostridium hathewayi, Clostridium symbiosum, Escherichia coli, Bacteroides vulgatus,*

Veilonella denticariosi, and *Lactobacillus* (W. Wu et al., 2022). Furthermore, the genera of *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia*, and *Roseburia* were negatively associated with T2DM, while the genera of *Ruminococcus, Fusobacterium,* and *Blautia* were positively associated with the disease (Gurung et al., 2020; Z. Zhou et al., 2022). Moreover, when analyzing individuals according to their glucose tolerance, T2DM patients have a higher abundance of *Lactobacillus* species and a lower abundance of *Clostridium* species than normal glucose tolerance individuals. While *Lactobacillus* has been positively related to fasting glucose, *Clostridium* species have been negatively correlated with it, which suggests these same bacterial taxa are related to the development of T2DM (Gurung et al., 2020; Z. Zhou et al., 2022).

iv. Obesity

According to several studies, obesity can be influenced by the imbalance of the *Firmicutes/ Bacteroidetes* ratio at the phylum level, showing a low amount of *Firmicutes*, such as *Blautia hydrogenotrophica, Coprococcus catus, Eubacterium ventriosum, Ruminococcus bromii,* and *Ruminococcus obeum,* with a prevalence of *Prevotella* in obese groups. (Castaner et al., 2018; Stojanov et al., 2020). Moreover, since *Methanobacteriales, Lactobacillus, Bifidobacteria* genera*,* and *Christensenellaceae* and *Akkermansia* families are used as probiotics to help this condition, it is essential to analyze their reduction, as it can be associated with obesity advance (Jacob, 2023).

1.3. Food Additives Related to Gut Health Issues

Nowadays, our products are often far from their natural state, mainly presented in altered forms. This is due to the pressure of ensuring certain quality and shelf-life parameters that must be maintained to increase food products' time-to-market. Food additives are crucial players since they are intentionally incorporated into products to achieve specific goals, whether preserving food, enhancing color, improving texture, or extending shelf-life.

According to the Codex Alimentarius, an additive is defined as any substance that would not be usually used as an ingredient but is applied in food for manufacture, processing, preparation, treatment, packing, packaging, transport, or holding purposes, which may, or not, maintain or improve its nutritional qualities (Carocho et al., 2014). Since they have become normalized in human consumption, the potential issues they might cause to human health have been studied. Some of them were discontinued for their hazardous outcomes, having the potential to cause gut dysbiosis. Considering this, more research has been conducted to understand the

significance of additives (still circulating) in our health (Abiega-Franyutti & Freyre-Fonseca, 2021b).

1.3.1. Food Additives Intended for Re-Evaluation Work Program

Many food additives could have effects like those discussed, but the additives listed below (Table 1.3) are currently under research by the European Food Safety Authority(Abiega-Franyutti & Freyre-Fonseca, 2021b). Within these additives, Carrageenin (E407) and Carboxymethyl cellulose (E466) have been shown as potential risk additives for the gut microbiota, being essential to deepen the knowledge on their possible toxicity and to understand how these may affect human gut microbiota potentially leading to more severe conditions due to dysbiosis.

Table 1.3 - Food additives intended for re-evaluation work program after 2020. (adapted from Abiega-Franyutti & Freyre-Fonseca, 2021b).

Food Additive Type	Name	EFSA Number
Antioxidant	Tartaric Acid	E334
	Sodium Tartrate	E335
	Potassium Tartrate	E336
	Sodic Potassium Tartrate	E337
	Calcium Tartrate	E354
	Metataric Acid	E353
Colorant	Calcium Carbonate	E170
	Vegetal Carbon	E153
	Esters of Acetic Acid	E472a, E472b, E472d, E472e, E472f
Emulsifiers	Stearyl Tartrate	E483
	Carrageenin	E407

i. Carrageenin

Carrageenin or Carrageenan (CGN) is an emulsifier additive from red seaweed. Emulsifiers reduce the interfacial tension between two immiscible liquids. This reduction in tension allows the liquids to mix evenly and remain dispersed, forming a stable emulsion that is only used in beverages (Williams et al., 2003). Several studies suggest that consuming food emulsifiers can decrease the thickness of the mucus barrier in the GIT and higher permeability in the intestinal barrier, which makes them capable of increasing intestinal inflammation (X. Zhou et al., 2023). Studies on this subject use *iota*-(ι-) and *kappa*-(κ-) CGN, which significantly differ in their properties. While ι-CGN can form soft and elastic (heat-reversible) gels, κ-CGN can form strong and rigid gels that are not easily affected by heat. Since it is an additive that needs re-evaluation, studies have been conducted to understand its possible adverse effects on human gut health, as shown in Table 1.4.

There are several assays regarding both ι-CGN and κ-CGN consumption, most of which are *in vivo*. The period of the experiments shows high variety, with the most minor period observed being 90 days (Necas & Bartosikova, 2013-a), while there are experiments that do lifelong administration. The studied concentration of CGN directly applied went up to 25% (w/v), with the smallest dose being 0.1% (w/v) (Nilson et al.,1959). The consumption of the higher concentrations has shown different results, with some studies indicating the development of hepatic cirrhosis, while others suggest it could lead to the animal's death (Food and Drug Research Labs., Inc. 1971). Testing with concentrations ranging from 0.1% to 5.0% concentrations presents effects such as ulcerative lesions, soft stools or diarrhea, and bowel lesions (Grasso et al., 1973, 1975; Marcus, 1971). There is a lack of assays *in vitro*, but the existent has more variable ranges of concentrations and periods of testing than *in vivo*. On the other hand, research involving cell models had the longest trials, taking about 96 h (Borthakur et al., 2012) and administering carrageenan at 1.0% (w/w), and the minimal dose being 0.1% (w/v), mainly showing signs of inflammation promoted by the presence of CGN (McKim et al., 2015)

Within all the different administered concentrations of CGN, and regardless of the testing type, the majority have shown that the presence of carrageenan can create an environment that favors inflammation (Bhattacharyya et al., 2017; Borthakur et al., 2012; W. Wu et al., 2022) although some of these studies have shown no effects (Benitz et al., 1973; McGill et al., 1977; McKim, 2014; McKim et al., 2015; Necas & Bartosikova, n.d.-b; Nilson et al., n.d.; Poulsen, 1973).

Table 1.4 - Studies on the effects of CGN consumption.

Reference	Type of additive	Test Type	Model	Period	Concentration	Effects
Food and Drug Research Labs. 1971	κ/ λ - CGN from *C. cripus*	Animal *(in vivo)*	Mouse	10 weeks	0.0, 5.0, 10 or 20%	Mice with highest dose died.
(Hawkins & Yaphe, 1964)	κ/ λ -CGN from *C. crispus*		Male and female rats	23–143 days	2.0, 5.0, 10, 15, or 20%	No effects on appearance or behaviour were observed in male and female Osborne- Mendel or Sprague-Dawley rats fed 5%.
(Tomarelli et al., 1974)	κ/ λ -CGN		Groups of 12 male and 25 female Sprague-Dawley rats	6 months	Diet containing 4.0% processed, heat sterilized	There was no effect on growth rate, and the caecum and colon were normal on gross and microscopic examination.
(Grasso et al., 1973)	ι-CGN from *E. spinosum*		10 male Wistar rats	56 days	5.0%	Slight diarrhea.
(Marcus, 1971)	Undegraded ι-CGN from *E. spinosum*		10 adult male albino guinea-pigs	20 days	1.0%	Two of four treated animals had ulcerative lesions in the caecum. The control group remained healthy.
(Grasso et al., 1973)	ι-CGN from E. spinosum		7 female guinea-pigs	56 days	5.0%	Formation of multiple pinpoint caecal and colonic ulcerations.
(Nilson et al., 1959.)	κ/ λ -CGN from *C. crispus* or *G. mamillosa*		5 male and 5 female mice of two unidentified strains	Lifelong administration	0.0, 0.1, 5.0, 15, or 25%	No adverse effects.
(Nilson et al., 1959.)	κ/λ -CGN from C. *crispus* or G. *mamil- losa*		Groups of 5 male and 5 female rats	Lifetime administration	0.0, 0.1, 5.0, 15, or 25%	Evidence of hepatic cirrhosis, only at the 25% concentration, with no effect on mortality.

(Rustia et al., 1980)	κ-CGN from *C crispus*	Groups of 30 male and 30 female MRC rats	Lifetime administration	0.5, 2.5, or 5.0%	Soft stool consistency in the begging of the experiment.
(Poulsen, 1973)		Groups of 3 male and 3 female Danish Landrace pigs	83 days	0.00, 50.0, 200, or 500 mg/kg bw per day	No compound-related deaths were seen, and the behaviour, appearance, and feed intake of the animals remained normal.
(Benitz et al., 1973)		Male and female rhesus monkeys	7–11 weeks	1.0%	No gross adverse effects were observed, and the microscopic changes were not attributed to carrageenan.
(McGill et al., 1977)	κ/ λ -CGN derived from *C. crispus*	Male and female infant baboons	112 days	0.0, 1.0, or 5.0%	No effect was seen on organ or body weights, characteristics of the urine and faeces.
(Necas & Bartosikova, 2013 - a)	ι-CGN from *E. spinosum* and κ- CGN from *E. cottonii*	Groups of 10 male and 10 female Sprague-Dawley rats	90 days	5.0%	No changes found directed to the additive consumption.
(Necas & Bartosikova, 2013- a)	κ-CGN from *Hypnea musciformis* or *Irideae crispata*	Groups of 15 male and female Sprague-Dawley rats	1 year.	1.0 or 5.0%	No changes were observed in the stools of rats receiving 1% of either carrageenan. At 5% concentration, rats had loose stools.
EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)	λ-CGN	Rats	--	3,400–3,900 mg/kg body weight (bw) per day	No observation of adverse effects.
(Martino et al., 2017)		Guinea pigs	8 weeks	2.0%	Bowel lesions first (from 2 to 6 weeks). Colonic lesions developed after 8 weeks.

(W. Wu et al., 2022)			Mice	90 days	λ-CGN (1.70 mg/ kg, CGN-L, n = 16.0; 8.30 mg/kg, CGN-M, n = 16; or 41.7 mg/kg, CGN-H, n = 24)	λ-CGN may create an environment that favors inflammation by altering gut microbiota composition and gut bacterial metabolism.
(Bhattacharyya et al., 2017)	Equal parts of ι- and κ- CGN were incorporated in a gelatin capsule		Humans	December 2009 to December 2013.	100 mg of food-grade carrageenan	Carrageenan consumption may aggravate ulcerative colitis (UC) disease activity and reduce the interval to relapse in patients who are in clinical remission.
(McKim et al., 2015)	λ -CGN	Cell Line. *(in vitro)*	Two human intestinal cell lines (HT-29 and HCT-8)	--	0.1, 1.0, and 10.0 mg/mL	No effect on oxidative stress was observed after 24 h.
(McKim, 2014)			A Caco-2 absorption model	--	100, 500 and 1000 mg/mL	Couldn't be observed cytotoxicity and no CGN permeability.
(Borthakur et al., 2012)			Human intestinal cells	1–96 h	1.0 µg/ml	Inflammation and colitis. Carrageenan triggers TLR-4 which mediates intestinal inflammation.

ii. Carboxymethyl Cellulose

The Carboxymethyl Cellulose (CMC) is a water-soluble polysaccharide derived from cellulose. It has a longstanding history of usage across various industries, and in the food sector, CMC serves as a dietary emulsifier, contributing to enhanced food quality and heightened food safety (Costa et al., 2023). However, as mentioned earlier, there has been a growing concern in recent years about the link between dietary emulsifiers, notably CMC, and gut inflammation, raising questions about its continued use. It is possible to observe the studies on CMC impacts below (Table 1.5):

Table 1.5 - Studies on the effects of CMC consumption.

Reference	Test Type	Model	Period	Concentration	Effects
(ZANGARA ET AL., 2022)	Animal *(in vivo)*	Mice	11 weeks	1.0 % w/w	Increased disease incidence, leading to chronic inflammation and colitis.
(CHASSAING ET AL., 2015)			12 weeks	1.0 % w/v	Alteration of the microbiota localization, composition, and pro-inflammatory potential.
(VIENNOIS ET AL., 2020)			13 weeks	1.0% w/v	It confirms the induction of low-grade inflammation.
(Mondal & Yeasmin, 2016)			--	2.5, 5.0 and 10 % w/v	No statistically significant or treatment-related adverse effects on any of the parameters evaluated in safety trials.
(BARAN ET AL., 2020)		Zebrafish embryos	--	The maximum CMC concentration is determined as 5000 ppm for microinjection application.	It can lead to important effects on lipid metabolism by causing changes in the expression of some genes associated with obesity.
(NAIMI ET AL., 2021)	Cells *(in vitro)*	MiniBioReactor Array model	--	0.1 % w/v	Induced a lasting seemingly detrimental impact on microbiota composition and function.
(CHASSAING ET AL., 2017)		HT29-MTX and Hep G2 cells	--	1.56 and 25.0 mg/mL	Presented a strong pro-inflammatory profile.
(CHASSAING ET AL., 2017)		(M-SHIME) model	--	1.00, 0.50, 0.25 or 0.10 % w/v	Acted directly upon human microbiota to increase its pro-inflammatory potential.

CMC has only been recently studied, so there is a lack of information concerning its effects. The results showed that the CMC administered was ranging from 0.1% to 10.0% (Mondal & Yeasmin, 2016; Naimi et al., 2021). The run-up to 13 weeks *in vivo* (Viennois et al., 2020), although there was no report of the period in *in vitro* studies, depended on the effects caused and the care of the cells used. Most of the assays have shown a high incidence of chronic inflammation and colitis, with alteration of the microbiota (Chassaing et al., 2015, 2017; Zangara et al., 2022).

iii. Molecular Properties of CGN and CMC

The ι-CGN and κ-CGN are both sulfated polysaccharides, each possessing distinct molecular properties that influence their behavior and applications. ι-CGN features a linear structure with alternating β-(1→3) and α-(1→4) glycosidic bonds and is characterized by the presence of sulfate groups. In contrast κ-CGN also consists of repeating units of galactose and 3,6-anhydrogalactose, but with a different sulfation pattern, featuring alternating β-(1→4) and α-(1→3) glycosidic bonds, with a molecular weight that ranges from approximately 200 to 500 kilodaltons (kDa) (Necas & Bartosikova, n.d.-b). The primary molecular difference between ι-CGN and κ-CGN is their sulfation patterns. ι-CGN possesses two sulfate groups, located at the 2nd and 4th position, while κ-CGN contains one sulfate group per disaccharide unit, attached at the 4th position of the galactose ring (Chauhan & Saxena, 2016). This structural variation results in κ-CGN being less sulfated than ι-CGN, leading to the mentioned difference in their rigidity, in section 1.3.1. i.

CMC is a water-soluble derivative of cellulose, distinguished by the substitution of certain hydroxyl groups (–OH) in the cellulose structure with carboxymethyl groups (–CH_2–COOH) . This chemical modification preserves the β-(1→4) -linked D-glucose units of the cellulose backbone, resulting in a linear polymer chain while significantly enhancing its solubility in water. The molecular weight of CMC typically ranges from approximately 90 to 700 kilodaltons (kDa). The molecular structure of these food additives can be found in Figure 1.1.

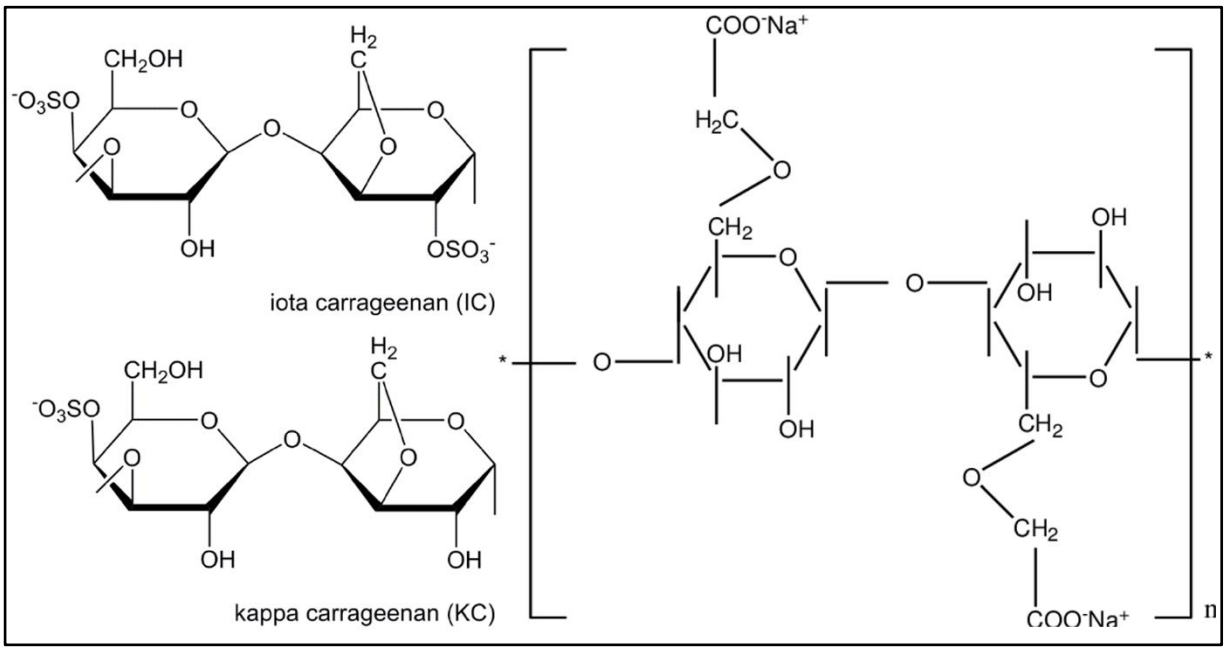

Figure 1.1 - ι-CGN, κ-CGN and CMC molecular structure. Source: (Necas & Bartosikova, 2013-b; Rani et al., 2014)

1.3.2. Gut Modulation by CGN and CMC: Insights and Implications

The continuous consumption of these additives, associated with the increased consumption of processed foods, may lead to several issues related to gut health, according to several *in vivo* and *in vitro* studies, as mentioned before. The ingestion of CGN or CMC has been shown to possibly lead to the development of hepatic cirrhosis, soft stools consistency, slight diarrhea, ulcerative lesions on the caecum, bowel, and colonic lesions, creating an environment that favors inflammation, changes in lipid metabolism, and colitis, which can lead to alterations in the microbiota localization, composition, and function. When understanding diseases that are affected by gut dysbiosis (see section 1.2.), in the majority of these, it can be observed that there is a dominance of the phyla *Bacteroidetes* and *Firmicutes,* being the most observed genera *Bacteroides*, *Faecalibacterium, Akkermansia, Enterococcus, Bacillales, Enterobacteriaceae*, *Fusobacterium, Parvimonas, Gemella, Leptotrichia and Roseburia w*ith a decrease in the *Lachnospiraceae* family and the *Clostridium, Bifidobacterium, Faecalibacterium,* and *Blautia* genera (Cohen & Ito, 2002; Komisarska et al., 2024). Still, several species have been studied and are promotors of dysbiosis, which are identified in Table 1.6. These species need to be prioritized when studying the additives' effect on the gut microbiota since they can create an environment that supports inflammation and have shown involvement with these additives' consumption (Ojo et al., 2021).

Table 1.6 - Dysbiosis by CGN and CMC consumption that can favor inflammation.

Increase	Decrease
E. coli	*F. prausnitzii*
Desulfovibrio	*Blautia faecis*
Clostridium cocleatum	*Roseburia inulinivorans*
Clostridium thermosuccinogenes	*Clostridium lavalense*
Coprobacillus catenaformis	*Clostridium coccoides*
Ruminococcus torques	*Clostridium leptum*
Ruminococcus bromii-like bacteria	
Helicobacter pylori	
Streptococcus bovis	
Fusobacterium nucletum	*Akkermansia muciniphila*
Enterotoxigenic *Bacteroides fragilis*	*Bifidobacterim genera*
Clostridium hatheway	
Clostridium symbiosum	
Bacteroides vulgatus	
Veilonella denticariosi	

SOURCES: Cohen, S. M., & Ito, N. (2002). A Critical Review of the Toxicological Effects of Carrageenan and Processed Eucheuma Seaweed on the Gastrointestinal Tract. In Critical Reviews in Toxicology (Vol. 32, Issue 5); Komisarska, P., Pinyosinwat, A., Saleem, M., & Szczuko, M. (2024). Carrageenan as a Potential Factor of Inflammatory Bowel Diseases. In Nutrients (Vol. 16, Issue 9); Ojo, O., Ojo, O. O., Zand, N., & Wang, X. (2021). The effect of dietary fiber on gut microbiota, lipid profile, and inflammatory markers in patients with type 2 diabetes: A systematic review and meta-analysis of randomized controlled trials. In Nutrients (Vol. 13, Issue 6). MDPI AG.

1.4. Bioaccessibility and Bioavailability of CGN and CMC

Bioaccessibility is defined as the maximum amount of a food additive released from the food matrix and becomes available for absorption by the epithelial cells of the intestine. In contrast, bioavailability refers to the proportion of an ingested compound that enters the bloodstream and is available to perform its physiological functions. Both ι-CGN and κ-CGN, as well as CMC, are not significantly degraded by human digestive enzymes, which cannot break them down (Lovegrove et al., 2017; MacArtain et al., 2007). Like other forms of cellulose, the CMC passes through the GIT largely intact, mimicking the behavior of dietary fiber, while CGN, with its complex linear structure, is also resistant to enzymatic breakdown (Cohen & Ito, 2002). Once these substances reach the intestine, they are not absorbed into the intestinal walls, leading to increased viscosity of intestinal contents and influencing nutrient absorption. Several studies have described that CMC remains within the gut and the intestine wall, while CGN is excreted in the feces, which has a significant impact on gut microbiota and its behavior throughout time and consumption (Gibson & Roberfroid, 1995; Komisarska et al., 2024b; Ojo et al., 2021b; Stephen et al., 2006). There is an urge to study the impact of these food additives on the

bioaccessibility and bioavailability within the GIT, which have been described as significantly low, and how these impact the bioaccessibility and bioavailability of the remaining compounds throughout digestion and absorption.

1.5. Microbiota Fermentation

The human gut microbiotas' metabolic functions are dependent on the subtrates available, whether they are exogenous, such as dietary fibers, or endogenous, which are produced by the host, making it possible to maintain a diverse microbiota environment (Bernalier-Donadille, 2010). One of the primary roles of the human gut microbiota is to metabolize carbohydrates, proteins, and peptides into essential SCFAs, including acetate, propionate, and butyrate (reaching concentrations of 50–200 mM in the human large intestine) or even intermediate metabolites, such as succinate, lactate, and formate, which are branched-chain fatty acids (BCFAs) obtained from proteolytic fermentation (Cummings & Macfarlane, 1991; Louis & Flint, 2017). Additionally, pH is a critical environmental factor that influences the gut microbiota community within the GIT. It regulates nutrient availability and extracellular enzyme activity, affecting microbial metabolism and growth. The pH in the GIT can range from 6.0 to 7.5 and is influenced by the production of SCFAs, which can cause fluctuations in pH values (Firrman et al., 2022).

1.6. Aim of the Thesis

ι-CGN, κ-CGN, and CMC are polysaccharides described as non-digestible molecules by humans, mainly due to their nature, which results in their low bioavailability. Consequently, these substances accumulate in the colon, potentially affecting essential physiological functions, including digestion and metabolism. Despite the common use of these additives, there is a paucity of information regarding their impact on gut microbiota, so it is crucial to investigate the potential impact and dysbiosis that these compounds might induce and assess whether such disturbances could lead to inflammatory responses. This thesis aims to clarify the interactions between the selected food additives and the human gut microbiota and to assess how these interactions impact the physiological functions of the microbiota.

CHAPTER II

2. Methods and Material

2.1. Samples Preparation

Several studies have used ι-CGN (Sigma-ALDRICH, Co., Spruce Street, St Louis), κ-CGN (Sigma-ALDRICH, Co., Spruce Street, St Louis), and CMC (Sigma-ALDRICH, Co., Spruce Street, St Louis), making it possible to establish the most appropriate concentrationfor this study. Since these emulsifiers have a high viscosity, 1.0% was found to be the most suitable concentration (sections 1.3.i. and ii.), as it easily reaches the required viscosity when diluted, while still maintaining significant concentration (Marcus, 1971; Viennois et al., 2020). Therefore, the samples were prepared at 1.0% in ultrapure water. About 0.5 g (≈ 0.5 mL) of ι-CGN, κ-CGN, and CMC (Sigma-ALDRICH, Co., Spruce Street, St Louis) was dissolved in 50 mL of ultrapure water volume.

2.2. Measurement of Saccharide Molecular Weight Using High-Performance Liquid Chromatography (HPLC-SEC)

The molecular weight (MW) of the polysaccharides and the simple sugars were measured using Size Exclusion Chromatography (SEC) on a Beckman & Coulter 168 series HPLC system equipped with a refractive index (RI) detector (Knauer, Berlin, Germany). The weight-based separation was carried out using two Waters Ultrahydrogel SEC columns (Waters, Milford, Massachusetts, USA) packed with hydroxylated polymethacrylate-based gel: the UltraHydrogel 120 and UltraHydrogel 250, with pore sizes of 120 Å and 250 Å, respectively, both measuring 7.8 x 300 nm, according to Campos et al., 2020.

The saccharide quantification was performed using ultrapure water (Milli-Q, Merck, Darmstadt, Germany) under an isocratic gradient with a constant flow rate of 0.5 mL min^{-1} for 90 min at room temperature. The samples to analyze (1.0% ι-CGN, 1.0% κ-CGN, 1.0% CMC, INFOGEST samples, and Fermentation Samples) were filtered through 0.22 μm nylon membrane filters and transferred to 1 mL glass vials. About 30 μL of each sample was injected into the HPLC-SEC system and eluted with an aqueous mobile phase of sodium nitrite ($NaNO_2$; 0.1 M) and sodium azide (NaN_3, 0.2 g/L), which had been previously filtered and degassed. Data acquisition and analysis were performed using Karat32 software. The peak evaluation was done by comparing retention times to two calibration curves: one generated with Shodex Pullulan P-82 standards (Showa Denko K. K., Tokyo, Japan), ranging from P-5 (5.9 kDa) to P-

800 (708 kDa), and another using pure monosaccharides with varying molecular weights (150.13, 180.6, 342.3, 414.4, 546.5 Da).

2.3. Determination of Simple Sugars

The supernatants from the fermentation samples were filtered through 0.20 μm membranes, and chromatographic analysis was performed as outlined in Section 2.2. The separation was achieved using an Aminex HPX-87H column (BioRad, Hercules, CA) maintained at 50 °C, with a mobile phase of 0.003 mol/L Sulfuric Acid (H_2SO_4) at a flow rate of 0.6 mL/min. Aliquots of the filtered samples were subsequently analyzed for simple sugars (D-glucose, fructose, sucrose, and mixture) using an Agilent 1200 series HPLC system fitted with both an RI and a UV detector (Agilent, Germany), as performed by (Campos et al., 2020b).

2.4. *In Vitro* Gastrointestinal Digestion

The method applied for the digestion simulation of the ι-CGN, κ-CGN, and CMC was the INFOGEST 2.0 protocol by Brodkorb et al. (2019), which replicates the physicochemical and physiological environments of the GIT, including the Oral, Gastric, and Intestinal phase, followed by Dialysis. It was previously prepared 10 g of each sample, and an additional 10 g of ultrapure water for a control (CSGI). Firstly, for the Oral phase, the 10 g samples were dissolved in 8 mL of simulated salivary fluid and 1 mL of a 21.14 mg/mL salivary bacterial α-amylase solution (Sigma Amylase A1031; Sigma-ALDRICH, Co., Spruce Street, St. Louis; 70.94 U/mg measured activity). Then, 50 μL of 0.3 M $CaCl_2$ (previously prepared) and 0.95 mL of ultrapure water was added, obtaining 20 mL of Oral phase volume, being formerly incubated in an orbital (Orbital Shaker MaxQ 6000) for 2 min at 37 ºC and 200 rpm. Afterward, in the Gastric phase, the prepared volume was dissolved in 16 mL of simulated gastric fluid, 10 μL 0.3M $CaCl_2$ and 1 mL of an 82.89 mg/mL Pepsin solution (Sigma Pepsin P7125; Sigma-ALDRICH, Co., Spruce Street, St. Louis; 965 U/mg measured activity). Then, the pH was adjusted to 3.0, and ultrapure water was added to achieve a 1x concentration of simulated gastric fluid. The final gastric volume was then incubated at 37 ºC and 130 rpm for 2 h. A 1 mL aliquot was taken of each and left at 90 ºC for 5 min for enzyme inactivation (ThermoBlock, HLC BioTech, Model TH 21).

For the Intestinal phase, the volume was dissolved in 17 mL of simulated intestinal fluid, 80 μL 0.3M $CaCl_2$, 10 mL of a 100 mg/mL Pancreatin (Sigma Pancreatin P7545; Sigma-ALDRICH, Co., Spruce Street, St. Louis; 8 U/mg of unit measured) and 5 mL of a 90.4 mg/mL Bile Salts

solutions (Sigma Bile B863; Sigma-ALDRICH, Co., Spruce Street, St. Louis; 1.77 mmol/g of concentration measured). The pH was then adjusted to 7.0 by adding NaOH, and ultrapure water was added to achieve a 1x concentration of simulated intestinal fluid. The final volume was 80 mL, incubating at 37 ºC and 45 rpm for 2 h. A 1 mL aliquot was taken and put at 90 ºC for 5 min for enzyme inactivation.

The dialysis allows us to understand the bioactive compounds' absorption of the samples using 3.5 kDa membranes (dialysis tubing cellulose membrane). The final volume obtained from the intestinal phase was placed inside the membrane and well-locked. Each membrane is then individually placed in shotts with 900 mL of ultrapure water, being then incubated overnight at 45 rpm and 37 ºC. The next day, 80 mL of the permeate (the water outside the membrane) and the remaining retentate (volume remaining inside the membrane) were saved for each INFOGEST sample, which will be used for later analysis.

2.5. *In Vitro* Fecal Fermentation in Fed-Batch

Fresh human fecal samples from five healthy volunteers without antibiotic therapy (Universidade Católica Portuguesa, Escola Superior de Biotecnologia, Porto) were collected after acquiring consent, as required according to the Declaration of Helsinki. For the inoculum preparation, 2 g of each fecal sample was equally measured and pooled together, obtaining 10 g, being then washed with 100 ml of PBS 0.1 M inside an anaerobic chamber (Don Whitley Scientific, West Yorkshire, UK; 10% CO_2, 5% H_2, and 85% N_2). After agitation, a 1:10 (m/v) inoculum was obtained. The pool was formerly preserved with 5% (v/v) DMSO at -80 ºC.

In order to prepare the medium used in the fecal fermentation, it was dissolved 5.0 g/L of trypticase soy broth (TBS) without dextrose (Sigma-ALDRICH, Co., Spruce Street, St. Louis), 5.0 g/L of peptone bacteriological (HiMedia Laboratories, Maharashtra, India), 5.0 g/L of yeast nitrogen base (Sigma-ALDRICH, Co., Spruce Street, St. Louis) and 0.5 g/L of cysteine hydrochloride (Merk KGaA, Darmstadt, Germany) were dissolved in 500 mL of distilled water. It was then added to the 500 mL solution, 5 mL of a separately prepared 1.0 % (v/v) salt solution. The salt solution was prepared by dissolving 2 g of 100 g/L NH_4Cl (E. Merk, Darmstadt, Germany), 0.2 g of 10 g/L MgCl2.6H2O (Dasitgroup, Val de Reuil Cedex, France) and 0.151 g of 10 g/L $CaCl_2.2H_2O$ (Fluka, Seelze, Germany) in 20 mL of distilled water. It was added 1 mL to a second salt solution with 0.2% (v/v), with 2 g of 200 g/L $K_2HPO_4.3H_2O$ (Sigma-ALDRICH, Co., Spruce Street, St Louis) in 10 mL of distilled water. Lastly, 1 mL of 0.2% (v/v) of 0.5 g/L of resazurin anaerobic indicator (Sigma-ALDRICH, Co., Spruce Street, St. Louis) and 5 mL of

trace minerals (ATTC) 1% (v/v) (Manassas, VA 20108 USA) were added. The final solution pH was adjusted to 6.8 and bubbled with N_2, being sterilized before application.

The fermentation occurred in dynamic bioreactors (Laboratory Glass, HamiltonLabGlass, United Kingdom), which contain an entrance for a potentiometer (pH measurement), an inlet (entrance of O_2-free gases), an outlet (release of gas produced during fermentation), acid (0.5 M of HCl) and base (1.0 M of NaOH) addition, and an entrance for taking samples (Figure 2.1). The potentiometer, the acid, and base entries were connected to a controller (ElectroLab FerMac 260 pH Regulator) so that the pH adjustment was reliant on the one measured inside the bioreactor, according to the gap set (6.7 – 6.9 pH). Six cycles were performed in batch (with medium and sample renewed every 48 h). Each cycle lasted nine days, and two bioreactors were run in parallel. The bioreactors were maintained at 37 ºC.

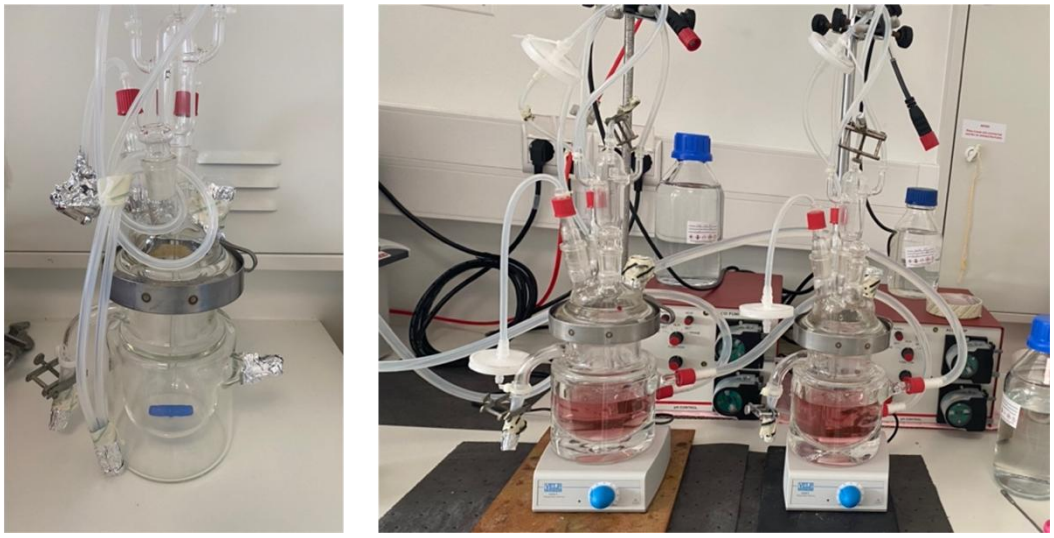

Figure 2.1 – Bioreactor prepared for sterilization and Bioreactors Display, respectively.

Before each cycle, the bioreactors were previously decontaminated in an autoclave at 120 ºC for 2 h, then added 170 mL of the growth medium (under sterile conditions) to each sterilized bioreactor, with continuous mixing and slow sparging of O_2-free, 10% CO_2, 5% H_2, and 85% N_2 overnight (Figure 2.1). On the first day, the inoculum was defrosted and washed with sterilized PBS 0.1M in anaerobic conditions (Anaerobic chamber; Don Whitley Scientific, West Yorkshire, UK; 10% CO_2, 5% H_2, and 85% N_2) and centrifugated at 4696 *g*, 4 ºC for 5 min, being the process repeated two more times. In the fourth cycle, the anaerobic chamber was not working correctly, so the inoculum cleanse with PBS was made under the flame, which might impact future results. Once properly washed, 5% (w/w) inoculum was added to the bioreactor, along with 10% (w/w) of the sample (aseptically), obtaining a total of 200 mL volume. We

tested four digested samples (ι-CGN, κ-CGN, CMC, and CSGI, at 1%), a positive control (FOS), containing 1% (w/w) (0.2g in 20 mL) FOS (Fructooligosaccharides; Megazyme, Bray, Ireland), and a negative control (NC), being each condition duplicated. The process of renewing the medium and samples was made at 48 h, 96 h and 144 h time-points. We recorded the volumes of acid and base added for pH regulation, as well as the volumes taken during sampling, to determine the amount of liquid to be removed from the bioreactor in order to achieve half the reactor volume. Then, 80 mL of growth medium and 20 mL of sample were added (20 mL of growth medium for the NC), obtaining 200 mL of final volume inside the bioreactor. Fermentation samples of 10 mL were collected from each bioreactor at 10 time points (0, 12, 24, 48, 72, 96, 120, 144, 168, and 192 hours). The samples were immediately placed on ice to halt the fermentation process, followed by centrifugation for 5 minutes at 2795 ×g at 4 ºC.The supernatant was stored in 15 mL and 1.5 mL tubes, and then the pellet was washed with PBS 0.1M 2x for 5 min at 5000 rpm at 4 ºC, both stored at -80 ºC. The supernatant was later used to quantify organic acids and saccharides, and the pellet was used for the metagenomic analysis.

2.6. Evaluation of SCFAs with Gas Chromatograph (GC-FID)

The SCFAs detection was conducted using an Agilent 8860 gas chromatograph (Agilent, USA) equipped with a flame ionization detector and a BPX70 capillary column (60 m × 0.25 mm × 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). The following conditions were applied: injector settings were split 25:1 with an injection volume of 1 μL, and the injector and detector temperatures were maintained at 250 °C and 275 °C, respectively. Hydrogen was the carrier gas at a 1 mL/min flow rate. The oven temperature was initially set to 60 °C and then increased to a final temperature of 225 °C (Salsinha et al., 2023). The SCFAs detected by GC-FID was acetic acid (C2), propionic acid (C3), butyric acid (C4), iso-butyric acid (iC4), iso-valeric acid (iC5), valeric acid (C5), iso-caproic acid (iC6) and caproic acid (C6). The process was first optimized to perform the GC and test the organic acids. The ideal sample mass to add (150 mg) was first defined by applying the SCFAs protocol (without the internal standard). The SCFAs extraction protocol was applied to the fermentation samples from 0, 12, 24, 48, 72, 96, 120, 144, 168, and 192 h time-points. In each glass tube, it was added 150 mg of fermentation sample, 200 μL of 50% sulfuric acid (kept at 4 ºC), 10 μL of standard solution (heptanoic acid), and 800 μL of ethyl ester, being vortexed for 1 min and then centrifuged at 4696 *g* for 5 min. The supernatant was removed to a separate glass tube with a glass Pasteur pipette, and 800 μL

of ethyl ester was added to the first tube, then vortexed and centrifuged two more times in the same conditions as before. The final supernatant achieved was then changed into vials and placed into the GC for assessment. To ensure sample analysis reproducibility, GC extracts were kept stored at 4ºC (Scortichini et al., 2020). Also, according to Scortichini et al. (2020), C5 was usually used as the internal standard solution when doing GC analysis with fermentation fluids. Still, it was possible to observe its presence in all fermentation tested, possibly related to the growth medium applied in the fermentation process. While testing, there was no production of C7, so it was tested to be applied as the standard, regardless of being a medium-chain acid. For this, it was prepared a solution of C7 in 1750 μM, which was serial diluted 5x, obtaining solutions of 875, 437.50, 218.75, 109.35 and 54.69 μM. For each dilution, 1 mL of samples was added into individual glass tubes, and the SCFAs extraction protocol was applied without sample application, followed by GC assessment. A calibration curve was developed, demonstrating that a C7 is a feasible standard. The data acquisition and analysis were performed, comparing the observed acids in samples with a calibration curve, which was obtained by preparing a solution with a concentration of $7.00x10^3$ μM for C2, C3, and C4 and a concentration of $1.00x10^3$ μM for iC4, iC5, C5, iC6, and C6.

2.7. Metagenomics

Key fermentation time points were selected based on the observed production rate of SCFAs, leading to the assessment of all fermentation cycles at 12, 24, 72, 120, and 168 h. The 0-hour time point of each cycle was used as a control. The pellet obtained from each selected time-point (previously washed) was then suspended in 5 mL of TE Buffer (Tris EDTA buffer; 1x) and vortexed, passing then 1 to a 2 mL microcentrifuge tube, being centrifuged at 4000 ×g for 10 min, discarding then the supernatant. It was applied 180 μL of previously prepared lysozyme (10 mg/mL in 1x TE Buffer), and the samples were incubated at 37 ºC for 2 h. After incubation, the microcentrifuge tube was again centrifuged at 4000 ×g for 10 min, discarding the supernatant. From then on, the NZYTissue gDNA isolation kit was applied according to the manufacturer's instructions. After the extraction it was measured the concentration and purity of the DNA by Thermo Scientific μDrop Plate coupled with a Thermo Scientific Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) to guarantee the required quality to be sent to NovoGene for 16S rDNA amplicon sequencing. Novogene follows a meticulous workflow to ensure data quality when analyzing DNA samples. Quality control measures were applied at every step, including sample testing, PCR amplification with barcode-

linked primers, and agarose gel electrophoresis for size selection. Equal amounts of PCR products were pooled, end-repaired, A-tailed, and ligated with Illumina adapters. Libraries were quantified using Qubit and real-time PCR, followed by size distribution analysis with a Bioanalyzer. Sequencing was performed on an Illumina platform, and raw data were processed to remove low-quality sequences through splicing and filtering, resulting in clean data. Noise reduction was achieved using DADA2 (Li et al., 2020). The annotated Amplicon Sequence Variants (ASVs) were analyzed for species abundance and diversity. Phylogenetic trees were constructed, and community structure differences were explored through dimensionality reduction analyses and statistical tests like T-tests and LEfSe. Additionally, functional predictions of microbial communities were performed using Tax4Fun 1.

2.8. Statistical Analysis

For the statistical analysis of the data, Prism 10 software was utilized. The normality and homogeneity of the data distribution were assessed using the Shapiro-Wilk test and the Brown-Forsythe test, respectively. Since the data did not follow a normal distribution, the Kruskal-Wallis test, accompanied by multiple comparisons, was employed to determine the significance of the effects of food additives on bacterial populations, SCFAs production, and molecular weight at each time point. Differences were considered statistically significant for p-values ≤ 0.05.

CHAPTER III

3. Results and Discussion

3.1. Stabilization of Polysaccharides During Degradation

During fermentation, various sugars, including glucose, fructose, and sucrose, are broken down and utilized as energy sources by the resident bacteria, producing SCFAs. The specific type of SCFAs produced depends on the microorganisms involved and the available substrates. In numerous fermentation pathways, the initial step is glycolysis, wherein glucose is converted into pyruvate. This pyruvate can subsequently undergo further metabolism through various pathways, depending on the type of microorganism and the specific fermentation route (Silva et al., 2020).

Based on the chromatograms for saccharide identification (Figure 3.1), a peak at 7.8 min is observed in all samples and controls, which is typically associated with sucrose. However, it is chemically not possible for ι-CGN, κ-CGN and CMC to be degraded into sucrose due to their distinct molecular compositions, as detailed in section 1.3.1.iii. This This peak appears consistently across all samples, including controls, indicating that the presence of this peak is unlikely to be a result of the degradation of these compounds. Therefore, it is reasonable to conclude that the 7.8-minute peak is most likely a base signal produced by the equipment itself, rather than an actual metabolic byproduct of the tested substances. This would suggest that the observed signal is an artifact of the chromatographic process rather than a result of the sample compositions. . During the fermentation of ι-CGN, κ-CGN, and CMC, different sugar utilization patterns reflect the structural complexities and microbial preferences for each polysaccharide, having a common peak between 5-7 min. CMC fermentation showed an early increase in peak levels up to 24 h, achieving the highest concentration observed among samples, followed by a steady decline by 72 h and near depletion by 92 h This behavior is expected due to CMC's simpler structure, which is readily accessible and easily broken down by microbes, leading to a rapid initial consumption phase (Rani et al., 2014; Yu et al., 2022). However, the peak's resurgence at 120 h indicates secondary fermentation activity. In this phase, microbes likely utilize the breakdown products generated during the initial CMC degradation, leading to continued fermentation (Leeuwendaal et al., 2022). Bacteria associated with this process include species such as *Clostridium*, *Eubacterium*, *Lactobacillus*, *Ruminococcus*, and *Bacteroides*, each contributing uniquely to SCFAs production depending on the availability of intermediate substrates (Louis & Flint, 2017b; G. T. Macfarlane & Macfarlane, 2012).

In contrast, ι-CGN displays a different fermentation profile. The initial high peak followed by a decrease at 24 h suggests that while microbes can initially access ι-CGN, it requires

adaptations to continue breaking it down due to its more complex structure (Chauhan & Saxena, 2016). This is evidenced by the increases observed at 48 and 120 h. This is expected, pointing to shifts in microbial populations or metabolic pathways as the community adjusts to the complex sulfated polysaccharide structure in ι-CGN (Davis, 2016; Fu et al., 2022). This process often involves *Bacteroides* and certain *Firmicutes* species, which are adept at metabolizing sulfated and structurally complex polysaccharides but require time to upregulate specific enzymatic pathways (Portincasa et al., 2022).

Lastly, κ-CGN follows another unique pattern, presenting the lowest concentrations among all samples due to the most complex structure, making its degradation difficult (Sun et al., 2019). The peak increases from 0 h to 12 h, showing rapid initial consumption, but it fluctuates in a stable pattern every 24 h after 72 h. This periodicity is expected since it may indicate that the microbial community efficiently degrades the available κ-CGN but requires specific metabolic shifts to access it continuously, resulting in alternating sugar utilization phases (Davis, 2016; Fu et al., 2022). NC and CSGI samples, both acting as controls, showed a steady pattern of low fermentation activity with distinct differences in sugar utilization compared to FOS. For NC, a gradual decline in peak intensity from 0 h to 24 h is observed, stabilizing until around 144 h, followed by a decrease and an unexpected increase at 192 h. This pattern suggests minimal fermentation as expected in the absence of additional polysaccharides, with possible late-stage microbial adjustments or cross-feeding on remaining substrates (Duncan et al., 2004). CSGI similarly lacks added polysaccharides yet demonstrates a distinctive fermentation profile. The initial growth phase peaks around 48 h, decreases by 120 h, and fluctuates every 24 h thereafter, possibly indicating microbial adaptation to a low-substrate environment, where remaining metabolic byproducts undergo periodic utilization, possibly creating fluctuations in the activity (Flint, Scott, Louis, et al., 2012). FOS, in contrast, displays a typical profile for rapidly fermentable oligosaccharides, starting with a high peak that declines sharply by 48 h. This is followed by cyclical fluctuations every 24 h, highlighting sugar depletion and microbial adaptation phases. This cycle reflects the well-documented rapid fermentation and recycling of simpler sugars in FOS, which support continuous microbial activity (Rossi et al., 2005). These differences highlight how polysaccharide structure influences microbial succession and metabolic pathways during fermentation. With its simpler structure, CMC is more readily accessed and broken down by microbes, leading to rapid initial consumption and SCFAs production. In contrast, the more complex structures of κ-CGN and ι-CGN require specific microbial adaptations and enzymatic pathways, resulting in slower and less efficient fermentation (González-Figueredo et al., 2019; McKim et al., 2019; Sharma et al., 2020). The

chromatograms also reveal distinct peaks with low concentration after the 10-min mark, which cannot be effectively analyzed with the current referenced standards, indicating the need for additional assays to identify these substances accurately.

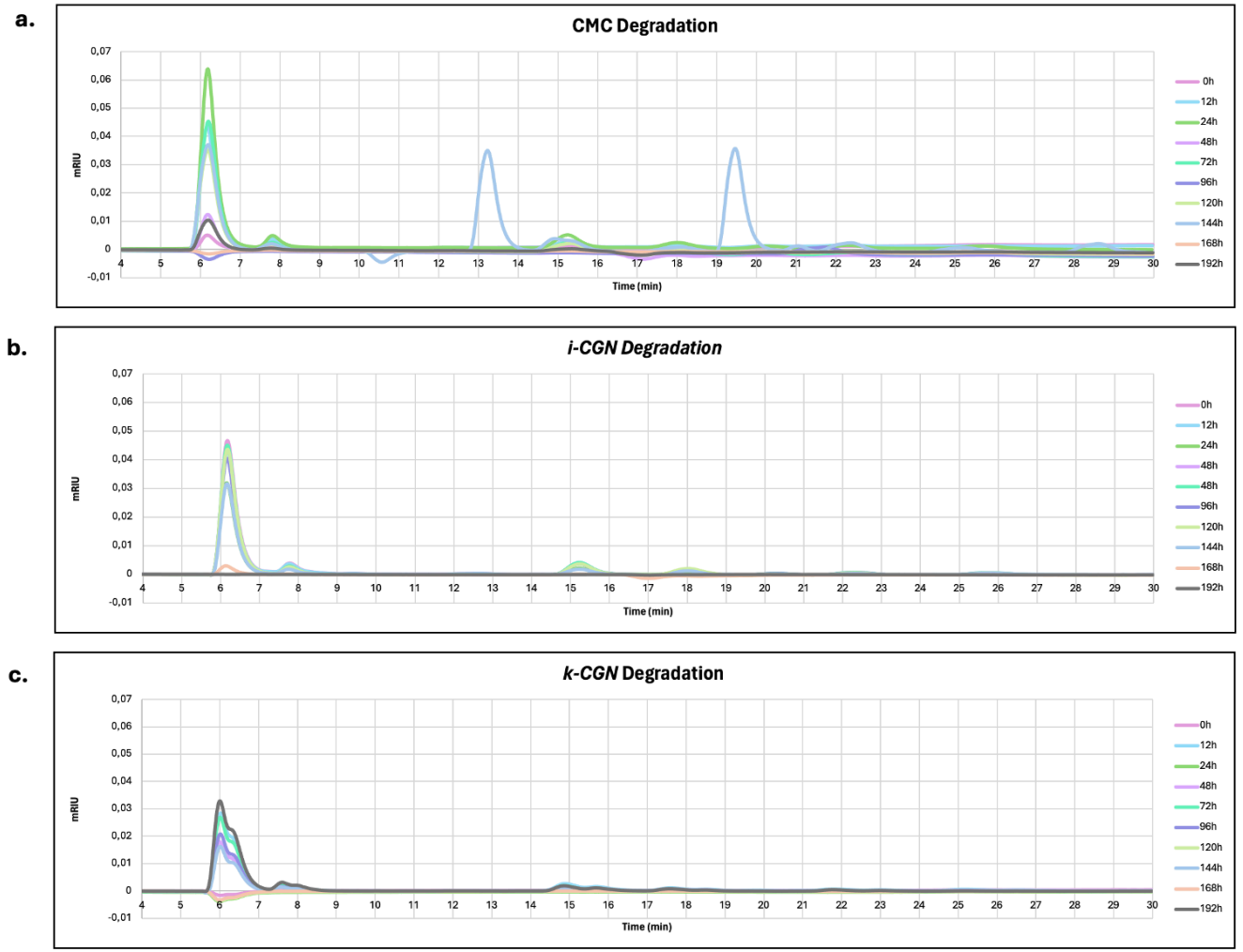

Figure 3.1- Saccharide identification chromatogram showing the concentration of saccharides from from 12 h to 192 h, during in vitro fermentation, with two biological replicates and two analytical replicates for each condition: CMC (a), ι-CGN (b), κ-CGN (c).

3.2. Molecular Weight Profile

The molecular weight (MW) of the selected additives was profiled both before and after digestion, as well as after fermentation, to gain a better understanding of the digestion process.

After the digestion simulation, the samples of ι-CGN and κ-CGN, presented peaks at 5000 Da, accompanied by a peak 342.3 Da, while CMC and CSGI presented a peak only at 5000 Da. In both ι-CGN and κ-CGN samples, after fermentation, initial measurements at 0 h showed a predominant peak at 5000 Da, accompanied by smaller peaks (342.3 Da for ι-CGN and 180.16 Da for κ-CGN), indicating similar initial MW distributions. The MW of 180.16 Da corresponds to galactose, a monosaccharide that is one of the fundamental building blocks of CGN, which forms the core of the CGN structure—the MW of 342.3 Da matches that of a galactose disaccharide (Conte et al., 2021). By 24 h, both samples experienced a significant reduction in MW, suggesting partial degradation of the polysaccharide chains. Notably, at this point, ι-CGN showed a new peak at 414.4 Da, while κ-CGN retains a low MW at 180.16 Da with a minor emergence at 342.3 Da, indicating differing degradation pathways. At 72 h, both samples showed an increase in the 5000 Da peak, possibly indicating polymer re-aggregation or incomplete degradation products reforming larger structures. However, the remaining peaks show reduced MWs in both samples, highlighting further degradation in smaller chains. By 168 h, nearly all peaks for both ι-CGN and κ-CGN diminished to near-negligible MWs, suggesting extensive degradation across both types of carrageenan. For CMC, initial data at 0 h displayed peaks at 5000 Da and 342.3 Da. At 24 h, these peaks drop significantly in MW. By 72 h, the MW of the 5000 Da and 342.3 Da peaks increases, with an additional peak appearing at 180.16 Da. After 168 h, in contrast to the CGN samples, the 5000 Da peak shows a slight decrease, but the MW of the remaining peaks remains broadly consistent.

When comparing the additive-treated samples with CSGI, at 0 h it exhibited peaks at 5000 Da and 342.3 Da. By 24 h, both peaks showed a marked decrease in molecular weight. At 72 h, the 5000 Da peak increased again, but by 168 h, it decreased, with the 342.3 Da peak reappearing. Also, at 0 h, NC and FOS showed a main peak at 5000 Da, along with peaks at 342.3 Da and 414.4 Da, although FOS had slightly higher MWs across all peaks than NC. After 24 h, the 5000 Da peak in NC decreases, the 414.4 Da peak rises slightly, and the 342.3 Da peak remains steady. In FOS, all peaks decreased slightly. By 72 h, NC's peak profile remains stable, while in FOS, the 5000 Da peak increased, and the other peaks are no longer visible. At 168 h, all NC peaks decreased slightly. In FOS, the 5000 Da peak decreased slightly, while the 342.3 Da peak reappears, and the 414.4 Da peak showed a significant increase.

According to the literature, ι-CGN MW ranges between 200.000 and 400.000 Da, while κ-CGN ranges from 300.000 to 500.000 Da (Hoffmann et al., 1996; Uno et al., 2001). CMCs' MW can range from 90.000 to 700.000 Da, with most commonly used grades around 200.000 to 400.000 Da (Y. Chen et al., 2023). According to the observed results, all samples present signs of degradation after digestion, with further degradation after fermentation. Additionally, CSGI, CMC, and CGNs exhibited re-aggregation of the 5000 Da peak; however, CMC and CSGI demonstrate a more stable re-aggregation pattern than the fragmented behavior observed in the CGNs. By the conclusion of the fermentation period, both CGNs showed nearly complete degradation, whereas CMC and FOS exhibited some degree of resistance, maintaining higher MW peaks. In contrast, NC and CSGI gradually decrease their molecular weight profiles. Additionally, ι-CGN and κ-CGN developed distinct intermediate peaks at 414.4 Da and 180.16 Da, respectively, which suggest unique degradation pathways. Notably, FOS also showed a significant increase in the 414.4 Da peak by the end of the fermentation period, indicating reformation behaviors distinct from those observed in CMC and NC. If the samples re-aggregate into larger, less accessible structures, this may limit the effectiveness of certain microbial strains, potentially resulting in lower SCFAs yields.

Conversely, if fragmentation leads to a greater diversity of accessible substrates, it may stimulate a broader range of microbial activity, thereby promoting higher SCFAs production (Flint, Scott, Duncan, et al., 2012). Additionally, unstable re-aggregation can lead to fluctuations in substrate availability, which may affect the consistency of SCFAs production over time. While stable re-aggregation could support more predictable fermentation outcomes, it might also imply that specific substrates are less available for fermentation at certain time points (Flint, Scott, Duncan, et al., 2012). Thus, balancing re-aggregation and fragmentation is crucial for optimizing SCFAs yields during fermentation. Therefore, the specific behaviors of CMC, κ-CGN, ι-CGN, FOS, NC, and CSGI during fermentation directly influenced SCFAs production by affecting substrate availability, microbial community dynamics, and metabolic pathways. CMC may lead to predominantly acetate production while suppressing propionate and butyrate yields, which goes according to literature (Frolova et al., 2022a; S. Macfarlane & Macfarlane, 2003; Undiandeye et al., 2024). In contrast, CGNs promote a more balanced SCFAs profile, particularly enhancing butyrate production. Some studies indicate that complex polysaccharides such as CGNs, can enhance butyrate production by promoting the growth of butyrate-producing bacteria, including those within the *Clostridium* cluster, such as *Faecalibacterium*, *Roseburia*, and *Eubacterium* (Frolova et al., 2022a; Karim et al., 2024; Singh et al., 2023). These bacteria are proficient in breaking down complex carbohydrates,

however, while CGNs may contribute to a balanced profile, their effect on butyrate production may not be as significant as that of FOS. FOS supports rapid fermentation, favoring balanced acetate and butyrate productions as observed by Bhadra et al., 2022.

3.3. pH Control

A stable pH value is vital for fostering a diverse and balanced gut microbiome, essential for effective digestion and the prevention of dysbiosis. An acidic environment is a barrier against the proliferation of pathogenic bacteria, fungi, and viruses, thereby mitigating the risk of infections and gastrointestinal disorders (Jabłońska-Ryś et al., 2022). Furthermore, stable pH values are crucial for the fermentation processes carried out by gut bacteria. A stable and optimal pH environment promotes the growth of beneficial bacteria that generate SCFAs through the fermentation of dietary fibers. In addition, a stable pH significantly influences nutrient absorption, playing a key role in the solubility and assimilation of various essential nutrients (Jin & Kirk, 2018). The need for acidification or alkalinization within the bioreactor provides valuable insights into the type of fermentation occurring in each sample and its potential impact.

This relationship is illustrated in Figure 3.2.

It is shown that the only samples requiring more alkalinization within the fermenter are those treated with FOS and ι-CGN. This is expected for FOS-treated samples, as noted in section 3.4 where a significantly higher production of SCFAs was observed compared to other samples ($p < 0.05$). A similar trend is seen in ι-CGN-treated samples, though the increase is not statistically significant. In contrast, samples treated with CMC, κ-CGN, CSGI, and NC resulted in alkaline fermentations, where acidification was needed. This observation is consistent with the low levels of acid production observed in the samples in section 3.4, which can contribute to an environment that tends toward alkalinity. Comparing samples, FOS-treated samples required significantly higher milliequivalents of OH^- compared to CSGI and CMC-treated samples ($p < 0.05$). No significant differences were observed between other samples. This is expected since ι-CGN, κ-CGN, and CMC generated low acid production, having no overall significant differences in concentration.

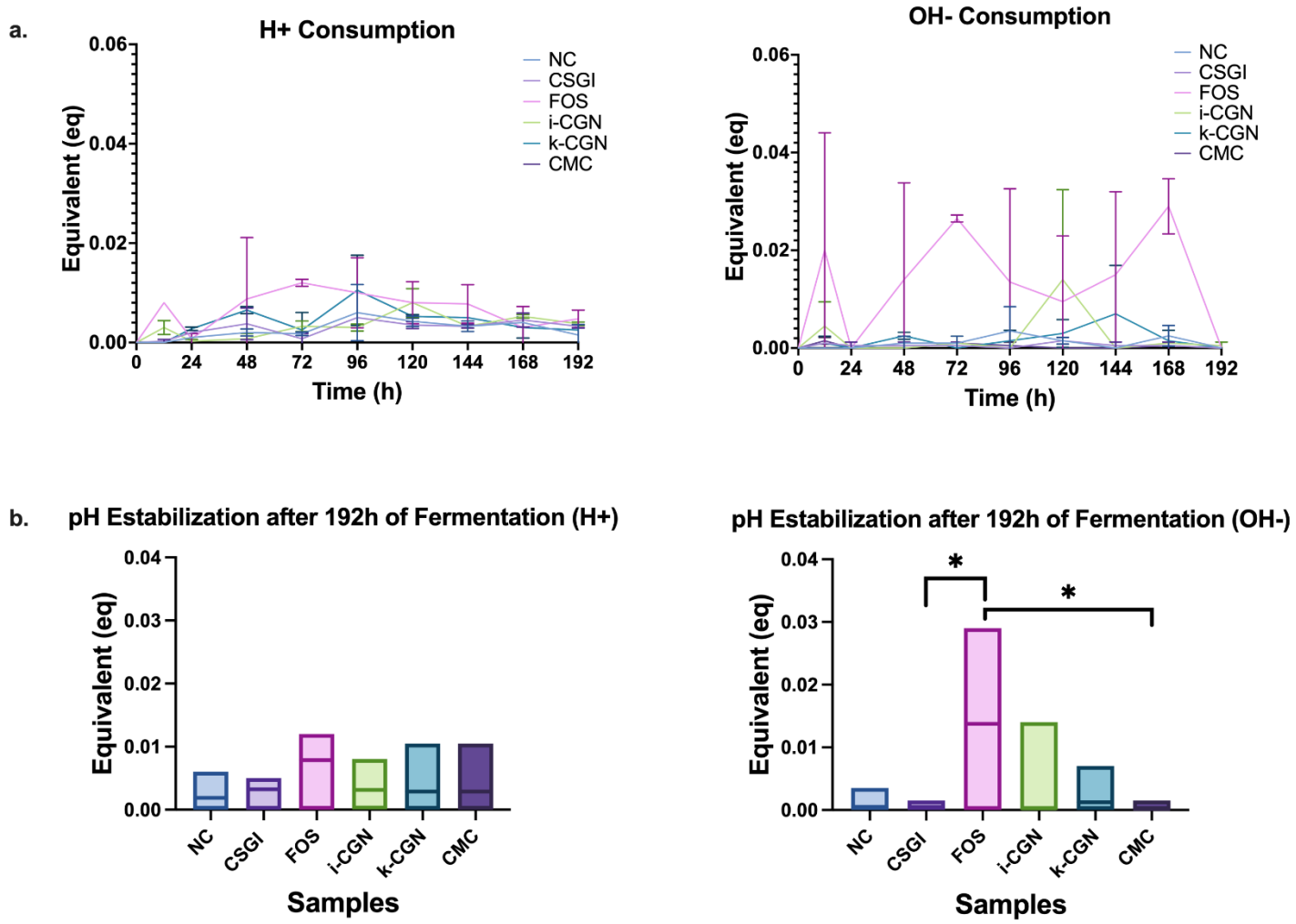

Figure 3.2 - Spaghetti plot (MEAN ± SD) (a) and Floating bars (min to max) (b) representation of milliequivalents transferred of NaOH (1 M) and HCl (0.5 M) to each sample condition, in the presence of two replicas and two analytical replicates with: ι-CGN, κ-CGN, CMC, CSGI, FOS and NC throughout 192 h of *in vitro* fermentation. Different letters mark statistically significant ($p < 0.05$) differences

3.4. Impact of Food Additives on SCFAs Production

The production of SCFAs by the selected food additives, such as acetic, propionic, butyric, valeric, and caproic acid, was analyzed to understand the impact that these can have on the metabolic production of the GIT. In κ-CGN, no production of acetic acid (C2, Figure 3.3a) was observed during the first 12 h. However, production commenced at 24 h, with a subsequent increase at 48 h. Following the medium change, there was a marked reduction in production, a trend that persisted throughout the fermentation process until 192 h. Notably, C2 production declined sharply at 168 h. Meanwhile, for ι-CGN, production was observed at 12 h, with an increase recorded over the first 48 h, followed by a decline at 72 h, mirroring the trend seen in

κ-CGN. Both κ- and ι-CGN exhibited peak production at 96 h. As for CMC, production began at 12 h and showed a significant increase up to 96 h, at which point the highest peak was reached. This was followed by a slight decrease that continued until the end of the fermentation process. Overall, the CMC sample demonstrated higher, but not significant, C2 production than CGN treated samples, with production remaining relatively constant even after the medium change and until the end of the fermentation process. FOS exhibited significantly high peaks throughout fermentation ($p = 0.0168$)st peaks were recorded at 96 h and 168 h. CSGI started acid production at the very beginning - 0 h, with a concentration not significantly different than other samples during the first 48 h. However, its concentration decreased sharply as the fermentation process continued, and the sample control stabilized at levels similar to the NC. Overall (Figure 3.3a), during the first 48 h of fermentation, significantly higher production of C2 was observed in FOS samples compared to all other tested groups ($p = 0.0222$), with no significant differences among the other samples. However, at 96 h, the trend shifted, showing a significant difference between NC and both ι-CGN and CMC ($p = 0.0267$; $p= 0.0137$), as well as between CMC and κ-CGN ($p < 0.0489$). By 144 h, these trends overlap, and at 192 h, with the decline in C2 concentration, significant differences remain only between ι-CGN and CSGI ($p < 0.0375$). The observed trends in the production of C2 during the fermentation of polysaccharides, such as κ- and ι-CGN, and CMC, align with general findings in SCFAs fermentation studies. Specifically, the delayed production of C2 in κ-CGN until 24 h, with a notable peak around 96 h, is consistent with the slower fermentation rates seen in complex, high-molecular-weight polysaccharides. This delay in C2 production is often attributed to the need for initial microbial adaptation and enzyme activation to break down complex polysaccharides into fermentable substrates (Maryati et al., 2021). This can also be due to an

altered *Firmicutes-Bacteroidota* ratio since lower *Bacteroidota* levels will lead to lower production of C2 or depending on the presence of *Bifidobacterium* (Han et al., 2024). The increase in C2 production over the first 48 hours, as seen with ι-CGN and CMC, highlights the microbial fermentation processes associated with intermediate complexity substrates, which tend to reach a peak before beginning to decline (Guo et al., 2022). This decline suggests substrate depletion or microbial composition shifts, favoring other metabolic pathways (Maryati et al., 2021; Z. Zuo et al., 2019). Meanwhile, FOS, a simpler and more readily fermentable substrate, supports consistently high C2 production throughout the process, as Rossi et al. (2005) reported.

For propionic acid (C3, Figure 3.3b), in κ-CGN, there is minimal production throughout the fermentation process, except at 192 h, where the highest peak is observed. In contrast, ι-CGN acid production began at 12 h, followed by a slight but steady decrease until 72 h. Production then increased and remained stable until the point of 144 h, after which it declined, although significant production persisted until the time point of 192 h. The highest concentrations of C3 production occurred for CMC fermentation and were recorded between 72 and 120 h, mirroring the trend seen with the fermentation of ι-CGN. The positive control, FOS, exhibited a C3 increase between 12 and 96 h, achieving its higher peaking at 96 h. A balance of available carbon sources was reached, leading to a sharp decrease. At 192 h, C3 production was similar to the tested samples. The CSGI showed barely any C3 production, with only a slight presence at 72 h and 192 h. During the first 12 h, FOS demonstrated markedly higher C3 production than CMC and NC ($p = 0.0202$). However, this difference becomes non-significant up to the 72-h mark, where FOS once again exhibits significantly higher production than all other tested groups (FOS-NC $p = 0.0239$; FOS-CSGI $p = 0.0004$, FOS- κ-CGN $p = 0.0203$, FOS-, ι-CGN $p = 0.0001$). The absence of significant differences continued until 192 h when FOS showed an elevated production compared to CSGI ($p < 0.0337$). The observed C3 production patterns in κ-CGN, ι-CGN, CMC, and FOS align with known patterns in polysaccharide fermentation studies, where substrate complexity and microbial activity influence SCFAs production profiles. In κ-CGN, minimal C3 production throughout fermentation, with a peak only at 192 h, aligns with studies that highlight how complex, sulfated polysaccharides like κ-CGN are slower for microbes to break down, often resulting in delayed SCFAs production (Z. Zuo et al., 2019). In contrast, ι-CGN, which has fewer sulfate groups, began producing C3 earlier at 12 h, showing fluctuations. This early onset suggests that ι-CGN's reduced complexity allows certain microbes to initiate fermentation sooner, producing SCFAs like C3 more readily than κ-CGN.

This staggered production aligns with findings that indicate microbial access is influenced by polymer composition and structural accessibility (Yu et al., 2022), being also possibly due to a lack of *Bacteroides* and *Prevotella* species, which specialize in C3 production (Louis & Flint, 2017b). CMC showed peak C3 production between 72 and 120 h, following a gradual increase typical for cellulose-based polysaccharides (Mikkelsen et al., 2011). Meanwhile, FOS, a simpler oligosaccharide, led to high early C3 production at 96 h and a decline as the carbon source was depleted. This pattern aligns with studies showing that FOS is rapidly fermented due to its simpler structure, leading to earlier C3 peaks than in more complex polysaccharides (Mikkelsen et al., 2011; Rossi et al., 2005).

The iso-butyric acid (iC4) concentrations remained very low, resulting in a significant standard deviation. Both κ- and ι-CGN showed an increase in iC4 production from 12 to 48 h, followed by sequential decreases and increases every 24 h until the end of fermentation, with the highest peak occurring at 96 h. In contrast, CMC and CSGI exhibited growth until 72 h, then decreased until 120 h, and subsequent alternating increases and decreases until 196 h. The NC displayed a pattern of periodic increase and decrease every 24 h until 192 h, with the initial production of iC4 observed at 12 h. FOS displayed a similar behavior, though production only started at 24 h and ceased by 168 h. During the first 48 h, FOS showed significantly higher production than NC and CSGI, as expected, since there was an available carbon source, leading to fermentation by the microbiota. Overall, while there were observable differences in iC4 production between the types of CGNs, the lack of statistically significant differences suggests that while microbial communities respond differently to these substrates, the variations may not be pronounced enough to achieve statistical significance. This is consistent with findings in microbial fermentation literature, where various factors, including the complexity of the polysaccharide, can influence SCFAs production (Yu et al., 2022). Still, there is a lack of studies on the impact of polysaccharide fermentation on iC4 production.

Butyric acid (C4) production became detectable at 12 h (Figure 3.3c). For samples with κ-CGN, a decrease in butyric acid concentration was noted at 24 h. Following the medium renewal at 48 h, at which the highest concentration was observed, a marked reduction in production occurred, continuing until 168 h, with a slight resurgence noted at 192 h. ι-CGN exhibited a similar trend throughout the fermentation process, being expected due to their similar composition. In contrast, CMC showed an initial increase in production at 12 h, followed by a decrease at 24 h, an increase up to 72 h, and a subsequent decrease until the 168-h time point. NC exhibited a progressive increase in C4 production from the onset, peaking at 96 h, followed

by alternating decreases and increases until 192 h. FOS consistently exhibited the highest concentration across all time points, mirroring the behavior of ι-CGN throughout the fermentation process. Notably, CSGI demonstrated an inverse pattern to FOS and ι-CGN, with its highest concentration observed at 72 h. In general, by 96 h the samples containing FOS continued to display significantly higher production of C4 compared to NC and ι-CGN (p = 0.0206; p = 0.0249), while a significant difference is also seen between CMC and CSGI (p = 0.0386). At 144 h, more pronounced differences emerged, with NC differing significantly from CMC, CSGI, and FOS, while FOS showed significant differences from CMC and CSGI (p = 0.0218; p = 0.410). By 192 h, only the difference between κ-CGN and FOS remained (p = 0.0025). The described production patterns C4 are generally expected. The initial detection of C4 at 12 h aligns with the metabolic activity of certain bacteria, such as *Clostridium* and *Firmicutes* species, known for C4 production (Louis & Flint, 2017b; Qureshi et al., 2022). The fluctuations in concentration, particularly the decrease after the medium change and subsequent resurgence, are consistent with how microbial populations adapt to changing nutrient availability over time (Mikkelsen et al., 2011). The higher concentrations observed in FOS compared to other samples fit expectations as FOS is a readily fermentable substrate, often leading to increased SCFAs production (Rossi et al., 2005). In contrast, the varying trends of CMC, κ-CGN, and ι-CGN suggest that the complexity of polysaccharide structures influences microbial fermentation rates and outcomes (Z. Zuo et al., 2019).

Production of iso-valeric acid (iC5) was first detected at 12 h. All samples exhibited a consistent pattern, characterized by an increase in production until 48 h, followed by alternating increments and decreasing every 24 h cycles until 144 h, after which production generally declined until the end of fermentation (192 h). Specifically, differences between 12 h and 24 h of NC with k-CGN and FOS (p = 0.0201; p = 0.0005) were observed. After 48 h, only NC and ι-CGN display significant differences (p < 0.0198). By 96 h, there was a more pronounced distinction, with NC differing significantly from FOS, κ-CGN, and ι-CGN (p = 0.006; p = 0.0235; p = 0.0211). and further differences were noted between ι-CGN, FOS, and κ-CGN (ι-CGN-FOS p = 0.0012; ι-CGN-κ-CGN p = 0.0015). At 144 h, the only significant difference was between κ-CGN and ι-CGN (p = 0.0278). By the end of fermentation, FOS exhibits significantly higher production compared to NC, CMC, and κ-CGN (p = 0.0087; p = 0.0203; p = 0.0056), with a significant difference also observed between κ-CGN and CMC (p = 0.0202). Regarding valeric acid (C5, Figure 3.3d), NC displayed notably high concentrations (p < 0.05), with production increasing 24h after each medium change until 192h. This pattern was similarly

observed across all samples, except that production ceased at 144h, after which a general decline was noted. At 24 h, a significant difference is observed between κ-CGN and CSGI (p = 0.0085), with minimal changes until 96 h, where CMC and CSGI diverge (p = 0.0388). By 168 h, FOS shows significantly higher production than NC and κ-CGN (p = 0.0178; p = 0.0375). At the end of fermentation, significant differences are noted between ι-CGN and both NC and κ-CGN (p = 0.0087; p = 0.0074). The described production patterns for iC5 are generally expected based on fermentation dynamics. The initial detection at 12 h and the observed increase until 48 h aligns with the typical metabolic activity of gut microbiota utilizing available substrates (Guo et al., 2022), increasing in production when in the presence of *Clostridium, Bacteroides,* and *Faecalibacterium* species. Still, there is a lack of information on the impact of polysaccharide fermentation on iC5 production. The significant differences noted at various time points, particularly between NC, κ-CGN, and FOS, indicate the varying fermentability of these substrates. The trends for C5 production, including the significant differences between samples, also align with what is expected in polysaccharide fermentation, showing distinct patterns based on the substrate complexity utilized (W. Wu et al., 2024), which was observed along the abovementioned acid production.

Iso-caproic acid (iC6) was primarily detected in samples with FOS and the NC, though its concentrations remained low (<50 μM). iC6 shows minimal production, though significant differences emerge after 120 h between FOS and all other samples (FOS-NC p = 0.0146; FOS-CSGI p =0.0407, FOS- κ-CGN p = 0.0245, FOS-, ι-CGN p = 0.0132; FOS-CMC p = 0.0439) and between CMC and CSGI (p = 0.0121). This trend continues until the end of fermentation, except for NC. Caproic acid (C6, Figure 3.3e), despite typically serving as an internal standard (Scortichini et al., 2020), was detected across all samples. Notably, its concentration was particularly elevated in the negative control at 12 h and 72 h, and in the CMC samples at 168 h and 192 h. This presence is not expected, and its appearance may be due to the nutrition medium applied to provide the microbiota composition of the fecal inoculum prepared, leading to C6 production. After 48 h, NC exhibited a significantly higher production than the other samples NC-FOS p = 0.0273; NC-CSGI p =0.0215, NC- κ-CGN p = 0.0253, NC-, ι-CGN p = 0.0147; NC-CMC p = 0.0134). Typically, C6 production can be due to *Clostridium, Bacteroides,* and *Fusobacterium* (King, 2007). By 96 h, this distinction remained evident only between NC and κ-CGN(p = 0.0211) and between FOS and CMC (p = 0.0367). At 144 hours, notable differences emerged between CSGI and both κ-CGN and ι-CGN (p = 0.0211; p = 0.0165), with significant variation also observed between κ-CGN and ι-CGN (p = 0.0426). By 192 h, FOS demonstrated

significantly higher production of C6 than all other samples (FOS-NC $p = 0.0219$; FOS-CSGI $p = 0.0052$, FOS- κ-CGN $p = 0.0233$, FOS-, ι-CGN $p = 0.0180$), except for CMC, due to its' high standard deviation.

A notable overall reduction in acetic, propionic, and butyric acid production was observed when comparing the samples treated with additives to the NC and those treated with FOS. This aligns with research indicating that the consumption of CMC can lead to decreased levels of these SCFAs, as mentioned in section 1.3.1. ii., as well as the effects of ι- and κ-CGN on SCFAs production studies, which suggest that their association with chronic inflammation may contribute to lower levels of acetic, propionic, and butyric acid, mentioned in section 1.3.1.i. Nevertheless, there is no discernible difference between the additive-treated samples and CSGI at specific time points. This observation suggests that the conditions established by the digestion conditions may impact the low production of these SCFAs. Additionally, valeric and caproic acid and their iso-form have not been as extensively studied as the aforementioned SCFAs. While SCFAs like acetic and butyric acid are well-documented for their beneficial effects on gut health (Han et al., 2024b, 2024a; Louis & Flint, 2017b; G. T. Macfarlane & Macfarlane, 2012; Qureshi et al., 2022; Yu et al., 2022; T. Zhang et al., 2018), less is known about the physiological roles of valeric and caproic acids. Studies discuss the significance of SCFAs in gut health but do not extensively cover longer-chain fatty acids like valeric and caproic acids. It indicates that most research has focused on the well-established roles of SCFAs in human health, implying a knowledge gap regarding longer-chain fatty acids (Chen et al., 2016; Han et al., 2024; Maryati et al., 2021; Z. Zuo et al., 2019). The concentrations of SCFAs such as acetic, propionic, butyric acid, and valeric acids exhibit similarities across most samples, except FOS, which demonstrates significantly higher concentrations of acetic and butyric acid production. The observed lower concentrations of these acids may be attributed to an altered *Firmicutes*-to-*Bacteroidota* ratio in the gut microbiome, as well as the structural complexity of polysaccharides, which can hinder their availability as substrates for fermentation, ultimately leading to reduced acid production.

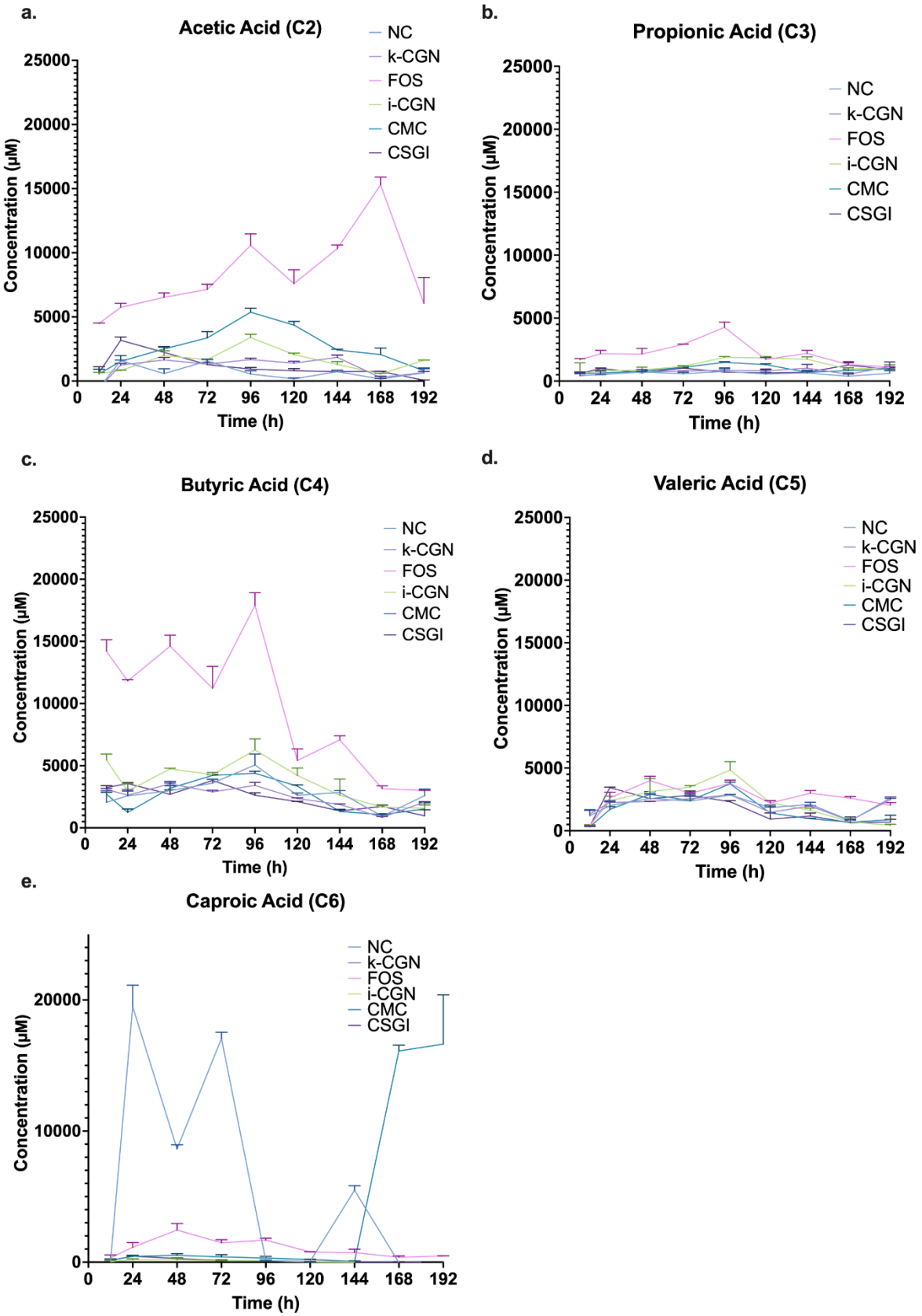

Figure 3.3 - Spaghetti plot depicting the concentration (MEAN ± SD) of SCFAs produced (a-e), in µM, from 12h to 192h, in the presence of two replicas and two analytical replicates with ι-CGN, κ-CGN, CMC, FOS, NC and CSGI throughout *in vitro* fermentation.

3.5 Microbiota Population Analysis

A technique involving DNA extraction and amplification of the 16S ribosomal RNA (rRNA) gene was employed to obtain information on the microbiota population, as referenced in section 2.6. This approach, utilizing polymerase chain reaction (PCR) and metagenomic sequencing, allows for the characterization of microbial dynamics.

3.5.1. Relative Abundance

The main taxa present in each tested condition ι-CGN, κ-CGN, CMC, FOS, NC, and CSGI, and fermentation initial time-point 0h (Fecal Inoculum - FI). The relative taxonomic abundances for phyla, genera, and species observed under each tested condition are depicted accordingly (Figure 3.4a, 3.4b, and 3.4c).

In Figure 3.4a, a clear impact on the *Firmicutes*-to-*Bacteroidota* ratio can be observed at the phylum level, along with notable variations in the relative abundance (RA) of *Proteobacteria*, *Actinobacteria*, and *Synergistota*. These shifts are more pronounced in digested, suggesting that these samples may influence the gut environment and microbial competition. Within the *Bacteroidota* phylum, only the *Bacteroides* genus was detected (Figure 3.4b), showing an increase in RA across all samples compared to FI. The genera *Streptococcus*, *Enterococcus*, *Dorea*, *Ruminococcus*, *Blautia*, and *Agathobacter* were identified in the *Firmicutes* phylum. *Streptococcus* exhibited consistent levels across most samples, with an increase observed in the FOS sample, which was also the only sample where *Enterococcus* was detected. There was a marked decrease in *Ruminococcus*, *Blautia*, and *Agathobacter* across all samples, while *Dorea* showed an increase compared to FI. Within the *Actinobacteria* phylum, the genus *Collinsella* displayed an increase in the FOS sample. As for the *Synergistota* phylum, the genus *Cloacibacillus* was absent in both the initial and FOS samples but exhibited a significant increase in the digested samples. *Collinsella* is known for its ability to utilize complex carbohydrates, including oligosaccharides like FOS, which serve as a substrate for fermentation. This can lead to enhanced growth of this *genus* when such fermentable substrates are present in the gut environment (Gomez-Arango et al., 2018).

On the other hand, the absence of *Cloacibacillus* in both initial and FOS samples, with a significant increase in digested samples, suggests that this genus may thrive under specific fermentation conditions created by the simulated digestion conditions. So, *Cloacibacillus* may be sensitive to the gut environment's overall composition or require specific fermentation byproducts. The increase of *Cloacibacillus* might also suggest an imbalance in the gut microbiota, particularly as it has been associated with opportunistic pathogenic behavior,

indicating a potential imbalance in microbial communities following altered dietary conditions (Domingo et al., 2015).

At the species level, the most detailed taxonomic resolution achieved in this study, the analysis revealed nuanced effects of the tested food additives on specific bacterial species. Most detected changes occurred within the *Firmicutes* and in the *Synergistota* phylum. Digested samples exhibit a marked increase in *Cloacibacillus evryensis* (phylum *Synergistota*), a bacterium absent in the initial samples. Its presence in the negative control suggests that the simulated digestion conditions may have facilitated its growth. In contrast, within the *Firmicutes* phylum, *Ruminococcus* sp. N15/MGS-57 is detectable for FI but becomes undetectable in all subsequent samples. Both *Dorea formicigenerans* and *Clostridium scindens* show a modest increase, with the most pronounced growth observed in the κ-CGN and CSGI samples. *Streptococcus anginosus* and *Coprococcus catus* are only present in the FOS sample, followed by a slight increase in *Blautia* sp. GD9.

As a general observation, throughout the fermentation time points, the digested samples exhibited disturbance in the microbiota, particularly affecting the *Firmicutes*-to-*Bacteroidota* ratio, which may lead to reduced production of SCFAs (Abiega-Franyutti & Freyre-Fonseca, 2021), as can be observed in section 3.1. The effects were less pronounced, although the FOS and NC samples were also impacted. This impact can be due to changes in the population dynamics of other bacteria, which may create niches or alter the competitive landscape, allowing *Bacteroidota* to flourish (Bäckhed et al., 2004).

Additionally, digested samples demonstrated a high relative abundance of *Cloacibacillus evryensis*, a potential pathogen previously linked to bacteremia (Domingo et al., 2015). In contrast, the FOS-treated samples showed an increase in *Streptococcus anginosus*. This typically benign gut inhabitant contributes to maintaining microbiome balance alongside *Coprococcus catus*, known for its capacity to produce SCFAs, particularly butyrate, through the fermentation of dietary fibers, which can be corroborated with the data at section 3.1. (Companys et al., 2021; Domingo et al., 2015; Lu et al., 2020; Uppakarn et al., 2021).

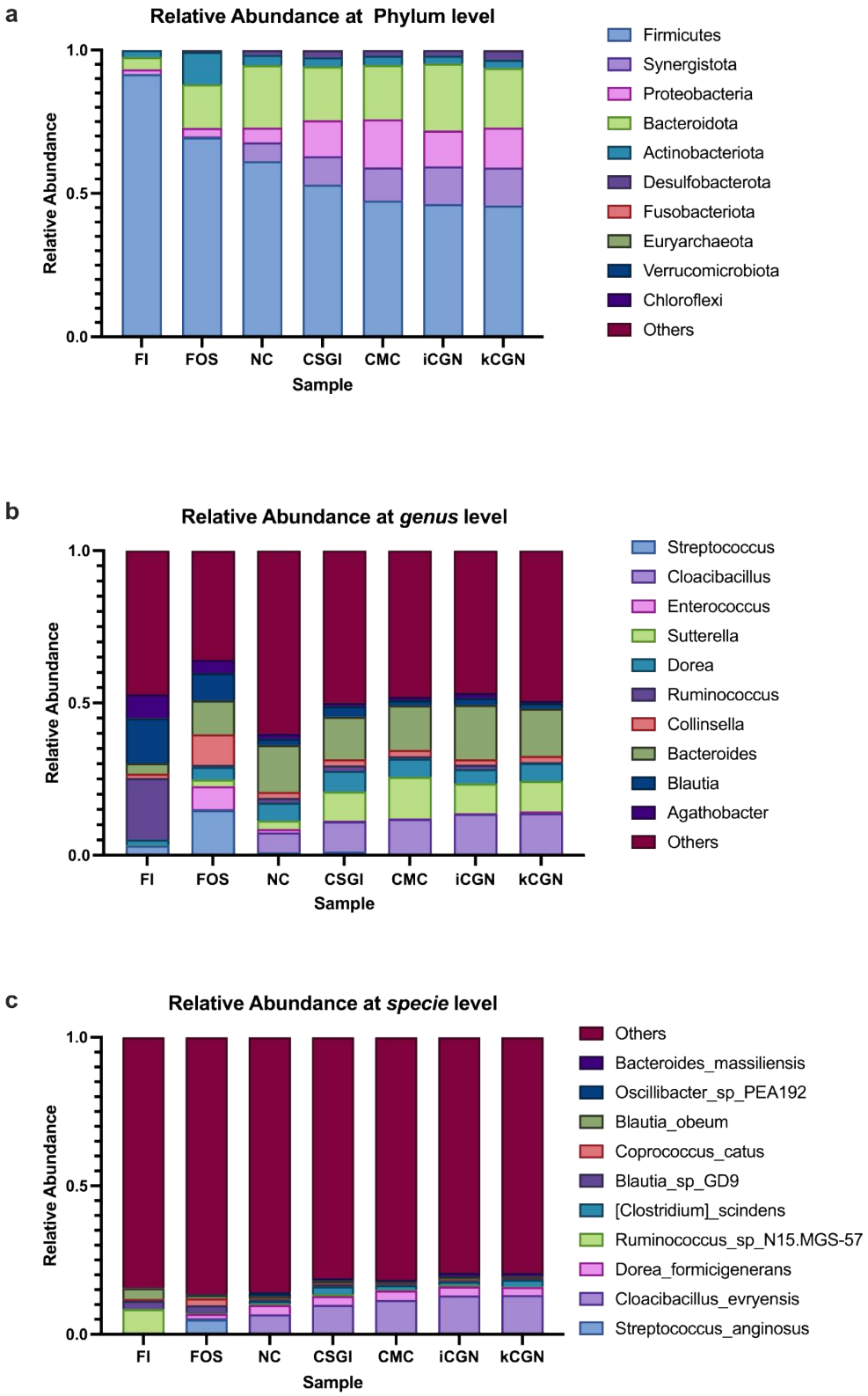

Figure 3.4. - Barplot representation of phyla (a), genera (b) and specie (c) relative taxonomic abundance from each sample condition, in the presence of two replicas and two analytical replicates with FI, NC FOS, CSGI, ι-CGN, κ-CGN and CMC throughout 192 h of *in vitro* fermentation.

3.5.2. Predominant Taxa Abundance

To identify the differences in dominant taxa among the three samples groups at phylum level, the top 10 taxa with the highest average abundance across the three groups were selected to generate a ternary plot. The three vertices of the ternary plot correspond to the three groups: ι-CGN, κ-CGN and CMC (Figure 3.5). Each circle on the plot represents a dominant taxon, with its size reflecting the relative abundance of that taxon. A circle closer to a vertex indicates that the associated phylum is more prevalent in that group (Mitter et al., 2017).

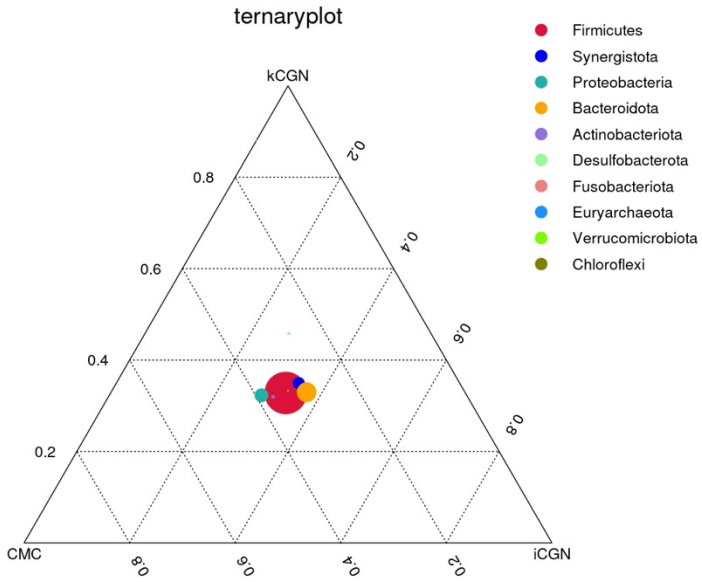

Figure 3.5 - Ternary plot of fermentation samples treated with ι-CGN, κ-CGN and CMC.

Figure 3.5 shows that the most abundant phyla are *Firmicutes, Bacteroidota, Proteobacteria,* and *Synergistota*, listed in decreasing order of abundance. The overlapping regions of these phyla suggest that their quantities are nearly equivalent across the different groups. The overlapping regions of these dominant phyla on the ternary plot suggest that their relative abundances are comparable across the three groups, indicating minimal variation among them. This lack of differentiation implies that none of the groups exhibited a significantly higher abundance of any of these phyla, suggesting an absence of distinct, group-specific dominance patterns at the phylum level (Luo et al., 2019; Z. Wu et al., 2024). Furthermore, if the phyla display similar abundance across groups, it is plausible that the functional roles they support may also be conserved (K. Wu et al., 2019). Since ι-CGN, κ-CGN, and CMC show no

differences in their microbial community structure, the observed SCFAs production is similar across these samples, which can be observed in section 3.4. This is expected since the production of SCFAs is primarily determined by the bacterial composition This is expected since the influenced of SCFAs is primarily determined by the bacterial composition and their associated metabolic pathways. In the absence of significant differences in microbial community composition, SCFAs profiles will likely remain consistent, as observed and their associated metabolic pathways. In the absence of significant differences in microbial community composition, SCFAs profiles are likely remain consistent, as observed (Frolova et al., 2022b; Harris et al., 2021).

3.5.3. Unique Features

Figure 3.6 presents a flower diagram, where each petal is color-coded to represent a distinct sample group, with the number of unique feature sequences corresponding to that group. The central core of the diagram illustrates the number of feature sequences shared across all groups (Wang et al., 2022). According to the diagram, the sample groups share 267 feature sequences. Notably, the FI, NC, and FOS samples exhibited the highest number of unique feature sequences, indicating a greater presence of distinct bacterial species. This suggests that these three groups have higher bacterial diversity than the other conditions. FI is anticipated to possess a highly diverse microbial community, generally containing a broad array of bacterial species, which reflects the complex and diverse composition of the gut microbiota (de Carvalho

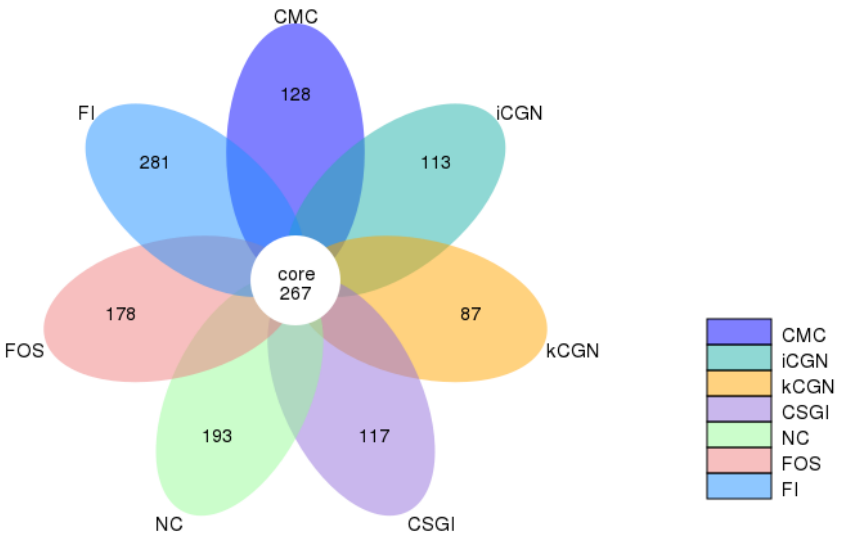

Figure 3.6 - Flower Diagram of the fermentation samples treated with FI, FOS, NC, CSGI, ι-CGN, κ-CGN and CMC.

et al., 2021), as well as FOS, which as a prebiotic, can enhance overall microbial diversity by selectively promoting the growth of beneficial bacterial species (Mahalak et al., 2023a).

NC samples generally exhibit lower microbial diversity than those supplemented with prebiotics or other substrates. This difference arises because the NC lacks the additional nutrients that promote the growth of a broad range of bacterial species.

Nonetheless, the fecal inoculum used in the NC still contains a diverse microbial community representative of the gut microbiota. (Brodkorb et al., 2019b). Also, diverse microbial communities are generally more efficient in degrading complex carbohydrates and fibers, resulting in increased production of SCFAs (D. Zhang et al., 2023). Regarding SCFAs production, the NC shows lower levels of SCFAs than samples with FOS, which can be observed in section 3.4, which is expected (Brodkorb et al., 2019b). Consequently, samples characterized by high microbial diversity and the presence of FOS are expected to exhibit elevated levels of these SCFAs (Fehlbaum et al., 2018).

3.5.4. Alpha Diversity Analysis

Alpha (α)-diversity is applied for assessing the diversity of microbial communities within a sample. It is typically tested using indices such as Shannon, Simpson, Observed Features, and Chao1, all based on Operational Taxonomic Units (OTUs) and their relative abundances. The Shannon index gauges the number of different species and the evenness of their distribution within the sample. Conversely, the Simpson index estimates the likelihood that two randomly selected individuals belong to different species, with a higher Shannon index and a lower Simpson index reflecting greater diversity (Nagendra, 2002). Unlike these indices, the Observed Features index counts the distinct species present.

Meanwhile, the Chao1 index offers a more comprehensive estimate of species richness by predicting how many additional species might be discovered with further sampling (Z. Zhou, Wu, et al., 2022). These metrics enable a comprehensive analysis of species richness (the number of species present) and evenness (the distribution of individuals among species). Shannon, Simpson, Chao1, and Observed Features indices are illustrated in the box plots, respectively (Figures 3.7a, 3.7b, 3.7c, and 3.7d).

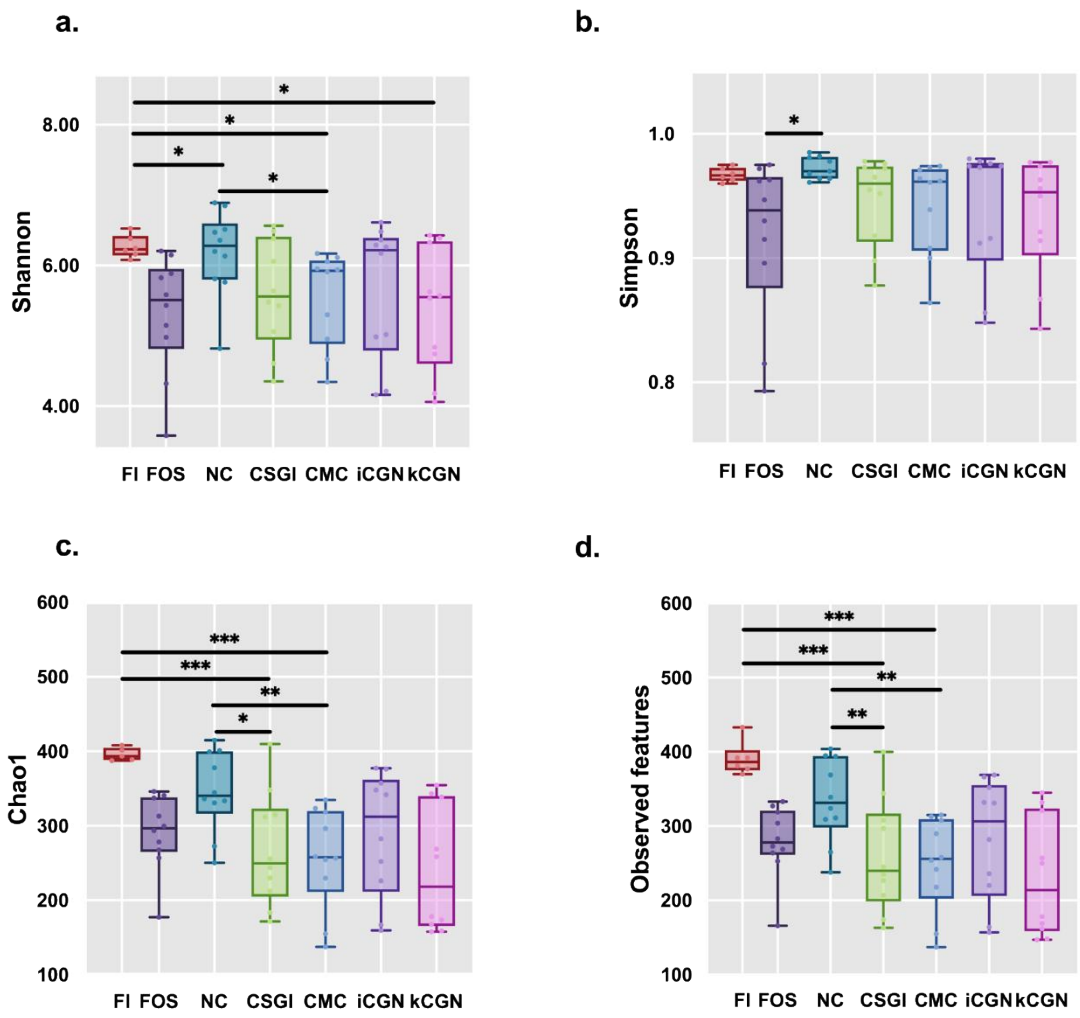

Figure 3.7 - Boxplot for the alpha (α)-diversity Shannon (a), Simpson (b), Chao1(c), and Observed Features (d) indices, of samples with FI, FOS, NC, CSGI, ι-CGN, κ-CGN and CMC, applying the Kruskal-Wallis test. Significant differences were obtained ($p < 0.05$).

The Kruskal-Wallis test on the Shannon index showed that microbial diversity significantly increased in the CMC sample ($p < 0.05$) compared to the NC. Additionally, microbial diversity in the CMC, κ-CGN, and FOS samples significantly increased ($p < 0.05$) compared to FI. However, no significant difference in diversity was found between the samples and CSGI. Regarding the Simpson index, the analysis indicates a significant difference in microbial community diversity between the FOS and NC samples alone ($p < 0.05$). This means that samples treated with CMC, κ-CGN, and FOS enhance microbial diversity, which is often beneficial for gut health and ecosystem stability. FOS significantly alters the community composition compared to the NC, which is expected since FOS can serve as a prebiotic, which

affects the host by selectively stimulating the growth and/or activity of beneficial microorganisms (Rossi et al., 2005). The structural complexity and diversity of CMC and κ-CGN provide a range of fermentation substrates that various microbial species can utilize, which encourages a broader range of microbial activities and interactions (Udayakumar et al., 2024; W. Wu et al., 2020). The Chao1 index reveals that the CSGI exhibited the highest median richness, with a wide range of values. Both CMC and CSGI samples showed significantly higher richness ($p < 0.05$) than the initial time point and the negative control ($p < 0.05$). Overall, all samples demonstrated greater species richness than FI, a trend similarly reflected in the Observed Features index, demonstrating alterations along the fermentation time points.

3.5.5. Beta-Diversity Analysis

Beta (β)-diversity of microbial communities illustrates the relative differences in species composition between habitats, being further used to calculate UniFrac, which relies on a phylogenetic tree where each branch reflects evolutionary relationships between species or Operational Taxonomic Units (OTUs). UniFrac comes in two forms: unweighted and weighted. Unweighted UniFrac focuses solely on the presence or absence of taxa, measuring the fraction of unique evolutionary lineages found in one sample but not in another. In contrast, weighted UniFrac incorporates both phylogenetic relationships and the relative abundance of taxa, offering a more nuanced understanding of community differences by considering both the presence and abundance of species. Several tests were performed to understand the relative differences in species composition deeply. The Kruskal-Wallis test was applied to the unweighted and weighted UniFrac data. Unweighted UniFrac results (Figure 3.8) reveal significant differences in microbial community composition between several groups. Notably, FI shows a significant difference compared to both CGI and κ-CGN ($p < 0.05$). CSGI displays a marked difference in species composition compared to ι-CGN. NC also exhibits significant differences from CMC, κ-CGN, and CSGI ($p < 0.05$).

Furthermore, there is a notable difference between ι-CGN and κ-CGN among the samples. The marked difference between κ-CGN and ι-CGN may arise from the substrates' differing complexities. Since ι-CGN, being less complex, it may be more readily fermented by a broader range of bacteria, thereby facilitating a more diverse microbial community. Moreover, studies have indicated that easily fermentable polysaccharides enhance microbial richness and diversity, contributing to healthier gut microbiota profiles (Matzaras et al., 2022). In contrast,

more complex polysaccharides may not be as effectively utilized, potentially leading to lower diversity if fewer bacterial species can metabolize them.

Regarding the Weighted UniFrac results, several significant differences were observed. The FI sample displayed significant differences from all other samples ($p < 0.05$). The FOS sample showed significant differences when compared to NC and CMC ($p < 0.05$), while the CSGI sample differed significantly from NC, ι-CGN, and CMC ($p < 0.05$). No significant differences were observed among the remaining sample groups. The significant differences in microbial community structure observed in the FI, FOS, and CSGI samples suggest that each treatment distinctly impacts microbial composition (de Carvalho et al., 2021; Roldán Ahumada & Avendaño Garrido, 2019). In contrast, the lack of significant differences among the remaining groups suggests a shared microbial profile, which could be observed in section 3.5.3.

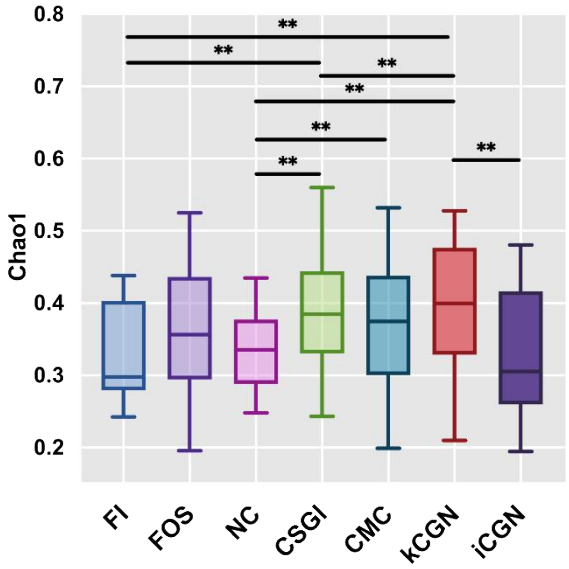

Figure 3.8 - Boxplot for the beta (β)-diversity of the Unweighted Data with the Chao1 index, of samples with FI, FOS, NC, CSGI, ι-CGN, κ-CGN and CMC, applying the Kruskal-Wallis test. Significant differences were obtained ($p < 0.05$).

The Principal Coordinates Analysis (PCoA) based on Weighted and Unweighted UniFrac distances at the genus level visually represents beta diversity among microbial communities, illustrating the dissimilarity between samples.

Figure 3.9a and 3.9b depict the PCoA plot by Weighted and Unweighted UniFrac.The PCoA plot based on weighted UniFrac (Figure 3.9a) reveals minimal community differences among the digested samples and between these samples and the negative control. In contrast, the FOS and FI groups form distinct clusters, which is expected given the diversity already observed in

sections 3.5.3 and 3.5.4 The PCoA1 axis accounts for 47.41% of the variation, while the PCoA2 axis explains 15.63%, indicating that these two axes capture a substantial portion of the variability in the data. For unweighted UniFrac (Figure 3.9b), the PCoA1 axis explains 30.05% of the variation, and the PCoA2 axis explains 11.1%. together, these two axes account over 41% of the total variation, which is substantial. The plot shows a clear separation of the FOS and FI groups from the other samples, along with a distinct separation of the NC group from the ι-CGN, κ-CGN, and CMC samples. However among the digested samples, no notable differences are observed.

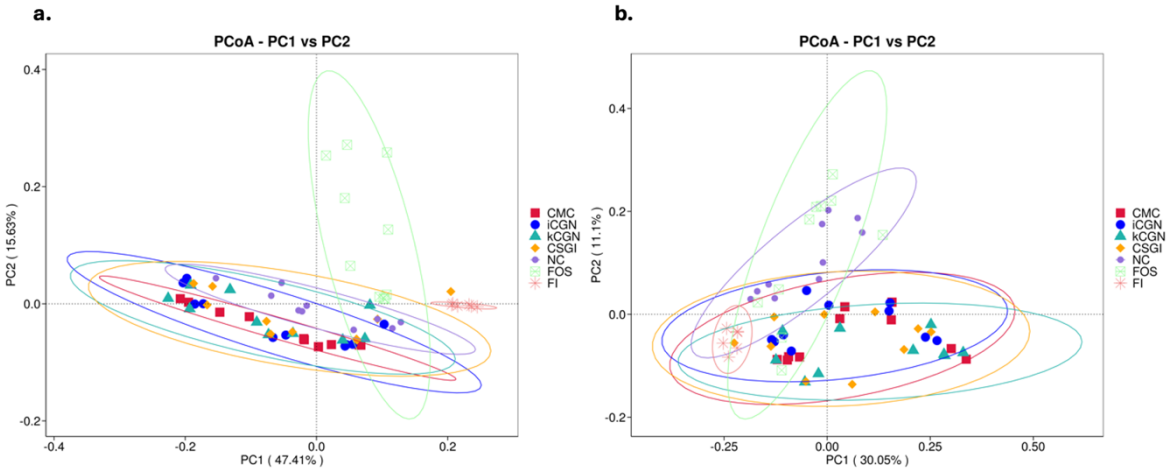

Figure 3.9 - PCoA plot comparison by Weighted (a) and Unweighted (b) UniFrac, of the fermentation samples treated with FI, FOS, NC, ι-CGN and κ-CGN, and CMC.

The UPGMA cluster tree, based on Unweighted UniFrac distances, offers a hierarchical view of the similarities and differences in microbial community composition across samples. The dendrogram in Figure 3.10 visually illustrates the relationships between the treatment groups (along selected fermentation time-points), with shorter branch lengths reflecting greater similarity among microbial communities.

As observed, the FOS group samples cluster closely at the start and end of the fermentation process, suggesting a high degree of similarity among samples within specific time intervals. However, differences are shown between the beginning and end of the fermentation. The CMC, ι-CGN, κ-CGN, and NC groups exhibited similarities within the first 24 h, but later time points show increasing divergence, indicating changes within the same sample over time. Digested samples form distinct clusters at the 72 h and 120 h time points, highlighting the similarity

between these samples at those stages, with the clusters positioned closely. CMC, ι-CGN, and κ-CGN samples at 168 h form a cluster, joined by κ-CGN at 120 h, while showing a clear separation from the CSGI and NC samples at 168 h, underscoring the differences between these groups and the controls at the end of the fermentation. NC samples form a tight cluster after 72 h, indicating substantial similarity across these later time points. In contrast, while the control samples were taken at time 0 h cluster closely with CSGI at 168 h, signifying a high degree of similarity between these two sample sets at these specific time points.

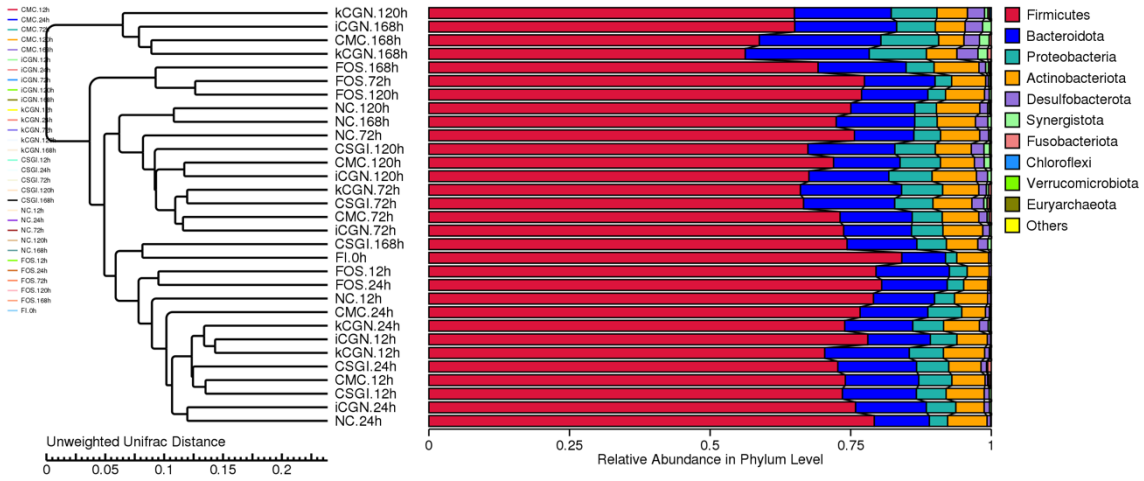

Figure 3.10 - UPGMA cluster tree based on the Unweighted UniFrac distance of the fermentation samples treated with FI, FOS, NC, ι-CGN, κ-CGN, and CMC.

3.5.6. Statistical Tests

Linear Discriminant Analysis Effect Size (LEfSe) is a powerful statistical tool to identify biomarkers and highlight the differences between microbial communities. The histogram of LDA scores highlights the most distinctive taxa associated with each treatment group.

When comparing the samples to the negative control (Figure 3.11), the absence of ι-CGN suggests that, within the limits of this conservative analysis, its impact on the microbiota may be either subtler or less pronounced than other treatments, or it may influence taxa at levels below the detection threshold. This could be attributed to various factors, such as the nature of ι-CGNs' interaction with the microbiota or its overall effect on the gut environment.

It is possible to observe the most abundant genera and phyla in each sample. For CMC, the most predominant phylum is *Proteobacteria*.

In comparison to the controls, it is evident that *Firmicutes* primarily dominate the NC, while the CSGI-treated samples show significant representation from both *Proteobacteria* and *Firmicutes*. The primary differences are observed at the genus level; CMC exhibits higher abundances of *Sutterella* and *Enterobacteriaceae*, while the NC displays greater levels of *Oscillobacter* and *Peptoniphilus*, particularly within the *Lachnospiraceae* family, and CSGI presents abundance in *Dorea*, *Lachnoclostridium*, and *Morganella*. In contrast, for ι-CGN and κ-CGN, the dominant phylum remains *Firmicutes*, similar to the NC. However, κ-CGN presents distinct genus-level variations, showcasing notable abundances of *Morganella*, *Desulfovibrio*, *Acidaminococcus*, and members of *Enterobacteriaceae*. Samples treated with FOS exhibited a distinct abundance of bacteria belonging to the *Firmicutes* phylum, which play a crucial role in fermentation, SCFAs production, and the maintenance of a balanced gut microbiome—factors essential for overall health and well-being. In contrast, the bacterial composition in digested samples is strongly associated with inflammatory diseases, as discussed in section 3.2.1. Conversely, the bacteria in both the NC and treatments are recognized for their capacity to ferment dietary fibers, particularly resistant starches, thereby generating beneficial SCFAs.

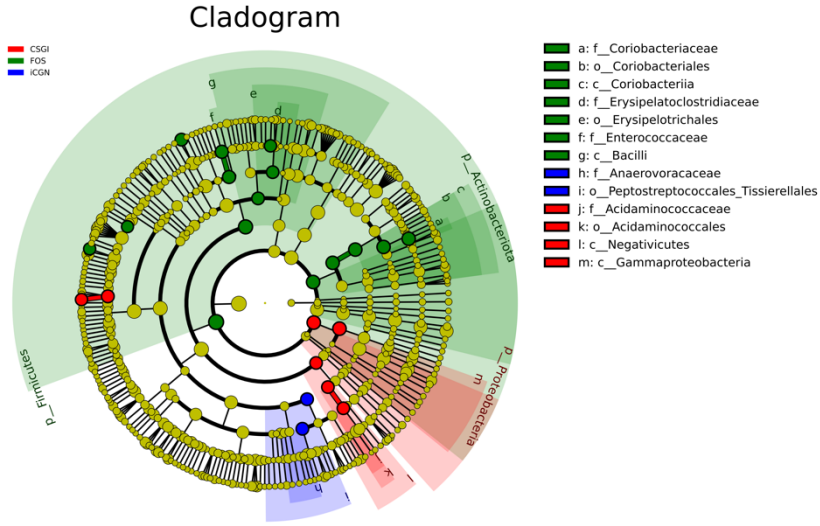

Figure 3.11 - Cladogram representation for compositional analysis of the fermentation samples treated with FI, FOS, NC, ι-CGN, κ-CGN, and CMC.

The Analysis of Similarity (ANOSIM) is a statistical test used to assess whether significant differences exist between two or more groups based on their composition or structure. By utilizing a distance or dissimilarity matrix, such as UniFrac, ANOSIM measures the degree of dissimilarity between samples. The test produces an R-value ranging from -1 to 1, where a value

closer to 1 indicates greater dissimilarity between groups, with more variation occurring between them than within each group, while closer to 0 indicates that there is little to no linear relationship between the group, and closer to -1, indicating an inverse relationship, whereas one group increases in a particular characteristic, the other decreases (Chang et al., 2011).

Additionally, the test provides a *p*-value to indicate the statistical significance of these differences (typically $p < 0.05$). The results are summarized in Table 3.1. It was observed that FOS shows significant differences when compared to ι-CGN, κ-CGN, CMC, CSGI, NC, and FI ($p < 0.05$; R≈1). Similarly, FI differs significantly from ι-CGN, κ-CGN, CMC, NC, and CSGI ($p < 0.05$; R≈1). The NC group also presents significant differences from ι-CGN, κ-CGN, and CSGI ($p < 0.05$; R≈1). However, CSGI does not exhibit any significant differences compared to samples treated with food additives, and no significant differences were observed between the food additives.

Table 3.1 - ANOISM Assessment representation. Significant differences were obtained ($p < 0.05$).

Group	R-value	p-value
CMC-iCGN	0.00689	0.321
CMC-kCGN	-0.00367	0.385
CMC-CSGI	-0.00456	0.368
CMC-NC	0.31511	0.011
CMC-FOS	0.58844	0.001
CMC-FI	0.98	0.001
iCGN-kCGN	-0.00711	0.334
iCGN-CSGI	-0.02489	0.518
iCGN-NC	0.17889	0.041
iCGN-FOS	0.50333	0.001
iCGN-FI	0.81639	0.001
kCGN-CSGI	-0.07833	0.976
kCGN-NC	0.22778	0.021
kCGN-FOS	0.56722	0.001
kCGN-FI	0.885	0.001
CSGI-NC	0.15044	0.039
CSGI-FOS	0.42	0.001
CSGI-FI	0.61889	0.003
NC-FOS	0.44367	0.001
NC-FI	0.86111	0.001
FOS-FI	0.42667	0.007

3.5.7. Functional Prediction

Tax4Fun is a specialized tool designed to predict the functional potential of microbial communities based on 16S rRNA gene sequencing data. It enables analyzing how microbial communities contribute to essential functions like nutrient cycling, SCFAs production, and pathogen resistance, offering valuable insights into how microbial dynamics impact health and disease. This study focused on the fermentation of FOS, κ-CGN, ι-CGN, and CMC with appropriate controls to understand their impact on the gut microbial community at both general (Level 1) and specific (Level 2 and Level 3) functional levels.

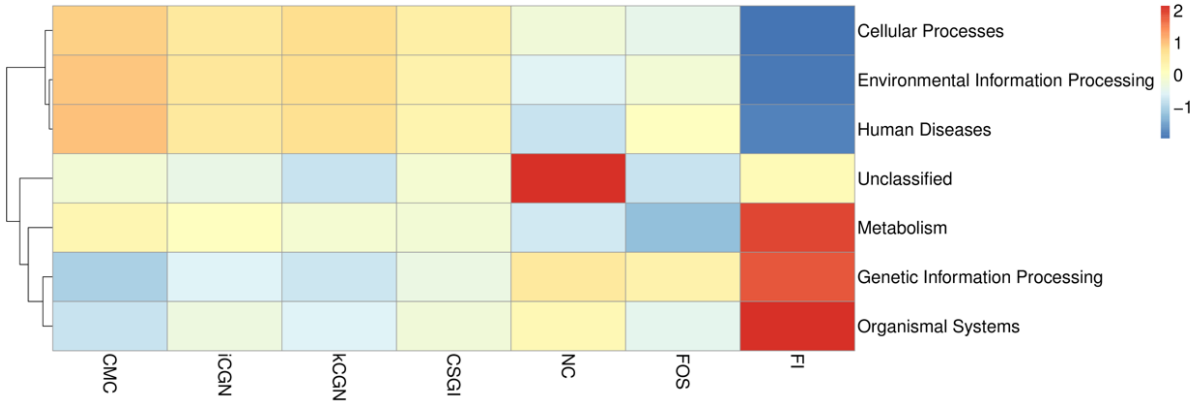

Figure 3.12 - Heatmap representation of Level 1 KEGG Pathways in with FI, FOS, NC, ι-CGN, κ-CGN, and CMC throughout 192 h of *in vitro* fermentation.

At the general level (Level 1 KEGG Pathways; Figure 3.12), it was observed that the FI sample exhibited high metabolic activity prior to fermentation, indicative of a metabolically versatile microbial community. In contrast, samples treated with CMC, κ-CGN, and ι-CGN demonstrated lower metabolic activity, suggesting that these food additives were associated with dysbiosis throughout the fermentation process, leading to a less versatile microbial community. This could be due to the low abundance of *Firmicutes*, resulting in a reduced capacity for the microbial community to metabolize certain complex substrates (Hassan et al., 2022).

FOS also demonstrated significant differences from the other samples, showing enhanced genetic information processing pathways relative to ι-CGN and κ-CGN ($p < 0.05$). Specifically, FOS fermentation led to a significant increase in pathways related to DNA replication, transcription, and repair ($p < 0.05$). This suggests that FOS stimulates microbial proliferation and supports genomic maintenance by serving as a fermentable energy source. This enhanced

microbial growth can indirectly support genomic maintenance, as increased metabolic activity promotes DNA replication, transcription, and repair (Mahalak et al., 2023).

Furthermore, pathways associated with human diseases were significantly reduced in the FOS fermentation compared to CMC ($p = 0.0006$), underscoring the potential health benefits of FOS by potentially inhibiting pathogenic bacterial functions (Bhadra et al., 2022; Mahalak et al., 2023b). While CMC itself is not inherently pathogenic, its influence on gut microbiota and the gut environment can create conditions that favor dysbiosis, decreasing the abundance of *Firmicutes* (Section 3.5.1.), and the growth of *Enterobacteriaceae* (Chassaing et al., 2015), leading to an increase in harmful microbial functions, including those related to human diseases.

At a more detailed functional level, based on Level 2 KEGG pathways (Figure 1 in Appendixes), FOS fermentation significantly enhanced carbohydrate metabolism pathways ($p = 0.001$) compared to CMC, κ-CGN, and ι-CGN. This finding is consistent with FOS's established prebiotic properties, serving as a substrate for beneficial bacteria such as *Bifidobacteria* and *Lactobacilli*, which ferment FOS to produce SCFAs that promote host health (Bhadra et al., 2022), which is observed in section 3.1.

FOS samples present significantly higher energy metabolism ($p < 0.05$), indicating that the organisms present are highly capable of efficiently generating and utilizing energy, which is not the case for CMC, κ-CGN, and ι-CGN. Lipid metabolism abilities, encompassing that the microorganisms within the sample are highly efficient at breaking down, processing, and utilizing lipids as a source of energy or for other biological functions, is also significantly higher at FOS samples when compared to CMC, κ-CGN, and ι -CGN ($p < 0.05$). Research suggests that κ-CGN can inhibit lipid digestion and absorption, reducing lipid bioavailability. Similarly, CMC has been shown to influence lipid metabolism. Due to its emulsifying properties, CMC may interfere with normal lipid digestion processes, potentially affecting lipid absorption and altering metabolic outcomes (Huang et al., 2023; Ma et al., 2017). The microorganisms involved in these additives fermentation, such as in the *Cloacibacillus, Sutterella,* and *Bacteroides* genus (Section 3.5.1.), are more specialized in breaking down carbohydrates rather than lipids, as their primary metabolic focus tends to be on fermenting polysaccharides and other complex carbohydrates.

Translation pathways, encompassing ribosome biogenesis and protein synthesis, were elevated in FOS fermentations ($p < 0.001$), suggesting enhanced protein production necessary for microbial proliferation and metabolic functions. FOS fermentation promotes the production of SCFAs, which serve as energy sources for the host and stimulate various metabolic processes within the microbial community, including those related to protein synthesis and cell growth.

In contrast, CMC and CGNs fermentations did not significantly enhance these pathways. The limited fermentability of CGNs is reflected in the minimal changes observed in carbohydrate metabolism and genetic information processing. CMC showed some moderate effects but did not reach the levels induced by FOS. CMC has been associated with metabolic changes that enhance the synthesis of proteins and ribosomal components, promoting cellular growth and proliferation. Conversely, when exposed to these compounds, CGNs' effects may not have the same impact on these translation pathways, suggesting a potentially different metabolic profile in microbial communities. These findings suggest that while the gut microbiota may partially utilize CMC and CGNs, they do not substantially promote microbial growth or metabolic activity.

Translation pathways, which include ribosome biogenesis and protein synthesis, were significantly elevated during fermentations involving FOS ($p < 0.001$). This increase suggests a heightened production of proteins necessary for microbial proliferation and various metabolic functions. FOS fermentation promotes the production of SCFAs, which serve as energy sources for the host and stimulate multiple metabolic processes within the microbial community, particularly those related to protein synthesis and cellular growth (Bhadra et al., 2022).

In contrast, fermentations involving CMC and CGNs did not yield significant enhancements in these pathways. The limited fermentability of CGNs is observed in the minimal alterations in carbohydrate metabolism and genetic information processing. While CMC exhibited moderate effects on metabolic activity, these did not approach the levels induced by FOS. Specifically, CMC has been linked to metabolic changes that enhance the synthesis of proteins and ribosomal components, thereby promoting cellular growth and proliferation. On the other hand, CGN appears to have a lesser impact on translation pathways, indicating a potentially distinct metabolic profile in microbial communities exposed to these substances (Rahman et al., 2021; Udayakumar et al., 2024). These findings imply that, although the gut microbiota may partially utilize CMC and CGNs, they do not significantly enhance microbial growth or metabolic activity compared to FOS.

As for specific metabolic pathways (Level 3 KEGG Pathways; Figure 2 in Appendixes), FOS fermentation resulted in significant upregulation of glycolysis/gluconeogenesis pathways ($p < 0.05$), highlighting the enhanced energy production from carbohydrate fermentation. In the FOS condition, the amino acid metabolism pathways, particularly those involving alanine, aspartate, and glutamate, were also significantly increased ($p = 0.0021$). This suggests that FOS may support carbohydrate fermentation and protein and amino acid utilization, contributing to a more diverse metabolic profile (Mahalak et al., 2023b). FOS samples also presented elevated

galactose, fructose, and mannose metabolism abilities compared to CMC, κ-CGN, and ι -CGN ($p < 0.05$). Research demonstrates that the fermentation process significantly enhances the activity of metabolic pathways involved in utilizing galactose, fructose, and mannose. This enhancement contributes to overall improvements in gut health and metabolic functions. These findings underscore the importance of FOS as a prebiotic, fostering beneficial microbial communities that facilitate the efficient metabolism of these sugars (Dominguez et al., 2014; Le Bourgot et al., 2018).

In short, the varying effects of different dietary substrates underscore their distinct capabilities to modulate gut microbiota function. CMC demonstrated moderate effects on microbial functions, although not as pronounced as those observed with FOS. Nevertheless, CMC did not significantly enhance core metabolic pathways, indicating its limited fermentability and low production of SCFAs, as observed in section 3.4.

Conversely, CGNs, including ι-CGN and κ-CGN, exhibited minimal impacts on microbial metabolic functions. The observed increases in environmental information processing and cellular processes during CGNs fermentations imply that these substrates may impose challenges on the microbiota, potentially inducing stress responses or necessitating additional energy for adaptation (Bellanco et al., 2024; Kimilu et al., 2024). The lack of significant enhancements in carbohydrate metabolism and genetic information processing suggests that gut microbiota do not efficiently utilize CGNs (Mostafavi Abdolmaleky & Zhou, 2024; W. Wu et al., 2020).

CHAPTER IV

4. Conclusions

This thesis analyzes the interactions between select food additives— ι-CGN, κ-CGN, CMC— and the intestinal microbiota, focusing on their effects on microbial community composition and SCFAs production. The findings reveal that the structural complexity of these additives profoundly influences their fermentability, microbial community adaptation, and SCFAs synthesis. Due to its simpler structure, gut microbes rapidly access and metabolize CMC, resulting in acetic acid production, but limited contributions to propionic and butyric acid yields. In contrast, the structurally complex and sulfated CGNs, ι-CGN and κ-CGN, demonstrated slower and more sustained fermentation patterns, likely due to the adaptive requirements of the microbial community. These CGNs ultimately promote a more balanced SCFAs profile, emphasizing butyric acid production, consistent with the metabolic needs of specialized gut bacteria that utilize complex polysaccharides. Still, it was verified that there was an overall low production of SCFAs.

Moreover, this study highlights significant shifts in the *Firmicutes*-to-*Bacteroidota* ratio and changes in the relative abundance of *Proteobacteria*, *Actinobacteria*, and *Synergistota*. These microbial shifts, in tandem with pH alterations observed during fermentation, suggest that additive-induced changes to gut microbiota can affect microbial dynamics and SCFAs synthesis. The MW analysis supports the conclusion that structural and molecular characteristics of ι-CGN, κ-CGN, and CMC directly impact their fermentative breakdown, substrate availability, and microbial metabolic pathways. This study, therefore, suggests that food additives like CMC and CGNs may meaningfully alter gut microbial composition and activity, with potentially harmful implications for long-term gut health. Regardless, this study also revealed that the simulated digestion conditions can influence the microbiota's fermentation capacity and SCFAs output, underscoring the importance of simulating realistic digestive environments *in vitro* and further research to understand its impact deeply.

CHAPTER V

5. Future Perspectives

Given the limited research on the effects of food additives like CGN and CMC on microbiota dysbiosis, particularly regarding their widespread use in processed foods, the long-term impacts of chronic exposure on human health remain uncertain. Further studies on long-term consumption are essential to thoroughly investigate how these additives influence gut microbiota composition and functionality, as evidence suggests that they may contribute to shifts in microbial populations and promote dysbiosis.

For this, exploring the mutagenic, anti-mutagenic, and cytotoxic effects, as well as the impact on the integrity of the epithelial barrier promoted by these additives, would be valuable. Assessing their potential mutagenicity and cytotoxicity could provide a clearer understanding of any long-term health risks associated with chronic exposure, offering essential insights for risk assessment in dietary use.

Moreover, examining the effects of these additives within more complex food matrices would simulate real-world consumption conditions more closely, as CMC and CGNs are typically consumed in food rather than in isolation. Understanding their behavior within a food matrix could provide a more accurate assessment of their influence on gut microbiota, SCFAs production, and digestion. Such studies could also reveal any interactions between these additives and other food components, which may alter microbial metabolism or affect the bioavailability of nutrients.

Additionally, enhancing the protocols for sugar detection following degradation is essential. By incorporating a broader range of standards, future studies could enable a more accurate qualitative analysis of the breakdown sugars that were currently undetectable.

6. References

Abiega-Franyutti, P., & Freyre-Fonseca, V. (2021a). Chronic consumption of food-additives lead to changes via microbiota gut-brain axis. In *Toxicology* (Vol. 464). Elsevier Ireland Ltd. https://doi.org/10.1016/j.tox.2021.153001

Abiega-Franyutti, P., & Freyre-Fonseca, V. (2021b). Chronic consumption of food-additives lead to changes via microbiota gut-brain axis. In *Toxicology* (Vol. 464). Elsevier Ireland Ltd. https://doi.org/10.1016/j.tox.2021.153001

Adak, A., & Khan, M. R. (2019). An insight into gut microbiota and its functionalities. In *Cellular and Molecular Life Sciences* (Vol. 76, Issue 3, pp. 473–493). Birkhauser Verlag AG. https://doi.org/10.1007/s00018-018-2943-4

Bä ckhed, F., Ding, H., Wang, T., Hooper, L. V, Young Koh, G., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). *The gut microbiota as an environmental factor that regulates fat storage*. www.pnas.orgcgidoi10.1073pnas.0407076101

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences*, *101*(44), 15718–15723. https://doi.org/10.1073/pnas.0407076101

Baran, A., Sulukan, E., Türkoğlu, M., Ghosigharehagaji, A., Yildirim, S., Kankaynar, M., Bolat, I., Kaya, M., Topal, A., & Ceyhun, S. B. (2020). Is sodium carboxymethyl cellulose (CMC) really completely innocent? It may be triggering obesity. *International Journal of Biological Macromolecules*, *163*, 2465–2473. https://doi.org/10.1016/j.ijbiomac.2020.09.169

Belkaid, Y., & Hand, T. W. (2014). Role of the microbiota in immunity and inflammation. In *Cell* (Vol. 157, Issue 1, pp. 121–141). Elsevier B.V. https://doi.org/10.1016/j.cell.2014.03.011

Bellanco, A., Félix, J., Díaz Del Cerro, E., Martínez Cuesta, M. C., De la Fuente, M., & Requena, T. (2024). Influence of consumption of the food additive carrageenan on the gut microbiota and the intestinal homeostasis of mice. *Food and Function*, *15*(12), 6759–6767. https://doi.org/10.1039/d4fo01418a

Benchabane, A., & Bekkour, K. (2008). Rheological properties of carboxymethyl cellulose (CMC) solutions. *Colloid and Polymer Science*, *286*(10), 1173–1180. https://doi.org/10.1007/s00396-008-1882-2

Benitz, K.-F., Golberg, L., & Couuton, F. (1973). Intestinal Effects of Carrageenans in the Rhesus Monkey (Macaca mulatta)*. In *Fd Cosmet. Toxicol* (Vol. 11). Pergamon Press.

Bernalier-Donadille, A. (2010). Activités métaboliques du microbiote intestinal humain. *Gastroenterologie Clinique et Biologique*, *34*(SUPPL. 1). https://doi.org/10.1016/S0399-8320(10)70016-6

Bhadra, S., Chettri, D., & Verma, A. K. (2022). Microbes in fructooligosaccharides production. In *Bioresource Technology Reports* (Vol. 20). Elsevier Ltd. https://doi.org/10.1016/j.biteb.2022.101159

Bhattacharyya, S., Shumard, T., Xie, H., Dodda, A., Varady, K. A., Feferman, L., Halline, A. G., Goldstein, J. L., Hanauer, S. B., & Tobacman, J. K. (2017). A randomized trial of the effects of the no-carrageenan diet on ulcerative colitis disease activity. *Nutrition and Healthy Aging*, *4*(2), 181–192. https://doi.org/10.3233/NHA-170023

Borthakur, A., Bhattacharyya, S., Anbazhagan, A. N., Kumar, A., Dudeja, P. K., & Tobacman, J. K. (2012). Prolongation of carrageenan-induced inflammation in human colonic epithelial cells by activation of an NFκB-BCL10 loop. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1822*(8), 1300–1307. https://doi.org/10.1016/j.bbadis.2012.05.001

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019a). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019b). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Campos, D. A., Coscueta, E. R., Vilas-Boas, A. A., Silva, S., Teixeira, J. A., Pastrana, L. M., & Pintado, M. M. (2020a). Impact of functional flours from pineapple by-products on human intestinal microbiota. *Journal of Functional Foods*, *67*. https://doi.org/10.1016/j.jff.2020.103830

Campos, D. A., Coscueta, E. R., Vilas-Boas, A. A., Silva, S., Teixeira, J. A., Pastrana, L. M., & Pintado, M. M. (2020b). Impact of functional flours from pineapple by-products on human intestinal microbiota. *Journal of Functional Foods*, *67*. https://doi.org/10.1016/j.jff.2020.103830

Cao, Y., Liu, H., Qin, N., Ren, X., Zhu, B., & Xia, X. (2020). Impact of food additives on the composition and function of gut microbiota: A review. In *Trends in Food Science and Technology* (Vol. 99, pp. 295–310). Elsevier Ltd. https://doi.org/10.1016/j.tifs.2020.03.006

Carocho, M., Barreiro, M. F., Morales, P., & Ferreira, I. C. F. R. (2014). Adding molecules to food, pros and cons: A review on synthetic and natural food additives. In *Comprehensive Reviews in Food Science and Food Safety* (Vol. 13, Issue 4, pp. 377–399). Blackwell Publishing Inc. https://doi.org/10.1111/1541-4337.12065

Castaner, O., Goday, A., Park, Y. M., Lee, S. H., Magkos, F., Shiow, S. A. T. E., & Schröder, H. (2018). The gut microbiome profile in obesity: A systematic review. *International Journal of Endocrinology*, *2018*. https://doi.org/10.1155/2018/4095789

Center, M. M., Jemal, A., & Ward, E. (2009). International trends in colorectal cancer incidence rates. *Cancer Epidemiology Biomarkers and Prevention*, *18*(6), 1688–1694. https://doi.org/10.1158/1055-9965.EPI-09-0090

Chang, Q., Luan, Y., & Sun, F. (2011). Variance adjusted weighted UniFrac: a powerful beta diversity measure for comparing communities based on phylogeny. *BMC Bioinformatics*, *12*(1), 118. https://doi.org/10.1186/1471-2105-12-118

Chassaing, B., Koren, O., Goodrich, J. K., Poole, A. C., Srinivasan, S., Ley, R. E., & Gewirtz, A. T. (2015). Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature*, *519*(7541), 92–96. https://doi.org/10.1038/nature14232

Chassaing, B., Van De Wiele, T., De Bodt, J., Marzorati, M., & Gewirtz, A. T. (2017). Dietary emulsifiers directly alter human microbiota composition and gene expression ex vivo

potentiating intestinal inflammation. *Gut*, *66*(8), 1414–1427. https://doi.org/10.1136/gutjnl-2016-313099

Chauhan, P. S., & Saxena, A. (2016). Bacterial carrageenases: an overview of production and biotechnological applications. In *3 Biotech* (Vol. 6, Issue 2). Springer Verlag. https://doi.org/10.1007/s13205-016-0461-3

Chen, C., Huang, Q., Fu, X., & Liu, R. H. (2016). In vitro fermentation of mulberry fruit polysaccharides by human fecal inocula and impact on microbiota. *Food & Function*, *7*(11), 4637–4643. https://doi.org/10.1039/C6FO01248E

Chen, Y., Chen, Y., & Zhang, L. (2023). Effect of the Molecular Weight of Carboxymethyl Cellulose on the Flotation of Chlorite. *Materials*, *16*(9), 3356. https://doi.org/10.3390/ma16093356

Cohen, S. M., & Ito, N. (2002). A Critical Review of the Toxicological Effects of Carrageenan and Processed Eucheuma Seaweed on the Gastrointestinal Tract. In *Critical Reviews in Toxicology* (Vol. 32, Issue 5).

Companys, J., Gosalbes, M. J., Pla-Pagà, L., Calderón-Pérez, L., Llauradó, E., Pedret, A., Valls, R. M., Jiménez-Hernández, N., Sandoval-Ramirez, B. A., Del Bas, J. M., Caimari, A., Rubió, L., & Solà, R. (2021). Gut microbiota profile and its association with clinical variables and dietary intake in overweight/obese and lean subjects: A cross-sectional study. *Nutrients*, *13*(6). https://doi.org/10.3390/nu13062032

Conte, F., van Buuringen, N., Voermans, N. C., & Lefeber, D. J. (2021). Galactose in human metabolism, glycosylation and congenital metabolic diseases: Time for a closer look. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1865*(8), 129898. https://doi.org/10.1016/j.bbagen.2021.129898

Costa, E. M., Silva, S., Pereira, C. F., Ribeiro, A. B., Casanova, F., Freixo, R., Pintado, M., & Ramos, Ó. L. (2023). Carboxymethyl Cellulose as a Food Emulsifier: Are Its Days Numbered? *Polymers*, *15*(10). https://doi.org/10.3390/polym15102408

Cummings, J. H., & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. In *Journal of Applied Bacteriology* (Vol. 70).

Davis, C. D. (2016). The Gut Microbiome and Its Role in Obesity. *Nutrition Today*, *51*(4), 167–174. https://doi.org/10.1097/NT.0000000000000167

de Carvalho, N. M., Oliveira, D. L., Saleh, M. A. D., Pintado, M., & Madureira, A. R. (2021). Preservation of human gut microbiota inoculums for in vitro fermentations studies. *Fermentation*, *7*(1). https://doi.org/10.3390/fermentation7010014

Domingo, M. C., Yansouni, C., Gaudreau, C., Lamothe, F., Lévesque, S., Tremblay, C., & Garceau, R. (2015). Cloacibacillus sp., a potential human pathogen associated with bacteremia in Quebec and New Brunswick. *Journal of Clinical Microbiology*, *53*(10), 3380–3383. https://doi.org/10.1128/JCM.01137-15

Dominguez, A. L., Rodrigues, L. R., Lima, N. M., & Teixeira, J. A. (2014). An overview of the recent developments on fructooligosaccharide production and applications. In *Food and Bioprocess Technology* (Vol. 7, Issue 2, pp. 324–337). Springer New York LLC. https://doi.org/10.1007/s11947-013-1221-6

Duncan, S. H., Louis, P., & Flint, H. J. (2004). Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied and Environmental Microbiology*, *70*(10), 5810–5817. https://doi.org/10.1128/AEM.70.10.5810-5817.2004

Fehlbaum, S., Prudence, K., Kieboom, J., Heerikhuisen, M., Van den Broek, T., Schuren, F. H. J., Steinert, R. E., & Raederstorff, D. (2018). In Vitro Fermentation of Selected Prebiotics and Their Effects on the Composition and Activity of the Adult Gut Microbiota. *International Journal of Molecular Sciences*, *19*(10), 3097. https://doi.org/10.3390/ijms19103097

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes*, *3*(4), 289–306. https://doi.org/10.4161/gmic.19897

Flint, H. J., Scott, K. P., Louis, P., & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology & Hepatology*, *9*(10), 577–589. https://doi.org/10.1038/nrgastro.2012.156

Frank, D. N., St Amand, A. L., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). *Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases*. www.pnas.org/cgi/content/full/

Frolova, M. S., Suvorova, I. A., Iablokov, S. N., Petrov, S. N., & Rodionov, D. A. (2022a). Genomic reconstruction of short-chain fatty acid production by the human gut microbiota. *Frontiers in Molecular Biosciences*, *9*. https://doi.org/10.3389/fmolb.2022.949563

Frolova, M. S., Suvorova, I. A., Iablokov, S. N., Petrov, S. N., & Rodionov, D. A. (2022b). Genomic reconstruction of short-chain fatty acid production by the human gut microbiota. *Frontiers in Molecular Biosciences*, *9*. https://doi.org/10.3389/fmolb.2022.949563

Fu, J., Zheng, Y., Gao, Y., & Xu, W. (2022). Dietary Fiber Intake and Gut Microbiota in Human Health. *Microorganisms*, *10*(12). https://doi.org/10.3390/microorganisms10122507

Gao, R., Gao, Z., Huang, L., & Qin, H. (2017). Gut microbiota and colorectal cancer. In *European Journal of Clinical Microbiology and Infectious Diseases* (Vol. 36, Issue 5, pp. 757–769). Springer Verlag. https://doi.org/10.1007/s10096-016-2881-8

Gibson, Y., & Roberfroid, M. B. (1995). Dietary Modulation of the Human Colonie Microbiota: Introducing the Concept of Prebiotics. In *J. Nutr* (Vol. 125). https://academic.oup.com/jn/article-abstract/125/6/1401/4730723

Gomez-Arango, L. F., Barrett, H. L., Wilkinson, S. A., Callaway, L. K., McIntyre, H. D., Morrison, M., & Dekker Nitert, M. (2018). Low dietary fiber intake increases *Collinsella* abundance in the gut microbiota of overweight and obese pregnant women. *Gut Microbes*, *9*(3), 189–201. https://doi.org/10.1080/19490976.2017.1406584

González-Figueredo, C., Alejandro Flores-Estrella, R., & A. Rojas-Rejón, O. (2019). Fermentation: Metabolism, Kinetic Models, and Bioprocessing. In *Current Topics in Biochemical Engineering*. IntechOpen. https://doi.org/10.5772/intechopen.82195

Grasso, P., Gangolli, S. D., Butterworth, K. R., & Wright, M. G. (1975). *STUDIES ON DEGRADED CARRAGEENAN IN RATS AND GUINEA-PIGS* (Vol. 13). XII. Pergamon Press.

Grasso, P., Sharratt, M., Carijanini, F. M. B., & Gangolli, S. D. (1973). Studies on Carrageenan and Large-bowel Ulceration in Mammals. In *Fd Cosn~t. Toxicol* (Vol. 11). Pergamon Press.

Guo, Y., Chen, X., Gong, P., Wang, M., Yao, W., Yang, W., & Chen, F. (2022). *In vitro* digestion and fecal fermentation of *Siraitia grosvenorii* polysaccharide and its impact on human gut microbiota. *Food & Function*, *13*(18), 9443–9458. https://doi.org/10.1039/D2FO01776H

Gurung, M., Li, Z., You, H., Rodrigues, R., Jump, D. B., Morgun, A., & Shulzhenko, N. (2020). Role of gut microbiota in type 2 diabetes pathophysiology. In *EBioMedicine* (Vol. 51). Elsevier B.V. https://doi.org/10.1016/j.ebiom.2019.11.051

Han, D., Yang, Y., Guo, Z., Dai, S., Jiang, M., Zhu, Y., Wang, Y., Yu, Z., Wang, K., Rong, C., & Yu, Y. (2024a). A Review on the Interaction of Acetic Acid Bacteria and Microbes in Food Fermentation: A Microbial Ecology Perspective. In *Foods* (Vol. 13, Issue 16). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/foods13162534

Han, D., Yang, Y., Guo, Z., Dai, S., Jiang, M., Zhu, Y., Wang, Y., Yu, Z., Wang, K., Rong, C., & Yu, Y. (2024b). A Review on the Interaction of Acetic Acid Bacteria and Microbes in Food Fermentation: A Microbial Ecology Perspective. In *Foods* (Vol. 13, Issue 16). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/foods13162534

Harris, H. C., Morrison, D. J., & Edwards, C. A. (2021). Impact of the source of fermentable carbohydrate on SCFA production by human gut microbiota *in vitro* - a systematic scoping review and secondary analysis. *Critical Reviews in Food Science and Nutrition*, *61*(22), 3892–3903. https://doi.org/10.1080/10408398.2020.1809991

Hassan, N. E., El Shebini, S. M., El-Masry, S. A., Ahmed, N. H., Kamal, A. N., Ismail, A. S., Alian, K. M., Mostafa, M. I., Selim, M., & Afify, M. A. S. (2022). Brief overview of dietary intake, some types of gut microbiota, metabolic markers and research opportunities in sample of Egyptian women. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-21056-z

Hawkins, W. W., & Yaphe, W. (n.d.). *CARRAGEENm AS A DIETARY CONSTITUEm FOR THE RAT: FAECAL EXCRETION, NITROGEN ABSORPTION, AND GROWTH1*. www.nrcresearchpress.com

Hoffmann, R. A., Russell, A. L., & Cidley, M. J. (1996). *Gums and Stabilizers f'or the Food Industry B Phillips* (Vol. 137). eds fRL Press.

Hou, H., Chen, D., Zhang, K., Zhang, W., Liu, T., Wang, S., Dai, X., Wang, B., Zhong, W., & Cao, H. (2022). Gut microbiota-derived short-chain fatty acids and colorectal cancer: Ready for clinical translation? In *Cancer Letters* (Vol. 526, pp. 225–235). Elsevier Ireland Ltd. https://doi.org/10.1016/j.canlet.2021.11.027

Huang, Z., Ma, Y., Xie, Y., Zhao, D., & Li, C. (2023). Carrageenan in meat: improvement in lipid metabolism due to Sirtuin1-mediated fatty acid oxidation and inhibited lipid bioavailability. *Food & Function*, *14*(11), 5404–5416. https://doi.org/10.1039/D3FO00906H

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H. H., Earl, A. M., Fitzgerald, M. G., Fulton, R. S., Giglio, M. G., Hallsworth-Pepin, K., Lobos, E. A., Madupu, R., Magrini, V., Martin, J. C., Mitreva, M., Muzny, D. M., Sodergren, E. J., … White, O. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Jabłońska-Ryś, E., Sławińska, A., Skrzypczak, K., & Goral, K. (2022). Dynamics of Changes in pH and the Contents of Free Sugars, Organic Acids and LAB in Button Mushrooms during Controlled Lactic Fermentation. *Foods*, *11*(11). https://doi.org/10.3390/foods11111553

Jacob, A. , M. J. and 28. N. S. H. (2023). Frontmatter. In *The Gut Microbiota in Health and Disease*. Wiley. https://doi.org/10.1002/9781119904786.fmatter

Jin, Q., & Kirk, M. F. (2018). pH as a primary control in environmental microbiology: 1. thermodynamic perspective. *Frontiers in Environmental Science*, *6*(MAY). https://doi.org/10.3389/fenvs.2018.00021

Karim, Md. R., Iqbal, S., Mohammad, S., Morshed, Md. N., Haque, Md. A., Mathiyalagan, R., Yang, D. C., Kim, Y. J., Song, J. H., & Yang, D. U. (2024). Butyrate's (a short-chain fatty acid) microbial synthesis, absorption, and preventive roles against colorectal and lung cancer. *Archives of Microbiology*, *206*(4), 137. https://doi.org/10.1007/s00203-024-03834-7

Karsa, L. V., Lignini, T. A., Patnick, J., Lambert, R., & Sauvaget, C. (2010). The dimensions of the CRC problem. *Best Practice and Research: Clinical Gastroenterology*, *24*(4), 381–396. https://doi.org/10.1016/j.bpg.2010.06.004

Kimilu, N., Gładyś-Cieszyńska, K., Pieszko, M., Mańkowska-Wierzbicka, D., & Folwarski, M. (2024). Carrageenan in the Diet: Friend or Foe for Inflammatory Bowel Disease? In *Nutrients* (Vol. 16, Issue 11). https://doi.org/10.3390/nu16111780

King, T. C. (2007). Inflammation, Inflammatory Mediators, and Immune-Mediated Disease. In *Elsevier's Integrated Pathology* (pp. 21–57). Elsevier. https://doi.org/10.1016/B978-0-323-04328-1.50008-5

Komisarska, P., Pinyosinwat, A., Saleem, M., & Szczuko, M. (2024a). Carrageenan as a Potential Factor of Inflammatory Bowel Diseases. In *Nutrients* (Vol. 16, Issue 9). https://doi.org/10.3390/nu16091367

Komisarska, P., Pinyosinwat, A., Saleem, M., & Szczuko, M. (2024b). Carrageenan as a Potential Factor of Inflammatory Bowel Diseases. In *Nutrients* (Vol. 16, Issue 9). https://doi.org/10.3390/nu16091367

Krogius-Kurikka, L., Lyra, A., Malinen, E., Aarnikunnas, J., Tuimala, J., Paulin, L., Mäkivuokko, H., Kajander, K., & Palva, A. (2009). Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. *BMC Gastroenterology*, *9*. https://doi.org/10.1186/1471-230X-9-95

Le Bourgot, C., Apper, E., Blat, S., & Respondek, F. (2018). Fructo-oligosaccharides and glucose homeostasis: A systematic review and meta-analysis in animal models. In *Nutrition and Metabolism* (Vol. 15, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s12986-018-0245-3

Leeuwendaal, N. K., Stanton, C., O'Toole, P. W., & Beresford, T. P. (2022). Fermented Foods, Health and the Gut Microbiome. *Nutrients*, *14*(7). https://doi.org/10.3390/nu14071527

Louis, P., & Flint, H. J. (2017a). Formation of propionate and butyrate by the human colonic microbiota. In *Environmental Microbiology* (Vol. 19, Issue 1, pp. 29–41). Blackwell Publishing Ltd. https://doi.org/10.1111/1462-2920.13589

Louis, P., & Flint, H. J. (2017b). Formation of propionate and butyrate by the human colonic microbiota. *Environmental Microbiology*, *19*(1), 29–41. https://doi.org/10.1111/1462-2920.13589

Lovegrove, A., Edwards, C. H., De Noni, I., Patel, H., El, S. N., Grassby, T., Zielke, C., Ulmius, M., Nilsson, L., Butterworth, P. J., Ellis, P. R., & Shewry, P. R. (2017). Role of polysaccharides in food, digestion, and health. *Critical Reviews in Food Science and Nutrition*, *57*(2), 237–253. https://doi.org/10.1080/10408398.2014.939263

Lu, D., Huang, Y., Kong, Y., Tao, T., & Zhu, X. (2020). Gut microecology: Why our microbes could be key to our health. In *Biomedicine and Pharmacotherapy* (Vol. 131). Elsevier Masson s.r.l. https://doi.org/10.1016/j.biopha.2020.110784

Luo, Z., Liu, J., Zhao, P., Jia, T., Li, C., & Chai, B. (2019). Biogeographic Patterns and Assembly Mechanisms of Bacterial Communities Differ Between Habitat Generalists and Specialists Across Elevational Gradients. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00169

Ma, N., Liu, X., Kong, X., Li, S., Jiao, Z., Qin, Z., Dong, P., Yang, Y., & Li, J. (2017). Feces and liver tissue metabonomics studies on the regulatory effect of aspirin eugenol eater in hyperlipidemic rats. *Lipids in Health and Disease*, *16*(1). https://doi.org/10.1186/s12944-017-0633-0

MacArtain, P., Gill, C. I. R., Brooks, M., Campbell, R., & Rowland, I. R. (2007). Nutritional value of edible seaweeds. In *Nutrition Reviews* (Vol. 65, Issue 12, pp. 535–543). Blackwell Publishing Inc. https://doi.org/10.1301/nr.2007.dec.535-543

Macfarlane, G. T., & Macfarlane, S. (2012). Bacteria, Colonic Fermentation, and Gastrointestinal Health. *Journal of AOAC INTERNATIONAL*, *95*(1), 50–60. https://doi.org/10.5740/jaoacint.SGE_Macfarlane

Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society*, *62*(1), 67–72. https://doi.org/10.1079/PNS2002207

Magne, F., Gotteland, M., Gauthier, L., Zazueta, A., Pesoa, S., Navarrete, P., & Balamurugan, R. (2020). The firmicutes/bacteroidetes ratio: A relevant marker of gut dysbiosis in obese patients? In *Nutrients* (Vol. 12, Issue 5). MDPI AG. https://doi.org/10.3390/nu12051474

Mahalak, K. K., Firrman, J., Narrowe, A. B., Hu, W., Jones, S. M., Bittinger, K., Moustafa, A. M., & Liu, L. (2023a). Fructooligosaccharides (FOS) differentially modifies the in vitro gut microbiota in an age-dependent manner. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.1058910

Mahalak, K. K., Firrman, J., Narrowe, A. B., Hu, W., Jones, S. M., Bittinger, K., Moustafa, A. M., & Liu, L. S. (2023b). Fructooligosaccharides (FOS) differentially modifies the in vitro gut microbiota in an age-dependent manner. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.1058910

Marcus, R. (1971). Carrageenan-induced ulceration of the large intestine in the guinea pig. In *Gut* (Vol. 12). http://gut.bmj.com/

Mariat, D., Firmesse, O., Levenez, F., Guimarăes, V. D., Sokol, H., Doré, J., Corthier, G., & Furet, J. P. (2009). The firmicutes/bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiology*, *9*. https://doi.org/10.1186/1471-2180-9-123

Martino, J. V., Van Limbergen, J., & Cahill, L. E. (2017). The role of carrageenan and carboxymethylcellulose in the development of intestinal inflammation. In *Frontiers in Pediatrics* (Vol. 5). Frontiers Media S.A. https://doi.org/10.3389/fped.2017.00096

Maryati, Y., Nuraida, L., & Hariyadi, R. D. (2021). Production of Organic Acid and Short-Chain Fatty Acids (SCFA) from Lactic Acid Bacteria Isolate on Oligosaccharide Media. *Jurnal Kimia Sains Dan Aplikasi*, *24*(6), 213–221. https://doi.org/10.14710/jksa.24.6.213-221

Matzaras, R., Nikopoulou, A., Protonotariou, E., & Christaki, E. (2022). Gut Microbiota Modulation and Prevention of Dysbiosis as an Alternative Approach to Antimicrobial Resistance: A Narrative Review. *The Yale Journal of Biology and Medicine*, *95*(4), 479–494.

McGill, H. C., McMahan, C. A., Wigodsky, H. S., & Sprinz, H. (1977). Carrageenan in formula and infant baboon development. *Gastroenterology*, *73*(3), 512–517. https://doi.org/10.1016/s0016-5085(19)32132-8

McKim, J. M. (2014). Food additive carrageenan: Part I: A critical review of carrageenan in vitro studies, potential pitfalls, and implications for human health and safety. *Critical Reviews in Toxicology*, *44*(3), 211–243. https://doi.org/10.3109/10408444.2013.861797

McKim, J. M., Wilga, P. C., Pregenzer, J. F., & Blakemore, W. R. (2015). The common food additive carrageenan is not a ligand for Toll-Like- Receptor 4 (TLR4) in an HEK293-TLR4 reporter cell-line model. *Food and Chemical Toxicology*, *78*, 153–158. https://doi.org/10.1016/j.fct.2015.01.003

McKim, J. M., Willoughby, J. A., Blakemore, W. R., & Weiner, M. L. (2019). Clarifying the confusion between poligeenan, degraded carrageenan, and carrageenan: A review of the chemistry, nomenclature, and *in vivo* toxicology by the oral route. *Critical Reviews in Food Science and Nutrition*, *59*(19), 3054–3073. https://doi.org/10.1080/10408398.2018.1481822

Mikkelsen, D., Gidley, M. J., & Williams, B. A. (2011). In vitro fermentation of bacterial cellulose composites as model dietary fibers. *Journal of Agricultural and Food Chemistry*, *59*(8), 4025–4032. https://doi.org/10.1021/jf104855e

Mitter, E. K., de Freitas, J. R., & Germida, J. J. (2017). Bacterial Root Microbiome of Plants Growing in Oil Sands Reclamation Covers. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00849

Mondal, M. I. H., & Yeasmin, M. S. (2016). Toxicity study of food-grade carboxymethyl cellulose synthesized from maize husk in Swiss albino mice. *International Journal of Biological Macromolecules*, *92*, 965–971. https://doi.org/10.1016/j.ijbiomac.2016.08.012

Mostafavi Abdolmaleky, H., & Zhou, J. R. (2024). Gut Microbiota Dysbiosis, Oxidative Stress, Inflammation, and Epigenetic Alterations in Metabolic Diseases. In *Antioxidants* (Vol. 13, Issue 8). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/antiox13080985

Nagendra, H. (2002). Opposite trends in response for the Shannon and Simpson indices of landscape diversity. *Applied Geography*, *22*(2), 175–186. https://doi.org/10.1016/S0143-6228(02)00002-4

Naimi, S., Viennois, E., Gewirtz, A. T., & Chassaing, B. (2021). Direct impact of commonly used dietary emulsifiers on human gut microbiota. *Microbiome*, *9*(1). https://doi.org/10.1186/s40168-020-00996-6

Necas, J., & Bartosikova, L. (n.d.-a). Carrageenan: a review. In *Veterinarni Medicina* (Vol. 58, Issue 4).

Necas, J., & Bartosikova, L. (n.d.-b). Carrageenan: a review. In *Veterinarni Medicina* (Vol. 58, Issue 4).

Nilson et al. (n.d.). nilson1959. *1959*.

Ojo, O., Ojo, O. O., Zand, N., & Wang, X. (2021a). The effect of dietary fibre on gut microbiota, lipid profile, and inflammatory markers in patients with type 2 diabetes: A systematic review and meta-analysis of randomised controlled trials. In *Nutrients* (Vol. 13, Issue 6). MDPI AG. https://doi.org/10.3390/nu13061805

Ojo, O., Ojo, O. O., Zand, N., & Wang, X. (2021b). The effect of dietary fibre on gut microbiota, lipid profile, and inflammatory markers in patients with type 2 diabetes: A systematic review and meta-analysis of randomised controlled trials. In *Nutrients* (Vol. 13, Issue 6). MDPI AG. https://doi.org/10.3390/nu13061805

Portincasa, P., Bonfrate, L., Vacca, M., De Angelis, M., Farella, I., Lanza, E., Khalil, M., Wang, D. Q.-H., Sperandio, M., & Di Ciaula, A. (2022). Gut Microbiota and Short Chain Fatty Acids: Implications in Glucose Homeostasis. *International Journal of Molecular Sciences*, *23*(3), 1105. https://doi.org/10.3390/ijms23031105

Poulsen, E. (1973). Short-term Peroral Toxicity of Undegraded Carrageenan in Pigs. In *Fd Cosmet. ToxicoL* (Vol. 11). Pergamon Press.

Quaglio, A. E. V., Grillo, T. G., De Oliveira, E. C. S., Di Stasi, L. C., & Sassaki, L. Y. (2022). Gut microbiota, inflammatory bowel disease and colorectal cancer. In *World Journal of*

Gastroenterology (Vol. 28, Issue 30, pp. 4053–4060). Baishideng Publishing Group Inc. https://doi.org/10.3748/wjg.v28.i30.4053

Qureshi, N., Liu, S., & Saha, B. C. (2022). Butyric Acid Production by Fermentation: Employing Potential of the Novel Clostridium tyrobutyricum Strain NRRL 67062. *Fermentation*, *8*(10). https://doi.org/10.3390/fermentation8100491

Rahman, M. S., Hasan, M. S., Nitai, A. S., Nam, S., Karmakar, A. K., Ahsan, M. S., Shiddiky, M. J. A., & Ahmed, M. B. (2021). Recent developments of carboxymethyl cellulose. In *Polymers* (Vol. 13, Issue 8). MDPI AG. https://doi.org/10.3390/polym13081345

Rani, M., Rudhziah, S., Ahmad, A., & Mohamed, N. (2014). Biopolymer Electrolyte Based on Derivatives of Cellulose from Kenaf Bast Fiber. *Polymers*, *6*(9), 2371–2385. https://doi.org/10.3390/polym6092371

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C. (2019). What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms*, *7*(1). https://doi.org/10.3390/microorganisms7010014

Roldán Ahumada, J. A., & Avendaño Garrido, M. L. (2019). A Commentary on Diversity Measures UniFrac in Very Small Sample Size. In *Evolutionary Bioinformatics* (Vol. 15). SAGE Publications Ltd. https://doi.org/10.1177/1176934319843515

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., & Matteuzzi, D. (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: A comparative study of pure and fecal cultures. *Applied and Environmental Microbiology*, *71*(10), 6150–6158. https://doi.org/10.1128/AEM.71.10.6150-6158.2005

Rustia, M., Shubik, P., & Patila, K. (n.d.). *LIFESPAN CARCINOGENICITY TESTS WITH NATIVE CARRAGEENAN IN RATS AND HAMSTERS*.

Salsinha, A. S., Cunha, S. A., Machado, M., Rodríguez-Alcalá, L. M., Relvas, J. B., & Pintado, M. (2023). Assessment of the bioaccessibility and bioavailability prediction of omega 3 and conjugated fatty acids by in vitro standardized digestion model (INFOGEST) and cell model. *Food Bioscience*, *53*. https://doi.org/10.1016/j.fbio.2023.102635

Scortichini, S., Boarelli, M. C., Silvi, S., & Fiorini, D. (2020). Development and validation of a GC-FID method for the analysis of short chain fatty acids in rat and human faeces and

in fermentation fluids. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1143*. https://doi.org/10.1016/j.jchromb.2020.121972

Sharma, R., Garg, P., Kumar, P., Bhatia, S. K., & Kulshrestha, S. (2020). Microbial Fermentation and Its Role in Quality Improvement of Fermented Foods. *Fermentation*, *6*(4), 106. https://doi.org/10.3390/fermentation6040106

Silva, Y. P., Bernardi, A., & Frozza, R. L. (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. In *Frontiers in Endocrinology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fendo.2020.00025

Singh, V., Lee, G., Son, H., Koh, H., Kim, E. S., Unno, T., & Shin, J.-H. (2023). Butyrate producers, "The Sentinel of Gut": Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.1103836

Stephen, A. M., Phillips, G. O., & Williams, P. A. (2006). *Food Polysaccharides and Their Applications, Second Edition*.

Stojanov, S., Berlec, A., & Štrukelj, B. (2020). The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. In *Microorganisms* (Vol. 8, Issue 11, pp. 1–16). MDPI AG. https://doi.org/10.3390/microorganisms8111715

Sun, Y., Cui, X., Duan, M., Ai, C., Song, S., & Chen, X. (2019). In vitro fermentation of κ-carrageenan oligosaccharides by human gut microbiota and its inflammatory effect on HT29 cells. *Journal of Functional Foods*, *59*, 80–91. https://doi.org/10.1016/j.jff.2019.05.036

Tobacman, J. K. (2001). Review of Harmful Gastrointestinal Effects of Carrageenan in Animal Experiments. In *Environmental Health Perspectives • VOLUME* (Vol. 109, Issue 10). http://ehpnet1.niehs.nih.gov/docs/2001/109p983-994tobacman/abstract.html

Tomarelli et al. (1974). *tomarelli1974*.

Udayakumar, S., Girigoswami, A., & Girigoswami, K. (2024). Biological Activities of Carrageenan from Red Algae: a Mini Review. In *Current Pharmacology Reports* (Vol. 10, Issue 1, pp. 12–26). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s40495-023-00348-6

Undiandeye, J., Gallegos, D., Bonatelli, M. L., Kleinsteuber, S., Bin-Hudari, M. S., Abdulkadir, N., Stinner, W., & Sträuber, H. (2024). Medium-chain carboxylates production from plant waste: kinetic study and effect of an enriched microbiome. *Biotechnology for Biofuels and Bioproducts*, *17*(1), 79. https://doi.org/10.1186/s13068-024-02528-y

Uno, Y., Omoto, T., Goto, Y., Asai, I., Nakamura, M., & Maitani, T. (2001). Molecular weight distribution of carrageenans studied by a combined gel permeation/inductively coupled plasma (GPC/ICP) method. *Food Additives and Contaminants*, *18*(9), 763–772. https://doi.org/10.1080/02652030117235

Uppakarn, K., Bangpanwimon, K., Hongpattarakere, T., Wanitsuwan, W., & Article, R. (2021). *Comparison of the Human Gut Microbiota between Normal Control Subjects and Patients with Colonic Polyps and Colorectal Cancer*. https://doi.org/10.21203/rs.3.rs-1172479/v1

Viennois, E., Bretin, A., Dubé, P. E., Maue, A. C., Dauriat, C. J. G., Barnich, N., Gewirtz, A. T., & Chassaing, B. (2020). Dietary Emulsifiers Directly Impact Adherent-Invasive E. coli Gene Expression to Drive Chronic Intestinal Inflammation. *Cell Reports*, *33*(1). https://doi.org/10.1016/j.celrep.2020.108229

Wang, Y., Wu, J., Yan, J., Guo, M., Xu, L., Hou, L., & Zou, Q. (2022). Comparative genome analysis of plant ascomycete fungal pathogens with different lifestyles reveals distinctive virulence strategies. *BMC Genomics*, *23*(1), 34. https://doi.org/10.1186/s12864-021-08165-1

Wexler, A. G., & Goodman, A. L. (2017). An insider's perspective: Bacteroides as a window into the microbiome. In *Nature Microbiology* (Vol. 2). Nature Publishing Group. https://doi.org/10.1038/nmicrobiol.2017.26

Williams, P. A., Phillips, G. O., Royal, T., Kennedy, J. F., & Zhang, Z. (2003). Book Review Gums and Stabilisers for the Food Industry 11. In *Carbohydrate Polymers* (Vol. 52). www.elsevier.com/locate/carbpol

Wu, K., Zhao, W., Wang, Q., Yang, X., Zhu, L., Shen, J., Cheng, X., & Wang, J. (2019). The Relative Abundance of Benthic Bacterial Phyla Along a Water-Depth Gradient in a Plateau Lake: Physical, Chemical, and Biotic Drivers. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.01521

Wu, W., Wang, Y., Yi, P., Su, X., Mi, Y., Wu, L., & Tan, Q. (2024). Various steaming durations alter digestion, absorption, and fermentation by human gut microbiota outcomes of

Polygonatum cyrtonema Hua polysaccharides. *Frontiers in Nutrition*, *11*. https://doi.org/10.3389/fnut.2024.1466781

Wu, W., Zhou, J., Chen, J., Han, H., Liu, J., Niu, T., Chen, H., & Wang, F. (2020). *Dietary κ-carrageenan facilitates gut microbiota-mediated intestinal inammation*. https://doi.org/10.21203/rs.3.rs-56671/v1

Wu, W., Zhou, J., Xuan, R., Chen, J., Han, H., Liu, J., Niu, T., Chen, H., & Wang, F. (2022). Dietary κ-carrageenan facilitates gut microbiota-mediated intestinal inflammation. *Carbohydrate Polymers*, *277*. https://doi.org/10.1016/j.carbpol.2021.118830

Wu, Z., Li, M., Qu, L., Zhang, C., & Xie, W. (2024). Metagenomic insights into microbial adaptation to the salinity gradient of a typical short residence-time estuary. *Microbiome*, *12*(1), 115. https://doi.org/10.1186/s40168-024-01817-w

Yu, C., Ahmadi, S., Shen, S., Wu, D., Xiao, H., Ding, T., Liu, D., Ye, X., & Chen, S. (2022). Structure and fermentation characteristics of five polysaccharides sequentially extracted from sugar beet pulp by different methods. *Food Hydrocolloids*, *126*, 107462. https://doi.org/10.1016/j.foodhyd.2021.107462

Zangara, M. T., Ponti, A. K., Miller, N. D., Engelhart, M. J., Ahern, P. P., Sangwan, N., & McDonald, C. (2022). Maltodextrin Consumption Impairs the Intestinal Mucus Barrier and Accelerates Colitis Through Direct Actions on the Epithelium. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.841188

Zhang, D., Jian, Y.-P., Zhang, Y.-N., Li, Y., Gu, L.-T., Sun, H.-H., Liu, M.-D., Zhou, H.-L., Wang, Y.-S., & Xu, Z.-X. (2023). Short-chain fatty acids in diseases. *Cell Communication and Signaling*, *21*(1), 212. https://doi.org/10.1186/s12964-023-01219-9

Zhang, T., Yang, Y., Liang, Y., Jiao, X., & Zhao, C. (2018). Beneficial effect of intestinal fermentation of natural polysaccharides. In *Nutrients* (Vol. 10, Issue 8). MDPI AG. https://doi.org/10.3390/nu10081055

Zhou, X., Qiao, K., Wu, H., & Zhang, Y. (2023). The Impact of Food Additives on the Abundance and Composition of Gut Microbiota. In *Molecules* (Vol. 28, Issue 2). MDPI. https://doi.org/10.3390/molecules28020631

Zhou, Z., Sun, B., Yu, D., & Zhu, C. (2022). Gut Microbiota: An Important Player in Type 2 Diabetes Mellitus. In *Frontiers in Cellular and Infection Microbiology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fcimb.2022.834485

Zhou, Z., Wu, H., Li, D., Zeng, W., Huang, J., & Wu, Z. (2022). Comparison of gut microbiome in the Chinese mud snail (*Cipangopaludina chinensis*) and the invasive golden apple snail (*Pomacea canaliculata*). *PeerJ*, *10*, e13245. https://doi.org/10.7717/peerj.13245

Zuo, T., & Ng, S. C. (2018). The Gut Microbiota in the Pathogenesis and Therapeutics of Inflammatory bowel disease. In *Frontiers in Microbiology* (Vol. 9, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.02247

Zuo, Z., Zheng, M., Xiong, H., Shi, H., Huang, X., & Liu, Y. (2019). Short-chain fatty acid (SCFA) production maximization by modeling thermophilic sludge fermentation. *Environmental Science: Water Research & Technology*, *5*(1), 11–18. https://doi.org/10.1039/C8EW00572A

7. Appendixes

Table 1 - Microorganisms most present on the gut Microbiota **-** Microorganisms most present on the gut Microbiota

Phylum	Class	Order	Family	Genus	Gut Microbiota Species
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*Alistipes finegoldii*
			Bacteroidaceae	*Bacteroides*	*Bacteroides vulgatus* *Bacteroides uniformis* *Bacteroides fragilis*
			Tannerellaceae	*Parabacteroides*	*Parabacteroides distasonis*
				Tannarella	
			Prevotellaceae	*Prevotella*	*Prevotella* spp.
	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Sphingobacterium*	
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Faecalibacterium*	*Faecalibacterium prausnitzii*
				Clostridium	*Clostridium spp.*
			Lachnospiraceae	*Roseburia*	*Roseburia intestinalis*
			Ruminococcaceae	*Ruminococcus*	*Ruminococcus faecis*
	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus reuteri*

SOURCE: Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H. H., Earl, A. M., Fitzgerald, M. G., Fulton, R. S., Giglio, M. G., Hallsworth-Pepin, K., Lobos, E. A., Madupu, R., Magrini, V., Martin, J. C., Mitreva, M., Muzny, D. M., Sodergren, E. J., … White, O. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214.

Table 2 - Pathogenic Microorganisms to the gut Microbiota.

Phylum	Class	Order	Family	Genus	Pathogenic Species
Actinobacteria	Actinomycetia	Actinomycetales	Actinomycetaceae	*Actinomyces*	*Actinomyces odontolyticus* *Actinomyces viscosus*
		Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	*Bifidobacterium dentium*
Apicomplexa	Conoidasida	Eucoccidiorida	Crypto bacterium curtum	*Cryptobacterium*	*Cryptobacterium*
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*Alistipes putredinis*
			Bacteroidaceae	*Bacteroides*	*Bacteroides vulgatus* *Bacteroides thetaiotaomicron* *Bacteroides fragilis*
			Tannerellaceae	*Parabacteroides*	*Parabacteroides distasonis*
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Capnocytophaga*	*Capnocytophaga ochracea*
Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Brucella*	*Brucella ceti*
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*	*Campylobacter curvus* *Campylobacter concisus*
	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Enterobacteriaceae*	*Enterobacter cancerogenus* *Escherichia coli* *Klebsiella pneumoniae* *Escherichia coli* *Shigella dysenteriae* *Shigella boydii* *Shigella sonnei*
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	*Clostridium*	*Clostridium difficile* *Clostridium perfringens*

SOURCE: Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H. H., Earl, A. M., Fitzgerald, M. G., Fulton, R. S., Giglio, M. G., Hallsworth-Pepin, K., Lobos, E. A., Madupu, R., Magrini, V., Martin, J. C., Mitreva, M., Muzny, D. M., Sodergren, E. J., … White, O. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214.

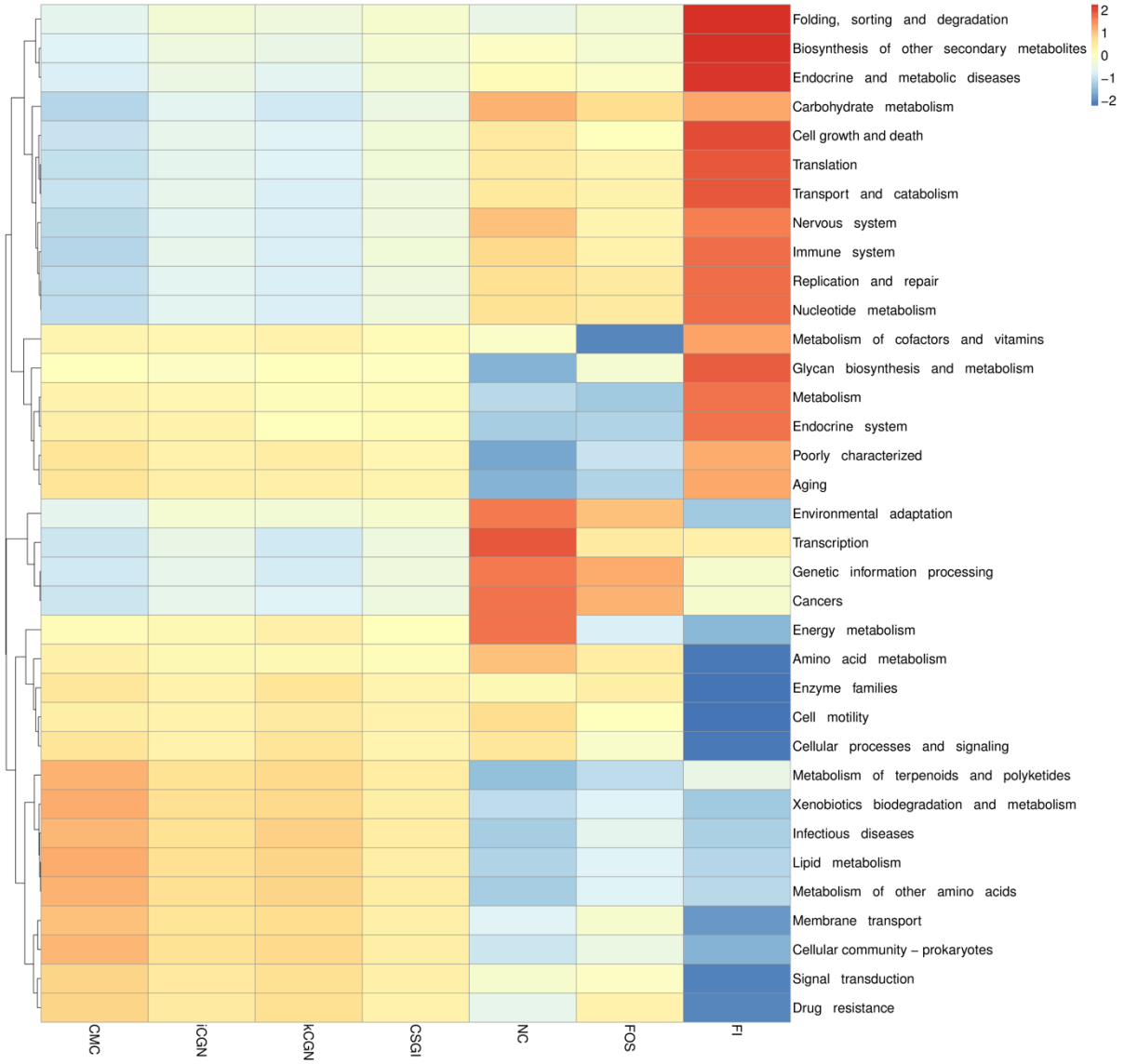

Figure 1 - Heatmap representation of Level 2 KEGG Pathways in with FI, FOS, NC, ι-CGN, κ-CGN, and CMC throughout 192 h of *in vitro* fermentation.

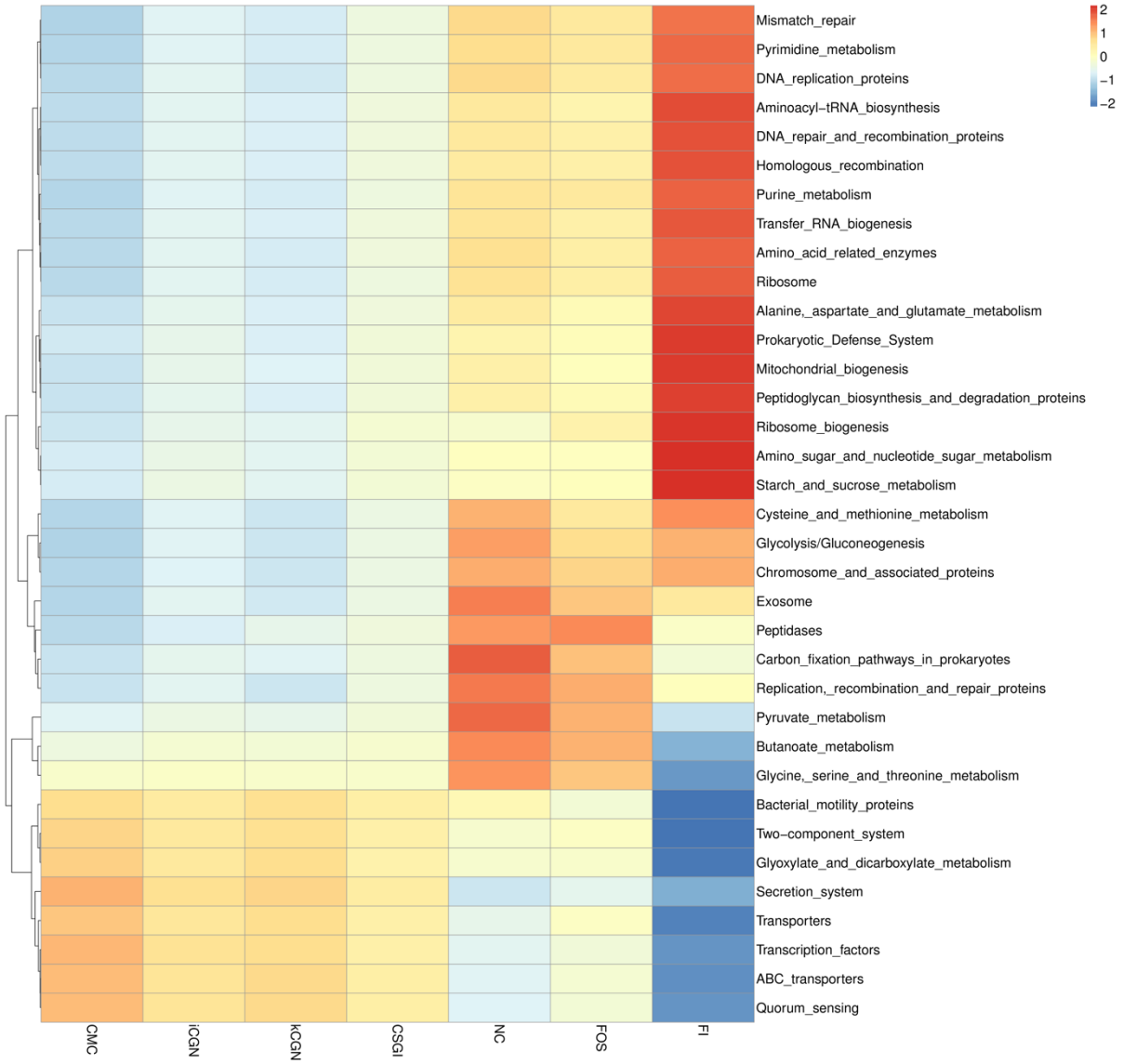

Figure 2 - Heatmap representation of Level 3 KEGG Pathways in with FI, FOS, NC, ι-CGN, κ-CGN, and CMC throughout 192 h of *in vitro* fermentation.