J Appl Biol Chem (2021) 64(1), 49–55
https://doi.org/10.3839/jabc.2021.008

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Food Science

Effect of *Chlorella vulgaris* on gut microbiota through a simulated *in vitro* digestion process

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Received: 11 January 2021 / Accepted: 15 January 2021 / Published Online: 31 March 2021

Abstract The diet plays a fundamental role in the formation of the gut microbiota, determining the interrelationship between the gut microbiota and the host. The current study investigated the effect of *Chlorella vulgaris* on the gut microbiota by using simulated *in vitro* digestion and colonic fermentation. Bioaccessibility was measured after *in vitro* digestion, and SCFAs and microbial profiling were analyzed after colonic fermentation. The bioaccessibility of *C. vulgaris* was 0.24 g/g. The three major SCFAs (acetate, propionate, and butyrate) increased significantly when compared to the control group. In microbial profiling analysis, microorganisms such as *Faecalibacterium*, *Dialister*, *Megasphaera*, *Dorea*, *Odoribacter*, *Roseburia*, *Bifidobacterium*, *Butyricmonas*, and *Veillonella* were high in *C. vulgaris* group. Among them, *Faecalibacterium*, *Dialister*, *Megasphaera*, *Roseburia*, and *Veillonella* were thought to be closely associated with the increased level of SCFAs. Finally, it can be expected to help improve gut microbiota and health through ingestion of *C. vulgaris*. However, further studies are vital to confirm the changes in the gut microbiota in *in vivo*, when *C. vulgaris* is ingested.

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Keywords Bioaccessibility · Gut microbiota · Microbial profiling · Short chain fatty acids

Introduction

Microorganisms that live in the human gut make important contributions to human metabolism and are able to be potential substances for new therapeutic agents [1]. Gut microorganisms perform several beneficial functions in the body's metabolic, immunological, structural and neurological environment, and have a significant impact on an individual's physical and mental health [2]. One of the main features of the gut microorganisms is to ferment undigested food ingredients into short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate [3]. In particular, acetate, propionate, and butyrate are SCFAs most often discovered in feces and play a very different but important role in the human body [4].

Diet plays a fundamental role in the formation of the gut microbiota and determines the interrelationship between the gut microbiota and the host [5]. Studies of gut microbiota through the diet of animals or humans give the most reliable results, but are time consuming and expensive. *In vitro* studies are an alternative solution to animal or human models because they screen food ingredients in a short time [6].

Chlorella, a single-celled green alga that grows primarily in aquatic environments, provided the antioxidant activity [7]. *Chlorella vulgaris* is the most known *Chlorella* species and rich in dietary fiber, amino acids, proteins, minerals, vitamins, and unsaturated fatty acids [7]. However, how they can be changed or modulated during digestion and fermentation in human body is still not clear. Thus, the current study investigated the effect of *C. vulgaris* on the gut microbiota after applying *in vitro* digestion and colonic fermentation to imitate digestion.

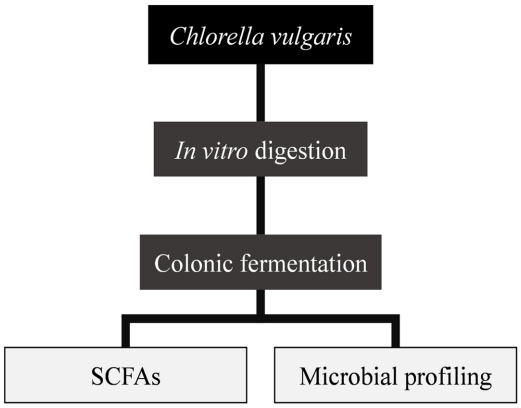

Fig. 1 Overall experimental design. The oral and gastrointestinal stages were simulated during *in vitro* digestion. Short chain fatty acids (SCFAs) were analyzed using gas chromatography-frame ionization detector (GC-FID). Microbial profiling analysis was carried out through mi-seq

Materials and Methods

The overall experimental procedure is shown in Fig. 1.

Chlorella vulgaris

C. vulgaris was received from Daesang Corporation (Seoul, Korea). Samples were obtained in powder form and kept in a refrigerator at 4 °C.

In vitro digestion

In vitro digestion was carried out using the method of Perez-Burillo [8]. Three solutions (simulated saline fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)) were prepared in advance by referring to Perez-Burillo's protocol. In the experiment, each 1 g *C. vulgaris* was transferred to a 50 mL conical tube, and then combined with 4 mL distilled water. After injecting 5 mL SSF solution at the oral stage, it was mixed with α-amylase (150 U/mL) and 3 M $CaCl_2$ (25 μL). Thereafter, the solution was incubated for 2 min in a shaking water bath (Model BS-21, JEIO TECH, Daejeon, Korea) under conditions of 37 °C and 95 rpm. In gastrointestinal stage, the cultured solution and SGF (10 mL) solution containing pepsin (4000 U/mL) were mixed with 4 N HCl to lower the pH to 2.5. Then, the solution was incubated in a shaking water bath under the conditions at 37 °C and 95 rpm for 2 h. In small intestinal stage, SIF (20 mL) solution containing 20 mM bile salt, 26.74 mg/mL pancreatin, and 1 mL of 0.01 g/mL pancreatin lipase was mixed with 8 N NaOH to make it between pH 6 and 6.5. Then, the solution was incubated for 2 h in shaking water bath under conditions of 37 °C and 95 rpm. Finally, the solution was centrifuged (4 °C, 2000× *g*, 30 min) to separate the precipitate, which was stored in −20 °C freezer.

Estimation of bioaccessibility

Bioaccessibility was measured by investigating differences in weight changes after *in vitro* digestion. The sample weight was measured using an electronic balance, and after *in vitro* digestion, the weight was calculated by measuring the moisture through Moisture Analyzer HE78 (Mettler-Toledo, Columbus, OH, USA). Bioaccessibility was calculated through the following equation.

$$\text{Bioaccessibility (g/g)} = 1 - \frac{\text{Sample weight after } \textit{in vitro} \text{ digestion (g)} \times \text{Moisture content}}{\text{Sample weight before } \textit{in vitro} \text{ digestion (g)}}$$

Colonic fermentation

Colonic fermentation was carried out using Long's method [9]. It was the control group that performed colonic fermentation without adding anything. Briefly, NaCl (8 g/L), KCl (0.2 g/L), Na_2HPO_4 (1.15 g/L), KH_2PO_4 (0.2 g/L), and L-cysteine (0.5 g/L) were dissolved in distilled water and then autoclaved (phosphate-buffered saline (PBS) medium). Fecal samples were gained from three healthy donors (20-30 years old; mean body mass index 22.3) who did not take any probiotics or antibiotics for 3 months prior to the experiment. Written consent was received from each donor, and the research was approved by the Institutional Review Board (IRB No. 2015-003) of the Korea Institute of Science and Technology. Fecal samples were diluted using PBS solution to a concentration of 10% (w/v) in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, USA). After *in vitro* digestion, a portion of the precipitate (0.5 g) was diluted with an anaerobic PBS solution (10 mL). Then, the two solutions (10 mL each) were mixed. Colonic fermentation of all solutions was performed at 37 °C using AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). All samples (1 mL) were taken at 0, 6, 12 and 24 h after fermentation and stored in a freezer (−20 °C).

Profiling of short chain fatty acids (SCFAs)

All steps from sample preparation to gas chromatography-frame ionization detector (GC-FID) analysis were carried out according to David's method [10]. First, the supernatant of the fecal mixture cultured for 0, 6, 12, and 24 h was separated by centrifugation (18,000× *g*, 4 °C, 10 min). Then, the supernatant (500 μL) was combined with 50% internal standard (1% 2 methylpentanoic acid), sulfuric acid (50 μL), and diethyl ether (500 μL). In addition, quantitative data were obtained using a 10 mM Volatile free acid mix (Sigma-Aldrich, St. Louis, MO, USA). The mixture was moved to a vial for gas chromatography analysis. Gas chromatography equipment (300-MS, Bruker, Madison, WI, USA) was utilized for GC-FID analysis. The oven temperature was kept at 170 °C and the both injector and detector were set at 225 °C. A Nukol column (Supelco, Merck, Darmstadt, Germany; a 30 m ×0.25 mm fused silica capillary column coated with a 0.25

μm film thickness) was used for GC-FID analysis. Data were analyzed together with internal standard (1% 2-methyl pentanoic acid) and Volatile free acid mix data to acquire quantitative data.

16S rRNA gene sequencing

DNA was extracted from the precipitate acquired by centrifugation (18,000× *g*, 4 °C, 10 min) of colonic fermented samples for 24 h using QIAamp DNA Stool Mini Kit (QIAGEN, Germantown, MD, USA) with bead-beating. The V3-V4 region (319F/806R) of the 16S rRNA gene was amplified by improved dual-indexing amplification [11]. Polymerase chain reaction products were purified using AMPure XT beads (Beckman, Danvers, MA, USA) and quantified using Qubit dsDNA high sensitivity reagent (Invitrogen, Carlsbad, CA, USA). Sequencing was carried out on the MiSeq platform using a paired-end 2× 300-bp reagent kit (Illumina, San Diego, CA, USA). Raw reads were demultiplexed and assembled. The quality was filtered by QIIME 2 (v2018.6) using the default settings [12]. To classify the filtered reads into taxonomic groups, a Naïve Bayes classifier was trained using the 16S rRNA region (V3-V4), a primer set, read length (469 bp), and the Greengenes 97% reference set (v13.5) [13]. This trained feature classifier was used to assign a classification to each read using the default settings of QIIME. MicrobiomeAnalyst [14] was used to analyze specific levels of microbial composition and β-diversity. β-diversity analysis method under the following conditions; Ordination method: PCoA, distance method: Jaccard Index, Taxonomic level: Feature-level, Statistical method: Permutational MANOVA (PERMANOVA). Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was performed under the following conditions for classification of human microbial community data; P-value cutoff: 0.1, FDR-adjusted, log LDA score: 2.0, number of top features: 22.

Statistical analysis

The grouped data were statistically analyzed using GraphPad Prism 9. To statistically analyze the results of SCFAs, significance was measured using the unpaired t test (p <0.05). In addition, MicrobiomeAnalyst was used to perform microbial data processing

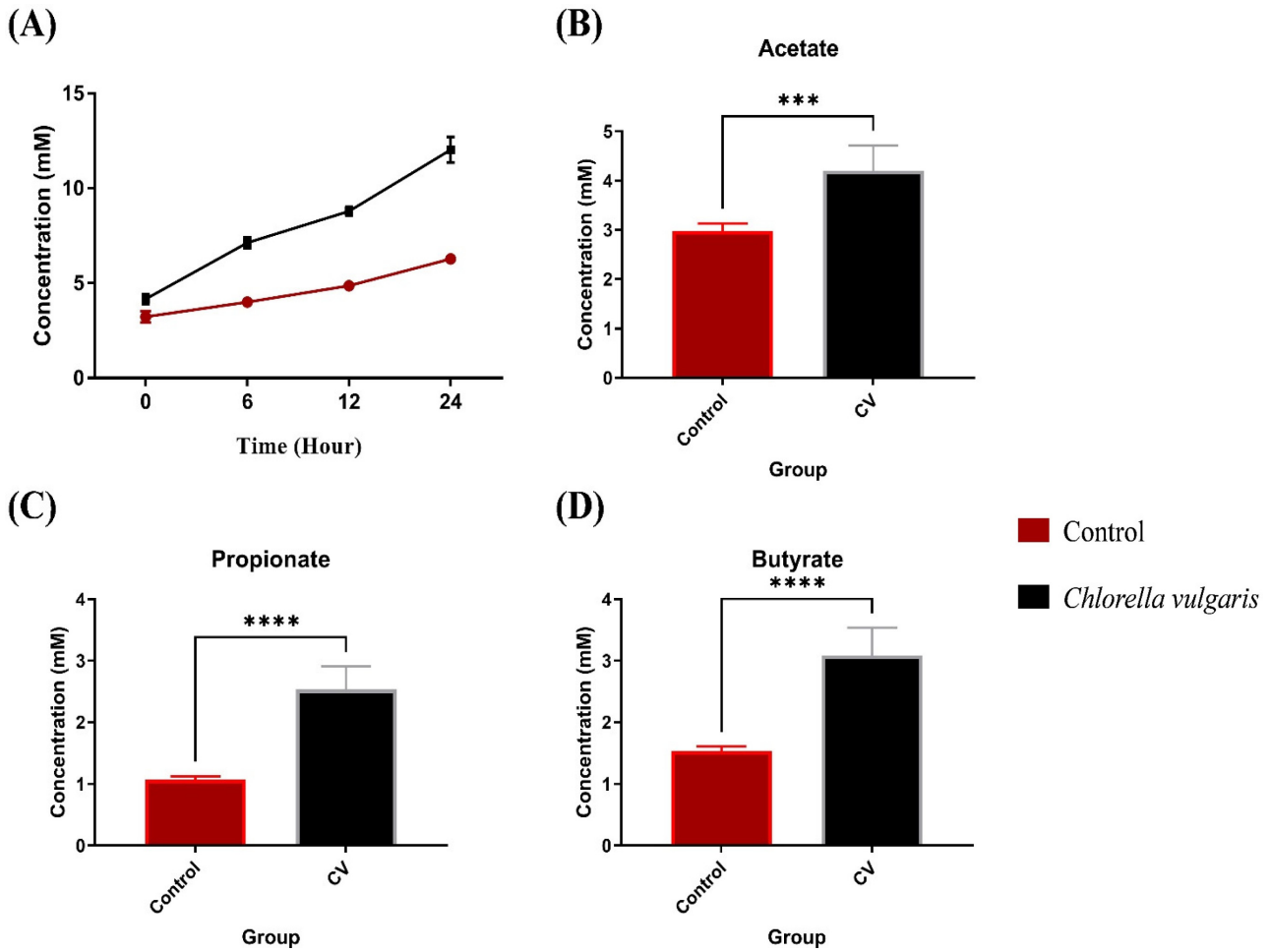

Fig. 2 Changes in the amount of (A) total of three major SCFAs, (B) acetate, (C) propionate, and (D) butyrate after 24 h colonic fermentation of *C. vulgaris* (n =6). Control: a group subjected to colonic fermentation without adding anything. (CV): a group subjected to colonic fermentation by adding *C. vulgaris*. Significance was determined using the unpaired *t* test (***p <0.001, ****p <0.0001)

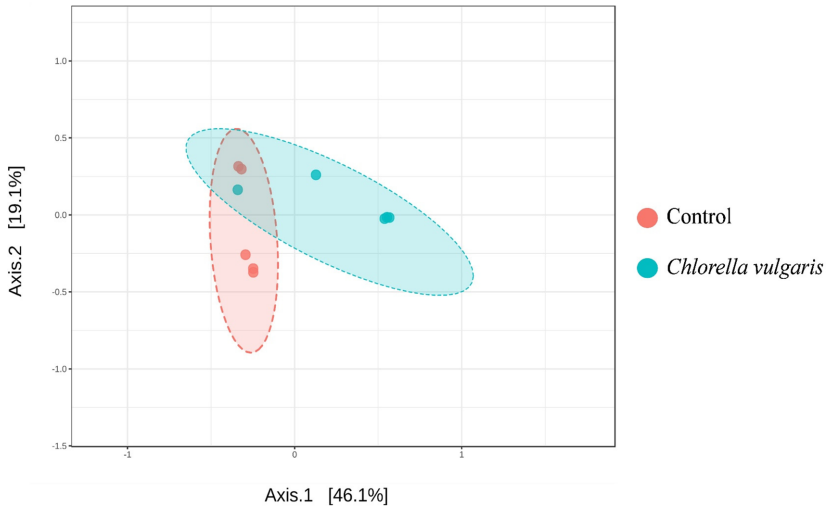

Fig. 3 β-diversity analysis for two colonic fermentation groups (control and *C. vulgaris*). Ordination method: PCoA, Distance method: Jaccard Index, Taxonomic level: Feature-level, Statistical method: Permutational MANOVA (PERMANOVA), F-value: 3.3868; R-squared: 0.29743; p <0.053. Control: a group subjected to colonic fermentation without adding anything. *Chlorella vulgaris*: A group subjected to colonic fermentation by adding *C. vulgaris*.

and statistical analysis. Permutational multivariate analysis of variance (PERMANOVA) was carried out to test the difference in overall microbial composition between the control group and the *C. vulgaris*-treated group based on PCoA.

Results and Discussion

Estimation of bioaccessibility

The bioaccessibility (g/g) of *C. vulgaris*, relative amount measured before entering colon stage after *in vitro* digestion, was 0.24 g/g. It implies that 0.76 g of residual component could reach the large intestine, possible to interact with gut microbes when 1 g of *C. vulgaris* is consumed.

Profiling of SCFAs

After colonic fermentation, SCFAs which are major products of gut microorganisms were analyzed. As the fermentation time passed, the difference in concentration of three major SCFAs between the control and *C. vulgaris* group increased more and more (Fig. 2A). After 24 h colonic fermentation, the difference in concentration of acetate, propionate, and butyrate was also significant (p <0.05) (Fig. 2B-D); acetate: control (2.979 mM ± 0.063, n =6), *C. vulgaris* (4.199 mM ±0.210, n =6), p <0.001; propionate: control (1.069 mM ±0.023, n =6), *C. vulgaris* (2.536 mM ±0.152, n =6), p <0.0001; butyrate: control (1.535 mM ± 0.030, n =6), *C. vulgaris* (3.085 mM ±0.184, n =6), p <0.0001. It was predicted that there is a clear difference in the microbial community because the difference in SCFAs is significant.

Measurement of β-diversity

Among the microbial profiling analysis results, β-diversity was analyzed to decide whether or not there is a difference between the microbial communities of two groups (control and *C. vulgaris*) (Fig. 3). As a result of the analysis, it was confirmed that there was a difference between the microbial communities in two groups.

LEfSe analysis

Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was performed to find the microorganisms that influenced the difference between the microbial communities for the two groups (Fig. 4). LefSe analysis was conducted under the following conditions; P-value cutoff: 0.1, FDR-adjusted, log LDA score: 2.0, number of top features: 22. In LefSe analysis, all results were analyzed at genus level. 9 microorganisms such as *Faecalibacterium*, *Dialister*, *Megasphaera*, *Dorea*, *Odoribacter*, *Roseburia*, *Bifidobacterium*, *Butyricmonas*, and *Veillonella* were high in *C. vulgaris* group. 13 microorganisms such as *Lactococcus*, *cc_115*, *Streptococcus*, *Mucispirillum*, *Anaerotruncus*, *Blautia*, *Dehalobacterium*, *Coprobacillus*, *Enterococcus*, *Coprobacillus*, *Adlercreutzia*, *Allobaculum*, and *Prevotella* were high in the control group. Microorganisms which are high in *C. vulgaris* group were predicted to have an effect on the increase in the concentration of SCFAs.

Analysis of discriminative microorganisms from *C. vulgaris* group

The association between the microbes and SCFAs production was examined. Five microorganisms (*Faecalibacterium*, *Dialister*, *Megasphaera*, *Roseburia*, and *Veillonella*) were selected by comparing the results obtained from LEfSe analysis and the result by Reichardt et al. [15] in which the microorganisms related to propionate or butyrate production are summarized. After that, the relative abundance of those microbes was shown in Fig. 5 by

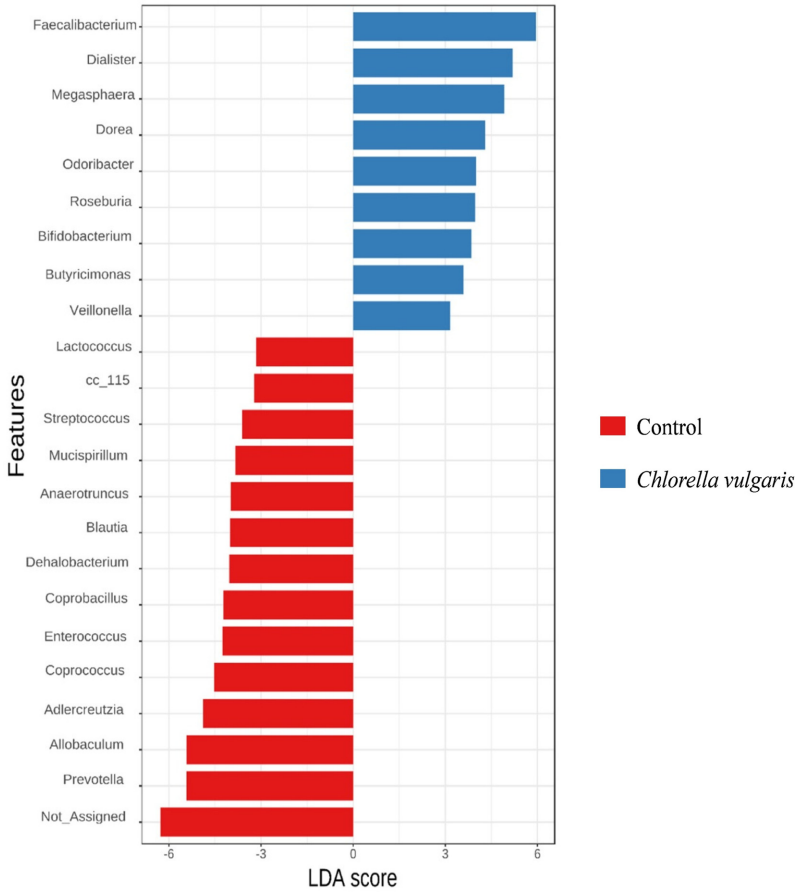

Fig. 4 LEfSe analysis for two colonic fermentation groups. *p*-Value cutoff: 0.1, FDR-adjusted, log LDA score: 2.0, number of top features: 22. Control: A group subjected to colonic fermentation without adding anything. *Chlorella vulgaris*: A group subjected to colonic fermentation by adding *C. vulgaris*.

comparing the control group with the *C. vulgaris* group. Microorganisms associated with propionate production are *Dialister*, *Veillonella*, and *Megasphaera*. Propionate is primarily metabolized in the liver and plays a role in reducing the concentration of blood sugar and serum cholesterol [16]. In addition, propionate is vital in regulating endocrine production of adipose tissue and preventing the metabolic diseases such as obesity [17]. Microorganisms associated with butyrate production are *Faecalibacterium* and *Roseburia*. Butyrate is a main energy source for colon cells and has a direct effect on cell growth and differentiation [18]. In addition, butyrate is known to be effective in preventing colon cancer [19].

Probiotics are defined as living microorganisms that, when administered in appropriate amounts, provide a health benefit to the host [20]. In general, *Lactobacillus* and *Bifidobacterium* have been used as probiotics [21]. *Bifidobacteria* are known to have beneficial influences on humans through protection against pathogens, synthesis of important vitamins, inhibition of potential toxicity, digestion of plant oligosaccharides/polysaccharides and carcinogenic metabolites, and stimulation of host immune response [22]. As a result of the LEfSe analysis in Fig. 4 and 5, *Bifidobacterium* was higher in the *C. vulgaris* group compared to the control group. Therefore, it was expected that the ingestion of *C. vulgaris* helped improve gut microbiota community and human health.

This study suggests that taking *C. vulgaris* may improve the intestinal microbial flora. The bioaccessibility of *C. vulgaris* was obtained through *in vitro* digestion, and the amount of *C. vulgaris* in the colon was predicted. After colonic fermentation, SCFAs analysis confirmed an increase in high SCFAs in the group to which *Chlorella vulgaris* was added. Microbial profiling analysis using MiSeq confirmed that the gut microbiota was changed by addition of *C. vulgaris*. Based on our results, ingestion of *C. vulgaris* can help people's health through improving the intestinal microbial flora. However, further studies are vital to confirm the changes in the gut microbiota in *in vivo*, when *C. vulgaris* is ingested.

Acknowledgment This work was supported by the Ministry of Oceans and Fisheries, Korea [grant number 20170488].

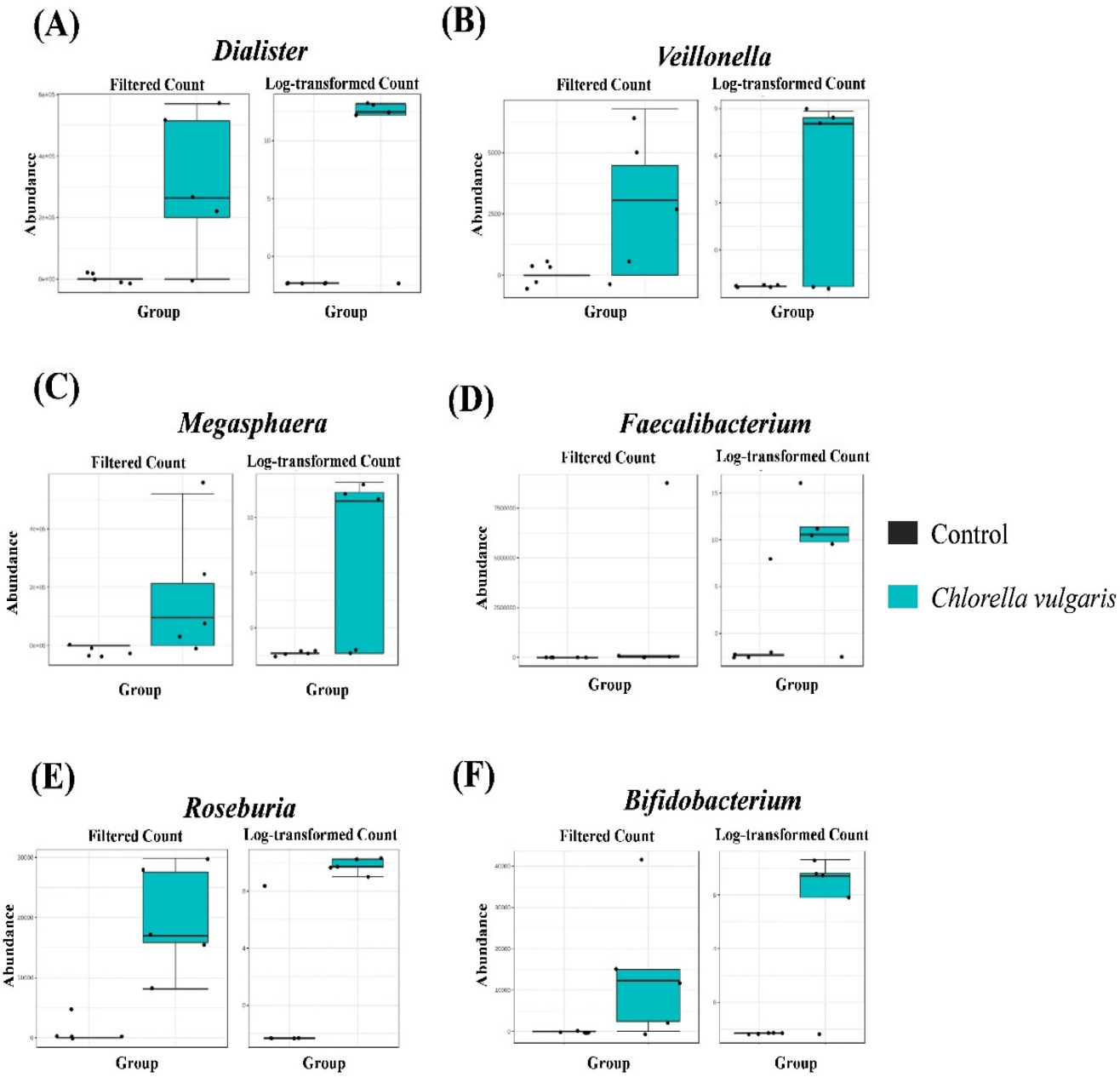

Fig. 5 The relative abundance of six discriminative taxa, *Dialister*, *Veillonella*, *Megasphaera*, *Faecalibacterium*, *Roseburia*, and *Bifidobacterium*.

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