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Engineering probiotics for multiple interventions on intestinal diseases

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Engineering probiotics for multiple interventions on intestinal diseases

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Editorial: Engineering probiotics for multiple interventions on intestinal diseases

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Editorial on the Research Topic:
Engineering probiotics for multiple interventions on intestinal diseases

Gut microbiota, known as an important "organ" of the human body, plays an important role in regulating the host immune response, repairing the intestinal barrier, and resisting pathogenic bacteria invasion. The imbalance of intestinal microbiota is closely related to digestive system diseases, accelerating the occurrence and development of inflammatory bowel disease (IBD), colorectal cancer (CRC), irritable bowel syndrome (IBS), acute or chronic radiation bowel disease, colonic constipation, diarrhoea and other intestinal diseases. Microecological therapy targeting the structure and function of gut microbiota has attracted extensive attention in the biomedical scientific community (Cani, 2018) . The network meta-analysis (NMA) conducted by Zhang et al. suggested that *B.coagulans* had prominent efficacy in treating IBS patients. Thus incorporating *B.coagulans* into a probiotic combination, or genetically engineering the strain to amplify its biological function may be potential routes to treat IBS. Lyu et al. highlighted the mechanisms of *SpA* by which the gut microbiota impact gut inflammation and trigger the immune responses and discussed the potential of probiotics being an adjunctive therapy for *SpA*. Hao et al. evaluated the efficacy of probiotics in combination with prebiotics to treat patients suffering from hypothyroidism complications with small intestinal bacterial overgrowth during the second trimester of pregnancy. Yin and Zhu's systematic review on the meta-analysis of clinical trials suggested probiotics have potential value in the treatment of Parkinson's disease (PD)-related constipation.

With the development of multi-omics technologies, the genetic and metabolic characteristics of the gut microbiota have been deeply explored to develop new therapeutic interventions for the host (Agrawal et al., 2022). Modelling the spatial interaction network of gut microbiota has been built to reveal the causal relationship between spatial variability and changes in health states (Cao et al., 2022). Intestinal homeostasis is maintained in a dynamic equilibrium by balancing the contribution of different players, including diet and drug use. Traditional Chinese medicine and natural products play an important role in this process. Gut microbiota act as important regulators in inflammation and metabolic disorders (Wang et al., 2021a), relying on microbial metabolites and their interactions with receptors on host cells to activate or inhibit signalling pathways (Wang et al., 2021b). Che et al. elucidated

the mechanism of the bidirectional interaction between traditional Chinese medicine and intestinal flora, as well as repairing the intestinal mucosal barrier and protecting the barrier function through various modalities. Thus, multiple interventions based on the modulation of the gut microbiota or the use of specific prebiotics and probiotics might contribute to the design of microecological agents.

Isolating and identifying microbes that can interact with probiotics provides an important basis for evaluating the efficacy of probiotics and clarifying their mechanisms. Yin et al. developed a single-cell droplet approach to obtain the isolates of the beneficial gut bacteria, which complements culture-independent metagenomic investigations of living bacteria therapy. Moreover, emerging technologies, such as Raman spectroscopy, flow cytometry and microfluidic technologies, have provided powerful tools to study microbiome function at the single-cell level (Yuan et al., 2017) and sorting cells (McIlvenna et al., 2016; Lee et al., 2019; Lyu et al., 2020). Wee et al. showed the feasibility of Raman spectroscopy and flow cytometry for phenotypic studies in long-term antibiotic treatment or when investigating new antibiotic classes.

Engineered probiotics are the next generation of live biotherapeutics that have been modified to target specific diseases. In recent years, engineered probiotics served as live biotherapeutics have been continuously created due to the rapid development of synthetic biology (Ozdemir et al., 2018). When disease marker molecules were detected, probiotics were programmed to release therapeutic effectors such as SCFAs (Bai et al., 2020; Wang et al., 2022), 5-HT (Li et al.) and active ingredients from plant sources. In this way, engineered probiotics have been used to improve metabolic disorders, behavioral disorders and cancer efficacy (Gurbatri et al., 2022). In addition to bacteria and fungi, bacteriophage engineering promises to generate phage variants with unique properties for prophylactic and therapeutic applications (Kortright et al., 2019; Dhanoa et al.).Researchers are mining the key components of bacteriophages to build synthetic biological systems (Xu et al., 2020).

The artificial flora designed and synthesized with the concept of synthetic biology is expected to overcome the existing shortcomings and achieve high efficiency, precision and control of microecological therapy (Wang et al.). On the other side, researchers use material or chemical strategies to modify probiotics to achieve therapeutic efficacies for treating intestinal diseases (Song et al., 2022). Fecal Microbiota Transplantation (FMT) is one of the recommended treatments for recurrent *Clostridioides difficile* infection, but endoscopy and available oral formulations still have several limitations in their preparation, storage, and administration. Aira et al. used microcrystalline cellulose as the main excipient to maintain the viability of gut microbiota for a long time.

In conclusion, this research topic showcases the emerging multidisciplinary approaches, including gene editing, single-cell technology, and faecal microbiota formulation, for engineering and evaluating probiotics as potential therapeutical agents to treat intestinal diseases. We hope that readers find these articles informative and look forward to an exciting future for engineered probiotics.

Author contributions

HH, HY and XZ wrote the manuscript. Both authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Efficacy of Probiotics for Irritable Bowel Syndrome: A Systematic Review and Network Meta-Analysis

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Background: Irritable bowel syndrome (IBS) is a common gastrointestinal condition. Studies regarding the treatment of IBS with probiotics have not yielded consistent results, and the best probiotics has not yet been confirmed. Therefore, we performed a network meta-analysis (NMA) to assess the relative rank order of different probiotics for IBS.

Method: We searched for RCTs on the efficacy of probiotics for IBS until August 25, 2021. The primary outcome was the symptom relief rate, as well as global symptoms, abdominal pain, bloating, and straining scores. The NMA was conducted using Stata 15.0. We also used meta-regression to explore whether the treatment length and dose influenced the efficacy.

Results: Forty-three RCTs, with 5,531 IBS patients, were included in this analysis. Firstly, we compared the efficacy of different probiotic species. *B.coagulans* exhibited the highest probability to be the optimal probiotic specie in improving IBS symptom relief rate, as well as global symptom, abdominal pain, bloating, and straining scores. In regard to the secondary outcomes, *L.plantarum* ranked first in ameliorating the QOL of IBS patients, but without any significant differences compared with other probiotic species in standardized mean differences (SMD) estimates. Moreover, patients received *L.acidophilus* had lowest incidence of adverse events. The meta-regression revealed that no significant differences were found between participants using different doses of probiotics in all outcomes, while the treatment length, as a confounder, can significantly influence the efficacy of probiotics in ameliorating abdominal pain (Coef = -2.30; p = 0.035) and straining (Coef = -3.15; p = 0.020) in IBS patients. Thus, we performed the subgroup analysis on treatment length subsequently in these two outcomes, which showed that efficacy of *B.coagulans* using 8 weeks ranked first both in improving the abdominal pain and straining scores. Additionally, *B. coagulans* still had significant efficacy compared to different types of probiotic combinations in present study.

Conclusions: The findings of this NMA suggested that *B.coagulans* had prominent efficacy in treating IBS patients, and incorporating *B.coagulans* into a probiotic combination, or genetically engineering it to amplify its biological function may be a

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future research target to treat IBS patients. With few direct comparisons available between individual therapies today, this NMA may have utility in forming treatment guideline for IBS with probiotics.

Keywords: irritable bowel syndrome, probiotics, network meta-analysis, efficacy, adverse events

INTRODUCTION

Irritable bowel syndrome (IBS) is a common and chronic gastrointestinal (GI) condition characterized by abdominal pain, bloating, and changes in bowel habits associated with altered stool form, which can affect the quality of life and work productivity of patients (Mearin et al., 2016; Camilleri, 2021). In terms of clinical epidemiology, the prevalence of IBS varies substantially among different countries and different diagnostic criteria, ranging from 1.1% to 45% (Black and Ford, 2020); Furthermore, there is a higher prevalence of IBS in women than in men (12% vs. 8.6%) (Oka et al., 2020). IBS can be diagnosed by reviewing the clinical findings based on the Rome Criteria rather than basing the diagnosis on definite biological markers and organic lesions in patients with IBS (Lacy and Patel, 2017).

There are trillions of microbes residing in the human GI tract, which is over 150 times the number of genes in the human genome (Qin et al., 2010; Raskov et al., 2016). Beneficial commensal bacteria, which play an important role in healthy individuals, can contribute to the upregulation of anti-inflammatory genes and downregulation of pro-inflammatory genes (Plaza-Diaz et al., 2014). In IBS cases, the reduction of microbiome diversity, gut barrier deficiency, gut-brain signaling disorders, and immune disorders are significantly related to the abnormal function of the GI tract (Raskov et al., 2016). Liu et al. (2016) found that when compared with healthy controls, the diarrhea predominant IBS (IBS-D) group had lower biodiversity of microbial communities, which were dominated by *Bacteroides* and *Prevotella* genera. Moreover, a decrease in probiotic species and an increase in pathogenic species were also found to be common in IBS cases (Ringel and Ringel-Kulka, 2015).

Probiotics, available in various dietary components or by prescription, contain live microorganisms in which most bacteria are similar to the beneficial bacteria that are naturally present in the human GI tract (Wilkins and Sequoia, 2017). *Lactobacillus* and *Bifidobacteria* are often used in probiotic products and have been studied in clinical trials (Kligler and Cohrssen, 2008; Raskov et al., 2016). The efficacy and safety of probiotic products for the treatment of IBS are supported by an increasing number of clinical studies. A meta-analysis (Ford et al., 2018) with 53 randomized controlled trials (RCTs) involving 5,545 patients provided data regarding the potential efficacy of probiotic combinations and specific probiotic species or strains for improving global IBS symptoms and abdominal pain. In addition to relieving symptoms, probiotics have been demonstrated to improve the quality of life (QOL) and diversify the microbial community of IBS cases in several studies (Sun et al., 2018; Preston et al., 2018).

To the best of our knowledge, although the efficacy and safety of probiotics have been confirmed by numerous studies, the best species for probiotics used in the treatment of IBS have not been identified yet (Gwee et al., 2019). Therefore, in the present study, we performed a systematic review and network meta-analysis (NMA) to compare the efficacy of probiotics for IBS to identify the best interventions.

Abbreviations: IBS, Irritable bowel syndrome; GI, gastrointestinal; IBS-D, diarrhea predominant IBS; RCTs, randomized controlled trials; QOL, quality of life; NMA, network meta-analysis; OR, odds ratio; 95% CI, 95% confidence interval; SMD, standardized mean difference; SUCRA, surface under the cumulative ranking curve; AEs, adverse events; USFDA, US Food and Drug Administration.

METHODS

A systematic review and NMA were carried out in accordance with the Preferred Reporting Items for Systematic Review and Meta-analysis extension statement, including NMA (PRISMA-NMA) (Page et al., 2021).

Search Strategy

The databases, including PubMed, Cochrane Library, Web of Science, and Medline, were searched systematically by two independent researchers on August 25, 2021, to identify RCTs exploring the efficacy of probiotics for patients with IBS. The search terms in PubMed were as follows: *(irritable bowel syndrome) OR (IBS) AND (probiotics) OR (probiotic) OR (Saccharomyces) OR (Escherichia) OR (Bifidobacterium) OR (Bacillus) OR (Lactobacillus) OR (Clostridium) AND ([randomized controlled trial{Publication Type}] OR [clinical trial{Publication Type}])*. In addition, the lists of references from the previous systematic review and meta-analysis in this field were also reviewed to identify any missing literature.

Eligible Criteria

Studies that met the following criteria were eligible for NMA.

1. RCTs that compared the efficacy and tolerability of probiotic with placebo or another probiotic for patients with IBS were eligible.
2. The patients included in all RCTs had a well-established diagnosis of IBS, and there were no limitations on age, sex, countries, types of IBS, and the publication year of the RCTs.
3. The probiotics included the following species: *Saccharomyces boulardii* (*S. boulardii*), *Saccharomyces cerevisiae* (*S. cerevisiae*), *Escherichia coli* (*E. coli*), *Bifidobacterium bifidum* (*B. bifidum*), *Bacillus coagulans* (*B. coagulans*),

Lactobacillus acidophilus (*L. acidophilus*), *Lactobacillus GG* (*LGG*), *Lactobacillus paracasei* (*L. paracasei*), *Lactobacillus salivarius* (*L. salivarius*), *Lactobacillus plantarum* (*L. plantarum*), *Bifidobacterium longum* (*B. longum*), *Lactobacillus casei* (*L. casei*), *Lactobacillus gasseri* (*L. gasseri*), *Bifidobacterium infantis* (*B. infantis*), *Clostridium butyricum* (*C. butyricum*), *Lactobacillus reuteri* (*L. reuteri*), and *Bifidobacterium lactis* (*B. lactis*), etc.;

4. The dosages of the probiotics and the duration of each intervention were recorded in detail.
5. The patients were required to be followed up for at least 1 week, and the studies had to report the outcome of symptom relief rate, assessment of global and individual symptom scores, QOL, and adverse events.

Exclusion Criteria

The exclusion criteria included the following.

1. Duplicated studies and studies that were not related to our research topic were excluded.
2. Non-RCTs, observational studies, single-arm studies, case reports, reviews, meta-analyses, letters, protocols, and other such sources were excluded.
3. Papers published in a language other than English were excluded.
4. Papers without full text (or in which only the abstract was available) or the data of our target outcomes were excluded.
5. Participants with other comorbidities, such as inflammatory bowel disease, celiac disease, lactose intolerance, were excluded from the study.

Data Extraction and Risk of Bias

Two authors independently extracted the following information from each study: author, year of publication, country, sample size, age of patients, subtypes of IBS, comparison, and treatment details (types and dosages of probiotics, response rate of placebo, duration of treatment, and outcome measures).

Two authors evaluated the risk of bias for each included RCT with the help of measures displayed in the Cochrane Handbook for Systematic Reviewers (version 5.1.0), which includes seven indicators: 1) random sequence generation (selection bias), 2) allocation concealment (selection bias), 3) blinding of patients and personnel (performance bias), 4) blinding of outcome assessment (detection bias), 5) incomplete outcome data (attrition bias), 6) selective reporting (reporting bias), and 7) other bias. Each indicator contained three levels: low risk, unclear risk, or high risk of bias.

If there were any inconsistencies or disagreements in the process of data extraction and quality assessment, the two authors discussed these issues or an independent expert in this field was consulted to reach a consensus.

Statistical Analysis

NMA was performed using the Stata software version 15.0. For categorical data, we estimated the summary odds ratio (OR) with a 95% confidence interval (95% CI), and for continuous data, we estimated the summary standardized mean difference (SMD) with 95% CI. We showed the direct comparison between different interventions using a network diagram, in which the size of the nodes represents the sample size of each intervention, and the thickness of the continuous lines connecting the nodes indicates the number of studies directly comparing the two interventions. Subsequently, global inconsistency was evaluated, and the local inconsistency assessment was performed using the node-splitting method to check whether the estimated effects from the direct comparisons were consistent with those from the indirect comparisons. $P>0.05$ indicates that there were no significant differences of estimated effects between direct and indirect comparisons, thus the consistency model was used; otherwise, the inconsistency model was used. We assessed network heterogeneity across all treatment contrasts using I^2 statistics, and loop-specific heterogeneity using the τ^2 statistics. To rank the efficacy and safety of the interventions, we calculated the probabilities of the surface under the cumulative ranking curve (SUCRA) between all interventions for the primary and secondary outcomes. League tables containing both direct and indirect comparisons were also performed to summarize the outcomes of each indicator. Additionally, we also conducted a meta-regression analysis to explore whether the lengths and doses of interventions were associated with efficacy and adverse events of probiotics in IBS, if so, a subgroup analysis was performed.

RESULTS

Study Selection and Characteristics

As is shown in **Figure 1**, we identified a total of 676 articles in our initial search of databases and review of the lists of references. A total of 253 papers were included after accounting for the presence of duplicate papers. Furthermore, we reviewed the titles and abstracts of these papers carefully, and 162 of them were excluded because they were not relevant to our research topic. The full texts of the remaining 91 papers were further analyzed, and 48 articles were excluded (the detailed reasons for exclusion are shown in **Figure 1**). Ultimately, 43 RCTs were included in the present study.

Among the included studies, 29 RCTs (Niedzielin et al., 2001; Bauserman and Michail, 2005; Niv et al., 2005; O'Mahony et al., 2005; Whorwell et al., 2006; Sinn et al., 2008; Enck et al., 2009; Ligaarden et al., 2010; Choi et al., 2011; Guglielmetti et al., 2011; Kabir et al., 2011; Dapoigny et al., 2012; Ducrotté et al., 2012; Kruis et al., 2012; Abbas et al., 2014; Lyra et al., 2016; Majeed et al., 2016; Pineton et al., 2015; Spiller et al., 2016; Stevenson et al., 2014; Thijssen et al., 2016; Pinto-Sanchez et al., 2017; Sun et al., 2018; Sudha et al., 2018; Madempudi et al., 2019; Andresen et al., 2020; Gayathri et al., 2020; Martoni et al., 2020; Gupta and Maity, 2021) were related to 15 probiotic species, including the following species: *L. plantarum* (4 RCTs), *L. acidophilus* (4 RCTs), *B. coagulans* (4 RCTs), *S. boulardii* (3 RCTs), *S. cerevisiae* (3 RCTs), and *L. casei* (2 RCTs).

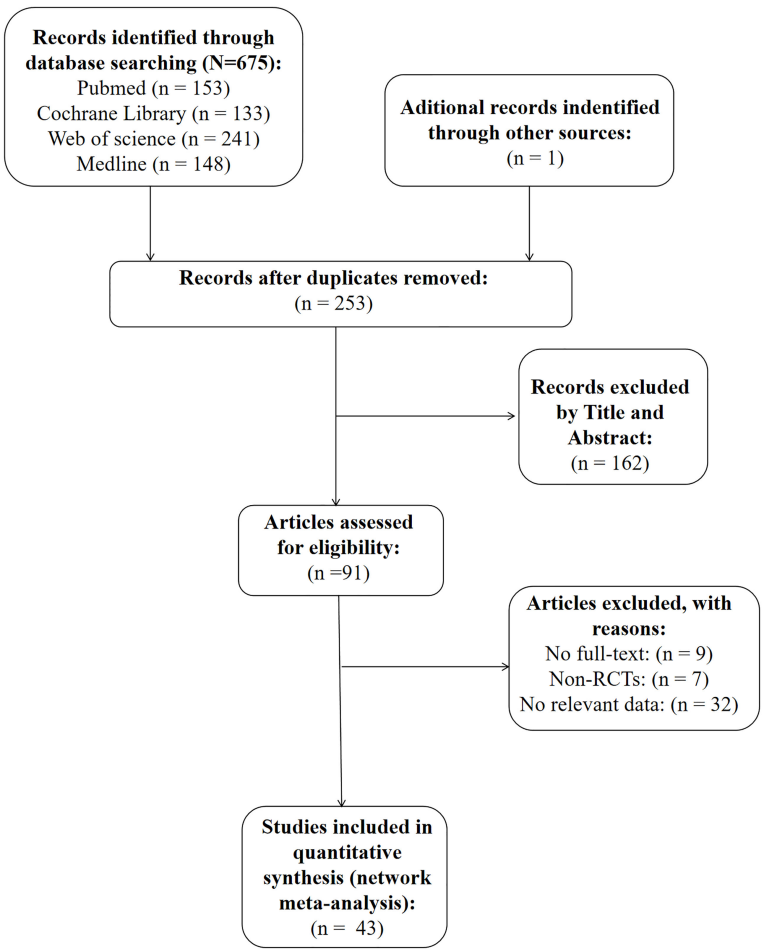

FIGURE 1 | Flow diagram of assessment of studies.

One RCT (Sadrin et al., 2020), related to two different strains of *L. acidophilus*, was also identified as a RCT exploring the efficacy of a probiotic specie (*L. acidophilus*) on IBS. Unfortunately, there was only one article with respect to *B. lactis*, *L. GG*, *L. salivarius*, *B. longum*, *C. butyricum*, and *L. reuteri*.

13 RCTs (Kim et al., 2003; Kajander et al., 2005; Kim et al., 2005; Guyonnet et al., 2007; Kajander et al., 2008; Drouault-Holowacz et al., 2008; Zeng et al., 2008; Agrawal et al., 2009; Søndergaard et al., 2011; Begtrup et al., 2013; Roberts et al., 2013; Jafari et al., 2014; Wong et al., 2015) were associated with 5 types of probiotic combinations that frequently used in clinical trials as follows: 1) 1 strains of *Bifidobacterium*, 1 strains of *Lactobacillus*, and 1 strains of *Streptococcus (*hereinafter referred to as 1B1L1S*);* 2) 1 strains of *Bifidobacterium* and 2 strains of *Lactobacillus* (1B2L); 3) 1 strains of *Bifidobacterium*, 2 strains of *Lactobacillus*, and 1 strains of *Propionibacterium* (1B2L1P); 4) 1 strains of *Bifidobacterium*, 2 strains of *Lactobacillus*, and 1 strains of *Streptococcus* (1B2L1S*);* 5) 3 strains of *Bifidobacterium*, 4 strains of *Lactobacillus*, and 1 strains of *Streptococcus* (3B4L1S). The study sample size ranged from 25 to 443, and a total of 5,531 participants were included in the NMA. The detailed patient characteristics are shown in **Table 1**.

Risk of Bias

The risk of bias assessment for all the included RCTs is presented in **Supplementary Figure 1** (**Figure S1**). Overall, twelve trials were judged to have a low risk of bias across all domains. Two trials were judged to have a high risk of bias for blinding of the outcome assessment. Almost all the RCTs were judged to have a low risk of bias for allocation concealment, blinding of participants and personnel, incomplete outcome data, and selective reporting, except for two trials that had unclear risk in the domain of allocation concealment, one trial that had an unclear risk for blinding of participants and personnel, and one trial that had an unclear risk for incomplete outcome data.

Primary Outcomes: Effect of Different Probiotic Species on Symptom Relief Rate

A total of 19 RCTs explored the efficacy of probiotics on symptom relief rate, and the network plots are presented in

TABLE 1 | Characteristics of RCTs about the efficacy of probiotics in irritable bowel syndrome.

Study	country	Criteria used	IBS Subtypes	Intervention				Control (PLA)			#Outcome measures used in NMA
				Sample size	*Age	Probiotic used	Dose and duration	Sample size	*Age	Response rate	
(Abbas et al., 2014)	Pakistan	Rome III criteria	IBS-D	37	37.7 ± 11.6	*S.boulardii*	750mg/day, 6w	35	33.0 ± 12.0	N.A.	C, D, E, G
(Choi et al., 2011)	Korea	Rome II criteria	IBS-D and IBS-M	45	40.2 ± 13.1	*S.boulardii*	$4x10^{11}$ live cells/day, 4w	45	40.6 ± 12.9	N.A.	B, C, D, E, F, G
(Kabir et al., 2011)	Bangladesh	Rome II criteria	IBS-D	35	NA	*S.boulardii*	500mg/day, 4w	35	NA	N.A.	C, D
(Gayathri et al., 2020)	India	Rome III criteria	IBS-C, IBS-D, IBS-M	52	42.25 ± 15.44	*S.cerevisiae* CNCMI-3856	$4×10^9$CFU/day, 8w	48	39.6 ± 12.79	N.A.	C, G
(Pineton et al., 2015)	France	Rome III criteria	IBS-C, IBS-D, IBS-M	86	42.5 ± 12.5	*S.cerevisiae* CNCMI-3856	$4×10^9$ CFU/day, 8w	93	45.4 ± 14	47%	A, C, G
(Spiller et al., 2016)	France	Rome III criteria	IBS-C	192	45.3 ± 15.7	*S.cerevisiae* CNCMI-3856	$8x10^9$CFU/day, 12w	187	45.4 ± 14.1	26.90%	A, G
(Enck et al., 2009)	Germany	Kruis scale	N.A.	148	49.8(19–70)	*E.coli*	(1.5-4.5x10^7 CFU/mL) 0.75mL drops t.i.d. for 1 week, then 1.5mL t.i.d. for weeks 2 to 8	150	49.4(18–76)	4.67%	A
(Kruis et al., 2012)	Germany	Rome II criteria	N.A.	60	46.3 ± 12.1	*E.coli* Nissle1917	(2.5-25x10^9 CFU/capsule) o.d. for 4 days then b.d. for 12 weeks	60	45.1 ± 12.7	41.70%	A, G
(Andresen et al., 2020)	Germany	Rome III criteria	IBS-C, IBS-D, IBS-M, IBS-U	221	40·1 ± 12·8	*B.bifidum* MIMBb75	$1 × 10^9$ CFU/day, 8w	222	42·6 ± 13·8	30%	A, G
(Guglielmetti et al., 2011)	Germany	Rome III criteria	IBS-C, IBS-D, IBS-M	60	36.65 ± 12.42	*B.bifidum* MIMBb75	$1x10^9$ CFU/day,4w	62	40.98 ± 12.80	21%	A, G
(Gupta and Maity, 2021)	India	Rome IV criteria	N.A.	20	36.20 ± 9.81	*B.coagulans* LBSC	$6 × 10^9$ CFU/day, 80d	20	34.80 ± 11.06	N.A.	C, D, E, J, K
(Majeed et al., 2016)	India	Rome III criteria	IBS-D	18	36.2 ± 11.07	*B.coagulans* MTCC5856	$2×10^9$ CFU/day, 90d	18	35.4 ± 10.75	N.A.	C, D, J, K
(Madempudi et al., 2019)	India	Rome III criteria	N.A.	53	44.4	*B.coagulans* Unique IS2	$2×10^9$CFU/day, 8w	55	42.3	10.91%	A, B, C, D, H, I, J, K
(Sudha et al., 2018)	India	Rome III criteria	IBS-C, IBS-D, IBS-M	72	7.86	*B.coagulans* Unique IS2	$2×10^9$CFU/day, 8w	69	7.89	21.74%	A, B, C, D, E, H, I, J, K
(Lyra et al., 2016)	Finland	Rome III criteria	IBS-C, IBS-D, IBS-M, IBS-U	131 129	47.2 ± 12.5 47.1 ± 13.3	*L. acidophilus* NCFM	$1 × 10^{10}$ CFU/day, 12w $1 × 10^9$ CFU/day, 12w	131	49.4 ± 12.9	28.40%	A, C, D, F, G
(Martoni et al., 2020)	India	Rome IV criteria	N.A.	111 110	39.41 ± 11.80 41.60 ± 11.11	*L. acidophilus* DDS-1 *B.lactis* UABla-12	$1 × 10^{10}$ CFU/day, 6w $1 × 10^{10}$ CFU/day, 6w	109	37.61 ± 10.12	15.60%	A
(Sadrin et al., 2020)	France	Rome III criteria	N.A.	40	48.9 ± 8.4	*L.acidophilus* NCFM and *L.acidophilus* subs *p.helveticus* LAFTIL10	$1×10^{10}$ CFU/day, 8w	40	48.9 ± 8.0	N.A.	B, C, D, G
(Sinn et al., 2008)	Korea	Rome III criteria	IBS-C, IBS-D, IBS-M	20	41.9 ± 14.4	*L.acidophilus*-SDC2012,2013	$4×10^9$ CFU/day, 4w	20	47.5 ± 11.0	35%	A
(Bauserman and Michail, 2005)	USA	Rome II criteria	N.A.	25	11.6 ± 3.2	*L.GG*	$2×10^{10}$ CFU/day, 6w	25	12.4 ± 2.9	40%	A
(O'Mahony et al., 2005)	Eire	Rome II criteria	IBS-C, IBS-D, IBS-M	26 24	NA NA	*L.salivarius* UCC4331 *B.infantis* 35624	$1 × 10^{10}$ CFU/day, 8w $1 × 10^{10}$ CFU/day, 8w	25	NA	N.A.	B, C, D
(Ligaarden et al., 2010)	Norway	Rome II criteria	N.A.	19	50 ± 11	*L.plantarum* MF1298	$1x10^{10}$ CFU/day, 3w	19	50 ± 11	N.A.	B, C, D, E
(Ducrotté et al., 2012)	India	Rome III criteria	IBS-D (63.89%) and other types	108	36.53 ± 12.08	*L.plantarum* 299v(DSM 9843)	$1×10^{10}$ CFU/day, 4w	106	38.40 ± 13.13	8.10%	A, C
(Stevenson et al., 2014)	South Africa	Rome II criteria	IBS-C, IBS-D	54	48.15 ± 13.48	*L.plantarum 299v(DSM 9843)*	$1×10^{10}$ CFU/day, 8w	27	47.27 ± 12.15	N.A.	B, F
(Niedzielin et al., 2001)	Poland	Clinical diagnosis	N.A.	20	48 ± 18	*L.plantarum 299v(DSM 9843)*	$2× 10^{10}$ CFU/day, 4w	20	42 ± 15	15%	A
(Pinto-Sanchez et al., 2017)	Canada	Rome III criteria	IBS-D, IBS-M	22	46.5 (30-58)	*B.longum* NCC3001	$1x10^{10}$ CFU/day, 6w	22	40.0 (26-57)	35%	A, B, G
(Dapoigny et al., 2012)	France	Rome III criteria	IBS-C, IBS-D, IBS-M, IBS-U	25	48.0 ± 10.8	*Lactobacillus casei rhamnosus* LCR35	$6x10^8$ CFU/day, 4w	25	48.0 ± 10.8	40%	A
(Thijssen et al., 2016)	Netherlands	Rome II criteria	IBS-C, IBS-D, IBS-M, IBS-U	39	41.1 ± 14.8	*L.casei* iShirota	$1.3×10^{10}$ CFU/day, 8w	41	42.4 ± 13.5	29%	A
(Whorwell et al., 2006)	UK	Rome II criteria	IBS-C, IBS-D, IBS-M, IBS-U	90 90	40.8 ± 10.44 42.7 ± 10.44	*B.infantis*35624	$1×10^6$ CFU/day, 4w $1×10^8$ CFU/day, 4w	92	42.4 ± 10.45	About 40%	A, B, C, D, E

(Continued)

TABLE 1 | Continued

Study	country	Criteria used	IBS Subtypes	Intervention				Control (PLA)			#Outcome measures used in NMA
				Sample size	*Age	Probiotic used	Dose and duration	Sample size	*Age	Response rate	
				90	41.8 ± 10.44		1×10^{10} CFU/day, 4w				
(Sun et al., 2018)	China	Rome III criteria	IBS-D	105	43.00 ± 12.45	*C.butyricum*	5.67×10^{7} CFU/day, 4w	95	44.91 ± 13.01	35%	A, B, C, D, F, G
(Niv et al., 2005)	Israel	Rome II criteria	IBS-C, IBS-D, IBS-M	27	45.7 ± 14.2	*L.reuteri* ATCC55730	2×10^{8} CFU/day, 6m	27	45.6 ± 16.1	N.A.	B, G
(Agrawal et al., 2009)	UK	Rome III criteria	IBS-C	17	42(24,69)	*B. lacti* DN-173010, *S.thermophilus, and L. bulgaricus*	7.35×10^{10} CFU/day, 4w	17	37(20,59)	N.A.	J, K
(Begtrup et al., 2013)	Denmark	Rome III criteria	IBS-C, IBS-D, IBS-M, IBS-U	67	31.63 ± 10.05	*L. paracasei ssp paracasei* F19, *L. acidophilus* La5, *and B. lactis* Bb12	$5.2x10^{10}$ CFU/day, 6m	64	29.38 ± 8.64	29%	H, I, J, K
(Søndergaard et al., 2011)	Denmark and Sweden	Rome II criteria	N.A.	27	53.9(29–67)	*L.paracasei ssp paracasei* F19, *L.acidophilus* La5, *and B.lactis* Bb12	$2.5x10^{10}$ CFU/day, 8w	25	48.5(29–67)	About 25%	H, K
Guyonnet 2007 (Guyonnet et al., 2007)	France	Rome II criteria	N.A.	135	49.4 ± 11.4	*B.animalis* DN173 010, *S.thermophilus, and L.bulgaricus*	2.98×10^{10} CFU/day,6w	132	49.2 ± 11.4	56.80%	H, I, J, K
(Drouault-Holowacz et al., 2008)	France	Rome II criteria	IBS-C, IBS-D, IBS-M	48	47 ± 14	*B. longum* LA 101, *L. acidophilus* LA 102, *L. lactis* LA 103, *and S. thermophilus* LA 104	1×10^{10} CFU/day, 4w	52	44 ± 14	42.30%	H, J
(Jafari et al., 2014)	Iran	Rome III criteria	N.A.	54	36.6 ± 12.1	*B.animalis subsp. lactis* BB-12®, *L.acidophilus LA-5®, L.delbrueckii subsp. bulgaricus* LBY-27, *S.thermophilus* STY-31	8×10^{9} CFU CFU/day, 4w	54	36.6 ± 12.1	47%	H
(Kim et al., 2003)	USA	Rome II criteria	IBS-D	12	48 ± 5.7	VSL#3: (Threes trains of *Bifidobacterium* (*B.longum, B.infantis and B.breve)*; four strains of *Lactobacillus* (*L.acidophilus, L.casei, L.bulgaricus* and *L.plantarum*); and one strain of *Streptococcus* (*S.salivarius subspecies thermophilus*)	4.5×10^{11} bacteria/day, 8w	13	38 ± 3.4	38%	H, I, J, K
(Zeng et al., 2008)	China	Rome II criteria	IBS-D	14	44.6 ± 12.4	*S.thermophilus, L.bulgaricus, L.acidophilus, and B.longum*	2.6×10^{10} CFU/day, 4w	15	45.8 ± 9.2	N.A.	I, J, K
(Kajander et al., 2005)	Finland	Rome I and II criteria	IBS-C, IBS-D, IBS-M	52	46(23–65)	*L.rhamnosus* GG, *L.rhamnosus* Lc705, *P.freudenreichii, and B.breve* Bb99	$8–9\times10^{9}$/CFU/day, 6m	51	45(21–65)	33.33%	H, J, K
(Kajander et al., 2008)	Finland	Rome II criteria	IBS-C, IBS-D, IBS-M	43	50 ± 13	*L.rhamnosus GG, L.rhamnosus Lc705 DSM 7061, P.freudenreichii, and B.animalis*	4.8×10^{9} CFU/day, 20w	43	46 ± 13	N.A.	I
(Kim et al., 2005)	USA	Rome II criteria	N.A.	24	40 ± 14.70	VSL#3: Threes trains of *Bifidobacterium* (*B.longum, B.infantis and B.breve)*; four strains of *Lactobacillus* (*L.acidophilus, L.casei, L.bulgaricus* and *L.plantarum*); and one strain of *Streptococcus* (*S.salivarius subspecies thermophilus*)	9×10^{11} CFU/day; 8w	24	46 ± 14.70	33%	H, J, K
(Roberts et al., 2013)	England	Rome III criteria	IBS-C, IBS-M	88	44.66 ± 11.98	*B.lactis I-2494, S.thermophilus, and L.bulgaricus*	2.98×10^{10} CFU/day, 12w	91	43.71 ± 12.76	68.30%	H
(Wong et al., 2015)	Singapore	Rome III criteria	N.A.	20	53.35 ± 18.56	VSL#3: Threes trains of *Bifidobacterium* (*B.longum, B.infantis and B.breve)*; four strains of *Lactobacillus*	9×10^{11} CFU/day, 6w	22	40.86 ± 16.46	N.A.	J, K

(Continued)

TABLE 1 | Continued

Study	country	Criteria used	IBS Subtypes	Intervention				Control (PLA)			#Outcome measures used in NMA
				Sample size	*Age	Probiotic used	Dose and duration	Sample size	*Age	Response rate	
						(*L.acidophilus, L.casei, L.bulgaricus* and *L.plantarum*); and one strain of *Streptococcus* (*S.salivarius subspecies thermophilus*)					

*Mean ± sd or mean (range).

A: Symptom relief rate; B: global symptom scores; C: abdominal pain scores; D: bloating scores; E: straining scores; F: QOL; G: AEs; and H-K refers to the outcome relevant to the comparisons of B. coagulans with different probiotic combinations: H: Symptom relief rate; I: global symptom scores; J:abdominal pain scores; K: bloating scores.

N.A., Not reported or not available.

Figure 2A. The results of the global and local inconsistency tests are presented in **Figure S2** and **Table S1**. Both tests showed that there was no significant inconsistency between the direct comparisons and indirect comparisons; thus, the consistency model was used. The NMA revealed that *B. coagulans* (OR 60.73, 95% CI, 14.83 to 248.61), *L. plantarum* (OR 15.62, 95% CI 2.90 to 84.21), and *L. acidophilus* (OR 3.00, 95% CI 1.03 to 8.68) had a greater effect on symptom relief rate in patients with IBS compared with placebo (PLA). The SUCRA analysis (**Table S2** and **Figure S3**) and league table (**Table 2**) showed that *B. coagulans* had the best rank among all the treatment interventions; meanwhile, *L. plantarum* ranked second, *L. acidophilus* ranked third, and PLA ranked last.

Significant heterogeneity was observed across all treatment contrasts (I^2 = 85.5%), but no evidence of loop-specific heterogeneity was found (τ^2 = 0). The meta-regression analysis by treatment dose and length did not significantly influence the SMD estimates for this outcome (**Figures 3A, B**).

Primary Outcomes: Effect of Different Probiotic Species on Global Symptom Scores

A total of 13 RCTs comparing the effect of probiotics on the global symptom scores of patients with IBS were included. The network plot is shown in **Figure 2B**. Both the global and local inconsistency tests revealed a significant inconsistency between direct and indirect comparisons (**Figure S4** and **Table S3**), which indicated that the inconsistency model should be used. The result of NMA revealed a significant improvement in the global symptom scores in patients who received *B. coagulans* (SMD −1.99, 95% CI −2.39 to −1.59) and *Bifidobacterium infantis* (SMD −0.74, 95% CI −1.47 to −0.01) compared with those who received PLA. Based on the SUCRA analysis (**Table S4** and **Figure S5**) and league table (**Table 3**), *B. coagulans*, *C. butyricum*, and *Bifidobacterium longum* ranked as the top three interventions in improving the global symptom scores of patients with IBS, while *L. plantarum* ranked last.

In this outcome, we found obvious heterogeneity across all treatment contrasts (I^2 = 91.2%), but no heterogeneity (τ^2 = 0) in the loop of NMA. Meta-regression analysis showed that treatment dose and length did not significantly influence the SMD estimates for global symptom scores (**Figures 3C, D**).

Primary Outcomes: Effect of Different Probiotic Species on Abdominal Pain Scores

A total of 16 RCTs reported the effect of probiotics on abdominal pain scores in patients with IBS, and the network diagram is shown in **Figure 2C**. Both the global inconsistency test (**Figure S6**) and node-splitting assessment (**Table S5**) showed no significant inconsistency between direct and indirect comparisons; therefore, the consistency model was used. The results of NMA (**Table 4**) revealed a significant improvement in the abdominal pain scores in patients who received *B. coagulans* (SMD −1.71, 95% CI −2.15 to −1.27) and *S. cerevisiae* (SMD −0.54, 95% CI −1.08 and −0.00) than those who received PLA. The SUCRA analysis (**Table S6** and **Figure S7**) demonstrated that *B. coagulans* ranked first in improving the abdominal pain scores of patients with IBS, while *S. cerevisiae* ranked second, *C. butyricum* ranked third, and *S. boulardii* ranked last.

There was a significant heterogeneity across all treatment contrasts (I^2 = 90.4%), but no loop-specific heterogeneity (τ^2 = 0) in this outcome. The meta-regression analysis (**Figures 3E, F**) showed that treatment duration, as a confounder, can significantly influence the efficacy of probiotics in improving the symptoms of abdominal pain (Coef = −2.30; p= 0.035) in patients with IBS.

Subsequently, we performed a subgroup analysis of treatment duration (**Figure 2D**). No evidence of loop-specific heterogeneity was found (τ^2 = 0). The consistency model was used based on the results of the global inconsistency test (**Figure S8**) and node-splitting assessment (**Table S7**), both of which showed that there was no significant inconsistency between the direct and indirect comparisons. The results of the NMA indicated that the patients who received *B. coagulans* (8 w) (SMD −2.13, 95% CI −2.84 to −1.41), *B. coagulans* (11 w/13 w) (SMD −1.61, 95% CI −2.46 to −0.76), and *S. cerevisiae* (10 w) (SMD −1.00, 95% CI −2.00 to −0.00) had lower abdominal pain scores than those who received PLA. Based on the results of the SUCRA (**Table S8** and **Figure S9**) and league table (**Table S9**), we found that *B. coagulans* (8 w), *B. coagulans* (11 w/13 w), and *S. cerevisiae* (10 w) ranked as the top three among all the interventions, while *S. boulardii* (4 w/6 w) ranked last.

Primary Outcomes: Effect of Different Probiotic Species on Bloating Scores

The effect of probiotics on abdominal bloating was reported in 13 RCTs, and the network plot is presented in **Figure 2E**.

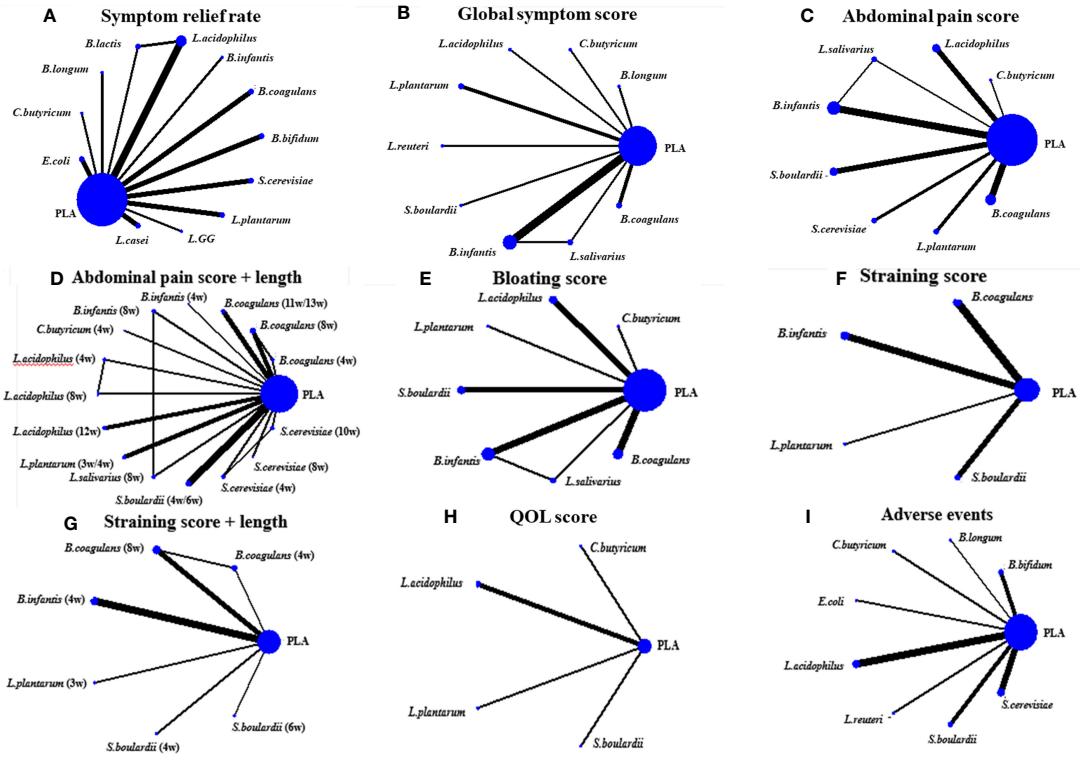

FIGURE 2 | The network plots. **(A)** was the network plot about the effect of probiotics on improving the symptom relief rate of IBS patients; **(B)** Global symptom score; **(C)** Abdominal pain score; **(D)** Bloating score; **(E)** Straining score; **(F)** QOL score; **(G)** Adverse events; **(H)** Subgroup analysis on treatment length of probiotics for improving the abdominal pain of IBS patients; **(I)** Subgroup analysis on treatment length of probiotics for improving the straining scores of IBS patients.

The consistency model was used as there was no significant inconsistency found in both the global and local inconsistency tests (**Table S10** and **Figure S10**). Only patients who received *B. coagulans* (SMD −1.42, 95% CI −1.87 to −0.96) had a significant improvement in abdominal bloating scores compared with those who received PLA. The SUCRA analysis (**Table S11** and **Figure S11**) and league table (**Table S12**) indicated that *B. coagulans* ranked best among all the other interventions, while *B. infantis* ranked second, *L. acidophilus* ranked third, and *L. plantarum* ranked last.

In this primary outcome, the heterogeneity across all treatment contrasts was significant (I^2 = 90.0%), which was in contrast to the loop-specific heterogeneity (τ^2 = 0). Additionally, the meta-regression analysis using treatment length and dose did not influence the SMD estimates for bloating sores significantly (**Figures 3G, H**).

Primary Outcomes: Effect of Different Probiotic Species on Straining Scores

There were only seven RCTs involved when comparing the effect of probiotics on straining scores. The network plot is shown in **Figure 2F**. Due to a lack of inconsistency resources, the consistency model was used. The results of the NMA showed that the patients who were administered *B. coagulans* (SMD −1.29, 95% CI −1.63 to −0.94) had lower straining scores than those who were administered PLA. Based on the SUCRA analysis in **Table S12** and **Figure S13** and the league table in **Table S14**, *B. coagulans* had the best rank among all other interventions in improving straining scores of patients with IBS, followed by *B. infantis* and *L. plantarum*; meanwhile, PLA ranked last.

The heterogeneity was significant across all treatment contrasts (I^2 = 88.7%) and the meta-regression analysis (**Figures 3I, J**) showed that treatment duration can significantly influence the efficacy of probiotics in improving the symptoms of straining (Coef = −3.15; p = 0.020) in patients with IBS. Therefore, we performed a subgroup analysis of treatment lengths (**Figure 2G**). Both the global and local inconsistency tests showed a significant inconsistency (**Table S13** and **Figure S15**), which indicated that the inconsistency model should be used. The NMA results revealed a significant improvement in symptoms of straining in the patients who received *B. coagulans* (8 w) (SMD −1.60, 95% CI −2.12 to −1.08) compared to those who received PLA. Additionally, the SUCRA (**Table S16** and **Figure S14**) and league table (**Table S17**) showed that *B. coagulans* (8 w) ranked first, *S. boulardii* (4 *w*) ranked second, *B. coagulans* (4 w) ranked third, while *S. boulardii* (6 w) ranked last.

Secondary Outcomes: Effect of Different Probiotic Species on QOL

The effect of probiotics on the QOL of patients with IBS was only reported in four RCTs. The network plot is shown in **Figure 2H**.

TABLE 2 | Odds ratio (OR) with 95% confidence interval on symptom relief rate.

B.coagulans												
3.89 (0.43,34.97)	**L.plantarum**											
20.27	5.21	**L.acidophilus**										
(3.47,118.40)	(0.72,37.92)											
23.13	5.95	1.14	**B.bifidum**									
(3.57,149.71)	(0.74,47.62)	(0.23,5.76)										
24.02	6.18	1.19	1.04	**B.longum**								
(2.04,283.25)	(0.44,86.09)	(0.12,11.67)	(0.10,11.08)									
26.05	6.70	1.29	1.13	1.08	**E.coli**							
(3.86,175.58)	(0.80,55.75)	(0.24,6.81)	(0.19,6.66)	(0.10,11.94)								
32.93	8.47	1.62	1.42	1.37	1.26	**C.butyricum**						
(3.56,304.61)	(0.76,94.16)	(0.21,12.29)	(0.17,11.78)	(0.10,19.55)	(0.15,10.84)							
40.65	**10.45**	2.01	1.76	1.69	1.56	1.23	**B.lactis**					
(4.83,341.86)	**(1.03,106.03)**	(0.41,9.74)	(0.24,13.13)	(0.13,22.30)	(0.20,12.12)	(0.12,12.92)						
43.74	**11.25**	2.16	1.89	1.82	1.68	1.33	1.08	**S.cerevisiae**				
(6.87,278.61)	**(1.42,89.04)**	(0.43,10.73)	(0.34,10.52)	(0.17,19.17)	(0.29,9.76)	(0.16,10.83)	(0.15,7.93)					
49.97	**12.85**	2.47	2.16	2.08	1.92	1.52	1.23	1.14	**B.infantis**			
(5.53,451.25)	**(1.18,139.74)**	(0.33,18.16)	(0.27,17.43)	(0.15,29.08)	(0.23,16.04)	(0.14,16.93)	(0.12,12.57)	(0.14,9.08)				
51.53	13.25	2.54	2.23	2.14	1.98	1.56	1.27	1.18	1.03	**L.GG**		
(4.56,581.63)	(0.99,177.27)	(0.27,23.89)	(0.22,22.71)	(0.13,36.21)	(0.19,20.83)	(0.11,21.44)	(0.10,16.03)	(0.12,11.85)	(0.08,13.84)			
66.15	**17.01**	3.26	2.86	2.75	2.54	2.01	1.63	1.51	1.32	1.28	**L.casei**	
(9.28,471.33)	**(1.94,149.18)**	(0.58,18.48)	(0.46,17.96)	(0.24,31.69)	(0.39,16.60)	(0.22,18.09)	(0.20,13.33)	(0.25,9.33)	(0.15,11.64)	(0.12,14.14)		
60.73	**15.62**	**3.00**	2.63	2.53	2.33	1.84	1.49	1.39	1.22	1.18	0.92	**PLA**
(14.83,248.61)	**(2.90,84.21)**	**(1.03,8.68)**	(0.77,8.95)	(0.33,19.15)	(0.64,8.44)	(0.33,10.31)	(0.30,7.37)	(0.42,4.61)	(0.22,6.59)	(0.16,8.47)	(0.23,3.60)	

Lower left triangle refers to the OR from the network meta-analysis. (e.g.,the OR [95%CI] of symptom relief rate between B.coagulans and placebo is 60.73[14.83-248.61]). The data in bold indicates that the effect size is statistically significant (P < 0.05).

The consistency model was used due to the lack of inconsistent resources. The NMA results showed that there are no treatment interventions better than PLA in improving the QOL of patients with IBS. The results of the SUCRA analysis, available in **Table S18, 19** and **Figure S15**, showed that *L. plantarum* ranked first in improving the QOL of patients with IBS; meanwhile, *S. boulardii* ranked second, *L. acidophilus* ranked third, and PLA ranked last. No significant heterogeneity was observed across all treatment contrasts (I^2 = 0.0%). Meta-regression by treatment length and dose did not significantly influence the SMD estimates for QOL (**Figures 3K, L**).

Secondary Outcomes: Adverse Events (AEs)

Total AEs were reported in 13 RCTs, and the network plot is presented in **Figure 2I**. The consistency model was used due to a lack of inconsistent resources. The NMA results revealed that only patients who received *L. acidophilus* had a lower incidence of AEs compared with patients who received PLA (OR 0.47, 95% CI 0.32, 0.67). Based on the SUCRA analysis (**Table S20 and Figure S16**) and league table (**Table S21**), *L. acidophilus* ranked first among all the other interventions, PLA ranked second, *L. reuteri* ranked third, and *C. butyricum* ranked last. Additionally, we observed significant heterogeneity (I^2 = 59.3%) across all studies in this outcome. Meta-regression by treatment duration and dose did not significantly influence the SMD estimates for the incidence of AEs (**Figures 3M, N**).

Comparisons of *B. coagulans* With Different Probotic Combinations for the Treatment of IBS

The evidences above revealed that *B. coagulans* was more effective in improving several IBS related symptoms than other probiotic species, thus, we further explored its efficacy compared to different types of probiotic combinations. Interestingly, based on the results from SUCRA analysis (**Figures S17-20** and **Tables S22-25**), we found that *B. coagulans* had the best rank among all the probiotic combinations in improving symptom relief rate, as well as global symptom, abdominal pain, and bloating scores. Simultaneously, the probiotic combinations 1B2L1S (with 1 strains of *Bifidobacterium*, 2 strains of *Lactobacillus*, and 1 strains of *Streptococcus*) ranked second in improving global symptom and abdominal pain scores.

Comparisons of Different Strains of *B. coagulans* for the Treatment of IBS

As for the symptom relief rate and global symptom scores, only *B. coagulans* Unique IS2 was involved, thus it is not difficult to conclude that *B. coagulans* Unique IS2 ranked first in improving the symptom relief rate and global symptoms of IBS relatively among all interventions. In terms of the ability to alleviate abdominal pain of IBS patients, the league table (**Table S26**) showed that *B.coagulans* MTCC5856 ranked first and *B.coagulans* Unique IS2 ranked second, which was consistent with the result of abdominal bloating scores (**Table S27**). Lastly, *B.coagulans* Unique IS2 also exhibited the highest probability to be the optimal strains in improving the symptom of straining (**Table S28**).

DISCUSSION

To date, the guidelines on the treatment of IBS with probiotics remain controversial. The British Society of Gastroenterology guidelines (Vasant et al., 2021) on the management of IBS, which

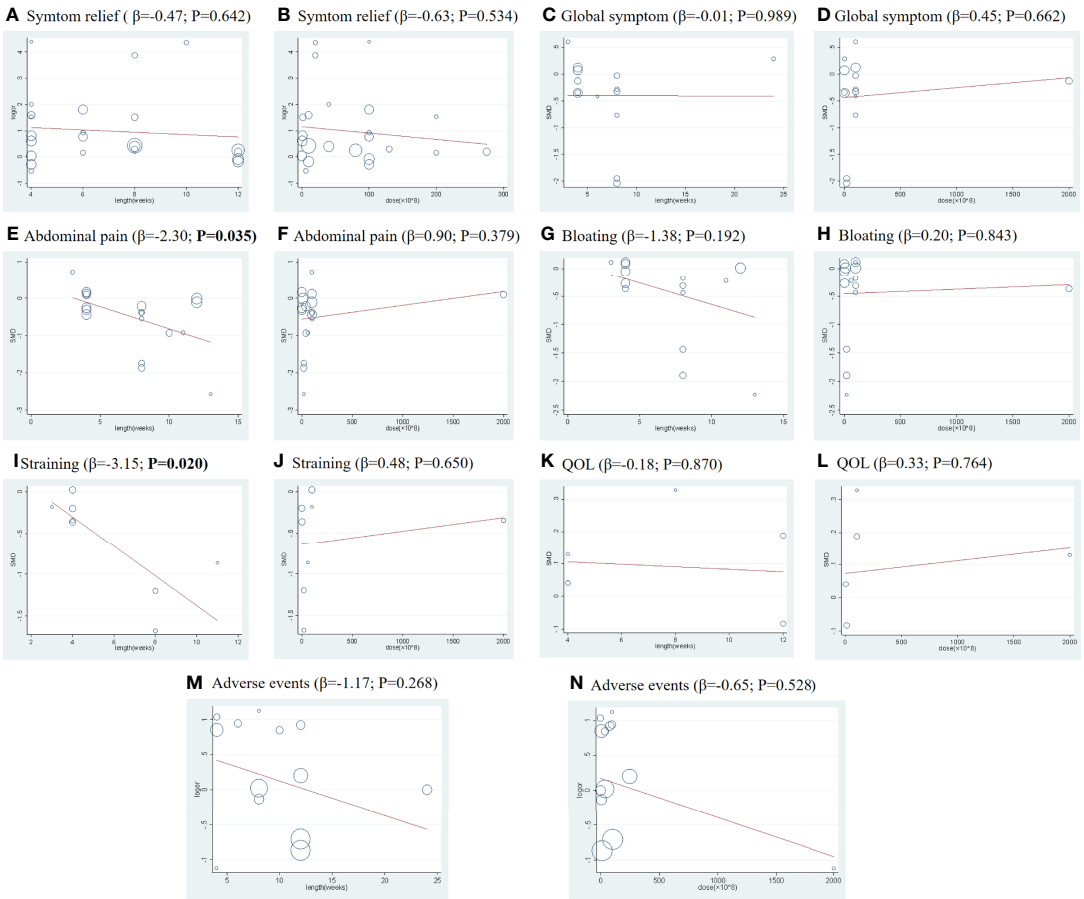

FIGURE 3 | Meta-regression by treatment lengths and doses for all primary and secondary outcomes: **(A, C, E, G, I, K, M)** indicate the meta-regression analysis by treatment lengths for the outcomes of symptom relief rate, global symptom score, abdominal pain score, bloating score, straining scores, QOL, and adverse events, respectively; **(B, D, F, H, J, L, N)** indicate the meta-regression analysis by doses for the outcomes of symptom relief rate, global symptom score, abdominal pain score, bloating score, straining scores, QOL, and adverse events, respectively. The abscissa (X) represents the duration (weeks) or dose (×108), and the ordinate (Y) represents the SMD.

was updated in 2021, reported that probiotics may be an effective treatment for improving global symptoms and abdominal pain in patients with IBS, which was consistent with the recommendations of the Canadian Association of Gastroenterology (Moayyedi et al., 2019) and the Japanese Society of Gastroenterology (Fukudo et al., 2021). In contrast, the guidelines from the American College of Gastroenterology (Lacy et al., 2021) suggest against the use of probiotics for the treatment of global IBS symptoms. Despite the controversies among different clinical practice guidelines, the effectiveness of probiotics in treating patients with IBS has not been completely validated before (Gwee et al., 2019) due to significant heterogeneity, publication bias, and inconsistent results in some meta-analyses, as well as several small sample size RCTs without rigorous endpoints based on US Food and Drug Administration (USFDA), and multiple types of probiotics without adequate validations, which may also contribute to the low level of evidence in the guidelines (Lacy et al., 2021).

To the best of our knowledge, this is the first study to simultaneously compare the efficacy of different probiotic species used for the treatment of IBS. The strength of this systematic review and NMA is that we performed a meta-regression analysis on the duration and doses of different treatments to explore whether these factors influence the outcomes, and if there were any influences, a subgroup analysis was conducted, adding rigor to our results. The main findings of our NMA were that *B. coagulans* was effective in increasing the symptom relief rate of patients with IBS, as well as in improving global symptoms, abdominal pain, bloating, and straining. Moreover, the meta-regression on treatment duration can significantly influence the SMD estimates of abdominal pain and straining scores, which indicates that increased treatment duration was a factor that negatively influenced both outcomes. The subgroup analysis of treatment durations indicated that the administration of *B. coagulans* for 8 weeks increased the effectiveness in the improvement of symptoms of abdominal pain and straining in patients with IBS. Unfortunately, due to insufficient original data of the included RCTs, NMA could not be performed to determine its efficacy in improving the QOL of

TABLE 3 | Standardized mean differences (SMDs) and 95% CI on global symptom scores.

B.coagulans									
-1.65 (-2.37,-0.93)	**C.butyricum**								
-1.59 (-2.63,-0.55)	0.06 (-1.07,1.19)	**B.longum**							
-1.67 (-2.37,-0.97)	-0.02 (-0.84,0.81)	-0.08 (-1.20,1.04)	**L.acidophilus**						
-1.70 (-2.47,-0.92)	-0.05 (-0.94,0.84)	-0.11 (-1.28,1.06)	-0.03 (-0.91,0.85)	**L.salivarius**					
-1.86 (-2.55,-1.18)	-0.21 (-1.03,0.60)	-0.27 (-1.38,0.84)	-0.20 (-0.99,0.60)	-0.17 (-1.03,0.70)	**S.boulardii**				
-1.94 (-2.42,-1.45)	-0.29 (-0.94,0.36)	-0.35 (-1.35,0.65)	-0.27 (-0.91,0.37)	-0.24 (-0.96,0.47)	-0.07 (-0.69,0.54)	**B.infantis**			
-1.99 (-2.39,-1.59)	-0.40 (-1.36,0.57)	-0.05 (-0.32,0.22)	-0.34 (-0.93,0.26)	0.28 (-0.45,1.01)	-0.29 (-0.95,0.37)	**-0.74 (-1.47,-0.01)**	**PLA**		
-2.27 (-3.10,-1.43)	-0.62 (-1.56,0.32)	-0.68 (-1.89,0.53)	-0.60 (-1.53,0.33)	-0.57 (-1.56,0.41)	-0.41 (-1.32,0.51)	-0.33 (-1.11,0.45)	-0.21 (-0.68,0.25)	**L.reuteri**	
-2.20 (-2.82,-1.58)	-0.55 (-1.31,0.20)	-0.61 (-1.68,0.46)	-0.53 (-1.27,0.21)	-0.50 (-1.31,0.31)	-0.34 (-1.06,0.39)	-0.26 (-0.80,0.28)	0.32 (-0.25,0.89)	0.07 (-0.80,0.94)	**L.plantarum**

Lower left triangle refers to the SMD from the network meta-analysis. (eg.,the SMD [95%CI] of global symptom scores between B.coagulans and placebo is -1.99[-2.39, -1.59]). The data in bold indicates that the effect size is statistically significant (P<0.05).

patients with IBS and the AEs associated with this disease. Additionally, *B. coagulans* still had significant effects in improving symptom relief rate, as well as global symptom, abdominal pain, and bloating scores compared to different types of probiotic combinations in present study, which further validated the pronounced efficacy of *B. coagulans*.

B. coagulans is a spore-forming bacteria widely used in commercial probiotic formulations owing to its outstanding properties which are partly associated with its encapsulated coating that can protect it from drought conditions and allow it to survive and proliferate in various secretions of the GI tract, such as gastric acid, pepsin, pancreatin, digestive enzymes, and bile (Mu and Cong, 2019). Additionally, it can produce a range of proteins, antimicrobial substances, and vitamins, as well as modulate the gut microbiome, strengthen the body's immunity (Elshaghabee et al., 2017; Maity et al., 2020), and treat various ailments such as *Helicobacter pylori* infection, gingivitis, and IBD. Although there are only a few RCTs regarding the use of different strains of *B. coagulans* for patients with IBS, their efficacy and safety are apparent. In two different studies, Madempudi et al. (Sudha et al., 2018; Madempudi et al., 2019) demonstrated that *B. coagulans* Unique IS2 was effective in relieving IBS-associated symptoms, such as abdominal pain, bloating, urgency, and straining, in improving stool consistency, and in increasing the serum anti-inflammatory factor IL-10 in children and adults with acceptable tolerability. Majeed et al. (2016) and Gupta et al. (Gupta and Maity, 2021) found that *B. coagulans* can improve the QOL of patients with IBS-D and significantly relieve the symptoms of diarrhea and constipation in the patients. To the best of our knowledge, our study is the first to compare the effectiveness of *B. coagulans* with other interventions and confirms the significant efficacy of *B. coagulans* in patients with IBS, especially at 8 weeks. Nevertheless, it is valuable to note that the benefits provided by probiotics are strain-specific rather than species-specific and

TABLE 4 | Standardized mean differences (SMDs) and 95% CI on abdominal pain scores.

B.coagulans								
-1.17 (-1.87,-0.47)	**S.cerevisiae**							
-1.45 (-2.31,-0.60)	-0.29 (-1.19,0.62)	**C.butyricum**						
-1.52 (-2.42,-0.63)	-0.35 (-1.30,0.59)	-0.07 (-1.13,1.00)	**L.salivarius**					
-1.56 (-2.18,-0.94)	-0.39 (-1.08,0.30)	-0.11 (-0.95,0.74)	-0.04 (-0.93,0.85)	**L.acidophilus**				
-1.60 (-2.18,-1.01)	-0.43 (-1.09,0.23)	-0.14 (-0.97,0.68)	-0.07 (-0.85,0.70)	-0.03 (-0.61,0.54)	**B.infantis**			
-1.71 (-2.45,-0.97)	-0.54 (-1.34,0.26)	-0.25 (-1.19,0.69)	-0.19 (-1.16,0.79)	-0.15 (-0.88,0.59)	-0.11 (-0.82,0.60)	**L.plantarum**		
-1.71 (-2.15,-1.27)	**-0.54 (-1.08,-0.00)**	-0.26 (-0.99,0.47)	-0.19 (-0.97,0.59)	-0.15 (-0.58,0.28)	-0.12 (-0.50,0.27)	-0.00 (-0.59,0.59)	**PLA**	
-2.03 (-2.68,-1.39)	**-0.86 (-1.58,-0.15)**	-0.58 (-1.45,0.29)	-0.51 (-1.42,0.40)	-0.47 (-1.11,0.17)	-0.44 (-1.04,0.17)	-0.33 (-1.08,0.43)	-0.32 (-0.79,0.15)	**S.boulardii**

Lower left triangle refers to the SMD from the network meta-analysis. The data in bold indicates that the effect size is statistically significant (P<0.05).

genus-specific (Majeed et al., 2016); therefore, the health benefits may vary based on different strains of *B. coagulans*. Thus, we compared the efficacy of different strains of *B. coagulans* for the treatment of IBS, which revealed that *B. coagulans* Unique IS2 exhibited the highest probability to be the optimal strains in improving symptom relief rate, global symptom scores, and the symptom of straining. Meanwhile, *B.coagulans* MTCC5856 ranked first in alleviating abdominal pain and abdominal bloating.

Increasing evidence, including the biopsychosocial model of IBS, suggests that in patients with IBS, psychosocial factors (anxiety, stress) can be secondary to abdominal symptoms (bottom-up); in turn, intestinal (physiological) functions, such as visceral sensitivity, motility, and stress reactivity of the gut can be impacted by psychosocial factors (top-down) (Fond et al., 2014). It is believed that both the gut microbiome and the gut-brain axis play an important role in the bidirectional signaling between the brain and the gut (Schmidt, 2015) through the neurological, endocrine, and immune pathways (Carabotti et al., 2015), especially *via* the former two pathways (Ng et al., 2018). It has also been reported that the gut microbiome has a direct influence on stress reactivity by stimulating the vagus nerve and the enteric nervous system (Ng et al., 2018), as well as by synthesizing and modulating neurotransmitters (Yano et al., 2015). Thus, in IBS cases, the disturbed QOL attributed to comorbidity of abdominal symptoms, extra-intestinal symptoms, and psychiatric symptoms (Creed et al., 2001; Spiegel et al., 2004) can be improved by alleviating IBS-related pain (abdominal symptoms) (El-Serag and Olden, 2002) by regulating the gut microbiome with probiotic therapies.

Interestingly, a previous study found that some probiotics, such as *Lactobacillus acidophilus* NCFM, can modify the expression of pain-associated receptors, such as μ-opioid and cannabinoid receptors, in the GI tract in mice and humans (Rousseaux et al., 2007; Ringel-Kulka et al., 2014), thereby improving the symptoms of abdominal pain. Some bacterial species, such as *Enterobacteriaceae* and *Clostridia*, are more prone to producing intestinal gas and generating abnormal patterns of short-chain fatty acids than others; thus, the imbalance in gut microbiota may exacerbate the symptoms of bloating (King et al., 1998; O'Sullivan and O'Morain, 2000). The modification of microbiota attributed to probiotics may improve bloating symptoms by decreasing the production of intestinal gas and promoting gut motility. Despite the presence of ample data regarding this issue, the precise mechanism of action of specific probiotic species or strains in improving the symptoms of IBS is still speculative and remains to be confirmed.

It is notable that the efficacy of probiotic combinations are not necessarily better than mono-strain probiotics in present study, which was consistent with the outcomes of a research performed by Ringel-Kulka et al. (2014). Due to the different probiotic combinations used in many studies, it is difficult for us to determine which probiotic combination is more effective for IBS patients. Therefore, multi-center clinical trials with large sample sizes are still needed. Moreover, incorporating *B. coagulans* into a probiotic combination, or genetically engineering it to amplify its biological function may be a future research target to treat IBS patients.

Our NMA has several limitations. First, although we investigated all RCTs with synthesizable data, a lack of available trials or trials with large sample sizes for direct comparisons remains, which may have influenced our results. Second, due to the limited original data, we were unable to evaluate more clinical indicators, such as bowel habits, stool consistency, gut motility, serum inflammation-related factors, and the gut microbiome. Third, the methodologies of included RCTs vary in design, population, diagnosis criteria, IBS subtypes, and durations, and the outcome measures were different, making it difficult to draw robust conclusions. Therefore, the results of this NMA should be interpreted with caution.

CONCLUSIONS

The findings of our NMA suggest that *B. coagulans* was particularly effective in improving symptom relief rate, as well as global symptoms, abdominal pain, bloating, and straining scores. Furthermore, patients with IBS who received *L. acidophilus* had a lower incidence of AEs than those who received other treatments. Although some of the included RCTs are underpowered due to limited number of cases and different outcome measures, the results of our study may be useful in establishing treatment guidelines for IBS using probiotics, considering that there are only a few reports in the literature that have made direct comparisons between individual therapies for IBS.

AUTHOR CONTRIBUTIONS

Guarantor of the article: LD is guarantor. Author contributions: TZ, CZ, JZ, FS, and LD conceived and drafted the study. CZ screened abstracts, TZ collected all data. TZ, CZ, JZ, and FS analyzed and interpreted the data. TZ and CZ drafted the manuscript. LD acquired the funding and performed critical revisions of the manuscript. All authors have approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.859967/full#supplementary-material

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REVIEW
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Mechanisms by Which Traditional Chinese Medicines Influence the Intestinal Flora and Intestinal Barrier

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The effect of a drug on the intestinal flora and the intestinal barrier is an important evaluation index for drug safety and efficacy. Chemical synthetic drugs are widely used due to their advantages of fast efficacy and low doses, but they are prone to cause drug resistance and inhibit proton pumps, which may harm intestinal health. Traditional Chinese medicine (TCM) has been applied clinically for thousands of years, and how TCMs regulate intestinal health to achieve their effects of disease treatment has become a hot research topic that needs to be resolved. This paper reviews the recent research on the effects of TCMs on intestinal microorganisms and the intestinal mucosal barrier after entering the intestine, discusses the interaction mechanisms between TCMs and intestinal flora, and details the repair effect of TCMs on the intestinal mucosal barrier to provide a reference for the development, utilization, and modernization of TCM.

Keywords: traditional Chinese medicine, intestinal flora, intestinal mucosal barrier, effect mechanism, review

INTRODUCTION

In recent years, with the rise in public attention to intestinal health, the intestine has become a research hotspot in the field of traditional Chinese medicines (TCMs). The intestinal flora and intestinal barrier are likely to be important targets through which most Chinese medicines exert their effects and treat diseases. Studies have shown that the balance of intestinal flora and the stability of the intestinal barrier system are the basis for the physiological functions of the intestinal tract. Intestinal flora is a general term for the bacteria living in the human intestine. They help maintain a good environment in the intestine, and their physiological function has become an indispensable part of the physiological function of the host, playing an active role in material anabolism, catabolism, etc (Valdes et al., 2018). The intestinal barrier system refers to a functional isolation belt that prevents harmful substances in the intestinal lumen from entering the blood circulation, to maintain the relative stability of the body's internal environment and maintain the normal life activities of the body (Duan et al., 2019). In the process of evolution, the intestine and its microbiota have come to complement each other and jointly maintain the health of the body, but when they are stressed by factors from the environment, diet, or drugs, their function will be seriously affected, thereby inducing intestinal metabolic disorders, raising the chances of pathogens invading the body, and increasing the risk of diseases such as diabetes, obesity, and metabolic syndrome (Lin et al., 2021). At present, chemical synthetic drugs dominate in the treatment of diseases caused by intestinal problems, but their antibiotics, proton pump inhibitors and other

components also subtly endanger human intestinal health in the process of treating diseases. TCMs, with the advantages of mild antibacterial activity, reparative action, and the fact that humans do not easily develop resistance to them, have gotten much attention for the possibility of microflora balance regulation and intestinal barrier repair. However, due to the complexity and diversity of the active ingredients of TCMs, their interactions with the intestinal flora and their repair mechanisms of the intestinal barrier are not fully clear. This paper summarizes the mechanisms by which TCMs influence the intestinal flora and the intestinal barrier system as discovered in recent years to provide a reference for the development, utilization, and modernization of TCM. (The article map is shown in **Figure 1**)

INTERACTION MECHANISM OF TCM AND INTESTINAL FLORA

The interaction between TCMs and intestinal flora is bidirectional: on the one hand, TCMs can regulate the structure and metabolic function of the flora by selectively inhibiting or promoting the growth of different types of intestinal microorganisms, thereby promoting human health. On the other hand, the intestinal flora will metabolize TCMs, which may increase efficacy or reduce toxicity, or may generate toxic metabolites.

Mechanisms by Which TCMs Influence the Intestinal Flora

Normal flora mainly plays a positive physiological role in the gastrointestinal tract in the following four aspects. (1) Maintain the stability of the intestinal environment: the normal flora has the physical barrier function to resist the invasion of foreign bacteria and prevent the translocation of opportunistic pathogenic bacteria, and can also provide some nutrients for intestinal mucosal cells (Pushpanathan et al., 2019). (2) Involved in substance metabolism: Intestinal microflora contains more metabolic enzymes than the host genome, so it has more powerful metabolic functions. Its metabolites, such as short-chain fatty acids, secondary bile acids, sulfides and indoles, are key factors for improving the progress and prognosis of diseases based on intestinal flora (Sittipo et al., 2019). (3) Enhance immune function: the intestine is one of the organs with the most immune cells in the human body. Intestinal bacteria and their metabolites can regulate the immune system through various ways to promote the maturity of immune cells and the normal development of immune function (Belkaid and Harrison, 2017). (4) Affect brain function and behavior: gut microbiota can be associated with the central nervous system, affecting brain function and host behavior by regulating anxiety, emotion, cognition and pain (Xu et al., 2020). When the environment, diet, drugs and other factors cause the flora imbalance, the above functions may be affected, and severe cases may cause various system diseases as shown in **Figure 2**. According to the relevant data, dysbacteriosis can lead to significant changes in the expression of functional genes of flora and the activity of metabolic related enzymes, thereby affecting their normal physiological functions (Nogacka et al., 2019). At the same time, it can also cause the destruction of intestinal barrier, leading to the occurrence of toxic metabolites into blood and bacterial translocation, resulting in the body's inflammatory reaction to occur or worsen. In addition, the number and function of regulatory T cells are also hindered by intestinal flora disturbance, resulting in weakened response regulation of T cells to Th1, Th2 and Th17 effector cells, affecting the normal immune function of the body (Matsuoka and Kanai, 2015). These changes will have a serious impact on host health.

Clinical aseptic models or fecal transplantation have proved that intestinal flora can be used as targeted bacteria to achieve the purpose of TCM treatment of diseases, mainly reflected in the influence of the active ingredients of TCMs on the structure, composition and metabolites of intestinal flora. For example, Wuyao extract can regulate intestinal flora disturbance and affect bile acid metabolism, thereby improving hyperlipidemia (Jiang et al., 2021). Through PCR-DGGE and LC-MS detection, it was found that the number of *Bifidobacterium* and *Lactobacillus* and the content of short-chain fatty acids in the cecum of the constipation model rats treated with hemp seed oil increased, while the proportion of harmful bacteria decreased significantly, which effectively improved the constipation symptoms of rats (Hanbing et al., 2018). Other TCMs also showed good

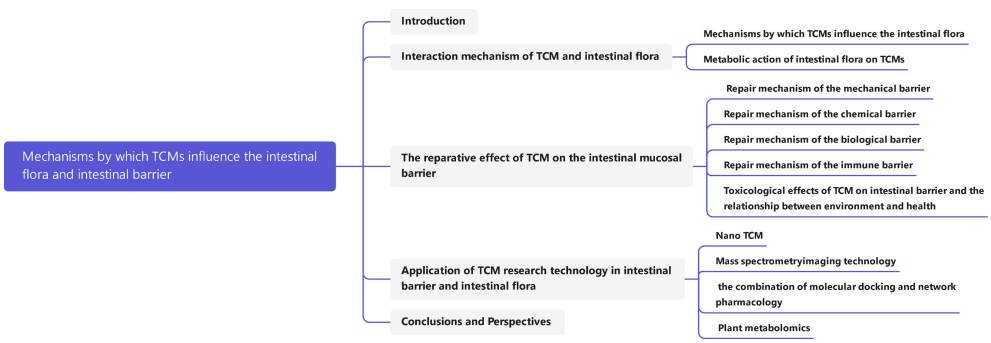

FIGURE 1 | Article Mind Map.

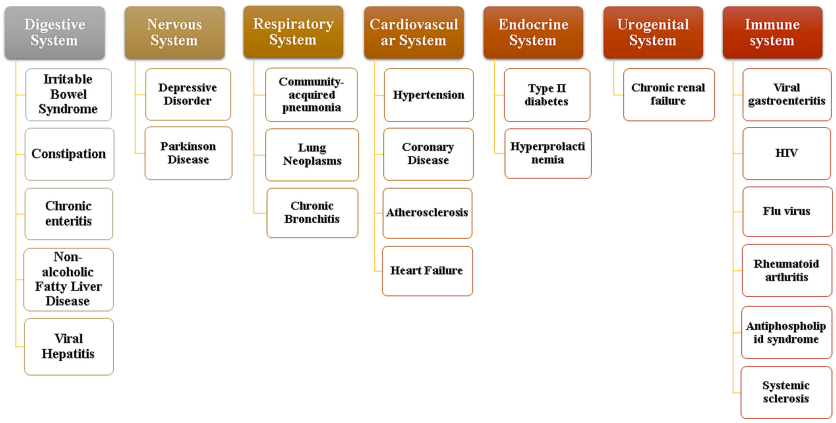

FIGURE 2 | System diseases involving intestinal flora.

therapeutic effects on improving corresponding diseases based on intestinal flora and its metabolites, as shown in **Table 1**. At the same time, relevant studies have shown that the interaction between TCM and TCM can achieve the effect of reducing toxicity, for example, *Aconitum carmichaeli Debx* has great toxicity, often combined with *Radix ginseng* for the treatment of cardiovascular system, respiratory system and nervous system diseases. Compared with *Aconitum carmichaeli Debx* alone, the combination of *Aconitum carmichaeli Debx* and *Radix ginseng* can improve the intestinal flora of normal rats and promote the proliferation of beneficial bacteria, and when the ratio of the two was 1:2, the content of *Lactobacillus* in the intestinal tract was higher than that of the *Aconitum carmichaeli Debx* alone and the ratio of 1:1 compatibility group (Tang et al., 2018). But when the side effect of TCM is strong or the combination of TCM is wrong, it may not achieve the therapeutic effect or even aggravate the disease. For example, Yuanhuapin is both as active and toxic ingredient in *Genkwa flos*. It can cause intestinal flora disorder in normal rats, resulting in significant changes in the contents of phenylacetylglycine, maleic acid and 3-ethyldioxyindole, the metabolites of intestinal flora, thereby affecting amino acid metabolism, lipid and glucose metabolism and other metabolic pathways, leading to toxic reactions in intestine and liver (Chen et al., 2016). Qianjinzi is similar to *Euphorbia* in "the eighteen incompatible medicaments" in terms of efficacy, basis and chemical composition. Using it in combination with *Glycyrrhiza uralensis Fisch* increased the number of harmful bacteria such as *Enterococcus* and S24_7_ukn, and enhanced the metabolic capacity of intestinal flora at the same time, which increases the content of toxic substances such as indole and p-cresol, thereby aggravating the intestinal injury in mice. (Tao et al., 2018).

Metabolic Action of Intestinal Flora on TCMs

Scientific research has shown that the intestinal microbiome encodes about 3.3 million genes, far more than humans. They have many enzymes that the human body does not have, and play an important role in the metabolism and transformation of TCM (Qin et al., 2010). Relevant studies have proved that intestinal flora can produce low polarity and relatively stable molecular mass of TCM metabolites through hydrolysis, oxidation, reduction and isomerization reactions, which can accelerate the intestinal absorption and improve the bioavailability of TCM (Xu et al., 2017). For example, Most glycosides in Huangqin Decoction can be digested and absorbed by the body through the catalytic deglycosylation of intestinal flora (Zuo et al., 2002). The ginsenosides can be transformed into hydrophobic compounds under the combined action of gastric juice and intestinal microorganisms, such as protopanaxadiol-type ginsenosides are mainly converted into compounds K and ginsenoside Rh2. Compared with protopanaxadiol-type ginsenosides, the transformed metabolite compound K exhibits more effective pharmacological effects such as antitumor, anti-inflammatory, antidiabetic, antiallergic and neuroprotective (Kim et al., 2018). At the same time, intestinal flora also has the effect of reducing toxicity to TCMs. For example, *Aconitum carmichaeli Debx* is widely used in clinical practice, but it must be used with caution because of its high toxicity. According to reports, in addition to processing and compatibility to achieve attenuation, it can also achieve attenuation through intestinal flora metabolism.

On the other hand, the side effects of intestinal flora on TCM are also worthy of further study. In recent years, many cases of bitter amygdala poisoning have been also reported. For example, the normal rats receiving 600 mg/kg amygdalin showed symptoms of drowsiness, convulsions, and death within 2 to 5 hours, while sterile rats did not show obvious symptoms of poisoning at the same dose, when both groups were received at a non-toxic oral dose of 50 mg/kg, the recovery rate of amygdalin in normal rats was lower than that of sterile rats, and only amygdalin was detected in sterile rat feces (Carter et al., 1980). Further studies have shown that oral amygdinoside was hydrolyzed by β-glucosidase of the intestinal flora to produce the toxic substance hydrocyanic acid, which triggers a serious toxic reaction (Liu et al., 2017). Of course, the intestinal flora also

TABLE 1 | Regulation of intestinal flora and its metabolites by TCMs.

TCM	Animal models	Effect on intestinal flora abundance	Effects on gut microbiota metabolites	Therapeutic effect	Mechanism of action	Document
Barley leaf	Colitic mice	*Proteobacteria*↑*Enterobacteriaceae*↓	Inosine, Guanosine↑	Reduces the severity of disease and microbial imbalance	Adenosine is produced by gut microbes to activate PPARγ signalling	(Li et al., 2021)
Gegen cenlian decoction	Type 2 diabetic rat	*Faecalibacterium, Roseburia*↑	SCFAs↑	Reduces systemic and local inflammation in rats	Increases the content of butyric acid	(Xu et al., 2020)
Ginseng	Obese mice	*E.faecalis*↑	Nutmeg oleic acid↑	Antiobesity action	Activates BAT and form brown fat to increase energy metabolism	(Quan et al., 2020)
Pu-erh tea	Hyperlipidaemia mice	*Lactobacillus, Bacillus, Enterococcus, Lactococcus, Streptococcus*↓	Cholesterol, Fat↓	Decreases liver and serum cholesterol levels	Inhibits microorganisms associated with bile salt hydrolase activity and increases ileal binding bile acid levels	(Huang et al., 2019)
Rhubarb	Ulcerative colitis mice	*Lactobacillus*↑	Uric acid↓	Alleviates dextran sulfate sodium-induced ulcerative colitis	Reduces the concentration of uric acid, the end product of intestinal purine metabolism	(Wu et al., 2020)
Luteolin	Ulcerative colitis mice	*Lactobacillus/Prevotella_9*↓	Amino acids, Starch, Sucrose↑	Colonic injury significantly reduced and inflammation effectively improved	Inhibition of inflammatory factors expression	(Li et al., 2021)
Radix Paeoniae Alba	Autoimmune thyroiditis rats	*Lactobacillus, Prevotellaceae, Romboutsia*↑ *Firmicutes*↓	SCFAs↑	Adjusts the composition and diversity of intestinal flora and improves intestinal mucosal injury	Regulation of inflammatory factors and sIgA to alleviate thyroid follicular injury and colonic mucosal lesion	(Mu et al., 2021)
GeGen QinLian decoction	Influenza virus infectious mice	*Akkermansia_muciniphila, Desulfovibrio_C21_c20, Lactobacillus_salivarius*↑ *Escherichia_coli*↓	—	Effectively protecting mice from influenza virus-infected pneumonia	affect systemic immunity, at least in part, through the intestinal flora, thereby protect the mice against influenza virus infectious pneumonia	(Deng et al., 2021)
Wenyang Jiedu Huayu prescription	HBV related liver failure	*bifidobacterium*↑ *enterobacteria*↓	Endotoxin↓	Effectively reduce endotoxemia and improve clinical efficacy	Reversing intestinal flora imbalance	(Wen-Fang et al., 2014)
Ephedra sinica	H1N1 virus infected mice	*Lactobacillales, Bifidobacteriaceae*↑	SCFAs↑	Significantly treats acute lung injury caused by H1N1	Regulate the type of bacteria and metabolites and inhibit the release of inflammatory factors	(Xiaoting et al., 2020)

↓ refers to decline, ↑ refers to increase, — refers to no effect.

has an improving effect on the toxic side effects of TCMs. For example, aconitine Chinese medicines such as Chuanwu and Caowu have been confirmed that they can be converted into mono- and di-lipids with lower toxicity under the acylation and esterification of intestinal microorganisms (Zhao et al., 2008).

To sum up, the interaction between TCMs and intestinal flora is both positive and negative, and the two complement each other and are closely related (**Figure 3**), showing great potential in the treatment and prevention of diseases. In addition, the recent application of TCMs in the treatment of novel coronavirus and influenza viruses has achieved good efficacy. Some studies have reported that the treatment of virus-related diseases with TCM may be related to the intestinal microecology, such as Houttuynia cordata can reverse the composition change of intestinal microbiota caused by H1N1 infection, with significantly reduced relative abundances of Vibrio and Bacillus, the pathogenic bacterial genera (Chen et al., 2019). The latest researches have shown that the gut microbiota plays an important role in the progression of COVID-19, and that gut microbiota imbalance and endotoxemia may accelerate the progression of COVID-19 (Gou et al., 2020). Many TCMs can support virus-affected body organs and systems, restore the structure of the gut flora, and exhibit properties relevant to COVID-19 treatment (Chen et al., 2021). In-depth exploration of the interaction mechanism between TCM and intestinal flora may provide new research directions and therapeutic targets for diseases including the COVID-19.

THE REPARATIVE EFFECT OF TCM ON THE INTESTINAL MUCOSAL BARRIER

The intestinal mucosal barrier is a highly selective functional barrier system present in the intestine (Kraehenbuhl et al., 1997) that can prevent harmful substances such as pathogenic microorganisms and endotoxins from passing through the intestinal mucosa while selectively absorbing nutrients in the intestine. It plays an important role in immune defence and maintaining intestinal mucosal integrity. Under normal conditions, the intestinal mucosal barrier can be divided into a mechanical barrier, biological barrier, chemical barrier, and immune barrier, which jointly exert the function of the

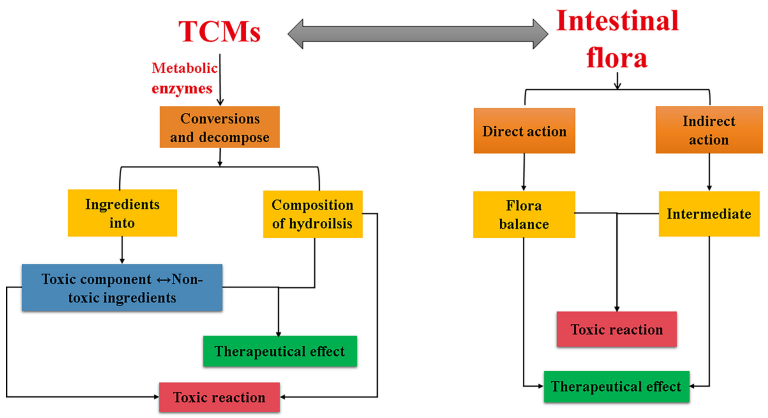

FIGURE 3 | Interaction between TCM and intestinal flora.

intestinal mucosal barrier. TCM can repair the intestinal mucosal barrier in many ways to protect barrier function.

Repair Mechanism of the Mechanical Barrier by TCM

The mechanical barrier, also known as a physical barrier, is the front-line guard that prevents harmful substances penetrating the intestinal mucosa and is the most important of the intestinal mucosal barriers. Normally, the mechanical barrier is mainly composed of a tight connection between the intestinal mucosal epithelium and cells. When the blood supply of the intestinal mucosa is insufficient, that is, a microcirculation disorder of the intestinal mucosa occurs, the integrity of the intestinal mucosa will be harmed, increasing the permeability of the intestinal mucosa, thereby weakening the selective permeation and barrier function. Many studies have shown that TCMs can maintain the mechanical barrier function by improving the microcirculation of the intestinal mucosa, repairing the integrity of the intestinal mucosa, and reducing the permeability of the intestinal mucosa. Under normal circumstances, the mammalian intestine lacks enzymes that decompose D-lactic acid (D-LA), and the level of diamine oxidase (DAO) in the body is also low; these substances are very rare in the blood. When the mechanical barrier of the intestinal mucosa is broken, the intestinal mucosa will be fully permeable, so they can enter the blood through the intestinal mucosa, and the integrity of the intestinal mucosa can be accurately reflected by measuring these indicators (Le, 2019). The intestinal mucosa also contains tight junction proteins such as occludin, which are important components in the integrity of the intestinal mucosa. Huanglian Jiedu Decoction combined with electroacupuncture can significantly reduce serum DAO levels and serum d-lactic acid levels in critically ill patients undergoing abdominal surgery, thus accelerating the repair of the intestinal mucosal mechanical barrier (Wang et al., 2015). Clinical studies have shown that TNF-α, IL-6 and other inflammatory factors can increase vascular endothelial cell permeability and weaken intestinal mucosal barrier function (Xie et al., 2019). A study also demonstrated that *Bletilla striata* polysaccharide can upregulate the expression of the occludin protein in mice with ulcerative colitis, thereby improving the function and integrity of the epithelial barrier (Li et al., 2021).

Repair Mechanism of the Chemical Barrier by TCM

The intestinal mucosa chemical barrier is composed of various chemicals, such as mucus, glycoproteins, various digestive enzymes, and lysozymes, which are secreted by epithelial cells of the intestinal mucosa, and bacteriostatic substances, which are secreted by the intestinal flora. These secretions can change the attack site of pathogenic bacteria or opportunistic pathogenic bacteria, affect the colonization ability of bacteria, and mainly play a role in inhibiting bacteria and regulating the intestinal environment (Zhai, 2014). TCM can affect the secretion of mucus and the composition of the mucus layer by regulating the number and secretion capacity of intestinal mucosal epithelial cells and creating a suitable living environment for some intestinal microorganisms while inhibiting others. Study has confirmed that Gegenqinlian Decoction (GQ) regulated the activity of Notch signalling by a bidirectional mechanism, promoted the proliferation and differentiation of goblet cells to accelerate the secretion of viscoelastic gels, and helped complete the repair of the intestinal mucosal epithelium (Zhao et al., 2020). *Aloe vera* significantly up-regulated the expression of mucins (such as MUC2 and MUC5AC) in ulcerative colitis rats, and increased the thickness of mucous layer in colon, thereby accelerating the repair of intestinal mucosa (Shi et al., 2021). In addition, *Blautia* can protect the intestine by producing antibacterial substances and compete for intestinal adhesion sites to inhibit pathogenic bacteria, thereby enhancing intestinal barrier function (Jiang et al., 2018). And ellagic acid can enhance the activities of digestive enzymes such as lactase, sucrase and alkaline phosphatase in jejunum of mice, so as to promote intestinal development and improve antioxidant capacity of mice (Xu et al., 2021). Another study confirmed that *Rhodiola crenulata* can reduce blood endotoxemia by

increasing the expression of tight junctions (zonula occlusion-1 and agglutinin) and antimicrobial proteins (Reg3g and lysozyme C) in the small intestine, and improve obesity in mice by regulating the balance of flora (Chang et al., 2018).

Repair Mechanism of the Biological Barrier by TCM

The intestinal mucosal biological barrier is a layer of the bacterial membrane barrier formed by the attachment of intestinal microorganisms to the intestinal mucosa. It has important value in nutrient absorption, immune defence, and metabolic balance. The intestinal microbiota is a unique and diverse ecosystem, and it is also one of the systems with the highest known cell densities (Lei, 2012). The balance of this system is closely related to obesity, hypertension, and other diseases, and regulating the balance of the microbiota has become a key point in the prevention and control of diseases today. According to relevant studies, external or internal factors are highly likely to affect the attachment sites of certain bacteria on the intestinal surface by altering the glycan structure of the intestinal mucosa, thereby indirectly selectively stimulating the instantaneous growth of certain bacteria to destroy the balance of flora, and eventually lead to the occurrence of intestinal diseases. The repair of biological barriers by TCM is mainly achieved by adjusting the balance of microbial groups or increasing the relative abundance of dominant microbes and the balance between flora and host. Studies have found that the active ingredient in *Pogostemon cablin* can significantly increase the relative abundance of probiotics such as *Lactobacillus* and *Bifidobacterium* and reduce the relative abundance of harmful bacteria such as *Parabacillus*, *Bacteroides*, and *Helicobacter pylori*, thus regulating the balance of the flora and maintaining the intestinal mucosa biological barrier function (Wu et al., 2020). There are many kinds of intestinal microorganisms. Now people only know about the tip of the iceberg, and there are many strains that have not been found. It is an urgent problem to study the metabolic mechanism of intestinal microorganisms in the field of medicine.

Repair Mechanism of the Immune Barrier by TCM

The main function of the intestine is to realize the digestion and absorption of substances. According to clinical studies, it has more higher concentration of lymphocytes than lymphoid tissues, and they are extremely important immune barriers in the gastrointestinal mucosa (Malaisé et al., 2020). They resist the damage of pathogenic antigen attack by humoural immunity and cellular immunity and are one of the main sites of the body's immune defence actions. Secretory immunoglobulin (S-IgA) is synthesized and secreted by plasma cells in the intestinal mucosa and is the most secreted immunoglobulin in the body. S-IgA can prevent or weaken the invasion and colonization abilities of antigenic substances and can also promote phagocytosis of antigens by phagocytes (Liu et al., 2015). The body's immune defence process is inseparable from the existence of such immune substances. TCM can improve the body's immunity to resist the invasion of external germs and can also fight intestinal inflammation by regulating the levels of pro-inflammatory and anti-inflammatory factors in the body. Research confirmed that Hetiao Jianpi Decoction can reduce plasma DAO and lactic acid levels and increase SIgA levels in antibiotic-associated diarrhea (AAD) rats to repair intestinal mucosal permeability and immune function (Li et al., 2020). Clinical studies have shown that GQD can reduce mouse PD-1, increase IL-2, and restore T cell function (Lv et al., 2019), thereby exerting immune function. In a study of the effect of Astragalus polysaccharide (APS) on mucosal immunity, it was confirmed that TCM can improve the overall immunity level of mice at the nonspecific immunity, humoural immunity, cellular immunity, and mucosal immunity levels (Xia et al., 2011). Another study showed that TCM can stimulate the body's immunity by regulating intestinal flora. The Sonnenberg team in the United States performed faecal bacterial transplantation in mice to observe the relationship between ILC3 cells and the body's immunity, and their results confirmed that ILC3 cells interact directly with TH17 to promote the production of TH1 cells and CD8+ T cells, and this interaction occurs under the action of specific intestinal microorganisms that exert intestinal-specific immune functions to resist tumourigenesis (Goc et al., 2021).

In the theory of Chinese medicine, the TCM could be used as a trigger or an enhancer to start the immune vitality of organs such as the spleen, and improves the body's immune defence ability. Regarding the four layers of the intestinal mucosal barrier (**Figure 4**), basically maintain integrity of the intestinal mucosa, block the invasion of the harmful material. Studies have shown that Shaoyao Decoction can repair the intestinal mucosal barrier by regulating the expression of the Muc1, Muc2, Muc4, and Tff3 genes in the mucus layer and the epithelial barrier genes ZO-1 and Occludin. Shaoyao Decoction can also reduce the levels of proinflammatory cytokines, improve the anti-inflammatory ability of colon tissue, and increase the secretion of mucus to repair the mucosal epithelium (Chi et al., 2021). Many research results have supported that TCMs can direct effects on the intestinal tract, activate the expression of related genes and signaling pathways, or repair intestinal mucosa by regulating microbial metabolites (**Figure 5**). But it is unknown whether the intestinal parts other than the intestinal flora are related to TCM. From the perspective of intestinal absorption, the effect of intestinal tract on TCM is likely to be reflected by affecting the absorption of medicinal ingredients.

Toxicological Effects of TCM on Intestinal Barrier and the Relationship Between Environment and Health

Most TCMs have a good repair effect on intestinal mucosal barrier and intestinal flora, but some TCMs have also brought certain damage to the intestine while achieving the effect of treating certain diseases. It has been confirmed that soya-saponins can reduce the mucosal fold height, induce the proliferation and apoptosis of intestinal cells, increase epithelial

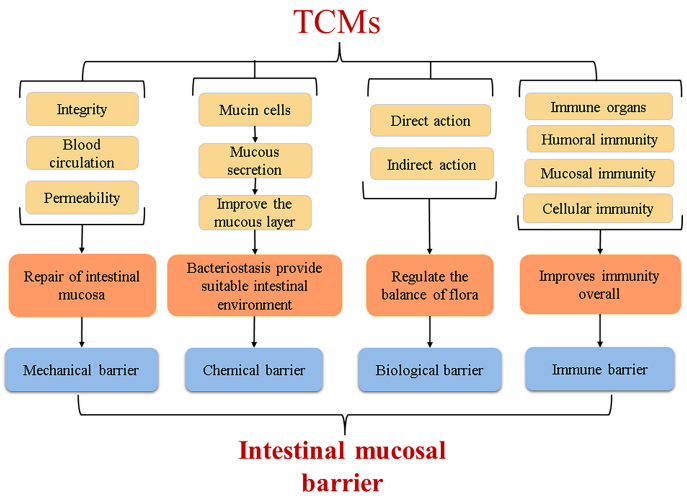

FIGURE 4 | The effect of TCMs on the intestinal mucosal barrier.

permeability, destroy intracellular connections and damage intestinal antioxidant system, eventually lead to intestinal mucosal damage (Gu et al., 2018). Soybean lectin (SBA) can bind to small intestinal epithelial cells, change the glycan structure of small intestinal mucosa, and further change the attachment sites of certain bacteria on the intestinal surface, thereby selectively stimulating the instantaneous growth of certain bacteria. On another level, SBA induction provides bacteria with rich nutrients (e.g. loss of serum proteins and increased intestinal cell loss). Besides, SBA can also destroy the intestinal mucosal immune system and reduce the secretion of immunoglobulin A (IgA), thereby inhibiting bacterial proliferation and aggravating the deterioration of intestinal mucosa (Pan et al., 2018). In addition, the planting environment of TCM has a certain influence on the effect of intestinal therapy. Toxicity of TCMs is caused not only by toxic medicinal compounds, but also by pollutants such as pesticides, herbicides and heavy metals, which can adversely affect the intestines through the human body (Feng et al., 2021). For example, aluminum accumulated in plants can promote the apoptosis of intestinal epithelial cells, destroy the structure of tight junction proteins, increase intestinal permeability;induce the activation of immune cells to secrete inflammatory factors and trigger immune response;regulate intestinal composition and enzyme activity; induce the imbalance of intestinal flora, inhibit the growth of beneficial bacteria, promote the proliferation of harmful bacteria, and then damage the four barrier of intestinal mucosa in an all-round way (Hao et al., 2022). The environment is closely related to human health. In the great cycle of nature, we should also deal with the relationship between nature and human beings, so as to achieve a win-win situation between environment and health.

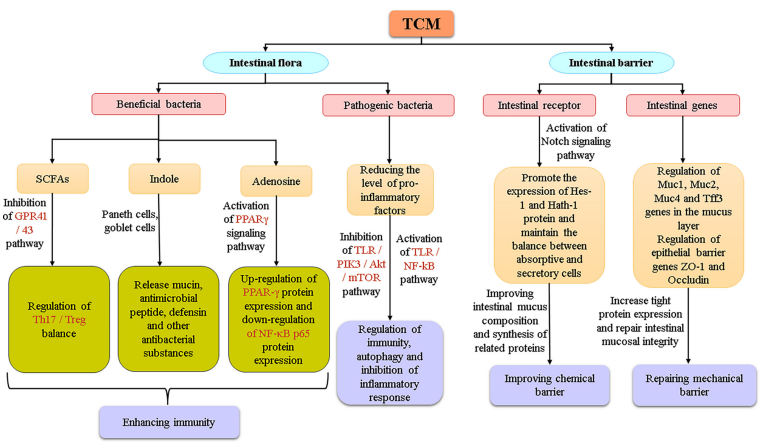

FIGURE 5 | The relationship between intestinal flora and the intestinal barrier system under the action of TCM.

APPLICATION OF TCM RESEARCH TECHNOLOGY IN INTESTINAL BARRIER AND INTESTINAL FLORA

The material basis of TCM for disease prevention and treatment comes from biological active parts or active chemical components, but the intestinal absorption, metabolism and excretion of drugs by biological organism is an extremely complex process. TCM is expected to develop into nano drug delivery system through careful design of nanotechnology, which comprehensively improve the medicinal value of TCM for curing and preventing diseases (Zheng et al., 2021). Nano traditional Chinese medicine (Nano TCM) refers to the effective components, original drugs and compound preparations of TCM made by nanotechnology with diameter less than 100 nm. Nano TCM includes many techniques, such as nano carrier, solid dispersion and so on. It is not only used to crush the drug to nanometer level, but also to process the prescription composition of the effective part or active component of the drug through nanotechnology, giving TCM new functions. Although nanotechnology is widely used and gradually improved, the nanotechnology of TCM is still in its infancy, and its development space needs to be excavated.

Mass spectrometry imaging technology is a high-throughput method to detect and image the metabolic changes of various components of TCMs. As a new analytical imaging technology, this method is fast and sensitive which does not require complex TCM extraction and separation. In addition, this technology can also be used to further focus on potential biomarkers, which has laid a certain foundation for the study of the interaction mechanism between TCMs and intestinal flora, and has unique characteristics in metabolic analysis, quality control and mechanism of action exploration. Advantages to help establish quality standards, explore the safety and toxicology of TCMs (Jiang et al., 2022).

Furthermore, the combination of molecular docking and network pharmacology has greatly promoted the prediction of bioactive components and shed a light on the mechanism study of TCM affecting on the intestine. Molecular docking is a computer technology based on structural design, while network pharmacology has established a powerful and comprehensive database to understand the relationship between TCM and disease networks. The combination of this two provides a theoretical basis and technical support for the construction of modern TCM based on component compatibility, and also provides a new way for the exploration of the repair mechanism of TCM on the intestinal barrier and intestinal flora (Jiao et al., 2021).

In addition, a newly developed technology can also identify the effect parts of TCM. Plant metabolomics, which could explore the interactions between plant metabolites by addressing key network components among plant small molecules, has made significant contributions to understanding the relationship between genotype and metabolic output (Hong et al., 2016). It can characterize the dynamic changes of plant metabolites and has the ability of holistic analysis, which conforms to the holistic view theory of TCM and can reflect the holistic effect of exogenous substances on organisms. This approach is good suitable for analyzing complex systems such as TCM, and is conducive to the exploration of various factors in intestinal repair (Zhang et al., 2019).

At present, the research on the mechanism of intestinal repair by TCM remains on the surface. However, based on the continuous improvement of science and technology, it is gradually systematic to explore the pathways, targets and repair mechanisms of TCM in the treatment of intestinal tract. With the continuous development of technology and the continuous improvement of scientific level, the specific mechanism behind it will eventually be clearly explored.

CONCLUSIONS AND PERSPECTIVES

TCM has a plentiful supply of natural biological components, which can comprehensively regulate the organs of the body, strengthen the spleen and lungs, regulate yin and yang, and have a remarkable effect on the defense and treatment of clinical diseases such as neurology and metabolic disorders. According to studies, 90% of the human body's diseases are related to the intestine, and intestinal regulation is increasingly recognized as the foothold and breakthrough point of diseases. From the perspective of TCM, people's various organs are not independent but are all interconnected and together influence the whole-body physiology. Therefore, TCMs regulate intestinal function through very complex mechanisms. Although the intestinal mucosal repair mechanisms of TCMs have not been completely and systematically detailed, their repair effect is obvious by now. There is also nanoimaging technology that can be used to track TCMs in the intestine, another avenue researchers are using to discover their mechanisms of action. We believe that the comprehensive regulatory mechanisms of TCMs in intestinal health will be gradually revealed in the future.

AUTHOR CONTRIBUTIONS

D-LX conceived, supervised and writing-reviewed the manuscript, QC and TL originally wrote and writing-reviewed the draft, JS and YH cofounded and co-administrated the project. All authors approved the final version.

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New Procedure to Maintain Fecal Microbiota in a Dry Matrix Ready to Encapsulate

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Fecal microbiota transplantation (FMT) is one of the recommended treatments for recurrent *Clostridioides difficile* infection, but endoscopy and available oral formulations still have several limitations in their preparation, storage, and administration. The need for a viable oral formulation that facilitates the implementation of this highly effective therapy in different settings has led us to test the microcrystalline cellulose particles as an adsorbent of concentrated filtered fresh feces in comparison to lyophilized feces. This free-flowing material can provide protection to bacteria and results in a dried product able to maintain the viability of the microbiota for a long time. Adsorbate formulation showed a stabilizing effect in gut microbiota, maintaining bacteria viability and preserving its diversity, and is a competitive option for lyophilized capsules.

Keywords: FMT, gut microbiota, adsorbate, lyophilization, capsules

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INTRODUCTION

The use of fecal microbiota transplantation (FMT) to restore a recipient's gut microbial composition is one of the recommended treatments for recurrent *Clostridioides difficile* infection (Johnson et al., 2021; van Prehn et al., 2021). It is usually performed using 50 g of fresh feces from healthy donors, filtrated, and administered *via* the lower or upper gastrointestinal route such as endoscopy or colonoscopy or after additional processing to be administered as oral capsules with high success rates (McDonald et al., 2018; Mullish et al., 2018; Cammarota et al., 2019). Current efforts are focused on the development of new oral formulations that facilitate the implementation of this highly effective therapy in different settings. The most widely tested options include direct encapsulation of the filtered feces (fresh or previously frozen) or processing the feces to obtain a dried product containing viable microbiota (i.e., lyophilization) that is easy to encapsulate (Reigadas et al., 2018; Fadda, 2020). These options have shown good tolerability and high success rates, and consequently have increased the use of FMT, but still have several limitations in its preparation, storage, and administration (Youngster et al., 2016; Staley et al., 2017; Reigadas et al., 2020).

There is a need for a simple and cheaper process to obtain an effective dried product from feces while maintaining microbiota viability, reducing the odor and the number of capsules per 50 g of feces, and being easy to store and transport. This has led to search other processes and materials

including the use of microcrystalline cellulose particles, which are a free-flowing material that does not form particle agglomerates and can provide protection to bacteria. This material is used to protect and encapsulate probiotics and is one of the most useful tablet and capsule diluent with adsorbent properties (Kocherbitov et al., 2008; Nofrerias et al., 2019; Sánchez-Portilla et al., 2020; Sheskey et al., 2020). We hypothesized that the use of microcrystalline cellulose particles could act as an adsorbent of concentrated filtered fresh feces to obtain a dried product able to maintain the microbiota viability for a long time at room temperature or at 4°C.

Our aim was to evaluate the use of microcrystalline cellulose in combination with an excipient as an adsorbent to obtain a dried product from feces in comparison to a lyophilized formulation. We analyzed the different formulations' capacity to preserve bacterial viability and diversity over time.

MATERIALS AND METHODS

Sample Processing

Feces were collected from healthy donors in a specific recipient for this purpose (Fecotainer®, AT Medical B.V., Netherlands) with a BD Gaspak™ EZ anaerobe system (Becton Dickinson and Company, USA) attached to the lid to maintain anaerobiosis. The samples, with a minimum of 50 g, were brought to the lab, maintained at 4°C, and processed as a pool within 4–6 h from collection. Pooled feces were transferred to a stomacher bag, sterile saline was added (10:1), and the mix was homogenate in Stomacher 400 circulator (Seward Ltd., United Kingdom) for 1 min at 230 rpm. Then, the pool was transferred to falcon tubes; 10% of pure glycerol was added and frozen at −80°C.

When required, samples were thawed overnight at 4°C and 20% of pure glycerol was added. They were centrifuged at 4°C in a Heraeus Megafuge 16R Centrifuge (Thermo Fisher Scientific Inc., USA) for 20 min at 400 g, with slow deceleration to remove sample debris. The supernatant was filtered with a conventional sieve to eliminate possible detritus, transferred into high resistant tubes, and centrifuged at 4°C for 30 min at 10,000 *g* (Sorvall Evolution RC Centrifuge, Thermo Fisher Scientific Inc., USA). The pellet with the concentrated microbiota was recovered with a spatula after decantation of the supernatant avoiding any remaining liquid.

For lyophilized capsules, the pellet was disposed into empty petri dishes and frozen at −80°C for at least 1 h. Then, they were introduced in the lyophilizator Telstar Liomega 3 (LI1) (Telstar, Spain) with a starting shelf temperature of −40°C and secondary drying for 10 h at +25°C. All steps were done under 90 to 150 mbar vacuum, and the whole process took 48 h. The lyophilized product was manually encapsulated due to its viscosity and morphological characteristics into 00 acid-resistant capsules (Capsugel®, Lonza, Switzerland) obtaining between 3 and 5 capsules per treatment (from 50 g of feces).

For the new formulation capsules (patent application WO2020212297A1), named adsorbate capsules, the pellet volume equivalent to 50 g of feces was mixed manually in a mortar with microcrystalline cellulose Vivapur-101® (JRS Pharma, Germany) and magnesium stearate in a proportion of 50:1 until a final homogeneous powder-like product is obtained. Vivapur-101® acted in the sample as a water adsorbent, and magnesium stearate was added to facilitate the flowability of the product into capsules. The powder was kept overnight at 4°C in a fridge surrounded by Silica gel plaques to reduce the humidity around the mixture. Then, the powder was encapsulated with a semi-automated encapsulator FagronLAB™ FG (Fagron Iberica, Spain) into 00 acid-resistant capsules, obtaining between 14 and 20 capsules per treatment (from 50 g of feces). Both lyophilized and adsorbate capsules were kept at 4°C with Silica gel bags and labeled for its traceability until analysis.

Bacterial Viability in Lyophilized and Adsorbate Capsules

We processed a 600-g pool of feces to obtain the pellet as previously described and separated it into two parts for lyophilization and adsorption experiments. Each part was divided into six identical replicates containing an equivalent of 50 g of feces each. Three replicates from each experiment (lyophilization and adsorption) were encapsulated to test the evolution of the product into the capsules (**Figure 1**).

We tested bacterial viability using two different methods: (1) flow cytometry with LIVE/DEAD™ Baclight™ Bacterial Viability and Counting Kit (Thermo Fisher Scientific, USA), and (2) quantitative bacterial culture of sample dilutions 1:10000, 1:1000, and 1:100 in Columbia Agar with 5% sheep blood (BD GmbH, Germany) incubated overnight at 37°C in aerobic and anaerobic conditions. The cytometer used was BD FACSCantoII

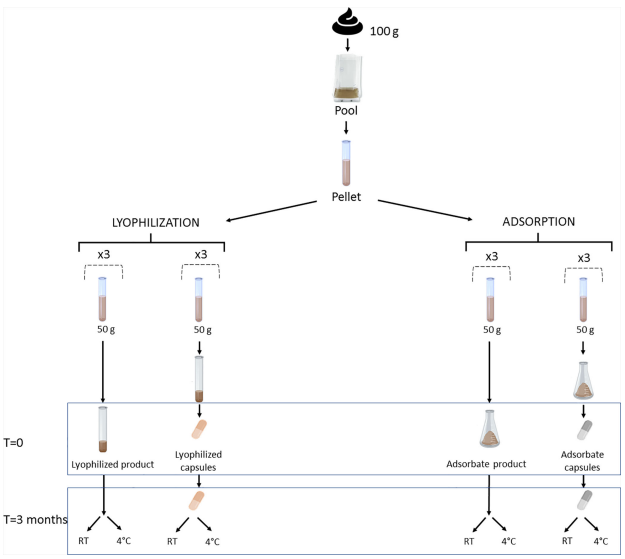

FIGURE 1 | Experiment design to compare bacterial viability in lyophilized and adsorbate capsules. Three replicates of each processing formulation were encapsulated, and three more were kept as lyophilized and adsorbate products. The analysis was performed in each aliquot at time point 0 and 3 months after being stored at room temperature (RT) and at 4°C. Created with BioRender.com.

(BD Biosciences, USA) and software was BD FACSDiva 8.0 following the manufacturer's instructions. Measurements were performed in the original pool and capsules from each replicate at time 0 (just after preparation) and 3 months after in duplicate, keeping samples at room temperature (RT) and at 4°C.

For flow cytometry analysis, encapsulated and non-encapsulated lyophilized and adsorbate aliquots were diluted to 1:10000 using 0.9% NaCl solution and vortexed vigorously. SYTO9 (1:1, 0.1 µl), propidium iodide (1:1, 0.1 µl), and microspheres (1:2, 10 µl) were added, for a final volume of 250 µl. The concentration of live bacteria was determined following the protocol equation (Molecular Probes Inc., 2004).

Bacterial Stability With or Without Magnesium Stearate in Adsorbate Formulation

In order to evaluate the impact of magnesium stearate in adsorbate formulation, three feces from healthy donors were obtained and processed in parallel as previously described. Pellets from each fecal sample were separated into two identical aliquots representing 50 g of feces. One part was mixed with Vivapur-101® (named V capsules) whereas the other was mixed with Vivapur-101® and magnesium stearate (named VMs capsules). Both products were semi-automatically encapsulated into 00 acid-resistant capsules (**Supplementary Figure S1**).

We tested bacterial viability in the initial samples and capsules using flow cytometry and bacterial culture at time 0, and 3 and 6 months after storage at 4°C.

Analysis of Microbial Composition

Samples from all experiments were stored at −80°C at different time points until they are processed for microbial analysis. We determined taxonomical composition and alpha diversity in order to check product stability in terms of microbial composition.

DNA was extracted using the PureLinkTM Microbiome DNA Purification Kit (Invitrogen, USA). The 16S rRNA gene V3–V4 region was amplified and sequenced on an Illumina MiSeq platform (2 × 300 bp) following the Illumina 16S Metagenomic Sequencing Library Preparation protocol using KAPA HiFi HotSart polymerase (Roche, Switzerland). The obtained sequences were filtered and demultiplexed using the DADA2 pipeline. Diversity metrics, compositional, and statistical analyses were performed using QIIME (QIIME 2 version 2020.2) and R version 3.4.4. Taxonomy was assigned using Silva version 132. Samples with <1,000 sequence reads were removed. Singletons and features with a relative frequency <0.01% were also removed. Finally, samples were rarefied to 4,300 read sequencing depth for alpha diversity, beta diversity, and compositional calculations.

For microbial diversity analysis, evenness (Pielou index) and Faith indices were calculated and clustering analysis was performed using Bray–Curtis dissimilarity distances at the feature level.

Macroscopic and Humidity Analysis

Visual inspection of capsules was evaluated at each time point, including size measurement and macroscopic aspect of the capsules. The humidity of the encapsulated adsorbate product was evaluated after storage at 4°C with or without Silica gel, using the Karl-Fischer method (899 coulometer, Metrohm, Switzerland) according to Pharmacopoeia 9.4., section 2.5.12. The humidity was tested in three capsules individually for each condition using Hydranal-Coulomat AG (Thermo Fisher Scientific, USA) as a reactive. From the content of each capsule, 100 mg was taken as aliquot and was analyzed with an agitation parameter rate of 10.

Scanning Electron Microscope Observation

For scanning microscope analysis, the content of an adsorbate capsule was fixed in a solution consisting of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in osmium tetroxide (1%) in the same phosphate buffer, dehydrated in graded alcohol, and dried for critical point drying using Emitech K850. Samples were covered with a carbon thin film in order to improve their electrical conductivity. The samples were observed with a Jeol JSM-7001F (Jeol, Japan) operated at 15 kV. We used a preparation of mixed VMs excipients as control.

Statistical Analysis

Continuous variables were analyzed using a two-sided *t*-test using R 3.6.2 version and considering a $p < 0.05$ to be statistically significant. Graphs were obtained with GraphPad Prism 9.2.0 and R 3.6.2.

RESULTS

The adsorbate product presented a homogenous powder-like appearance that was easy to manipulate in contrast to the viscosity of the lyophilized product. By using a scanning electron microscope, we observed that, in the adsorbate product, the bacteria were homogenously attached to Vivapur-101® microfibrils (**Figure 2**).

Comparison of Bacterial Viability in Lyophilized and Adsorbate Capsules

The bacterial viability results in pool and both capsule formulations studied by flow cytometry are depicted in **Table 1** and **Figure 3**. The comparisons of bacterial viability in formulations were made using the original pool as control. The bacterial viability in the lyophilized formulation non-significantly decreased just after preparation ($p = 0.05$) but differed significantly after 3 months of storage at room temperature ($p = 0.0009$). This was not observed when stored at 4°C ($p = 0.52$), showing a greater loss in bacterial viability when lyophilized capsules were stored at RT compared to 4°C ($p = 0.02$).

On the other hand, no significant changes on bacterial viability were observed in the adsorbate formulation just after preparation ($p = 0.14$), after 3 months of storage at room

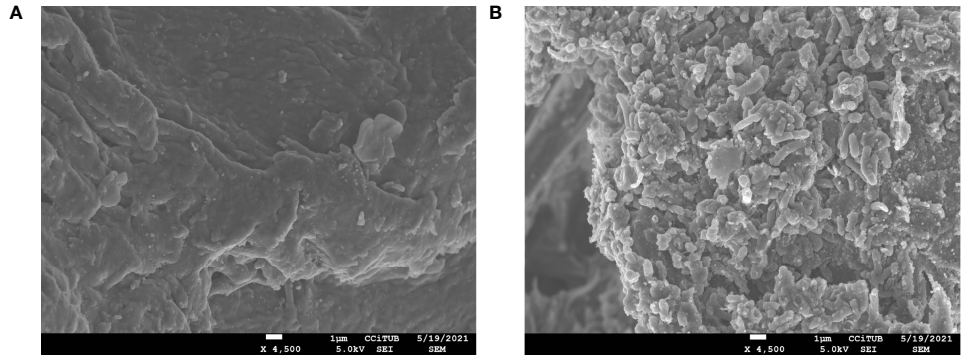

FIGURE 2 | Image from scanning electronic microscope of Vivapur-101® + magnesium stearate (×4,500) before **(A)** and after **(B)** mixing with concentrated filtered feces. In **(A)**, we observed the surface of Vivapur-101® microfibrils, and in **(B)**, the surface was completely covered by a film of bacteria with different morphologies.

temperature ($p = 0.26$), or at 4°C ($p = 0.16$) in comparison to the original pool.

When comparing the two formulations, no significant differences were found at time point 0 ($p = 0.17$), 3 months at RT ($p = 0.39$), or 3 months at 4°C ($p = 0.21$). The results from quantitative culture supported the results from flow cytometry (**Supplementary Data 1**).

From genetic analysis, the samples from both formulations corresponding to 3 months after storage at RT could not be recovered after DNA extraction protocol and sequencing was performed for lyophilized and adsorbate capsules at time point 0 and after 3 months at 4°C. The relative abundances at the family level are shown in **Figure 4**. From the original pool to capsules, there was a reduction in the relative abundance of *Bacteroidaceae*, which was more pronounced in the adsorbate formulation compared to the lyophilized formulation. We also observed an overrepresentation of some families such as *Enterococcaceae* and *Streptococcaceae* in both types of formulations. However, the relative abundances of families such as *Bifidobacteriaceae*, *Lactobacillaceae*, and *Ruminococcaceae* were conserved after lyophilization and adsorbate processing and storage for 3 months.

The lyophilized product showed a better maintenance of the relative abundances at the family level compared to the original sample, but after 3 months of storage, it showed a shift towards less *Bacteroidaceae*. In comparison, the adsorbate product had a change in relative abundances from day 0 but no changes were observed after 3 months.

These observations were reflected in the Faith diversity index where the adsorbate product at time point 0 showed a reduction

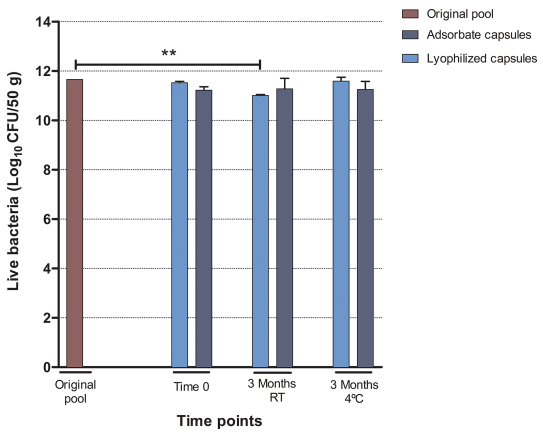

FIGURE 3 | Results from flow cytometry analysis corresponding to the number of viable bacteria expressed as the mean of Log_{10} CFU/50 g of feces (SD) in each step and after 3 months of storage at room temperature (RT) or 4°C. Statistical significance **<0.005.

($p = 0.01$) that was not observed in the lyophilized product ($p = 0.20$) compared to the original pool. After 3 months of storage, both formulations have shown a reduction in alpha diversity (adsorbate $p = 0.02$; lyophilized $p = 0.01$). On the other hand, focusing on the Pielou index, we did not observe any significant change in any of the formulations just after preparation (adsorbate $p = 0.08$; lyophilized $p = 0.84$), but after 3 months,

TABLE 1 | Results from flow cytometry of lyophilized and adsorbate capsules.

		Pool	Lyophilized capsules	*p*	Adsorbate capsules	*p*
$T = 0$		11.66	11.52 (0.06)	$p = 0.05$*	11.22 (0.14)	$p = 0.14$**
$T = 3$ months	RT	NA	11.01 (0.03)	$p = 0.0009$*	11.28 (0.43)	$p = 0.26$**
	4°C	NA	11.59 (0.16)	$p = 0.51$*	11.25 (0.33)	$p = 0.16$**

**Compared to pool results. **Compared to pool results.*
Data are presented as the mean of live bacteria (Log_{10} CFU/50 g of feces) from replicates and standard deviation (SD).
NA, Non Applicable.

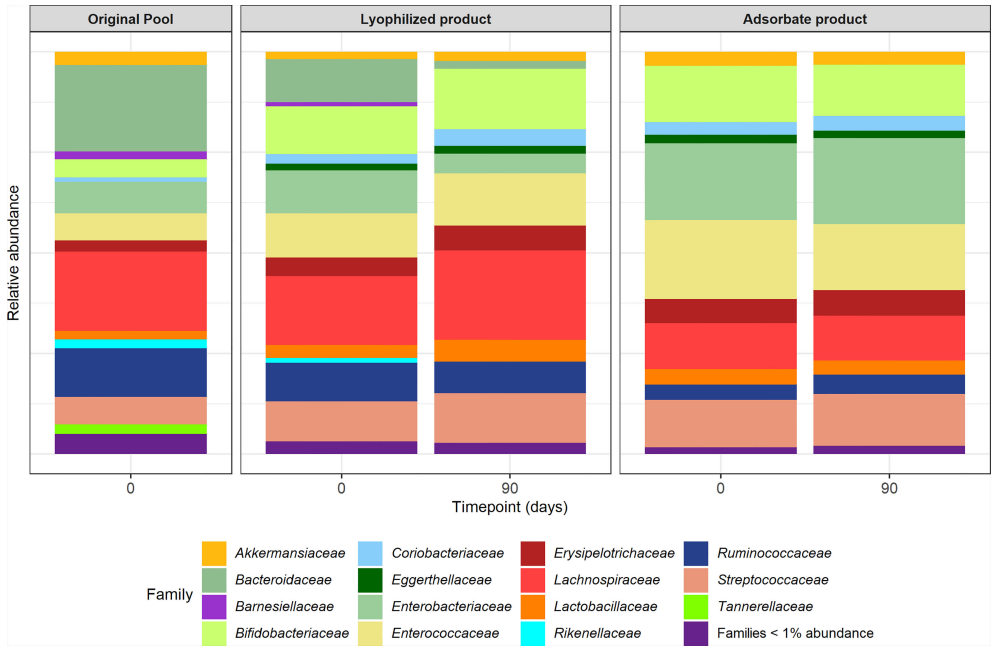

FIGURE 4 | Relative abundances of bacterial taxonomical composition of original pool and lyophilized and adsorbate capsules at time point 0 and 3 months.

there was a reduction only in the adsorbate evenness index (adsorbate $p = 0.03$; lyophilized $p = 0.50$). Analyzing Bray–Curtis dissimilarity distances at the feature level (**Supplementary Figure S2**), both formulations were separately distributed on their own clusters (ADONIS $p = 0.019$), but no differences were observed in terms of time of storage.

Macroscopic and Humidity Analysis

The capsule morphology was stable during the storage independently of temperature conditions and no odor was detected in lyophilized or adsorbate formulations. A humidity study for the adsorbate formulation after direct encapsulation showed 27.83% (SD 0.98) of water content in capsules versus 9.76% (SD 0.68) if encapsulation was preceded by a desiccation with Silica gel.

Viability Analysis of Encapsulated Adsorbate Formulation Using Magnesium Stearate or Not at 4°C up to 6 Months

Comparisons of bacterial viability in formulations were made using the mean of original pools as control. Results from flow cytometry (**Table 2** and **Figure 5**) did not show significant differences in viable bacteria at the time of capsule production (V $p = 0.12$; VMs $p = 0.25$) or at 3 months of storage between any of the formulations and the original pool (V $p = 0.07$; VMs $p = 0.05$). At month 6, we observed a slight reduction in the number of viable bacteria in both formulations that achieved significance in VM capsules ($p = 0.02$) but not in V capsules ($p = 0.06$). The results from quantitative bacterial culture supported the results from flow cytometry (**Supplementary Data 2**).

The genomic analysis of the product (**Figure 6**) showed a greater loss of anaerobic bacteria over time. However, some well-known families of anaerobic bacteria that are characteristically found in the gut microbiota of healthy individuals were present in the samples up to 6 months. These genera comprised *Bifidobacteriaceae, Ruminococcaceae, Lachnospiracea, Prevotellaceae*, and *Bacteroidaceae*. On the other hand, some bacterial families such *Streptococcaceae* and *Rikenellaceae* disappeared at the final time point.

In addition, the alpha diversity Faith index did not significantly change between the original pool (mean 8.88, SD 0.67) and the adsorbate capsules after 3 months (V $p = 0.33$; VMs

TABLE 2 | Results from flow cytometry of Vivapur-101® (V) or Vivapur-101®+magnesium stearate (VM) adsorbate capsules.

	Pool	V capsules	*p*	VMs capsules	*p*
$T = 0$	11.46 (0.09)	11.14 (0.15)	$p = 0.12$*	11.20 (0.20)	$p = 0.25$**
$T = 3$ months	NA	10.93 (0.16)	$p = 0.07$*	11.05 (0.08)	$p = 0.05$**
$T = 6$ months	NA	10.90 (0.27)	$p = 0.06$*	10.98 (0.06)	$p = 0.02$**

*Compared to pool results. **Compared to pool results.*
Data are presented as the mean of live bacteria (Log_{10} CFU/50 g of feces) from replicates and standard deviation (SD).
NA, Non Applicable.

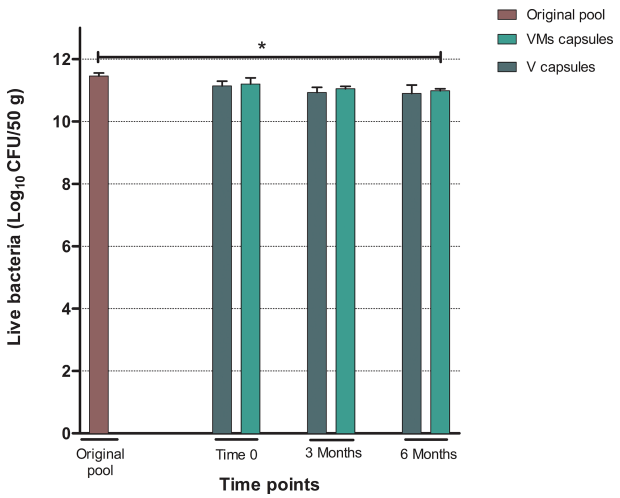

FIGURE 5 | Results from flow cytometry analysis corresponding to the number of viable bacteria expressed as the mean of Log_{10} CFU/50 g of feces (SD). VM capsules: adsorbate capsules with Vivapur-101® in combination with magnesium stearate. V capsules: adsorbate capsules with Vivapur-101® only. Statistical significance *<0.05.

p = 0.37) or after 6 months (V p = 0.92; VMs p = 0.45). The Pielou index was slightly reduced between the original pool (mean 0.93, SD 0.02) and the adsorbate capsules after 3 months (V p = 0.72; VMs p = 0.66), which was closer to significance after 6 months (V p = 0.06; VMs p = 0.06). Observing Bray–Curtis dissimilarity distances at the feature level (**Supplementary Figure S3**), we identified that samples were not clustered by the excipient used or by time of storage, but they were clustered by the variability of each replicate (ADONIS p = 0.001).

DISCUSSION

FMT has become a first-line treatment for recurrent *C. difficile* infection (McDonald et al., 2018; Mullish et al., 2018; Cammarota et al., 2019; Johnson et al., 2021; van Prehn et al., 2021), and its administration with oral capsules has proved to be as effective as traditional invasive methods (Reigadas et al., 2020). This strategy has several advantages: patients can take the FMT in an ambulatory manner, endoscopic procedures can be avoided, and hospitals will be able to save money (Kao et al., 2017; Reygner et al., 2020). The main challenge of oral formulations is to maintain not only bacterial viability but also its diversity in the minimum number of capsules to keep the functionality of gut microbiota once introduced in the new host. In this study, we compared a previously described encapsulated lyophilized formulation (Staley et al., 2017) with a new encapsulated formulation based on an adsorbate that could potentially be an alternative way for oral FMT administration.

From the first step, our analysis showed that at 4°C, both formulations maintained the bacterial viability for at least 3 months according to flow cytometry results. Despite the limitation of bacterial culture due to the large number of unculturable bacteria in feces, the results from quantitative culture supported the results from flow cytometry. In terms of microbial composition, lyophilized formulation maintained the relative abundances of most bacterial families present in the original sample. This included Bacteroidaceae, one of the key families in healthy gut microbiota (Rinninella et al., 2019), which was not represented in the adsorbate formulation from the beginning of the process. At this point, we hypothesize that the mixing step, performed under aerobic conditions, in the adsorbate formulation procedure exposes more bacteria to oxygen than in the lyophilization procedure. Nevertheless, this step could be optimized by performing the whole process under anaerobic conditions.

Analyzing the results of the second experiment, we observed that the addition of magnesium stearate in the adsorbate formulation did not represent a change in the encapsulation process, although it could imply a reduction of bacterial viability after 6 months. In microbial composition analysis, the relative abundance of other important genera including *Bifidobacteriaceae, Lachnospiraceae*, and *Ruminococcaceae* was maintained after 6 months of storage at 4°C compared to original pools. These families have been associated with a healthy status and the maintenance of gut mucosal health participating in the development of the immune system and the control of inflammatory processes, preventing pathogen colonization or the production of vitamins and metabolites such as short-chain fatty acids (La Rosa et al., 2019; Pittayanon et al., 2019; Rinninella et al., 2019; Vacca et al., 2020; Duranti et al., 2021).

The advantages of lyophilized capsules include the low final volume that reduces the number of capsules per treatment, less odor than frozen products, and stability at 4°C, avoiding the necessity of ultralow-temperature storage (Jiang et al., 2018). However, this is a costly procedure, the standardization of the

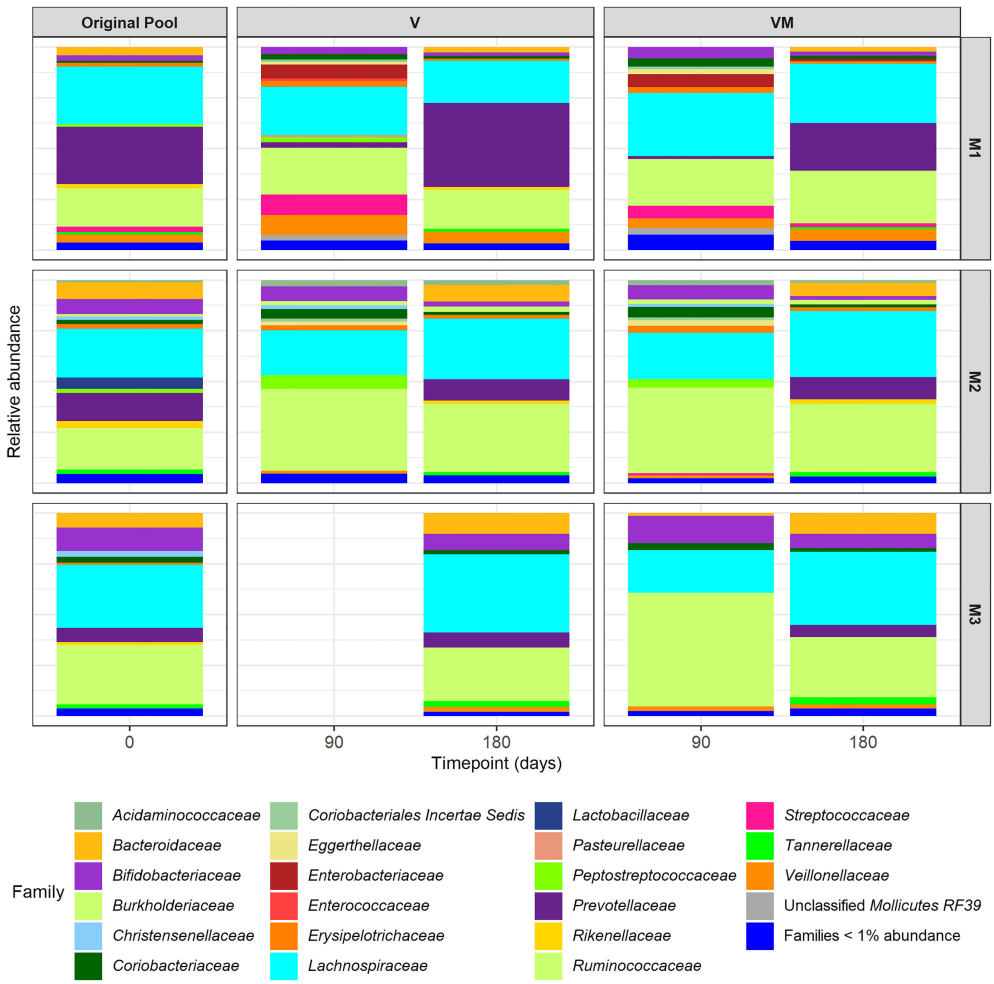

FIGURE 6 | Relative abundances of bacterial taxonomical composition at the family level of original pool and capsules using Vivapur-101® (V) or Vivapur-101® +magnesium stearate (VM) of the three experiment samples (M1, M2, and M3). The analysis was performed at 3 months (90 days) and 6 months (180 days) in the 3 individual samples analyzed. The M3 V capsules at 3 months were excluded due to low sequence quality.

process is a challenge, and we also found some difficulties in the encapsulation process, described also by other groups (Staley et al., 2017). The adsorbate formulation is a powder that has practical advantages regarding its processing compared to lyophilization. The manufacturing process is faster, does not consume energy, is significantly cheaper since the excipients have a low acquisition cost, and has no odor, and its organoleptic properties make the encapsulation process easier. These characteristics make this new formulation potentially incorporated into industrial processes, expanding FMT accessibility. Additionally, this study showed competitive results in bacterial viability and the stability of microbial composition after 6 months of storage at 4°C, which facilitates its transportation and storage. In contrast, previously described oral capsules for FMT (Youngster et al., 2016; Staley et al., 2017; Reigadas et al., 2020; Reygner et al., 2020) require frozen steps to produce it or maintain stability of the product, hindering the facilities where this product can be available.

Our study has several limitations. First, our results are subject to unrecognized bias because the number of samples was small, and feces have an inherent inter-sample variability. Furthermore, our genomic analysis has a limited resolution and lower sensitivity compared to metagenomic data in feces composition.

In conclusion, the adsorbate formulation performed using microcrystalline cellulose as the main excipient seemed to have a stabilizing effect in gut microbiota, maintaining bacteria viability and preserving its diversity. In the future, it is necessary to improve the early management of the fecal material to reduce the loss of anaerobic bacteria as well as during the mixing process of the adsorbent formulation and to test the encapsulated adsorbent formulation for recurrent CDI treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: SRR19025779 - SRR19025792.

AUTHOR CONTRIBUTIONS

AA, JS-N, and AS: conceptualization. AA, ER, AR, AV, and JS-N: methodology. AA, ER, AR, and AV: data analysis. AA: writing—original draft preparation. AA, ER, AV, CC-P, VR, JS-N, and AS: writing—review and editing. All authors reviewed the results and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.899257/full#supplementary-material

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REVIEW
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Evolutionary Insights Into Microbiota Transplantation in Inflammatory Bowel Disease

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The intestinal microbiome plays an essential role in human health and disease status. So far, microbiota transplantation is considered a potential therapeutic approach for treating some chronic diseases, including inflammatory bowel disease (IBD). The diversity of gut microbiota is critical for maintaining resilience, and therefore, transplantation with numerous genetically diverse gut microbiota with metabolic flexibility and functional redundancy can effectively improve gut health than a single probiotic strain supplement. Studies have shown that natural fecal microbiota transplantation or washing microbiota transplantation can alleviate colitis and improve intestinal dysbiosis in IBD patients. However, unexpected adverse reactions caused by the complex and unclear composition of the flora limit its wider application. The evolving strain isolation technology and modifiable pre-existing strains are driving the development of microbiota transplantation. This review summarized the updating clinical and preclinical data of IBD treatments from fecal microbiota transplantation to washing microbiota transplantation, and then to artificial consortium transplantation. In addition, the factors considered for strain combination were reviewed. Furthermore, four types of artificial consortium transplant products were collected to analyze their combination and possible compatibility principles. The perspective on individualized microbiota transplantation was also discussed ultimately.

Keywords: microbiota transplantation, artificial consortium transplantation, combination principles, clinical study, inflammatory bowel disease

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1 INTRODUCTION

Inflammatory bowel disease (IBD) is an inflammatory disease of the intestine, including ulcerative colitis (UC) and Crohn's disease (CD). The disease has complex etiology, but it is generally related to genetics, immune response, environmental factors and the gut microbiome (West et al., 2017). The current IBD treatment relies on anti-inflammatory agents, immunosuppressants, and biologic agents (Van Assche et al., 2013; Gomollón et al., 2016). Even so, these agents do not achieve

satisfactory outcomes in some patients, underlining the need for alternatives. Microbiome dysbiosis is an essential feature of IBD (Maloy and Powrie, 2011), which makes regulating the gut microbiome as one of the potential strategies for IBD treatment.

The human gut is home to numerous microbiota, which form a complex gut microbiome community. The gut microbiome is a highly dynamic and intricate ecosystem that differs among individuals, influenced by host genetics, age, diet, drug use, and other factors (Costello et al., 2009; Yatsunenko et al., 2012; Duffy et al., 2015; Xie et al., 2016). In a healthy state, maintaining a dynamic balance of gut microbes exists in healthy individuals. Disrupting this balance can cause several human diseases such as metabolic syndrome (Lim et al., 2017), obesity (Liu et al., 2017), infections (Petrof et al., 2013), gastrointestinal diseases [irritable bowel syndrome (IBS) (Pimentel and Lembo, 2020) and IBD (Chu et al., 2016)]. Introducing microorganisms into the intestinal tract can rapidly reverse diseases related to gut microbial diversity and abundance imbalance. Replacing the missing symbiotic microbes in the gut with corresponding strains or a mix of specific strains may prevent or treat such conditions. The gut microbiome includes bacteria, fungi, archaea and viruses. Recently, Underhill et al. reviewed the important role of fungal microbiome regulation in the development and severity of IBD in some patients (Underhill and Braun, 2022). The bacterial microbiome accounts for a large proportion of the intestinal tract which plays a major role in intestinal disturbance. Therefore, in this review, we only focused on the treatment of IBD through bacterial microbiome. Gut microbe-based therapies such as fecal microbiota transplantation (FMT) (Paramsothy et al., 2017), washed microbiota transplantation (WMT) (Zhang et al., 2020), and live biotherapeutic products (LBP) (Ye et al., 2021)have been used to treat IBD related to microbial alteration.

The essential characteristics of the gut microbiome are stability and resilience (Lozupone et al., 2012). Without interference, the gut microbiome remains stable. The gut microbiota is generally highly resilient to disturbances, and thus, the abundance of numerous key species remains stable in the host for a period of time. The stability and resilience of gut microbiota are closely related to their diversity. Higher microbial diversity increases the functional redundancy levels. It is generally thought to play a critical role in stabilizing microbial community function during disturbances (Fassarella et al., 2021). Therefore, transplantation with the combination of multiple microorganisms is more effective in modulating gut health than with a single probiotic strain supplement.

Studies have shown that fecal microbiota transplantation can alleviate IBD (Moayyedi et al., 2015; Sokol et al., 2020). However, FMT also causes adverse reactions in some patients due to the complex components in transplants (Wang et al., 2016; DeFilipp et al., 2019). A washed microbiota for transplantation that minimizes the adverse reactions caused by natural FMT has been developed (Zhang et al., 2020). Nevertheless, the precise composition of the transplantation flora is unclear, and the procedure has potential health risks. The recent technology has deepened our understanding of microbial community and its application for IBD therapy. FMT can be performed through enema, orally through freeze-dried bacterial capsules (Crothers et al., 2021), or non-freeze-dried bacterial suspension capsules (Khanna et al., 2021). Beneficial bacteria can be isolated from fermented foods or feces or engineered to obtain desirable biological characteristics (Kurtz et al., 2019; Puurunen et al., 2021).

The influence factors of microbiota transplantation outcome include recipient factors and transplant factors. The recipient parameters, such as genetics, immunity, microbiota and lifestyle, affect the efficacy of microbiota engraftment (Danne et al., 2021). Moreover, the matching between donors and recipients is essential for the long-term maintenance of disease remission (Wilson et al., 2019; Okahara et al., 2020). Given the limitation of the length of the article, we only discussed the diverse microbiota transplantation for IBD treatment.

To sum it up, numerous gut microbiota is vital to human health. Numerous studies have confirmed that intestinal flora participates in intestinal maturation and homeostasis through multiple functions, while symbiosis and interacting with human cells and organs (Backhed et al., 2005; Human Microbiome Project Consortium, 2012). Gut microbiota therapy can be performed using FMT and WMT, and this procedure effectively alleviates microbiome-related disorders, including IBD. However, those are not the best choices due to undefined composition. Microbial therapy with clear composition and standard quality monitoring may be the direction of microbiota transplantation. This review focuses on the current research on different artificial consortium transplant products and their improvement for IBD treatment. We discussed the characteristics of the strains used for combination and their possible compatibility principles, which may be necessary for the better development of individualized microbiota transplantation.

2 UNDEFINED CONSORTIUM TRANSPLANTATION

This refers to a community of microbiome with unknown composition.

2.1 Natural Microbiota Transplantation

Natural microbiota transplantation, also known as fecal microbiota transplantation (FMT), is a procedure in which stool from a healthy donor is delivered to the intestines of a recipient patient through enema or oral capsules (Gupta and Khanna, 2017). FMT is currently used primarily to treat recurrent *Clostridioides difficile* infections (Hvas et al., 2019). However, even though the mechanism of action of FMT is not well understood, existing findings show that it generally restores the abnormal composition and abundance of the gut microbiota. FMT is effective against microecological disorders, including IBD, hepatic encephalopathy (HE), metabolic syndrome, IBS, autism, and cancer (Bajaj et al., 2017; de Groot et al., 2017; Kang et al., 2017; Fong et al., 2020; Skrzydło-Radomańska et al., 2021).

2.1.1 Clinical Studies of FMT in IBD

Using a randomized controlled trial, Moayyedi et al. revealed that FMT induced remission of active UC. FMT is even more

effective against early UC because it is easier to restore the early gut microbial imbalance (Moayyedi et al., 2015). Another study confirmed that a 2-donor fecal microbiota preparation (FMP) was also a safe and effective method for restoring the normal intestinal microbial diversity in patients with active UC (Jacob et al., 2017). In addition to direct colon FMT, oral capsule FMT (cFMT) is well tolerated in mild to moderate UC patients (Crothers et al., 2021). Also, there are currently several registered trials investigating the efficacy of cFMT in IBD, as the previous Halaweish et al. described (Halaweish et al., 2022). Oral cFMT is a more acceptable alternative for UC treatment than the direct colon FMT, and it may enhance the potential of long-term microbial-based treatment strategies. Sokol et al. conducted a pilot randomized controlled study showed that FMT significant decreased endoscopic activity and C relative protein level of CD patients (Sokol et al., 2020). A systematic review evaluated the efficacy of FMT in Crohn's disease, involved 13 cohort studies and two RCTs between 2014 and 2020, showed that FMT may be an effective and safe therapy for CD and needed large controlled trials to confirm (Fehily et al., 2021).

The efficacy and safety of FMT have been studied in adult with IBD and children with UC and CD. Nikhil Pai et al. conducted a 6-week randomized, placebo-controlled pilot study using FMT in children with UC and CD to evaluate the safety and effectiveness of FMT supplement, providing preliminary evidence for the clinical application of FMT in children with IBD (Pai and Popov, 2017; Pai et al., 2019). Katarzyna et al. further demonstrated that FMT is also a safe and effective alternative for treating cytomegalovirus colitis in children with UC (Karolewska-Bochenek et al., 2021).

2.1.2 The Limitations of FMT

Even though FMT is effective against IBD remission, its effectiveness cannot be controlled by humans and is related to the diversity of the fecal microbiota in the donor individual, and there are no reliable and stable sources of the feces. The donor fecal microbiota mixture has many unknown ingredients, including bacteria, yeasts, parasites and viruses. It is unclear which one is responsible for beneficial effects and which may pose a risk by transferring antibiotic resistance or producing genotoxic metabolites. In addition, given the inter- and intra-individual differences in gut microbiota, the transplantation effects are not uniform even with the same donor.

Due to the complex composition of FMT, some adverse reactions often occur. Mild to moderate adverse reactions include abdominal pain, flatulence, increased stool frequency, constipation, vomiting, belching, fever, whereas serious adverse effects include viral and bacterial infections, relapse of IBD, and death (Wang et al., 2016). In 2019, the Food and Drug Administration (FDA) reported two cases of serious adverse events of *Escherichia coli* bacteremia that produces extended-spectrum beta-lactamase (ESBL) after FMT. Genetic sequencing revealed that both patients received FMT from the same donor. one of the patients died (DeFilipp et al., 2019). Moreover, optimized screening of fecal bacteria transplantation donors did not seem to improve the efficacy of FMT in the treatment of active UC (*ECCO 2022 abstract OP03*). Although studies have shown the safety and efficacy of multi-donor FMT for diseases, including IBD and obesity (Jacob et al., 2017; Wilson et al., 2021), there is no clear method for selecting multiple donors, and there is no study directly comparing the efficacy of single donor and multi-donor FMT.

2.2 Processed Microbiota Transplantation

Processed microbiota transplantation, known as washed microbiota transplantation (WMT), is the microfiltration of feces to remove fecal solids, parasites, and fungi from feces suspension. Pro-inflammatory metabolites such as leukotriene B4, corticosterone and prostaglandin G2, are also removed from the feces. Regarding safety, quality control and precise bacteria enrichment, Zhang et al. first revealed that WMT is superior to FMT through clinical results, animal experiments and *in vitro* trials (Zhang et al., 2020). The incidence of WMT-related adverse events in CD patients (since April 2014) was 8.7%, significantly lower than 21.7% in patients with manual FMT (from 2012 to April 2014) (Wang et al., 2018).

2.2.1 Clinical Studies of WMT in IBD

Based on a single-center, open-label prospective study, Chen et al. revealed that washed-treated FMT safely and effectively achieved a clinical response in 77.8% (7/9) of the assessed UC patients in just two weeks. At week 12, achieved clinical remission in 55.6% (5/9), whereas the endoscopic response rate was 33.3% (3/9) (Chen et al., 2020). A separate study showed that clinical remission was achieved in 53.7% of IBD patients after WMT therapy, and this therapy significantly increased the colonization rate of Akkermansia, a beneficial bacteria. Thus, the efficacy of WMT in treating IBD may be closely related to the abundance of Akkermansia bacteria (Zhang et al., 2020). Zhang and colleagues reported a case study of UC patients with recurrent fungal infections in which the antifungal therapy had failed. Interestingly, repeated WMT therapy remarkably and rapidly decreased the serum concentration of inflammatory makers and cleared the fungal during hospitalization. The fungal infection had not recurred after 6-month follow-up. However, the clinical application of WMT for recurrent fungal infections treatment needs further investigation (Wu et al., 2021). A randomized, open clinical study showed that enteral nutrition in combined with early WMT could rapidly improve the nutritional status and induce clinical remission in CD patients with malnutrition (Xiang et al., 2021).

3 ARTIFICIAL CONSORTIUM TRANSPLANTATION

We defined artificial consortium (AC) in a narrow sense as a combination of microbiome with clear composition in a specific manner. And the way AC are transplanted into the gut is called artificial consortium tranplantation (ACT).

3.1 Advances in IBD

3.1.1 *In Vitro* Studies of ACT

Geirnaert et al. investigated the therapeutic potential of a mix of six butyric-producing bacteria against IBD, given the beneficial

effects of butyric acid on epithelial barrier function and intestinal health. They found that the bacterial mix significantly enhanced the colonization of related butyric-producing bacteria and improved the integrity of the epithelial barrier *in vitro (*Geirnaert et al., 2017). Pistol et al. found that a combination of grape pomace (GP) extract and a mixture of Lactobacillus bacteria modulated inflammation by regulating the expression of related genes (Pistol et al., 2019). Palócz et al. reported the effect of chlorogenic acid in combination of *Lactobacillus plantarum* 2142 in reducing the lipopolysaccharide (LPS)-induced intestinal inflammation in porcine IPEC-J2 cells (Palócz et al., 2016).

Cuffaro et al. developed a method of evaluating the function of 21 strains isolated from neonatal and adult gut microbiota. They found that the isolated strains regulated the immune response and enhanced the functioning of the epithelial barrier. Also, 33% of the isolates exerted various benefits (Cuffaro et al., 2021). Even so, more *in vitro* studies are needed to identify the specific species, strains, or metabolites important for health, which extends the selection of a limited number of bacteria considered to have clinical importance and potential health-beneficial properties.

3.1.2 Preclinical Studies of ACT

VSL#3 is the most studied probiotic combination used against IBD. Each VSL#3 dose contained 450 billion freeze-dried bacteria (*Streptococcus thermophilus, Bifidobacterium longum, B. breve, B. infantis, Lactobacillus acidophilus, L. plantarum, L. casei, L. bulgaricus*) and corn starch (Reiff et al., 2009). Current, VSL#3 is being used to IBD clinical treatment. Vivomixx® in EU, Visbiome® in USA, similar component to VSL#3, also alleviated canine colitis by increasing mucosal polyamine levels and TJP expression (White et al., 2017; Rossi et al., 2018). Biagioli et al. formulated a new probiotic combination by adding Bacillus subtilis to Vivomixx®. This formulation enhanced the beneficial effects of Vivomixx® on DSS and TNBS-induced colitis (Biagioli et al., 2020).

Lactobacillus and Bifidobacterium are the most common probiotics. The efficacy of Lactobacillus in combination with Bifidobacterium against IBD, such as Ultrabiotique® (*Lactobacillus acidophilus, Lactobacillus Plantarum, Bifidobacterium lactis and Bifidobacterium breve*), Citogenex (*L. casei, Bifidobacterium lactis*), PM2 (*Lactobacillus acidophilus, Lactobacillus paracasei, Lactobacillus rhamnosus, and Bifidobacterium lactis*) has been evaluated. Ultrabiotique® alleviated intestinal inflammation and maintained the mucosal barrier in mice with DSS-induced colitis (Toumi et al., 2013). Pre-administration of Citogenex can alleviate TNBS-induced colon injury (Traina G, 2016). PM-2 attenuated 5-FU-induced mucositis, increasing villus/crypt ratio while decreasing inflammation in the intestine (Quaresma et al., 2020). Bacterial strains from different isolated sources can be combined. Je et al. evaluated the efficacy of *Lactobacillus johnsoniil* DCC9203 in combination with *Bifidobacterium animal subspecies lactis* IDCC4301 isolated from the feces of infants with *Lactobacillus plantarum* IDCC3501 isolated from the pickle at a ratio of 1:1:1 to form ID-JPL934, was applied to DSS-induced colitis model. It was found that ID-JPL934 could reduce mucosal and submucosal immune cell infiltration and decrease intestinal cell loss (Je et al., 2018).

In addition to the common Lactobacillus and Bifidobacterium combinations, some specific strain combinations have also been studied in animal models of IBD. Ming Li et al. demonstrated that a combination of 10 fecal bacteria, called bacterial consortia transplantation (BCT), was comparable to FMT in reestablishing mucosal barrier function in mice with intestinal disorders and that BCT was more stable and controllable than FMT (Li et al., 2015). Further investigation revealed that BCT rapidly restored intestinal microbiome balance, renovated the interaction between symbiotic flora and intestinal γδT17 cells, and improved mucosal barrier function (Li et al., 2016; Li et al., 2017). van der Lelie et al. further reported that gut-103, a cocktail of 17 bacterial strains, rapidly colonized mice intestines and alleviated experimental colitis established in germ-free mice. They further modified unsuitable bacterial strains, including antibiotic resistance, pathogenic, and strict anaerobes, to formulate a complex cocktail of 11 bacterial strains named GUT-108. This bacterial formulation induced stable and prolonged intestinal tract colonization, providing redundancy protection (van der Lelie et al., 2021). Clinical trials of similar probiotic preparation named I3.1 and comprising *Lactobacillus plantarum* (CECT7484, CECT7485) and *Pediococcus acidilactici* (CECT7483) alleviated IBS. Lorén et al. further demonstrated that I3.1 probiotic protected against DSS-induced colitis and IL-10-deficient colitis in mice (Lorén et al., 2017). GI7, composed of four lactobacillus, two Bifidobacterium species, and *Streptococcus thermophilus*, significantly inhibited the production of innate pro-inflammatory cytokines and relieved DSS-induced colitis (Kim et al., 2017). Commercial products Aviguard®, which comprise ten different bacterial species, alleviated acute enterocolitis induced by campylobacter bacteria (Heimesaat et al., 2021).

3.1.3 Clinical Studies of ACT

Clinical trials must be conducted to verify the safety and efficacy of treatment formulations, including bacterial combination therapy (**Figure 1**). Several studies have demonstrated the effect of VSL#3 on IBD in mice, rats, and dogs in last decade. VSL#3 prevented the apoptosis of intestinal epithelial cells, promoted the expression of the intestinal tight junction protein (TJP), reduced the production of pro-inflammatory factors, regulated the functioning of T cells and macrophages, and changed the composition of intestinal microorganisms (Reiff et al., 2009; Mennigen et al., 2009; Hormannsperger et al., 2010; Uronis et al., 2011; Isidro et al., 2017; Liu et al., 2019). VSL#3 was effective in preventing pouch colitis and inducing remission of ulcerative colitis in clinical trials (Gionchetti et al., 2003). A double-blind, randomized, placebo-controlled study showed that VSL # 3 could be used as adjunctive therapy for IBD and can be used in combination with standard 5-ASA or immunosuppressant therapy for the remission of relapsing mild-to-moderate ulcerative colitis (Tursi et al., 2010). In a meta-analysis of 23 randomized controlled trials, Shen et al. reported that VSL#3 significantly increased the remission rates of active UC. [P¼0.01, risk ratio (RR)¼1.51], and also considerably

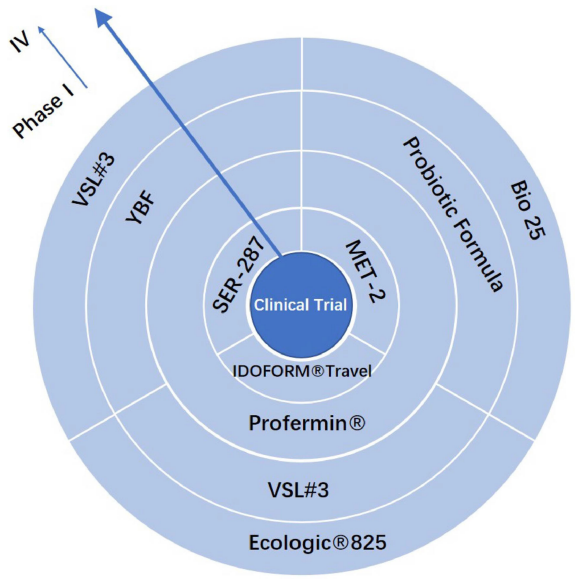

FIGURE 1 | Artificial consortium transplantation products with clinical trials.

reduced the clinical recurrence rate of pouch colitis (P, 0.00001, RR¼0.18), without additional adverse events (Shen et al., 2014). Although VSL#3 reduced the levels of mucosal inflammation in patients with CD, there was no significant difference in the rate of endoscopic recurrence between patients who received VSL#3 and placebo. Therefore, whether VSL#3 can prevent the recurrence of Crohn's disease remains to be validated (Fedorak et al., 2015).

Persborn et al. applied Ecologic®825 to UC patients with severe pouchitis founded that it normalized the permeation of *E. coli* K12, which was associated with active pouchitis, improved mucosal permeability, but had no effect on mucosal pouch microbiota composition (Persborn et al., 2013). Furrie et al. found that short-term synbiotic therapy (*Bifidobacterium longum*/Synergy 1) alleviated active UC by reducing inflammation and promoting epithelial tissue regeneration (Furrie, 2005). Steed et al. further confirmed that synbiotic therapy (*Bifidobacterium longum*/Synergy 1) effectively alleviated active Crohn's disease (Steed et al., 2010). Fujimori et al. also found a combination of probiotics and prebiotics (*Bifidobacterium breve, Lactobacillus casei, Bifidobacterium Longum* and Psyllium) was effective against active Crohn's disease without causing adverse events. Specifically, complete remission was observed in six patients, partial remission was observed in one patient, whereas three patients were non-responsive (Fujimori et al., 2007). In their clinical trial, Krag et al. found that Profermin® (*Lactobacillus plantarum*299V, fermented oats, barley malt and lecithin) was fairly tolerable and promoted remission of UC without causing serious adverse reactions. The estimated reduction mean score was 5.0 points (95% CI: 4.1-5.9, P < 0.001) (Krag, 2012). SER-287, composed of Firmicutes polyspores, is safe and well-tolerated. SER-287 induced a high remission of moderate UC after vancomycin treatment and promoted bacterial colonization of gut microbiota (Henn et al., 2021). Besides prolonged active IBD remission, microbial combination therapy can modulate inflammation and maintain remission in patients with asymptomatic or quiescent IBD. Bjarnason et al. found that a multi-strain probiotic Symprove decreased intestinal inflammation in patients with asymptomatic UC (Bjarnason et al., 2019). Yoshimatsu et al. used Bio-Three tablets containing *Streptococcus faecalis, Clostridium butyricum* and *Bacillus mesentericus* to maintain clinical remission in patients with quiescent UC (Yoshimatsu et al., 2015). Caviglia et al. found that FEEDColon® (consists of *Bifidobacterium bifidum, Bifidobacterium lactis*, calcium butyrate and oligosaccharides) was an effective adjunctive therapy for prolonged UC remission. Further analysis revealed that the remission rate of 5-ASA + FEEDColon® was greater than of 5-ASA alone (95% > 57%) (P = 0.009) (Caviglia et al., 2021).

There are also several bacterial combinations against IBD, such as bacterial ecosystem therapeutic-2 [MET-2] (ClinicalTrials.gov, 2019) and IDOFORM TRAVEL® (ClinicalTrials.gov, 2020a), under development (**Table 1**).

4 SELECTION AND COMBINATION

4.1 Selection

Therapies relying on bacterial combinations usually use beneficial bacteria that have a research basis to support their benefits, most of which are recognized probiotics, such as Lactobacillus and Bifidobacterium (Araya et al., 2002). The bacteria used in combination therapy should be culturable and adaptable to the unique gastrointestinal environment. The bacteria must also stably colonize the intestinal tract.

4.1.1 Environmental Adaptability

To reach therapeutic levels, the bacteria used in microbiota transplantation must be resistant to gastric acidity and bile acid toxicity (Daliri and Lee, 2015). Low pH is a primary host defense against ingested microorganisms. Compared with Bifidobacteria, Lactobacillus is more resistant to low pH (Tripathi and Giri, 2014). Acid resistance is not only genus-specific but also species-specific. For example, *L.casei* and *L. acidophilus* are better resistant to the low pH than *L.delbruekiis* sp.*bulgaricus*. Interestingly, the different Bifidobacterium strains vary in their resistance to gastrointestinal tract acidity (Daliri and Lee, 2015), with *B. animalis* the most acid-resistant strain (Saarela et al., 2006).

Acid tolerance is also linked to bacterial genetics. For instance, the loss of the urec gene encoding a protein associated with acid resistance reduced the ecological adaptability of *L. ruteri* 100-23 (Krumbeck et al., 2016). Bacterial bile salt hydrolase (BSH) protects intestinal bacteria from bile salts by breaking down conjugated bile salts into conjugated bile acids. Several bacterial genera, including Lactobacillus (Corzo and Gilliland, 1999; Wang et al., 2012), Bifidobacterium (Kim et al., 2004), Enterococcus (De Filippo et al., 2010) and Clostridium spp (Coleman and Hudson, 1995), secrete BSH. Therapeutic strains must be safe for use, even in immunocompromised individuals. And to avoid elimination by the gut immune response, probiotic strains usually have mild (not pro-inflammatory) immunomodulatory effects (Daliri and

TABLE 1 | Ongoing clinical trials of artificial consortium transplantation products.

Name	Components	Indication	ClinicalTrials.gov Identifier	Ref.
MET-2	comprises 40 different strains of gut bacteria from a healthy donor	Mild to moderate ulcerative colitis	NCT03832400	(ClinicalTrials.gov, 2019)
IDOFORM®Travel	*Lactobacillus rhamnosus* (LGG), *Lactobacillus acidophilus* (LA-5), *Bifidobacterium* sp. (BB-12), *Lactobacillus bulgaricus* (LBY-27), and *Streptococcus thermophilus* (STY-31)	patients with ulcerative colitis undergoing anti-TNF treatment with insufficient clinical response	NCT04241029	(ClinicalTrials.gov, 2020a)
Synbiotic	three Bifidobacterium spp.(*Bifidobacterium longum* spp. *longum* R0175, *Bifidobacterium animalis* spp. *Lafti* B94, *Bifidobacterium bifidum* R0071) plus three dietary fibers	Post-op Crohn's Disease	NCT04804046	(ClinicalTrials.gov, 2021)
Probiotic Mixture	contains 8 different strains of bacteria, the specific composition is unclear	Quiescent Inflammatory Bowel Disease	NCT03266484	(ClinicalTrials.gov, 2017)
Probiotic Formula	*Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus paracasei, Lactobacillus casei, Lactobacillus gasseri, Lactobacillus plantarum, Bifidobacterium lactis, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium infantis*	Ulcerative colitis	NCT04223479	(ClinicalTrials.gov, 2020b)
Peptidic+ Probiotic	Oligomeric oral nutritional supplement (Bi1 peptidic), *Bifidobacterium animalis subsp. lactis* BPL1, *Lactobacillus rhamnosus* BPL15, *Lactobacillus rhamnosus* CNCM i-4036 *Bifidobacterium longum* ES1	Crohn's Disease	NCT04305535	(ClinicalTrials.gov, 2020c)

Lee, 2015). The environmental adaptability of strains also includes sensitivity to oxygen and utilization of nutrients.

4.1.2 Colonization Characteristic

Stable colonization is a vital beneficial bacterial trait. Probiotics must adhere to the human intestinal cells and intestinal mucins to colonize and proliferate in these areas and compete with potential pathogens that adhere to mucosal surfaces. Recent studies have shown that the pattern of establishment, colonization and persistence of bacteria in certain gut sites are species-and strain-specific and are related to the host factors, strains characteristics, microbial interactions, and the diet (Xiao et al., 2021).

Introducing strains early in life or after antibiotic treatment promotes colonization and persistence of the bacteria in the intestines (Denou et al., 2008; Xiao et al., 2021). From the ecological perspective and coevolution, it is possible to determine the colonization and persistence potential of a particular bacterial species in human intestines. For example, judging from the natural history, compared with Lactobacillus, Bifidobacteria can colonize the intestinal tract more easily (Xiao et al., 2020). *B. longum* is a perfect example of bacterial species with long-term gut colonization potential, and it is dominant in the gut throughout the human lifespan (Odamaki et al., 2018). Bacterial genes, such as luxS (Tannock et al., 2005; Christiaen et al., 2014), pili (Turroni et al., 2013), and BSH (Corzo and Gilliland, 1999), are essential in host-microbe interactions. For instance, the luxS gene in *B. breve* UCC2003 (Christiaen et al., 2014)and *L. reuteri* 100–23 (Tannock et al., 2005) participated in producing the interspecies signaling molecule autoinducer-2 (AI-2), which mediates colonization of the bacteria in the human gut. Pili is a strain-specific colonization factor in LGG (but not in LC705) support the intestinal colonization ability of bacteria was strain-specific (Kankainen et al., 2009). In addition to the bacterial characteristics that contribute to its colonization, specific strains display reciprocal colonization effect and may influence the colonization of other strains. The success of FMT in intestinal flora reconstruction after antibiotic treatment suggests that a flora with rich genetic diversity and metabolic interactions is more likely to thrive in the intestine (Suez et al., 2018). Moreover, cross-feeding of Bifidobacterium strains (*B. bifidum*PRL2010, *B. breve*12L, *B. adolescentis*22L, and *B. Infantis*ATCC15697) improved the persistence of each strain in the cecum (Turroni et al., 2016). Therefore, exploring the interaction behavior of probiotic strains can enhance the development of effective co-colonization combination strains. The core effect of dietary is by adding prebiotics to provide privileged nutrition pathway for intake of strains. Prebiotics including tryptophan, GOS and polysaccharide, which enhanced the colonization of specific bacterial strains. High levels of tryptophan increased the abundance of *L. reuteri* in the stomach and stool (Zelante et al., 2013), whereas high levels of GOS in the human intestine significantly enrich the abundance of Bifidobacterium in the gut in a dose-dependent manner (Krumbeck et al., 2015). Despite these findings, additional synergistic dietary and specific bacterial combinations that enhance the colonization of beneficial bacteria in the gut need to be identified.

4.2 Bacterial Combination

4.2.1 Types of ACT Products

In this article, the current bacterial combinations for IBD were divided into four types (**Table 2**). The first type is a combination of probiotics combined with prebiotics, named synbiotic. Prebiotics are dietary fiber supplementations that stimulate the growth of specific, putatively beneficial bacteria already present in the gut. Prebiotics promote the metabolism and colonization of probiotics. The most commonly used prebiotics are fermentable carbohydrates such as inulin, oligosaccharides, galactose oligosaccharides and resistant starch. In addition, polyphenols and polyunsaturated fatty acids, which act on gut microbes, are also classified as prebiotics. Example of this type of

combination include Profermin® (Krag, 2012), FEEDColon® (Caviglia et al., 2021) and YBF (ClinicalTrials.gov, 2010).

The second type of ACT products comprises beneficial bacteria of different phyla or genera paired based on their social interactions, is a mutual effect combination (mutualbiotic). The most common match includes a combination of Bifidobacteria or Lactobacillus and other strains. The strains establish a symbiotic interaction that promotes reciprocal colonization in the harsh and dynamic human gut environment, including ID-JPL934 (Je et al., 2018), GI7 (Kim et al., 2017), VSL#3 (Tursi et al., 2010) and IDOFORM TRAVEL® (ClinicalTrials.gov, 2020a).

The third type of diverse bacterial combination consists of strains from multiple phyla, named diversified combination (diverbiotic). These strains are isolated from feces then combined in an optimum manner to form a diverse community. This type of consortia includes BCT (Li et al., 2016), GUT-103, GUT-108 (van der Lelie et al., 2021) and MET-2 (ClinicalTrials.gov, 2019).

The fourth type of bacterial consortia includes a combination of bacteria grouped together according to metabolic characteristics, named ejusdem combination (ejusbiotic), comprises bacteria that produce the same metabolic products beneficial for IBD patients. An example is the butyrate-producing bacteria (Geirnaert et al., 2017).

4.2.2 The Principles of the ACT Products

ACT shall be developed in accordance with the principles of complementarity, reciprocity, specificity and stability (CRSS).

The function of each strain is limited, and the strains complement the effects of each strain. For example, the butyrate-producing bacteria consortium competed with resident microbiota for substrates (e.g., acetate) and, thus, promoted the production of butyrate (Geirnaert et al., 2017).

The gut microbiome must establish a reciprocal interaction to remain stable and functional in the dynamic gut environment. Given that exogenous acetate is critical in maintaining butyrate production, the cross-feed between the acetic acid-producing bacteria and butyrate-producing bacteria enhances the continued functioning of butyrate-producing bacteria (Duncan et al., 2004). The cross-feeding observed among the intestinal microbiota suggests the inter-dependence of the strains. Lactobacillus and Bifidobacterium, the most commonly used probiotics, are the most common artificial consortium bacteria (**Table 3**; **Figure 2**. each corresponding). Most Bifidobacteria display reciprocal carbohydrate metabolism capability *in vitro* (Riviere et al., 2018) and persistence in the gut *in vivo* (Turroni et al., 2016). In addition to their symbiosis association within the genus, Bifidobacterium promotes the activities of other gut bacteria (e.g., Bacteroidetes), including carbohydrate metabolism, thus, enhancing the environmental adaptability of these bacteria (Sonnenburg et al., 2006; Turroni et al., 2016).

The strain-specific and disease-specific effects of bacteria on disease have been extensively reported. In addition to the inter-species differences, the diversity within species should be considered. For example, in describing two different *F. prausnitzii* phylogroups (Lopez-Siles et al., 2012), Lopez-Siles et al. found that whereas the abundance of phylogroup I was significantly low in the gut of CD, UC, and colorectal cancer patients, depletion of phylogroup II was explicitly related to CD (Lopez-Siles et al., 2016). A decrease in Lactobacillus is predominant in active ulcerative colitis, whereas a similar phenomenon is observed for Bifidobacteria in Crohn's disease.

Conditions may also influence the effect of the final ACT products, such as production method or strain ratio. Even if the composition of strains in the artificial consortium products is similar, their effect may differ. For example, VSL#3 and Visbiome® are two products composed of the same bacteria species. However, Visbiome® activated Treg cells (CD4+FoxP3+) and T lymphocytes to produce anti-inflammatory cytokines IL-10 and short-chain fatty acids more effectively than VSL#3, which may be due to different production methods or composition ratios of individual bacterial species (Biagioli et al., 2019). Therefore, more stable production standards should be maintained.

5 PERSPECTIVES OF ENGINEERED CONSORTIUM TRANSPLANTATION

Naturally isolated strains aside, genetically modified bacteria can be used for microbiota transplantation (Gao et al., 2022). The engineered strains perform different functions from the original strain or possess modified metabolic characteristics. For example, Puurunen et al. inserted genes encoding phenylalanine ammonia-lyase and L-amino acid deaminase into the *E. coli Nissle* 1917 genome, generating the modified SYNB1618 strain. The engineered strain could degrade phenylalanine in the gastrointestinal tract (Puurunen et al., 2021). A butyrate-producing bacteria, recombinant *B. subtilis* BsS-RS06550 with high butyric acid production was constructed using synthetic biological strategies could effectively regulate body metabolism and intestinal flora disruption (Bai et al., 2020; Wang et al., 2022).The modified strains can increase the variety and number of available strains to the bacterial combination. So far, the engineered strain alone against certain diseases, such as phenylketonuria (Puurunen et al., 2021), liver cirrhosis (https://clinicaltrials.gov/ct2/show/NCT03447730?term=NCT03447730&draw=2&rank=1), has been assessed.

Lactococcus lactis (LL-Thy12), in which the thymidylate synthase gene was replaced with a synthetic sequence encoding mature human interleukin-10 (Braat et al., 2006), was found to alleviate Crohn's disease. Yeast expressing human P2Y2 purinergic receptor and ATP-degrading enzyme, creating self-regulating yeast probiotics system capable of sensing pro-inflammatory molecules inhibits intestinal inflammation in IBD mice (Scott et al., 2021). However, clinical trials on the efficacy of engineered strains against IBD are limited, and even few engineered strains have been transplanted together.

Bacterial combinations promote metabolic characteristics and colonization and increase species diversity. Therefore, the efficacy of the combination of engineered bacteria is a promising research direction for microbiota transplantation.

TABLE 2 | Four categories of artificial consortium transplantation products.

Type	Name	Components	Producer	Ref
Synbiotic	Lactobacillus sp. +prebiotic	grape pomace extract, *L. rhamnosus* (IDIBNA02), *L. paracasei* (ID13239), *L. acidophilus* (ID11692)	Gina Cecilia Pistol et al.	(Pistol et al., 2019)
	Bifidobacterium longum/Synergy 1	Fructo-oligosaccharide/inulin mix, *B. longum*	H. Steed et al	(Steed et al., 2010)
	Profermin®	Fermented oats, barley malt, lecithin, *L.plantarum* 299v	Nordisk Rebalance	(Krag, 2012)
	Symprove™	Barley extract, *L.rhamnosus* NCIMB 30174, *L. plantarum* NCIMB 30173, *L. acidophilus* NCIMB 30175, *Enterococcus faecium* NCIMB 30176	Symprove Ltd	(Bjarnason et al., 2019)
	Bio-Three tablets	Potato starch, lactose, *Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A, *Bacillus mesentericus* TO-A	Toa Pharmaceutical Co.	(Yoshimatsu et al., 2015)
	FEEDColon®	Calcium butyrate, fructo-oligosaccharides, *B. bifidum*, *B. lactis*	Princeps	(Caviglia et al., 2021)
	YBF	Yogurt, soluble fiber, Bifidobacteria	Instituto Lala	(ClinicalTrials.gov, 2010)
Mutualbiotic	VSL#3	*L. plantarum, L. paracasei, L. delbrueckii subsp. bulgaricus, L. acidophilus, B. longum, B. breve, B. infantis*, *Streptococcus thermophilus*	VSL#3, Pharma	(Reiff et al., 2009)
	Visbiome®	*L. plantarum* DSM 24730, *L. paracasei* DSM 24733, *L. delbrueckii subsp. bulgaricus* DSM 24734, *L. acidophilus* DSM 24735, *B. longum* DSM 24736, *B. breve* DSM 24732, *B. infantis* DSM 24737, *Streptococcus thermophilus* DSM 24731	A blend produced under Prof. De Simone's control	(Rossi et al., 2018)
	Five strains probiotics	*L. casei, B. breve, B. animalis subsp. Lactis*, *Streptococcus thermophilus, Bacillus subtilis*	Michele Biagioli et al.	(Biagioli et al., 2020)
	Ultrabiotique®	*L. acidophilus, L. plantarum, B. lactis, B. breve*	Laboratoire Nutrisante	(Toumi et al., 2013)
	Citogenex	*L. casei, B. animalis subspecies lactis*	G Traina et al.	(Traina G, 2016)
	PM-2	*L. acidophilus, L. paracasei, L. rhamnosus, B. lactis*	Marielle Quaresma et al.	(Quaresma et al., 2020)
	ID-JPL934	*L. johnsonii*IDCC9203, *L. plantarum*IDCC3501, *B. animalis subspecies lactis*IDCC4301	In-Gyu Je et al.	(Je et al., 2018)
	I3.1 probiotic	*L. plantarum* (CECT7484, CECT7485), *Pediococcus acidilactici* (CECT7483)	AB-Biotics S.A	(Lorén et al., 2017)
	GI7	*L. acidophilus* LA1 (KCTC 11906BP), *L. plantarum* LP3 (KCTC 10782BP), *L. rhamnosus* LR5 (KCTC 12202BP), *L. lactis* SL6 (KCTC 11865BP), *B. bifidum* BF3 (KCTC 12199BP), *B. breve*BR3 (KCTC 12201BP), *Streptococcus thermophilus* ST3 (KCTC 11870BP).	M.S. Kim et al.	(Kim et al., 2017)
	Ecologic®825	*L. acidophilus, L. casei, L. paracasei, L. plantarum*, *L. salivarius, B. bifidum, B. lactis, Lactococcus lactis.*	Winclove Probiotics BV	(Persborn et al., 2013)
	IDOFORM®Travel	*L. rhamnosus* (LGG), *L. acidophilus* (LA-5), *L. bulgaricus* (BY-27), *Bifidobacterium* sp. (BB-12), *Streptococcus thermophilus* (STY-31)	Pfizer	(ClinicalTrials.gov, 2020a)
	SYNBIO ®	*L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®	Synbiotec S.r.l.	(Coman et al., 2020)
	LAB mixture	*L. plantarum* CRL 2130, *Streptococcus thermophilus* (CRL 807, CRL 808)	Romina Levit et al.	(Levit et al., 2019)
	Bifico	Bifidobacterium, Lactobacillus, Enterococcus	Shanghai Sine Pharmaceutical	(Zhang et al., 2018)
	Bio 25	*L. rhamnosus* LR5, *L. casei* LC5, *L. paracasei* LPC5, *L. plantarum* LP3, *L. acidophilus* LA1, *L. bulgaricus* LG1, *B. bifidum* BF3, *B. longum* BG7, *B. breve* BR3, *B. infantis* BT1, *Streptococcus thermophilus* ST3, *Lactococcus lactis* SL6	Supherb Ltd	(ClinicalTrials.gov, 2013)
Diverbiotic	BCT	Lactobacillus, Eubacterium, Pediococcus, Veillonella, Streptococcus, Staphylococcus, Bifidobacterium, Bacteroide, Escherichia, Fusobacterium	Ming Li et al.	(Li et al., 2015)
	GUT-103	*Megamonas funiformis* DSM19343, *Megamonas hypermegale* DSM1672, *Acidaminococcus intestini* DSM21505, *Bacteroides massiliensis* DSM17679, *Bacteroides stercoris* ATCC43183/DSM19555, *Barnesiella intestinihominis* DSM21032, *Faecalibacterium prausnitzii* DSM17677, *Subdoligranulum variabile* DSM15176, *Anaerostipes caccae* DSM14662, *Anaerostipes hadrus* DSM3319/ATCC 29173, *Clostridium symbiosum* ATCC14940, *Akkermansia muciniphila* ATCC BAA-835, *Clostridium scindens* ATCC35704, *Clostridium bolteae* ATCC BAA-613, *Blautia*	Daniel van der Lelie et al.	(van der Lelie et al., 2021)

(Continued)

TABLE 2 | Continued

Type	Name	Components	Producer	Ref
		producta DSM2950, *Blautia hydrogenotrophia* DSM10507, *Marvinbryantia formatexigens* DSM14469		
	GUT-108	*Bacteroides xylanisolvens* GGCC_0124, *Clostridium butyricum*GGCC_0151, *Clostridium scindens* GGCC_0168, *Intestinimonas butyriciproducens* GGCC_0179, Extibacter sp.GGCC_0201, *Eubacterium callanderi* GGCC_0197, *Akkermansia* sp. GGCC_0220, *Clostridium symbiosum* GGCC_0272, *Bacteroides uniformis*GGCC_0301, *Bitterella massiliensis* GGCC_0305, *Barnesiella* sp. GGCC_0306	Daniel van der Lelie et al.	(van der Lelie et al., 2021)
	SER-287	Spores of Firmicutes	Matthew R. Henn et al.	(Henn et al., 2021)
	MET-2	40 different strains of gut bacteria from a healthy donor	NuBiyota	(ClinicalTrials.gov, 2019)
Ejusbiotic	Butyrate-producing bacteria	*Butyricicoccus pullicaecorum* 25-3T (LMG 24109 T), *Butyricicoccus pullicaecorum* 1.20, *Faecalibacterium prausnitzii* (DSM 17677), *Roseburia hominis* (DSM 16839), *Roseburia inulinivorans* (DSM 16841), *Anaerostipes caccae* (DSM 14662) and *Eubacterium hallii* (DSM 3353)	Annelies Geirnaert et al.	(Geirnaert et al., 2017)

L., Lactobacillus; B., Bifidobacteriu.

6 DISCUSSION

Autochthonous strains are more likely to exert beneficial effects, while allochthonous strains may stimulate the immune system to some extent (Tannock et al., 2000; Yamashita et al., 2020). The sources of the strains are various, such as fermented food and feces. While from the co-evolution perspective, it is better to select strains isolated from human feces for transplantation into the human intestine. Different species display distinctly varied gut fitness. For instance, autochthonous lactobacilli showed better gut colonization ability than the allochthonous (Duar et al., 2017). Microbiota transplantation research is gravitating toward using specific FMT or engineered fecal microbiota, which generates a superior effect to the natural fecal microbiota.

The efficacy of strain combinations can be assessed based on the degree and duration of *in vivo* colonization. Most of the data on intestinal colonization of bacteria have been derived from animal models. Given the differences between animal and human systems, more clinical trials are needed to validate the effectiveness of combined microbiota transplantation. Moreover, the functional characterization of most symbiotic strains in the gut is still in infancy, and more research is needed to identify new strains with high potential for health benefits. Identification of novel health-associated gut bacteria allows better insight into the functionality of the different species and strains (Cuffaro et al., 2021). The findings extend the number of potential candidates for personalized probiotics, taking individual host variations and specific responses into account.

The diversity of gut microbiota is critical for maintaining resilience, and therefore, the transplantation of microbiota combinations is a potentially effective alternative for IBD treatment. Previous articles on microbiota transplantation were mainly limited to FMT, and most of them focused on the application of FMT in IBD, or emphasized the importance of the gut microbes in the pathogenesis and treatment of IBD (Zuo and Ng, 2018; Ooijevaar et al., 2019; Tan et al., 2020; Lee and Chang, 2021; Underhill and Braun, 2022; Halaweish et al., 2022). We mainly analyzed different microbiome-based interventions currently applied in IBD clinical trials, including FMT, WMT (a method that removes adverse factors in natural FMT by special washing manner), as well as ACT, which combines different and limited microorganisms, and analyzed the possible combination principles of ACT. In particular, engineered single bacteria have been used against IBD in recent years (Braat et al., 2006; Scott et al., 2021), we imaged that the artificial consortium combined with engineered bacteria is expected to bring revolutionary mutations to microbiota transplantation.

Given the enormous prospective of microbiota transplantation, the review of different combinations and principles will help to provide a theoretical basis for the generation of more artificial consortium transplantation in the future. The application of microbiota combination in different disease states is differ, even distinct in different individuals. The artificial consortium should not just be a simple combination of strains. However, it should be oriented by engineering ideas and form a systematic whole with the combined characteristics of strains, classification of recipient microbes, disease stages and other factors. Such a consortium could be the next generation of microbiota transplants. Finding rules or principles on the basis of existing research is helpful in exploring the optimal solution of the microbiota approach applied to IBD patients, that is, fewer adverse effects and better clinical outcomes. Recently, Gianluca Ianiro published a comment on the treatment of recurrent *Clostridioides difficile* Infection by SER-109, an artificial microbiome consortium Product, which was similar to our idea in this review, that ACT, to a certain extent, overcome issues related to donor safety and maintenance associated with classical FMT. However, still needed more studies to compare synthetic microbial complexes with standard FMT. If these are better or equivalent to classic FMT, then this will herald the era of FMT2.0 (Feuerstadt et al., 2022; Ianiro, 2022).

The limitations of this review are that neither the fungal microbiome is taken into account nor receptor factors are combined. In most clinical trials, only limited information about the receptors has been mentioned. Generally, there is only a classification of IBD severity. However, the classification of the

TABLE 3 | A list of each product components of mutulbiotics applied in IBD.

Phylum	Firmicutes													Actinobacteria				
Genus	Lactobacillus								Lactococcus	Entero-coccus	Streptococcus	Bacillus	Pediococcus	Bifidobacterium				
Species/ Name	johnsonii	plantarum	casei	acidophilus	paracasei	rhamnosus	salivarius	delbrueckii	lactis	faecalis	thermophilus	subtilis	acidilactici	animalis	breve	bifidum	longum	infantis
VSL#3 Visbiome®		O DSM 24730		O DSM 24735	O DSM 24733			O Bulgaricus DSM 24734			O DSM 24731				O DSM 24732		O DSM 24736	O DSM 24737
Five strains probiotics			O (30%)								O(30%)	O(10%)		lactis15%	O(15%)			
Ultrabiotique®		O		O										lactis	O			
Citogenex			O											lactis				
PM-2				O	O	O								lactis				
ID-JPL934 (1:1:1)	DCC9203	DCC3501												lactisDCC4301				
I3.1probiotic formula		CECT7484 + CECT7485											CECT7483					
GI7		LP3(KCTC 10782BP)		LA1(KCTC 11906BP)		LR5 (KCTC 12202BP)		LactisSL6 (KCTC 11865BP)			ST3(KCTC 11870BP)				BR3 (KCTC12201BP)	BF3 (KCTC12199BP)		
Ecologic®825		W62	W56	W22	W20		W24		W19					lactisW51 +W52		W23		
IDOFORM® Travel				LA-5		O		bulgaricusLBY-27			STY-31			BB-12				
SYNBIO®					IMC50®	IMC501®												
LAB mixture		CRL 2130									CRL808+ CRL 807							
Bifico Bio 25		LP3	LC5	O LA1	LPC5	LR5		LG1	SL6	O	ST3				BR3	BF3	O BG7	BT1

"O" indicates the existence of the strain in this product, but the specific strain is not clear.

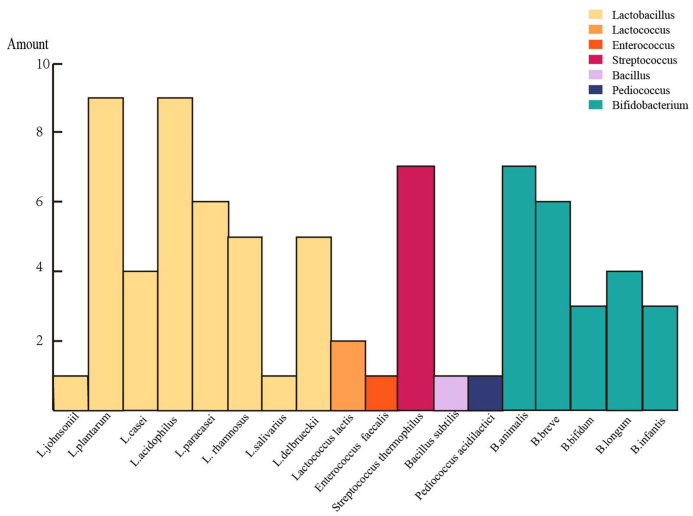

FIGURE 2 | A chart listed the number of occurrences of each strain in these mutualbiotics (one color represents a genus).

intestinal microbiota of the recipients is always absent and the age and gender information is also ominous, which is not conducive to our more comprehensive analysis. Therefore, we appeal to record more complete and comprehensive information in clinical trials which could provide a foundation for more comprehensive analysis of microbiota transplantation in the future.

7 CONCLUSION

In this review, we summarized clinical studies on various microbiota combinations applied to IBD (**Figure 3**) and emphasized the application of artificial microbiota combination transplantation against IBD. The advantages of the bacterial combination were discussed, the types and the possible principles of ACT products were summarized, while the prospect of microbiota transplantation was discussed. The combination of microbiota needs to take the complementary relevance between strains, individual strains' specificity and stable conditions into account. Future research should identify combinations of strains that display metabolic interactions based on ecological knowledge, bacterial genomic data and *in vitro* experimental results, and then validate them *in vivo*, ultimately contributing to mutually beneficial human implantation. The transplantation of microbiota combinations is a potentially safe and effective alternative for IBD treatment. Compared with classical FMT, ACT reduces safety concerns and diversified the options available for different disease states to some extent. Furthermore, the artificial consortium combined with engineered bacteria is expected to bring revolutionary mutations to the microbiota transplantation, which has a bright foreground against IBD. These will probably herald the arrival of the new era of microbial therapy!

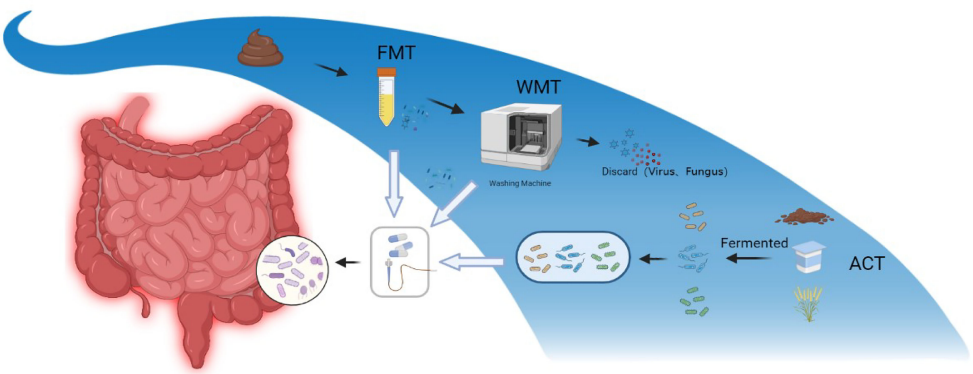

FIGURE 3 | Diagram of three different modes of microbiota transplantation. FMT, fecal microbiota transplantation; WMT, washed microbiota transplantation; ACT, artificial consortium transplantation.

AUTHOR CONTRIBUTIONS

XC, GK, XW, and JZ contributed to the conception of this review. XW and JZ wrote the first draft of the manuscript. YF, ZF, YY, and LL is responsible for literature retrieval. XC, GK, XW, JZ, YF, ZF, YY, and LL wrote sections of the manuscript. XC and GK supervise the project administration. All authors contributed to the article and approved the submitted version.

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Investigating the effect of bacteriophages on bacterial FtsZ localisation

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Escherichia coli is one of the most common Gram-negative pathogens and is responsible for infection leading to neonatal meningitis and sepsis. The FtsZ protein is a bacterial tubulin homolog required for cell division in most species, including *E. coli*. Several agents that block cell division have been shown to mislocalise FtsZ, including the bacteriophage λ-encoded Kil peptide, resulting in defective cell division and a filamentous phenotype, making FtsZ an attractive target for antimicrobials. In this study, we have used an *in vitro* meningitis model system for studying the effect of bacteriophages on FtsZ using fluorescent *E. coli* EV36/FtsZ-mCherry and K12/FtsZ-mNeon strains. We show localisation of FtsZ to the bacterial cell midbody as a single ring during normal growth conditions, and mislocalisation of FtsZ producing filamentous multi-ringed bacterial cells upon addition of the known inhibitor Kil peptide. We also show that when bacteriophages K1F-GFP and T7-mCherry were applied to their respective host strains, these phages can inhibit FtsZ and block bacterial cell division leading to a filamentous multi-ringed phenotype, potentially delaying lysis and increasing progeny number. This occurs in the exponential growth phase, as actively dividing hosts are needed. We present that ZapA protein is needed for phage inhibition by showing a phenotype recovery with a ZapA mutant strain, and we show that FtsI protein is also mislocalised upon phage infection. Finally, we show that the T7 peptide gp0.4 is responsible for the inhibition of FtsZ in K12 strains by observing a phenotype recovery with a T7Δ0.4 mutant.

KEYWORDS

bacteriophages, FtsZ, microscopy, inhibitors, filamentation, human cells, cell division

1 Introduction

Antimicrobial resistance is a growing problem worldwide, and infections caused by Gram-negative bacteria are particularly concerning, as these organisms are highly efficient at acquiring genes for antibiotic drug resistance (Peleg and Hooper, 2010; ECDC, 2019), in addition to permeability issues presented by the outer membrane. This has led to the need for novel antibacterial agents and model systems in which to test them. A potential target for new molecules could be the bacterial cell division machinery, as this is essential for bacterial propagation and survival and is directly related to bacterial cell wall biosynthesis, which is a validated target for natural products and semisynthetic clinically used antibiotics (Araya et al., 2019).

Escherichia coli is one of the most common Gram-negative pathogens and is responsible for many different diseases. For example, *E. coli* O18:K1:H7 is responsible for secondary infections in burn patients, neonatal meningitis (Baud and Aujard, 2013), and sepsis, which can rapidly lead to shock and mortality (Busch et al., 2000). Approximately 80% of the *E. coli* strains that are able to cause meningitis are of the K1 capsule type (Kaper et al., 2004). The K1 antigen produced by these strains makes up a thick polysaccharide capsule, which aids pathogenicity by immune system evasion, giving bacterial cells the ability to cross certain barriers (such as the blood–brain barrier) and defend against certain bacteriophages (Scholl and Merril, 2005).

Bacteriophages (phages) are viruses that infect bacteria. Their potential to kill bacteria was first discovered in 1917 (D'Herelle, 1917), but despite their undaunted therapeutic potential, they were widely disregarded as therapeutic agents in Western nations due to the discovery of small-molecule antibiotics (Martha et al., 2011). However, the worldwide rise of multi-drug resistant bacteria has led to a reinvestigation of phage therapy as an alternative to antibiotics in human and animal infections. T7 is a small bacteriophage with a genome of ~40 kbp encapsulated in a 55-nm icosahedral head (Xu et al., 2018), which infects *E. coli* and related enteric bacteria, encoding a tail fibre that specifically binds to lipopolysaccharide and recognises many *E. coli* K12 strains (Scholl et al., 2005). Laboratory strains of K12 are similar to and share many genes and phenotypes with pathogenic strains, for example, *E. coli* O157:H7, so inhibitors of these strains are also likely effective against pathogens (Koli, 2021; Browning et al., 2013; Mahata et al., 2021). The K1F phage is a natural T7-like phage that infects *E. coli* O18:K1:H7. It is similar to T7 at the genome scale, although rather than the T7 tail fibre protein, it incorporates the endosialidase enzyme into its tail structure, allowing attachment to and degradation of the K1 polysaccharide capsule of its host (Scholl and Merril, 2005).

The bacterial filamentous temperature sensitive Z (FtsZ) protein is a tubulin homolog required for cell division in most species, including *E. coli* (Prahathees and Eswara, 2017). It is comprised of a globular domain, made up of two subdomains, which can fold independently (Moore et al., 2017). FtsZ monomers in the cytoplasm undergo GTP-dependent polymerisation into single-stranded protofilaments, which bundle together through lateral interactions (Haeusser et al., 2014). FtsZ polymerises to assemble a ring structure (called the Z-ring) at the division site, which recruits other cell division components, such as FtsI and ZapA (Szwedziak and Löwe, 2013), ready to initiate cytokinesis (Prahathees and Eswara, 2017). Since FtsZ is the first protein in the divisome and is needed for downstream recruitment, it would make a good target for novel antibiotics (Broughton et al., 2016; Araya et al., 2019), specifically aiming to inhibit FtsZ polymerisation (Dow et al., 2015). Changes in the polymerisation of FtsZ or GTPase activity would prevent the formation of the Z-ring and septum formation, which would in turn block cell division and cause cell death, making FtsZ a potential target for new antibacterial agents, as demonstrated by the mode of action of PC1900723 (Haydon et al., 2008). The Kil peptide of bacteriophage λ prevents FtsZ polymerisation and therefore Z-ring formation, leading to filamentation of *E. coli* cells and cell death (Haeusser et al., 2014). Kil has been shown to block FtsZ from forming protofilaments during the lytic cycle, and it has been suggested that Kil can directly interact with FtsZ *in vitro* for ZipA-dependent inhibition of the Z-ring (Haeusser et al., 2014). At high concentrations, Kil may be able to sequester FtsZ subunits and reduce their GTPase activity (Hernandez-Rocamora et al., 2015). The gene product (gp) 0.4 protein expressed by bacteriophage T7 prevents FtsZ assembly to give elongated bacterial cells, thereby enhancing T7's competitiveness (Kiro et al., 2013). Gp0.4 is a non-essential gene transcribed approximately 2 min after infection with the early genes (Studier, 1972). It has previously been shown that purified FtsZ is inhibited by purified gp0.4 to block Z-ring assembly *in vitro*, along with *in vivo* studies, which found that gp0.4 specifically binds to and interacts with FtsZ (Kiro et al., 2013).

Several features of FtsZ make it an attractive drug target: it performs an essential set of functions in the majority of bacterial pathogens (Broughton et al., 2016); it has a specific role in prokaryotic cell division related to cell wall biosynthesis, which is a proven target for antimicrobial agents; it is conserved across the vast majority of bacterial and archaeal species (Prahathees and Eswara, 2017); it is absent in human and animals, so it should not exhibit adverse effects on host cells (Tripathy and Sahu, 2019).

The aim of this study was to investigate the effect of bacteriophages and their peptides on the localisation of FtsZ during infection. We used a previously developed *in vitro* meningitis model system (Moller-Olsen et al., 2020) to study the effect of bacteriophages on FtsZ using fluorescent *E. coli* EV36/FtsZ-mCherry (Vimr and Troy, 1985; Galli and Gerdes, 2010) and K12/FtsZ-mNeon (Moore et al., 2017) strains in the

hCMEC human brain cell line. We investigate FtsZ localisation in both intracellular and extracellular bacteria within a human cell environment, in order to understand better the cell biology mechanisms during phage therapy. We show localisation of FtsZ to the bacterial cell midbody as a single ring during normal growth conditions in the absence and presence of human cells and mislocalisation of FtsZ to give filamentous multi-ringed cells upon addition of the known inhibitor Kil peptide (Haeusser et al., 2014). We show that bacteriophages K1F-GFP (Moller-Olsen et al., 2018) and T7-mCherry are able to inhibit FtsZ and block bacterial cell division in the exponential growth phase only since an actively dividing host is needed. We show that inhibition of the divisome proteins FtsI and ZapA occurs, and potentially the inhibition of MinC. Finally, we show that the T7 peptide gp0.4 is responsible for inhibition of FtsZ in K12 strains by observing a phenotype recovery with a T7Δ0.4 mutant (Kiro et al., 2013), and this peptide potentially acts to delay host cell lysis and increase phage progeny numbers.

2 Materials and methods

2.1 Human cell culture

The human cerebral microvascular endothelial cell (hCMEC) line (Merck, London, UK) was cultured in EndoGRO-MV Complete Media (Merck) supplemented with 1% penicillin–streptomycin and grown at 37°C with 5% CO_2 in culture vessels coated with 5 μg/cm^2 Collagen Type 1 (Merck). Prior to the experiment, the cells were seeded onto coverslips in a 6-well plate or a fluorodisc in culture media at a density of 4 × 10^4 cells/ml and left for 24 h to settle. The culture media was then replaced with Leibovitz L-15 media (Lonza), and the cells were moved to a 37°C incubator suitable for bacterial infection.

2.2 Bacterial cultures

Bacterial cultures were grown from a single colony in lysogeny broth (LB), supplemented with the appropriate antibiotic if needed, and grown in a 37°C shaking incubator. The seven bacterial strains used in this study are listed in Table 1. During experiments, bacterial cells were added during the exponential growth phase at OD_{600} 0.3 (unless otherwise stated as stationary) and incubated for 1 h prior to fixation or phage addition.

2.3 Bacteriophage propagation and purification

The bacteriophages used in this study are listed in Table 2. *E. coli* EV36 was used as a host to grow and purify K1F-GFP phage, and K12/FtsZ-mNeon was used as a host to grow and purify T7 phage, T7-mCherry phage, and T7Δ0.4 phage. After host clearance, each phage propagation culture was centrifuged at 3,220 × *g* for 15 min. The resulting supernatant was filter sterilised and incubated on ice with 0.2 M of NaCl for 1 h and then centrifuged at 3,220 × *g* for 15 min at 4°C. The supernatant was then incubated with 10% w/v PEG8000 overnight at 4°C to precipitate the phage and then centrifuged at 3,220 × *g* for 15 min at 4°C. The resulting phage pellet was resuspended in an SM buffer 1 (1 M of NaCl, 8 mM of $MgSO_4$•$7H_2O$, and 25 mM of Tris-HCl). A CsCl density gradient was set up with three solutions of densities of 1.7, 1.5, and 1.4 g/ml, along with a phage solution with CsCl added to give a density of 1.3 g/ml. The solutions were added in equal volumes to a centrifuge tube, starting with the heaviest, and centrifuged at 125,000 × *g* for 20 h at 4°C. The resulting phage band was extracted and placed in dialysis tubing (molecular weight cutoff (MWCO) of 14 kDa) and dialysed overnight in SM buffer 1 at 4°C, followed by dialysis in SM buffer 2 (100 mM of NaCl, 8 mM of $MgSO_4$•$7H_2O$, and 25 mM of Tris-HCl) for 2 h at room temperature twice. After dialysis, the purified phage was retrieved and stored at −20°C, and the phage titre was found by plaque assay. WT T7, T7-mCherry, and K1F-GFP phage stocks were diluted to approximately 10^9 pfu/ml for use in experiments. For the experiments, phages were incubated for 1 h prior to fixation.

2.4 Engineering of T7-mCherry phage

2.4.1 Plasmid construction

The pSB1C3 plasmid (Registry of Standard Biological Parts http://parts.igem.org/Main_Page) contained chloramphenicol resistance. The synthetic gBlock was ordered from Integrated DNA Technologies, with *Eco*RI and *Spe*I sites to ligate to the corresponding restriction sites of the pSB1C3 plasmid. The gBlock (Figure S1) was designed to contain the mCherry gene (Shaner et al., 2004) flanked by 150-bp homology regions of the C-terminus (excluding the stop codon) of the T7 minor capsid protein (gene 10), along with a linker sequence. A PCR was performed to amplify the insert using primers AG005 and AG006 (Table S1). The insert and vector were digested with restriction enzymes at 37°C for 1 h, purified using a GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), and then ligated (150 ng of insert and 50 ng of vector) with DNA T4 ligase overnight at 4°C.

2.4.2 Sequencing the plasmid

After ligation, the resulting vector was transformed into electrocompetent K12 cells and plated on chloramphenicol agar overnight, and plasmid was miniprepped from the colonies using a QIAprep Spin kit. The miniprepped DNA was sent for Sanger sequencing by GATC using the AG005 primer. One

TABLE 1 Bacterial strains used in this study.

Strain	Description	Source
Escherichia coli EV36	*E. coli* K1/K12 hybrid suitable for Class 1 laboratory work while maintaining phenotypic properties of a pathogenic K1 strain	Dr Eric R. Virm (Vimr and Troy, 1985)
E. coli EV36/ FtsZ-mCherry	Derivative of EV36 transformed with a pBAD30FtsZ-mCherry plasmid and cultured under 35 μg/ml chloramphenicol selection	Plasmid kindly provided by Dr Kenn Gerdes (Galli and Gerdes, 2010), transformed in this study
E. coli EV36/ Kil	Derivative of EV36 transformed with a pBAD33-Kil plasmid and cultured under 35 μg/ml chloramphenicol selection	Plasmid kindly provided by Dr William Margolin (Haeusser et al., 2014), transformed in this study
E. coli K12 FtsZ-mNeon	*E. coli* BW27783 strain, which has had its wild-type *ftsZ* gene replaced with FtsZ-mNeonGreen	Dr Harold Erickson (Moore et al., 2017)
E. coli K12/ FtsZ-mNeon/ Kil	Derivative of K12/FtsZ-mNeon transformed with a pBAD33-Kil peptide plasmid and cultured under 35 μg/ml chloramphenicol selection	Plasmid kindly provided by Dr William Margolin (Haeusser et al., 2014), transformed in this study
E. coli K12ΔzapA	*E. coli* BW25113 K12 strain from the Keio collection with a deleted *zapA* gene that has been replaced by a kanamycin resistance cassette. Cultured under 50 μg/ml kanamycin selection	OEC4987-200828117, Keio collection (Baba et al., 2006)
E. coli K12ΔminC	*E. coli* BW25113 K12 strain from the Keio collection with a deleted *minC* gene that has been replaced by a kanamycin resistance cassette. Cultured under 50 μg/ml kanamycin selection	OEC4987-213605848, Keio collection (Baba et al., 2006)

plasmid returned a matching sequence and was used for engineering the phage.

2.4.3 Homologous recombination

Wild-type T7 phages were propagated with electrocompetent K12/FtsZmNeon transformed with the donor plasmid at a low OD_{600} of 0.2. Homologous recombination occurred between the homology regions of the plasmid and genome of some phages, resulting in a mixed population lysate, which was centrifuged at 3,220 × *g* for 15 min and then filter sterilised.

2.4.4 Screening for engineered phage

The presence of engineered phage was found by PCR analysis of the lysate using the primers mCherry-forward and mCherry-rev (Table S1). The positive lysate was then plated out on the host lawn at an appropriate dilution to give individual phage plaques. Ten plaques were chosen and used as a template for a second PCR screen. The plaques showing positive bands were propagated as before, the lysate was plated for a second plaque assay, and plaques were again checked by a PCR screen. Positive phage was propagated, CsCl purified, and further checked for mCherry fluorescence by confocal and electron microscopy.

2.5 Bacterial and phage infections of human cells

Cultures of hCMECs were incubated with the relevant bacterial strain at OD_{600} of 0.2–0.4, which were added to the Leibovitz media for 60 min (unless otherwise stated) along with antibiotics or plasmid induction if needed, and if phages were being used, then the phage was added at 1×10^7 pfu/ml and incubated for a further 60 min. Control cultures were incubated with bacteria or phage alone for 60 min in parallel.

To investigate if strains acted intracellularly, hCMEC cultures were infected with bacterial cultures at OD_{600} 0.3 for 1 h and then incubated with 100 μg/ml of gentamicin for a further 2 h to kill extracellular bacteria, performed in triplicate for each condition. For each condition, a minimum of 300 human cells were counted to calculate the internalised bacteria percentages.

TABLE 2 Phage strains used in this study.

Phage	Description	Source
K1F-GFP	A K1F phage derivative engineered to express GPF, which shows a high specificity towards K1 capsule bacteria	K1F from Dr Dean Scholl (Scholl and Merril, 2005), K1F-GFP from Dr Antonia Sagona (Moller-Olsen et al., 2018)
T7	Extensively used strain showing high specificity to commensal *E. coli* K12 strains	Dr Ian Molineux, Texas USA (Studier, 1969)
T7-mCherry	A T7 phage derivative engineered with genomic integration of mCherry, showing high specificity to commensal *E. coli* K12 strains	This study
T7Δ0.4	T7 phage derivative engineered with a knockout of gp0.4 showing high specificity towards commensal *E. coli* K12	Dr Udi Qimron (Kiro et al., 2013)

To quantify human cell death after bacterial infection, hCMEC cultures were infected with bacterial cultures at OD_{600} 0.3 for 1 h, in triplicate conditions. For each condition, a minimum of 500 human cells were counted.

2.6 Immunofluorescent confocal microscopy

After bacterial and/or phage infection, hCMEC cultures were fixed with 4% paraformaldehyde (Thermo Fisher Scientific) in phosphate-buffered saline (PBS) for 15 min and then washed in PBS. Cells were then permeabilised in ice-cold PEM/0.05% saponin for 5 min, washed, incubated with 50 mM of NH_4Cl in PBS for 15 min, and then washed in PBS/0.05% saponin. The fixed cells were stained overnight at 4°C with anti-Prokaryotic Cell Division GTPase (FtsZ) antibody (Agrisera, Vännäs, Sweden) or rabbit anti-*E. coli* (strain K12) FtsI Polyclonal antibody if no fluorescent proteins were present and FtsZ or FtsI staining was needed, with diluted 1:100 in 0.05% saponin in PBS. This was followed by conjugation with secondary Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) diluted 1:500 in 0.05% saponin in PBS at room temperature for 45 min. For samples with fluorescent proteins, the fixed cells were stained with the following antibodies diluted in PBS/0.05% saponin for 60 min at room temperature: 5 µg/ml of GFP-booster (ChromoTek, Planegg, Germany), 5 µg/ml of RFP-booster (ChromoTek), and 5 µg/ml of phalloidin CF680R conjugate (Biotium, Fremont, CA, USA).

After staining, the coverslips were washed, mounted on slides with DAPI-containing Fluoroshield Mounting Medium (Abcam, Cambridge, UK), and sealed. The slides were then imaged using a Zeiss LSM800 confocal microscope using the following excitation wavelengths: DAPI at 405 nm, GFP/mNeon at 488 nm, mCherry at 561 nm, and phalloidin at 633 nm.

Quantification was performed by manually counting Z-rings of 75 bacterial cells per condition and measuring cell length using the Fiji (ImageJ) software for phage filamentation experiments. Data were plotted with error bars showing one standard deviation of uncertainty. For significant difference comparisons, Tukey's tests were performed, and the calculated probability values (p-values) are displayed as $p < 0.05$ (*), $p < 0.01$ (**), and not statistically significant $p > 0.05$ (ns).

2.7 Live-cell imaging

Cultures of bacteria alone expressing fluorescent proteins were imaged live on 2% agarose pads. Cultures of K12/FtsZ-mNeon were grown to OD_{600} 0.5 and imaged immediately. For EV36/FtsZ-mCherry, cultures were grown to OD_{600} 0.3 and induced with 0.2% arabinose for 5 min (stopped using 0.2% glucose) and incubated for 45 min to allow time for protein folding before imaging. Agarose pads were made by dissolving 0.2 g of agar into 10 ml of sterile water by heating, and a drop was placed onto a glass microscope slide and left to dry. Culture measuring 10 µl was then placed onto the agarose layer and covered with a coverslip once dry. Cells were imaged using the Zeiss LSM 880 confocal microscope, with mNeon excitation at 488 nm and mCherry at 561-nm wavelengths, and transmitted light was used to visualise the cell outline.

2.8 Correlative light and electron microscopy

T7-mCherry phage was added to a K12/FtsZ-mNeon culture (OD_{600} 0.3) and incubated at 37°C with shaking for 60 min; 300 mesh copper grids (FC300Cu) were hydrophilised by glow discharge. The sample was added to the grid and incubated for 2 min, and then 2% uranyl acetate was dropped into the grid and incubated for 2 min. A wash step was performed three times and blotted; 0.1% trehalose was added to the grid and incubated for 2 min. This was left to air-dry. The sample was imaged using Zeiss LSM 880 confocal microscope (565 nm for T7-mCherry and 488 nm for K12/FtsZ-mNeon) and Jeol 2100 transmission electron microscope, and ImageJ was used for overlay.

3 Results

3.1 Visualisation of the FtsZ ring and Kil peptide-mediated inhibition of FtsZ

We first sought to observe Z-rings in bacterial cells, using the *E. coli* K12/FtsZ-mNeon (Moore et al., 2017) strain, which has its genomic copy of *FtsZ* gene replaced with FtsZ-mNeon. A crucial aspect of the design of this FtsZ construct is that mNeon protein has been placed on an internal loop of FtsZ so that the head-to-tail mode of polymerisation required for the function was not impaired (Moore et al., 2017), and the mNeon proteins were prevented from forming aggregates with each other. The Z-rings were visualised by placing exponential-phase bacterial cultures onto 2% agarose pads for visualisation by confocal microscopy. FtsZ-mNeon assembled at the cell midbody as a single Z-ring (Figure S2A). This visualisation was repeated in the *E. coli* K1/K12 hybrid strain EV36 (Vimr and Troy, 1985), by transforming it with an arabinose-inducible FtsZ-mCherry plasmid (Galli and Gerdes, 2010), and FtsZ-mCherry forms a Z-ring at the EV36 midbody (Figure S2B).

We next observed the phenotype of the Z-rings in a meningitis infection model to see if FtsZ localisation changed during bacterial infection of human cells. hCMEC brain cells were infected with K12/FtsZ-mNeon culture for 1 h, fixed, and stained before visualising by confocal microscopy. During human cell infection, FtsZ rings were still visible at the midbody of K12 cells as a single ring per cell (Figure 1A), suggesting cells can divide normally, and this was also found for

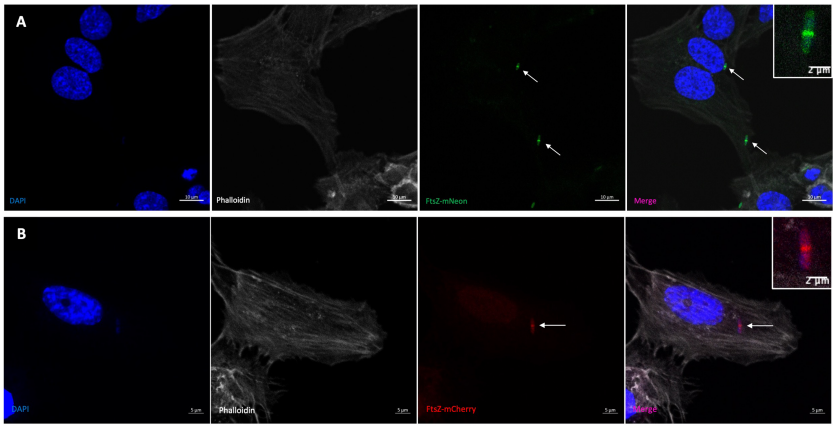

FIGURE 1
Visualisation of the FtsZ Z-ring in normal growth conditions by confocal microscopy. **(A)** K12/FtsZ-mNeon cells fixed during human cell infection. An internalisation experiment using gentamicin to kill extracellular bacteria showed this strain had an infection rate of 4.1% in hCMECs. **(B)** EV36/FtsZ-mCherry cells fixed during human cell infection. Arrows pointing to bacterial Z-rings. DAPI stain is shown in blue, phalloidin stain is shown in grey, FtsZ-mNeon is shown in green, and FtsZ-mCherry is shown in red.

the EV36/FtsZ-mCherry strain (Figure 1B). These data confirm that FtsZ assembles as a single ring at the cell midbody under normal bacterial growth conditions and during bacterial infection of human cells. The EV36 strain has previously been shown to have an infection rate of 20%–50%, depending on the human cell line (Moller-Olsen et al., 2018; Moller-Olsen et al., 2020). Since the K12 strain is non-pathogenic and not expected to act intracellularly (Meier et al., 1996; Sahu and Kar, 2012), an internalisation experiment was done using gentamicin to kill extracellular bacteria and to confirm if K12 would be present inside human cells. This showed that only 4.1% of hCMECs infected with K12/FtsZ-mNeon had intracellular bacteria. Since the infection rate is low, for the following experiments, we chose to focus on both intracellular and extracellular bacteria in the infection model for both strains. Nevertheless, both extracellular and intracellular bacteria are toxic to human cells and have a variety of mechanisms to cause disease (Mak et al., 2014). We confirm this by quantifying human cell death by confocal microscopy after bacterial infection of human cells and found that 5.3% of human cells died after infection with the K12 strain, and 12.1% of human cells died after infection with the EV36 strain (Figure S3). Recent studies have presented the efficiency of bacteriophages in targeting both intracellular and extracellular bacteria in the human cell environment (Śliwka et al., 2022), and bacteriophages have been shown to be more efficient in the presence of human cells (Shan et al., 2018). It is therefore necessary to test the proposed system in infection human cell models, in order to understand better the interplay of phage and bacteria in the presence of human cells.

We then applied the known FtsZ inhibitor Kil peptide to the model system to investigate how FtsZ inhibition would appear in these conditions. Kil stops FtsZ from polymerising, leading to filamentation of cells that are unable to divide, so a Kil peptide plasmid (Haeusser et al., 2014) was transformed into both strains. For these experiments, the plasmid was induced with 0.2% arabinose for 2 h. Imaging in Figure S4A confirmed that induction of Kil in K12/FtsZ-mNeon resulted in long filamentous cells, with varying FtsZ phenotypes that ranged from distinct multiple rings (42.2% of cells) to diffuse FtsZ spread (30.1%) and cells in an intermediate phenotype (27.7%) of faint rings within a diffuse background. Previous studies have shown a diffuse FtsZ spread upon Kil inhibition using antibody staining (Haeusser et al., 2014), which was confirmed in Figure S5; however, here we present that when using a fluorescent protein stably expressed, multiple distinct Z-rings still form along the cell body, potentially due to Kil not having fully acted on these cells yet.

To support these findings, images for the induction of Kil were quantified in K12/FtsZ-mNeon under the following conditions: control (no Kil induction), 1-h induction, and 2-h induction. The averages (Figures S4C, D) showed an increase in the number of Z-rings and cell length after 1 h of Kil induction, with a further increase after 2 h, confirming that the cells are not dividing and continually elongating in the presence of Kil, and formation of multiple Z-rings suggests cell division had been unsuccessfully attempted. The increase seen after 2 h compared to 1 h suggests that the effect and phenotypes seen may be dependent on the amount of Kil peptide present in the cell.

These data support previous findings that the phage λ Kil peptide causes filamentation of *E. coli* cells by blocking cell division with diffuse FtsZ spread by antibody staining, as FtsZ is unable to polymerise to form rings and further show that multiple Z-rings can

form along the body of the filamentous cell, as it fails to divide at new division sites when using fluorescent proteins.

3.2 Genetic engineering of a T7-mCherry phage

The K1F-GFP phage (Moller-Olsen et al., 2018) has been previously engineered to express GFP, and this was first visualised in human cell vacuoles (Figure 2A) to confirm fluorescence compared to no-phage controls as a visual control for use in confocal experiments. In order to visualise the T7 phage in the K12/FtsZ-mNeon model system, we attached a fluorescent protein to the C-terminal of the phage's minor capsid protein so that it is displayed at the surface, using homologous recombination from an engineered plasmid. The presence of mCherry gene in the phage lysate was confirmed by PCR screening of the recombinant phage genome using the primers in Table S1 and associated plaque assays (Figures S6A–D), prior to purification of positive lysate by caesium chloride gradient purification (Figures S6E, F). Fluorescence of the mCherry tag was further tested by confocal microscopy (Figure 2B) showing mCherry fluorescence inside human cells after phage infection, alongside no-phage controls (Figure S7), performed to show the fluorescence seen was from the phage label, confirming successful engineering of the T7-mCherry phagemid, which was able to be taken up by human cells.

3.3 T7 phage blocks cell division in K12 by FtsZ inhibition

K1F-GFP phage was confirmed to target the EV36/FtsZ-mCherry strain (Figure S8A), and T7-mCherry phage was confirmed to target the K12/FtsZ-mNeon strain (Figure S8B) by using growth curves. This was further confirmed by correlative light and electron microscopy (CLEM) showing the formation of a septum at the midbody of cells, as well as colocalisation of FtsZ-mNeon and T7-mCherry signals within K12 cells (Figure S9), confirming the specificity of the phage.

Next, the effect of T7-mCherry on FtsZ was examined by infecting hCMEC with K12/FtsZ-mNeon, followed by incubation with T7-mCherry. Confocal microscopy showed that T7 phage infection caused a change from the single midbody ring previously seen and led to the formation of filamentous, multi Z-ring *E. coli* cells (Figures 3A, B), which were unable to divide, suggesting that T7 phage can block cell division. We were then interested to see how the Z-rings and cell length of *E. coli* changed over time in control conditions (no phage) and the presence of T7 phage by using a time-series experiment. For the control, hCMECs were infected with exponential K12/FtsZ-mNeon and fixed at 15-min intervals for 2 h (Figure S10). For the phage time series, hCMECs were infected with K12/FtsZ-mNeon for 1 h, and then T7 phage was added (Figure S11). For each condition, bacterial cells were quantified using confocal microscopy (Figures 3C, D). Since K12/FtsZ-mNeon cells were incubated prior to phage addition, this meant phage time of 0 min was equivalent to control 60 min, so these were plotted to overlap. Larger error bars were due to the variation in phenotypes, as some cells were fully filamentous, whereas others were yet to be infected. In the first 60 min of the control series, each cell averages one Z-ring at the midbody as seen earlier in Figure 1, but later we unexpectedly observed two 2 polar rings in the majority of cells. Upon phage addition, the number of Z-rings started to increase after 15–30 min, and after 1 h, they reached an average of four rings, double compared to the control. After 1 h of incubation with phage, there was a

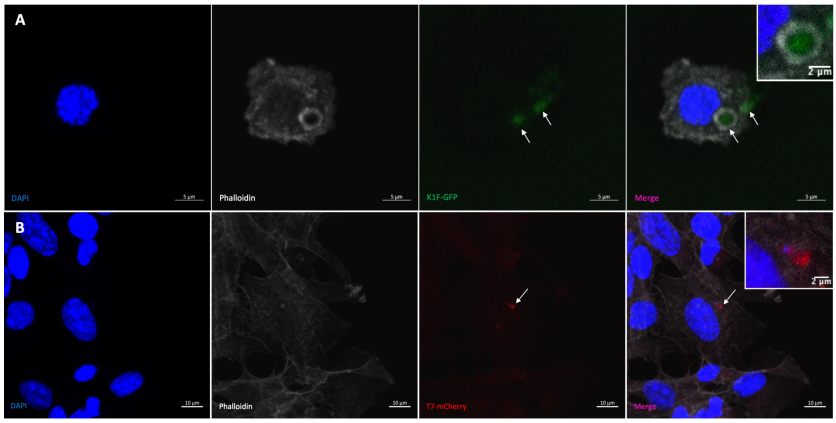

FIGURE 2
Visualising bacteriophage in hCMEC brain cells. **(A)** K1F-GFP phage inside vacuole of hCMEC cell after infection. **(B)** T7-mCherry phage inside hCMECs after infection. Arrows pointing to phage clusters. DAPI stain is shown in blue, phalloidin stain is shown in grey, K1F-GFP is shown in green, and T7-mCherry is shown in red.

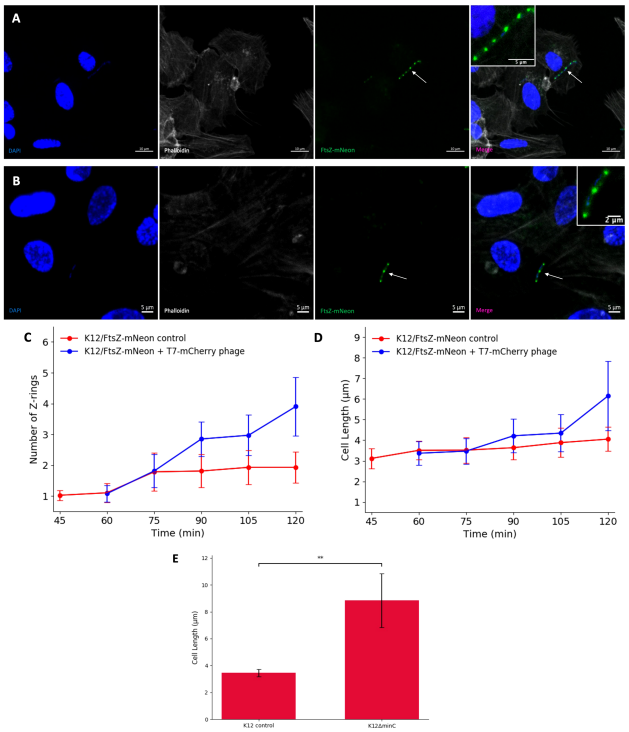

FIGURE 3
T7 phage inhibition of FtsZ in K12 *Escherichia coli* cells. **(A, B)** Fluorescent images of K12/FtsZ-mNeon cells after T7-mCherry phage infection fixed in a human cell model. Arrows point to filamentous cells. DAPI stain is shown in blue, phalloidin in grey, and FtsZ-mNeon in green. **(C, D)** Quantification of Z-ring number and cell length for 75 K12/FtsZ-mNeon bacterial cells infected with T7-mCherry phage at 15-min intervals and compared to a no-phage control. Averages plotted with error bars showing one standard deviation of uncertainty. **(E)** Quantification of cell length for 75 K12 control and K12ΔminC bacterial cells. Tukey's tests were performed, and the calculated probability values (p-values) are displayed as $p < 0.01$ (**).

notable increase in cell length showing filamentation, but interestingly, this did not start until about 30–45 min after infection, suggesting that the number of rings started to increase first and then filamentation followed.

To investigate the cause of the filamentation, confocal microscopy was performed using a K12ΔminC strain from the Keio collection with *minC* gene knocked out, alongside a K12 control. Cultures were incubated for 2 h in no-phage conditions, then stained with an FtsZ antibody, and imaged (Figure S12). The images were quantified, and average cell length was plotted (Figure 3E), showing a significant increase in cell length of K12ΔminC compared to the control, suggesting that inhibition of MinC could be causing the filamentation seen upon phage infection of cells.

Taken together, these data confirm the specificity of engineered T7-mCherry for K12 strains and show that during infection, the phage causes a filamentous (potentially due to MinC inhibition) non-dividing phenotype with multiple Z-rings formed where the division had been attempted, similar to what was seen with the known FtsZ inhibitor Kil, suggesting that T7 phage can inhibit FtsZ to block division.

3.4 K1F phage blocks cell division in EV36 K1 by FtsZ inhibition

To investigate the effect of K1F-GFP phage on FtsZ localisation, hCMECs were infected with host EV36/FtsZ-mCherry and K1F-GFP phage. The EV36/FtsZ-mCherry cells showed a long filamentous phenotype with multiple Z-rings and DNA along the cell after K1F-GFP infection (Figures 4A, B), unlike control samples, suggesting that the K1F phage can also inhibit FtsZ to block host cell division.

To further confirm this, bacterial cells were quantified for control conditions and K1F-infected conditions (Figures 4C, D). The control showed an average of 1.027 rings per cell as expected, and after K1F-GFP infection, this increases to 4.120 rings per cell, suggesting cells had attempted to divide two to three times unsuccessfully. There was an increase in average cell length, showing cells were starting to elongate to confirm filamentation. These data show that similar to phages λ and T7, the K1F phage is able to inhibit FtsZ to block host cell division.

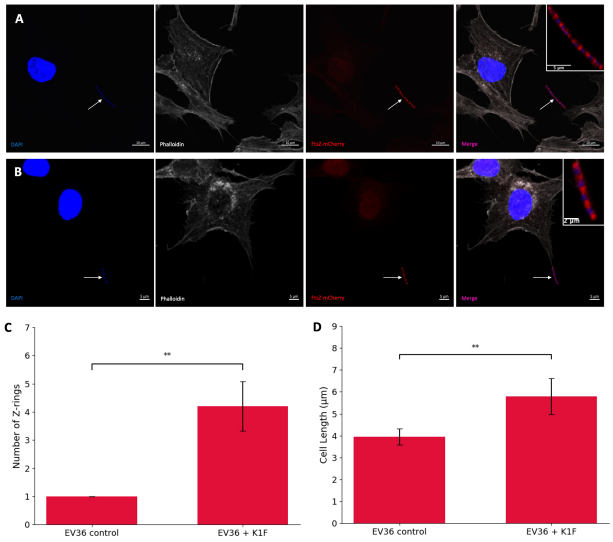

FIGURE 4
K1F phage inhibition of FtsZ in K1 *Escherichia coli*. **(A, B)** Fluorescent images of EV36/FtsZ-mCherry cells after K1F-GFP phage infection fixed in a human cell model. Arrows point to filamentous cells. DAPI stain is shown in blue, phalloidin in grey, and FtsZ-mCherry in red. **(C, D)** Quantification of Z-ring number and cell length for 75 EV36/FtsZ-mCherry bacterial cells infected with K1F-GFP phage and compared to a no-phage control. Averages plotted with error bars showing one standard deviation of uncertainty. Tukey's tests were performed, and the calculated probability values (p-values) are displayed as $p < 0.01$ (**).

3.5 Effects of T7 bacteriophage on ZapA and FtsI divisome proteins

We hypothesised that the observed effect of FtsZ inhibition by phages may not be specific to just FtsZ and that other divisome proteins may be involved in the infection process. To test this, a K12ΔzapA strain from the Keio collection with *zapA* gene knocked out was used, alongside a K12 control. Cultures were incubated for 1 h, then infected with T7 phage for a further hour, alongside a no-phage control, and imaged by light microscopy to observe cell filamentation (Figure 5A). The images were quantified, and average cell lengths for the conditions were plotted (Figure 5B). As previously shown, there was an increase in cell length upon T7 phage infection of K12 cells as they became filamentous, but this phenotype appears to be absent during T7 phage infection of K12ΔzapA cells, suggesting that ZapA is essential for cell filamentation and the phenotype seen during phage inhibition of cell division.

Next, the localisation of FtsI protein in this system was investigated. Cultures of K12 were grown for 1 h, infected with T7 phage, alongside a no-phage control, and stained with an FtsI antibody. Under normal growth conditions in the no-phage control, we observed that FtsI is localising to the midbody of the cell as a single ring (Figure 5C) as expected after recruitment by FtsZ as the divisome forms. After T7 phage infection of K12, we observed mislocalisation of FtsI (Figure 5D). This suggests that other divisome proteins are mislocalised upon phage infection as cell division is inhibited, potentially being recruited by mislocalised FtsZ.

3.5.1 T7 Gp0.4 targets FtsZ to increase titres and delays lysis

It has been previously shown by light microscopy that the T7 phage peptide gp0.4 directly inhibits FtsZ and causes host cell filamentation (Kiro et al., 2013), and in this study, we confirm this observation by confocal microscopy, to allow visualisation of the Z-rings in the human cell model after infection with a T7Δ0.4 mutant phage (Kiro et al., 2013). hCMECs were treated with K12/FtsZ-mNeon followed by infection with T7-mCherry or T7Δ0.4 phage, along with a control. Cells were imaged to see the resulting phenotypes (Figures 6A–C), and for each condition, cells were quantified and averages were plotted (Figures 6D, E). Upon T7Δ0.4 addition, unlike the WT T7, the mutant-infected *E. coli* showed recovery back to a phenotype similar to the control, confirming that T7's gp0.4 targets FtsZ and causes filamentation of the cells as well as multiple Z-rings along the cell body. After 2 h, the control sample had an average of 1.920 rings per cell with an average length of 3.426 µm, and for the WT T7-infected cells, this nearly doubled to an average of 3.827 rings and 6.143-µm length. Infection with T7Δ0.4 resulted in cells averaging 2.200 rings with a length of 3.513 µm, which closely resembles control measurements and suggests the host can divide normally with the mutant, so gp0.4 plays a role in blocking cell division *via* FtsZ inhibition.

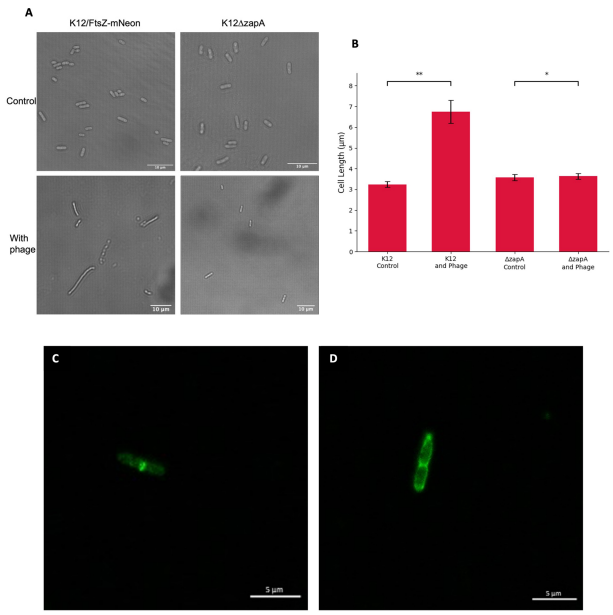

FIGURE 5
Effects of T7 bacteriophage on ZapA and FtsI divisome proteins. **(A)** Light microscopy images of K12 control and K12ΔzapA cells after T7 phage infection. **(B)** Quantification of cell length for 75 K12 control and K12ΔzapA bacterial cells infected with T7 phage and compared to a no-phage control. Averages plotted with error bars showing one standard deviation of uncertainty. Tukey's tests were performed and the calculated probability values (p-values) are displayed as $p < 0.05$ (*), $p < 0.01$ (**), and not statistically significant $p > 0.05$ (ns). **(C)** *Escherichia coli* K12 cell stained with an FtsI antibody showing localisation of FtsI to the cell midbody as a single ring. **(D)** *E. coli* K12 cell after T7 phage infection stained with an FtsI antibody showing mislocalisation of FtsI to three rings.

We then investigated why phages may have evolved these peptides, and it has been shown that WT phage progeny is higher than T7Δ0.4 progeny *via* PCR (Kiro et al., 2013), so we further verified this by performing plaque assays to titre phage lysate grown in the presence and absence of gp0.4 (Figure 7A). There was nearly a 10-fold decrease in titre with the T7Δ0.4 mutant, supporting previous PCR findings and suggesting gp0.4 plays a role in increasing progeny numbers, potentially by enabling the formation of long undivided cell factories. Growth curves were done to compare phage infection and cell lysis of WT T7 and T7Δ0.4 (Figures 7B, C), and we found that T7Δ0.4 lysed host cells after 45 min, whereas the WT T7 took 105 min, suggesting that the gp0.4 peptide has a role in delaying host cell lysis by approximately 1 h (43%).

3.5.2 Phage inhibition of FtsZ is limited in the stationary phase and under bacteriostatic conditions

We then wanted to apply the same phages to the model system during the stationary phase of cell growth to test the hypothesis that phage proteins need hosts that are actively dividing and producing an FtsZ target to inhibit FtsZ and block cell division. hCMECs were infected with K12/FtsZ-mNeon cells from overnight cultures to give cells in the stationary phase, followed by incubation with either T7-mCherry or T7Δ0.4 phage, along with a no-phage control. Bacterial cells were quantified and plotted alongside the exponential phase quantification for comparison (Figures 8A, B).

The average number of Z-rings in the stationary phase control was 1.160, unlike the exponential control of 1.920 rings per cell, suggesting no movement of FtsZ to confirm cells are not dividing. When the phages are applied, there is an increase in ring number for both phages compared to the control, but there are fewer rings produced compared to the exponential phase, suggesting some inhibition is taking place, but it is limited in the stationary phase. There is no change in length upon either phage addition in the stationary phase, so cells are unable to become filamentous. These data are consistent with the hypothesis that T7 inhibition of bacterial cell growth requires the presence of a functional FtsZ engaged in cell division. This was further confirmed by performing growth curves with bacteriostatic concentrations of chloramphenicol (Figure S13), which showed the phage was unable to lyse static host cells. Finally, we investigated whether the concentration of FtsZ changed in the stationary phase and upon phage infection by live-cell imaging calculation of corrected total cell fluorescence (CTCF) (Figure 8C and Figure S14). FtsZ-mNeon

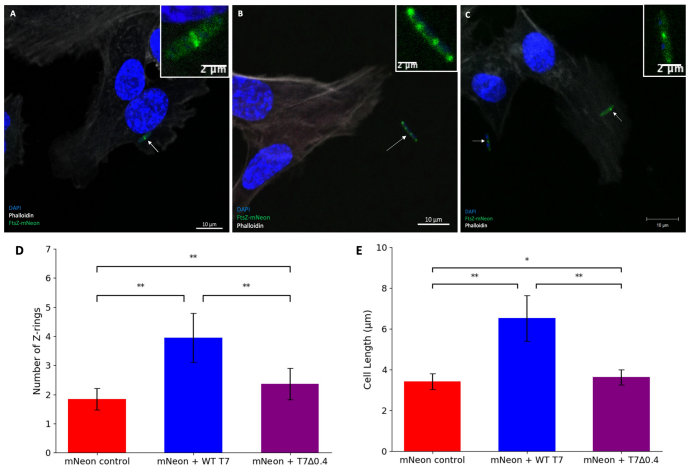

FIGURE 6
T7Δ0.4 mutant phage microscopy. **(A)** K12/FtsZ-mNeon control cells fixed in human cells. **(B)** K12/FtsZ-mNeon cells infected with T7-mCherry phage fixed in human cells. **(C)** K12/FtsZ-mNeon cells infected with T7Δ0.4 phage fixed in human cells. For fluorescent images, DAPI stain is shown in blue, phalloidin in grey, and FtsZ-mNeon in green. Arrows pointing to bacterial cells of interest. **(D,E)** Quantification of Z-ring number and cell length for 75 K12/FtsZ-mNeon bacterial cells infected with T7-mCherry or T7Δ0.4 phage during exponential growth and compared to a no-phage control. Averages plotted with error bars showing one standard deviation of uncertainty. Tukey's tests were performed, and the calculated probability values (p-values) are displayed as $p < 0.05$ (*), $p < 0.01$ (**), and not statistically significant $p > 0.05$ (ns).

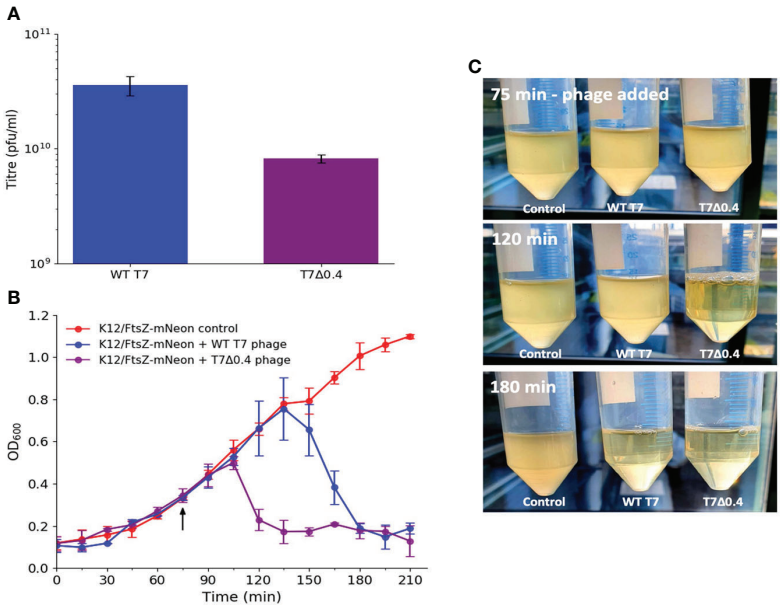

FIGURE 7
T7Δ0.4 mutant experiments showing that gp0.4 delays lysis and increases progeny. **(A)** WT T7 and T7Δ0.4 phages were propagated and titred by plaque assays in triplicate. Averages plotted on a log scale with error bars showing one standard deviation of uncertainty. **(B)** Growth curves were performed on cultures of K12/FtsZ-mNeon only, K12/FtsZ-mNeon and WT T7, K12/FtsZ-mNeon, and T7Δ0.4 phage. Average readings across replicates at 15-min time intervals were plotted with error bars showing one standard deviation unit of uncertainty. Arrow shows when phage was added. **(C)** Photo time series of growth curves with images of cultures taken upon phage addition at 75 min, at 120 min showing T7Δ0.4 lysis, and 180 min showing WT T7 lysis.

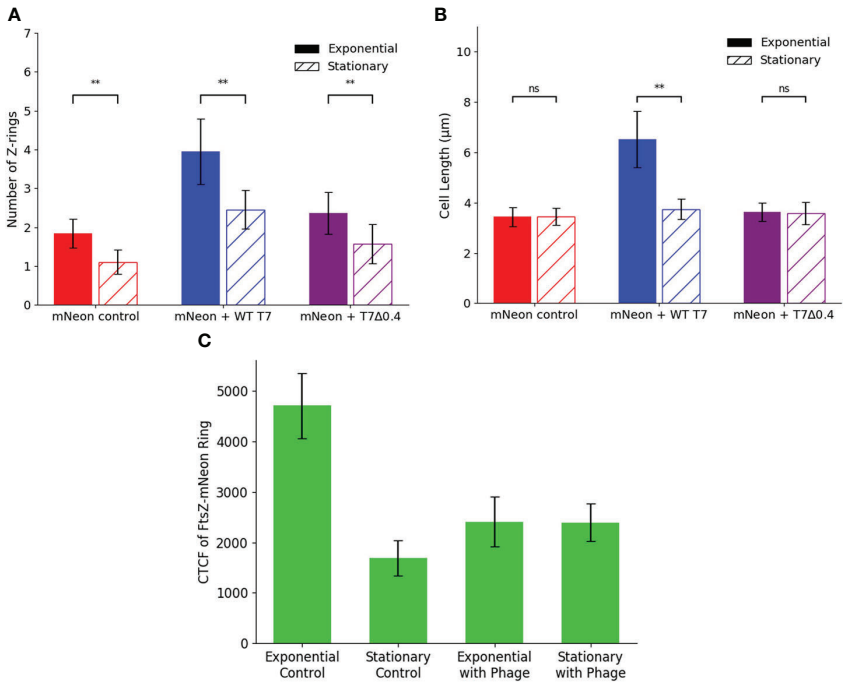

FIGURE 8
Stationary phase microscopy quantification of WT T7 and T7Δ0.4 infection. Quantification of **(A)** Z-ring number and **(B)** cell length for 75 K12/FtsZ-mNeon bacterial cells infected with T7-mCherry or T7Δ0.4 phage during the stationary phase of growth, along with a no-phage control, compared to exponential phase data. Tukey's tests were performed, and the calculated probability values (p-values) are displayed as $p < 0.05$ (*), $p < 0.01$ (**), and not statistically significant $p > 0.05$ (ns). **(C)** Exponential phase control, stationary phase control, exponential phase with T7 phage, and stationary phase with T7 phage cultures of *Escherichia coli* K12/FtsZ-mNeon imaged on agarose pads, and the corrected total cell fluorescence (CTCF) of 50 Z-rings per sample was averaged and plotted. Error bars show one standard deviation of uncertainty ns, not significant.

imaging shows a 2.8-fold decrease in the intensity per Z-ring in the stationary phase compared to exponential. Interestingly, upon phage addition to exponential culture, the average intensity per ring almost halves, potentially due to FtsZ being spread through the elongated cell.

4 Discussion

In this study, we used an *in vitro* meningitis model (Moller-Olsen et al., 2020) to investigate the effect of phage on bacterial FtsZ localisation during treatment, using hCMEC human brain cell cultures infected with *E. coli* EV36/FtsZ-mCherry or K12/FtsZ-mNeon strains and initially observed FtsZ localising to the cell midbody as a single ring. It has been previously shown that the bacteriophage λ Kil peptide can block FtsZ polymerisation and Z-ring formation, leading to a filamentous cell phenotype as a result of cells being unable to complete division (Haeusser et al., 2014). Here we confirm this phenotype and further show for the first time that Kil also leads to the formation of multiple Z-rings in the filamentous cell.

We have engineered a fluorescent T7-mCherry phage by homologous recombination, which specifically targets K12 strains of *E. coli*. This has allowed us to observe the invasion of hCMEC human brain cells by this phage and show that during T7 infection of K12, there is a mislocalisation of FtsZ into multiple rings along a filamentous cell that is unable to divide, suggesting that T7 phage inhibits FtsZ to block cell division. These multiple rings may represent attempts at cell division, where the inhibitor has stalled cytokinesis, creating multiple midbodies since the new upcoming cells are unable to separate. The filamentation seen may be due to MinC inhibition, since we show a MinC mutant strain becomes filamentous in normal growth conditions.

We have also shown that actively dividing cells are needed for phage inhibition of FtsZ, as inhibition is limited in the stationary phase. Since phage is still able to bind and infect these cells, we hypothesise that the decrease in average cell length and ring number is due to the small number of cells that are still dividing at a slower rate, leading to less of an effect seen from gp0.4 or any other inhibitor.

The T7 phage inhibition of FtsZ is likely to have a similar effect on other divisome proteins since proteins such as ZipA and FtsA have been shown to be recruited to the FtsZ ring site, so it is likely they will also mislocalise away from the midbody as new Z-rings form along the cell (Haeusser et al., 2014; Chen et al., 2017). Here we present that the ZapA protein is needed for phage inhibition of FtsZ by showing a phenotype recovery with a ZapA mutant strain, and we show that FtsI protein is also mislocalised upon phage infection. We propose that this effect is specific to the divisome due to the phenotype seen; for example, if the elangosome (Szwedziak and Löwe, 2013) protein MreB was inhibited and mislocalised, we would expect to see lemon-shaped cells (Molshanski-Mor et al., 2014) rather than elongation; therefore, these other proteins appear to be functioning as expected.

A recent study has shown that the T7 phage peptide gp0.4 directly inhibits FtsZ by binding the protein and preventing Z-ring assembly, giving *E. coli* cells an elongated phenotype under light microscopy, to enhance the phage competitive ability (Kiro et al., 2013). In this study, we further investigated how and why phage proteins might target FtsZ, using the T7Δ0.4 mutant to confirm that the T7 peptide gp0.4 is responsible for inhibiting FtsZ by showing a reversal in phenotype to normal single central ring growth compared to the filamentous multi-ring phenotype seen with the wild type. Plaque assays were performed to titre propagated T7Δ0.4 and wild-type T7 phages and found a 10-fold decrease in titre with the mutant, suggesting that the gp0.4 peptide assists in increasing phage progeny, confirming what was previously shown by Kiro et al. using PCR analysis (Kiro et al., 2013), to give the phage a competitive advantage by producing more progeny per burst cycle.

Here we present for the first time that gp0.4 delays host cell lysis, showing that the mutant T7Δ0.4 phage lysed bacterial culture approximately 60 min before the wild-type T7 containing lysed gp0.4. The Kil peptide has similarly been reported to delay lysis by 30% (Haeusser et al., 2014), so it seems that both these phage peptides targeting FtsZ are able to cause this delay. Lysis is initiated when cell signals such as lysins reach a certain threshold concentration towards the end of the infection cycle (Cahill and Young, 2019). Here we hypothesise that the delay in lysis is due to the cell filamentation increasing the volume of the infected cell, and therefore, it is take longer for a similar number of signals to reach the threshold concentration. This could also explain why the reduced titre seen for the mutant since lysis occurs faster, so the host cell bursts prematurely and the phage does not fully exploit the entire resources.

We report for the first time that the K1F phage is also able to target FtsZ and leads to a multi-ringed filamentous phenotype to block cell division, suggesting multiple phages have evolved this strategy.

K1F is a natural T7-like phage (Scholl and Merril, 2005). Interestingly, it lacks the 0.4 genes found in T7 but still shows the same phenotype, so it has evolved another peptide with the same function, which is currently unknown. Further studies on K1F phage to find this inhibitory peptide could lead to the discovery of a new antimicrobial compound. A BLAST search on the National Center for Biotechnology Information (NCBI) database showed that the K1F phage genome had no sequence similarity to the *kil* or *gp0.4* genes, so the inhibitory peptide is likely to be novel from these.

We observed that after about 75 min into normal growth the K12 bacterial cells seem to form two polar Z rings instead of the characteristic single midbody ring in the absence of phage infection. The location of the Z-ring is restricted by regulatory systems such as Min in *E. coli* (Shen and Lutkenhaus, 2011), so two Z-rings may demonstrate a time within the cell in which the Min system has not started to discriminate against FtsZ at the poles (Hu et al., 1999) and has also been previously shown that competition between FtsZ and the Min system could be the cause for these polar sites (Hu et al., 1999). We further show that a knockout mutant lacking the MinC protein displays a filamentous phenotype under normal growth conditions.

In conclusion, we have shown that FtsZ assembles as a single ring at the cell midbody under normal bacterial growth conditions during infection of human cells and shown that this localisation is disrupted to give a filamentous phenotype with multiple Z-rings along the cell body *via* phage peptides such as Kil, gp0.4, and an unknown K1F phage protein during phage infection. This inhibition may function to delay host lysis and increase phage progeny numbers and occurs during the exponential phase of bacterial growth when cells are dividing. This furthers our understanding of the mechanism that phages use to control their bacterial host, allowing them to be considered future antimicrobials.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

GD designed and performed experiments and wrote and revised the manuscript. IK performed experiments. UQ revised the manuscript. DR advised on experimental design and setup and revised the manuscript. AS conceived the idea, designed the experiments, supervised the study, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.863712/full#supplementary-material.

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A droplet-based microfluidic approach to isolating functional bacteria from gut microbiota

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Metabolic interactions within gut microbiota play a vital role in human health and disease. Targeting metabolically interacting bacteria could provide effective treatments; however, obtaining functional bacteria remains a significant challenge due to the complexity of gut microbiota. Here, we developed a facile droplet-based approach to isolate and enrich functional gut bacteria that could utilize metabolites from an engineered butyrate-producing bacteria (EBPB) of anti-obesity potential. This involves the high throughput formation of single-bacteria droplets, followed by culturing "droplets" on agar plates to form discrete single-cell colonies. This approach eliminates the need for sophisticated s instruments to sort droplets and thus allows the operation hosted in a traditional anaerobic chamber. In comparison to the traditional culture, the droplet-based approach obtained a community of substantially higher diversity and evenness. Using the conditioned plates containing metabolites from the EBPB supernatant, we obtained gut bacteria closely associated or interacting with the EBPB. These include anaerobic *Lactobacillus* and *Bifidobacterium*, which are often used as probiotics. The study illustrates the potential of our approach in the search for the associated bacteria within the gut microbiota and retrieving those yet-to-be cultured.

KEYWORDS

gut microbiota, microfluidics, droplet, anaerobic culture, probiotics

Introduction

The gut microbiota plays a vital role in human health (Brody, 2020). Differences in the composition and function of gut microbiota are associated with a wide range of chronic diseases, from gastrointestinal inflammatory to metabolic, neurological, cardiovascular and respiratory diseases (Vijay and Valdes, 2021). With the rapid

development of multi-omics technologies, such as metagenomics and amplicon sequencing, the complexity of the human gut microbiota and its role in disease are gradually being understood (Miyoshi et al., 2020). However, such methods are challenged by high cost, easily contaminated samples, and difficulties in reproducing experimental results (Kim et al., 2017). Moreover, its incapacity of obtaining and culturing isolates limits studying the interactions between species (Bilen et al., 2018). In contrast, the strains obtained by the culture methods can be used in *in vitro* and *in vivo* experiments, which are essential for the in-depth understanding of diseases and validation of potential therapeutics (Lagier et al., 2018). Therefore, in addition to culture-independent techniques, it is often necessary to isolate and obtain pure bacteria isolates for functional studies and verification.

Obesity refers to the condition in which excessive accumulation of body fat harms health, leading to shortened life expectancy and various problems (Hurt et al., 2010; Khan, 2016). Butyrate, a short-chain fatty acid, can decrease the pH of the intestine and promote the growth of beneficial bacteria in the gut (Chen and Walker, 2005). However, oral administration of butyrate compounds usually results in a low bioavailability (Bai et al., 2020). On the other hand, bacteria have been used as therapies for centuries, and recent advances in synthetic biology have unlocked tremendous opportunities for engineered bacteria in diagnosis and therapies (Riglar and Silver, 2018). Previously, we have engineered butyrate-producing bacteria (EBPB) and confirmed its potential anti-obesity effects on mice (Wang et al., 2022). Through metagenomic analysis of the gut microbiota, we found that after the long-term use of the EBPB bacteria, the abundance of beneficial bacteria, such as *Bifidobacterium*, *Lactobacillus*, and *Akkermansia*, increased. This indicates that the EBPB bacteria could regulate the microbiota composition, promoting the growth of beneficial gut bacteria. However, despite the knowledge of the genetic identity of these potential beneficial bacteria, obtaining these bacteria from gut microbiota *via* traditional culture has been futile and encountered well-known limitations, such as fast-growing bacteria dominating the culture plate. New approaches allowing enriching bacteria with different abundance and growth rates are highly desirable.

Droplet-based microfluidics has emerged as a powerful tool to control a small volume of fluid (e.g., pico- to nano-litre) in a high throughput manner and has found increasing applications in many fields (Sohrabi and Moraveji, 2020; Amirifar et al., 2022). Encapsulating individual cells in tiny droplets provides a protective environment for cells to grow without competition from others (Shang et al., 2017). In addition, thousands of single-cell microdroplets can be cultured in parallel, significantly enhancing the throughput (Mahler et al., 2021). It has been shown that droplet-based culturing enabled the growth of low-abundance bacteria (Watterson et al., 2020) and increased the diversity of cultured strains (Mahler et al., 2018). Furthermore, the surrounding oil can be pre-treated to tune the relative aerobic or anaerobic environment (Villa Max et al., 2020). Thus, the prospect of using droplet-microfluidic platforms to isolate and culture bacteria from the gut microbiota is very attractive. However, sorting desirable droplets requires bulk, sophisticated instruments, which are difficult to accommodate in a traditional anaerobic chamber.

Here, we developed a facile, droplet-based microfluidic approach to isolate and enrich individual bacteria cells from gut microbiota. This involves single-cell encapsulation in droplets followed by culturing the droplets on agar plates in an anaerobic chamber (which effectively "sorts" empty droplets). This approach can easily interface with conventional operations. Importantly, it can increase the diversity of obtained anaerobic species compared to traditional methods. Using desirable metabolites containing culture media, i.e. the supernatant from the engineered butyrate-producing bacteria (EBPB), we obtained metabolically functional species (e.g. *Lactobacillus* and *Bifidobacterium*), which could be used for further investigations, mining probiotics and constructing artificial flora to develop bacterial therapies.

Materials and methods

Strains and growth conditions

Engineered butyrate-producing BsS-RS06551 strain based on *Bacillus subtilis SCK6* host (EBPB) were created in-house previously (Wang et al., 2022). The butyrate yield reached 1.5 g/l, and the supernatant was weakly acidic. Green fluorescent protein (GFP) producing *Escherichia coli* BL21 was used to determine the most suitable cell loading density for single-cell droplet formation. *E. coli* BL21 and EBPB were routinely cultured in Luria-Bertani (LB) liquid medium and LB agar plates at 37°C. All strains obtained from faecal samples were cultured anaerobically in Yeast Casitone Fatty Acids (YCFA) liquid medium at 37°C and stored in a YCFA medium with 24% glycerol at -80°C. *Escherichia coli* Nissle 1917 (Biobw) and *Bifidobacterium pseudocatenulatum* (China General Microbiological Culture Collection Center, CGMCC) were routinely cultured in De Man, Rogosa and Sharpe (MRS) medium and MRS agar plates.

Conditioned medium plates

A freshly transformed EBPB single colony on the LB agar plate was inoculated into 5 mL of liquid medium and cultivated at 37°C until the OD_{600nm} value reached 1.0. Then 2 ml of the bacterial broth was transferred into 200 mL of fresh LB medium and cultured at 37°C, 220 rpm for 24 h. The fermentation supernatants were collected by centrifugation of the culture at

12000 rpm for 10 min and filtering through 0.22 μm PES membrane to remove all bacterial cells. To form conditional medium plates (CMPs), an autoclaved YCFA medium with 3% agar was heated to 60°C, then mixed with the supernatant at a ratio of 1:1 and dispensed into disposable plastic plates. These plates were stored at 4°C after agar solidification.

Fabrication of microfluidic chip

The microfluidic chip was designed using the AutoCAD 2016 software. A SU8 silicon mould was fabricated using the standard photolithography at the James Watt Nanofabrication Centre at the University of Glasgow, UK. Polydimethylsiloxane (PDMS) and curing agent (SYLGARD 184, Dow Corning Co., UK) mixture at a 10:1 ratio was poured onto the mould, degassed under a vacuum, and cured at 80°C for two hours. The PDMS replica was cut from the mould, and a biopsy punch (1.5 mm) was used to create both inlets and outlets for connecting tubes. After that, PDMS and a glass slide were ultrasonically cleaned with acetone, methanol, and isopropanol for five minutes and dried with filtered nitrogen gas. The channel side of the PDMS chip and the glass slide was treated in a Zepto plasma cleaner (Diener, Germany) for 20 seconds [p(O_2):0.3~0.4 mbar] and immediately assembled. Finally, the chips were heated overnight in the oven at 80°C.

Stool bacteria community preparation

The stool samples were collected from the previous animal experiments (Bai et al., 2020). Before each experiment, the anaerobic chamber (Shanghai Yuejin medical instruments Co. Ltd., HYQX-II) was filled with an anaerobic gas mixture (85% N_2/10% CO_2/5% H_2) the day before. The stool samples stored at -80°C were taken out and transferred to the pre-set anaerobic chamber. After anaerobic treatment for two hours, the stool samples were dissolved and suspended in a pre-anaerobic treated YCFA medium. To prevent clogging of the microfluidic chip, the bacterial suspension was filtered through a 40 μm sieve to remove large food residues and particles.

Bacteria encapsulation in droplets

The diluted bacterial suspension was used as the dispersed phase, and mineral oil (Sigma-Aldrich, light mineral oil) was used as the continuous phase. Various parameters (e.g., flow rates, cell loading density) have been evaluated to achieve robust droplet formation and single-cell encapsulation. The formed droplets were collected in an Eppendorf tube filled with mineral oil to prevent droplets from breaking. The microfluidic operation was conducted in the anaerobic chamber. In a typical experiment, a 0.03 g stool sample was dissolved in 4 ml of YCFA medium and filtered through a 40 μm membrane to remove large particles. Then the samples were washed three times, and the live cell density was measured using the Live/Dead BacLight Bacterial Viability kits (Invitrogen) and found to be $2.04 \pm 0.06\times10^7$/ml. Since the fluorescent dyes require oxygenation of the surrounding medium to fluoresce, we exposed the aliquot to air, which may affect the measured number of live cells.

Inoculation of single-cell droplets and diluted cell solution

The number of samplings needed to characterize a microbiome completely can be addressed through Coupon Collector's Problem (Morgan and Huttenhower, 2012). The approximate solution is given by Sampling cell number = N*(log(N) + 0.577216) + 1/2, where N is the total number of unique species in the microbiota. Recently the mouse gut microbial biobank revealed less than 150 species (Liu et al., 2020); thus, at least 414 cells are needed to characterize this level of diversity completely. To ensure sufficient sampling size while avoiding overseeding, the initial cell seeding number was chosen to be ~6.0×10^3 cells per plate for all the conditions.

Thus, the concentration of droplets in the collection tube was measured using microscopy and adjusted to ~6.0×10^4 droplets per μl with mineral oil. Since ~ 1% of droplets are single-cell droplets whilst the rest are empty droplets, 10 μl of droplets/oil solution was taken from the Eppendorf tube and spread on an agar plate for 72 h culture in an anaerobic chamber. Similarly, 10 μl of cell solution at 6.0×10^5 live cells/ml was spread onto an agar plate in parallel and cultured for 72 hours in the anaerobic chamber. YCFA plates and YCFA plates containing the supernatant of EBPB were used, and five replicas per condition were conducted.

16S rRNA sequencing of gut microbiota

After 72 hours of culture, colonies cultivated at each plate were all scraped for 16S rRNA sequencing with primers targeting the V3-V4 regions to evaluate the diversity of the cultivated cells. The CTAB/SDS method was used to extract the total genome DNA in samples. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL with sterile water. 16S rRNA genes in distinct regions (16S V3-V4) were amplified with specific primer and barcodes. All PCR mixtures contained 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of each primer and 10 ng target DNA, and cycling conditions consisted of a first denaturation step at 98°C for 1 min, followed by 30 cycles at 98°C (10s), 50°C

(30s) and 72°C(30s) and a final 5 min extension at 72°C. Mix an equal volume of 1X loading buffer (contained SYB green) with PCR products and perform electrophoresis on 2% agarose gel for DNA detection. The PCR products were mixed in equal proportions, and then Qiagen Gel Extraction Kit (Qiagen, Germany) was used to purify the mixed PCR products. Following the manufacturer's recommendations, sequencing libraries were generated with NEBNext® Ultra™ IIDNA Library Prep Kit (Cat No. E7645). The library quality was evaluated on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated. The raw Illumina sequence data have been deposited in the NCBI database under BioProject accession number PRJNA861917.

Bioinformatics & statistics

We have used VSEARCH to generate OTUs at 97% similarity using the protocol given in our previous study (Trego Anna et al., 2020), with one modification, we have used the latest version of SILVAMOD v138 reference database. Furthermore, as a prefiltering step, we removed typical contaminants such as those matching *Chloroplast* and *Mitochondria* and those that are unassigned (https://docs.qiime2.org/2022.2/tutorials/filtering/), which resulted in a final OTU table comprising of a total of 1,387 unique sequences for n = 17 samples. Statistical analyses were performed in R using the tables and data generated above, as well as the metadata associated with the study. We used the vegan package Field for alpha and beta diversity analysis (Oksanen et al., 2012). In particular, we used Rarefied Richness, a commonly used index, to measure the estimated number of OTUs within a sample after rarefying to the minimum library size. For beta diversity analysis, we have used Bray-Curtis distance in Principle Coordinate Analysis (PCoA) by using the cmdscale() function. Vegan's adonis() function was used to perform an analysis of variance (PERMANOVA) of sources of variations (groups in this study) against Bray-Curtis distance as mentioned above. To find genera (OTUs collated at genus level) that are significantly different between multiple categories considered in this study, we have used DESeqDataSetFromMatrix() function from DESeq2 (Love et al., 2014) package with the adjusted p-value significance cut-off of 0.05 and log fold change cut-off of 2.0. This function uses negative binomial GLM fitting to obtain maximum likelihood estimates for the genera log fold change between the two conditions. Then Bayesian shrinkage is applied to obtain shrunken log fold changes, subsequently employing the Wald test for obtaining significances. For visualisation of results, we have used R's package ggplot2 (Wickham, 2016).

Genetic sequencing of selected isolates

To have a better resolution of species identification, 100 colonies formed at each condition were randomly picked for the full-length 16S rRNA gene sequencing (Sanger sequencing). Each picked colony was cultivated anaerobically in a 20 mL medium at 37°C for 12 hours. The genome of each single-cell colony was extracted using TIANamp Bacteria DNA Kit as a template for PCR. Universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify nearly the full length of the 16S rRNA sequence. PCR reactions were proceeded in 50 µL volumes, each containing 2 µL of 10 µM forward and reverse primers, respectively, 25 µL 2× Phanta Max Buffer, 17 µl ddH_2O, 1 µl Phanta Max Super-Fidelity DNA Polymerase, 1 µl dNTP Mix and 2 µL DNA template. The thermocycling was performed as follows: 41 cycles (95°C, 15 s; 55°C, 15 s; 72°C, 60 s) after an initial denaturation at 95°C for 3 min, following a final extension at 72°C for 5 min. Then, the PCR products were purified and sequenced (Sanger sequencing) to get the gene sequences. Finally, the gene sequence was submitted to NCBI to identify the isolates. Sequence annotation and the database searches for sequence similarities were performed using the BLAST tool available online. Generally, these isolates' 16S rRNA genes nucleotide sequences with homology between 99% and 100% with the reference strain in NCBI GenBank, belong to different strains of the same species (Janda and Abbott, 2007).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.3.0(538) (GraphPad Software, San Diego, California, USA, www.graphpad.com). Average data were given from five plates in each condition. For each condition, at least three independent repeated experiments were conducted.

Results

Production of stable and uniform droplets

The overall strategy is illustrated in Figure 1, and the setting for droplet formation is shown in Figure S1. A pre-filtered faecal bacterial suspension was injected into the microfluidic device as the aqueous phase to form water-in-oil droplets. A certain amount of the collected droplets were spread on an agar plate. In principle, every droplet containing a single cell could result in a single-cell derived colony if the cell is cultivable. Compared with the conventional series dilution-based culture, this method would offer simplicity and speed in obtaining single-cell derived colonies.

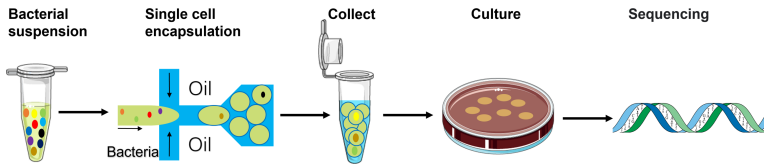

FIGURE 1
Schematic of the workflow using the single-cell droplet culture approach to search for functional bacteria from gut microbiota. Faecal samples were dissolved in media to extract gut microbiota into liquid. The pre-filtering bacterial suspension was injected into the microfluidic device for cell encapsulation in droplets. Stable and uniform droplets were collected into an Eppendorf tube containing mineral oil. Droplets were then spread on a culture plate and would burst eventually. The bacteria in the droplets could continue to grow on the plates. 16S rRNA sequencing technology was applied to identify growth colonies' species.

A flow-focused microfluidic chip was designed for droplet formation since it offers excellent flexibility to tune droplet size by varying the ratio between the continuous phase (oil) and the dispersed phase (aqueous) (Anna et al., 2003). Considering the size of bacteria cells (~ 1 µm), the cross-section of the chip had a dimension of 10µm (width) × 65µm (length) × 20µm (height) to facilitate small droplet formation and hence single-cell encapsulation (Figure 2A). We firstly evaluated conditions for the generation of stable and uniform droplets, which was essential for single-cell encapsulation. In this regard, the surfactant is an important factor since surfactants are adsorbed between the dispersed and continuous phases, thereby reducing the interfacial tension (Baret, 2012). This prevents droplets from coalescing with each other, therefore stabilizing the droplets in emulsion for a relatively long period (Mazutis et al., 2013). With 2% Span80 in the mineral oil phase, droplet coalescence occurred frequently; the droplet size had a wide distribution with an average value of 18.24 ± 5.54 µm (Figures 2B, C). Increasing Span80 concentration to 5% in mineral oil resulted in the generation of stable and uniform droplets at a throughput of 5500 droplets per second. Furthermore, hardly any coalescence was observed

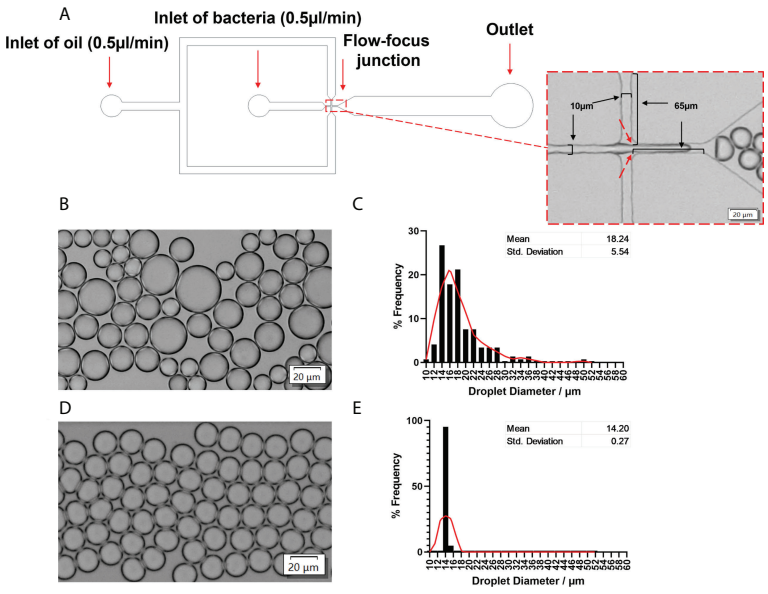

FIGURE 2
Microfluidic device to generate stable and uniform droplets. **(A)** Channel dimensions of the microfluidic chip. The dotted rectangle insert showed the bright-field image of the flow-focusing junction. A bacterial solution was the disperse phase, and oil was the continuous phase. Droplets were produced at the flow-focusing junction (indicated by red arrows). **(B)** Bright-field images and **(C)** the frequency distribution of droplet sizes produced by 2% Span80 in mineral oil. The average diameter was 18.24 ± 5.54 µm. **(D)** Bright-field images and **(E)** the frequency distribution of droplet sizes produced by 5% Span80 in mineral oil. The average diameter was 14.20 ± 0.26 µm. 94.2% of droplets diameter values fell into the bin of 14.05 µm. Randomly selected 150 droplets were measured using the cellSens imaging software. The relative frequency distribution (percentage) was analysed using GraphPad Prism 8.3.0(538). The red Lowess curve showed the trend of the data.

(Figure 2D), and more than 80% of droplets are 14.20 ± 0.27 μm in diameter (Figure 2E).

Optimizing single-cell encapsulation

Cell loading density is an effective way to control the encapsulated cell number in droplets. Previous studies show that the number of cells encapsulated in droplets follows the Poisson distribution (Collins et al., 2015; Villa Max et al., 2020), which is given by $P(x, \lambda) = e^{-\lambda}(\lambda^x/x!)$, where x is the number of cells per droplet, P is the proportion of droplets containing a given cell number x, and λ is the average number of cells per droplet volume (i.e. $\lambda=\rho V$, where V is the droplet volume and ρ is cell loading density). We simulated P as a function of λ for empty droplets, single-cell droplets and multiple-cell droplets (Figure 3A). To ensure no more than one cell in each droplet (important for single-cell colony formation), we selected λ at 0.01, corresponding to ~ 1% of single-cell droplets and 99% of empty droplets. Based on the droplet dimension (14.20 ± 0.27 μm), the cell loading density was around ~7×10^6 cells/mL. To validate the condition, GFP *E. coli* BL21 was used as the model strain to aid the detection of individual bacteria cells in a droplet *via* fluorescence imaging. No droplets with >1 cell were found (Figure 3B). The percentage of single-cell droplets is close to the theoretical value of 1%.

Droplet-aided culture enhances the diversity of obtained species

The stability of the droplets spread on agar plates was monitored at 37°C over a time course (Figure S2). Most droplets remained intact for up to 8 hours (some were stable for up to 13 hours), indicating a single bacteria cell could grow first in droplets until the droplets broke. To test this, *E. coli* Nissle 1917 and *Bifidobacterium pseudocatenulatum* were cultured using the single-cell droplet culture and traditional dilution approach at the same seeding density for 48 hours. *Bifidobacterium pseudocatenulatum* is known to be strictly anaerobic and difficult to culture on agar plates. It was found that colonies of *B. pseudocatenulatum* only formed *via* the single-cell droplet culture (Figure S3). However, colonies of *E. coli Nissle 1917* formed under both conditions.

Furthermore, the colony formation rate of *E. coli via* the single-cell droplet culture is substantially higher than that of traditional culture. It is known that traditional plate cultures select fast-growing bacteria over slow-growing bacteria (i.e., many bacteria do not grow on commonly used culture plates) (Jannasch and Jones, 1959; Staley and Konopka, 1985; Olsen and Bakken, 1987). Similarly, in the case of faecal samples, substantially more colonies were formed *via* the single-cell droplet approach method (Figure S4). These results illustrated that our droplet approach can enhance cell growth, especially for slow-growing species, and challenge anaerobic gut bacteria.

To understand the composition and diversity of the cultivated cells, all colonies under each condition were scraped for 16S rRNA sequencing with primers targeting the V3-V4 region. A total of 1,387 unique OTU sequences were found (n=17 independent experiments). Statistical analysis shows that the α – diversity (richness) of both the original faecal sample (denoted as **C**) and the diluted loading sample (denoted as **L**) was marginally significantly different from that of the cultured cells from the traditional culture (denoted as **T**) ($P<0.05$). However, the α–diversity of the cells from the droplet culture (denoted as **D**) is significantly different from that of the traditional culture **T** ($p<0.001$). Notably, there are no significant differences between either **C** and **D** or **L** and **D;** this suggests that the alpha diversity (richness) is conserved and makes us believe that the communities obtained from the

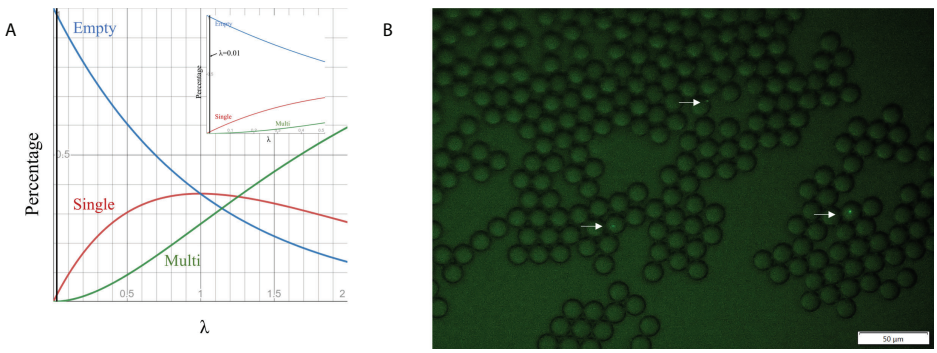

FIGURE 3
Cell encapsulation in droplets. **(A)** Relationship between the percentage of droplets containing different cell numbers and λ, which is the average number of cells per droplet volume. **(B)** Fluorescence images of droplet occupancy at 7×10^6 cell numbers per ml (the most frequently used cell loading densities).

droplet method are similar to the original communities we started with (Figure 4A).

We have also used Bray-Curtis distance for beta diversity analysis, which only considers the composition of community members. It was noticed that C and L were very close to each other, suggesting there was minimal loss of beta diversity between them. The samples from the traditional culture and those from the droplet method formed distinct clusters and did not overlap (Figure 4B), and PERMANOVA analysis showed 65% variability between all groups. The top 25 most abundant genera observed in all samples differed (Figure 4C). The differentially expressed genera, explaining differences between the droplet culture approach and the traditional culture, are given in Figure 5, with most of them increasing in abundance in the droplet method. It is worth noting that the single-cell droplet approach recovered largely uncultured *Thiotrichaceae* (i.e., the reference sequence obtained through genomics), illustrating its promise in the discovery of yet-to-be-cultured species.

Because we have taken a shorter amplicon region for 16S rRNA sequencing, to obtain a better taxonomic resolution of the species, we randomly picked 100 colonies from three plates under each condition for Sanger sequencing with universal primers 27F and 1492R (Tables 1, 2). A total of 7 species were found from the traditional culture (Table 1), but 14 were found

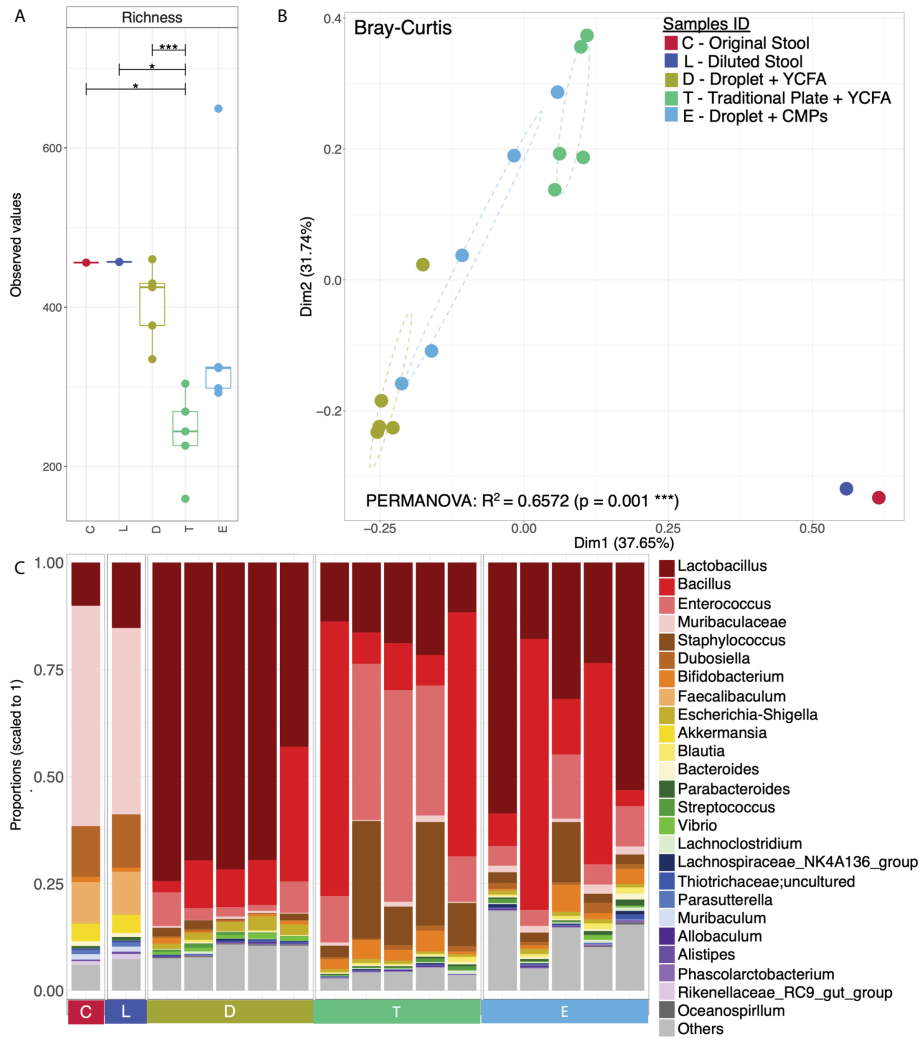

FIGURE 4
Microbial diversity and community structure. **(A)** Rarefied richness with lines connecting two categories where the differences were significant (ANOVA), i.e., * ($p < 0.05$), *** ($p < 0.001$); **(B)** Principle Coordinate Analysis (PCoA) using Bray-Curtis distance with the axis showing the percentage variability explained by each axis, and ellipses representing 95% confidence interval of standard error for each group (Sample IDs). PERMANOVA's R^2 represented percentage variability explained by the groups, i.e., 65.72%; and **(C)** Top 25 most abundant genera observed in all samples grouped by categories, where "Others" contain those genera which didn't make the cut.

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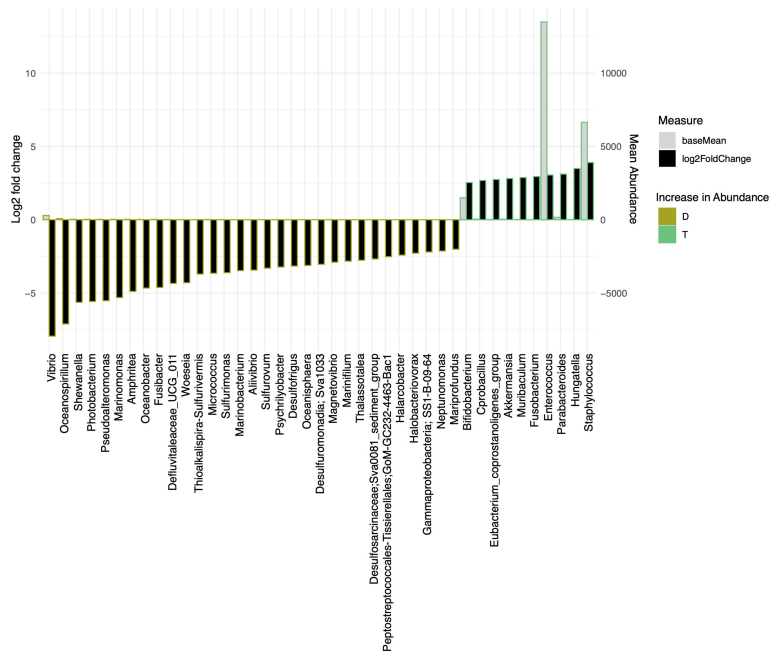

FIGURE 5
The bar charts show Log2 fold change in abundance of significant genera between groups (y-axis on the left and black bar) and the mean abundance across all the samples (y-axis on the right and light grey bar). Taxa increased in D (Droplet + YCFA) have bars with a mustard border (negative log2 fold change) meanwhile taxa increased in T (Traditional Plate + YCFA) have bars with a green border (positive log2 fold change).

from the droplet culture (Table 2). There were 5 species common between the two groups (Figure S5). These results are in excellent agreement with the overall 16S rRNA characterisation. Together, they show that our single-cell droplet culture approach provides a facile and effective platform for culturing gut microbiota and preserving its diversity.

Isolating gut bacteria metabolically associated with EBPB

With the advantages demonstrated by the single-cell droplet culturing approach, we next exploited this platform to isolate gut bacteria capable of using butyrate or other metabolites produced by the EBPB bacteria (Bai et al., 2020). Oral administration of the EBPB bacteria alleviated obesity symptoms, and the

TABLE 1 Result of the obtained species using the traditional method.

Isolates ID[a]	Total isolates[b]	Species	Genus	Similarity[c]
1, 2, 5, 18, 34, 47, 51, 63, 90, 91	10	*E. faecalis*	*Enterococcus*	100%
13, 14, 17, 46, 81, 82, 92	7	*E. gallinarum*		99.72%
3, 27	2	*L. reuteri*	*Lactobacillus*	99.31%
7, 10, 12, 15, 19, 60, 68, 85, 86, 87, 93, 94, 95	13	*L. johnsonii*		99.79%
6, 25, 26, 29, 30, 31, 32, 33, 35, 36, 38, 39, 40, 41, 42, 43, 44, 45, 48, 49, 50, 52, 53, 55, 56, 58, 59, 61, 62, 65, 66, 67, 69, 70, 71, 72, 80, 96	38	*P. aquatica*	*Pelomonas*	99.93%
28, 37	2	*P. saccharophila*		99.78%
64	10	*S. roterodami*	*Staphylococcus*	99.72%

[a]: Isolates ID. – the reference number of each colony.
[b]: The total isolates obtained that belong to the same species.
[c]: A similarity is a number used to describe how similar the query sequence is to the target sequence. The higher the similarity, the more significant the matching (Pearson, 2013).
The 16S rRNA gene sequences of the isolates in Table 1 have been deposited in GenBank databases under the accession numbers ON974135-ON974208.

TABLE 2 Result of the obtained species with droplet encapsulation.

Isolates ID	Total isolates	Species	Genus	Similarity
5, 8, 24, 29	4	*B. paraconglomeratum*	*Brachybacterium*	100%
36	1	*B. cereus*	*Bacillus*	100%
42, 46	2	*B. licheniformis*		99.12%
17, 41	2	*B. mojavensis*		99.90%
6, 7, 10, 45	4	*B. casei*	*Brevibacterium*	99.93%
44	1	*B. sanguinis*		99.69%
84, 94, 26	3	*E. faecalis*	*Enterococcus*	100%
23	1	*E. gallinarum*		99.65%
70, 71	1	*P. aquatica*	*Pelomonas*	99.93%
52, 85, 88	3	*L. johnsonii*	*Lactobacillus*	99.79%
74, 75, 76, 77, 78, 80, 81, 82, 83, 86, 87, 89, 90, 91, 92, 93, 47, 48, 49, 50, 51, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68	36	*L. murinus*		99.72%
79, 55	1	*L. reuteri*		99.66%
1, 4, 19, 27, 40, 43	6	*M. endophyticus*	*Micrococcus*	99.93%
2, 12, 13, 15, 16, 18, 20, 21, 22, 28, 30, 31, 32, 33, 35, 37	16	*M. luteus*		99.93%

The 16S rRNA gene sequences of the isolates in Table 2 have been deposited in GenBank databases under the accession numbers ON974317-ON974397.

metagenomic study revealed an altered gut microbiota (Wang et al., 2022). To illustrate the mechanism, it is necessary to understand who interacts with EBPB in the gut microbiota.

Here, we spread the collected droplets on the conditioned medium plates (CMP, i.e. containing the EBPB supernatants) under the same seeding condition as that on the YCFA plate Similarly, all colonies (denoted as **E**) were scraped for 16S rRNA amplicon sequencing. Although the alpha diversity of the obtained community **E** appears different from the others, there was no significant difference (Figure 4A) from the original communities **C** or **L**. Beta-diversity analysis reveals an independent cluster for community **E**, suggesting their unique composition (Figure 4B). Interestingly, *Lactobacillus* is the most abundant genus in the community, and *Bifidobacterium* is within the top 10 (Figure 4C). These confirm our previous *in vivo* study, implanted EBPB in mice enriched *Lactobacillus* and *Bifidobacterium* (Bai et al., 2020; Wang et al., 2022).

Similarly, we further characterised randomly picked 100 single-cell colonies for species identification *via* Sanger sequencing. 11 species under four genera (namely, *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Enterococcus*) were identified (Table 3). Among the assigned 86 isolates, 42 belong to *Lactobacillus* (i.e. the top genus), and one belongs to *Bifidobacterium* (i.e. *Bifidobacterium pseudocatenulatum*), which agrees well with the 16S rRNA study. It is worth noting that *Bacillus subtili genus* is the 2nd largest group (33 isolates). It

TABLE 3 Result of the obtained isolates with droplet encapsulation using the CMPs.

Isolates ID	Total isolates	Species	Genus	Similarity
17,18,21,22,23,36,37,38,49, 56, 57, 70, 71, 75, 76,77, 78, 79, 80, 84, 85, 86, 88, 90, 91, 97	26	*L. murinus*	*Lactobacillus*	99.93%
50, 58	2	*L. intestinalis*		100%
51, 61, 64, 65, 66, 67	6	*L. vaginalis*		100%
53,54, 59, 60	4	*L. reuteri*		100%
48, 55, 62, 63	4	*L. johnsonii*		99.79%
52	1	*B. pseudocatenulatum*	*Bifidobacterium*	99.79%
1, 3, 41	3	*B. cereus*	*Bacillus*	100%
5, 7, 10, 11, 12, 15,16,19,20, ,24,25,26,28,29,30, 31,32,33,35, 39,40,42,72,73,74,81,82	27	*B. nealsonii*		100%
8	1	*B. paramycoides*		100%
44, 83	2	*B. circulans*		100%
27, 34, 46,47,87, 89, 92, 94, 98, 99	10	*E. faecalis*	*Enterococcus*	100%

The 16S rRNA gene sequences of the isolates in Table 3 have been deposited in GenBank databases under the accession numbers ON974744-ON974829.

has been shown recently that *Bacillus subtilis* can produce bifidogenic factors that promote the growth of *Bifidobacterium* species (Hatanaka et al., 2020).

Discussions

The human gut is inhabited by diverse microbes that play a fundamental role in maintaining the health of the host (Clemente et al., 2012). An increasing amount of evidence reveals that many diseases often involve significant variations in the diversity and composition of gut microbiota (Marchesi et al., 2016; Liu et al., 2021). Understanding the underlying process requires the knowledge of "who does what in the gut microbiota" and "how they interact with each other and the host" (Miyoshi et al., 2020). However, the complexity of gut microbiota *in vivo* is prohibitively challenging. To date, our insights come mainly from extensive research of faecal samples, which are used as a surrogate for the gut. Although molecule-based approaches can reveal the genetic composition of the gut microbiota, obtaining pure gut bacteria isolates is indispensable for deciphering the role of specific bacteria and their interactions (Bäckhed et al., 2012). However, traditional culture is time-consuming and biased toward dominant, fast-growing bacteria in the community (Watterson et al., 2020). Even the recent development of "culturomics", which uses multiple culture conditions, has discovered hundreds of new microorganisms (Lagier et al., 2015), substantially amount of bacteria in gut microbiota have yet-to-be-cultured to allow *in vitro* investigations of their physiologic functions.

With the ability to isolate single cells in a confined environment, droplet-based microfluidic has rapidly become a promising, high throughput tool for microbial cell culture. However, the implementation of this technology for anaerobic bacteria studies is restrained by the difficulties of operating bulky instrumentation in an anaerobic workstation (Kaminski et al., 2016; Liu and Walther-Antonio, 2017). To overcome those problems, we have developed an easy-to-operate microfluidic approach for isolating functional bacteria from gut microbiota. The workflow of single-cell encapsulation in droplets and droplet culture on standard plates can be easily carried out in an anaerobic chamber without sophisticated and bulky instrumentation. This approach also simplifies the interface between the microscale world with the conventional macroscopic operation and thus can be readily implemented in microbiology labs. We showed that the single-cell droplet culture promoted cell growth, especially for the slow-growing and challenging anaerobic cells, which resulted in a significantly higher diversity of the obtained community compared with the traditional approach and preserved the diversity of the original gut microbiota.

The flexibility of our method for obtaining interactive or metabolically associated bacteria in gut microbiota was illustrated using the butyrate-producing EBPB bacteria, which demonstrated potential in preventing obesity and improving metabolic function (Bai et al., 2020). Our previously metagenomic studies showed that oral administration of EBPB in mice seemed to increase the abundance of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus, in vivo* (Bai et al., 2020; Wang et al., 2022). Here, the single-cell droplet approach enabled us to obtain the isolates of these beneficial bacteria, providing solid evidence for observed therapeutical benefits. The isolates could be used for further investigations, mining probiotics and constructing artificial flora to develop bacterial therapies against obesity. Taken together, the single-cell droplet culture approach complements culture-independent metagenomic investigations in studying living bacteria therapy. While the metagenomic analysis of the gut microbiota could reveal overall shifts in microbiota composition, isolating gut bacteria and those closely associated are important for further research to understand their function and interaction with the host (Figure 6).

Conclusions

We developed a single-cell droplet on plate culture to isolate and enrich functional gut bacteria from faecal microbiota. The

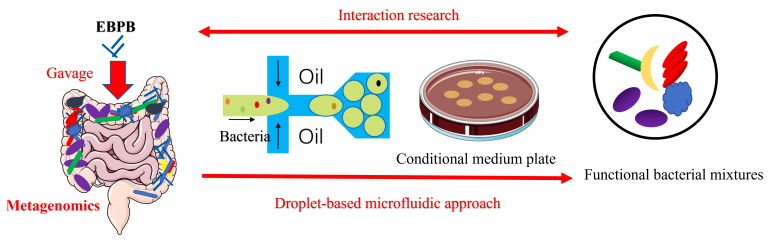

FIGURE 6
Schematic of the combined metagenomics and the single-cell droplet culture approach to investigate the potential anti-obesity potential of EBPB. The metagenomic analysis of the gut microbiota revealed that the abundance of beneficial bacteria increased after the long-term use of the EBPB. However, isolating, culturing, and analysing the associated bacteria allow us to further study the interaction between EBPB and host, revealing its therapeutic potential. The information will open avenues to develop future living bacteria therapies (e.g., probiotics).

whole process can be easily operated in an anaerobic chamber, allowing the search for obligate anaerobic bacteria. We show the reliable formation of single-cell colonies and significantly improved diversity and evenness of the obtained species. The approach integrates the capability of microfluidics for high throughput and precise cell manipulation with the simplicity of plate culture and can be easily implemented in traditional microbiology labs. With this approach, we have successfully obtained pure gut bacteria isolates that are metabolically associated with the engineered EBPB bacteria, shining a light on the mechanism of its therapeutical potential. We show that our approach, in combination with metagenomic studies, will provide a powerful tool to study gut microbiota and develop potential therapeutics (e.g., probiotics).

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: GenBank ON974135-ON974208; ON974317-ON974397; ON974744-ON974829; NCBI - BioProject PRJNA861917

Author contributions

HH, HY, PW, GK, YS, XL, JY, and XC conceived and designed the experiment; YS fabricated the silicon mould. XC and JY performed the experiments; XC, JY, and UI analyzed the data. JY, XC, HH, and HY wrote the manuscript. XC and JY contributed equally to this work. All authors read and approved the final manuscript.

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The DNA model elements in Figure 1 were sourced from Scidraw.io. Furthermore, the part original elements of the Figures 1, 6 were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

Conflict of interest

Authors XL and PW were employed by Tianjin Modern Innovative TCM Technology Co. Ltd.

The remaining authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.920986/full#supplementary-material

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Emerging story of gut dysbiosis in spondyloarthropathy: From gastrointestinal inflammation to spondyloarthritis

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As a set of inflammatory disorders, spondyloarthritis (SpA) exhibits distinct pathophysiological, clinical, radiological, and genetic characteristics. Due to the extra-articular features of this disorder, early recognition is crucial to limiting disability and improving outcomes. Gut dysbiosis has been linked to SpA development as evidence grows. A pathogenic SpA process is likely to occur when a mucosal immune system interacts with abnormal local microbiota, with subsequent joint involvement. It is largely unknown, however, how microbiota alterations predate the onset of SpA within the "gut-joint axis". New microbiome therapies, such as probiotics, are used as an adjuvant therapy in the treatment of SpA, suggesting that the modulation of intestinal microbiota and/or intestinal barrier function may contribute to the prevention of SpA. In this review, we highlight the mechanisms of SpA by which the gut microbiota impacts gut inflammation and triggers the activation of immune responses. Additionally, we analyze the regulatory role of therapeutic SpA medication in the gut microbiota and the potential application of probiotics as adjunctive therapy for SpA.

KEYWORDS

spondyloarthritis, inflammation, gut-joint axis, probiotics, gut dysbiosis

Introduction

Spondyloarthritis (SpA) is a family of clinical disorders with some featured manifestations, etiopathological characteristics and genetic factors (Moz et al., 2017; Duba and Mathew, 2018; Terenzi et al., 2018). SpA primarily features sacroiliitis, namely, axial SpA (axSpA), and the peripheral joint can also be involved (pSpA) (Sieper et al., 2009; Rudwaleit et al., 2011; Stolwijk et al., 2012; Dubreuil and Deodhar, 2017; van der

Heijde et al., 2017). Back pain is a major symptom of axSpA, and exercise can improve pain and stiffness (Rudwaleit and Sieper, 2012; Bidad et al., 2017). Additionally, there are other musculoskeletal manifestations (e.g., arthritis, enthesitis, dactylitis, etc.) and extra-articular manifestations (e.g., psoriasis, anterior uveitis, etc.) for axSpA (Duba and Mathew, 2018; Magrey et al., 2020). In recent years, the term "axSpA" has been used more commonly to cover this group of SpA diseases (Sieper and Poddubnyy, 2017; Ritchlin and Adamopoulos, 2021; Robinson et al., 2021; Danve and Deodhar, 2022).

Based on the sacroiliac joint radiological results, axSpA can be classified further into radiologically positive axSpA with definite imaging damage (r-axSpA) and radiologically negative axSpA, namely, non-radiographic axSpA (nr-axSpA) (Sieper and Poddubnyy, 2017; Ritchlin and Adamopoulos, 2021; Robinson et al., 2021; Danve and Deodhar, 2022). There are some patients with nr-axSpA who progress to r-axSpA (Protopopov and Poddubnyy, 2018). Ankylosing spondylitis (AS) is the best studied subtype of axSpA at the later stage (Dougados and Baeten, 2011; Sieper and Poddubnyy, 2017). Enteropathic arthritis (EPA), reactive arthritis (ReA), and psoriatic arthritis (PsA) are the common types of pSpA (Wilson and Folzenlogen, 2012; Taams et al., 2018). New imaging modalities, biomarkers, and genetic data may be available to update the classification criteria of SpA (Sieper et al., 2009; Rudwaleit et al., 2011; Dubreuil and Deodhar, 2017; van der Heijde et al., 2017). Emerging evidence supports the idea that SpA and inflammatory bowel disease (IBD) share similarities in their genetic predisposition and pathogenesis (Olivieri et al., 2014; Karreman et al., 2017; Fragoulis et al., 2019; Qaiyum et al., 2021). Thus, IBD-associated SpA was a concern in this study.

Based on the most recent research on the relationship between SpA and intestinal inflammation, we summarize the evidence that supports intestinal dysbiosis in SpA. Specifically, the mechanisms by which dysbiosis contributes to subclinical intestinal inflammation and immune response activation to SpA will help us to understand the idea of the "gut-joint axis". To this end, we outline the effects of current SpA medication treatment options on the gut microbiota with a focus on research findings in the field of microbiota-targeted precision therapy. Lastly, some thoughts were provided on the underlying mechanisms and modulation of the intestinal microbiota as potential new treatment approaches to SpA.

Intestinal microbial dysbiosis in SpA

The human microbiota contains a plethora of parasitic or symbiotic microorganisms, such as bacteria, viruses, and fungi (Miller et al., 2021). The intestine is crucial for maintaining homeostasis between the microbiota and the host, which involves the effective coordination of different immune cells (Tiffany and Bäumler, 2019). The number of cells in the typical flora of the human colon has been estimated to reach 10^{12} colony forming units (CFU) (Sekirov et al., 2010). The intestinal flora primarily consist of *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (Honda and Littman, 2012). The gut microbiota is associated with human metabolic and physiological activities, including balancing the immune response and regulating intestinal endocrine function and amino acid metabolism (Honda and Littman, 2012; Lynch and Pedersen, 2016; Dupont et al., 2020). Dysbiosis means the occurrence of a microbiome imbalance when environmental factors or host-related factors alter the composition and function of microbial communities, which is related to a series of clinical disorders (Brandl and Schnabl, 2015; Tiffany and Bäumler, 2019).

Emerging publications report the relationship between the gut microbiota and SpA. We summarized the recent findings on the changes in the gut microbiota under SpA in Table 1. There is no consensus on which bacterial species are linked to the development of SpA. Several candidate microbes were identified as potential drivers of gut inflammation in experimental SpA induced by HLAB27 (Gill et al., 2019). Prevotella and Blautia (Lewis rats) and Akkermansia muciniphila, rc4-4, Lachnospira, and Lachnospiraceae (Fischer rats) were found to be closely related to the dysregulated inflammatory pathways (Gill et al., 2019). The emergence of joint damage resembling Reiter's syndrome in a ship's crew sick with bacterial dysentery prompted researchers to investigate the link between the intestine and arthritis (Noer, 1966). Mounting evidence supports the functional links of SpA and gastrointestinal inflammation (Rizzo et al., 2018), which are closely related to intestinal microbial dysbiosis (Ni et al., 2017). Animal experiments have revealed that transgenic rats harboring HLA-B27 displayed clinical symptoms comparable to human SpA but without further development under sterile conditions, which indicates that intestinal microbes are essential for the onset of SpA (Taurog et al., 1994).

Mechanisms of microbiota-derived intestinal inflammation in SpA

Microbial dysbiosis of gut commensals has been implicated in SpA and is highly related to gut inflammation (Gill et al., 2015). Patients with SpA frequently suffer from IBD (Fragoulis et al., 2019), and there are similarities in the aberrations of the gut microbiota under IBD and AS (Klingberg et al., 2019). The mechanism underlying how intestinal inflammation causes immune damage to peripheral organs is currently inconclusive. Three points are under consideration. (1) There may be no inherent relationship, but rather a simple overlap of

TABLE 1 Recent findings on changes in the SpA gut microbiota.

	Study Subjects	Key findings	Ecological changes in the flora	Strains with increased abundance	Strains with reduced abundance
1	Microbial profiles for terminal ileum biopsy specimens obtained from patients with recent-onset tumor necrosis factor antagonist-naive AS (Costello et al., 2015)	Microbial communities in AS differ significantly from those in healthy control subjects, driven by a higher abundance of 5 families of bacteria	The microbial composition was demonstrated to correlate with disease status	*Lachnospiraceae, Ruminococcaceae, Rikenellaceae, Porphyromonadaceae, Bacteroidaceae*	*Veillonellaceae, Prevotellaceae*
2	Stool specimens from 150 AS patients (Klingberg et al., 2019)	There is a distinct fecal microbiota profile, which is associated with the fecal calprotectin levels.	87% of patients with ecological disorders	*Proteobacteria, Enterobacteriaceae, Bacilli, Streptococcus* spp., *Actinobacteria*	*Bacteroides, Lachnospiraceae*
3	Chinese AS patient cohort (Wen et al., 2017)	Reduced abundance of melanin-producing *Prevotella*, *Prevotella* spp. and *Anaphyllobacter* spp.	Ecological disorders		*Bacillus* spp., *Prevotella, melanogaster, Prevotella* spp.
4	Stool samples from 22 patients with AS (Li et al., 2019)	Increased abundance of *Bacillus variegatus* and reduced *Bacillus mimicus*	Lower biodiversity ratios; significant reduction in the diversity of intestinal fungi	*Ascomycota, Cysticercus*	*Basidiomycota, Stretchers*
5	Stool samples from two AS cohorts (Breban et al., 2017)	*Ruminal cocci* may be a potential marker of disease activity	A unique ecological disorder	align="left">*Rumenococcus*	
6	27 patients with SpA (Tito et al., 2017)	*Dialister* may be a potential microbial marker of disease activity	Significant differences in the microbiological composition of the gut in patients with microscopic intestinal inflammation	*Dialister*	
7	Macrogenome sequencing of stool samples from patients with IBD (Hall et al., 2017)	Significant increase in the abundance of parthenogenic anaerobic bacteria tolerant to oxidative stress; dramatic but transient rumen cocci blooms coinciding with increased disease activity	Low diversity	*Rumenococcus*, Parthenogenic anaerobic bacteria	
8	A total of 174 mucus samples from 43 UC and 26 CD patients (Nishino et al., 2018)	Significant increase in the Metaplasma phylum and significant decrease in the phylum *Firmicutes* and *Bacteroidetes*; CD and UC have different microbial community structures associated with mucous membranes.	Significant reduction in alpha diversity	Phylum *Metaplasma*	Phylum *Firmicutes*, *Bacteroidetes*
9	Stool analysis of patients with IBD (Alam et al., 2020)	The changed bacterial groups are those that do not co-exist well with the common intestinal commensal bacteria	Low microbiome diversity	*Coriobacteriaceae, Prevotellaceae, Burkholderiaceae, Veillonellaceae, Streptococcaceae, Pseudomonadaceae, Acidaminococcaceae*	
10	Recent-onset, DMARD-naive PsA (Scher et al., 2015)	Low relative abundance of *Akkermansia* and *Ruminoccocus* hb as a characteristic of the PsA gut microbiota	Reduced diversity of the gut microbiota due to the low relative abundance of several taxa.		*Akkermansia*, *Ruminoccocus*
11	52 psoriasis patients (Codoner et al., 2018)	Type 2 patients have a higher frequency of bacterial translocation and more frequent inflammatory states		*Faecalibacterium*	*Bacteriodes*

clinical characteristics, between axSpA with subclinical intestinal inflammation and IBD with sacroiliitis. (2) A-specific factor causes SpA to experience both intestinal and joint inflammation in parallel. Genetic, immune, and environmental factors all have a direct impact on the immune system, which in turn induces the clinical immune feature of SpA. This may partly explain why some individual SpA cases have only joint immune changes but no intestinal symptoms. (3) The "gut-joint axis" hypothesis, which is currently very popular, indicates possible links between intestinal and joint pathology. Genetic, immune, and environmental factors first affect the gut and induce dysbiosis in the microbiota through a series of regulatory mechanisms

(e.g., arthritic peptide recognition, clonal expansion, etc., and ultimately lead to the inflammation of other sites, such as joints (Gracey et al., 2020; Zaiss et al., 2021).

Mounting evidence supports the functional link between the gut dysbiosis-related immune system response and SpA. Th17 cells are vital in host defense against external microbes and fungi, and IL-23 signaling is required for the maturation and stability of the pro-inflammatory Th17 phenotype (Schinocca et al., 2021). The IL-23/IL-17 axis and associated cytokines have been implicated in the etiology of SpA (Tsukazaki and Kaito, 2020; Lukasik et al., 2021). In addition to Th17 cells, some innate-like T-cell subsets that express the IL-23 receptor, such as mucosa-associated invariant T (MAIT) cells, T cells, invariant natural killer T cells (iNKT), and type 3 innate lymphocytes (ILC3), boost the type 3 immune response and play a role in the pathogenesis of SpA (Sherlock et al., 2012; Mauro et al., 2019). For instance, upon stimulation with bacterial products, ILC3s can induce an inflammatory response through the production of IL-17 and link the gut microbiota and local/systemic immunity (Annunziato et al., 2015; Asquith et al., 2019). Other immune cells, including mast cells and macrophages, can further amplify the inflammatory effect by secreting cytokines such as IL-23, IL-17, IL-1, IL-22, and tumor necrosis factor (TNF)-α (Annunziato et al., 2015). MAIT cells are capable of producing pro-inflammatory cytokines, such as IL-17, IL-22, TNF, or interferon (IFN)-γ (Gracey et al., 2016; Toussirot and Saas, 2018; Li et al., 2022). Taken together, these findings show that various immune cell-mediated IL-23/IL-17 axes contribute to the migration of blood vessels.

Here, we propose an IL-23/IL-17 axis-based mechanistic model regarding intestinal inflammation driving immune damage in peripheral joints. As shown in Figure 1, our model includes three parts, namely, "immunological changes in the gut", "cytokine cascades initiated in blood vessels", and

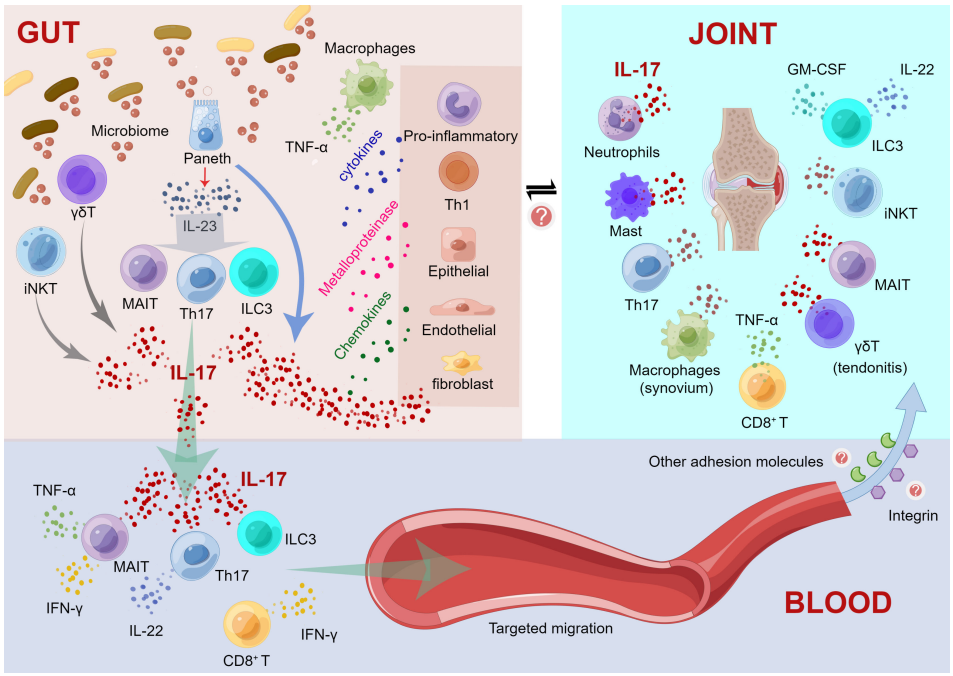

FIGURE 1
Mechanistic model regarding the intestinal inflammation driving immune damage in peripheral joints. **(1) Immunological changes in the gut**. a) Activated Paneth cells produce IL-23 and IL-17 after recognizing the altered microbiome; IL-23 causes the differentiation of Th17, ILC3 and MAIT cells; and these activated cells foster the production of elevated levels of IL-17. IL-17 acts as an inflammatory mediator to stimulate the production of cytokines by other proinflammatory cells and Th1 cells and promotes the production of metalloproteinases and chemokines by macrophages, epithelial cells, endothelial cells, and fibroblasts, thereby triggering and maintaining inflammation. b) γδ T and iNKT cells can recognize microbial antigens and release IL-17. c) Simultaneously, macrophage recruitment promoted the secretion of TNF-α. **(2) Cytokine cascades initiated in blood vessels.** a) IL-17, Th17 cells, ILC3 and MAIT cells can migrate through the intestinal mucosal barrier to the blood, and these cells may transfer inflammation to the joints. b) Th17, γδT, ILC3 cells and MAIT cells are induced to produce IL-17 in the bloodstream; Th17 cells also produce IL-22. c) MAIT cells also contribute to the production of TNF-α and IFN-γ. d) $CD8^+$ T cells produce IFN-γ. **(3) Targeting migration and immune response of extraintestinal joint sites**. a) Activated cytokines migrate to inflammatory sites, such as the axial/peripheral joints, for immune interference. b) Some adhesion molecules, such as integrins, may contribute to the target migration of these immune cells circulating in the blood to peripheral joints. c) In peripheral joints, neutrophils, mast cells, Th17 cells, $CD8^+$ T cells, MAIT cells and iNKT cells induce IL-17 production. ILC3 cells produce IL-22 and GM-CSF, whereas IL-17 is only produced in axial joints. d) γδT cells promote tendonitis through elevated IL-17. e) Macrophages induce TNF-α production in the synovium. This figure was drawn by Figdraw.

"targeting migration and immune response of extraintestinal joint sites". Detailed information is shown in the legend of Figure 1. Accumulating evidence suggests that impaired intestinal barrier structure and function, as characterized by altered tight junction protein density and high intestinal permeability, may increase the entry of bacterial and/or microbiota-derived inflammatory components into the circulatory system (Vaile et al., 1999; Gill et al., 2015; Ciccia et al., 2017). For instance, as a modulator of high intestinal permeability, zonulin protein is highly expressed and implicated in damage to the intestinal mucosal barrier and gut vascular barrier (Ciccia et al., 2017). Fluctuations in the amount, variety, and function of the core gut microbiome can cause increased gut permeability (Brandl and Schnabl, 2015). Herein, IL-23/IL-17 axis-mediated gut bacteria and metabolites influence gut immune mechanisms and disrupt the gut barrier integrity. The "aberrant cell trafficking hypothesis (recirculation)" mimics the recruitment of mucosal-derived cells to joints, in which T cells and macrophages in the gut activate and then recirculate to the joints (Qaiyum et al., 2021). Evidence on adhesion molecules (e.g., integrin, etc.) that determine the homing pattern of circulating lymphocytes may be involved in the mechanism and process of immune-competent cell migration from the intestinal mucosa. For instance, MAIT cells have been isolated from the synovial fluid of AS patients (Gracey et al., 2016). Integrin-expressing T cells proliferate in the inflamed joints of AS patients, and α4β7 integrin promotes T lymphocyte migration from the gut to the synovium (Qaiyum et al., 2019). Thus, cytokine cascades of IL-17 are initiated in blood vessels and migrate to the extraintestinal axial/peripheral joint sites for immune interference, probably through an adhesion molecular-involved mechanism.

SpA therapeutic medication and gut microbiome

Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are the first choice for pharmacological treatment of axSpA, according to the guideline panel (Sieper and Poddubnyy, 2017; Ritchlin and Adamopoulos, 2021; Robinson et al., 2021; Danve and Deodhar, 2022). Disease-modifying anti-rheumatic drugs (DMARDs) have some effect on axSpA patients with peripheral arthritis, but their efficacy in most SpA patients remains debatable (Ward et al., 2019). DMARDs can be further divided into several categories, including traditional DMARDs or conventional synthetic DMARDs (csDMARDs), biologic DMARDs (bDMARDs), and targeted synthetic DMARDs (tsDMARDs) (Buer, 2015; Smolen et al., 2020). bDMARDs and tsDMARDs are recommended for adults with active AS despite treatment with NSAIDs (Ward et al., 2019). Considering the close links between SpA and the gut microbiota (Table 1), we reviewed the potential association between different SpA therapeutic medications and the gut microbiome.

NSAIDs

For SpA patients, NSAIDs can effectively alleviate the symptoms of SpA, such as morning stiffness and joint pain (Queiro-Silva et al., 2021). Even so, different NSAID treatments can cause a distinct alteration in the bacterial composition, which contributes to intestinal damage during NSAID treatment (Rogers and Aronoff, 2016; Otani et al., 2017). A reduction in mouse NSAID-induced enteropathy can be achieved by inhibiting bacterial β-glucuronidase enzyme activity (Saitta et al., 2014).

csDMARDs

Even though csDMARDs [e.g., methotrexate (MTX), sulfasalazine (SSZ), hydroxychloroquine (HCQ), etc.] are not the first choice for axSpA, the subtypes of peripheral joint manifestations for PsA and EPA involve csDMARDs (Mease, 2012; Chimenti et al., 2019; Scher et al., 2020; Jacobs et al., 2021). The treatment efficacy of the MTX and SSZ combination was observed for active axSpA patients in a prospective cohort study (Ganapati et al., 2021). Mounting evidence supports the functional links of csDMARDs and gut microbiota. For instance, the gut microbiome is essential for the treatment effect and prognostic evaluation of MTX for patients with rheumatoid arthritis (RA) (Scher et al., 2020; Artacho et al., 2021). Restored gut dysbiosis symptoms were observed in a rat model of experimental colitis after treatment with SSZ (Zheng et al., 2017). Following treatment with a short-term high dose of HCQ, female C57BL/6J mice showed altered gut microbiota rather than immunological responses (Pan et al., 2021). However, there are few studies on the effect of csDMARDs on the intestinal flora of SpA, which merits further investigation.

bDMARDs

Tumor necrosis factor inhibitors (TNFi) and interleukin-17 inhibitors (IL-17i), two main types of bDMARDs, can be considered for patients who have failed or are intolerant to NSAID therapy (van der Heijde et al., 2017). TNF-α antagonists have specific therapeutic effects on SpA patients (Navarro-Compán et al., 2021) and exhibit considerable microbiota recovery (Chen et al., 2021; Ditto et al., 2021). Furthermore, there is a cross-influence between TNFi treatment and intestinal microbiota. For instance, SpA patients may respond to anti-TNF-α treatment more effectively if they exhibit a specific fecal microbiota signature (Bazin et al., 2018). Some microbes can

serve as indicators of the therapeutic responsiveness of TNFi treatment for AS patients (Zhang et al., 2020). The lowering of bacterial arthritic peptides has been demonstrated to contribute to the improvement of the gut microbiome following TNFi treatment (Yin et al., 2020). Recent studies targeting IL-17 have provided new ideas for treating refractory SpA that has not responded to previous treatments (Gladman et al., 2019; van der Heijde et al., 2020; Schett et al., 2021). The IL-17 axis has a great deal of promise in terms of enhancing SpA therapy options (Smith and Colbert, 2014), and the potential role of gut dysbiosis can be considered.

tsDMARDs

tsDMARDs, such as Janus kinase (JAK) inhibitors, have begun to be used in clinical trials with promising outcomes (Gladman et al., 2017; Toussirot, 2022). There have been limited studies of longitudinal changes in the gut microbiome with tsDMARD treatment in SpA. JAK inhibition has been found to have an indirect effect on the production of critical cytokines implicated in the pathogenesis of SpA as well as the triggering and maintenance of immunological responses (Veale et al., 2019; Ritchlin and Adamopoulos, 2021).

New microbiome therapies such as probiotics

Probiotics have emerged as a new topic of SpA study as research into the involvement of gut microorganisms in the pathophysiology of the disease continues to intensify. Probiotics are described as living microorganisms that provide health advantages to the host when given in sufficient amounts (Hill et al., 2014). *Lactobacillus*, *Bifidobacterium*, and yeast are the most prevalent probiotics (Ashraf and Shah, 2014; Cunningham et al., 2021). Probiotics can be made up of a single strain, a mixture of strains, or a combination of both.

Mechanism of action of probiotics

Probiotics have been researched *in vivo*, *in vitro*, and in animal models for their anti-inflammatory properties. Probiotics have been shown to improve the microbiota by modifying the gut environment, suppressing harmful bacterial growth, and preventing further immune system damage caused by inflammatory diseases (Shamoon et al., 2019). The mechanism of action of probiotics is usually strain-specific, interacting with the host and microbiome primarily through molecular effectors present on cell structures or secreted as metabolites (Cunningham et al., 2021). The basic mechanisms of action include promoting the growth of beneficial microorganisms in the intestinal microbiota, influencing immunological function, strengthening the intestinal barrier, competing with harmful microbes in the gut, and producing organic acids and antimicrobial compounds (Plaza-Diaz et al., 2019).

The immune response can be modulated by influencing cells involved in innate and adaptive immunity. Epithelial cells, dendritic cells (DCs), natural killer cells (NKs), macrophages, and lymphocytes are all affected by probiotics through the Toll-like activation of signaling pathways that regulate cell proliferation and cytokine production (Bermudez-Brito et al., 2012). The ability of probiotics to modulate the cytokine profile of DCs is strain-specific (Borchers et al., 2009; Cristofori et al., 2021)Furthermore, some probiotics can mediate the differentiation from B cells to IgA-producing plasma cells, and secretory IgA protects against infections by restricting bacterial attachment to the epithelium and inhibiting the penetration of host tissue (Liu et al., 2018).

Probiotics can also engage with the host immune system indirectly. Through the manipulation of the gut epithelial barrier and mucus layer properties, the release of antimicrobial compounds, and the management of competition with pathogenic bacteria, specific probiotic metabolites may exert anti-inflammatory and antibacterial effects. Immune responses and systemic inflammation are also influenced by probiotic-driven metabolites such as short-chain fatty acids (SCFAs), which modulate immune cell activity (Oliviero and Spinella, 2020). This characteristic may contribute to the correction of intestinal wall hyperpermeability in the "gut-joint axis". The major mechanism of probiotic action is illustrated in Figure 2.

Clinical studies of probiotics as adjunctive therapy in SpA

Probiotics can theoretically be used to manipulate the microbiome as a promising adjunct therapy for SpA. As mentioned above, there are multiple extraintestinal manifestations of IBD, of which arthritis is one of the most common and is defined as EPA, an important member of the SpA disease spectrum. In this study, we provide a summary of current clinical trials using probiotics for IBD in Table 2. The number of controlled studies on probiotics for SpA is small, and the results of the few studies that have been conducted are not encouraging (Sanchez et al., 2022). Sanges et al. reported that a probiotic mixture containing *Lactobacillus acidophilus* and *Lactobacillus salivarius* is able to reduce arthritis disease scores in SpA patients with quiescent colitis, which may help in the management of SpA in patients with ulcerative colitis (Sanges et al., 2009). The findings of Lowe et al. suggest that combining *Bifidobacterium* and *Lactobacillus* preparations may have benefits in terms of pain relief, lower C-reactive protein (CRP), and improved quality of life. However, compared to

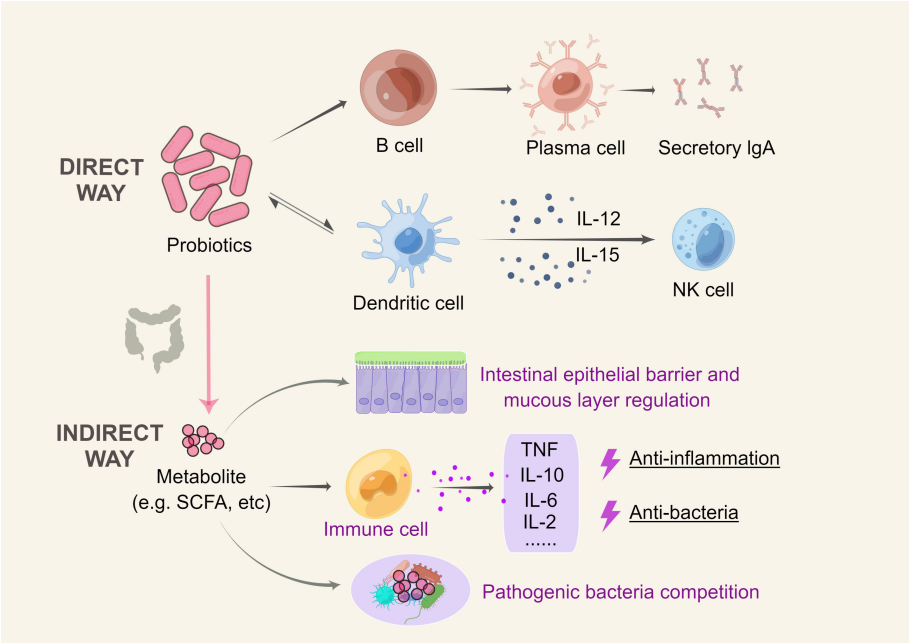

FIGURE 2
Mechanism of action of probiotics. **Direct mechanism:** probiotics can activate sentinel cells through the Toll-like activation of signaling pathways; DCs can drive NK cell activation by secreting cytokines such as IL-12 and IL-15, and probiotics can impact this pathway; probiotics can cause B cells to differentiate into IgA-producing plasma cells; and probiotics also interact with antigen-presenting cells to influence the reduction of proinflammatory cytokines, thereby triggering an adaptive response. **Indirect mechanism:** Through the manipulation of the gut epithelial barrier and mucus layer properties, probiotic release of antimicrobial compounds, and management of competition with pathogenic bacteria, specific probiotic metabolites may exert anti-inflammatory and antibacterial effects. This figure was drawn by Figdraw.

RA patients, SpA patients did not benefit as much from CRP reduction (Lowe et al., 2020).

The efficacy of probiotics in SpA is currently uncertain. There are several causes for this outcome. First, the quality of all current clinical data is poor, sample sizes are small, and the risk of bias and imprecision is significant, necessitating the performance of more randomized controlled trials. Second, the type of inflammatory change, illness severity, microbiota features, and potential confounding factors, such as age, sex, food, and individual microbiological characteristics, must all be considered. Lastly, the anti-inflammatory efficacy of probiotics is incredibly reliant on the dose and strain. It is possible that different bacterial strains have different functions in relation to their hosts.

Due to limited survival rates and/or competition with the indigenous gut microbiome, delivering live bacteria *via* probiotics is difficult. Probiotic effects can be mediated *via* their metabolites or biological components, such as postbiotics. Amino acid derivatives altered by the gut microbiota could be a type of postbiotic that has anti-inflammatory properties by attaching to specific receptors on intestinal epithelial cells (Żółkiewicz et al., 2020). Because of their stability, synbiotics, such as "nonviable" microbial cells or crude cell extracts, can benefit both humans and animals if present in sufficient amounts (Vallejo-Cordoba et al., 2020). Probiotic bacteria can be manipulated in an unlimited manner with emerging biological engineering tools. In response to externally supplied substrates, genetically modified bacterial/probiotic strains can be employed to detect early inflammatory markers as well as to distribute and generate therapeutic compounds to the mucosal surface, hence boosting the overall efficiency of the system. It is also worthy of in-depth investigation for other gut microbiota-targeted adjuvant therapies of SpA, such as fecal microbiota transplantation (FMT) and dietary treatment.

Some thoughts

1. It is time to rethink the heterogeneity of the SpA disease class and its underlying mechanisms. We only have a rudimentary understanding of the known associations with the microbiota in SpA-related diseases right now. However, the exact mechanisms through which the microbiome contributes to SpA in the axial or peripheral skeleton are still unknown, and the mechanisms by which the combined effects of the gut microbiome, immune system, and host genetics

TABLE 2 Summary of population-based clinical trials on probiotics for IBD.

Object	Probiotic strains	Study results	Reference
IBD in remission or with mild symptoms	*Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Enterococcus faecalis*	No difference in clinical symptoms after treatment	(Bjarnason et al., 2019)
CD	*Lactobacillus* GG	Not effective in preventing relapse	(Bousvaros et al., 2005)
UC	*E. coli* Nissle1917	Appears to be expected to maintain a period of remission	(Kruis et al., 2004)
UC	*Lactobacillus delbrueckii Lactobacillus fermentum*	Reduces NF-kB regulation and further reduces IL-6 and TNF- α levels	(Hegazy and El-Bedewy, 2010)
UC	*Bifidobacterium infantis*	CRP and TNF- α levels were reduced, but there was no significant effect on the course of UC; induces NF-kB regulation and further reduces IL-6 and TNF-α levels	(Groeger et al., 2013)
UC	*Bifidobacterium* Short *Bifidum perfringens* strain	Better endoscopic scores obtained, but no significant effect on UC	(Ishikawa et al., 2011)
UC	*Bifidobacterium breve, Lactobacillus acidophilus*	No significant improvement observed	(Matsuoka et al., 2018)
UC	*Lactobacillus acidophilus, Bifidobacterium animalis subsp. lactis*	Remission in 25% of the patient group, 8% of the placebo group. No significant difference	(Wildt et al., 2011)
Mild to moderate UC	*Bifidobacterium longum*	Reduced disease activity index, reduced rectal bleeding and clinical remission	(Tamaki et al., 2016)
UC	*Lactobacillus salivarius, Lactobacillus acidophilus, Bifidobacterium bifidum*	Have a positive effect	(Palumbo et al., 2016)
Mild to moderately active UC	VSL#3 (*Lactobacillus, Bifidobacterium, Streptococcus thermophilus*)	Significant improvements in rectal bleeding and stool frequency, mucosal appearance and overall assessment by the doctor	(Sood et al., 2009)
Mild to moderate UC that does not respond to conventional treatment	VSL#3	Remission/response rate of 77% with no adverse events.	(Bibiloni et al., 2005)

contribute to tissue damage will continue to be a hot topic for future research.

2. There is both commonality and specificity in dysbiosis among key disease subtypes, such as AS, IBD, and PsA, in SpA, implying a complicated relationship in the etiology of these heterogeneous disorders. The genetic background of these diseases has a high degree of overlap, but the clinical manifestations are significantly heterogeneous. It is not clear whether there is a connection between gut microbiota and joint lesions and whether there are other factors involved, weaving a relatively complex network of infection, immunity, and injury. Using SpA as an example to identify microbes that influence human disease susceptibility and phenotype will be a considerable challenge.
3. Does gut flora research have the potential to aid us in treating SpA with precision? Early diagnosis, early intervention, early treatment, and effective management of SpA have remained unsolved challenges in clinical practice, often leading to delayed diagnosis and precise individualized treatment. Advances in detection technologies such as sequencing and multiomics approaches such as metabolomics have allowed us to delve deeper into the links between ecological dysregulation, bacterial metabolites, and disease development and will provide new insights into the unraveling of this diverse group of diseases. Further research into mechanisms such as the 'gut-joint' axis may allow physicians to characterize SpA and disease progression with specific biomarkers to aid early diagnosis and provide individualized and precise treatment. The gut microbiome's plasticity has also prompted researchers to assess the viability of precision therapy based on the gut microbiome's SpA and to design new targeted therapies.

Conclusions

The gut microbiota has emerged as a major focus of investigation into the pathogenesis of SpA. Although definitive proof of causality is still lacking, dysbiosis is linked to the pathogenesis of HLA-B27-associated SpAs. Notably, there is an interaction between the gut flora and the efficacy of current conventional therapeutic agents, and detailed studies on the gut-joint axis suggest that more targeted bDMARDs are a promising

therapeutic area. Large-scale longitudinal studies and cross-sectional clinical trials are required to investigate the microbiota as a potential biomarker and its role in the prevention or treatment of SpA. According to the available literature, probiotics are extensively researched for use in SpA as adjunctive therapy. Nevertheless, the high heterogeneity in study design due to the use of different strains, quantities, and timing of supplementation makes it difficult to conclude whether probiotics are effective at this time. Engineered microbes, however, could be a more promising topic. Future microbiomics investigations and new analytical tools, such as bioinformatics, will allow for the more extensive study of host-microbiota interactions, providing new insights into the pathophysiology of SpA and, ideally, translating into therapeutically useful therapies.

Author contributions

XL, JC, XG, and JY conceived and drafted the study. JY screened abstracts, XL, JC, and XG collected all data. XL and JC drafted the manuscript. JY performed critical revisions of the manuscript. All authors have approved the final draft of the manuscript.

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Conflict of interest

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Efficacy evaluation of probiotics combined with prebiotics in patients with clinical hypothyroidism complicated with small intestinal bacterial overgrowth during the second trimester of pregnancy

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Objective: To explore the effect of probiotics combined with prebiotics on clinical hypothyroidism during pregnancy combined with small intestinal bacterial overgrowth.

Methods: (1) In total, 441 pregnant women were included in this study. A total of 231 patients with clinical hypothyroidism during the second trimester of pregnancy and 210 normal pregnant women were enrolled in the lactulose methane-hydrogen breath test. The positive rate of intestinal bacterial overgrowth (SIBO), gastrointestinal symptoms, thyroid function and inflammatory factors were compared between the two groups by chi-square test and two independent sample t-test. (2) SIBO-positive patients in the clinical hypothyroidism group during pregnancy (n=112) were treated with probiotics combined with prebiotics based on conventional levothyroxine sodium tablets treatment. The changes in the methane-hydrogen breath test, gastrointestinal symptoms, thyroid function and inflammatory factors were compared before treatment (G0) and 21 days after treatment (G21) by chi-square test and paired sample t test.

Results: (1) The positive rates of SIBO in pregnant women in the clinical hypothyroidism group and control group were 48.5% and 24.8%, respectively. (2) The incidence of abdominal distention and constipation in the clinical hypothyroidism group was significantly higher than that in the control group, and the risk of abdominal distention and constipation in SIBO-positive pregnant women was higher than that in SIBO-negative pregnant women. (3) The serum levels of hypersensitive C-reactive protein (hsCRP), IL-10, IL-6, TNF-α, low-density lipoprotein (LDL), total cholesterol (TC), free fatty

acids (FFAs) and apolipoprotein B (ApoB) in the hypothyroidism group during pregnancy were higher than those in the control group. (4) After 21 days of probiotics combined with prebiotics, the incidence of pure methane positivity in the methane-hydrogen breath test in the G21 group was significantly reduced, and the average abundance of hydrogen and methane at each time point in the G21 group was lower than that in the G0 group. (5) The incidence of constipation in the G21 group was significantly lower than before treatment. (6) The levels of serum TSH, hsCRP, IL-6, TNF-α, TC and LDL in pregnant women after probiotics combined with prebiotics were lower than those before treatment.

Conclusion: Probiotics combined with prebiotics are effective in the treatment of pregnant patients with clinical hypothyroidism complicated with SIBO, providing a new idea to treat pregnant patients with clinical hypothyroidism complicated with SIBO.

KEYWORDS

breath test, hypothyroidism, intestine, pregnancy, therapy

Introduction

Thyroid disease is one of the most common endocrine diseases in women of childbearing age, and the incidence of clinical hypothyroidism during pregnancy is 0.3%-0.5% (Casey and T.D.M.a.J.Q, 2020), which can cause adverse outcomes, such as neurodevelopmental disorders in children and fetal growth restriction, and has a significant impact on the growth and development of offspring (Ge et al., 2020; Kerver et al., 2021). In addition, the clinical manifestations of hypothyroidism are not specific and sometimes cannot be distinguished from common symptoms or signs of pregnancy, such as fatigue, constipation, weight gain, edema and dry skin (Casey and T.D.M.a.J.Q, 2020).

The basic concept of small intestinal bacterial overgrowth (SIBO) is that the colonization level of intestinal microorganisms is reduced and the balance of the bacterial community is significantly changed (Pimentel et al., 2020). One of the risk factors is reduced gastrointestinal motility, and hypothyroidism is related to changes in gastrointestinal motility, so hypothyroidism may lead to SIBO (Bohinc Henderson, 2021). The diagnosis of SIBO is based on the lactulose breath test (LBT), which has the advantages of being non-invasive, convenient, sensitive, accurate, and reproducible (Pimentel et al., 2020). Probiotics are active in the small intestine, and prebiotics provide nutrients for probiotics. A combination of both may produce a certain energy effect. In the small intestine, bacterial overgrowth, inflammatory bowel disease, irritable bowel syndrome and other diseases have considerable effects, such as inhibiting bacterial translocation and intestinal mucous membrane barrier function and reducing inflammation (Hamasalim, 2016; Markowiak and Śliżewska, 2017).

To further explore a new idea for the treatment of patients with clinical hypothyroidism complicated with intestinal bacterial overgrowth during pregnancy, this study used probiotics (bifidobacteria tetrad live tablets) combined with prebiotics (polysaccharide fiber powder) to treat patients with clinical hypothyroidism complicated with positive intestinal bacterial overgrowth during pregnancy and evaluated its efficacy.

Materials and methods

Experimental subjects

A total of 442 pregnant women who received perinatal care at the Obstetrics Clinic of the Third Affiliated Hospital of Zhengzhou University from July 2020 to December 2021 were included, including 231 pregnant women with clinical hypothyroidism during pregnancy and 210 pregnant women with normal pregnancy. All subjects signed informed consent forms, and the study was approved by the Ethics Committee.

The inclusion criteria for this study were age > 18 years old and < 35 years old, thyroid function in the second trimester met the reference range of clinical hypothyroidism established by the Laboratory of the Third Affiliated Hospital of Zhengzhou University (TSH > 4.32mIU/L and FT4 < 9.77pmol/L, commercial kit (Roche, Shanghai, China)). And the patients used levothyroxine sodium tablets regularly to control thyroid function.

The exclusion criteria included pregnant women with positive thyroid peroxidase antibody; used probiotics, prebiotics, antibiotics and other drugs affecting intestinal flora in the past three months; multiple pregnancies and artificial impregnation; gestational diabetes mellitus, gestational hypertension, systemic lupus erythematosus, thyroid dysfunction before pregnancy and other complications affecting the endocrine system and immune system.

Experimental method

A total of 231 pregnant women with clinical thyroidism during pregnancy were selected as the Hypothyroidism Group, and 210 pregnant women with normal thyroidism and no pregnancy complications were selected as the Control Group. Fasting blood was taken to detect thyroid function, inflammatory factors and lipid levels.

SIBO-positive patients in the clinical hypothyroidism group during pregnancy (n=112) were included in the further experimental study to explore the changes in parameters of probiotics (bifidobacterium quadrupectin viable tablets) combined with prebiotics (polysaccharide fiber powder) before treatment (G0) and 21 days after treatment (G21). The methane-hydrogen breath test was performed again after 21 days of treatment. Fasting blood was taken to detect thyroid function, inflammatory factors and lipid levels. The dosage of levothyroxine sodium tablets was appropriately adjusted according to the thyroid function and the patient's tolerance after 21 days of treatment. The dosage of levothyroxine sodium tablets at G0 and G21 was recorded respectively.

Participants were asked not to change their daily eating habits during the study and to avoid consuming foods or drugs containing probiotics or fermented products.

After 21 days of treatment, patients with persistent positive SIBO were treated with berberine based on continued treatment with probiotics combined with prebiotics until SIBO turned negative, and subsequent treatment was no longer included in the study.

Lactulose methane-hydrogen breath test

Before examination, patients should make proper preparations, such as avoid hydrogen-producing foods, such as dairy products, soy products, wheat flour products and high-fiber vegetables within 24 hours before exhalation. Rice, meat and eggs are edible. At least 12 hours before exhalation on an empty stomach, they were allowed to drink a small amount of water, the empty stomach should be checked on that day and teeth should be brushed first to avoid bacteria in the mouth affecting the results. Patients should be awake and quiet during exhalation, eliminate all beverages, and avoid chewing gum and a smoking environment.

On the morning of the examination day, subjects blew for the first time on an empty stomach. After blowing for the first time, they immediately drank the medicine (lactulose 15 g+warm water 50 ml) in one swallow. After drinking the medicine, they started timing, blew for the second time 20 min later, blew for the third time 20 min after the second time, etc.

Diagnosis of SIBO

The diagnostic criteria were based on the literature support of the North American consensus and the definition of Breath Tracker SC(QuinTron, USA), a lactulose methane-hydrogen breath test instrument (1) Intestinal bacterial overgrowth is diagnosed as a baseline increase of ≥20 ppm within 90 minutes from the beginning of substrate administration (2) methane concentration ≥10 ppm at any time point is considered to be positive for SIBO (3) if hydrogen and methane concentrations do not reach the above values within 90 minutes of breath test, the sum of the two values is higher than the sum of fasting hydrogen baseline values, and methane concentration exceeds 15 ppm, SIBO is considered positive (Rezaie et al., 2017). In this study, 20-minute intervals were used to reduce the false positive rate because Asian populations have shorter blinding times than Western populations and lactulose reduces the time of mouth blindness (Gwee et al., 2010; Rezaie et al., 2017).

Gastrointestinal symptoms

(1) Abdominal distension: the following two items must be included: ① Abdominal distension occurs at least 3 days in the last 3 months; ②The diagnosis of functional dyspesia(FD), Irritable Bowel Syndrome(IBS)or other functional gastro intestinal disorders (FGID)is not achieved. The past 3 months meet the diagnostic criteria, and symptoms must appear at least 6 months before diagnosis (Schmulson and Chang, 2011) (2). Constipation: two or more of the following six items (1): in more than a quarter (25%) of the time in the appearance of exertion; ② more than 25% of the time blocking or difficult defecation; ③anus rectum obstruction > 25% (4); incomplete defecation, time in defecation > 25%; ⑤ need manual help defecation time >25%; ⑥defecation < 3 times per week (Jani and Marsicano, 2018) (3); diarrhea: increased stool frequency (more than 3 times per day) and changes in stool properties (mushy liquid) (Schiller et al., 2017).

Probiotics

In this experiment, a Bifidobacterium quadruple living tablet (Sienkang, National drug approval: S20060010) was used, which is composed of intestinal probiotics and is a compound

preparation. The main ingredients include infant Bifidobacterium, Lactobacillus acidophilus, Enterococcus faecalis and Bacillus cereus. Usage: 1.5 g, 3 times a day.

Prebiotics

Polysaccharide fiber powder (Risikon®, production license number: SC13061011200721) is a dietary supplement made of various dietary fiber complexes, and the main components include inulin, ice threosaccharide, microcrystalline cellulose, and oat fiber. Usage: 5 g each time, 3 times a day.

Statistical analysis

The statistical analysis software used in this study was SPSS 26.0 (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp). The measurement data with a normal distribution are described as the mean ± standard deviation, and the measurement data with a nonnormal distribution are described as the median and quartile. Paired sample T test and independent sample T test were used for statistical analysis of quantitative data, and the chi-square test (Pearson chi-square and likelihood ratio) was used for statistical analysis of categorical variables. P<0.05 indicated a statistically significant difference.

Results

Comparison of the SIBO positive rate between the two groups: As shown in Table 1, the SIBO positive rate in the clinical hypothyroidism group and the control group during pregnancy was 48.5% and 24.8%, respectively. The positive rate of pure methane was 18.6%, which was significantly higher than that of the control group (P < 0.05). The average abundance of hydrogen and methane at each time point in the methane-hydrogen breath test of pregnant women in the clinical hypothyroidism group was higher than that in the control group, there were significant differences in hydrogen at 60, 80 and 100 time points and methane at 0, 40, 60, 80 and 100 time points (Figure 1).

Comparison of clinical symptoms between the two groups. As shown in Table 2, the incidence of abdominal distention and constipation in pregnant women in the clinical hypothyroidism group was 29.4%, higher than that in the control group. The probability of no obvious symptoms was 25.5%, which was significantly lower than that of the control group (P < 0.05). The incidence of abdominal distention and constipation in SIBO-positive patients was significantly higher than that in SIBO-negative patients during pregnancy.

The basic information and clinical indicators of the subjects were compared as follows:In Table 3, there were no significant differences in age, height, weight, BMI, IL-2, IL-4, TG, HDL or ApoA1 levels between the two groups (P >0.05). The levels of TSH, hsCRP, IL-10, IL-6, TNF-α, TC, LDL, FFA and ApoB in the pregnancy hypothyroidism group were higher than those in the control group (P <0.05). In Table 4, there was no significant difference in the levels of TSH and FT4 between SIBO positive and SIBO negative patients with clinical hypothyroidism during pregnancy (P > 0.05), while the dose of levothyroxine sodium tablets in SIBO positive patients was significantly higher than that in SIBO negative patients (P < 0.05).

Comparison of the methane-hydrogen breath test in pregnant patients with clinical hypothyroidism combined with SIBO positivity after 21 days of probiotics combined with prebiotics: As shown in Table 5, after 21 days of treatment, 46.4% of patients with clinical hypothyroidism in pregnancy combined with SIBO-positive patients turned negative, and the incidence of methane-positive patients was 3.6%, which was significantly lower than before treatment (P < 0.05). The average abundance of hydrogen and methane at each time point in The methane-hydrogen breath test after treatment was lower than that before treatment, there were significant differences in hydrogen at 80 and 100 time points and methane at 0, 20, 60, 80 and 100 time points (Figure 2).

Changes in gastrointestinal symptoms after 21 days of probiotics combined with prebiotics: As shown in Table 5, the incidence of constipation after 21 days of probiotics combined with prebiotics was 30%, significantly lower than before treatment. The probability of no obvious gastrointestinal symptoms was 40.2%, significantly higher than before treatment (P < 0.05).

TABLE 1 Comparison of the SIBO positive rate, pure hydrogen positive rate, pure methane positive rate and hydromethane positive rate between the clinical hypothyroidism group and the control group during pregnancy.

methane-hydrogen breath test	Control Group N = 210	Hypothyroidism Group N = 231	p
SIBO+ (%)	52 (24.8)	112 (48.5)	<0.001
hydrogen+ (%)	37 (17.6)	57 (24.7)	0.071
methane+ (%)	10 (4.8)	43 (18.6)	<0.001
hydrogen+methane+ (%)	5 (2.4)	12 (4.8)	0.098

p: values of patients in the control group and pregnant clinical hypothyroidism group.
p< 0.05 indicates statistical significance.

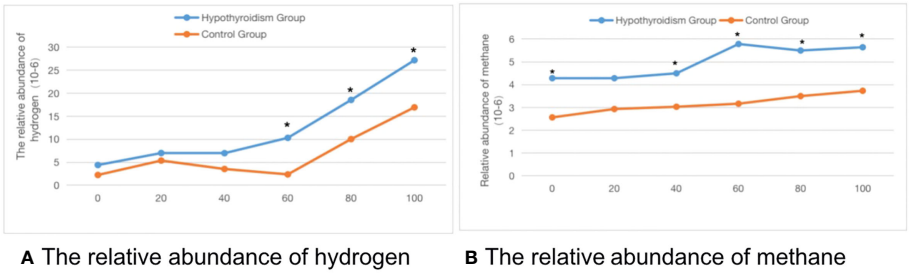

FIGURE 1
The time-abundance curve of hydrogen and methane in the control group and pregnant clinical hypothyroidism group. **(A)** The relative abundance of hydrogen. **(B)** The relative abundance of methane. *indicates statistical significance.

Comparison of clinical indicators of pregnant patients with clinical hypothyroidism combined with SIBO positivity before and after 21 days of treatment with probiotics combined with prebiotics: In Table 6, after 21 days of probiotics combined with prebiotics, the levels of TSH, hsCRP, IL-6, TNF-α, TC and LDL were significantly reduced compared with those before treatment ($P < 0.05$).

Discussion

In recent years, studying the intestinal microbiome has become a popular field. Some studies have shown that the intestinal microbiome is related to thyroid function, and its balance is of great significance to the stability of the human body, especially the function of the endocrine system (Zhang et al., 2019). Intestinal bacterial overgrowth (SIBO) is characterized by gastrointestinal symptoms resulting from the abnormal proliferation of bacterial species in the small intestine, mainly including Gram-negative aerobic and anaerobic bacteria, which can ferment gas-producing carbohydrates (Shanab et al., 2011; Pimentel et al., 2020). However, how to treat SIBO-positive pregnant women with clinical hypothyroidism during pregnancy is still unclear.

Our study found that the SIBO-positive rate during pregnancy was higher in the clinical hypothyroidism group than in the normal group. The dose of levothyroxine sodium in SIBO positive patients was higher than SIBO negative patients in the clinical hypothyroidism group during pregnancy, which further indicated that hypothyroidism during pregnancy is closely related to intestinal bacterial overgrowth. The results of *Lauritano et al.* (Lauritano et al., 2007) are consistent with our findings that nongestational hypothyroidism is closely related to bacterial overgrowth in the small intestine. Studies have found that nongestational hypothyroidism and intestinal flora may interact by slowing gastrointestinal motility, reducing the expression of the sodium-iodine cotransporter (NIS) and affecting the absorption of iodine (Ebert, 2010; Knezevic et al., 2020). We believe the possible mechanism is that smooth muscle dysfunction and gastric acid secretion are decreased in patients with clinical hypothyroidism during pregnancy. With the weakening of gastrointestinal motility,

TABLE 2 Comparison of gastrointestinal symptoms between the hypothyroidism group and the control group during pregnancy.

		diarrhea (n/%)	abdominal distension (n/%)	constipation (n/%)	No obvious symptoms (n/%)
Control Group		27 (12.9)	43 (20.5)	33 (15.7)	107 (51.0)
Hypothyroidism Group		36 (15.6)	68 (29.4)	68 (29.4)	59 (25.5)
$P_{c\text{-}H}$		0.306	0.03	0.001	<0.001
Control Group	SIBO+	10 (19.2)	11 (21.2)	9 (17.3)	5 (9.6)
	SIBO-	27 (17.1)	19 (12.0)	23 (14.6)	106 (67.1)
P_c		0.725	0.103	0.632	<0.001
Hypothyroidism Group	SIBO+	9 (8.0)	24 (21.4)	63 (56.3)	16 (14.3)
	SIBO-	8 (6.7)	5 (4.2)	24 (20.2)	82 (68.9)
P_H		0.702	<0.001	<0.001	<0.001

pc-H: P values of gastrointestinal symptoms in the control group and pregnant clinical hypothyroidism group.
pc:P values of gastrointestinal symptoms in SIBO-positive and –negative patients in the control group.
pH: P values of gastrointestinal symptoms in patients with SIBO-positive and SIBO-negative clinical hypothyroidism during pregnancy.
p<0.05 indicates statistical significance.

TABLE 3 Comparison of general data between pregnant women with clinical hypothyroidism during pregnancy and pregnant women in the control group.

parameters	Control Group (n = 210)	Hypothyroidism Group (n = 231)	F	P value
Age (years)	30.80 ±4.18	30.94 ± 4.76	3.118	0.744
High (cm)	161.60 ± 4.41	161.42 ± 3.96	4.247	0.659
Weigh (kg)	60.75 ± 8.30	60.08 ± 5.22	46.230	0.302
BMI (kg/cm2)	23.33 ± 3.53	23.08 ± 2.18	41.548	0.372
FT4 (pmol/L)	13.21 ± 1.56	11.61 ± 1.79	0.032	<0.001
TSH (mIU/L)	1.74 ± 0.90	2.48 ± 1.59	65.704	<0.001
hsCRP (mg/L)	2.71 ± 1.93	4.74 ± 3.54	34.046	<0.001
TC (mmol/L)	5.34 ± 1.11	6.28 ± 1.32	10.574	<0.001
TG (mmol/L)	2.45 ± 1.05	2.43 ± 1.11	0.191	0.819
HDL (mmol/L)	2.14 ± 0.49	2.18 ± 0.47	0.002	0.397
LDL (mmol/L)	3.13 ± 0.74	3.49 ± 1.01	26.264	<0.001
FFA (mmol/L)	0.33 ± 0.13	0.37 ± 0.13	0.265	<0.001
ApoA1 (g/L)	2.34 ± 0.36	2.32 ± 0.42	0.012	0.575
ApoB (g/L)	1.11 ± 0.59	1.32 ± 0.70	0.731	<0.001
IL-2	3.14 ± 2.62	3.56 ± 3.02	13.188	0.121
IL-10	2.29 ± 1.65	3.66 ± 2.55	14.343	<0.001
IL-6	3.22 ± 1.80	7.40 ± 5.10	160.690	<0.001
IL-4	3.99 ± 2.06	4.40 ± 2.97	45.384	0.100
TNF-α	1.92 ± 1.48	4.15 ± 3.56	104.295	<0.001

BMI, body mass index; FT4, free T4; TSH, thyroid stimulating hormone; hsCRP, serum hypersensitive C-reactive protein; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FFA, free fatty acid; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B.
p: p values of patients in the Control group and Hypothyroidism group.
$p < 0.05$ indicates statistical significance.

TABLE 4 Comparison of FT4, TSH levels and levothyroxine sodium tablets between SIBO positive and SIBO negative pregnant women with clinical hypothyroidism during pregnancy.

	Hypothyroidism Group (n = 231)			
parameters	SIBO+ (n = 112)	SIBO- (n = 119)	F	P-value
FT4 (pmol/L)	11.58 ± 1.92	11.66 ± 1.66	3.909	0.753
TSH (mIU/L)	2.40 ± 1.51	2.49 ± 1.64	1.28	0.850
LT4 (ug/d)	50.89 ± 25.54	36.97 ± 18.65	1.757	<0.001

FT4, free T4; TSH, thyroid stimulating hormone; LT4, levothyroxine.
$p < 0.05$ indicates statistical significance.

TABLE 5 Positive rates of pure hydrogen, pure methane and hydromethane in the G0 and G21 groups.

	G0	G21	p
Hydrogen-methane breath test			
SIBO+ (%)	112	52 (46.4)	–
hydrogen+ (%)	57 (50.9)	43 (38.4)	0.060
methane+ (%)	43 (38.4)	4 (3.6)	<0.001
hydrogen+methane+ (%)	12 (10.7)	5 (4.5)	0.077
gastrointestinal symptoms			
diarrhea (n/%)	17 (15.2)	14 (12.5)	0.562
abdominal distension (n/%)	27 (24.1)	23 (20.5)	0.521
constipation (n/%)	49 (43.8)	30 (26.8)	0.008
No obvious symptoms (n/%)	19 (17.0)	45 (40.2)	<0.001

P0-21 P values of patients before treatment (G0) and 21 days after treatment (G21).
$p<0.05$ indicates statistical significance.

the ability of intestinal bacteria removal is impaired, promoting the occurrence of intestinal bacterial overgrowth. Meanwhile iodine uptake in the gastrointestinal tract is mediated by the sodium-iodine cotransporter (NIS). When intestinal bacteria are disturbed, NIS expression may decrease, and iodine uptake may be affected, thus leading to the occurrence of gestational hypothyroidism.

Through the methane-hydrogen breath test, we found that the incidence of pure methane-positive pregnant women in the pregnancy clinical hypothyroidism group was significantly higher than that in the normal group, and the incidence of abdominal distention and constipation in the pregnancy clinical hypothyroidism group was also higher than that in the normal group. Hydrogen-producing bacteria are mainly *Clostridium, Enterobacter, Klebsiella* and *Bacillus*, and *Methanobrevicter Smithii* and *Methanosphaera Stadtmanae* have also been identified as the main methanogens in the human intestinal tract

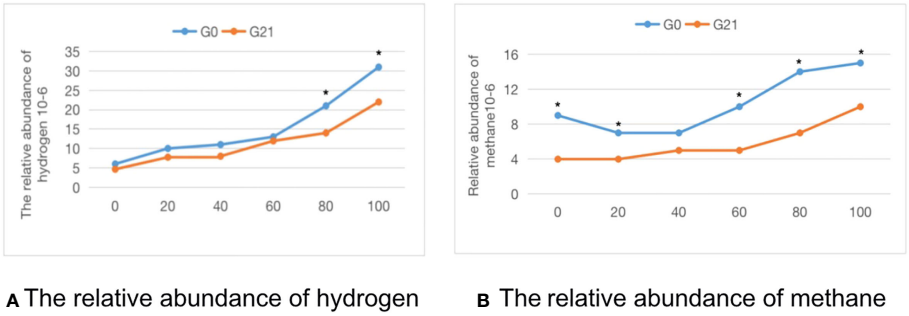

FIGURE 2
The time-abundance curve of hydrogen and methane in the G0 group and G21 group. **(A)** The relative abundance of hydrogen. **(B)** The relative abundance of methane. *indicates statistical significance.

(Su et al., 2018). Previous studies conducted by our research group on fecal 16S RNA sequencing found that hydrogen-producing *Clostridium* was relatively enriched in the intestinal flora of patients with clinical hypothyroidism during pregnancy, which was inconsistent with the results of our methane-hydrogen breath test. This fact may be due to the sample size. *Lepp et al.* believed that methanogens consume short-chain fatty acids when producing methane (Lepp et al., 2004). Meanwhile, studies have confirmed that short-chain fatty acids can stimulate the expression of thyroid hormone, and the ability of intestinal flora to produce short-chain fatty acids in patients with hypothyroidism is significantly decreased (Su et al., 2020). Therefore, we believe that the excessive growth of intestinal bacteria in patients with clinical hypothyroidism during pregnancy may increase the abundance of methanogens and lead to a decrease in the level of short-chain fatty acids, promoting the occurrence of clinical hypothyroidism during pregnancy. The increase in progesterone during pregnancy may inhibit the release of gastrin and slow gastrointestinal peristalsis, leading to constipation. Meanwhile, hypothyroidism leads to an increase in the abundance of methanogens, slowing down intestinal transport time and causing constipation and abdominal distension.

In this study, we found that the levels of hsCRP, IL-10, IL-6 and TNF-α in pregnant women with clinical hypothyroidism during pregnancy were significantly higher than those in the control group. *Tang C et al.* also believed that hypothyroidism was related to inflammatory factors, including IL-6, TNF-α and hs-CRP, and the concentration of inflammatory factors was relatively high in nonpregnant hypothyroidism patients (Abbas and Sakr, 2016; Tang et al., 2021), consistent with our research results. Our previous studies on fecal 16S RNA sequencing found that increased CRP was related to *Gammaproteobacteria, Pasteurellaceae* and *Firmicutes*, and the

TABLE 6 Parameter changes after 0 (G0) and 21 (G21) days of treatment.

parameters	G0	G21	P	95%CI
FT4 (pmol/L)	11.58 ± 1.92	11.39 ± 2.28	0.363	-0.22~0.61
TSH (mIU/L)	2.40 ± 1.51	1.72 ± 0.61	<0.001	0.07~0.73
hsCR (mg/L)	5.16 ± 3.71	3.33 ± 1.71	<0.001	1.23~2.44
TC (mmol/L)	6.31 ± 1.28	5.56 ± 0.75	<0.001	0.49~1.05
TG (mmol/L)	2.52 ± 1.14	2.30 ± 1.08	0.161	-0.09~0.51
HDL (mmol/L)	2.23 ± 0.47	2.11 ± 0.51	0.063	-0.01~0.25
LDL (mmol/L)	3.47 ± 1.01	2.57 ± 0.57	<0.001	0.69~1.13
FFA (mmol/L)	0.37 ± 0.14	0.36 ± 0.13	0.526	-0.20~0.04
ApoA1 (g/L)	2.37 ± 0.41	2.37 ± 0.41	0.924	-0.12~0.11
ApoB (g/L)	1.28 ± 0.72	1.40 ± 0.72	0.428	-0.28~0.12
IL-2	3.09 ± 2.86	2.80 ± 2.35	0.990	-0.43~1.01
IL-10	3.76 ± 2.74	3.76 ± 2.74	0.929	-0.74~0.73
IL-6	6.28 ± 4.40	4.09 ± 2.60	<0.001	1.12~3.26
IL-4	3.92 ± 3.07	4.20 ± 3.03	0.532	-1.17~0.61
TNF-α	4.13 ± 3.59	2.67 ± 2.61	0.003	0.44~2.05
LT4 (ug/L)	50.89 ± 25.54	48.88 ± 23.10	0.538	-4.40~8.42

FT4, free T4; TSH, thyroid stimulating hormone; HsCRP, serum hypersensitive C-reactive protein; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FFA, free fatty acid; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; LT4, levothyroxine.
P: p values of patients in the G0 group and G21 group.
$p < 0.05$ indicates statistical significance.

combined effect of CRP and phosphocholine on bacteria could activate inflammatory factors. Intestinal leakage is an important mechanism leading to the inflammatory state of the body. Intestinal epithelial cells are connected by tight junctions and seal cell bypass to act as a selective osmotic barrier (Zheng et al., 2021). When intestinal leakage occurs, disturbance of intestinal flora may lead to an increase in intestinal permeability, bacterial translocation, and the abundance of inflammation-related flora, such as *Gammaproteobacteria* and *Pasteurellaceae*. The accumulation of harmful metabolites of the flora in the intestine further activates the inflammatory response. Aggravating tissue damage affects the metabolism of thyroid follicular cells, leading to thyroid hormone synthesis disorder and clinical hypothyroidism during pregnancy.

The concentrations of TC, LDL, FFA and ApoB in pregnant women with clinical hypothyroidism were significantly higher than those in the control group. Many studies have reported that elevated TC and LDL levels are a significant feature of hypothyroidism (Zhu and Cheng, 2010; Peppa et al., 2011). According to the study of *Jung et al.* the decrease in the number of LDL receptors in the serum of nonpregnant hypothyroidism patients reduced the LDL clearance rate, resulting in an increase in LDL and ApoB levels (Jung et al., 2017). Our previous studies on lipid metabolism (Li et al., 2021) found that the infectious pathway of pathogenic Escherichia coli was significantly higher in the disease group than in the normal group, which may be related to the increased level of phosphatidylethanolamine (PE) in hypothyroidism patients during pregnancy. And PE can maintain the stability of the cell membrane, affect the function of membrane proteins, and stimulate the occurrence of inflammation. Changes in gut microbiome composition and function as a result of inflammation are markers of metabolic damage, and its metabolites, such as lipopolysaccharides and endotoxins, may reduce the integrity of cellular connections (Farzi et al., 2018). Therefore, we speculate that when clinical hypothyroidism occurs in pregnancy, the pathogenic Escherichia coli metabolic pathway is dominant, accompanied by increased PE levels, resulting in intestinal microflora disorder in pregnant women, reducing the stability of cell connections and reducing the number of LDL receptors, LDL, TC, ApoB and other lipid synthesis and degradation disorders.

After 21 days of probiotics combined with prebiotics, 46.4% of SIBO-positive pregnant women with clinical hypothyroidism in pregnancy turned negative, and the pure methane positive rate was significantly lower than before the treatment, but treatment after 21 days significantly reduced the expiratory hydrogen methane experiment and the average abundance of hydrogen and methane at all time points and significantly improved the patients with constipation. *Pimentel et al.* believe that probiotics can improve the composition of intestinal microbes in a beneficial way and relieve gastrointestinal symptoms of constipation (Pimentel et al., 2006; Zhang et al., 2010; Nickles et al., 2021), which is consistent with our research results. *Audrey et al.* did not find that the combination of probiotics and prebiotics had a significant effect on improving gastrointestinal symptoms in nonpregnant healthy subjects (Talebi et al., 2020), which is different from our study. This finding may be related to different factors, such as the study population, disease and treatment duration. We think that the effective treatment of probiotics and prebiotics may increase the number of *lactobacilli* and *Bifidobacterium* in the intestinal tract, reduce adverse metabolic products of gut microbes, and increase the content of short-chain fatty acids and methyl acetate to maintain the balance of intestinal flora and small intestine bacterial overgrowth. In addition, the short-chain fatty acids produced by probiotics in intestinal fermentation lead to osmotic stimulation, which can improve the frequency of intestinal peristalsis and stool characteristics, thus improving gastrointestinal symptoms such as constipation. At the same time, probiotics combined with prebiotics may improve the abundance and diversity of intestinal methanogens in patients with clinical hypothyroidism combined with SIBO positivity during pregnancy and improve constipation symptoms by reducing the production of intestinal methane and speeding up intestinal operation time.

Thyroid function of patients with clinical hypothyroidism complicated with SIBO during pregnancy was controlled within the normal range before and after treatment, and TSH level decreased within the normal range after 21 days of treatment compared with before treatment. *Sepide et al.* (Talebi et al., 2020) believed that the combination of probiotics and prebiotics did not significantly improve thyroid function. We found that this difference may be related to the sample size and the amount of oral levothyroxine sodium in patients. As a part of the intestinal barrier, the intestinal microbiota not only regulates the tight connection of cells and intestinal permeability but also regulates the characteristics and mucus components of intestinal epithelial cells. When the intestinal microbiota is disturbed, the absorption of thyroid hormones is significantly affected (Virili and Centanni, 2015). Our previous studies found that the abundance of *Roseburia, Pasteurellales, Lachnospira* and other bacteria in the intestinal flora of patients with clinical hypothyroidism during pregnancy was high, and the metabolites of these bacteria were often harmful to the body. Studies have shown that probiotics combined with prebiotics can improve the intestinal flora disturbance, maintain the stability of the intestinal permeability and maintain the TSH level (Sergeev et al., 2020; Mohamed et al., 2021). *Lactobacillus-* and *Bifidobacterium-related* bacteria can protect intestinal epithelial cells from the gut microbes' harmful metabolites qualitative damage, so probiotics combined with prebiotics may increase the abundance of beneficial bacteria groups, ensuring the integrity of the intestinal mucosal barrier can reduce TSH levels and improve thyroid function.

The levels of hsCRP, IL-6 and TNF-α in SIBO-positive pregnant women with clinical hypothyroidism after treatment with probiotics combined with prebiotics were significantly

lower than before treatment, and the concentrations of TC and LDL were also lower after treatment. The combination of probiotics and prebiotics is fermented by beneficial bacteria to produce short-chain fatty acids (SCFAs), which may signal through metabolism-sensitive G-protein-coupled receptors and free fatty acid receptors in the intestinal epithelium and inhibit tissue deacetylase, thus inhibiting systemic inflammatory responses (Vinolo et al., 2011; McLoughlin et al., 2017). Meanwhile, SCFA can reduce cholesterol in the blood by blocking the synthesis of cholesterol in the liver (Thandapilly et al., 2018). We believe the possible mechanism is that the combination of prebiotics and probiotics may regulate the disorder of intestinal flora, inhibit the increase in harmful flora, stabilize the integrity of the intestinal mucosal barrier, and thus downregulate inflammatory factors such as IL-6 and TNF-α. And the use of probiotics combined with prebiotics may increase the abundance of beneficial bacteria such as Bifidobacterium and Lactobacillus in the intestinal flora, which stimulate the production of SCFA, which in turn acts as a ligand to activate peroxisome proliferator-activated receptor (PPAR), which reduces the synthesis and absorption of LDL and TC.

Strengths and limitations of the study

The advantages of this study is that the methane-hydrogen breath test was used in this study to evaluate the intestinal microflora of patients with clinical hypothyroidism during pregnancy. This method is convenient, noninvasive and fast for evaluating the overgrowth of intestinal bacteria, and it provides a new idea for the treatment of patients with clinical hypothyroidism during pregnancy combined with SIBO positivity. However, some limitations of this study must be acknowledged, such as the relatively short follow-up period, and some insignificant changes may be statistically significant with the extension of follow-up time. In addition, in our study, only the methane-hydrogen breath test was used to evaluate the changes in intestinal flora in patients with clinical hypothyroidism combined with SIBO positivity during pregnancy, which had certain limitations. We will expand the sample size and further study the changes in intestinal flora by using 16S RNA sequencing technology.

Conclusion

In conclusion, probiotics combined with prebiotics can not only improve the overgrowth of intestinal bacteria but also adjust thyroid function and have a certain controlling effect on the body's inflammatory response, providing new ideas for the treatment of clinical hypothyroidism combined with intestinal bacterial overgrowth during pregnancy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception and design: YX and YH. Collection and assembly of data: YH, YB, JL, YC, QO, and BW. Data analysis and interpretation: YH, ZS, MZ, and YB. Manuscript writing: YX and YH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Engineered 5-HT producing gut probiotic improves gastrointestinal motility and behavior disorder

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Slow transit constipation is an intractable constipation with unknown aetiology and uncertain pathogenesis. The gut microbiota maintains a symbiotic relationship with the host and has an impact on host metabolism. Previous studies have reported that some gut microbes have the ability to produce 5-hydroxytryptamine (5-HT), an important neurotransmitter. However, there are scarce data exploiting the effects of gut microbiota-derived 5-HT in constipation-related disease. We genetically engineered the probiotic *Escherichia coli* Nissle 1917 (EcN-5-HT) for synthesizing 5-HT in situ. The ability of EcN-5-HT to secrete 5-HT *in vitro* and *in vivo* was confirmed. Then, we examined the effects of EcN-5-HT on intestinal motility in a loperamide-induced constipation mouse model. After two weeks of EcN-5-HT oral gavage, the constipation-related symptoms were relieved and gastrointestinal motility were enhanced. Meanwhile, administration of EcN-5-HT alleviated the constipation related depressive-like behaviors. We also observed improved microbiota composition during EcN-5-HT treatment. This work suggests that gut microbiota-derived 5-HT might promise a potential therapeutic strategy for constipation and related behavioral disorders.

KEYWORDS

***E. coli* Nissle 1917, engineered probiotic, 5-HT, gut microbiota, constipation**

Introduction

Chronic constipation (CC) is a common functional gastrointestinal (GI) disorder with a 15% global prevalence (Bharucha and Lacy, 2020). Slow transit constipation (STC), a most common type of chronic constipation in clinical practice, is characterized by markedly increased total bowel transit time (Jamshed et al., 2011). STC can cause abdominal distention, pain, nausea, vomiting, perianal illness, and even colon cancer

(Stern and Davis, 2016). The persistent occurrence of STC symptoms cause great distress to the patients and impairs their quality of life. Medical managements for STC include laxatives, motilin receptor agonist, prokinetic and other agents (Tillou and Poylin, 2017). However, the associated side effects, tolerance and dependencies of these drugs highlight the need for novel therapeutics (Ramkumar and Rao, 2005).

5-hydroxytryptamine (serotonin, 5-HT) is an important neurotransmitter and plays crucial roles in regulating host mood, memory, appetite, intestinal homeostasis and metabolism (Berger et al., 2009). The majority of 5-HT (up to 95%) is produced by enterochromaffin (EC) cells in the gut, despite its key roles in the central nervous system. (Gershon and Tack, 2007). Previous studies had proposed that endogenous 5-HT was an important enteric neurotransmitter. However, recent studies have shown that 5-HT antagonists still have the same or greater inhibitory effect on GI-motility and transit, even when all endogenous 5-HT has been genetically (Spencer et al., 2013) or pharmacologically (Yadav et al., 2010) ablated from the gut. However, exogenous 5-HT potently increases GI transit in many species tested (Spencer and Keating, 2022).

When 5-HT released by EC cells binds to different subtypes of 5-HT receptors (5-HTRs) in the intestinal lumen and lamina propria, a variety of important physiological activities are manipulated. The G-protein-coupled receptor (GPCR) 5-HTR4, which is present in the epithelium of the entire colon, is the most exposed 5-HTR to the lumen (Hoffman et al., 2012a). With its role in promoting motility and intestinal secretion control, 5-HTR4 has been targeted in diseases associated with slow GI transit, such as IBS-C (Cole and Rabasseda, 2004). Prucalopride, a very highly selective 5-HTR4 agonist, is developed as an orally administered, first-in-class drug for treatment of severe chronic constipation (Jiang et al., 2015).

Over recent decades, there has been a growing appreciation of the role of gut microbiota in the maintenance of human health. Recently, several studies have also shed light on the effect of microbiota in gut motility (Chandrasekharan et al., 2019; Obata et al., 2020; Wang et al., 2020). As probiotics can be delivered orally, enhance targeting drug efficacy and minimize systemic side effects, engineering them as therapeutics has garnered increasing interests. Since its discovery in 1917, *Escherichia coli* strain Nissle 1917 (EcN), a commensal bacterium in human gastrointestinal tract, has been successfully used in clinical applications to treat a variety of GI disorders (Henker et al., 2007; Schultz, 2008; Wassenaar, 2016). Owing to its excellent safety profile and genetic tractability, EcN has been modified as a versatile probiotic strain and proven to be effective for treating numerous diseases, including antitumor drug carriers, pathogens resistance, immunotherapy and metabolic abnormalities improvement (Hwang et al., 2017; Ho et al., 2018; Praveschotinunt et al., 2019).

In this study, we genetically inserted the rice (*Oryza sativa*) tryptophan decarboxylase gene *tdc(R)* into the chromosome of EcN (EcN-5-HT) to produce 5-HT. Then, we tested this system *in vivo* using a murine model of constipation. The oral delivered engineered probiotic showed therapeutic activities that efficiently alleviated constipation symptoms and related behaviour disorders. Furthermore, 5-HTR activation and microbiota regulation involved in the underline mechanisms were discussed.

Materials and methods

Strains and media

All bacterial strains and plasmid used in this study are listed in Supplementary Table 1 and Supplementary Table 2. EcN (non-pathogenic probiotic isolate, serotype O6: K5: H1) was kindly provided as a gift from Jun Zhu's lab (University of Pennsylvania).

Luria-Bertani (LB) medium with appropriate antibiotic selection (100 μg ml^{-1} ampicillin,100 μg ml^{-1} kanamycin) was used for cell cultivation. Cell growth was monitored by OD_{600} measurements. Modified M9 medium (M9Y) with 10 mM tryptophan, 1 mM BH4, 0.1% Casein Hydrolysate, 50 μg/mL FeCl3 and 0.2% ZYT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) was used for production of 5-HT in shake flasks. EcN-5-HT was incubated in LB at 37°C overnight. Then, the medium was centrifuged at 8,000×g for 10 min to obtain the supernatant. The production of 5-HT in fermentation supernatant was measured by UPLC-MS/MS.

Full-length of tryptophan decarboxylase (TDC) cDNAs from *Catharanthus roseus* (GenBank accession no. MG748691.1), *Oryza sativa* Japonica Group (GenBank accession no. AK069031) and *Bacillus atrophaeus* strain C89 (GenBank accession no. JQ400024.1) were codon optimized and synthesized by Genewiz (Suzhou, China). Then, flanking gene fragments were cloned into pACYC-araBAD plasmid backbone using NEBuilder HiFi DNA Assembly (NEB, Ipswich, USA) to create pACYC-araBAD-TDCs. Next, pACYC-araBAD-TDC expression systems were transferred to EcN-5-HTP strain using a MicroPulser electroporator (Bio-Rad, CA, USA) following the manufacturer's instructions. Clones were cultivated on LB agar supplemented with kanamycin (100 μg ml^{-1}) at 37°C. Modifications were verified by PCR and gene sequencing (Tsingke, Beijing, China) (Supplementary Figure 1).

For genome integration of TDC (R), a λ-Red recombination system was employed (Datsenko and Wanner, 2000). All primers for the integration used in this study are given in Supplementary Table 3.

Animal model

Specific pathogen-free (SPF) C57BL/6J mice (6 weeks old, male) were purchased from the Hubei Province Center for Disease Control and Prevention (Wuhan, China). The mice were housed (no more than four per cage) under humidity- and temperature-controlled conditions and a 12-hour light/dark cycle with free access to food and water. All animal procedures strictly conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

After a week of adaptive feeding, mice were randomly divided into five groups (n=8): a normal group, a model group, a EcN WT group, a EcN-5-HT group and a prucalopride group. The loperamide-induced constipation model was established by 7 days of twice-daily (9:00 and 18:00) intraperitoneal administration (*i.p.*) of loperamide hydrochloride (8 mg/kg body weight, 200 μL) suspended in physiological saline in all the groups except the normal group. After that, EcN WT/EcN-5-HT (1×10^9 CFU suspended in 100 μL of saline) was given through gavage every two days to the mice for 14 days. For the prucalopride group, 2 mg/kg body weight prucalopride was oral administered daily to the mice through gavage (Zhang et al., 2018), while mice from the normal group were treated with saline. Mice were sacrificed and samples were collected at the end of the treatment period.

GI motility

Stool water content was calculated as [(initial stool weight – dry stool weight)/initial stool weight] × 100%. Stool frequency was measured as the number of stool pellets extruded from each mice per hour. For defecation time measurement, mice were oral administrated of 10% activated carbon, and were given free access to food and water, the time between the gavage and the appearance of their first darkened feces was recorded (Liu et al., 2020). At the end of the experiment, each mouse was gavaged with 0.2 ml of activated carbon solution and sacrificed after 30 minutes. The GI transit time was determined by recording the length of the small intestine and the distance traveled by the activated carbon in the intestine.

UPLC–MS/MS

Bacterial supernatant or tissue homogenate was extracted with 70% methanol, vortexed and centrifuged at 14000×g for 15 min at 4°C. 5-HT was separated and detected on an AB Sciex 4500 UPLC-MS/MS system (AB Sciex, USA). Samples were injected (2 μl) and separated on a Waters BEH C18 column (Water, USA) (100mm×2.1mm×1.7μm). The mobile phase consisted of solution A (5mM ammonium formate, 0.1% formic acid) and solution B (0.1% formic acid in acetonitrile) at 0.3 ml min^{-1} at 40°C. The gradient elution was programed as follows: 0-1 min, 95% A; 1-2 min, 95-10% A; 2-4.5 min; 10% A; 4.5-4.6 min; 10-95% A and 4.6-7 min, 95% A. 5-HT was detected using selected reaction monitoring of compound-specific mass transitions in positive electrospray ionization mode: m/z 177 > 160 for the qualitative ion pair of 5-HT; m/z 177 > 132.1, 177 > 115.1 for the quantitative ion pair of 5-HT. Data acquisition and processing were performed with the analyst software MultiQuant 2.1.1.

Histological analysis

For immunofluorescence staining of 5-HT and CgA, frozen slices of the dissected colon tissues from different groups were blocked with 5% BSA in PBS for 60 min. Heat mediated antigen retrieval was performed in 0.01M citrate-buffer (pH 6.0). The slices were then incubated with a 1:500 dilution of anti-serotonin antibody (ab6336, Abcam, USA) and anti-Chromogranin A antibody (ab283265, Abcam, USA) rocked on an orbital shaker (Mini Roller, NEST Biotechnology, China) at 4°C in the dark overnight. Afterwards the slices were treated with HRP-conjugated secondary antibody, in PBS at room temperature in the dark for 60 min. Cell nuclei were stained with DAPI (Sigma, USA). Stained cells were then visualized by fluorescence microscopy (Nikon Eclipse CI, Japan).

Gene expression

RNA from harvested colonic tissues was extracted with TRIzol reagent (Invitrogen, USA). To generate cDNA, we used the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China) with 2 μg of RNA for each sample. mRNA relative expression was measured using a CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR was carried out with 10 μL of SYBR Green Master Mix (Yeasen, Shanghai, China), 2 μL of complementary DNA (cDNA), 0.4 μL of forward primer, 0.4 μL of reverse primer, and 7.2 μL of nuclease-free water. The samples were subjected to 40 cycles of amplification. Preincubation was for 30 seconds at 95°C, followed by denaturation at 95°C for 10 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for 30 seconds. The primers used in the present study are listed in Supplementary Table 4.

Behavior test

Open field test (OFT): Briefly, mice were gently placed in an open field, a white plastic box (46×46×40 cm). The center was located in 3/5 places of length and width. Mice were placed in the center of the arena tracked for 10 min. Elevated plus maze test

(EPMT): Mice were placed in the center part of the maze facing one of the two open arms. Mice behavior was tracked for 10 min. Forced swim test (FST): Mice were gently placed in transparent cylindrical tanks (30 cm height×20 cm diameters) containing water (23°C ± 2°C) with 15 cm in depth from the bottom. After 2 minutes for acclimation, the immobility time was recorded for 6 minutes. Tail suspension test (TST): Mice were suspended upside down by tails 40 cm above the floor by adhesive tape placed 1 cm from the tail tip and tracked for 6 minutes.

Before the behavioral tests, all mice were allowed to acclimate to the test room for at least 2 hours prior to starting the test. Movements of the subject mice were recorded and analyzed by SMART 3.0 video tracking software (Panlab Harvard, MA, USA).

Microbial DNA extraction and sequencing

At the end point of treatment, mice fecal samples were collected and frozen at -80°C immediately after collection. Total genomic DNA from approximately 200 mg of stool was extracted by a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The V3-V4 region of the bacterial 16S ribosomal RNA (rRNA) genes was amplified by PCR with universal primers (338F, ACTCCTACGGGAGGCAGCAG; 806R, GGACTACHVGGGTWTCTAAT) and FastPfu Polymerase. Amplicons were then purified by gel extraction (AxyPrep DNA Gel Extraction Kit, Axygen Biosciences, USA) and quantified using QuantiFluor-ST (Promega, USA). The purified amplicons were pooled in equimolar concentrations, and paired-end sequencing was performed using an Illumina MiSeq platform (Illumina, San Diego, USA).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8 statistical software. Comparisons between two groups were performed using unpaired two-tailed Student's t-test. One-way analysis of variance was used for comparisons of more than two groups. The results are presented as the mean ± SD. Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Results

Construction of 5-HT biosynthetic pathway in EcN

5-HT is natively produced from 5-HTP by tryptophan decarboxylase in animals and plants. The 5-HT biosynthetic pathway was introduced into a 5-HTP-producing EcN (Figure 1A, Supplementary Figure 2). To verify the decarboxylase activities and obtain desired products, we tested three tryptophan decarboxylases (TDCs) from *Catharanthus roseus*, *Oryza sativa* Japonica Group, and *Bacillus atrophaeus* strain C89. EcN was transformed with the protein expression plasmid pACYC-araBAD containing the genes encoding TDC under the inducible promoter (P_{BAD}). We first confirmed that the introduction of each *tdc* gene didn't influence the growth of EcN, comparing with the empty plasmid control (Figure 1B). Among them, EcN with pACYC-araBAD-*tdc(R)* showed the highest TDC protein yield (Supplementary Figure 3). The cell-free supernatants of the three transformed strains were collected separately and detected by UPLC-MS/MS. Results showed that all the supernatants contain 5-HT (Figure 1C). As expected, EcN with pACYC-araBAD-*tdc(R)* yield the highest 5-HT level (Figure 1B). Then, pACYC-araBAD-*tdc(R)* fragment was integrated into *malEK*, the intergenic region between *malE* and *malK* genes (Kurtz et al., 2019), of EcN using the λ-Red recombination system to ensure the stable expression *in vivo*. The recombinant strain (EcN-5-HT) generated higher production than the control strain (80.6 mg/L *vs*. 5.6 mg/L, Figure 1D), and performed a similar growth pattern as the wildtype strain (Figure 1E). These results together indicated that EcN-5-HT strain could efficiently secrete 5-HT to the extracellular culture without affecting its growth.

Gastrointestinal motility enhancement from engineered EcN

The roles of EcN-derived 5-HT in the gut were further evaluated in a constipation animal model. Constipation was induced by loperamide in six-week-old male C57BL/6 mice. Then, the mice were orally gavaged with the strain EcN-5-HT for two weeks (Figure 2A). The level of EcN in the fecal of treated mice were significantly increased at the endpoint of the experiment (Supplementary Figure 4). We observed that mice receiving EcN-5-HT exhibited improved gastrointestinal motility, as evident from an increase in stool water relative content (Figure 2B) and frequency of fecal defecations (Figure 2C). Notably, EcN-5-HT administration showed a more potent effect on increasing stool water content than prucalopride (Figure 2B). Time of the first black stool defecation following the administration of activated carbon is another indicator of the intestinal patency and peristalsis. We found that the time to first black stool defecation was significantly reduced in the EcN-5-HT treated group (Figure 2D). Meanwhile, reduction in whole gut transit time was observed after EcN-5-HT administration (Figures 2E, F). Besides, the body weight of mice was also monitored. At the endpoint, no acute body weight drop was observed from the above treatments throughout experiment (Supplementary

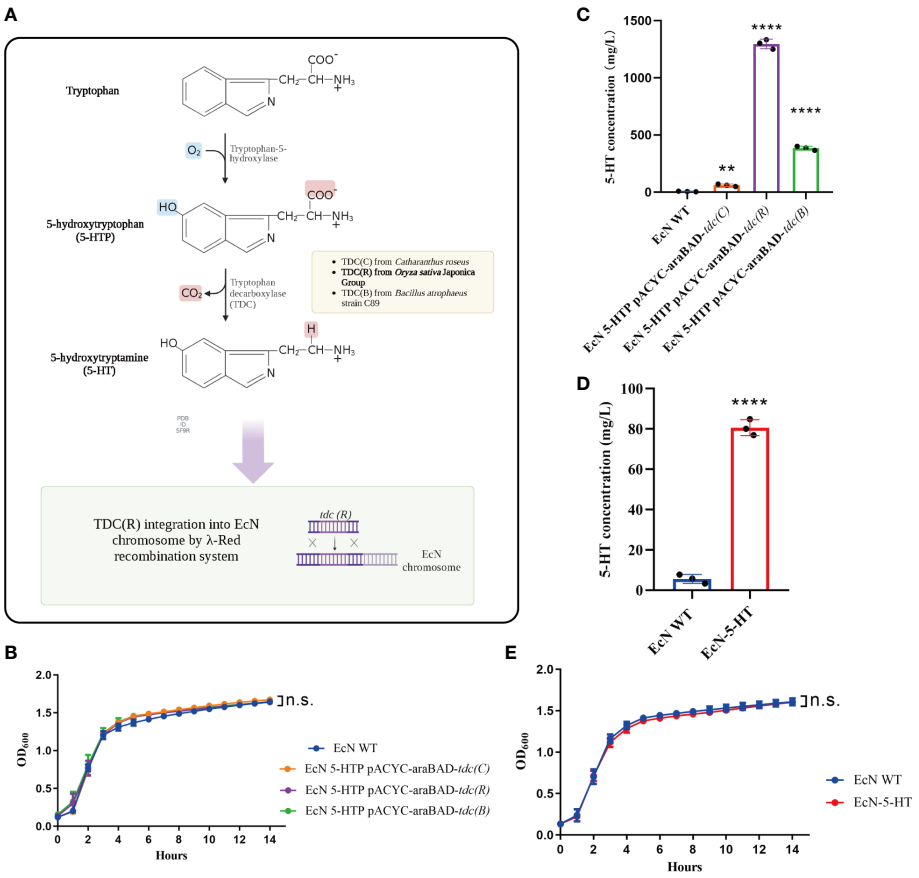

FIGURE 1
Engineer *Escherichia coli* Nissle 1917 (EcN) to synthesis of human neurotransmitter 5-HT. **(A)** Schematic summarizing the design strategy to engineer EcN-5-HT. **(B)** Growth curve of the three engineered EcN in LB medium. **(C, D)** Measurement of 5-HT production by UPLC-MS/MS. **(E)** Growth curve of the engineered EcN-5-HT in LB medium. Mean values ± SDs are presented, p values were calculated using unpaired t-test, **p < 0.01, ****p < 0.0001, n.s: not significant. Data are pooled from three independent experiments with n = 3 per group.

Figure 5). Together, our results suggest that administration of EcN-5-HT reverses loperamide-induced disorders in intestinal motility.

EcN-5-HT administration increases 5-HT accumulation and 5-HT receptors expression in mice

To further explore the mechanisms of EcN-5-HT strain in regulation of GI motility, we first detected the concentration of 5-HT *in vivo*. UPLC-MS/MS measurements revealed that engineered EcN treatment led to a significant increase of 5-HT yield in mice colon (Figure 3A). Colon tissue samples were further processed for immunofluorescence assay and confirmed that content of 5-HT in colon was increased by EcN-5-HT administration, whereas enteroendocrine cells identified by anti-chromogranin A showed no significant group differences in variances (Figure 3B). 5-HTR4 is an important therapeutic target for treatment of chronic constipation (Hoffman et al., 2012b; Gwynne and Bornstein, 2019). After different modalities of treatment in healthy or gastrointestinal function disturbed rodent models, the secretion of 5-HT increases, and the expression of 5-HTR4 receptor is upregulated, suggesting that 5-HT and 5-HTR4 receptors may be correlated (Orlando et al., 2020; Yaghoubfar et al., 2020; Zhu et al., 2020). Given the effect of EcN-5-HT on the 5-HT level in colon, we investigated the expression of 5-HTR4 gene. The results showed that expression of 5-HTR4 gene was significantly increased in EcN-5-HT group (Figure 3C). On the other hand, the concentration of 5-HT in the serum showed no significant increase in EcN-5-HT group, suggesting that the effect of EcN-5-HT is more significant locally in the intestine (Figure 3D). Collectively, the elevated level of 5-

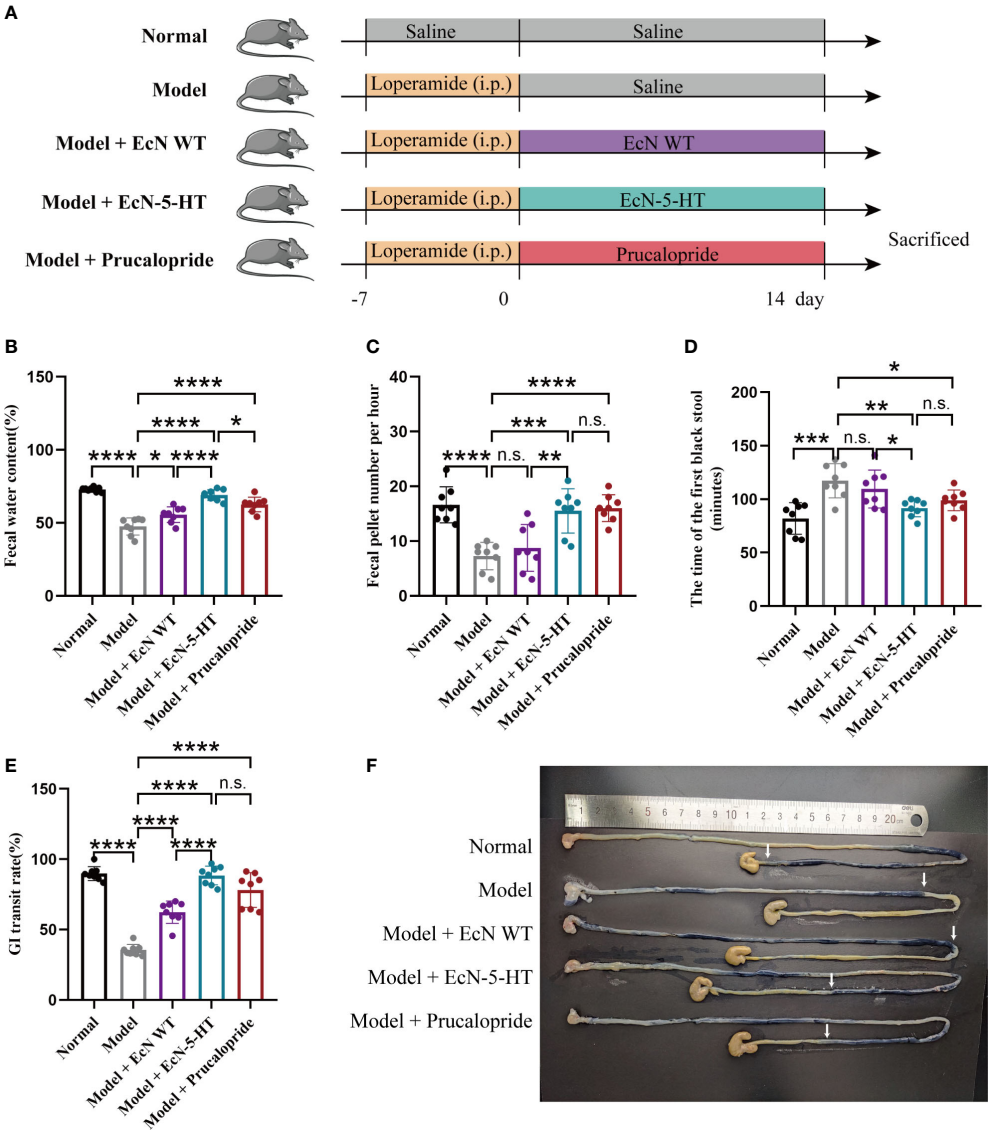

FIGURE 2
EcN-5-HT improved gastrointestinal motility in a loperamide-induced constipation model. **(A)** Experimental setup. **(B)** Fecal water relative content. **(C)** Fecal pellet number per hour. **(D)** Time to first black stool defecation. **(E)** GI transit. **(F)** Representative images of small intestine after treatment with activated carbon by gavage. Mean values ± SDs are presented, p values were calculated using unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 n.s, not significant. Data are pooled from three independent experiments with n = 8 mice per group.

HT and upregulated 5-HT receptors in EcN-5-HT treatment group leads to positive effects on intestinal motility.

Amelioration of depression-like behaviors in loperamide induced constipation mouse model

It has been reported that loperamide-treated mice exhibited significant depressive symptoms (Xu et al., 2018). Therefore, we also tested the behavioral parameters to evaluate the potential role of EcN-5-HT on depressive-like behaviour. Open field test, elevated plus maze test, tail suspension test, and forced swim test are widely used for assessing anxiety-like behaviors and cognitive function. As shown in Figures 4A, B, loperamide-treated mice exhibited significantly reduced movement and spent significantly less time in the central region of the open field compared to normal mice. Besides, the model group spent notable less time in the open arms in the EPMT (Figures 4C, D) and showed a significantly increased immobility time in the TST

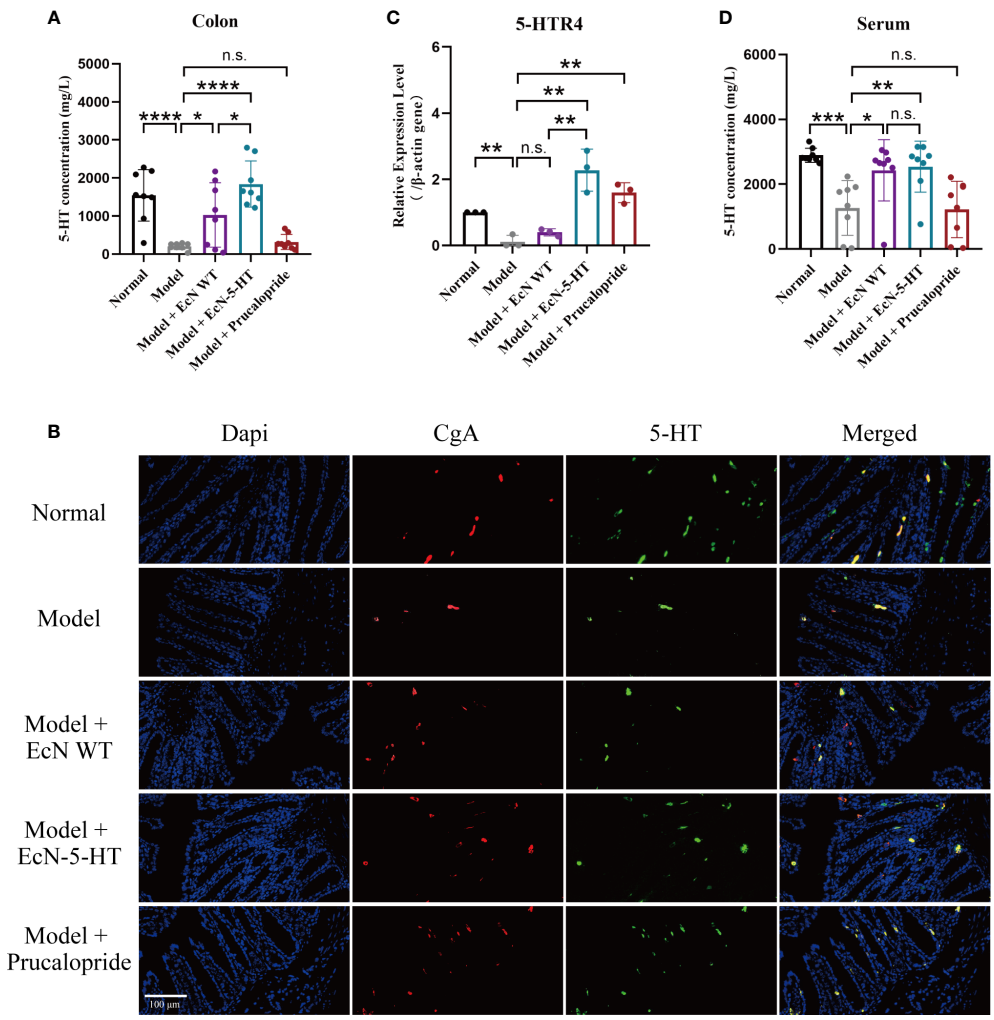

FIGURE 3
EcN-5-HT led to an increase of 5-HT concentration in constipation mice model. **(A)** Measurement of colon 5-HT by UPLC-MS/MS. n = 8 mice per group **(B)** Fluorescent microscope pictures of colon showing CgA antibody staining (red), 5-HT antibody staining (green) and cell nuclei (blue). **(C)** 5-HTR4 mRNA expression in colon tissue. n = 3 mice per group. **(D)** Measurement of serum 5-HT by LC-MS. n = 8 mice per group. Mean values ± SDs are presented, p values were calculated using unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. n.s, not significant.

(Figure 4E). No significant difference was observed between the model and normal groups in FST (Supplementary Figure 6). The administration of EcN-5-HT modulated locomotor activity in the OFT and restored the mobility of loperamide-treated mice to control levels (Figures 4A, B). On the EPMT, animals in EcN-5-HT group spent significantly more time in the open arms than saline and EcN WT-fed model animals (Figures 4C, D). Additionally, EcN-5-HT treatment led to decreased immobile time in TST compared to control mice (Figure 4E). Notably, EcN-5-HT showed a better anti-depression effect than prucalopride in TST, suggesting possibly different underlying mechanisms between them. These results indicate that EcN 5-HT ameliorated depression-like behaviors induced by loperamide in mice, suggesting that microbe derived 5-HT can perform anxiolytic effects in host gastrointestinal tract.

Improvement of gut microbiota dysbiosis by microbiota derived 5-HT

Increasing studies have reported that the microbiota plays important roles in gut motility (Chandrasekharan et al., 2019; Obata et al., 2020). To investigate the influence of microbiota derived 5-HT on gut microbiota composition, we collected the stools from mice at the end of the treatment. Then, microbial DNA extraction and 16S rRNA gene sequencing were

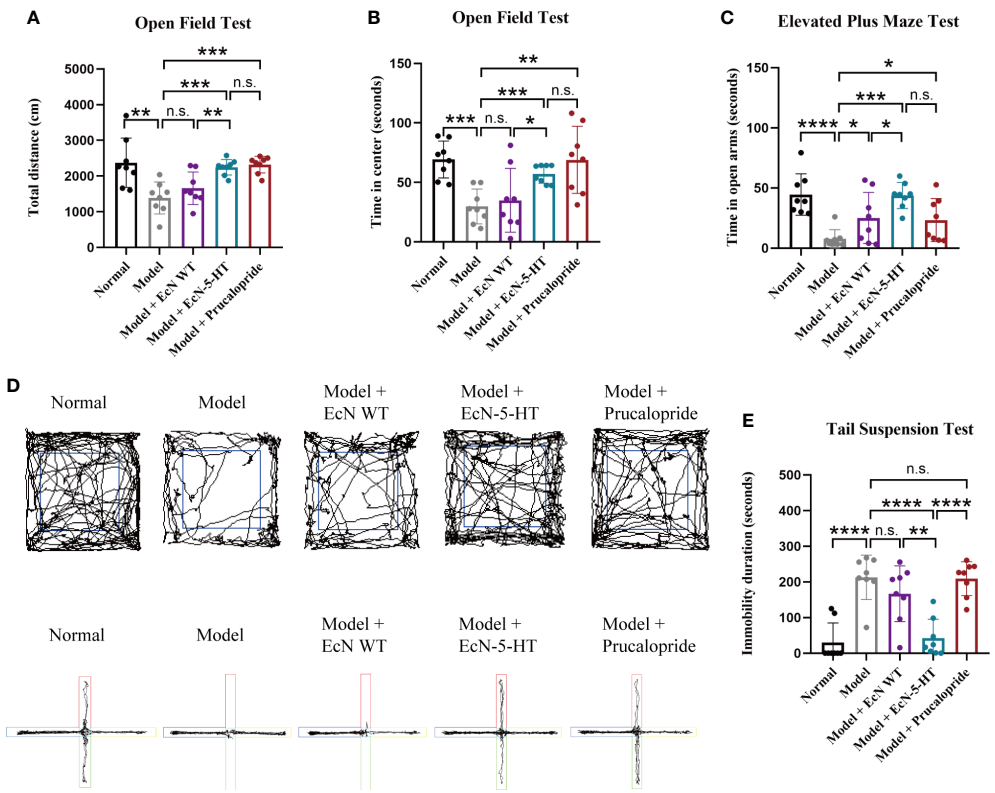

FIGURE 4
EcN-5-HT ameliorated loperamide-induced behavior disorders. **(A)** Open field test (OFT). **(B)** Representative tracking plots of the open field. **(C)** Elevated plus maze test (EPMT). **(D)** Representative tracking plots of the elevated plus maze test. **(E)** Tail suspension test (TST). Mean values ± SDs are presented, p values were calculated using unpaired t-test. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001, n.s.: not significant.

conducted. Interestingly, EcN-5-HT treatment significantly increased gut microbiota alpha diversity, including Shannon and Simpson diversity (Figure 5A), while the prucalopride treatment resulted in a significant lower alpha diversity (Figure 5A). Principal coordinate analysis (PCoA) on OTU levels was also performed to further examine the composition change of gut microbiota between different treatments. The results clearly showed an apparent clustering separation between the normal group and the model group (Figure 5B). After EcN-5-HT treatment, the abundance and composition of gut microbiota was more similar to that of the normal group (Figure 5B). Classification of OTUs at each phylogenetic level revealed distinct taxonomic patterns between normal mice and constipation mice (Figure 5C). To further elucidate the mechanisms of the effect exerted by altered gut microbiota after EcN-5-HT treatment, we performed LEfSe analysis to identify representative abundant bacterial communities among the groups (Figure 5D). Results showed that EcN-5-HT treated mice harbored distinctively higher abundances of the genera such as *Alistipes*, *Odoribacter* and *Clostridia* (Figure 5D). Relative abundance of *Alistipes* exhibited remarkable and negative correlations with the time of the first black stool and showed significant and positive correlations with GI transit rate, stool water relative content, and stool frequency (Figure 5E). Together, these data indicated that EcN-5-HT treatment can improve gut motility by regulating the intestinal microbiota composition.

Discussion

The role of 5-HT in human health and disease has been widely studied (Lesurtel et al., 2008; Manocha and Khan, 2012; Agus et al., 2018). However, most of the research have focused on host-derived 5-HT. Previous studies have reported that some gut microbes have the ability to produce 5-HT (Özoğul, 2004; Ozogul et al., 2012; O'mahony et al., 2015). The role of gut microbe-derived 5-HT in the gut has not been studied in detail. Although substantial recent evidence has now confirmed that ablation of endogenous 5-HT does not lead to major changes in gastrointestinal transit (Li et al., 2011; Sia et al., 2013; Spencer and Keating, 2022), the findings of the current study imply that

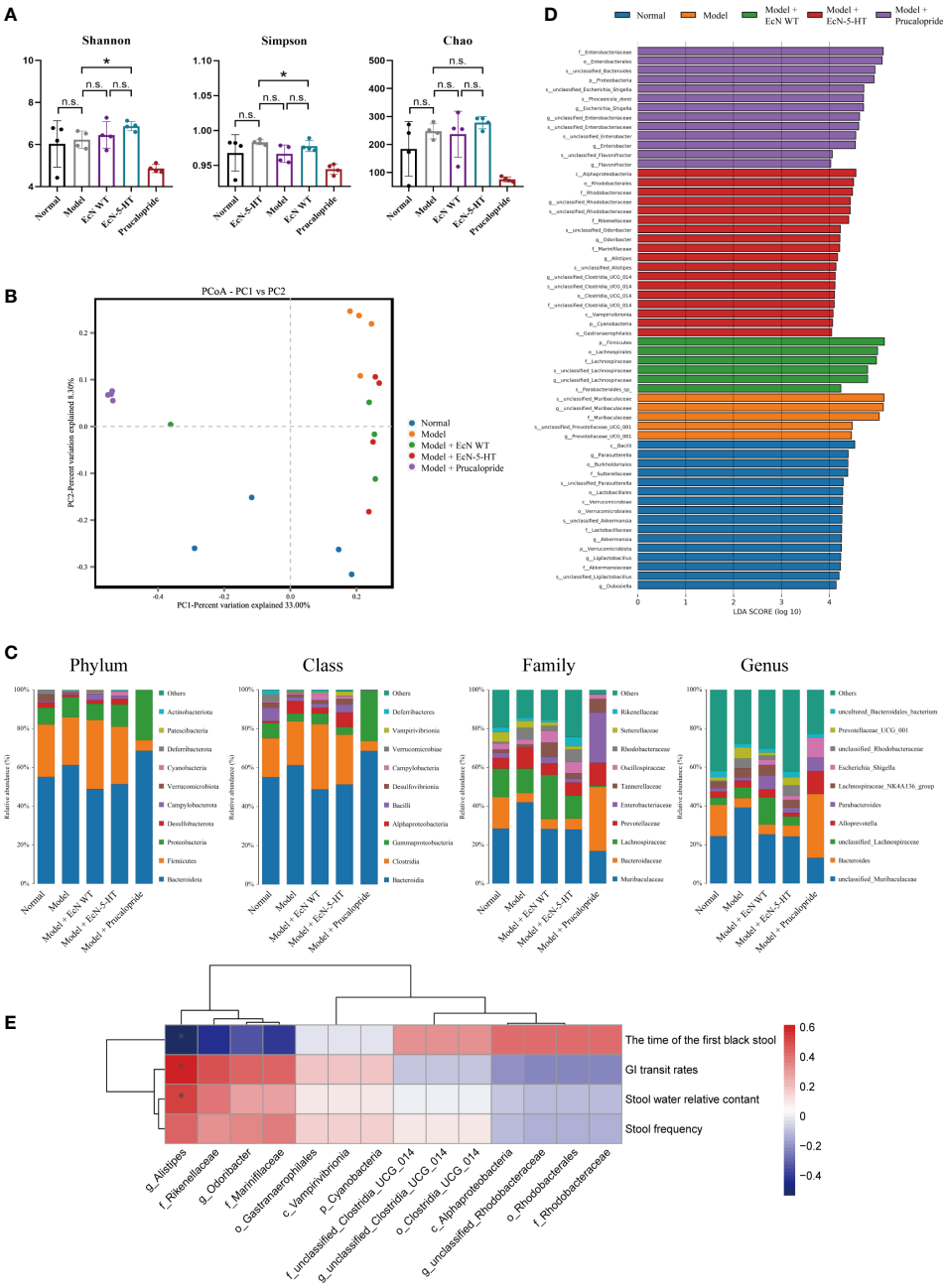

FIGURE 5
Effects of EcN-5-HT on intestinal microbiota in a constipation mice model. **(A)** Alpha diversity boxplot analysis. **(B)** Principal coordinate analysis (PCoA) profile of microbial diversity. **(C)** Relative abundance of microbial community at different taxonomic levels. **(D)** LDA score computed from features differentially abundant between the groups. **(E)** Spearman correlation analysis. Red and blue colors represent significant positive correlations and negative correlations. The color depth represents the correlation coefficient, and the darker the color, the greater the correlation coefficient. Mean values ± SDs are presented, p values were calculated using unpaired t-test, *p < 0.05, n.s: not significant. Data are pooled from three independent experiments with n = 4 mice per group.

synthesis of EcN 5-HT can lead to modifications in GI transit *in vivo*. The mechanisms by which this occurs remains unclear. In our present study, we proved that 5-HT-producing gut microbes can significantly impact gut motility. Our results suggest that microbial 5-HT metabolism could have more implications for GI health, which is barely discussed previously.

Since the 1950's from work of Bulbring & Crema (Bulbring and Crema, 1959) had provided circumstantial evidence that endogenous 5-HT maybe important in GI motility and transit. However, more recent studies have shown that in fact ablation of endogenous 5-HT has only minor or no effects on GI transit and motility (Spencer and Keating, 2022). Current evidence does not suggest endogenous 5-HT plays a major role, nor is required for control of gut motility or transit *in vivo*. Alterations in the 5-HT pathway are commonly reported in various constipation-related disease conditions. In patients with IBS-C, the content of mucosal 5-HT, the transcription expression of tryptophan hydroxylase 1 transcription and serotonin transporter transcription, and the immunoreactivity of serotonin transporter were all reduced significantly, without any change in the number of enterochromaffin cells (Coates et al., 2004; Wang et al., 2007). In IBS-C patients, postprandial levels of plasma 5-HT were also significantly decreased compared to controls and patients with IBS-D, which may result in significantly delayed gastrointestinal transit (Dunlop et al., 2005; Choi et al., 2014). In colonic inertia patients, lower serotonin receptors in muscular mucosa and circular muscle may contribute to delayed colonic transit (Zhao et al., 2003). In this study, we found no difference in the colonic CgA+ ECs between loperamide-treated mice and normal mice, suggesting that the decreased release of 5-HT by loperamide was not due to the density of ECs (Figure 3B).

A number of studies reported a decreased concentration of colon 5-HT in constipation patients, which is consistent with our results (Figures 3A, B). Alternatively, several studies also reported higher content of 5-HT in patients with constipation than in normal patients (Lincoln et al., 1990; Costedio et al., 2010). Circulating 5-HT, which represents the 5-HT that is not captured by serotonin transporter (SERT) in the epithelial cells, was used to evaluate the 5-HT availability in the mucosa. More studies on the SERT function in constipation patients are needed in order to guide precise medication of 5-HT-related drugs.

In addition, gut microbiota were involved in 5-HT-related physiology in host. Using antibiotics-depleted microbiota mice model, Ge et al. observed a decreased tryptophan hydroxylase 1 transcriptional expression, 5-HT production, and constipation-like symptoms (Ge et al., 2017). Fecal microbiota from constipation patients led to the same symptoms, including upregulated expression of SERT, and decreased concentration of 5-HT in mice (Cao et al., 2017). These studies suggest that gut microbiota is involved in host 5-HT biosynthesis, and intestinal dysbiosis may contribute to the development of chronic constipation. In this study, by comparing 5-HT producing microbe (EcN-5-HT) with its original strain (EcN WT), we show that gut microbiota-derived 5-HT could improve 5-HTR expression and ameliorated constipation symptoms (Figures 2, 3C). Meanwhile, we observed that EcN WT itself can also lead to an increase of 5-HT in colon and serum (Figures 3A, D). It has been reported that EcN is able to enhance host 5-HT bioavailability in intestinal tissues (Nzakizwanayo et al., 2015). This explanation may account for the increase of 5-HT concentration in EcN WT treated mice treated. As shown in Figure 3C, there are no significant differences in relative expression of 5-HTR4 between the model and the EcN WT group. It is possible that the colon concentration of 5-HT needs to be high enough in order to activate the 5-HT receptors. The improved GI motility by EcN WT (Figure 2E) suggested an additional mechanism independent of 5-HTR4.

Prucalopride, a highly selective 5-HTR4 agonist, is a first-in-class drug for severe chronic constipation treatment (Jiang et al., 2015). Prucalopride treatment can improve stool frequency and consistency, enhanced colonic transit in chronic constipation patients (Müller-Lissner et al., 2010). However, prucalopride side effects have been also reported, such as abdominal pain and diarrhea (Bassotti et al., 2016). In this paper, we observed that prucalopride treatment significantly reduced microbiota alpha diversity (Figure 5A) and disrupted microbiota homeostasis (Figure 5C). Our results showed that the effects of EcN-5-HT in relieving constipation symptoms are comparable to that of prucalopride (Figure 2), along with a positive regulation on the microbiota composition (Figure 5). Microbe-derived 5-HT has better effects than prucalopride in the improvement of depression and anxiety induced by constipation (Figure 4E), implying different mechanisms between pharmacologic treatment and microbial-derived 5-HT treatment, which requires further investigation.

Conclusions

Although recent studies have confirmed that endogenous 5-HT has a minor role in GI-motility and transit *in vivo*, our data here demonstrate that a genetically engineered probiotic strain (EcN-5-HT) producing 5-HT is able to significantly improve intestinal motility in a murine constipation model (Figure 6). EcN-5-HT treatment also greatly improved the gut microbiota homeostasis and significantly relieved depression-like behaviors. Our results suggested that engineered 5-HT producing microbe maybe a promising alternative to the treatment of constipation and related behavior disorders.

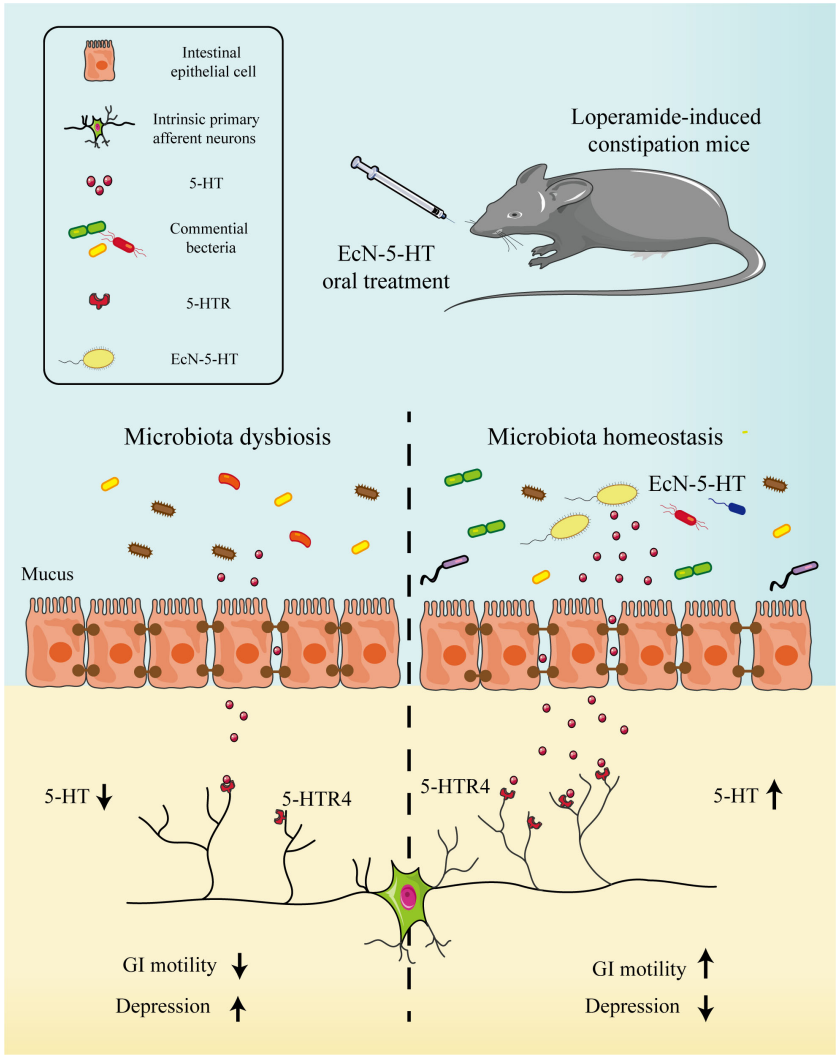

FIGURE 6
EcN-5-HT improved GI motility and ameliorate behavior disorder in loperamide-induced constipation mice model. EcN-5-HT increased the concentration of 5-HT in colon and activated 5-HT receptors, triggering the peristaltic reflex in the gastrointestinal tract and promoting the GI motility. Meanwhile, EcN-5-HT modified the composition of the intestinal microbiota in loperamide-treated mice.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://bigd.big.ac.cn/gsa/browse/CRA007693,PRJNA448831.

Ethics statement

The animal study was reviewed and approved by Animal Care Committee of Hubei Province.

Author contributions

ZL, WL, and BL participated in the study design; BL and YL performed the experiments and wrote the manuscript; ML and RL contributed to data analysis and figure drawing. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1013952/full#supplementary-material

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Probiotics for constipation in Parkinson's: A systematic review and meta-analysis of randomized controlled trials

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Background: Parkinson's disease (PD)-related constipation may affects both disease occurrence and disease progression. Probiotics, as a potential therapeutic intervention, have attracted the attention of researchers, but the evidence of their efficacy and safety has not been systematically reviewed.

Aim: A systematic review and meta-analysis of randomized controlled trials of probiotics in the treatment of PD constipation was conducted to determine the efficacy and safety of probiotics in the treatment of PD constipation.

Methods: Four databases (The Cochrane Central Register of Controlled Trials, Embase, PubMed, and Web of Science) were searched from their establishment to June 1, 2022. We included randomized controlled trials of probiotics for the treatment of constipation in patients with PD, with probiotics in the experimental group and a placebo, another treatment, or no treatment in the control group. The primary outcome was the number of bowel movements per week. Secondary outcomes included nonmotor symptoms (NMS), gut transit time (GTT), abdominal pain, abdominal distention, constipation, and quality of life scores. Stata15.1 was used to generate a summary of the data and perform a descriptive analysis if necessary. The GRADE tool was used to assess the quality of the evidence and the Cochrane guidelines to assess the risk of bias for each study.

Results: Finally, four qualified RCTs were included, comprising 287 participants. Compared with the control group, probiotics could effectively increase the frequency of defecation per week in PD patients (WMD = 1.02. 95%CI: 0.56–1.48, and $P < 0.00001$), but the heterogeneity was high, and the quality of the evidence was low. There was no significant difference in average stool consistency between patients with PD treated with probiotics and those given a placebo in (WMD = −0.08. 95%CI: −1.42–1.26, and $P = 0.908$). In addition, the results suggested that probiotics have no obvious effect on additional indicators of gastrointestinal dysfunction, such as GTT, abdominal pain, and abdominal distension, and there is insufficient evidence on their ability to improve NMS and Parkinson's disease Questionnaire 39 summary indices (PDQ39-SI). Safety issues should be carefully explained.

Conclusion: There is insufficient evidence supporting the use of probiotics to treat constipation in patients with PD. Taking all the results together, probiotics have potential value in the treatment of PD-related constipation.

Systematic Review Registration: PROSPERO CRD42022331325.

KEYWORDS

Parkinson, probiotics, constipation, systematic review, meta-analysis

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, and dyskinesia is a major feature of PD. In fact, a range of nonmotor symptoms (NMS) associated with autonomic nervous dysfunction, especially dysfunction of the gastrointestinal tract (Borek et al., 2006; Cloud and Greene, 2011; Fasano et al., 2015), may occur at all stages of PD (Chaudhuri and Schapira, 2009) and may even be closely related to the pathogenesis of PD (Braak et al., 2003; Holmqvist et al., 2014). NMS has a significant negative impact on clinical care and health-related quality of life (Hr-QoL) in patients with PD (Li et al., 2010; Lyons and Pahwa, 2011).

Constipation is one of the most common NMS in PD patients with autonomic system and gastrointestinal disorders (Sakakibara et al., 2003; Verbaan et al., 2007; Gao et al., 2011), even before the onset of motor symptoms (Abbott et al., 2001; Savica et al., 2009). It affects about 50%–80% of PD patients (Ashraf et al., 1997; Verbaan et al., 2007). The evidence shows that constipation is related to the duration and severity of PD (Krogh et al., 2008), and the frequency and severity of constipation are accelerated by the progression of PD (Edwards et al., 1993). Clearly, constipation and PD have reciprocal effects (Fu et al., 2022).

PD-related constipation is an active research field. Various studies have evaluated different drugs for the treatment of PD-related constipation, but there is no clear guideline recommendation so far (Poirier et al., 2016). Clearly, it is still necessary to explore effective and safe emerging drugs (Pohl et al., 2008). Previous studies have shown that probiotics can significantly improve the stool consistency and bowel habits of PD patients (Cassani et al., 2011); increase the number of complete bowel movements per week (CBM) and gut transit time (GTT) (Barichella et al., 2016; Ibrahim et al., 2020; Tan et al., 2021); and reduce abdominal pain, abdominal distension, and incomplete emptying in PD patients (Perez-Lloret et al., 2013; Georgescu et al., 2016).

However, evidence for the positive effects of probiotics on PD constipation is inconclusive. Therefore, this systematic review and meta-analysis included data from the results of several clinical trials evaluating the efficacy and safety of probiotics in the treatment of PD-related constipation. We aim to provide a comprehensive update of the clinical data for evidence-based guideline development.

Methods

Eligibility criteria

This study included all randomized controlled trials of probiotics in the treatment of PD-related constipation. PD participants meet internationally recognized diagnostic criteria and had constipation or gastrointestinal dysfunction. The experimental group was treated with probiotics, while the control group was treated with a placebo, other treatments, or no intervention. The main outcome was the number of bowel movements per week; secondary outcomes included average stool consistency, NMS, GTT, abdominal pain, abdominal distension, constipation, and quality of life scores.

Reviews, conference papers, comments, animal studies, retrospective studies, case-control studies, and self-controlled studies were excluded. RCTs that did not include constipation-related outcomes were also excluded.

Search strategy

RCTs were searched in The Cochrane Central Register of Controlled Trials, Embase, PubMed, and Web of Science from inception to June 1, 2022. In addition, a list of references included in the study was manually searched to identify relevant trials. There were no restrictions on language, year of publication, etc. Grey literature and data on the research registry platform were not within the scope of the search because we do not have access to these. Detailed search strategies are available in the Supplementary Materials.

Study selection

According to the strict retrieval strategy, reviewers used Endnote X9 and manual procedures to delete duplicate documents. Initial study selection was performed according to the title and abstract, followed by full-text reading to determine the final included studies. Two reviewers completed the literature search and screening independently. Any disagreement between the two reviewers was resolved by discussion. If no agreement was reached, the final decision was made by a third reviewer.

Data extraction

Data extraction was performed independently and cross-checked by two examiners according to a standardized form developed in advance. The main contents included the publication year, first author, country, study design, participants (age, sex), sample size, intervention details (formulation, dose, duration), results, etc. Any disagreement between the two reviewers was resolved by discussion. If no agreement was reached, the final decision was made by a third reviewer.

Data synthesis and statistical analysis

A meta-analysis was conducted on the same outcome indicators in two or more RCTs. Continuous variables were represented by the weighted mean difference (WMD) and 95% confidence interval (CI), a result was considered statistically significant at $P < 0.05$, and if $I^2 \geq 50\%$, a random effect model was used, and sensitivity analysis was conducted to observe the stability of the results. Due to the small number of RCTs included in this study, we did not test for publication bias. For individual outcome measures, data were summarized, and descriptive analysis was performed.

Assessment of the risk of bias and quality of evidence

A risk of bias assessment was conducted for each RCT according to the Cochrane Handbook. The evaluation areas included random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias. Each area was rated as a high, low, or unclear risk. Evaluation of the quality of evidence against Grading of Recommendations Assessment, Development, and Evaluations (GRADE) consists of five main factors: risk of bias, inconsistency, imprecision, indirectness, and publication bias. All evaluations were conducted independently by two reviewers, with unresolved differences determined by a third reviewer.

Results

Results of literature search and selection

We retrieved 53 related articles from 4 databases and removed 27 duplicates. Then, four qualified studies were included through title, abstract, and full text evaluation. The detailed flow chart is shown in Figure 1. The excluded list in the "full-text assessed for eligibility" phase are outlined in the Supplementary Materials.

Characteristics of included studies

Four RCTs with 287 participants were included in the study, which was conducted in Italy, Romania, and Malaysia between 2016 and 2021. Participants were over 60 years old on average, there were more men than women, and the duration of treatment ranged from four weeks to three months. Except for one study in which trimebutine was used in the control group, all the participants in control groups took a placebo with the same characteristics as the treatment given to the intervention group but without probiotics. Three RCTs showed adverse reactions, mainly manifested as abdominal pain, abdominal distension, and dizziness, and the experimental group was larger than the control group. Detailed literature features are shown in Table 1.

At the same time, we summarized the baseline information of severity of PD symptoms of participations and PD drugs used in the three RCTs included in the meta-analysis. Ibrahim et al. (2020) included idiopathic PD patients in Hoehn and Yahr stages 1–4, The proportion of participations with stage 3 and below in the experimental group and the control group was 59.3%/64.3%, and the proportion of participations with levodopa in the two groups was 92.6%/89.3% and dopamin agonist were 63%/57.1%; Barichella et al. (2016) also included idiopathic PD patients in Hoehn and Yahr stages 1–4, the proportion of participations with stage 3 and below in the experimental group and the control group was 76.3%/75%, and the proportion of participations who received dopamine-agonist therapy in the two groups was 63.8%/62.5%, and the daily dose of levodopa in the two groups was 691mg ± 315mg/624mg ± 289mg. Tan et al. (2021) used Movement Disorder Society Unified Parkinson's Disease Rating Scale to evaluate the severity of participations, the score of experimental group and control group was 27.9 ± 12.8/27.5 ± 12.6. The comparison of the proportion of participations taking drugs in the two groups was as follows: levodopa (97.1%/97.4%), caudate agonist (38.2%/39.5%), and anticholinergics (17.6%/13.2%). There was no significant difference in the severity of PD symptoms of

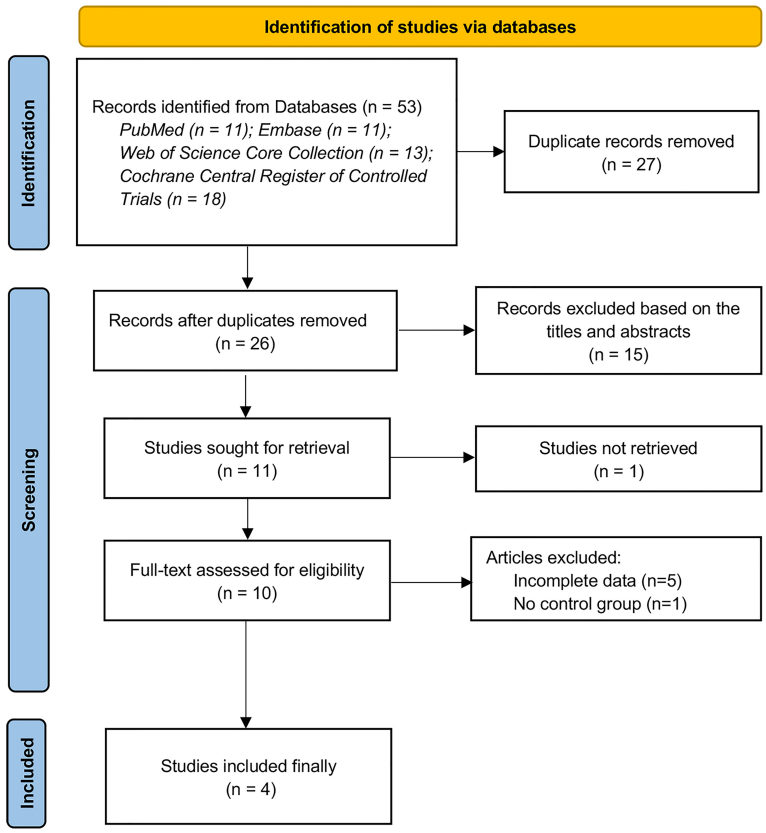

FIGURE 1
Flow chart of study selection.

participations and PD drugs used between the experimental group and the control group in the three RCTs.

Risk of bias

We assessed the risk of bias for four RCTs using the Cochrane Collaboration Handbook. The study by Georgescu et al. (2016) did not mention the allocation of hidden schemes, and the blinding was not sufficiently informative, so its risk of bias was rated as unclear. The risk of bias of all other studies was rated as low (Figure 2).

Results of the meta-analysis

The number of bowel movements per week was reported in three RCTs (Barichella et al., 2016; Ibrahim et al., 2020; Tan et al., 2021). A Meta-analysis suggested that probiotics could effectively increase the number of bowel movements per week in PD patients compared with the control group (WMD = 1.02, 95%CI: 0.56–1.48, and $P < 0.00001$), but the heterogeneity was high ($I^2 = 71.5\%$, P = 0.030), as shown in Figure 3. Two RCTs (Barichella et al., 2016; Tan et al., 2021) calculated the changes in the average stool consistency in PD patients, but the results were inconsistent, and meta-analysis results showed no statistically significant difference between the probiotic group and the control group (WMD = –0.08, 95%CI: –1.42 to 1.26, and $P = 0.908$), with high heterogeneity ($I^2 = 93.6\%$, P = 0.000). Detailed results are shown in Figure 4.

Summary of the outcomes

Georgescu et al. (2016) assessed gastrointestinal function (GI) in the NMS of PD patients. The results showed that probiotics showed the potential to relieve abdominal pain and abdominal distention in PD patients but had no significant effect on relieving constipation symptoms in PD patients. Overall, there was no statistically significant difference between the probiotic group and the trimebutine group. The results of a study by Ibrahim et al. (2020) showed that probiotics can shorten GTT and reduce the Non-motor Symptom Scale (NMSS) score in PD patients. There was a potential improvement in the scores on Parkinson's disease

TABLE 1 Detailed literature features.

Included studies	Country	Sample size (I/C)	Age [y, mean (SD)] (I/C)	Sex (male/female) (I, C)	Intervention	Comparison	Duration	Adverse events (I/C)
Barichella M, 2016	Italy	80/40	71.8 (7.7)/ 69.5 (10.3)	41/39, 24/16	Fermented milk containing probiotics and prebiotic fiber (125 g) Including the following strains: Streptococcus salivarius subsp thermophilus, Enterococcus faecium, Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus delbrueckii subsp bulgaricus, and Bifidobacterium (breve and animalis subsp lactis)/Qd	Placebo (a pasteurized, fermented, fiber-free milk)/Qd	4 w	1 (abdominal pain and bloating))/1 (abdominal pain and bloating)
Georgescu D, 2016	Romania	20/20	69.80 (5.64)/ 75.65 (9.66)	10/10, 7/13	Mixture of two lactic bacteria: Lactobacillus acidophilus and Bifidobacterium infantis, 60 mg/Bid	Trimebutine, 200mg/Tid	3 m	None
Ibrahim A, 2020	Malaysia	27/28	69.0/ 70.5	16/9, 17/10	Probiotic (Hexbio®) in orange flavouring containing microbial cell preparation of (MCP®BCMC®) at 30 x 109 colony forming units (CFU), 2% fructo-oligosaccharide (FOS), and lactose. The microbial composition of the probiotics were: Lactobacillus acidophilus (BCMC® 12130)– 107mg, Lactobacillus casei (BCMC® 12313) -107mg, Lactobacillus lactis (BCMC® 12451)-107 mg, (BCMC® 02290) -107mg, Bifidobacterium infantis (BCMC® 02129) -107mg and Bifidobacterium longum (BCMC® 02120)-107mg./Bid	Granulated milk of similar appearance to the probiotics containing lactose without fructo-oligosaccahride or microbial cells in orange flavouring/Bid	8 w	4 (abdominal bloating, n=2; dizziness, n=2)/0
Tan AH, 2021	Malaysia	34/38	63.1/ 61.5	42/29, 26/11	Probiotic capsule, contained 10 billion colony forming units (CFU) of eight different commercially available bacterial strains (Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus gasseri, Lactobacillus rhamnosus, Bifidobacterium bifidum, Bifidobacterium longum, Enterococcus faecalis, Enterococcus faecium)/Qd	Placebo capsul: containing an inactive substance (maltodextrin)/ Qd	4 w	1(lethargy)/ 0

I, intervention group; C, comparison group; F, Frequencies; m, months; y; w, week; Qd, Once a day; Bid, Twice a day; Tid, Three times a day.

Questionnaire 39 Summary Indices (PDQ39-SI), but there was no significant difference compared with the placebo group. Tan et al. (2021) showed that probiotics could significantly relieve the degree of constipation in PD patients. More detailed results are shown in Table 2.

Adverse reactions

A total of 7 adverse reactions were noted in the three RCTs, 6 of which occurred in the probiotic group, mainly abdominal pain, abdominal bloating, dizziness, and lethargy. Among them, Ibrahim et al. noted that side effects such as abdominal bloating and dizziness were transient, and symptoms resolved when probiotics were discontinued. No serious adverse reactions related to probiotic treatment were observed.

Sensitivity analysis and publication bias

We performed sensitivity analysis on the meta-analysis results of the number of bowel movements per week, and the results were stable (Supplementary Materials). Due to the small number of RCTs included in this study, we did not conduct a publication bias assessment.

Grade

Due to the high heterogeneity and small sample size in the meta-analysis of the number of bowel movements per week and average stool consistency in Parkinson's patients, the evidence level of the results was rated as low, as shown in Table 3.

Discussion

A total of four RCTs evaluating probiotics for PD constipation were included in this systematic review. A meta-analysis showed that probiotics increased the number of bowel movements per week in PD patients but had no effect on average stool consistency. Although the sensitivity analysis showed that the results were stable, subgroup analysis could not be carried out due to the small number of RCTs included, and the source of

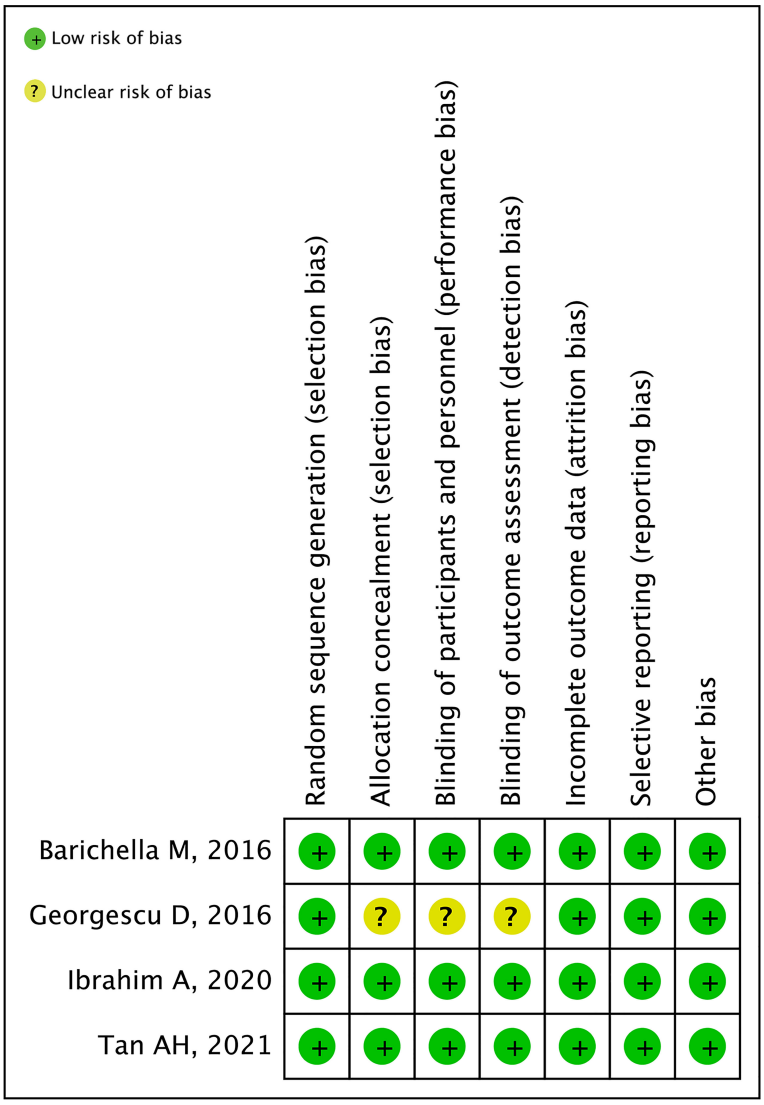

FIGURE 2
The risk of bias of studies.

heterogeneity could not be found. Different diagnostic criteria, different doses and types of probiotics taken, and the small number of included literatures may be the reasons for the high heterogeneity. High heterogeneity in the results and the small sample size of the meta-analysis resulted in a low quality of evidence. In addition, receiving probiotics or a placebo showed no significant difference in terms of alleviating abdominal pain, abdominal distention, GTT, and other gastrointestinal disorders in PD patients.

The gut-brain axis refers to the dynamic bidirectional interaction between the intestinal flora and the central nervous system. The interaction between the central nervous system and the gut mainly connects peripheral intestinal function to the emotional and cognitive brain centers through various neuro-immune-endocrine mediators (Naomi et al., 2021). An imbalance in the intestinal flora affects the occurrence and progression of neurodegenerative diseases and mental disorders, while supplementation with dietary fiber and probiotics can improve various cognitive functions (Barbosa and Vieira-Coelho, 2020; Barrio et al., 2022). Despite the popularity of probiotics as a treatment for neurodegenerative diseases in recent years, the results of studies on probiotics have been inconsistent.

The FAO/WHO defines probiotics as "living microorganisms beneficial to the health of the host when ingested in an appropriate amount" (Hill et al., 2014). A study summarized the evidence of the relationship between the intestinal microflora, cognitive function, and dementia

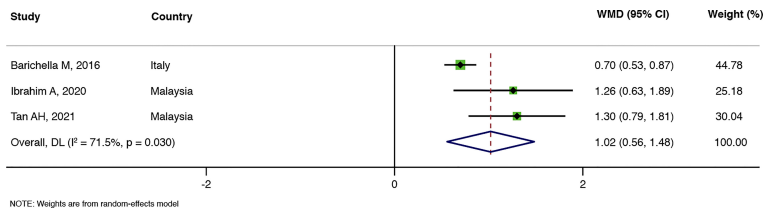

FIGURE 3
Meta-analysis of the number of bowel movements per week.

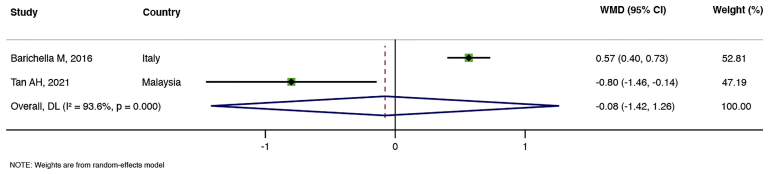

FIGURE 4
Meta-analysis of average stool consistency.

pathology in the elderly, and its conclusion supported the impact of intestinal microorganisms on cognitive function. In animal studies, prebiotics and probiotics had a positive effect on cognitive function (Neta et al., 2022; de Rijke et al., 2022), but the existing evidence is insufficient to support a clinical application (Ticinesi et al., 2018).

Gastrointestinal tract is closely related to the central nervous system, environmental pathogens may enter the central nervous system through the vagal connections in the gut, and eventually accelerate the progression of PD (Travagli et al., 2020). Constipation is a prevalen non-motor symptom in PD, its underlying mechanism and pathophysiology is complex, such as accumulation of alpha-synuclein originate from the myenteric plexus in the intestine may be one of the reasons (Fasano et al., 2015; Barrenschee et al., 2017). At the same time, the use of anti-parkinsonism drugs can also result in slow colonic transport or puborectalis dyssynergia and aggravate constipation symptoms (Stocchi and Torti, 2017). Moreover, PD patients are associated with lower short chain fatty acids (SCFAs), which have anti-inflammatory properties and are essential for gut mucosal lining repair, regulation of intestinal nervous system activity, and enhancement of gut motility (Unger et al., 2016; Aho et al., 2021). The mechanism by which probiotics improve PD constipation may be through the increase of SCFAs and mucin

TABLE 2 Summary of the outcomes.

Study	Sample size(I/C)	Outcomes	Intervention			Comparison		
			Baseline [mean (SD)]	After treatment [mean (SD)]	*P*-value	Baseline [mean (SD)]	After treatment [mean (SD)]	*P*-value
Georgescu D, 2016	20/20	Abdominal pain*	1.45 (0.51)	1.05 (0.69)	0.00432	1.55 (0.51)	0.6 (0.52)	<0.0001
		Bloating*	1.4 (0.5)	0.3 (0.47)	<0.0001	1.6 (0.5)	0.45 (0.51)	<0.0001
		Constipation*	1.35 (0.49)	1.15 (0.49)	0.2040	1.5 (0.51)	0.85 (0.67)	0.0014
Ibrahim A, 2020	27/28	GTT	125.26 (54.81)	77.32 (55.35)	<0.001	128.46 (53.68)	113.54 (61.54)	0.093
		NMSS	63.66 (35.22)	47.5 (30.07)	<0.001	71.6 (42.34)	63.5 (44.92)	0.007
		PDQ39-SI	33.1 (25.59)	26.87 (26.14)	0.013	40.1 (28.12)	36.17 (21.01)	0.341
Tan AH, 2021	34/38	Constipation severity score (0-15) †	8.4 (2.3)	5.2 (3.39)	–	7.5 (2.7)	5.9 (2.89)	–

GTT, Gut transit time; NMSS, Non motor Symptom Scale; PDQ39-SI, Parkinson's disease Questionnaire 39 summary indices.
*A scoring of the symptoms was set using a scale from 0 to 3, with 0 indicating no symptoms, 1 indicating mild symptoms, 2 indicating moderate symptoms, and 3 indicating severe symptoms.
†Based on the constipation severity questionnaire adapted from Rome IV criteria (higher scores indicate worse severity)

TABLE 3 The result of the GRADE.

Outcomes	No of Participants (studies) Follow up	Quality of the evidence (GRADE)	Relative effect (95% CI)	Anticipated absolute effects	
				Risk with Control	Risk difference (95% CI)
The number of bowel movements per week	240 (3 studies)	⊕⊕⊖⊖ **LOW**[1,2] due to inconsistency, imprecision			The mean the number of bowel movements per week in the intervention groups was 1.02 (0.56 to 1.48)
Average stool consistency	192 (2 studies)	⊕⊕⊖⊖ **LOW**[1,2] due to inconsistency, imprecision			The mean average stool consistency in the intervention groups was -0.08 (-1.42 to 1.26)

CI: Confidence interval.
GRADE Working Group grades of evidence
High quality: Further research is very unlikely to change our confidence in the estimate of effect.
Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.
Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.
Very low quality: We are very uncertain about the estimate.
[1] Serious inconsistency due to moderate heterogeneity with 50% < I2 and P value (chi-square test) < 0.10.
[2] Very serious imprecision due to the small sample size (< 400 individuals).

production in the gut thereby repairing the gut mucosal lining and enhancing gut motility (Dimidi et al., 2017; Suez et al., 2019). Whether and to what extent probiotics, while relieving constipation, also slow the progression of PD, remains to be investigated.

The authors of several systematic reviews and meta-analyses evaluating the use of probiotics for Alzheimer's disease (AD), mild cognitive impairment (MCI), and PD believe that probiotics and synbiotics supplements improve cognitive function in patients with AD, while no positive effect was seen in other biomarkers of oxidative stress or lipid profiles. Only insulin resistance could be improved in patients with AD (Krüger et al., 2021; Li et al., 2021), and dietary probiotics could improve cognitive function in MCI patients, but in another study, the effect on AD patients was limited (Zhu et al., 2021). However, studies by Leta et al. (2021) highlighted that probiotic therapy can increase glucose metabolism, reduce peripheral and central inflammatory responses (e.g., reduction of interleukin-6 (IL-6), hs-CRP, and tumor necrosis factor -α (TNF-α) in PD patients, and increase motor and non-motor function. The results of a meta-analysis by Xiang et al. (2022) suggest that probiotics can enhance the cognitive function of AD and MCI patients and improve the gastrointestinal symptoms of PD patients, for example, by relieving abdominal pain, abdominal distention, and constipation and increasing the number of bowel movements per week, with no significant effect on stool consistency. In addition, probiotics can also reduce biomarkers of inflammation and oxidative stress. The results of gastrointestinal symptoms were similar to those of this study.

Based on the gut-brain axis connection, patients with neurological diseases have a much higher risk of intestinal dysfunction. How to effectively manage intestinal disorders has always been a focus of the medical field, while intestinal management in the past was empirical with very little research basis (Coggrave et al., 2006). In the updated Cochrane Systematic Review, interventions to address constipation remain limited, and the quality of the evidence is very low due to differences in intervention and control approaches. At present, common methods to improve constipation mainly include catharsis, abdominal massage, electrical stimulation, an anticholinesterase anticholinergic drug combination (neostigmine glycopyrrolate), anal flushing, oral carbonated water, and lifestyle modification (Coggrave et al., 2014). Probiotic therapy has been well documented in patients with simple functional constipation, with multistrain probiotics significantly reducing GTT, increasing stool removal frequency, and improving stool consistency. Therefore, probiotics are considered safe and natural remedies for the relief of functional constipation in adults (Zhang et al., 2020).

However, The International Parkinson and Movement Disorder Society (MDS) Evidence-Based Medicine (EBM) Committee only recommended Macrogol, Lubiprostone, and Probiotics/Prebiotic fibers as three medicines/foods used to treat PD-related constipation (Hatano et al., 2022). Chronic constipation is the earliest symptom of PD prodrome and one of the universal NMS in PD (Kalia and Lang, 2015). This systematic review focused on the evaluation of probiotics for the treatment of constipation in PD patients. Probiotics increased the number of weekly defecations in PD patients compared with a placebo, but with high heterogeneity and a low quality of evidence. Our results also suggest that probiotics have no significant beneficial effect on stool consistency, GTT, NMSS, and PDQ39-SI, and there is no clear evidence that probiotics have a significant effect on additional symptoms of

gastrointestinal dysfunction, such as abdominal pain and bloating. In terms of safety, clinical studies have reported adverse reactions such as abdominal pain and abdominal distention in the probiotic group. In fact, gastrointestinal dysfunction in PD patients, as one of the common NMS, may include clinical symptoms such as abdominal pain and abdominal distension. Whether adverse reactions are caused by drugs requires careful consideration. In addition, the included studies also reported two adverse reactions of lethargy and dizziness in the probiotics group. Although the authors indicated that the symptoms disappeared after the cessation of probiotics and no serious adverse reactions occurred, the safety of probiotics still needs to be verified in subsequent studies. Meanwhile, clinical studies are limited, the overall sample size is small, and whether probiotics synthesized by different strains have different effects on intestinal function still needs further research. A large sample size and high-quality clinical evidence are still the top priority to clarify the efficacy and safety of probiotics in the treatment of PD-related constipation.

Strengths and limitations

The problem of constipation in PD patients is closely related to the progression of their own disease. With the gradual emergence of probiotics, it is clearly important to determine the effectiveness and safety of probiotics on PD-related constipation for clinical selection. Here, we must point out that the systematic review has some limitations. First, the number of clinical studies was limited. We only included four RCTs involving 287 participants and only conducted a meta-analysis on the two main results, the number of bowel movements per week and thin stool consistency, which had high heterogeneity. Secondly, due to the small number of RCTs included in this study, the composition, dosage, and frequency of probiotics were different, so we did not conduct publication bias assessment and subgroup analysis. In addition, the sensitivity analysis of the number of bowel movements per week was stable, but the results should be interpreted carefully. However, the advantages of our study are that (1) This is the first meta-analysis and systematic review of existing evidence to clarify the efficacy and safety of probiotics for constipation in PD patients, which will provide favorable evidence for evidence-based medicine. (2) The reviewers discussed the limitations of the included studies and proposed specific suggestions for future studies to provide reliable research results.

Conclusion

Although the evidence in this systematic review only supports the notion that probiotics have a significant effect on increasing the number of bowel movements per week in patients with PD constipation, probiotics have potential value in the treatment of PD-related constipation based on the overall results of existing clinical observational studies, animal research reviews, and clinical experience.

Author contributions

FZ and SY: study conception and design, data analysis, interpretation, and manuscript writing. All authors: final approval of manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1038928/full#supplementary-material

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Phenotypic convergence of bacterial adaption to sub-lethal antibiotic treatment

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Microorganisms can adapt quickly to changes in their environment, leading to various phenotypes. The dynamic for phenotypic plasticity caused by environmental variations has not yet been fully investigated. In this study, we analyzed the time-series of phenotypic changes in *Staphylococcus* cells during adaptive process to antibiotics stresses using flow cytometry and Raman spectroscopy. The nine antibiotics with four different mode of actions were treated in bacterial cells at a sub-lethal concentration to give adaptable stress. Although the growth rate initially varied depending on the type of antibiotic, most samples reached the maximum growth comparable to the control through the short-term adaptation after 24 h. The phenotypic diversity, which showed remarkable changes depending on antibiotic treatment, converged identical to the control over time. In addition, the phenotype with cellular biomolecules converted into a bacterial cell that enhance tolerance to antibiotic stress with increases in cytochrome and lipid. Our findings demonstrated that the convergence into the phenotypes that enhance antibiotic tolerance in a short period when treated with sub-lethal concentrations, and highlight the feasibility of phenotypic approaches in the advanced antibiotic treatment.

KEYWORDS

antibiotics, phenotype, bacterial adaptation, raman spectroscopy, flow cytometry

Introduction

Microorganisms can adapt rapidly to changes in their environment, resulting in a variety of phenotypes. Such emergence of phenotypic diversity is considered a result of gene expression changes in response to the environment. However, it remains unclear whether the phenotype continues to change in response to the adaptable abiotic stress or whether the phenotype converges at a certain time point (Jervis, 2006). Transcriptome and metabolome analysis by Horinouchi and his colleagues suggested that phenotypic diversity converges during adaptive evolution of *Escherichia coli* to ethanol stress

(Horinouchi et al., 2015). Such constraint of phenotype in the adaptation process is a ubiquitous phenomenon that also appears in the evolution of plants and animals (Rosenblum et al., 2010; Xu et al., 2020). Previous studies have observed phenotypic convergences from the perspective of long-term evolution where they focused on the changes in phenotype as an outcome of genetic evolution. Since environmental changes may occur in the form of temporary disturbance, it is also necessary to pay attention on the plasticity of phenotype without genetic alteration even in a short-term.

The emergence of antibiotic resistance bacteria is one of the world's most urgent public health problems. The prudent clinical and non-clinical use of antibiotics may slow the spread and emergence of new antibiotic resistant bacteria, but the threat will remain due to the increased range of antibiotic resistance and the rapid evolution of bacteria (Bernier and Surette, 2013). Antibiotic treatment is strongly associated with frameshift mutations resulting in multidrug resistance (Pérez-Capilla et al., 2005). Antibiotics resistance has been considered to occur in antibiotics that require high therapeutic levels, but there is evidence that low-level antibiotic treatment can also lead to mutation that cause resistance (Girgis et al., 2009). Concentrations below the minimal inhibitory concentrations (MICs) of certain antibiotics are present in the human body during antibiotic therapy and these sub-lethal concentrations can also be found in many natural environments, such as sewage water and sludge, rivers, and lakes (Hermsen et al., 2012; Andersson and Hughes, 2014). Such sub-lethal levels of antibiotics act as a stress inducer, allowing rapid bacterial adaptation through a variety of biological responses by the bacteria, such as gene expression as well as phenotypic changes without genetic alternation (Andersson and Hughes, 2014). Although studies on the convergence of phenotypes through genotype-phenotype mapping through re-sequencing have been performed to understand long term adaptive evolution, qualitative understanding of the phenotypic adaptation remains unclear (Andersson and Hughes, 2014; Suzuki et al., 2014). To address this issue, a physiological approach at high resolution is needed to monitor phenotypic changes at sub-lethal concentrations of antibiotic treatment.

Optic-based technologies, including Raman spectroscopy and flow cytometry, can offer new insight into cellular phenotype at the single cell level and further our understanding about how microorganisms respond to abiotic stress (Garcia-Timermans et al., 2020; Hatzenpichler et al., 2020). Raman spectroscopy has provided useful biomolecule information as fingerprints of single cells, including lipid, carbohydrate, nucleic acid, and protein composition (Garcia-Timermans et al., 2020; Hong et al., 2021). Many studies have recently been conducted to identify antibiotic-resistant and sensitive strains using Raman spectroscopy and predict antibiotic mechanisms. These allows to develop the rapid assay of the minimal inhibitory concentration of antibiotics and determine the multi-resistant clinical strains in hospital (Kirchhoff et al., 2018; Barzan et al., 2020; Nakar et al., 2022). Furthermore, the extent of cellular damage and resulting Raman spectral changes have been found to play an important role in distinguishing antibiotic exposure characteristics. This indicates that Raman spectroscopy has the potential for rapid bacterial identification and antibiotic susceptibility profiling (Schröder et al., 2015), and may also be suitable for investigating phenotypic changes caused by antibiotic treatment (Germond et al., 2018).

In addition, flow cytometry (FCM) allows high throughput analysis of phenotypic heterogeneity at the single cell level. Furthermore, FCM is a simple and sensitive technique and is not only provides information on the total cell count of suspended cells, including cell viability status, but also provides information regarding phenotype (Müller and Nebe-Von-Caron, 2010). These physiological approaches are suitable for investigating phenotypic changes during rapid adaptation of microorganisms in response to abiotic stresses.

In this study, we analyzed phenotypic changes of the opportunistic pathogen *Staphylococcus aureus* under antibiotic treatment with sub-lethal concentrations. I treated *S. aureus* with nine different antibiotics at concentrations at the MIC and monitored the time-series of their phenotypic changes using Raman spectroscopy and FCM. Then, we compared the phenotypic diversity and biomolecular information of single cells to analyze the phenotype convergence in bacterial adaptation after short-term cultivation under antibiotic stress conditions.

Material and methods

Bacteria growth conditions and antibiotic treatments

S. aureus NCTC 8325-4 was grown in tryptic soy broth (TSB; BD, NJ, USA) at 37°C with shaking (120 rpm). Amoxicillin (AMX; Sigma-Aldrich, MO, USA), vancomycin (VAN; Sigma-Aldrich, MO, USA), gentamycin (GEN; Sigma-Aldrich, MO, USA), chloramphenicol (CHL; TCI, Tokyo, Japan), tetracycline (TET; Sigma-Aldrich, MO, USA), ciprofloxacin (CIP; Sigma-Aldrich, MO, USA), norfloxacin (NOR; Sigma-Aldrich, MO, USA), and rifampicin (RIF; TCI, Tokyo, Japan) were used as the antibiotics in this study. *S. aureus* was treated with these antibiotics at MICs. The modes of action of these antibiotics include inhibition of cell wall synthesis, protein synthesis, and DNA gyrase or RNA synthesis inhibition. The detailed information of the antibiotics and their MICs are summarized in Table 1. Optical density (OD) measurements of bacterial cultures were performed in a SPARK 10M microplate reader (TECAN, Männedorf, Switzerland) in a 96-well plate (SPL, Seoul, South Korea), with 200 µL per well. Absorbance was

TABLE 1 Information of antibiotics and MIC of *Staphylococcus aureus* NCTC 8325-4.

Antibiotics	Class	Mode of action	Target	MIC (mg/L)	Reference
Amoxicillin (AMX)	Beta-lactam	Cell wall synthesis (CW)	Penicillin binding proteins (PBP)	0.06	(Stubbings et al., 2004)
Vancomycin (VAN)	Glycopeptides and glycolipopeptides	Cell wall synthesis (CW)	D-Ala-D-Ala moiety of NAM/NAG peptide subunits	1.25	(Hiramatsu et al., 1997)
Gentamycin (GEN)	Aminoglycosides	Protein synthesis Inhibitors (PS)	30S ribosomal protein S12 16S rRNA	0.25	(Stubbings et al., 2004)
Chloramphenicol (CHL)	Chloramphenicol	Protein synthesis Inhibitors (PS)	50S subunit of the ribosome and prevents the formation of peptide bonds	2	(Stubbings et al., 2004)
Tetracycline (TET)	Tetracycline	Protein synthesis Inhibitors (PS)	binding of aminoacyl-tRNA 30S ribosomal protein	0.06	(Stubbings et al., 2004)
Ciprofloxacin (CIP)	Quinolone	DNA gyrase (NA)	topoisomerase II and topoisomerase IV	0.25	(Schmidt et al., 2010)
Norfloxacin (NOR)	Quinolone	DNA gyrase (NA)	topoisomerase II and topoisomerase IV	1.25	(Kaatz et al., 2005)
Rifampicin (RIF)	Ansamycin	RNA synthesis inhibitors (NA)	DNA-dependent RNA polymerase	0.016	(Stubbings et al., 2004)

MIC, minimum inhibitory concentration.

measured at a wavelength of 600 nm and the average value was obtained for triplicate measurements. I sampled the bacterial cultures at 6, 12, and 24 h for measuring single-cell phenotypes using Raman spectroscopy and FCM.

Raman spectroscopy

The samples were prepared for Raman spectroscopy with the following steps: 1 mL of the bacterial culture sample was centrifuged at 15,928 ×g for 5 min at 4°C and the supernatant was discarded. The pellets were washed with the cold Phosphate-Buffered Saline (PBS) buffer (pH 7.4). The pellets were re-suspended in formaldehyde (4%, Sigma-Aldrich, MO, USA) and fixed for 2 h at 4°C in the dark. The samples were washed twice with the cold PBS buffer. Then, a 2 µL drop was spotted on an aluminum coated slide (LiMedlon Gmbh, Mannheim, Germany) and dried in air at room temperature. Single cell Raman spectra (SCRS) were obtained with a Confocal Raman Imaging System (Nanobase, Seoul, South Korea) equipped with a 532 nm DPSS laser (Leading tech, Shanghai, China), microscope body with MPLFLN 40X objective (Olympus, Tokyo, Japan), spectrometer (Nanobase, Seoul, South Korea), and charge-coupled device (Atik cameras, Bawburgh, UK). The spectrometer grating was 1,800 gr/mm. The laser power used on the sample was 2 mW. The total acquisition time for each spectrum was 25 s. Twenty single-cell Raman spectra were collected from the samples treated with antibiotics. The SCRS were analyzed in the 400–1800 cm^{-1} region, and processed using R software version 3.6.2 using the Chemospec package (Hanson, 2015). The function "baselineSpectra" with method "als" was used to correct the spectrum baseline using 2nd derivative constrained weighted regression. Normalization of the spectra was performed using the function "normSpectra" with the method "Totlnt". The "Totlnt" is a method of normalizing each y-value by dividing it by the sum of the y-values of a given spectrum.

Flow cytometry

1 mL of the cell suspension was collected by centrifugation (15,928 ×g, 5 min, 4°C). The pellet was resuspended in 1 mL of sterile PBS buffer (4°C). Each sample (100 µL) was diluted with 900 µL of sterile PBS buffer in a 1.5 mL amber colored microcentrifuge tube. Bacterial viability was assessed using the LIVE/DEAD^{TM} BacLight^{TM} bacterial viability kit (Invitrogen, MA, USA) as per the manufacturer's instructions. 987 µL of sterile PBS buffer was added to 10 µL of the prepared bacteria sample. These samples were immediately stained with 3 µL of a mixture of SYTO 9 (5 mM final concentration) and PI (30 mM final concentration) and incubated at room temperature for 15 min protected from light. All samples were measured using a CytoFLEX flow cytometer (Beckman coulter, CA, USA). The acquisition settings were as follows: intensity threshold for FSC channel, 10,000; gain value for FSC, 150; SSC, 60; FITC, 50; and PE, 20. Events were collected for 1 min at a flow rate of 10 µL min^{-1}. The green (fluorescein isothiocyanate [FITC], 525/40 nm) and red (phycoerythrin [PE], 585/42 nm) fluorescence and forward (FSC) and side (SSC) optical scattering were recorded. Two biological repeats and three technical repeats were performed for each sample. The bacterial cells were analyzed for FSC, SSC, and fluorescein isothiocyanate (FITC) channel to

observe changes in cell size, complexity, and nucleic acid composition following antibiotic treatment, respectively. The distinct bacterial populations (live and dead cells) were gated based on the different viability stages in density plots using the R package "alphahull". The events in the gated as a live cell were extracted and converted into four variables (FSC, SSC, FITC, and PE) using a standardized range of 0 to 1. The FSC and FITC variables reflected cell sizes and nucleic acid content, respectively, and there were plotted on a scatter plot. The scatter plots were divided into 10 x 10 bins. The events in each bin were counted and used to calculate the phenotypic diversity. The Shannon's diversity index (alpha diversity) and beta diversity were calculated using the R package "vegan".

Statistical analysis

Discriminant analysis of principal components (DAPC) was used for clustering SCRS according to antibiotic treatments. DAPC was performed by using the "dapc" function in the R package "adegenet", which first transforms the data using PCA and then performs a discriminant analysis on the retained principal component. The SCRS was converted using PCA by the "dudi.pca" function in the R package "ade4". The clusters were then identified using discriminant analysis (DA) using the "lda" function in the R package "MASS". (Jombart et al., 2010). The distances between clusters in the DAPC plots were expressed by computing the average distance between each centroid of the control and each antibiotic-treated group. A two-sample t-test was performed based on the results of a normality test with the Shapiro-Wilk test to calculate the significance of the difference in the ratio of live/dead cells between the control group and the antibiotic-treated sample. In addition, the significance of the difference in SCRS between the control group and the antibiotic-treated sample was calculated. Raman peaks with a significance of p-value < 0.01 were selected and visualized with a heatmap. The significance of the difference in the phenotypic alpha diversity (D_a) between control and antibiotic treated samples was investigated using a two sample t-test. The phenotypic beta diversity (D_b) was visualized using a Non-metric Multi-dimensional Scaling (NMDS) plot with Bray–Curtis dissimilarity distances using the R package "vegan". The analysis of similarity (ANOSIM) was completed using the function "anosim" within the R package "vegan" to calculate the significant difference between clusters in NMDS plot.

Results and discussion

Bacterial viability under antibiotic treatment

To elucidate the effect of antibiotics treatment at MIC on the growth of *S. aureus*, we first quantified the time-series of cell viability using flow cytometry. The viability varied slightly depending on individual antibiotics rather than the mode of action of antibiotics. The dead cell ratios ranged from 6.2% to 38.1% (mean: 16.8%) at 6 h (Figure 1A). When treated with CIP and RIF, the dead cell ratio was 38.1% and 31.6%, respectively, indicating a more marked effect on cell viability compared to other antibiotics. Cell viability was mostly recovered after 24 h under all antibiotic treatment, where the proportion of dead cells decreased on average from 16.8% to 8.7%. Although the dead cells treated with CIP and AMX decreased slightly compared to 6 h, it was observed that the number of dead cells in other antibiotic-treated samples decreased significantly after 24 h of antibiotic-treated (t-test, $P<0.01$). Since the effect of antibiotics is closely related to the fitness of bacteria, the effect of antibiotics may have been reduced in highly nutritious media such as TSB (Steixner et al., 2021). Taken together, these results suggest that the treatment of antibiotics at MIC results in the adaptation of *S. aureus* within 24 h.

Analysis of growth curves as a simple phenotypic test using live cell counts revealed that the initial growth rates at 6 h and 12 h were clearly different depending on the mode of action of antibiotics (Figure 1B). In general, antibiotics related to cell wall synthesis inhibition had little growth inhibition compared to the control, whereas significant inhibition of growth rate was seen for antibiotics with protein synthesis and nucleic acid inhibitory activity. Although the cells treated with TET, CIP, and NOR did not recover to their growth maximum even after 24 h, the cells treated with TET and NOR showed better recovery than CIP. These results are consistent with the data obtained by measuring the OD_{600} (Figure 1C). Despite cultures being grown in separate culture and treated with different antibiotics, both the cell viability and growth curves showed similar results among these samples.

Convergence of phenotypic diversity

To further characterize the phenotypic changes under antibiotic treatment, we compared the phenotypic diversity between control and treated samples using FCM. In the initial stages (6 h) of antibiotic exposure, a unique difference in the alpha diversity (D_a) was observed according to the modes of action (t-test, $P< 0.001$, Figure 2A). The D_a increased when antibiotics which inhibit cell wall synthesis and DNA gyrase were used, but decreased when antibiotics which inhibit protein and RNA synthesis were used. It was confirmed that significant changes in cell size and nucleic acid content were detected depending on the mechanism of action of the antibiotic even after short-term treatment at the sub-lethal concentration (Walberg et al., 1997). The difference in phenotypic diversity according to the modes of action of these antibiotics could be a result of differential gene expression of specific resistance mechanism related genes (Hanson, 2015; Knudsen et al., 2016).

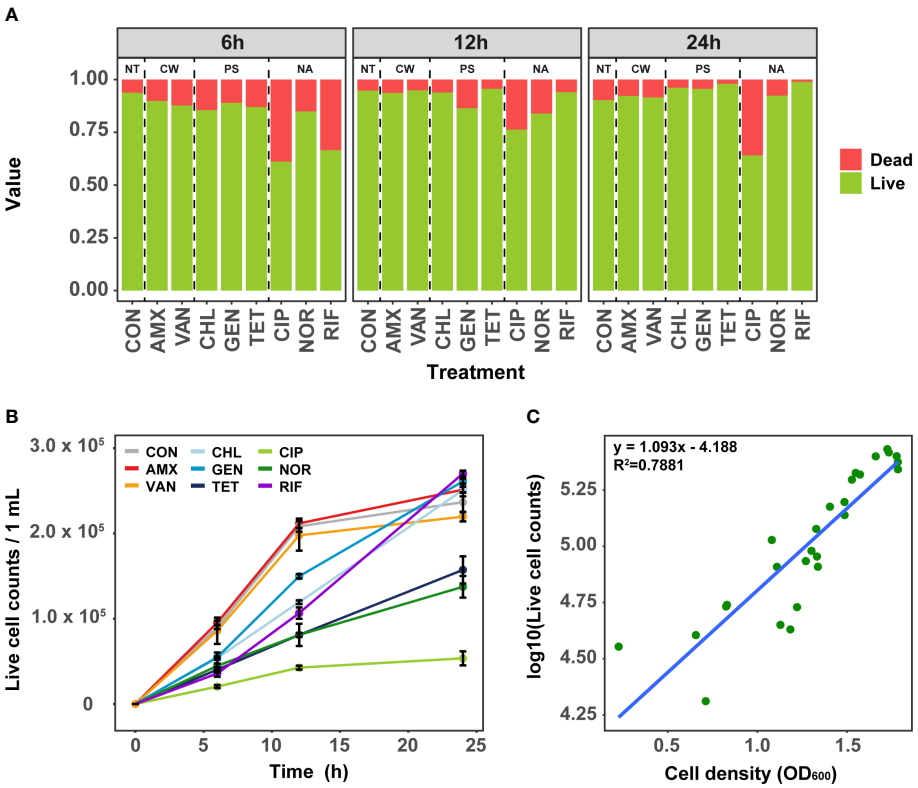

FIGURE 1
Analysis of cell viability in antibiotic-treated samples using Flow cytometry. **(A)** The change of ratio of live and dead cell over time. **(B)** Change of the live cell counts in samples treated with antibiotics over time. Error bars represent the mean ± SD. **(C)** The correlation between OD_{600} and bacterial live cell counts for each samples. NT, antibiotic non-treatment; CON, control.

Regardless of antibiotic class, the difference in the D_a decreased gradually over time compared to the control, and there was no significant difference at 24 h except for samples treated with CIP, which inhibits the bacterial cell growth and samples treated with CIP showed the lowest viability (Figure 1A). These results indicated that even though the different mode of actions influenced the D_a differently, the D_a converged into almost identical adapted states with similar orbits of phenotypes

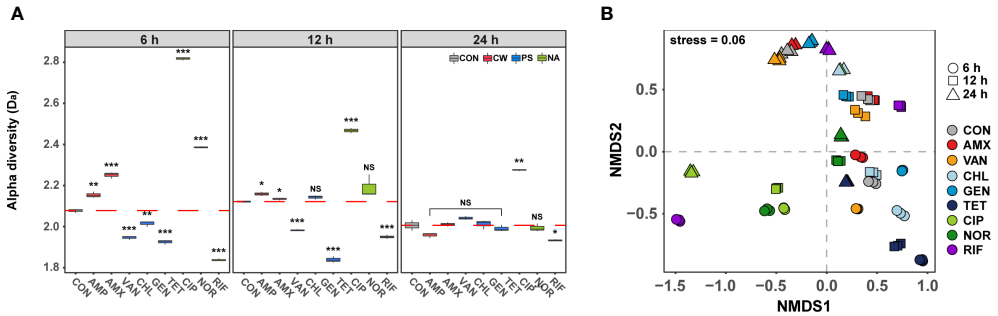

FIGURE 2
Differences in phenotype diversity between antibiotic treated samples over time by FSC-FITC data from flow cytometry. **(A)** Box plots depict alpha diversities (D_a) of live cell distribution by Shannon index. The red dashed line means the average of control. The difference was considered significant at a P-value (NS, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001, *t*-test). **(B)** The phenotypic beta diversity (D_b) analysis of the flow cytometry data separating samples according to the antibiotic treatment by non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distance metric.

if the concentration of antibiotics was adapted. These results are consistent with the similar genotype and phenotypes of bacteria that have shown adaptation in laboratory adaptive evolution experiments (Hanson, 2015; Jahn et al., 2017). Although the short-term cultivation did not produce sufficient genetic evolution, the temporal changes of gene expression due to the abiotic stress returns to a new steady-state level close to that of unstressed cells, leading to the congruence of phenotypes (Lopez-Maury et al., 2008). From this point of view, although the D_a of samples treated with CIP was still significantly different from the control at 24 h, considering the decreasing trend, the diversity difference from the control group is expected to disappear as the cultivation period is increased.

The phenotypic beta diversity (D_b) also demonstrated similar changes as seen for the D_a in the phenotype of subpopulations of *S. aureus* treated with the nine antibiotics (Figure 2B). Analyses of similarities (ANOSIM) were used to test for significant differences between samples according to the modes of action of antibiotics over time. The D_b of antibiotic-treated samples showed significant distance from the control after 6 h (ANOSIM, $R = 0.81$, $P < 0.01$), but most of those formed a tight cluster with the control after 24 h (ANOSIM, $R = 0.36$, $P < 0.01$). The phenotypic changes over time were found to exhibit high similarity under different antibiotics, although samples showed non-monotonic phenotypic changes even after 24 h. The observed phenotypic convergence to similar orbits clearly suggested that there is a phenotypic direction in which their fitness increases in microbial adaptation to antibiotics.

Convergence of single cell phenotypes

Raman spectroscopy can measure the single cell biomolecule composition (Hong et al., 2021), and thus determine the response of the phenotypic change to exposure to antibiotics (Athamneh et al., 2014). The SCRS results formed a tight cluster for each individual antibiotic at 6 h, suggesting that Raman spectroscopy is suitable for high-resolution analysis of phenotypes adapted to antibiotic exposure. These SCRS results were remarkably farther apart from the control as observed in a DA-PC plot (Figure 3A). These results were consistent with the fact that phenotypic diversity differed significantly from the control in the early stage of antibiotic treatment. Raman spectroscopy is a powerful approach for comparing the phenotypic difference of bacteria according to the treatment of various antibiotics with high resolution, and comparative research was also conducted at sublethal concentration of antibiotic treatment (López-Díez et al., 2005; Athamneh et al., 2014). Since ceftazidime, a cell wall inhibitor, inhibits the expression of *acrAB* encoding a multi-drug efflux pump, bacteria treated with ceftazidime were found to have decreased Raman peaks related to lipids and proteins (Athamneh et al., 2014; Peng et al., 2019). And CIP, a DNA gyrase inhibitor, inhibits the expression of *gyrAB* encoding DNA gyrase, thereby reducing nucleic acid related peaks in the bacterial SCRS (Athamneh et al., 2014; Germond et al., 2018). As is consistent with these previous studies, bacterial cellular biochemical biomolecules were clearly distinguished according to the defense mechanism against antibiotics, which indicates the

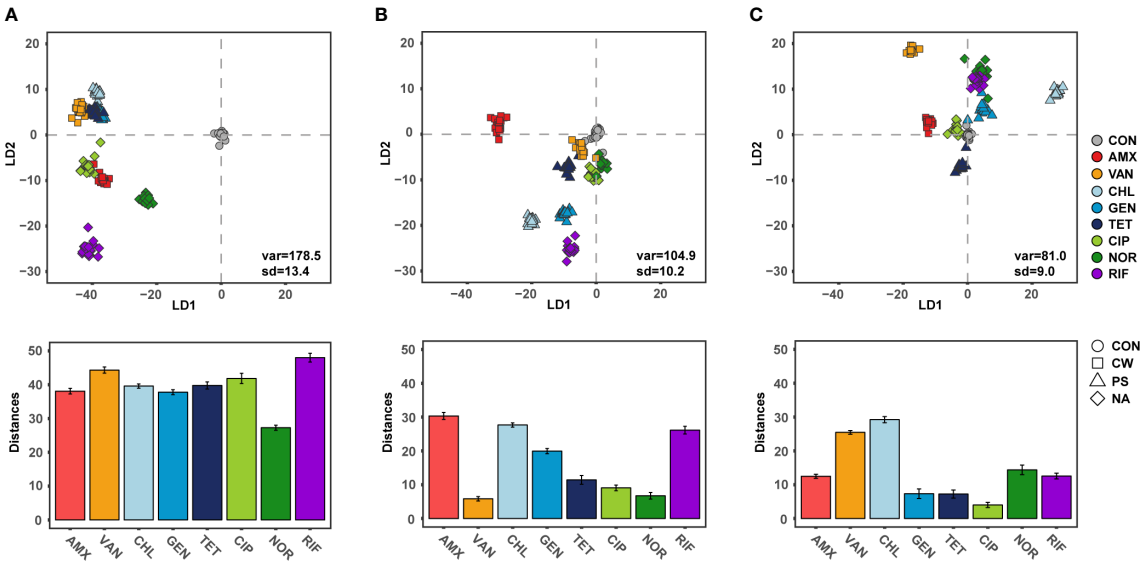

FIGURE 3
Discriminant of *S.aureus* according to the antibiotic treatments and Distance from centroids of control group to centroids of each groups. **(A)** 6 h treatment, **(B)** 12 h treatment, **(C)** 24 h treatment.

effect of antibiotics on the bacteria continues for 6 h after treatment, even at sub-lethal concentrations.

The distance between SCRS clusters with antibiotic treated cells, which differed from the control cells, gradually decreased as the cultivation periods increased (Figures 3B, C). The SCRS analysis showed a mean distance of 39.6 (± 5.8) for cultures after 6 h of cultivation and this was significantly decreased to 17.1 (± 9.5) and 14.1 (± 8.4) after12 h and 24 h, respectively (t-test, $P < 0.05$). These results indicate that cellular biochemical biomolecules progress in similar orbits through bacterial adaptation in response to antibiotic exposure within short-term periods, regardless of the type of antibiotic. In laboratory evolution experiments, the phenomenon of phenotypes or genotypes convergence in the process of adaptive evolution against abiotic stress for long generation is not a rare phenomenon (Horinouchi et al., 2015; Imamovic et al., 2018). While phenotypic convergence for long generation is decided by genetic evolutionary constraints in the adaptive evolution dynamics, other factors may be more important in the convergence of phenotypes in the short term by gene expression rather than genetic evolution. One explanation of these results is that there is selective pressure from exposure to antibiotics which gives rise to a sub-population of identical cells. In these results, phenotypic diversity or deviation from normal cellular biomolecules increased during the earlier stages of exposure to antibiotics which act as cell wall synthesis, DNA gyrase or RNA synthesis inhibitors, and then decreased as cultivation periods increased. Among single cells with various phenotypes due to subtle differences in gene expression, the phenotype can converge where changes offering a fitness benefit will be dominant. Divergence in fitness is a key strategy to drug resistance in bacteria (Melnyk et al., 2015). Although there is still a lack of research on divergence in fitness at the single cell level, these results suggested that phenotypic divergence contributes quickly to adaptation from antibiotics. Another possibility is that the role of antibiotics at sub-lethal concentrations changed from antibacterial agents to signaling molecule *via* bacterial memory, leading to a similar phenotype. Antibiotics at sublethal concentrations can serve as signaling molecules and cause alterations in biofilm formation, quorum sensing, and gene expression (Andersson and Hughes, 2014). When treated with protein synthesis inhibitor antibiotics, all three samples were initially clustered similarly with low phenotypic diversity. The convergence of phenotypes, while maintaining the similarity of phenotypes between samples along with the recovery of phenotypic diversity, could result in the reduced antibacterial effect of the antibiotics. Several studies demonstrated that in the case of similar constraints, due to a limited number of adaptations which are possible in response to a specific selection pressure, selection for similar traits seems to have led to similar responses (Losos, 2011; Germond et al., 2018).

In addition, signals detected when exposed to specific stimuli can trigger short-term memory or learning behaviors in which bacteria form memories for the stimuli and respond more rapidly or more broadly to the signals on subsequent exposures (Germond et al., 2018; Bhagirath et al., 2019). These responses are regulated through the expression intensity of genes without changes to the DNA sequence, and in genetic expression, these bacterial memories might contribute to the adaptation of antibiotics and should be considered in future studies.

Phenotypic changes in cellular biomolecules

Heat maps of the Raman intensities showed noticeable intensity changes for the major nucleic acids, proteins, lipids, and cytochrome Raman bands (Figure 4). The mean value of the spectral intensity of each antibiotic treated sample was colored by subtracting the average value of the spectral intensity of each time control group. Assignment of the Raman peaks was notated in Table 2. Regardless of the class of antibiotics, the antibiotic treatments in *S. aureus* at 6 h significantly changed the Raman intensities of cellular biomolecules including lipids, proteins, nucleic acids, cytochromes, and carbohydrates (t-test, P-value < 0.01). It is worth noting that the peaks attributing to the nucleic acids located at 1,338 cm^{-1} increased remarkably, whereas most Raman intensities were reduced by antibiotic stress after 6 h cultivation. Most of the nucleic acid related peaks were found to decrease with growth inhibition by antibiotic treatments. An exceptional increase at 1,338 cm^{-1} during the Raman peak associated with DNA can be explained by DNA damage (Sofińska et al., 2020). The SCRS under antibiotic treatments reveals a tendency for bacterial cells to decrease protein and lipid content when exposed to abiotic stress and this is enough to hinder growth (Teng et al., 2016; Germond et al., 2018).

In addition, large variability related to nucleic acid content could also be identified in the SCRS. Antibiotics resulted in changes to the various nucleic acid processes in bacterial cells, such as DNA fragmentation and gene expression for defense and repair (Moritz et al., 2010). Using Raman spectroscopy, we were able to distinguish changes in cellular biomolecules according to the modes of action of antibiotics, and confirmed common phenotypic changes that occur due to sublethal antibiotic treatment.

As the cultivation periods reached 24 h, most of the differences seen in the Raman intensities of biomolecules between control and treated samples were no longer observed. In particular, the Raman intensities associated with nucleic acids, proteins, and carbohydrates reverted back to levels similar to those of the control after 24 h cultivation, while cytochromes (1,123 cm^{-1}) and lipids (1,127 cm^{-1}) increased significantly in most of antibiotic treated samples except for AMX and CIP (t-test, P-value < 0.01). In particular, the bacterial cell membrane is essential in bacteria and serves as a significant barrier to preventing harmful chemicals from entering the cell

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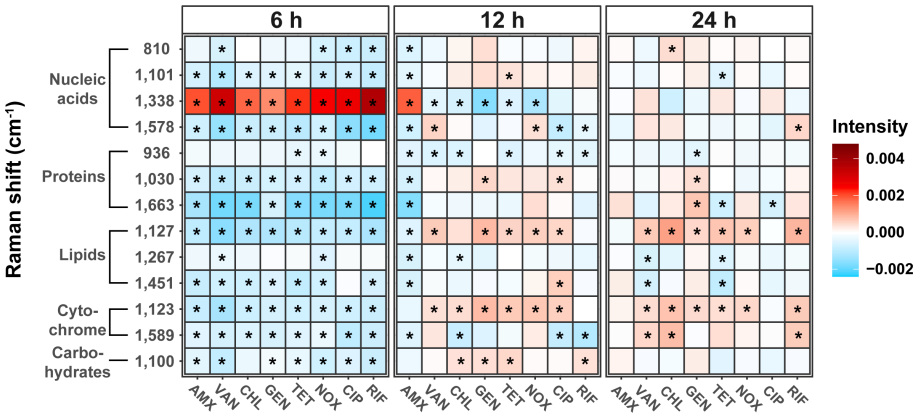

FIGURE 4
Changes in bacterial biochemical composition with different mechanisms of antibiotics and treatment time using Raman spectrum. Heat maps were generated to show the peak intensity trends over time in antibiotic treatment. Each antibiotic-treated sample's mean spectral intensity was colored by subtracting the average spectral intensity of each time control group. The difference from control was considered significant in P-value (*P < 0.01, *t*-test).

(Willdigg and Helmann, 2021). Bacteria induce a cell envelope stress response to resist antibiotics, which are regulatory pathways that detects threats and cause protective reactions, including modifications of the lipopolysaccharides, lipoteichoic acids, peptidoglycan (Macdermott-Opeskin et al., 2022). Changes in membrane composition has been previously reported as one of the most important adaptive mechanisms in bacteria when exposed to toxic compounds such as antibiotics (Ma et al., 2021). Antibiotic resistance can be induced by this lipid-mediated mechanism, and the increase of the lipid peak in clusters at 24 h is presumed to be caused by an increase in fatty acids in the cell membrane of the bacteria or a change in the composition of membrane lipids. Cytochromes are known to be required for biofilm formation and extracellular matrix production that can resist antibiotics and other external stressors. Bacteria can cope with oxidative or antibiotic-induced stress by increasing respiration through cytochromes and also enhance susceptibility to antibiotics by decreasing outer membrane permeability. (Beebout et al., 2021). Thus, the increase in lipids and cytochromes in most SCRS can be considered a phenotypic indicator that the bacterial tolerance to antibiotic-induced stress is improved. Although it is difficult to determine that *Staphylococcus* has evolved adaptation to antibiotic resistance over generations in this study, it is worth considering the possibility that repeated adaptation through antibiotic treatment at such sub-lethal concentrations will

TABLE 2 The Raman frequency of antibiotic-treated samples with significantly different intensities compared to antibiotic non-treated samples.

Raman shift (cm^{-1})	Assignment	Group	Reference
810	C-O-P-O-C in RNA backbone	Nucleic acids	(Schuster et al., 2000)
936	C–O–C linkage, C–C stretch., α-helix	Carbohydrate, protein	(De Gelder et al., 2007)
1,030	δ(CH) bend., Tyr, Phe	Aromatic compound	(Schuster et al., 2000)
1,100	mainly -C-C-(skeletal), C-O, def(C-O-H)	Carbohydrates	(Schuster et al., 2000)
1,101	Symmetric phosphate stretch. (DNA)	Nucleic acid	(Teng et al., 2016)
1,123	CH Phe	Cytochrome	(Notingher and Hench, 2006)
1,127	=C-C= (unsaturated fatty acids in lipids)	Lipids	(Huang et al., 2010)
1,209	$C–C_6H_5$ stretch., Phe, Trp	Protein	(Notingher and Hench, 2006)
1,267	Lipids	Lipids	(Van Manen et al., 2005)
1,338	Adenine, guanine, tryrosine, tryptophan	Nucleic acids	(Strola et al., 2014)
1,451	$C-H_2$ def, Lipids	Lipids	(Notingher and Hench, 2006)
1,578	Adenine, cytosine, guanine	Nucleic acid	(Maquelin et al., 2002)
1,589	ν(C–C)	Cyt c.	(Cui et al., 2018)
1,665	Amide I	Amide I	(Maquelin et al., 2002)

change the antibiotic susceptibility. Further research on how the repeated exposure to antibiotic leads to changes in antibiotic resistance and phenotype needs to be considered.

Conclusion

In this study, analyzed phenotypic changes of *S. aureus* cells that occurred during adaptation in response to antibiotic treatment at sub-lethal concentration using FCM and Raman spectroscopy. The similar phenotypic changes among independent cultures treated with different antibiotic classes, with different modes of action, indicate the existence of an adaptive direction in phenotype in short-term cultivation periods. These results provide a rapid transition to phenotypes in which fitness increases in the adaptation process, even if the initial response strategies to abiotic stress is different. This study also highlight the feasibility of phenotypic studies in long term antibiotic treatment or when investigating new antibiotic classes.

Data availability statement

The data presented in the study are deposited in the FlowRepository, accession number FR-FCM-Z56A.

Author contributions

GN and TK contributed to the conception of this study and designed the experiments. GN, ES, and J-KH conducted the experiments. GN, JK, and SB carried out the data analysis. GN and ES contributed to the interpretation of data. GN wrote the manuscript. TK supervised the project and provided critical comments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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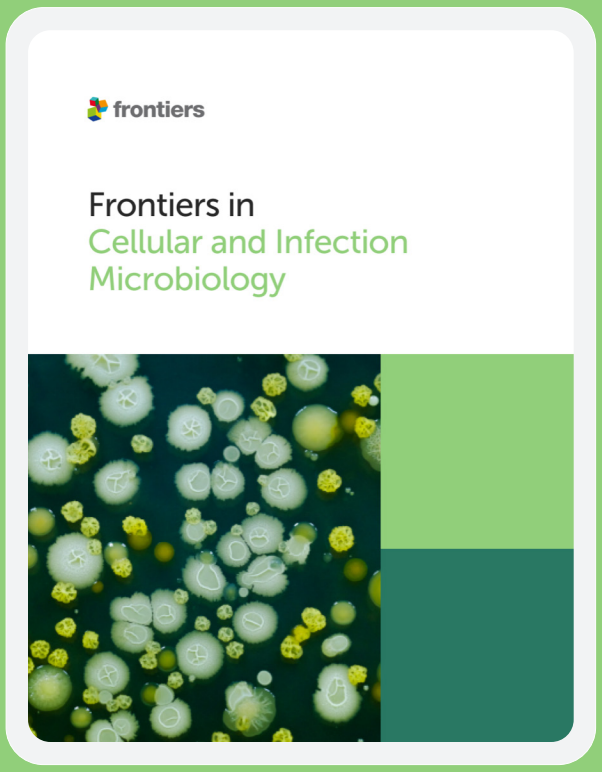

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