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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Combination of probiotics enhancing butyrogenesis in colonic microbiota model of patients with ulcerative colitis

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Abstract

Administering beneficial bacteria as probiotics to restore the intestinal microbiota and its metabolic functions, such as butyrogenesis, is a promising treatment strategy in ulcerative colitis (UC). This study aimed to investigate the effect of a combination of probiotics, consisting of the lactic acid bacterium *Weizmannia coagulans* SANK70258 and the lactate-utilizing butyrate-producing bacteria *Anaerostipes caccae* or *Clostridium butyricum*, on the colonic environment using an in vitro colonic microbiota culture model with fecal inoculums from seven patients with UC. Co-inoculated *W. coagulans* and *A. caccae* neither inhibited each other's growth nor significantly affected the relative abundance of other bacterial species; however, the growth of *W. coagulans* was significantly inhibited when co-inoculated with *C. butyricum*. The relative abundance of pro-inflammatory bacteria (*Escherichia* sp. and unclassified *Enterobacteriaceae*) and *Bifidobacterium* spp. significantly decreased in *W. coagulans-C. butyricum* co-inoculated cultures. Inoculation with any of the probiotics alone did not increase butyrate production, whereas co-inoculation of *W. coagulans* with *A. caccae* or *C. butyricum* significantly increased the butyrate levels. Overall, the results suggested that *W. coagulans* and lactate-utilizing butyrate-producing bacteria in combination have synergistic effects through cross-feeding and can effectively restore butyrogenesis in the colonic environment of patients with UC.

Key points

- *Effects of probiotics were evaluated using in vitro microbiota model of UC colon.*
- *W. coagulans and lactate-utilizing butyrate producers have synergistic effects.*
- *Co-inoculation of W. coagulans with A. caccae or C. butyricum enhanced butyrogenesis.*

Keywords Probiotics · Butyrogenesis · Ulcerative colitis · Colonic microbiota model · *Weizmannia coagulans* · Lactate-utilizing butyrate-producing bacteria

Introduction

Ulcerative colitis (UC) is a major subtype of chronic inflammatory bowel disease (IBD) (Tie et al. 2023). UC is characterized by dysregulation of the intestinal barrier function and of the immune system (Ramos and Papadakis 2019). It can lead to colorectal cancer and various extraintestinal inflammatory manifestations and complications, requiring expensive treatments and posing a significant burden to human health and the economic activity (Ungaro et al. 2017).

Although the pathogenesis of UC remains uncertain, several studies have shown that dysregulation of the intestinal microbiota, often referred to as dysbiosis, may be associated with the development of this disease (Sartor

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and Wu 2017). Compared to healthy individuals, patients with UC and animal models of experimental colitis have decreased diversity of microbiota and beneficial bacteria (such as the family *Ruminococcaceae*, known as *Clostridium* clusters IV; *Lachnospiraceae*, known as *Clostridium* clusters XIVa; the genera *Lactobacillus* and *Bifidobacterium*) and increased pro-inflammatory bacteria (such as *Escherichia coli* and *Fusobacterium*) (Nishida et al. 2018; Ramos and Papadakis 2019). Particularly noteworthy is the significant reduction in the abundance of major butyrate producers and butyrate concentration in the stools of patients with UC compared to that in healthy subjects (Machiels et al. 2014; Yamada et al. 2019). Among the short-chain fatty acids (SCFAs) produced by intestinal microbial fermentation, butyrate exhibits particularly distinctive properties in the gut, including anticarcinogenic, anti-inflammatory, antioxidant, and neuromodulatory effects and improves the function of colonic barrier (Hamer et al. 2008; Parada Venegas et al. 2019). A recent study reported the effectiveness of sodium butyrate in maintaining remission in patients with UC (Vernero et al. 2020). Therefore, decreased butyrate levels due to decreased butyrate producers may contribute to the etiology of UC (Kumari et al. 2013).

Probiotics have been defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). Previous studies have reported that administering beneficial bacteria as probiotics could reconstruct the balance of interaction between the intestinal microbiome and host immunity, thereby relieving intestinal inflammation and damage (Ganji-Arjenaki and Rafieian-Kopaei 2018). Studies in mouse models of colitis have revealed that the administration of certain probiotics can reduce the disease activity index (DAI) of colitis (Je et al. 2018), limit the recurrence of colitis (Fitzpatrick et al. 2012), alleviate gut microbiome imbalance (Jang et al. 2018), and prevent or delay the development of chronic colitis-associated cancer (Talero et al. 2015). Clinical studies have shown that probiotics can reduce DAI scores in patients with relapsing mild-to-moderate UC who are under treatment with 5-aminosalicylic acid and/or immunosuppressants (Tursi et al. 2010). Probiotics may induce clinical remission in active UC compared to placebo (Kaur et al. 2020) and can effectively suppress relapse in patients with inactive UC (Yoshimatsu et al. 2015).

The spore-forming lactic acid bacterium *Weizmannia coagulans* (synonym *Bacillus coagulans*) is a probiotic microorganism that has been studied for a long time (Cao et al. 2020). Compared to other traditional commercial probiotics, such as *Lactobacillus* and *Bifidobacterium* species, *W. coagulans* has considerably better survival and stability in extremely harsh environments owing to its spore-forming abilities. Thus, it has attracted great interest in recent years, particularly as a probiotic in functional foods (Fares et al. 2015; Hyronimus et al. 2000). *W. coagulans* influences the diversity, composition, and metabolic functions of the intestinal microbiota and has strong modulatory effects on host immune responses (Cao et al. 2020). Several studies have demonstrated the therapeutic and preventive effects for inflammation in colitis model mice using certain *W. coagulans* as probiotics (Fitzpatrick et al. 2012; Liu et al. 2022; Shinde et al. 2019). However, interventional clinical trials using these strains in patients with UC are limited, in part due to difficulties in patient recruitment (Rubin et al. 2021). Therefore, the mechanism of action of probiotic *W. coagulans* in UC would require further elucidation.

In recent years, several studies have been conducted using in vitro models to elucidate the mechanism of action of the probiotic *W. coagulans* (Keller et al. 2019; Maathuis et al. 2010; Sasaki et al. 2020). The Kobe University Human Intestinal Microbiota Model (KUHIMM) is an in vitro human colonic microbiota culture model comprising a single-batch anaerobic fermentation reactor (Sasaki et al. 2018). This model offers several advantages such as avoiding ethical concerns associated with human intervention, reducing cost and time compared to human trials, and allowing for experiments under various conditions. Additionally, it has a capacity to accurately reproduce the structure and diversity of the colonic microbiota and the metabolites in individual human donors including patients with UC from their fecal inoculum (Sasaki et al. 2019), thereby making it useful for evaluating the effects of probiotics in the colonic environment (Shintani et al. 2024). We had previously evaluated the effect of the administration of *W. coagulans* SANK70258 isolated from green malt (Mashita et al. 1964) on the colonic microbiota of healthy subjects and patients with UC using KUHIMM (Sasaki et al. 2020). In both healthy subjects and patients with UC, the administration of *W. coagulans* SANK70258 suppressed the bacteria related to the family *Enterobacteriaceae*, which are pro-inflammatory bacteria enriched in patients with IBD (Duvallet et al. 2017). In addition, in healthy subjects, the relative abundance of bacteria related to the family *Lachnospiraceae* and butyrate concentrations was increased due to the administration of *W. coagulans* SANK70258. Since certain members of *Lachnospiraceae*, such as *Anaerostipes caccae*, can assimilate lactate and produce butyrate (Louis and Flint 2009), the lactate produced by *W. coagulans* SANK70258 was speculated to be metabolized by *Lachnospiraceae* species, thereby contributing to their growth and butyrate production. However, these changes were not observed in KUHIMM inoculated with fecal samples from patients with UC, probably due to the low abundance of *Lachnospiraceae* species. Therefore, in order to elicit the beneficial effects of *W. coagulans* SANK70258 administration in patients with UC, increasing

the relative abundance of *Lachnospiraceae* by additional administration may be an effective strategy. However, to the best of our knowledge, there has been no study examining the effects of the co-administration of *W. coagulans* and butyrate-producing *Lachnospiraceae* species on the colonic environment.

In the present study, we aimed to investigate the possible effects of a combination of probiotics consisting of *W. coagulans* SANK70258 and *A. caccae*, a butyrate-producing *Lachnospiraceae* species, on the colonic environment of patients with UC. Fecal samples from seven patients with UC were individually cultivated in KUHIMM with the probiotics *W. coagulans* SANK70258 and *A. caccae*, both in combination and/or separately, and the microbial composition and concentration of SCFAs after cultivation were analyzed. Additionally, we conducted KUHIMM culture with *Clostridium butyricum*, a lactate-utilizing butyrate-producing bacterium of *Clostridiaceae* (Detman et al. 2019), to investigate the effects of a combination of probiotics consisting of *W. coagulans* SANK70258 and a non-*Lachnospiraceae* butyrate producer.

Materials and methods

Probiotics

W. coagulans SANK70258: Lacris®-S (Mitsubishi-Chemical Foods Corporation, Tokyo, Japan) was inoculated in Gifu anaerobic medium (GAM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at a concentration of 0.01 g/L according to a previously reported protocol (Sasaki et al. 2020). *W. coagulans* SANK70258 was cultivated aerobically at 37 °C and 160 rpm for 17 h in a shaker incubator. *A. caccae* NBRC 114412 and *C. butyricum* ATCC 19398 were inoculated in GAM at 0.01 g/L and cultivated anaerobically at 37 °C and 160 rpm for 17 h in a shaker incubator. After cultivation, the cells were collected by centrifugation at 5,000 rpm for 5 min, resuspended in PBS (Nacalai Tesque, Kyoto, Japan), and used in subsequent experiments.

Fecal samples

Fecal samples were collected from seven patients with UC who received no antibiotic treatment for at least 2 months before sampling, as described in an earlier report (Sasaki et al. 2019). Demographic data and UC characteristics are written down in Supplementary Table S1. Disease activity in patients with UC was assessed using the total Mayo score at the time of fecal sample collection. After sample collection, each fecal sample was stored under anaerobic conditions using a culture tube and used within 24 h.

Operation of the KUHIMM

For fecal sample cultivation, we used a Bio Jr.8 fermenter (ABLE, Tokyo, Japan) as previously described (Sasaki et al. 2018). Briefly, fecal samples (0.5 g) were suspended in 2 mL of PBS (Nacalai Tesque). Each vessel contained GAM broth (100 mL) and was inoculated with 500 µL of fecal suspension or fecal suspension and prebiotics (4×10^7 total cells/mL of each strain or a combination of 4×10^7 total cells/mL of *W. coagulans* and 4×10^7 total cells/mL of *A. caccae* or *C. butyricum*). Each vessel was then cultivated at 37 °C ($n=7$). The cultures were stirred at 200 rpm and continuously ventilated with an anaerobic gas (N_2:CO_2, 80:20) to preserve anaerobic conditions. After 6, 24, and 48 h, culture broths were collected and subsequently analyzed.

DNA extraction

We extracted the genomic DNA from fecal samples and broth from the KUHIMM, following previously described protocols (Sasaki et al. 2018).

Sequencing of 16S rRNA genes

The sequencing V3–V4 hypervariable regions of bacterial 16S rRNA genes were amplified as described previously, and extracted DNA samples served as templates, as described previously (Klindworth et al. 2013). Polymerase chain reaction (PCR) was performed using a Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) for attaching index adapters to the gene sequence, and the PCR amplicons were purified using AMPure XP (Beckman Coulter, Brea, CA, USA) per the manufacturer's protocol. The pooled amplicons of 16S rRNA genes were analyzed by MiSeq (Illumina) following previously described protocols (Callahan et al. 2016; Sasaki et al. 2023). The operational taxonomic units (OTUs) were analyzed using the Silva_138 99% OTU full-length sequence database (https://urldefense.com/v3/__https://www.arb-silva.de/documentation/release-138/__;!!NLFGqXoFfo8MMQ!tEaN_r1eBJixAtaPcmInq6bfol0Q2THpE-c1pTKQAfNBZN-KOQtyfI8LtiesYIx16jCz1pIvGEnHfcnko_7wFNv0gkTY$). Taxonomic metadata was used for α-diversity estimation.

Quantification of 16S rRNA genes

Quantitative real-time PCR to quantify total bacterial 16S rRNA genes copy number was performed as previously described (Sasaki et al. 2023). A primer set targeting all eubacteria (Matsuki et al. 2004; Rinttilä et al. 2004) was used with QuantStudio 3 real-time PCR systems (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Measurement of SCFA and lactate concentrations

The SCFAs (acetate, propionate, and butyrate) and lactate concentrations in the culture broth were measured using HPLC (Shimadzu, Kyoto, Japan), according to a previously reported protocol (Sasaki et al. 2023).

Statistical analyses

All statistical analyses were conducted using the Prism 9 software (GraphPad Software, Inc., San Diego, CA, USA). A p-value < 0.05 was considered statistically significant difference.

Results

Effect of probiotic inoculation on the bacterial species diversity and total eubacterial growth of colonic microbiota in patients with UC as simulated by KUHIMM

Fecal samples from seven patients with UC were individually cultivated anaerobically in KUHIMM with or without probiotics (4×10^7 total cells/mL of each strain or combination of 4×10^7 total cells/mL of *W. coagulans* SANK70258 and 4×10^7 total cells/mL of *A. caccae* NBRC 114412 or *C. butyricum* ATCC 19398) at 37 °C for 48 h. The microbial composition of the original fecal samples and samples cultivated in KUHIMM with or without each probiotic for 6, 24, and 48 h was analyzed by the bacterial 16S rRNA gene. The KUHIMM without probiotic inoculation was used as the control. Sequencing data of 16S rRNA gene and α-diversity values are summarized in Supplementary Table S2. On average, more than 100,000 high-quality reads were obtained for each sample. The bacterial OTU numbers, an indicator of species richness, were lower in the probiotic non-inoculated KUHIMM culture than in the original fecal samples ($p = 0.0156$; Wilcoxon matched-pairs signed-rank test). However, there was no significant difference in the OTU counts between non-probiotic-inoculated KUHIMM culture and the corresponding cultures inoculated with *W. coagulans* (WC: $p = 0.0781$), *A. caccae* (AC: $p = 0.7344$), combination of *W. coagulans* and *A. caccae* (WC, AC: $p > 0.9999$), *C. butyricum* (CB: $p = 0.7188$), and combination of *W. coagulans* and *C. butyricum* (WC, CB: $p = 0.5781$) (Wilcoxon matched-pairs signed-rank test). The Shannon index for species diversity was lower in the non-probiotic KUHIMM culture than in the original fecal sample ($p = 0.0312$; Wilcoxon matched-pairs signed-rank test), while no significant difference was observed in the values between the probiotic-inoculated and non-inoculated KUHIMM cultures (WC: $p = 0.4688$; AC: $p = 0.5781$; WC, AC: $p = 0.8125$; CB: $p = 0.3750$; WC, CB: $p = 0.3750$; Wilcoxon matched-pairs signed-rank test). The Simpson index for species diversity was lower in the probiotic non-inoculated KUHIMM culture than in the original fecal sample ($p = 0.0156$; Wilcoxon matched-pairs signed-rank test); however, no significant difference was observed in the values between the probiotic-inoculated and non-inoculated KUHIMM cultures (WC: $p > 0.9999$; AC: $p = 0.4688$; WC, AC: $p = 0.4688$; CB: $p = 0.5781$; WC, CB: $p = 0.1094$; Wilcoxon matched-pairs signed-rank test). The results confirmed that inoculation with 4×10^7 total cells/mL of each probiotic into KUHIMM did not change the diversity of colonic microbiota. In contrast, the number of total eubacteria tended to decrease with probiotic inoculation, and a significant decrease was observed in the KUHIMM culture inoculated with *W. coagulans* alone, *A. caccae* alone, and a combination of *W. coagulans* and *C. butyricum,* compared to that in the probiotic-non-inoculated culture (WC: $p = 0.0313$; WC, AC: $p = 0.0156$; CB: $p = 0.0469$; Wilcoxon matched-pairs signed-rank test, Fig. 1).

Effect of probiotic inoculation on the structure of colonic microbiota

Figure 2a shows the relative abundance of bacterial species in each sample. In the original fecal samples, *W. coagulans*, *A. caccae*, and *C. butyricum* were almost absent (< 0.1%, on average). In the non-probiotic-inoculated KUHIMM cultures, the relative abundances of these bacteria were very low (< 0.2%, on average) after 48 h of cultivation. When each of these probiotics was inoculated into KUHIMM alone, they remained even after 48 h of cultivation. When *W. coagulans* and *A. caccae* were co-inoculated into KUHIMM, no significant change was observed in the relative abundance of *W. coagulans* compared to when *W. coagulans* was inoculated alone, even after 48 h of cultivation. However, when *W. coagulans* and *C. butyricum* were co-inoculated, the relative abundance of *W. coagulans* decreased significantly (< 1%, on average) after 48 h of KUHIMM cultivation (Fig. 2b). No significant difference was detected in the relative abundance of *A. caccae* and *C. butyricum* after 48 h of KUHIMM cultivation when inoculated alone or in combination with *W. coagulans* (Fig. 2c and d). For the other major butyrate-producing species, such as *Eubacterium rectale* (unclassified *Lachnospiraceae*) and *Faecalibacterium prausnitzii* (*Faecalibacterium* spp.), no significant change in the relative abundance was observed between the probiotic-inoculated and non-inoculated KUHIMM cultures (Fig. 2e and f). Regarding the *Enterobacteriaceae* family, pro-inflammatory bacteria enriched in patients with IBD, a significant decrease in the relative abundance of *Escherichia* sp. and unclassified *Enterobacteriaceae* was

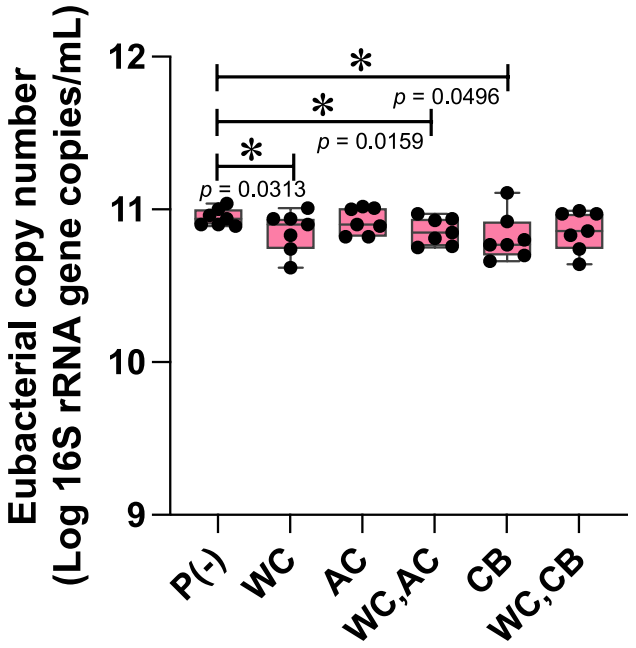

Fig. 1 Eubacterial copy number in original feces (FEC), in non-probiotic-inoculated KUHIMM culture (P(-)), and in the corresponding cultures inoculated with *W. coagulans* (WC), *A. caccae* (AC), combination of *W. coagulans* and *A. caccae* (WC, AC), *C. butyricum* (CB), and combination of *W. coagulans* and *C. butyricum* (WC, CB) after 48 h of cultivation. Data are shown as the median and interquartile range (25th–75th percentiles) of seven samples. $*p < 0.05$; Wilcoxon matched-pairs signed-rank test

observed only in the *W. coagulans* and *C. butyricum*-co-inoculated KUHIMM cultures ($p = 0.0313$ and $p = 0.0313$, respectively; Wilcoxon matched-pairs signed-rank test, Fig. 2g and h). For other species, the relative abundance of *Bifidobacterium* spp. in the KUHIMM cultures was significantly decreased due to co-inoculation with *W. coagulans* and *C. butyricum* compared to that in the non-probiotic-inoculated culture ($p = 0.0469$; Wilcoxon matched-pairs signed-rank test, Fig. 2i).

Effect of probiotic inoculation on SCFA production

To evaluate the effects of probiotics on SCFA production, the concentrations of acetate, propionate, and butyrate were measured after 48 h of cultivation in probiotic-inoculated and non-inoculated KUHIMM, with or without probiotics (Fig. 3). Acetate and propionate concentrations were not significantly affected by the addition of any probiotic combination (Fig. 3a and b). No significant increase in butyrate concentrations was detected when each probiotic was inoculated alone; however, a significant increase was observed when *W. coagulans* was co-inoculated with butyrate-producing bacteria (*A. caccae* or *C. butyricum*; $p = 0.0156$ and $p = 0.0313$, respectively; Wilcoxon matched-pairs signed-rank test, Fig. 3c).

Discussion

Several studies have shown that dysbiosis may be associated with the development of UC (Sartor and Wu 2017) and decreased butyrate levels due to decreased butyrate producers may contribute to the etiology of this disease (Kumari et al. 2013). Administering beneficial bacteria as probiotics to restore the intestinal microbiota and its metabolic function is a promising strategy for the remission and suppression of UC relapse (Ganji-Arjenaki and Rafieian-Kopaei 2018; Kaur et al. 2020; Yoshimatsu et al. 2015). Increasing evidence has shown that multi-strain probiotics have greater efficacy than single strains (Wang et al. 2020). However, effective probiotic combinations for restoring butyrate production in the dysregulated intestinal microbiota of patients with UC still remain unclear.

In the present study, we investigated the possible effect of a combination of probiotics consisting of the lactic acid bacterium *W. coagulans* SANK70258 and the lactate-utilizing butyrate-producing bacteria *A. caccae* (Sato et al. 2008) or *C. butyricum* (Detman et al. 2019) on the colonic environment of patients with UC using KUHIMM inoculated with their fecal samples. These bacteria were less abundant in fecal samples from patients with UC enrolled in this study (Fig. 2a), and inoculation of 4×10^7 total cells/mL of each strain alone into KUHIMM did not increase butyrate production compared to the probiotic non-inoculated KUHIMM culture (Fig. 3c). In contrast, butyrate concentrations significantly increased when *W. coagulans* was inoculated in combination with *A. caccae* or *C. butyricum* (Fig. 3c). Since no significant increase in the relative abundance of other major butyrate-producing bacteria was observed in these groups (Fig. 2e and f), the butyrate production was suggested to have been due to the contribution of the inoculated *A. caccae* and *C. butyricum*. The results suggested that administration of a combination of probiotics consisting of *W. coagulans* SANK70258 and lactate-utilizing butyrate-producing bacteria can alter microbial fermentation in the colons of patients with UC and promote the formation of butyrate, which is beneficial to the host.

The increased butyrate production by inoculation with a combination of probiotics used in this study could be explained by metabolic cross-feeding effects between lactic acid bacteria and lactate-utilizing butyrate-producing bacteria, as hypothesized in our previous study (Sasaki et al. 2020). The hypothesis was supported by the observation of a significant decrease in lactate concentration after 24 h of KUHIMM cultivation when *W. coagulans* was inoculated in combination with *A. caccae* or *C. butyricum* (Supplementary

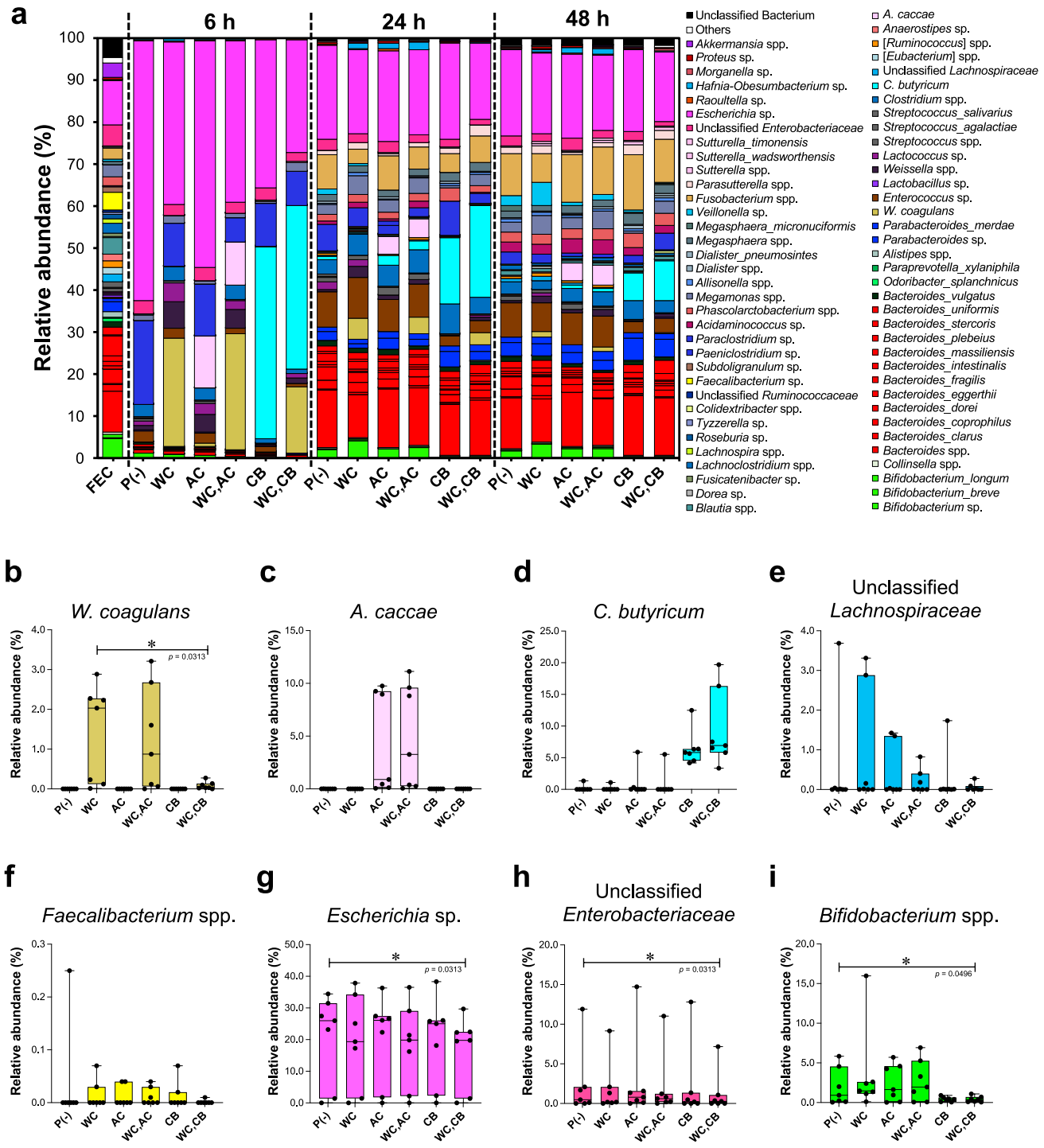

Fig. 2 Effect of probiotic inoculation on the relative abundance of microbiota in each sample. **a** Species-level compositional view of bacteria in original feces (FEC), in non-probiotic-inoculated KUHIMM culture (P(-)), and in the corresponding cultures inoculated with *W. coagulans* (WC), *A. caccae* (AC), combination of *W. coagulans* and *A. caccae* (WC, AC), *C. butyricum* (CB), and combination of *W. coagulans* and *C. butyricum* (WC, CB) after 6, 24, and 48 h of cultivation. Data are shown as the average relative abundances in seven samples. Species with lower abundance (< 1.0%) and lower levels of similarity (< 99%) were indicated as Others and Unclassified bacterium, respectively. **b–i** The relative abundance of *W. coagulans* (**b**), *A. caccae* (**c**), *C. butyricum* (**d**), unclassified *Lachnospiraceae* (**e**), *Faecalibacterium* spp. (**f**), *Escherichia* sp. (**g**), unclassified *Enterobacteriaceae* (**h**), and *Bifidobacterium* spp. (**i**) in KUHIMM. Data are shown as the median and interquartile range (25th–75th percentiles) of seven samples. *$p < 0.05$; Wilcoxon matched-pairs signed-rank test

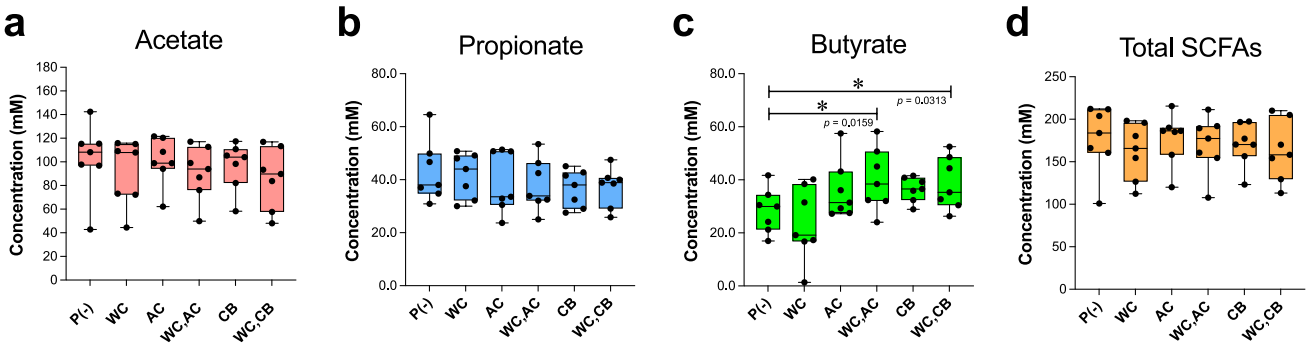

Fig. 3 Concentration of SCFAs in non-probiotic-inoculated KUHIMM culture (P(-)), and in the corresponding cultures inoculated with *W. coagulans* (WC), *A. caccae* (AC), combination of *W. coagulans* and *A. caccae* (WC, AC), *C. butyricum* (CB), and combination of *W. coagulans* and *C. butyricum* (WC, CB) after 48 h of cultivation. Data are shown as the median and interquartile range (25th–75th percentiles) of seven samples. $*p < 0.05$; Wilcoxon matched-pairs signed-rank test

Fig. S1). Many intestinal bacteria can produce butyrate through the cross-feeding of microbial metabolites such as acetate or lactate (Zhao et al. 2024). The cross-feeding phenomenon has mainly been reported between *Bifidobacterium*, a representative probiotic microorganism, and butyrate producers, such as *F. prausnitzii* (Rios-Covian et al. 2015), *E. rectale* (Rivière et al. 2015), *Megasphaera indica* (Zhao et al. 2024), and *A. caccae* (Chia et al. 2021). Our results showed, for the first time, the conversion of lactate into butyrate through cross-feeding between *W. coagulans* and *A. caccae* and between *W. coagulans* and *C. butyricum* and provided novel insights into butyrogenesis in colonic microbiota.

While significant recovery of butyrate production was observed in both groups inoculated with *W. coagulans* and *A. caccae*, and with *W. coagulans* and *C. butyricum*, microbial composition analysis revealed different effects of *A. caccae* and *C. butyricum* on the structure of colonic microbiota. After 48 h of KUHIMM cultivation co-inoculated with *W. coagulans* and *A. caccae*, the relative abundances of these bacteria did not change significantly compared to that when they were inoculated individually. This indicated that *W. coagulans* and *A. caccae* did not inhibit each other's growth in the simulated colonic environment of patients with UC. On the other hand, in the KUHIMM culture co-inoculated with *W. coagulans* and *C. butyricum*, although the coexistence of these bacteria was observed until 24 h of cultivation, the relative abundance of *W. coagulans* decreased significantly after 48 h. This indicated that the symbiotic relationship involving cross-feeding between *W. coagulans* and *C. butyricum* stopped 48 h later. Furthermore, co-inoculation with *W. coagulans* and *C. butyricum* significantly decreased the relative abundance of not only pro-inflammatory bacteria (*Escherichia* sp. and unclassified *Enterobacteriaceae*) but also *Bifidobacterium* spp., which are beneficial members of the intestinal microbiota exerting health-promoting effects (Hidalgo-Cantabrana et al. 2017), compared to the probiotic non-inoculated KUHIMM culture (Fig. 2g–i). These results suggested that *C. butyricum* has a more pronounced effect than *A. caccae* on the structure of colonic microbiota in patients with UC.

The decrease in the relative abundance of *Escherichia* sp. and unclassified *Enterobacteriaceae* in the *W. coagulans* and *C. butyricum*-co-inoculated KUHIMM cultures may be related to increased butyrate levels. Inhibitory effect of butyrate against the family *Enterobacteriaceae* has been suggested in a previous study using in vitro broiler chicken cecal microbiota fermentation model (Asare et al. 2023). On the other hand, it has been reported that administration of *C. butyricum* promotes bifidobacterial growth in humans and mice (Imase et al. 2008; Kong et al. 2011). The decrease in the relative abundance of *Bifidobacterium* spp. upon co-inoculation with *W. coagulans* and *C. butyricum* observed in the present study was inconsistent with these previous reports. This could be associated with competition for carbon sources and other nutrients, such as amino acids, minerals, or vitamins. In contrast to animal experiments and clinical trials in which host feeding continuously provides nutrients, KUHIMM is an in vitro batch culture in which nutrients are limited in the culture medium. *C. butyricum* inoculated at high doses might have a competitive advantage for the limited nutrients in the KUHIMM culture over *W. coagulans* and bifidobacteria, resulting in the growth inhibition of these bacteria. The decreasing trend in the number of total eubacteria in the KUHIMM culture inoculated with probiotics (Fig. 1) may be attributable to the competition for the limited nutrients. Further research, including clinical trials, would be required to verify the actual survival rate of these probiotics in the human colon and their modulatory effects on the colonic microbiota.

Given the recent restrictions on animal experiments, it would be important to understand the behavior of a

combination of probiotics within the human colonic microbiota prior to human clinical trials. In the present study, we investigated the possible effects of a combination of probiotics consisting of *W. coagulans* SANK70258 and two lactate-utilizing butyrate-producing bacteria belonging to different families on the colonic environment of patients with UC, using the KUHIMM. Inoculation with *W. coagulans*, *A. caccae*, or *C. butyricum* alone did not increase butyrate production in KUHIMM with fecal samples from patients with UC, whereas butyrate levels significantly increased when *W. coagulans* was inoculated in combination with *A. caccae* or *C. butyricum*. The results suggested the combination of probiotics consisting of *W. coagulans* SANK70258 and lactate-utilizing butyrate-producing bacteria to have synergistic effects through cross-feeding and to effectively restore butyrogenesis in the colonic environment of patients with UC. Since decreased butyrate levels have been suggested to contribute to the etiology of UC, the probiotic combination used in this study may be effective in preventing and treating UC. In contrast, the modulatory effect on colonic microbiota was more pronounced in *C. butyricum* than in *A. caccae*. Co-inoculated *W. coagulans* and *A. caccae* did not inhibit each other's growth and did not significantly affect the relative abundance of other bacteria, whereas co-inoculation of *W. coagulans* with *C. butyricum* significantly inhibited the growth of *W. coagulans*. Furthermore, a significant decrease in the relative abundance of pro-inflammatory bacteria (*Escherichia* sp. and unclassified *Enterobacteriaceae*) and *Bifidobacterium* spp. was observed in the *W. coagulans* and *C. butyricum*-co-inoculated KUHIMM culture. The results obtained in this study provided novel insights into the symbiotic relationship between probiotics through cross-feeding phenomena and their influence on dysregulated colonic microbiota of patients with UC. However, it should be noted that the KUHIMM does not account for metabolite absorption systems, crosstalk among epithelial cells, and immune cell responses (Sasaki et al. 2018; Shintani et al. 2024). Further studies, including clinical trials, would be required to verify the efficacy of combination probiotics in vivo.

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Author contribution KI wrote the manuscript. DS, TS, JI, RY, YM, YK, and AK conceived and designed the experiments. DS and TS operated and analyzed the model culture system. JI and KO contributed to data acquisition and analysis related to the model culture system. KI, DS, TS, JI, KO, YN, TM, and RY contributed to data interpretation. DS, TS, JI, and YN revised the manuscript. AK conceived and supervised the research. All authors read and approved the manuscript.

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Data availability The raw sequencing data reported in this paper have been deposited in the DDBJ Sequence Read Archive (DRA) as PRJDB17830 and DRR541750-DRR541882.

Declarations

Ethics approval The study design was approved by the Institutional Ethics Review Board of Shiba Palace Clinic (research code 154461_rn-36924, approval date Feb 22, 2024). The study was conducted in accordance with the principles of the Declaration of Helsinki, and all participants provided written informed consent prior to specimen collection.

Competing interests The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests: YN, TM, and RY are employed by the Mitsubishi Chemical Corporation. The remaining authors declare no competing interests.

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