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Article

In Vitro Fermentation of Carrot Juice by the Human Gut Microbiota: Potential Use of It as A Novel Prebiotic Agent

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Abstract: Carrot juice and its associated beverage products are well-known healthy drinks all over the world. However, what effect carrot juice has on the human gut microbiota and how it is fermented by the intestinal microbes have not been studied. Here, using an *in vitro* model of anaerobic fermentation, we demonstrated that carrot juice could be fermented into lactate and acetate by the human gut microbiota. 16S high-throughput sequencing and bioinformatic analyses indicated that fermentation of carrot juice could significantly change the composition of the human gut microbiome. Interestingly, carrot juice remarkably increased the abundances of beneficial bacteria, including *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus mucosae* and *Bacteroides uniformis* and decreased the population of opportunistic pathogenic bacteria, such as *Enterococcus faecium* in the gut. Collectively, our study illustrates a favorable effect of carrot juice on the human gut microbiota and lays a foundation for the development of carrot juice as a novel prebiotic agent.

Keywords: carrot juice; human gut microbiota; fermentation; prebiotic; lactate; *Lactobacillus fermentum*; *Lactobacillus salivarius*; *Lactobacillus mucosae*; *Bacteroides uniformis*; *Enterococcus faecium*

1. Introduction

Carrot juice and its associated beverage products are well-known healthy drinks all over the world. Carrot juice is high in vitamins, carotenoids and dietary fibers [1, 2]. Intake of fresh carrot juice is a simple yet effective approach to increase plasma total carotenoids and reduce oxidative stress [3]. Previous studies have indicated that drinking carrot juice could increase the total antioxidant status and decreases lipid peroxidation in adults [4]. Besides, carrot juice consumption was also found to reduce the body weight and body mass index in type 2 diabetic subjects [5]. However, although the biological and nutritional activities of carrot juice have been extensively studied, what effect it has on the human gut microbiota has not been investigated.

Gut microbiota plays a crucial role in the metabolism of the dietary nutrients [6, 7]. Besides, maintaining a balanced gut microbiome contributes significantly to the health status of the host [8, 9]. Preceding animal studies indicated that dietary intake of carrot juice could modulate the gut microbiota of Wistar rats with type 2 diabetes [10]. Specifically, carrot juice significantly increased the intestinal abundances of *Christensenellaceae R-7* spp., *Oscillibacter* spp., *Ruminococcaceae UCG-013* spp., *Lachnospiraceae NK4A136* spp. and *Akkermansia* spp. in diseased rats. Carrot juice has been proposed as a potential gut microbiota modulator [10-12] and these results rationalize the use of carrot juice as a new

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functional foods ingredient for human consumption [13, 14]. However, although we have made progresses in understanding the nutritional mechanisms of carrot juice from the perspective of gut microbiota, more detailed studies are still urgently needed to fully dissect the complex interactions between carrot juice and human gut microbiota.

In the present study, we investigated what effect carrot juice has on the human gut microbiota and how it is fermented by the intestinal microbes. Using an in vitro model of anaerobic fermentation and 16S high-throughput sequencing techniques, we demonstrated that carrot juice could be fermented into lactate and acetate by the human gut microbiota. Additionally, we showed for the first time that carrot juice could increase the abundances of beneficial bacteria, including *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus mucosae* and *Bacteroides uniformis* in the gut. Altogether, our study illustrates a favorable effect of carrot juice on the human gut microbiota and lays a foundation for the development of carrot juice as a novel prebiotic agent.

2. Materials and Methods

2.1. Chemicals and Reagents

The carrot juice used in the present research was prepared and provided by Tsingtao Brewery Co., Ltd.. The carrot juice contains the following nutrients per 100 mL: protein, 0.47g; fat, 0.20g; dietary fiber, 0.50g; carbohydrate, 9.70g; energy, 180kJ; sodium, 37.90mg; folic acid, 3.33μg; *β*-carotene, 3.50mg. All other chemicals and reagents of analytical or biochemical grade were obtained from Sigma (Shanghai, China).

2.2. In Vitro Fermentation of Carrot Juice

The fresh human fecal samples were collected from eleven healthy individuals living in Qingdao. All the participants have provided a signed consent form before sample collection. The experiments regarding to the fecal sample collection from humans were approved and supported by the Ethical Committee of Ocean University of China, School of Medicine and Pharmacy (Permission No. OUC-2020-1008-01) as previously described [15]. Batch fermentations of carrot juice by the human gut microbiota were performed at 37 °C in an anaerobic chamber (AW 500SG, Electrotek Ltd., Shipley, UK). The VI medium that contains glucose at a concentration of 8 g/L were used for the anaerobic fermentation. The carrot juice was added to the VI medium at a final concentration of 50%. Each fermentation was carried out with the help of Hungate tubes and the anaerobic environment was achieved using an 80% N_2, 10% H_2 and 10% CO_2 gas mix. To start the fermentation, 20 grams of the fecal samples were dissolved in 100 milliliters of phosphate-buffered saline and 1 milliliter of the bacterial suspension was quickly inoculated into 9 milliliters of the VI media (G group) and VI media plus 50% carrot juice (H group) at the anaerobic chamber. The batch fermentation was terminated after 48 hours and the resulted media were collected for subsequent analysis.

2.3. 16S High-Throughput Sequencing and SCFAs' Analysis

16S high-throughput sequencing was applied to analyze the gut microbiota after fermentation. Briefly, the bacterial cells were harvested from 6 milliliters of the media by centrifuging at 12,000× g for 3 minutes. The metagenomic DNA of the gut bacterial cells was extracted and purified using a Qiagen QIAamp DNA Stool Kit (Hamburg, Germany). The V3–V4 regions of the 16S rDNA were specifically amplified using 338F and 806R, two widely used universal primers for bacterial genes. The obtained V3-V4 amplicons of the 16S gene were sequenced on the Illumina PE300 (San Diego, CA, USA) platform from Shanghai Majorbio Biopharm Biotechnology Co., Ltd. (Shanghai, China). For the analysis of the short-chain fatty acids (SCFAs) in the fermentation media, a standard high-performance liquid chromatography (HPLC) method was applied. The HPLC system (Agilent 1260, Santa Clara, CA, USA) was equipped with an UV detector (210 nm) and an Aminex

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HPX-87H ion-exclusion column (Bio-Rad, Hercules, CA, USA) were used as previously described [15, 16].

2.4. Bioinformatic and Statistical Analyses

The α-diversity (Observed species, Chao1 index, Ace index, Shannon index and Simpson index), β-diversity (Venn diagram, PCA and NMDS analysis) and compositional analyses of the human gut microbiota during fermentation were performed with the help of the Majorbio Cloud platform (https://cloud.majorbio.com). Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to compare the gut microbiota profiles between different groups. The threshold of the linear discriminant analysis score was set at a default value of 2.0. Metabolic functions of the human gut microbiota were analyzed based on the 16S sequencing data using PICRUSt and Tax4Fun as previously described [16]. Data are expressed as mean ± SEM. Statistical analysis of SCFAs was performed using Student t-test (GraphPad, San Diego, CA, USA). Spearman's correlation analysis was applied to investigate the associations between changes of the human gut microbiota and productions of the SCFAs. The results in the present study were considered statistically significant at $p < 0.05$; * $p < 0.05$ and ** $p < 0.01$.

3. Results and Discussions

3.1. Carrot Juice Changed the Overall Structure of the Human Gut Microbiota

Previous animal studies indicated that dietary intake of carrot juice could modulate the gut microbiota of Wistar rats [10]. However, what effect carrot juice has on the human gut microbiota and how it is fermented by the intestinal microbes have not been studied. Here, using an *in vitro* anaerobic fermentation model, we investigated the effect of carrot juice on the human gut microbiota.

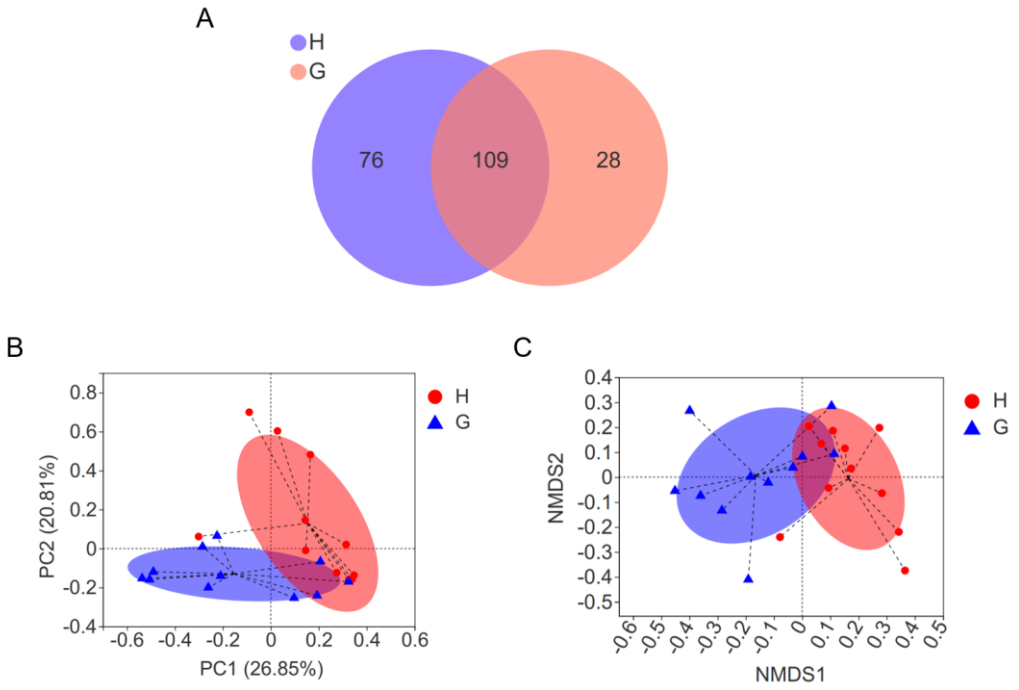

Figure 1. The β−diversity analysis of the human gut microbiota at the OTU level. Venn diagram analysis (A). PCA score plot analysis (B). NMDS score plot analysis (C).

The α-diversity analysis of the 16S sequencing data suggested that carrot juice had no effect on the Observed species, Chao1 index, Ace index, Shannon index and Simpson index of the gut microbiota (Table S1). However, despite that, carrot juice did change the

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structure of the human gut microbiota as indicated by β-diversity analysis (Figure 1). Venn diagram showed that 76 and 28 OTUs were separately identified in the carrot juice-treated group (H) and the glucose control group (G) (Figure 1A). Additionally, there was a clear separation of the two different groups of gut microbiota in both PCA and NMDS score plot analyses (Figure 1B and 1C). Altogether, our study extends previous animal results and demonstrates that carrot juice could change the structure of the human gut microbiota.

3.2. Carrot Juice Modulated the Composition of the Human Gut Microbiota at Different Taxonomic Levels and Increased the Abundances of Lactobacillus spp. and Bacteroides uniformis

Given that carrot juice could change the structure of the gut microbiota, we next sought to investigate what effect it had on the composition of the human gut microbiota at different taxonomic levels. The human gut microbiota in the present study was dominated by Actinobacteriota, Firmicutes, Proteobacteria, Bacteroidota and Fusobacteriota at the phylum level and *Bifidobacterium* spp., *Lactobacillus* spp., *Enterococcus* spp., *Lactococcus* spp., *Pediococcus* spp., *Streptococcus* spp. and *Escherichia-Shigella* spp. at the genus level (Figure 2). As revealed by the Heatmap analysis, carrot juice significantly changed the configuration of the human gut microbiota at both the phylum and genus levels (Figure 2).

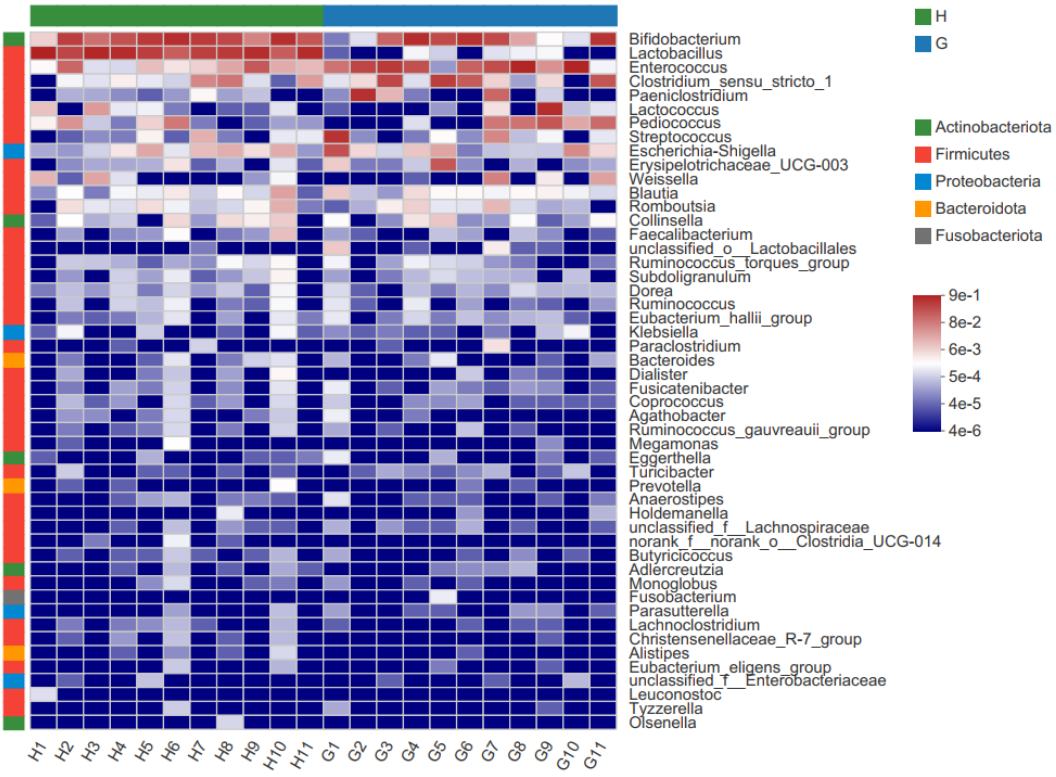

Figure 2. Heatmap analysis of the human gut microbiota at the phylum and genus levels.

LEfSe analysis was applied to identify the key bacteria that were modulated by carrot juice supplementation. Interestingly, carrot juice remarkably increased the abundances of beneficial bacteria, including *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus mucosae* and *Bacteroides uniformis* and decreased the population of opportunistic pathogenic bacteria, such as *Enterococcus faecium* in the gut (Figure 3). Lactic acid-producing bacteria including *Lactobacillus fermentum*, *Lactobacillus salivarius* and *Lactobacillus mucosae* are widely used probiotics that could confer health benefits to the host [17-19]. *Bacteroides uniformis* is a commensal bacterium in the human gut [20, 21]. Accumulating evidence indicates that *Bacteroides uniformis* could be used as a next-generation probiotic and

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previous studies have demonstrated a therapeutic effect of *Bacteroides uniformis* on high-fat diet-induced gut dysbiosis, metabolic and immune dysfunction [20, 21]. *Enterococcus faecium* is an opportunistic pathogenic bacterium commonly found in the human intestine [22]. Besides, *Enterococcus faecium* has been observed to cause opportunistic infections in severely ill patients [23].

Our results indicated that carrot juice could increase the abundances of probiotic bacteria, including *Lactobacillus* spp. and *Bacteroides uniformis* and decreased the population of opportunistic pathogenic bacteria, such as *Enterococcus faecium* in the gut. Collectively, our study illustrates a favorable effect of carrot juice on the human gut microbiota and lays a foundation for the development of carrot juice as a novel prebiotic agent.

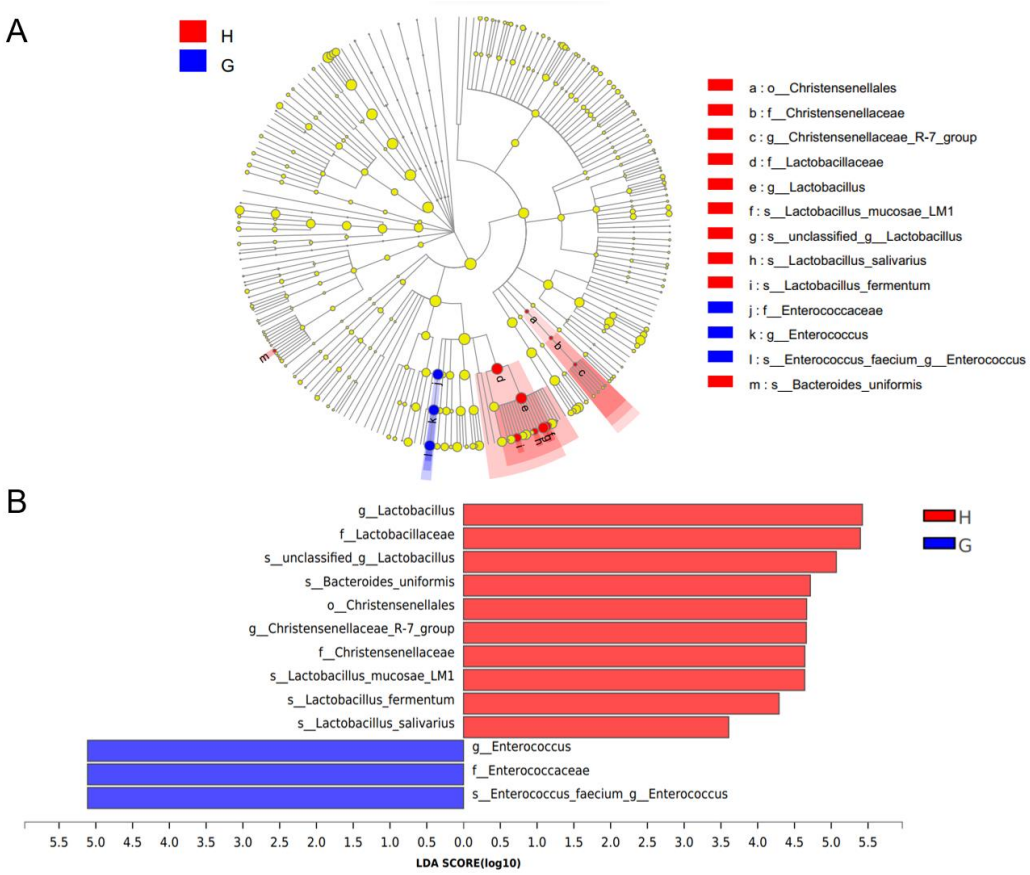

Figure 3. LEfSe analysis of the human gut microbiota in carrot juice-treated group (H) and the glucose control group (G). Only bacterial taxa with an LDA score of above 2.0 were listed.

3.3. Carrot Juice Changed the Metabolic Functions of the Human Gut Microbiota and Increased the Productions of Acetate and Lactate

In light of the fact that carrot juice modulated the composition of the human gut microbiota, we next wondered whether it could also change the metabolic functions of the gut microbiome. Interestingly, we found that carrot juice altered the metabolic functions of the human gut microbiota (Figure 4A). Specifically, carrot juice supplementation significantly increased the carbohydrate metabolism, lipid metabolism, transcription, replication and repair functions while decreased the amino acid metabolism and secondary metabolites biosynthesis functions of the human gut microbiota (Figure 4B-4G).

Carrot juice is high in vitamins, carotenoids and dietary fibers [1, 2]. The strengthened ability of the human gut microbiota to metabolize carbohydrate and lipid is likely due to the increased fermentation of dietary fibers and lipid-soluble vitamins from the carrot juice. Previous studies have indicated that drinking carrot juice could help to reduce the body weight and body mass index in type 2 diabetic subjects [5]. However, the

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detailed nutritional mechanism is largely unknown. Type 2 diabetes is characterized with severe microbial dysbiosis and abnormal metabolic functions of the microbes in the gut [24, 25]. Our results suggest that carrot juice might have gut microbiota as a primary target for the treatment of metabolic diseases, such as obesity and type 2 diabetes. However, more clinical studies are needed to verify this hypothesis.

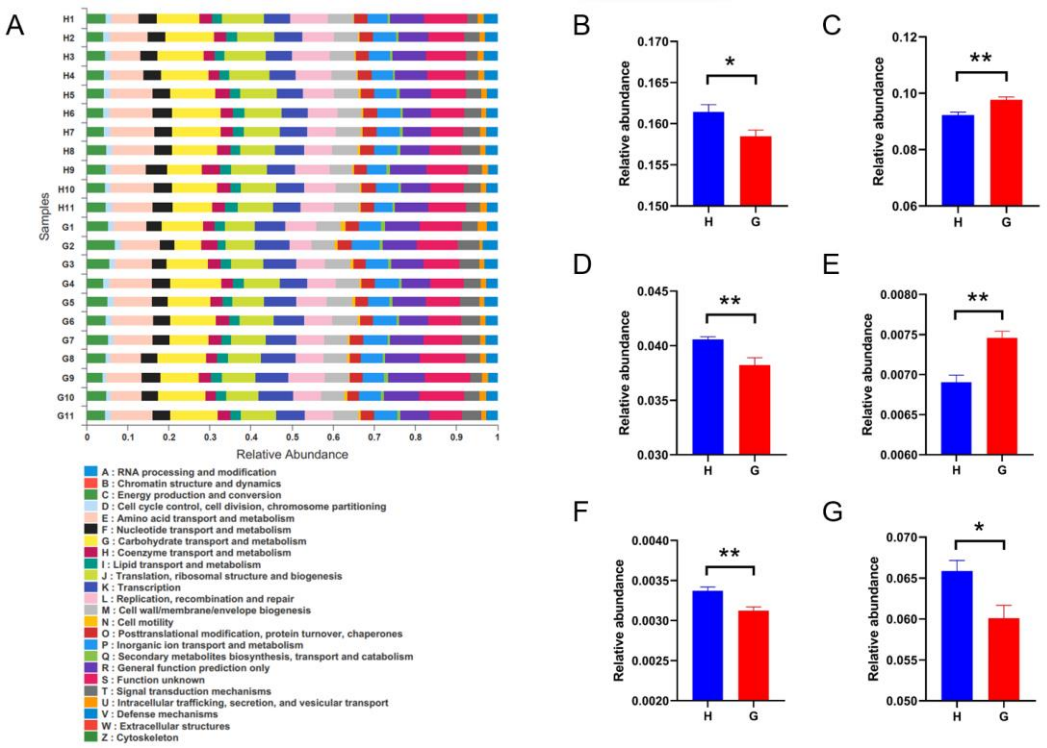

Figure 4. *In silico* analysis of the metabolic functions of the human gut microbiota. Clusters of orthologous genes (COG) function classification of the gut microbiota (A). Analysis of different COG functions including carbohydrate metabolism (B), amino acid metabolism (C), lipid metabolism (D), biosynthesis of other secondary metabolites (E), transcription (F) and replication and repair (G).

We next investigated the fermentation outcomes of carrot juice since it remarkably changed the composition and metabolic functions of the human gut microbiota (Figure 5). Short-chain fatty acids (SCFAs) are important fermentation products of the intestinal bacteria [26, 27] and, interestingly, we found that carrot juice supplementation profoundly increased the microbial production of acetate, lactate and total SCFAs (Figure 5A, 5C and 5D). It is of note to mention that although not statistically significant, carrot juice supplementation also tended to increase the production of succinate, isobutyrate and butyrate (Figure 5B, 5E and 5F).

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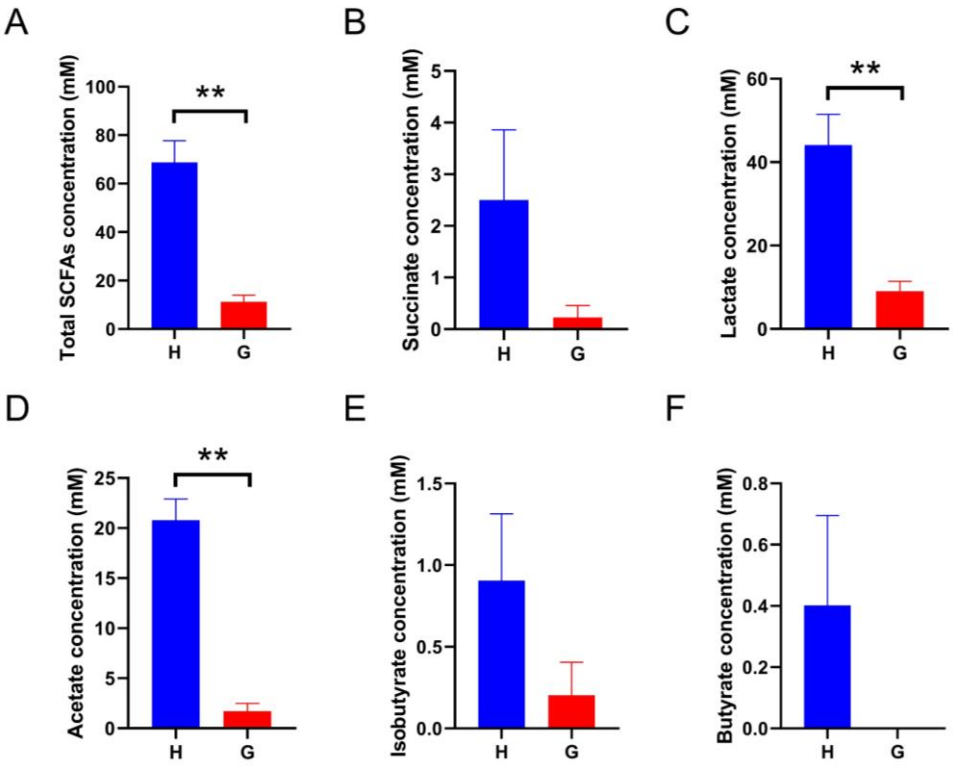

Figure 5. Analysis of the production of SCFAs during fermentation. Total SCFAs concentration (A), succinate concentration (B), lactate concentration (C), acetate concentration (D), isobutyrate concentration (E), butyrate concentration (F).

3.4. Carrot Juice Induced Production of Acetate and Lactate Was Positively Associated with Incresed Abundacne of Lactobacillus spp.

We next sought to find out which bacterium might drive the increase of the productions of SCFAs. Spearman's correlation analysis was applied to investigate the associations between changes of the human gut microbiota and productions of the SCFAs. Interestingly, the abundance of *Lactobacillus* spp. was positively associated with the productions of total SCFAs (R=0.76, $p < 0.001$), lactate (R=0.74, $p < 0.001$) and acetate (R=0.73, $p < 0.001$) (Figure 6).

Acetate and lactate play a pivotal role in maintaining the health status of the host [26-28]. Our results indicated that *Lactobacillus* spp. was a major contributor for the production of acetate and lactate during fermentation of carrot juice. It is highly possible that the dietary fibers and others nutrients were utilized and fermented by *Lactobacillus* spp. to produce SCFAs including acetate and lactate. However, more studies are warranted to verify this hypothesis.

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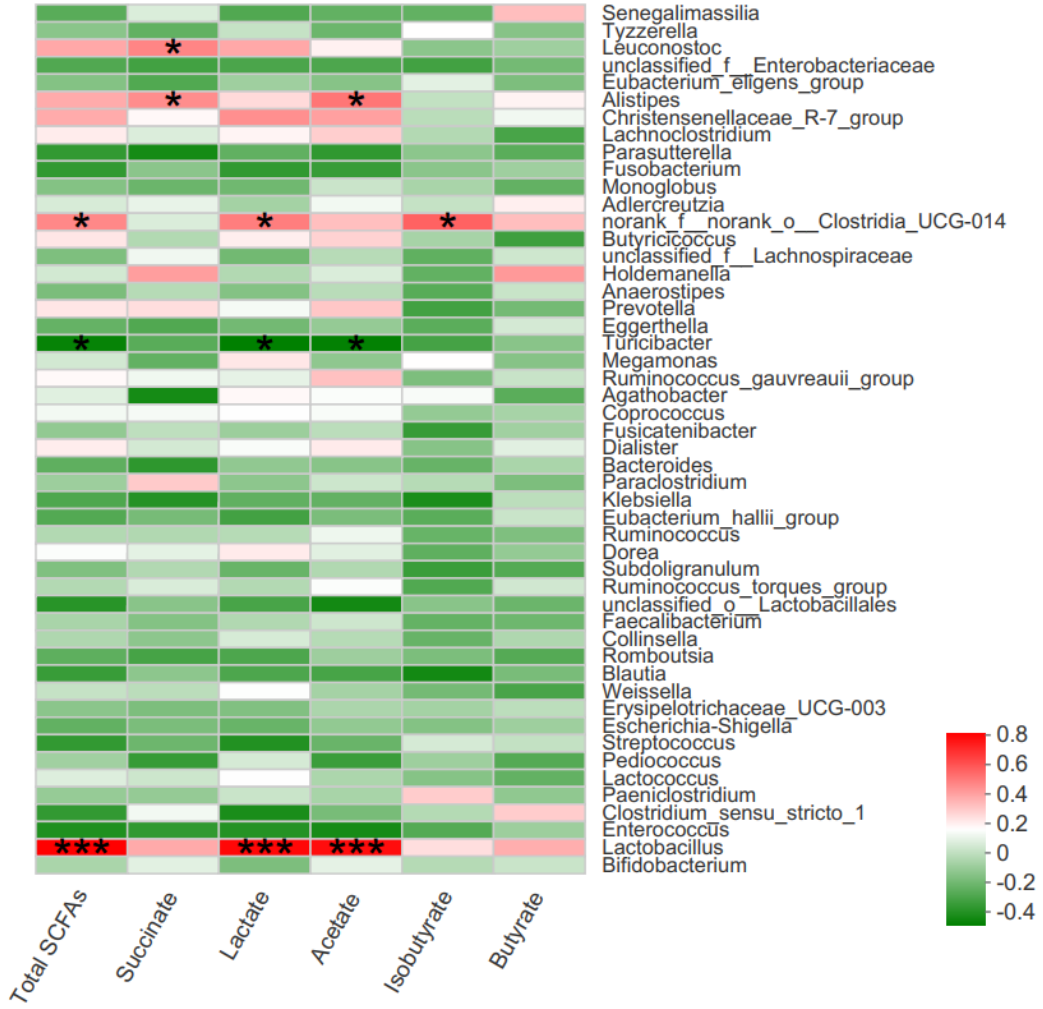

Figure 6. Spearman's correlation analysis of the associations between changes of the human gut microbiota and productions of the SCFAs. Correlations with an R value of above 0.4 or an R value below −0.4 were marked by asterisks. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

In conclusion, carrot juice supplementation changed the structure and metabolic functions of the human gut microbiota. Specifically, carrot juice remarkably increased the abundances of beneficial bacteria, including *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus mucosae* and *Bacteroides uniformis* and decreased the population of opportunistic pathogenic bacteria, such as *Enterococcus faecium* in the gut. Our study illustrates a favorable effect of carrot juice on the human gut microbiota and lays a foundation for the development of carrot juice as a novel prebiotic agent.

Supplementary Materials: Table S1: The α−diversity analysis of the gut microbiota.

Author Contributions: Conceptualization, Q.S. and H.Y.; methodology, Q.S. and H.Y.; software, Q.S.; validation, X.S. and Q.S.; formal analysis, X.S. and Q.S.; investigation, M.M., Y.W., L.C. and J.Y.; resources, M.M., Y.W., L.C. and J.Y.; data curation, X.S., M.M. and Q.S.; writing—original draft preparation, X.S. and Q.S.; writing—review and editing, Q.S.; visualization, X.S. and Q.S.; supervision, Q.S.; project administration, Q.S. and H.Y.; funding acquisition, Q.S. and H.Y.. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experiments regarding to the fecal sample collection from humans were approved and supported by the Ethical Committee of Ocean University of China, School of Medicine and Pharmacy (Permission No. OUC-2020-1008-01).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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