Anti-obesity effects of Chenpi: an artificial gastrointestinal system study

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Summary

The gut microbiota plays a significant role in human health; however, the complex relationship between gut microbial communities and host health is still to be thoroughly studied and understood. Microbes in the distal gut contribute to host health through the biosynthesis of vitamins and essential amino acids and the generation of important metabolic by-products from dietary components that are left undigested by the small intestine. Aged citrus peel (Chenpi) is used in traditional Chinese medicine to lower cholesterol, promote weight loss and treat various gastrointestinal symptoms. This study investigated how the microbial community changes during treatment with Chenpi using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Two preparations of Chenpi extract were tested: Chenpi suspended in oil only and Chenpi in a viscoelastic emulsion. Short-chain fatty acids (SCFAs) were measured during treatment to monitor changes in the microbial community of the colon presenting a decrease in production for acetic, propionic and butyric acid (ANOVA \(P < 0.001\)) during the 15 days of treatment. 16S rRNA sequencing of microbial samples showed a clear difference between the two treatments at the different sampling times (ANOSIM \(P < 0.003\); ADOSIM \(P < 0.002\) \([R^2 = 69\%]\)). Beta diversity analysis by PcoA showed differences between the two Chenpi formulations for treatment day 6. These differences were no longer detectable as soon as the Chenpi treatment was stopped, showing a reversible effect of Chenpi on the human microbiome. 16S rRNA sequencing of microbial samples from the descending colon showed an increase in *Firmicutes* for the treatment with the viscoelastic emulsion. At the genus level, *Roseburia, Blautia, Subdoligranulum* and *Eubacterium* increased in numbers during the viscoelastic emulsion treatment. This study sheds light on the anti-obesity effect of a polymethoxyflavone (PMFs)-enriched Chenpi extract and creates a foundation for the identification of ‘obesity-prevention’ biomarkers in the gut microbiota.

Introduction

Obesity is a growing problem that now affects one-third of the adult population and has become a major global health challenge (Engin, 2017). It is caused by a disparity between energy intake and energy expenditure, although genetic and environmental factors also influence this balance and modify metabolism (Bogardus, 2009). Obesity is associated with an excess of (white) adipose tissue mass, insulin resistance, liver fat accumulation, a chronic pro-inflammatory state and major health issues that include a plethora of comorbidities, such as type 2 diabetes, cardiovascular diseases, hypertension, stroke and certain types of cancer (Pi-Sunyer, 2009). Plant extracts may represent an additional option in support of weight management strategies, although the mechanisms of action can be very different. Chenpi has long been used in traditional Chinese medicine to treat many symptoms and has shown...
Chenpi’s beneficial effects on the gut microbiota

Chenpi is commonly used for promoting weight loss (Scott et al., 2013a, 2013b). It can have a particularly marked impact on the gut environment, affecting factors such as gut transit time and pH. Chenpi is made from the dry peel of the fruit of *Citrus reticulata* Blanco after an ageing process in a temperature and moisture-controlled environment. Polymethoxyflavones (PMFs), in particular nobelitin and tangeritin, are found exclusively in citrus fruits and primarily within the citrus peel. 5-OH PMFs are analogs of PMFs with a hydroxyl group replacement on C5. Interestingly, the concentration of 5-OH PMFs increases with ageing time, which coincides with a general observation that longer aged Chenpi seems to have better medicinal efficacy. In a recent study, it has been demonstrated that the administration of a Chenpi extract in high-fat diet-induced obese mice attenuated adiposity and hepatic steatosis. These results provide strong evidence that a 5-OH PMF-enriched Chenpi extract could be effective in preventing the progression of metabolic syndromes (Scott et al., 2013a, 2013b).

In this study, we used the Simulator of Human Intestinal Microbial Ecosystem (SHIME, ProDigest, Gent, Belgium) to explore the possible effects of Chenpi on the gut microbiota. The SHIME system consists of five reactors simulating the different regions of the GIT. The first two reactors simulate the stomach and small intestines. Peristaltic pumps allow for the addition of defined amounts of SHIME nutritional medium, pancreatic enzymes and bile in the small intestines. Retention time and the pH of the different vessels are carefully controlled to resemble *in vivo* conditions within each region of the gastrointestinal tract. The human fecal microbiota-inoculated SHIME system houses stable microbial communities within the three-colon compartments, which differ in compositions and functions, and to some degree, are representative of the different colon regions.

In this study, we used the TWIN-SHIME platform, consisting of two SHIME systems running in parallel. This model is suitable for the simultaneous comparison of two variables and has many advantages that allow for the investigation of potential mechanisms of action for both products and ingredients. With the SHIME system, one can work with volumes that closely match *in vivo* conditions, and it is possible to collect multiple samples from different regions of the colon at various timepoints. Furthermore, the modular set-up, which characterizes the SHIME, makes it possible to explore inter-individual variability in microbiome behaviour in response to specific treatments.

Despite the physiological relevance, *in vivo* experimental set-ups are inherently associated with some drawbacks. (Van den Abbeele et al., 2010; Ericsson and Franklin, 2015) First, apart from faecal analyses over time, most *in vivo* data are derived from endpoint measurements, thereby limiting the dynamic monitoring of the gut microbiota. Second, troublesome sampling of different gut regions makes it difficult to locate the effects of a given treatment. For mechanistic reasons, a third drawback of *in vivo* approaches is the inability to focus solely on gut microbial activity because there is always a host involved. Furthermore, for *in vitro* studies, the microbiota needs to be gut region specific, representative of the *in vivo* situation, and maintain a high level of diversity. The strength of an *in vitro* model thus relies on a good characterization of the associated microbiota.

This pilot study shows for the first time the effects of Chenpi on human GIT microbiota through the analysis of metabolites and 16S rRNA and also identifies potential biomarkers for the control of obesity.

**Results**

**Chenpi treatment**

The gut microbiome plays a significant role in human health, but the nature of the relationship between microbial ecology and host health continues to be a matter of controversy (Clemente et al., 2012). In this pilot study, we investigated the effect of aged citrus peel on the gut microbiome of a healthy subject. The ascending, transverse and descending regions of the colon were inoculated with human faecal samples from a healthy donor. Two different conditions were studied using a TWIN-SHIME system: SHIME 1 (S1) was treated with Chenpi oil, and SHIME 2 (S2) was treated with Chenpi in a viscoelastic emulsion (Fig. 1). HPLC analysis of the Chenpi oil and viscoelastic emulsion identified nobelitin, tangeretin and 5-OH nobiletin as the three dominant PMFs, and they accounted for about 40% by mass of the Chenpi extract. Since the distal part of the colon, represented in the SHIME model by the descending colon (DC), is the most interesting section in regard to microbiome changes, we studied how the microbial community in the distal colon is affected by the two treatments (Marchesi et al., 2016). We analysed the microbial biodiversity profiles obtained using a 16S rRNA genes bTEFAP (bacterial tag-encoded FLX amplicon pyrosequencing) assay, looking at the metabolically active fraction (rRNA-containing) of the microbiota (Dowd et al., 2008).

**SCFA production**

The TWIN-SHIME was monitored throughout the duration of the experiment by measuring SCFA concentrations three times a week. Samples 1, 3, 5 and 7 were taken on day 0, treatment day 6, treatment day 15 and washout day 14, respectively. These four samples were taken from SHIME 1 (S1) for treatment with the Chenpi oil formulation. Samples 2, 4, 6 and 8 were taken on day
0, treatment day 6, treatment day 15 and washout day 14, respectively. These four samples were taken SHIME 2 (S2) for treatment with the Chenpi in a viscoelastic emulsion. Sample 9 served as the control sample. Time-points for 16S rRNA analysis were chosen based on the results of SCFA measurements. Figure 2 shows the concentrations of SCFAs in the three different regions during treatment and washout. A decrease in the total amount of SCFAs was observed during treatment, with the lowest values detected at the end of the treatment (TrDay15) in the three different regions, respectively, for both treatments (S1 and S2). ANOVA analysis revealed statistically relevant differences between treatment 1 and treatment 2 at day 6 (TrDay6) and washout day 7 (WoDay7) in the ascending, transverse and descending colon, and also for washout day 14 (WoDay14) in the transverse colon only. At the end of the washout period, the total amount of SCFAs was restored to pretreatment levels.

Although to a different extent, a similar trend was found for the three different SCFAs analysed: acetic, propionic and butyric acid. Treatment 2 presented a more substantial effect in the synthesis of the SCFAs, with the lowest value found at TrDay6 for all three SCFAs in the three regions of the colon. A cumulative effect of the Chenpi treatments was also detected. It was enough to reach the undetectable values of SCFAs by the end of the treatment (TrDay15) in all regions analysed for propionic acid and butyric acid for both formulations. Acetic acid was the most resilient, and after the drop in concentration at TrDay15, the concentration was restored to pretreatment levels after just 7 days of washout (WoDay7). Propionic acid levels were highly sensitive to treatment, especially with Chenpi in a viscoelastic emulsion, as demonstrated by the remarkable difference in concentrations recovered between the two treatments that persisted during the washout period. The Chenpi extract completely inhibited the synthesis of butyric acid in both formulations and in all analysed regions. Butyric acid concentrations returned to being measurable only at the end of the washout period.

Changes in faecal bacterial alpha and beta diversity

The diversity of the gut microbiota monitored by 16S rRNA genes bTEFAP during the experiment was
influenced by the Chenpi extract in both formulations: SHIME 1 Chenpi oil and SHIME 2 Chenpi in a viscoelastic emulsion (Fig. 3).

Within each sample, alpha diversity indices (number of observed OTUs, the Chao1 estimator of species richness and the Shannon entropy and rarefaction curves) indicate a consistent decrease of microbial biodiversity at TrDay6 and TrDay15 with both formulations, whereas the number of observed species and chao1 at WoDay14 were comparable with values identified on the starting day (day 0) for both Chenpi oil and Chenpi in a viscoelastic emulsion (Table S1, Fig. S1).

Sample 9 represents an example of the ‘healthy’ faecal gut microbial obtained by a pool of 31 different healthy donors, provided by OpenBiome for reference purposes. Although both the rarefaction curve and alpha indices indicate that this representative pool is the most microbially diverse, the values obtained in the TWIN-SHIME, with both formulations, showed comparable results for both the starting and ending point of the experiment (Day 0 and WoDay14, respectively) for sample 9 (Table S1, Fig. S1). This confirms the adequate performance of the SHIME system, with no contamination during the experiment; the microbial communities were able to restore to the original profiles following treatment.

Beta diversity analyses showed a clear partitioning of microbial biodiversity according to time (day of treatment) for both Chenpi formulations (SHIME 1 and SHIME 2), as described with principal coordinate analysis (PcoA) using both an unweighted (qualitative) and weighted (quantitative) phylogenetic UniFrac distance

Fig. 2. Total SCFA measurement in the different regions: (A) Ascending colon (AC); (B) transverse colon (TC); c) descending colon (DC), during starting day (Day0), treatment day 6 (TrDay6), treatment day 15 (TrDay15), washout day 7 (WoDay7) and washout day 14 (WoDay14). ANOVA significance difference ($P < 0.001$) is shown by asterisks (*). S1 (1) Chenpi oil and S2 (2) Chenpi in a viscoelastic emulsion.
The relative abundance of the OTUs identified at the different sampling points was extremely important in describing the biodiversity as demonstrated by the value of PC1 found in a weighted (80.56%) vs. unweighted (29.69%) UniFrac distance matrix.

Samples 3 and 5 and samples 4 and 6 correspond to the Chenpi oil formulation (S1) and Chenpi in viscoelastic emulsion (S2) treatments, respectively (Fig. 3). Principal coordinate analysis (PcoA) showed a clear partitioning of these samples that clustered apart from both the starting time of the treatment (day 0) and the washout periods (WoDay14). These data demonstrate that both Chenpi formulations had a strong effect on microbial community composition.

Despite both ANOSIM and ADONIS analysis showing that the treatment was statistically ineffective as a factor (P > 0.856 and P > 0.923 for unweighted and weighted UniFrac distance matrix, respectively) (Table 1), PcoA showed a non-negligible difference between samples 3 and 4 corresponding to the two different Chenpi formulations at TrDay6. After 9 days of treatment, these differences were gradually reduced until they were no longer detectable by 16s rRNA analysis, as described by the convergence of samples 5 and 6 corresponding to TrDay15. At the other sampling times (day 0, WoDay14 and sample 9 out), the microbial populations in the two SHIME systems showed similar patterns.

Both ANOSIM and ADONIS showed a clear effect of time on the clustering of samples, with both unweighted and weighted UniFrac (P < 0.003) (Table 1), the samples corresponding to the treatment period (TrDay6 and TrDay15) clustered apart compared to the other sampling times. It is noteworthy that after 15 days of Chenpi administration and 14 days of washout period, the simulated gut microbiota was able to return to a pretreatment state, with samples 7 and 8 (WoDay14) closely clustered with S1 and S2 treatments (TrDay 0) and the control sample 9, demonstrating a reversible effect of both Chenpi formulations.

Changes in the relative abundance of faecal bacterial

In total, more than 405 OTUs were identified during this experiment, presenting a number of OTUs comparable to previous investigations (Tap et al., 2009; Deering et al., 2019), although only eleven OTUs were present at more than 1% of the total microbial biodiversity. After the stabilization period, the gut microbiome at the phylum level was distributed as follows: four major phyla were detected, Actinobacteria (0.1%), Bacteroidetes (75%), Firmicutes (17%) and Proteobacteria (6%). The two parallel units of SHIME showed overlapping results at day 0 before starting treatment with the different formulations (Fig. 4).

Table 1. ANOSIM and ADONIS values obtained using both an unweighted and weighted UniFrac distance matrix. Condition: treatment and time were analysed separately following the QIIME compare_categories.py.

| Condition | UniFrac | ANOSIM P | ANOSIM $R^2$ | ADONIS P | ADONIS $R^2$ |
|-----------|---------|----------|---------------|----------|---------------|
| Treatment | Unweighted | 0.881 | 0.856 ($R^2 = 21\%$) | 0.879 | 0.923 ($R^2 = 11\%$) |
|           | Weighted | 0.001 | 0.003 ($R^2 = 69\%$) | 0.003 | 0.002 ($R^2 = 69\%$) |
| Time      | Unweighted | 0.881 | 0.856 ($R^2 = 21\%$) | 0.879 | 0.923 ($R^2 = 11\%$) |
|           | Weighted | 0.001 | 0.003 ($R^2 = 69\%$) | 0.003 | 0.002 ($R^2 = 69\%$) |
After 6 days of treatment (TrDay6), an increase in *Firmicutes* and a decrease in *Bacteroidetes* were shown for both SHIME 1 and SHIME 2. Interestingly, SHIME 2 had a slightly different microbial population, with an increase in *Actinobacteria* and *Gammaproteobacteria* followed by a less pronounced bloom of *Firmicutes*.

According to the SIMPER test, a single OTU from the genus *Bacteroides* (18.7 Av. Dissim) accounts for more than 58% of sequences detected at day 0 for both SHIME1 and SHIME2. After virtually disappearing during both treatments at TrDay6 and TrDay15, this OTU accounted for nearly 70% of sequences detected by the 14th day of washout period for both treatments (WoDay14). The same OTU was also the most abundant in the control sample (43.4%).

An OTU belonging to *Gammaproteobacteria*, genus *Escherichia-Shigella*, presented a SIMPER value of (12.26 Av. Dissim). It was found to increase drastically by TrDay15 of the Chenpi treatments. This OTU was found primarily during treatment with the viscoelastic emulsion and accounted for 69.55% of collected sequences on TrDay15 for sample 6 (treatment with Chenpi in viscoelastic emulsion/S2). This OTU presented 98% similarity towards clones previously found in both the animal and human GIT microbiota (Li et al., 2012; Muhammad et al., 2017).

Within the *Firmicutes* phylum, an OTU classified as *Roseburia* spp. belonging to the *Clostridiales* family showed a remarkable increase during treatment with the Chenpi (3.814 Av. Dissim), reaching 33.81% of reads after 6 days of treatment in SHIME1. *Roseburia* spp. has been shown to be a good candidate as a health biomarker (Grootaert et al., 2009).

Six other OTUs within the *Firmicutes* phylum: *Blautia*, *Lachnocrastidium*, *Subdoligranulum*, *Eubacterium*, *Erysipelotrichaceae* and *Phascolarctobacterium* presented SIMPER values higher than 1 (Av. Dissim), although to varying extents. The majority of these OTUs showed an increase in concentration during treatment at TrDay6, in both SHIME (1-2) experiments (Table S1).

The SIMPER test evidenced three other OTUs that contribute significantly to microbial biodiversity with (Av. Dissim>1): two belonging to *Bacteroidetes* class, *Alistipes*, family *Rikenellaceae*, *Parabacteroides* family *Porphyromonadaceaeplus* and one *Actinobacteria* belonging to the *Bifidobacterium* genus. Interestingly, while the two OTUs related to the *Bacteroidetes* class were found mainly in the control sample (>5.5% of reads) and were nearly undetectable during both treatments, the OTU attributed to *Bifidobacterium* increased only during treatment with the Chenpi viscoelastic emulsion/S2. This OTU presented 98% similarity towards clones previously found in both the animal and human GIT microbiota (Li et al., 2012; Muhammad et al., 2017).
Discussion

The SHIME system allowed us to study the metabolites produced in each distinct region of the colon. As with any other in vitro model, the SHIME suffers from the absence of a physiological environment. Moreover, water and metabolite absorption are not routinely simulated in the colonic compartment. Movement and mixing along the GIT is performed by pumping and stirring and not by peristalsis. Despite these drawbacks, the possibility to study the gut microbiome in an in vitro model allows for the study of different regions of the colon without the ‘interference’ of the host background.

Both Chenpi dissolved in oil and in the viscoelastic emulsion showed the ability to change the composition of the gut microbiota. These data were confirmed by monitoring SCFA production and by evaluating changes in the relative abundance of key players in the gut microbiota (Fig. 3).

It remains extremely difficult to link the presence or absence of specific microbial taxa with the SCFA profiles produced by the same microbial communities. This is in part because the ‘active presence’ of specific bacterial clades obtained by 16s rRNA profiling cannot be linked directly to the synthesis of specific SCFAs. An integrated metagenomic and metatranscriptomic or metaproteomic investigation directly targeting the genomic pathways involved in SCFA production is necessary to fully elucidate an active gut microbiome’s metabolic profile. Nevertheless, in this study, a good correspondence between changes in the SCFA profile and microbial biodiversity was evidenced, with a decrease in SCFA quantity, especially propionic and butyric acid, during treatment in the same samples where a pronounced decrease in microbial biodiversity was noticed (Fig. 2). Despite many attempts having been made to describe the composition of a possible bacterial ‘phylogenetic core’ in the healthy adult faecal microbiota, important inter-individual biodiversity was described. Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria constitute the core set of microbial phyla prevalent in the human gastrointestinal tract (Tap et al., 2009).

The Bacteroidetes group has previously been described as a regular member of the human gut microbiome (Tap et al., 2009; Li et al., 2012; Scott et al., 2013b), although it is known that in obese subjects, the ratio of Bacteroides/Firmicutes changes (Andoh et al., 2016). Our study demonstrates the sensitivity of Bacteroidetes to Chenpi treatment, as this bacterial genus was nearly undetectable during both treatments (Qian et al., 2021). Noteworthy genera belonging to Bacteroidetes recovered immediately after the interruption of Chenpi treatment, demonstrating the high resilience of the gut microbiota to this treatment.

Within the Firmicutes group, 16S analysis found the most representative genera were as follows: Roseburia, Blautia, Subdoligranulum and Eubacterium. Roseburia spp. can be considered as a marker for health, previous papers showed an improvement in obese subjects after faecal transplantation (Li et al., 2012; Tamanai-Shacoori et al., 2017; Ma and Chen, 2019; Zhang et al., 2019).

Among the Firmicutes, a direct involvement in the obesity disorder has been documented for Blautia, Lachnoclostridium, Subdoligranulum and Eubacterium (Andoh et al., 2016; Wang et al., 2020). Blautia is a group of bacteria containing various acetate and butyrate producers (Bui et al., 2019). More importantly, Blautia spp. were also reported to be enriched by metformin during the amelioration of obesity and insulin resistance in rats (Cao et al., 2016).

Despite different studies on the correlation of microbial biodiversity with obesity or other metabolic diseases, there are no clear data showing the contribution that individual microbial groups can have on the formation and maintenance of a state of disorder or a return to optimal health conditions (Rosario et al., 2018; Abenavoli et al., 2019). It is also important to note the limitations and inherent biases that can be associated with 16s rRNA analysis, which may come from the PCR and sequencing-preparation steps, and can also be caused by varying PCR amplification frequencies and the use of incomplete reference databases during sequence analysis (Poretsky et al., 2014).

Many of the bacterial groups that appear to benefit from Chenpi treatment have been described as important short-chain fatty acid producers (Rosario et al., 2018) and could therefore be linked to a healthier status of the host (Abenavoli et al., 2019). An interesting ‘secondary effect’ was highlighted by the two different Chenpi formulations, with some bacterial groups presenting remarkable differences between the two treatments. Two Firmicutes-related OTUs (Roseburia and Subdoligranulum) were detected mainly in TrDay 6 with Chenpi in oil suspension (33% and 23% of reads, respectively), whereas the Actinobacteria Blifidobacterium reached 10% of reads at the same sampling time with the Chenpi in the viscoelastic emulsion. Few data are available on the bioavailability of PMFs extracted from Chenpi, especially depending on the extraction methodology and carrier used to make these compounds bioavailable in the aqueous matrix. Our data demonstrate that the carrier used for this experiment, oil versus viscoelastic emulsion, can influence the response of the microbiome, likely changing the bioavailability of the different compounds present in the Chenpi extract. The observed
The effect of Chenpi as a weight loss-promoting agent is already known, but its effect on the human gut microbiota has not been investigated. Even with the limitations associated with an artificial human GIT simulator, as well as the inability to investigate the host response to the treatment, it appears that the intake of Chenpi can have an influence on the gut microbiota of healthy subjects. In this experiment, Chenpi dietary introduction shifts the composition of the gut microbiota towards a healthier profile, which indicates potential protection from developing an obesity disorder. Further studies on obese subjects will be conducted to confirm the importance of aged citrus peel as a dietary intervention and its effect on human health. Further studies will need to add Chenpi to a more varied diet to look at the effect of a more realistic diet composition.

This pilot study lays the groundwork for future experiments in which the gut microbiome will be studied in vivo with obese subjects. Further replicates of this experiment will be necessary to confirm our data. In future studies, the effect of Chenpi over time in faecal samples coming from obese donors will be analysed directly, and changes to the gut community will be recorded both at taxonomic and functional levels.

Experimental procedures

Faecal samples

Faecal samples were purchased from OpenBiome (Cambridge, MA, USA). Faecal Microbiota Preparation for Research (FMP-R) Packs are aliquots of stool material from healthy human donors. The FMP-R Pack provides microbial communities from healthy human donors with accompanying molecular and clinical characterization data. It includes 25 1 ml preparations of concentrated faecal microbiota isolated from one or more qualified OpenBiome donors.

Chenpi preparation and fingerprint analysis

Commercial aged citrus peel was purchased from Xinhui, China. A continuous phase transition extraction method was used to extract PMFs and 5-OH PMFs from Chenpi, provided from a local market. The extraction was conducted at a pressure of 0.5 MPa at 50°C for 60 min with n-butane as a solvent. The obtained extract was analysed by HPLC to determine the compositional profile of the Chenpi extracts (Guo et al., 2016). The concentration of Chenpi oil used was 0.6 mg ml⁻¹. A viscoelastic emulsion of Chenpi was prepared as described previously (Ting et al., 2014). Briefly, Chenpi extract and emulsifier (lecithin) were dissolved in the dispersed oil phase (100% medium chain triglyceride (MCT)) in the water bath. Once they were completely dissolved, the oil

Conclusions

These data are the result of the first experiment investigating the effect of Chenpi and its active compounds on the human gut microbiota using the SHIME system. The effect of Chenpi as a weight loss-promoting agent is
phase was homogenized with an equal volume of preheated water phase (100% double deionized water) using a high-speed homogenizer (ULTRA–TURRAX T-25 basic, IKA Works Inc., Wilmington, NC, USA) at 24,000 rpm to form a crude emulsion. The crude emulsion was then subjected to high-pressure homogenization (Emulsiflex-C6, AVESTIN Inc., Ottawa, Canada) as described in Ting et al., 2014. The final emulsions consisted of approximately 50% MCT (v/v), 2% lecithin (w/v), 50% DI water (v/v) and 10% Chenpi extract (w/v).

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME)

Three times per day, 140 ml SHIME feed and 60 ml pancreatic juice were added to the stomach and small intestine compartments, respectively. The three-colon regions were kept within a specific volume and pH range: 500 ml and pH = 5.6–5.9 for the ascending colon, 800 ml, and pH = 6.15–6.4 for the transverse, and 800 ml and 600 ml and pH = 6.6–6.9 for the descending. All the actions performed by SHIME are controlled using a program included with the SHIME software.

The SHIME feed (ProDigest, Gent, Belgium) contained arabinogalactan 1.2 g l⁻¹, pectin 2.0 g l⁻¹, xylan 0.5 g l⁻¹, starch 4.0 g l⁻¹, glucose 0.4 g l⁻¹, yeast extract 3.0 g l⁻¹, peptone 1.0 g l⁻¹, mucin 1.0 g l⁻¹ and L-cysteine·HCl 0.5 g l⁻¹. The pancreatic juice contained NahCO3 12.5 g l⁻¹ BDH (VWR analytical, Radnor, PA), bile salts 6.0 g l⁻¹ (DifcoOxgall, Franklin Lakes, NJ, USA), and pancreatin, porcin pancreas 0.9 g l⁻¹, Alfa Aesar (Tewksbury, MA, USA). All vessels were kept anaerobic by flushing them with nitrogen (N₂), and they were continuously stirred and kept at 37°C. In order to obtain identical environmental conditions for both units, a TWIN-SHIME set-up with two SHIME units in parallel was chosen (Molly et al., 1993). In this experiment, the three-colon regions of each SHIME unit of a TWIN-SHIME were inoculated with human faecal samples from a healthy donor (OpenBiome, Cambridge, MA, USA). Reactor set-up and inoculum preparation were as previously described (Nollet et al., 1997; Zhang et al., 2015). The SHIME set-up includes an initial 2-week stabilization period, which allows the microbiota to adapt to the imposed in vitro conditions and to evolve from a faecal microbial community to one representative of a specific colon region. In the next phase, there is a control period of 1 week. After the control period, there were 2 weeks of treatment with Chenpi. A washout period was performed for two weeks following the treatment. During the washout period, the SHIME was fed regularly every day without the addition of Chenpi. Samples were taken every two days for metabolite and microbiome analysis.

Short-chain fatty acid (SCFA) analysis

SCFA extraction and quantification were performed as previously described (Nollet et al., 1997). Briefly, 2 ml of liquid samples was collected and frozen at –20°C for subsequent analysis. The SCFAs were extracted from the samples using diethyl ether and identified using an Agilent 6850 gas chromatography system (Agilent, Santa Clara, CA, USA). The GC was equipped with a DB-WAX capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) and connected with a flame ionization detector (FID).

16s rRNA extraction, cDNA synthesis and sequencing

Total RNA was isolated from collected aliquots of SHIME content from the reactor simulating the different regions of the colon using the PowerSoil RNA Isolation Kit (Qia gen, Hilden, Germany) following the manufacturer’s instructions. Then, 17 μl of total RNA was treated with Baseline-ZERO™ DNase (Epicentre/Illumina, Madison, WI, USA) to remove the DNA contamination following the manufacturer’s instructions, followed by purification and concentration with the RNA Clean & Concentrator-5 columns (Zymo Research, Irvine, CA, USA). DNA-free RNA samples were used for cDNA synthesis; cDNA preparation was completed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and random primers following the manufacturer’s instructions.

16S rRNA genes bTEFAP (bacterial tag-encoded FLX amplicon pyrosequencing)

Amplification and sequencing was performed using MR DNA (www.mrdnalab.com, Shallowater, TX, USA). The 16S rRNA gene V4 variable region was amplified using the PCR primers 515F/806R (bac515F GTGCCAGCMGCCGCGGTAA and bac806R GGACTACVGGGTATCTAAT) (barcodes sequences not showed) in a single-step 30 cycle PCR using the HotStarTag Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 min, followed by 28 cycles (five cycle used on PCR products) of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, with a final elongation step at 72°C for 5 min. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt AMPure beads (Agencourt Bioscience Corporation, MA, USA). Sequencing was performed on an Ion Torrent PGM following the manufacturer’s guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). In brief, sequences were depleted of barcodes and primers, then sequences <150 bp, and sequences with ambiguous base calls and with homopolymer runs exceeding 6bp were removed.

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**Sequence data analysis**

Demultiplexed sequences were further processed using both Mothur (Schloss et al., 2009) and Quantitative Insight Into Microbial Ecology (QIIME) v1.9.1 open-source software packages (Caporaso et al., 2010) using the following workflow: demnoised sequences were analyzed by Mothur (Schloss et al., 2009) (version 1.39.5) following the standard operating procedures from the website. The sequences were screened for having no ambiguous bases and maximum 8 of homopolymer. After the dereplication process, the sequences were aligned to a 50,000–column wide SILVA-based reference database (Silva version v132) (Quast et al., 2013). Next, chimeras were identified and removed using the vsearch program (Rognes et al., 2016) following default parameters. The preprocessed sequences were clustered using the OptiClust algorithm (Westcott and Schloss, 2017) at 97% of the similarity cut-off and classified to the SILVA-based reference database (Silva version v132) using the classify.seqs command with the Naive Bayesian classifier (Wang et al., 2007) using 60 as cut-off for the confidence in bootstrap values for phylogenetics. Taxonomy data were converted in a biome table V 1.0. To feed these data in QIIME for alpha and beta diversity tests, both the biom table and seqs_rep_set.fasta were reformatted using the script provided in the Fig. S1. Samples were rarefied using single_rarefaction.py (http://qiime.org/scripts/single_rarefaction.html) at the value of 8567 sequences based on the minimum number of sequences found in the most indigent sample in the data set. For successive statistical analysis, operational taxonomic unit (OTU) sequences were used to construct a phylogeny tree using make_phylogeny.py (http://qiime.org/scripts/make_phylogeny.html) and FastTree (Price et al., 2010) with default values.

**Sequencing data statistical analysis**

Statistical data analysis was done using the QIIME pipeline version 1.9.1 (Caporaso et al., 2010). Alpha diversity was calculated using observed OTUs, Chao1, and Shannon indexes with 10 sampling repetitions at each sampling depth by alpha_diversity.py (http://qiime.org/scripts/alpha_diversity.html). Analysis of similarity (ANOSIM) and the permutational multivariate analysis of variance using distance matrices (ADONIS) (Anderson, 2001) test were used to determine the statistical differences between samples (beta diversity) following the QIIME compare_categories.py script and using weighted and unweighted phylogenetic UniFrac distance matrices. Principal coordinate analysis (PCoA) plots were generated using the QIIME beta diversity plot workflow (Caporaso et al., 2010).

PAST PAleontological Statistics V3.25 (https://palaeo-electronica.org/2001_1/past/issue1_01.htm) was used to generate the Bray–Curtis similarity matrix obtained from the rarefied OTU abundance table. The same program was used to calculate the contribution of single OTUs to the observed difference between samples (SIMPER test) (Clarke, 1993).

Differences in total SCFA measurements in the different regions of the SHIME system were calculated with one-way ANOVA analysis of variance using SigmaStat software (https://systatsoftware.com/). The relative importance of each treatment group was investigated by pairwise multiple comparison procedures (Holm–Sidak method). The overall significance level $P < 0.01$ is shown by asterisks. Error bars (standard deviations) are based on at least three replicates.

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**Conflict of interest**

None declared.

**Data availability statement**

The Ion Torrent PGM high-throughput sequencing data are available in the NCBI database Sequence Read Archive (SRA) (BioProject PRJNA624330).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Rarefraction curve calculated for each sample. Samples 1, 3, 5, 7 SHIME 1 (S1) Chenpi oil formulation. Samples 2, 4, 6, 8; SHIME 2 (S2) Chenpi in viscoelastic emulsion. Samples 1 and 2 starting day (Day0); 3 and 4 treatment day 6 (TrDay6); 5 and 6 treatment day 15 (TrDay15); 7 and 8 wash out day 14 (WoDay14); sample 9 out (control sample, pool of 31 healthy donors).

Table S1. Alpha diversity index calculated for each sample. Samples 1, 3, 5, 7 SHIME 1 (S1) Chenpi oil formulation. Samples 2, 4, 6, 8; SHIME 2 (S2) Chenpi in viscoelastic emulsion. Samples 1 and 2 starting day (Day0); 3 and 4 treatment day 6 (TrDay6); 5 and 6 treatment day 15 (TrDay15); 7 and 8 wash out day 14 (WoDay14); sample 9 out (control sample, pool of 31 healthy donors).