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RESEARCH PAPER

Consumption of 85% cocoa dark chocolate improves mood in association with gut microbial changes in healthy adults: a randomized controlled trial

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Abstract

Dark chocolate has long been recognized for its mood-altering properties; however, the evidence regarding the emotional effects of daily dark chocolate intake is limited. Therefore, we aimed to investigate the effects of dark chocolate intake on mood in everyday life, with special emphasis on the gut-brain axis. Two different dark chocolates (85% and 70% cocoa content) were tested in this study. In a randomized controlled trial, healthy adults (20–30 y) consumed either 30 g/d of 85% cocoa chocolate (DC85, n=18); 70% cocoa chocolate (DC70, n=16); or no chocolate (control group, CON; n=14); for 3 weeks. Mood states were measured using the Positive and Negative Affect Schedule (PANAS). Daily consumption of dark chocolate significantly reduced negative affect in DC85, but not in DC70. To assess the association between the mood-altering effects of dark chocolate and the gut microbiota, we performed fecal 16S rRNA sequencing analysis for the DC85 and CON groups. Gut microbial diversity was significantly higher in DC85 than CON (P<.05). *Blautia obeum* levels were significantly elevated and *Faecalibacterium prausnitzii* levels were reduced in DC85 compared to CON (P<.05). Furthermore, we found that the observed changes in negative affect scores were negatively correlated with diversity and relative abundance of *Blautia obeum* (P<.05). These findings indicate that dark chocolate exerts prebiotic effects, as evidenced by its ability to restructure the diversity and abundance of intestinal bacteria; thus, it may improve negative emotional states via the gut-brain axis.

Keywords: Dark chocolate; Mood; Gut microbiota; Gut-brain axis.

1. Introduction

Mood disorders are a leading cause of disability worldwide [1]. Disturbances in a person's mood interrupts their personal well-being and the ability to participate in social interactions, leading to physical health problems such as chronic diseases [2]. The symptoms of mood disorders include ongoing feelings of sadness, helplessness, hopelessness, and irritability. These disorders are mainly treated with drugs that manipulate the monoaminergic neurotransmitter system in the brain [3]. However, the efficacy of such drugs is in doubt because the drugs are associated with a delayed onset of action and a low response rate [3,4]. Therefore, the need for a new paradigm to prevent and treat mood disorders has been raised, and relevant research has been conducted in fields including nutrition and microbiome science, with special attention to the relationship between diet and mood.

The role of diet as a mood regulator has received a great deal of interest. Certain dietary components have been shown to reduce anxiety and depression and improve quality of life [5]. In particular, cocoa products such as dark chocolate contain a number of nutritional compounds that have the potential to affect mood [6]. The health benefits of dark chocolate consumption, particularly the effects of polyphenols on mood, have been reported in several studies [6,7]. For example, animal studies demonstrated that cocoa polyphenolic extracts have antioxidant and anti-inflammatory effects that may reduce depressive behaviors [7,8]. Human studies showed that consumption of cocoa ameliorated negative emotions when stress and anxiety were experimentally induced [9–12]. However, this topic remains controversial since other studies reported no significant effects of cocoa-enriched beverages and dark

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chocolate consumption on mood [13]. More importantly, the evidence regarding the emotional effects of dark chocolate intake in everyday life is limited since most studies investigated its acute effects following experimentally induced psychological stress. Furthermore, the underlying molecular and physiological changes related to the possible mood-altering effects of dark chocolate have not been clearly elucidated. Therefore, well-designed clinical studies are required to better understand the effects of dark chocolate consumption on changes in physiological and psychological states.

The human intestinal microflora contains 100 trillion bacterial cells composed of 1000 bacterial species; there are 10 times more bacterial cells than human cells in the human body [14]. These microorganisms continuously interact with the host and affect human health by playing important roles in digestion, metabolism, and production of bioactive byproducts. In addition, recent studies have provided overwhelming evidence suggesting that the gut microbiota plays a major role in the central nervous system and regulates the host's moods via the gut-brain axis [15–19]. Therefore, over the past few years, manipulation of the gut-brain axis has been regarded as a novel technique for the prevention or treatment of mood disorders [19]. Recent studies showed that germ-free (GF) mice have reduced anxiety- and depression-like behaviors compared to control mice [20,21]. Additionally, GF mice given gut microbiota transplants from patients with major depressive disorder showed depression-like behavior, whereas "healthy microbiota" recipient mice did not [21], suggesting a relationship between the gut microbiome and mood.

Here, we aimed to test our hypothesis that dark chocolate has beneficial effects on gut bacterial profiles that may serve to improve mood states. We performed a randomized clinical trial using dark chocolate with different cocoa contents (85% and 70% cocoa content) and identified changes in the diversity and composition of the gut microbiota following dark chocolate consumption, as well as the association between those changes and participants' emotional states, in healthy adults.

2. Materials and methods

2.1. Study design

This was a randomized controlled trial conducted at Seoul National University (Seoul, Republic of Korea) from July to December 2017. Participants who met the criteria for eligibility were randomly assigned to one of three groups: (1) control group (CON, n=14); 2) 85% cocoa chocolate group (DC85, n=18); and 3) 70% cocoa chocolate group (DC70, n=16). Subjects in the DC85 and DC70 groups were instructed to eat 10 g of chocolate each time, three times per day, at the same times each day (7:00 – 9:00, 12:00 – 14:00, 17:00 – 19:00) for 3 weeks. CON participants were not supplied any chocolate. The dark chocolate that was provided to the DC85 and DC70 groups was Weinrich 1895 Fine Dark Chocolate 85% Cocoa (Ludwig Weinrich GmbH & Co. KG, Germany) and Weinrich 1895 Fine Dark Chocolate 70% Cocoa (Ludwig Weinrich GmbH & Co. KG, Germany), respectively. The chocolate was divided into 10 g portions using an electronic scale. The divided chocolate was portioned into individual serving sizes and placed in sterilized sealed packets (10 g/packet). On days 1, 8, and 15 of the experiment, participants received one week's worth of chocolate (21 packets/week). Participants stored chocolate packets in a cool, dry place as instructed. The nutritional composition of the study products is shown in Supplementary Table 1. Compliance was calculated on the basis of the number of chocolate pieces returned every week as a percentage of the number of chocolate pieces consumed during the intervention period. The investigators were not blinded to this assignment, but participants in the DC85 and DC70 group were blinded as to whether they received high-cocoa chocolate or low-cocoa chocolate. All study procedures were approved by the Seoul National University Institutional Review Board (IRB No. 1706/002-015). This work is registered with Clinical Research Information Service (CRiS; Registration ID: KCT0005582).

2.2. Participants

Participants were recruited using a recruitment flyer that was posted at Seoul National University (Seoul, Republic of Korea). Candidates were invited for onsite screening interviews. Eligible subjects were healthy men and women between 20 and 30 years of age. Individuals who met any of the following criteria were excluded from the study: (1) Routinely eat sweets such as candy, chocolate, or cake more than twice a day; (2) Had a history of gastrointestinal tract surgery or disease; (3) Received antibiotics within 3 months prior to the study; (4) Had taken pre-/pro-biotics within 6 months prior to the study; (5) Had diabetes; (6) Had Beck Depression Inventory (BDI) score $\geq$ 16. A total of 117 candidates were screened, of which a total of 48 participants were eligible and consented to participate in the study.

2.3. Randomization

Randomization was conducted using a stratified block design through SealedEnvelope (https://www.sealedenvelope.com/simple-randomiser/v1/lists) considering sex, which could influence the gut microbiome and psychological markers.

2.4. Outcome assessments

Gut microbiota analysis and mood testing to measure positive and negative affect were conducted before and after the experiment. Body composition analysis and dietary assessment were also performed before and after the experiment.

2.4.1. Body composition analysis

The weight, skeletal muscle mass, body fat mass, BMI, and percent body fat of participants were measured twice, before and after the experiment, by using InBody (InBody370S, InBody Co., Ltd., Seoul, Republic of Korea) based on the bioelectrical impedance analysis (BIA) method which measures body water by obtaining the impedance index.

2.4.2. Dietary assessment

A dietary survey was conducted using the 3-day food record (3DR) method, a standardized tool for dietary assessment, which included 3 random days (2 weekdays and 1 weekend day). Participants were asked to record the types, amounts, ingredients and cooking methods of the food that they consumed for the previous 3 days and the location of each meal. Intake of total calories, carbohydrates, proteins, and fats were analyzed using the Computer Aided Nutritional Analysis Program (CAN-Pro 5.0, Korean Nutrition Society). The participants in the control group (CON) were asked to take note of the snacks that they ate every day during the experimental period, in addition to the 3-day food record.

2.4.3. Positive and negative affect

The Korean version of the Positive and Negative Affect Schedule (PANAS), one of the most widely used measures of positive and negative affect, was used to assess multiple aspects of mood [22]. It is composed of twenty adjectives that indicate positive or negative mood states. Participants were asked to rate their feelings on a scale of one (very slightly or not at all) to five (extremely), and the scores on each item were averaged separately by affect.

2.4.4. Depression

The Korean version of the Beck Depression Inventory (K-BDI), which was developed for measuring the severity of depression, was used to measure depression [23]. The K-BDI is a 21-question multiple-choice self-report inventory. Participants who had a score of 16 or greater, which indicates moderate or severe depression, were excluded at the screening step.

2.5. Fecal sample collection and genomic DNA extraction

Participants self-collected fecal samples using the OMNIgene•GUT kit (OMR-200, DNA Genotek Inc., Ottawa, ON, Canada) containing a stabilizing solution for microbial DNA present in feces. Participants brought the fecal samples at room temperature to the laboratory at Seoul National University at their time of visit. A ~500 mg sample aliquot from each participant was immediately collected and stored at -80°C before analyzing the gut microbiome. Total bacterial DNA was isolated from stool with the following additional steps. Briefly, an ~200 mg stool sample was homogenized with a 5 mm sterilize steel bead in ASL buffer using TissueLyser (QIAGEN, Hilden, Germany) for 1 min at 30 Hz. We increased the heating temperature of the fecal lysate to 95°C to enhance lysis of the cell walls of gram-positive bacteria. In the last incubation step, we increased the incubation time from 1 to 5 min to increase DNA yield. Extracted genomic DNA was confirmed via gel electrophoresis and was quantified with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.6. 16s rRNA gene sequencing data analysis

The 16S rRNA gene from the extracted DNA samples was amplified by targeting the V3-V4 hypervariable region and sequenced using the Illumina Miseq Sequencing system (Illumina, CA, USA) according to the manufacturer's instructions; this work was done at Chunlab, Inc. (Seoul, Republic of Korea). The DADA2 pipeline [24] of the QIIME2 package [25] (version 2019.01, https://qiime2.org) was used to

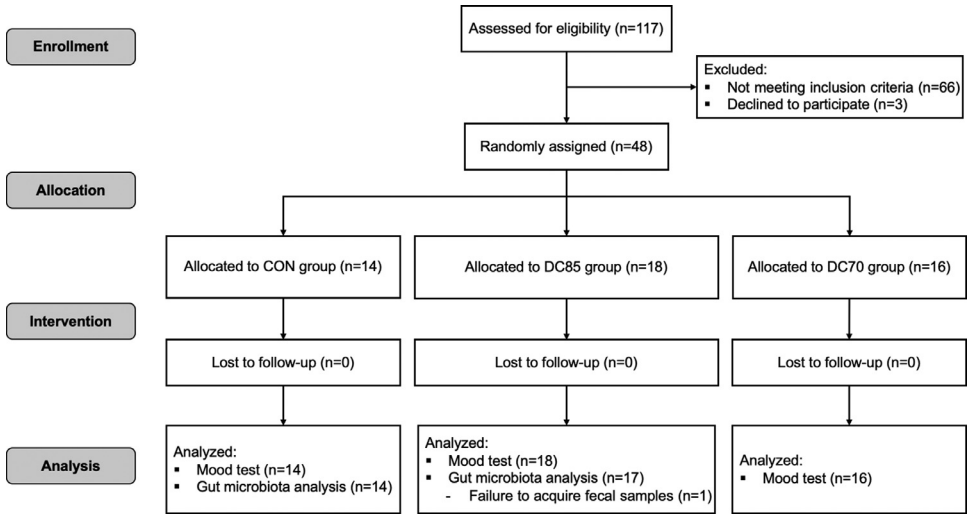

Fig. 1. Study flow diagram. CONSORT (Consolidated Standards of Reporting Trials) flow diagram of the phases of the randomized controlled trial. CON, control group; DC85, 85% cocoa chocolate group; DC70, 70% cocoa chocolate group.

generate unique sequence variants by filtering and removal of low-quality samples and chimera. For taxonomic analysis, taxonomy was assigned using a pre-trained naïve Bayes classifier based on the Greengene 13_8 99% operational taxonomic units (OTUs), which had been trimmed to only include the V3 and V4 region of the 16S rRNA gene. Chimeric reads were filtered from the sequencing data using UCHIME software [26]. Downstream analysis of alpha diversity was carried out by calculating Faith's phylogenetic diversity index (Faith's PD), the number of OTUs, and Shannon's diversity index (H) to assess differences in richness and evenness of the gut microorganisms. A comparison to reveal differences in microbial profiles involved measurement of the relative abundance of various microorganisms across the samples.

2.7. Statistical analysis

All statistical analyses were conducted based on intention-to-treat analysis. The Chi-squared test, Kruskal-Wallis test, one-way ANOVA, unpaired t-test or Mann-Whitney U test was used for between-group analysis. *P* values were corrected for multiple testing with the Benjamini–Hochberg method to control for the false-discovery rate (FDR). Spearman's correlation analysis was conducted to assess the correlation between gut microbiota composition and mood scores. $P<.05$ was considered statistically significant. The Statistical Package for Social Sciences software, version 23.0 (SPSS Inc., Chicago, IL, USA), and GraphPad Prism, version 8.4.3 (GraphPad Software, Inc., La Jolla, CA, USA) were used to assess statistical significance.

3. Results

3.1. General characteristics of participants at baseline

A total of 48 eligible participants who consented to participate in the study were randomly assigned to one of the following three groups, as presented in the flow diagram of the intervention study (Fig. 1): Control (CON, $n=14$); 85% dark chocolate (DC85, $n=18$); and 70% dark chocolate (DC70, $n=16$). The general characteristics of the study subjects at baseline are shown in Table 1. At baseline, there were no significant differences in sex ratio ($P=.903$), age ($P=.603$), weight ($P=.603$), BMI ($P=.601$), skeletal muscle mass ($P=.592$), body fat mass ($P=.447$), or percent body fat ($P=.927$) across the three groups. In addition, there were no significant differences in total energy (calorie), carbohydrate, fat, or protein intake among the three groups at baseline (Table 1), indicating that the participants were equally distributed into the three groups. Moreover, all participants completed the 3-week intervention, and the average compliance rates in the two chocolate intervention groups were not significantly different (97.0% in DC85 and 96.6% in DC70, $P=.838$, data not shown). Therefore, data from all participants were used in the statistical analyses to examine intervention effects, except that one DC85 sample was omitted from the gut microbial analysis due to a failure during sample collection.

3.2. Anthropometric parameters and dietary intake of participants

In order to determine whether anthropometric parameters and usual dietary intake were maintained during the trial, we conducted body composition analysis and 3DR-based dietary intake analysis. After the 3-week intervention, weight, skeletal muscle mass, body fat mass, BMI, and percent body fat were not significantly changed in all groups (Supplementary Table 2). In addition, there were no significant differences in the energy and macronutrient intake across the groups after the 3-week intervention period. The DC70 group had slightly less energy intake after the 3-week intervention period but the difference was not significantly different from other groups ($P=.421$) (Supplementary Table 2).

3.2. Effects of dark chocolate consumption on mood states

To determine whether dark chocolate consumption influences mood in everyday life, we measured positive and negative affect before and after the 3-week intervention period. We found that dark chocolate consumption had no significant impact on positive affect ($P=.498$, Table 2). However, negative affect was significantly altered by dark chocolate consumption (Table 2). The DC85 group showed a significant decrease in negative affect (-4.33 ± 5.91, $P=.029$), while the change in negative affect following the intervention was not significantly different in the DC70 group compared to the CON group (Table 2). Collectively, these results suggest that intake of dark chocolate with a higher cocoa content has a positive influence on negative emotional states.

3.3. Effects of dark chocolate consumption on gut microbial diversity

To evaluate the effect of dark chocolate intake on gut microbiota profiles in association with changes in mood states, we performed 16S rRNA sequencing analysis of bacterial genomic DNA from stool samples collected before and after the intervention. Our observation that daily DC85 intake was associated with a significant change in negative affect prompted us to examine any

Table 1
General characteristics of the study subjects at baseline

	CON (n=14)	DC85 (n=18)	DC70 (n=16)	P value†
Sex (men/women, n)	8/6	9/9	9/7	.903
Age (y)	22.86±1.75	24.94±4.58	24.06±2.84	.603
Anthropometric parameters				
Body weight (kg)	63.27±8.61	66.16±10.61	66.30±11.96	.603
BMI (kg/m^2)	22.29±2.72	22.37±2.37	22.66±2.96	.601
Skeletal muscle mass (kg)	25.98±4.59	28.21±5.76	27.31±6.05	.592
Body fat mass (kg)	16.33±5.45	15.46±4.74	17.08±5.25	.447
Percent body fat (%)	25.68±7.51	23.41±6.37	25.84±6.80	.927
Dietary intake				
Energy (kcal)	2034.37±467.95	1889.77±350.71	2061.85±386.88	.409
Carbohydrate (g)	251.08±53.60	228.58±71.27	253.13±54.17	.436
Fat (g)	70.63±21.28	66.71±19.15	77.28±21.37	.330
Protein (g)	79.70±27.16	82.19±22.57	81.38±24.97	.960

Values are mean ± SD.CON, control group; DC85, 85% cocoa chocolate group; DC70, 70% cocoa chocolate group. †P value by chi-squared test, Kruskal-Wallis test or one-way ANOVA.

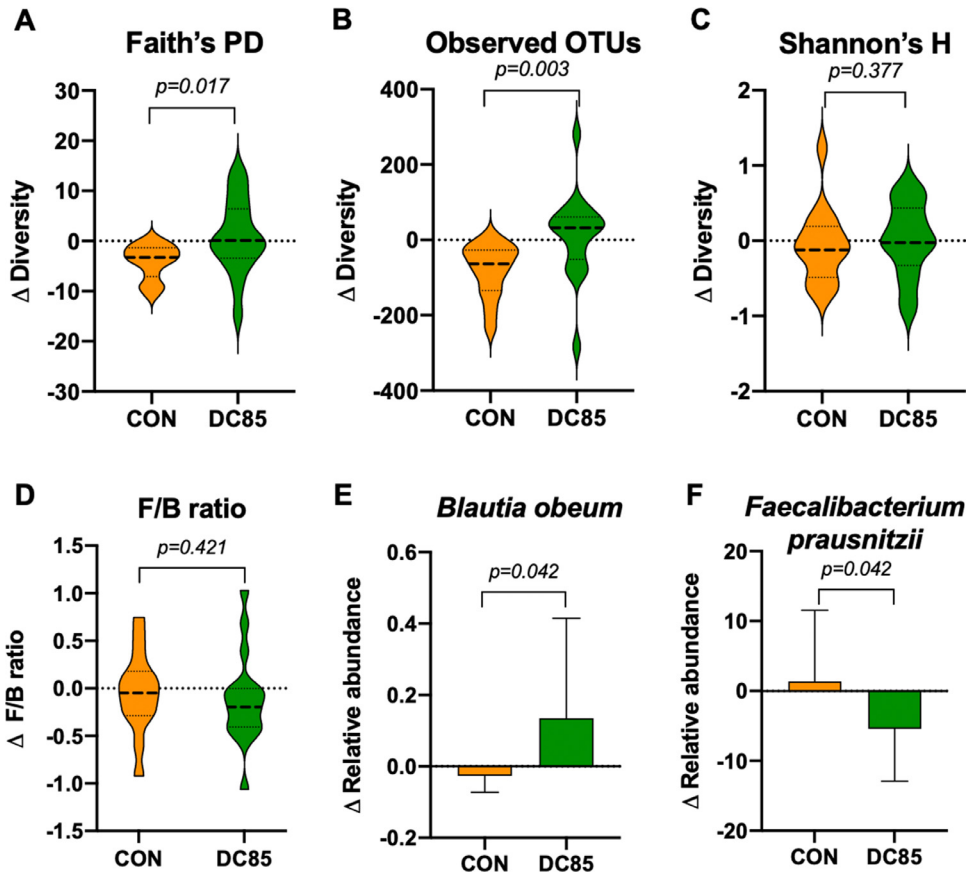

Fig. 2. Changes in the diversity and composition of the gut microbiota following dark chocolate consumption. Changes in alpha diversity index: (A) Faith's PD, (B) number of observed OTUs, and (C) Shannon's H index in the CON (control) and DC85 (85% cocoa chocolate) groups. (D) Changes in the Firmicutes-to-Bacteroidetes ratio (F/B ratio) and the relative abundance of significantly regulated gut bacterial species in the DC85 group compared to the CON group. Violin plots show the frequency distribution of the data, with lines at the median and quartiles. P values according to multiple t tests, corrected for multiple testing with the Benjamini-Hochberg method to control for the false discovery rate (FDR). PD, phylogenetic diversity; OTU, Operational taxonomic unit.

changes in gut microbiota that may have been induced by 85% dark chocolate consumption. First, we compared alpha diversity between groups as measured by Faith's phylogenetic diversity index (Faith's PD), number of OTUs, and Shannon's H index. Differences in the alpha diversity of the gut microbial community between baseline and the end of the intervention were measured and compared between the DC85 and CON groups (Fig. 2A-C). We found that Faith's PD and number of observed OTUs were significantly increased in the DC85 group compared to the CON group (P=.017 and P=.003, respectively; Fig. 2A, B), but Shannon's H index showed only a slight increase in the DC85 group, with no significant difference compared to the CON group (P=.377, Fig. 2C). These results indicate that daily intake of 85% dark chocolate increases the diversity of gut microbial communities.

Table 2
Positive and negative affect schedule scores

	CON (n=14)			DC85 (n=18)DC70 (n=16)						P value†	
	Before	After	Δ(After-Before)	Before	After	Δ(After-Before)	Before	After	Δ(After-Before)	Δ(After-Before)	
Positive affect	21.64±4.86[a]	23.86±4.04[a]	2.21±3.81	28.61±7.29[b]	28.7±5.13[b]	0.11±5.11	28.61±7.29[b]	28.72±5.13[b]	0.63±5.95	.498	
Negative affect	27.07±6.92	28.93±7.36[a]	1.86±6.22[a]	23.83±7.20	19.50±4.91[b]	-4.33±5.91[b]	25.00±8.17	22.69±7.11[b]	-2.31±6.85[a]	.029	

Values are mean ± SD. CON, control group; DC85, 85% cocoa chocolate group; DC70, 70% cocoa chocolate group. †P value by Kruskal-Wallis test or one-way ANOVA with Dunnett's multiple comparisons test. Different superscripts indicate a significant difference between groups in the same column.

3.4. Effects of dark chocolate intake on gut microbiota composition

Next, to further investigate the specific bacterial taxa that were significantly affected by dark chocolate consumption, we compared the composition of the gut microbiota between the CON and DC85 groups at various taxonomic levels. At the phylum level, there were no significant differences between groups (data not shown). However, the ratio of the two most abundant phyla in the human gut microbiome, the *Firmicutes*-to-*Bacteroidetes* ratio (F/B ratio), was slightly reduced in the DC85 group compared to the CON group, although the difference was not significant (*P*=.421, Fig. 2D). At the species level, the relative abundance of *Blautia obeum* and *Faecalibacterium prausnitzii* were significantly different in the DC85 group compared to the CON group (both *P*=.042, Fig. 2E-F). Taken together, these results identify the effects of dark chocolate consumption on compositional changes in gut microbiota.

3.5. Association between the gut microbiota and host mood status

To investigate the link between the gut microbiota and host mood, we conducted Spearman's correlation analysis between bacterial diversity and significantly altered taxa and PANAS scores (Fig. 3A). Positive affect score was positively associated with the F/B ratio (r = -0.425, *P*=.017; Fig. 3A), although there were no significant changes in positive scores in the DC85 group. We assume that this was because of the response bias (social desirability bias) shown in the CON, which resulted in a minor increase in the positive affect score, ultimately contributing to its positive association with the F/B ratio. Moreover, negative affect score was negatively correlated with the number of observed OTUs (r = -0.402, *P*=.025; Fig. 3B). In addition, among the bacterial taxa that were significantly altered by dark chocolate consumption, the relative abundance of *Blautia obeum* was negatively associated with negative affect score (r = -0.383, *P*=.034; Fig. 3C), although *Faecalibacterium prausnitzii* levels were not significantly correlated with mood status. These results suggest that the mood-altering effect of 85% dark chocolate consumption may be mediated by changes in the diversity and abundance of intestinal bacteria.

4. Discussion

Dark chocolate has long been recognized by psychologists for its mood-altering properties, which include increasing feelings of pleasure and enjoyment [11]. However, the previous evidence regarding the positive affect of chocolate on mood was obtained under experimentally induced stressful conditions [9–12], and the results of different experiments were sometimes inconsistent. Thus, there is limited evidence on the emotional effects of dark chocolate intake in everyday life. Additionally, the molecular and physiological changes corresponding to the mood-altering effects of dark chocolate intake have not yet been explored. Relatively recent studies have shown that gut microorganisms have a wide range of biological effects, including effects on brain function and behavior, via the gut-brain axis [27,28]. In fact, a few human studies demonstrated that humans with altered gut microbiota exhibited certain psychiatric changes that were related to modulation of the gut-brain axis [29,30]. Therefore, with special reference to the gut-brain axis, the present randomized controlled trial was conducted to examine whether dark chocolate consumption influences mood in healthy adults and whether changes in mood are associated with regulation of the gut microbiota.

In the present study, daily intake of dark chocolate significantly reduced negative affect in the 85% cocoa chocolate group (DC85), but not in the 70% cocoa chocolate group (DC70). Based on the results of a previous study, it is likely that the effects of cocoa

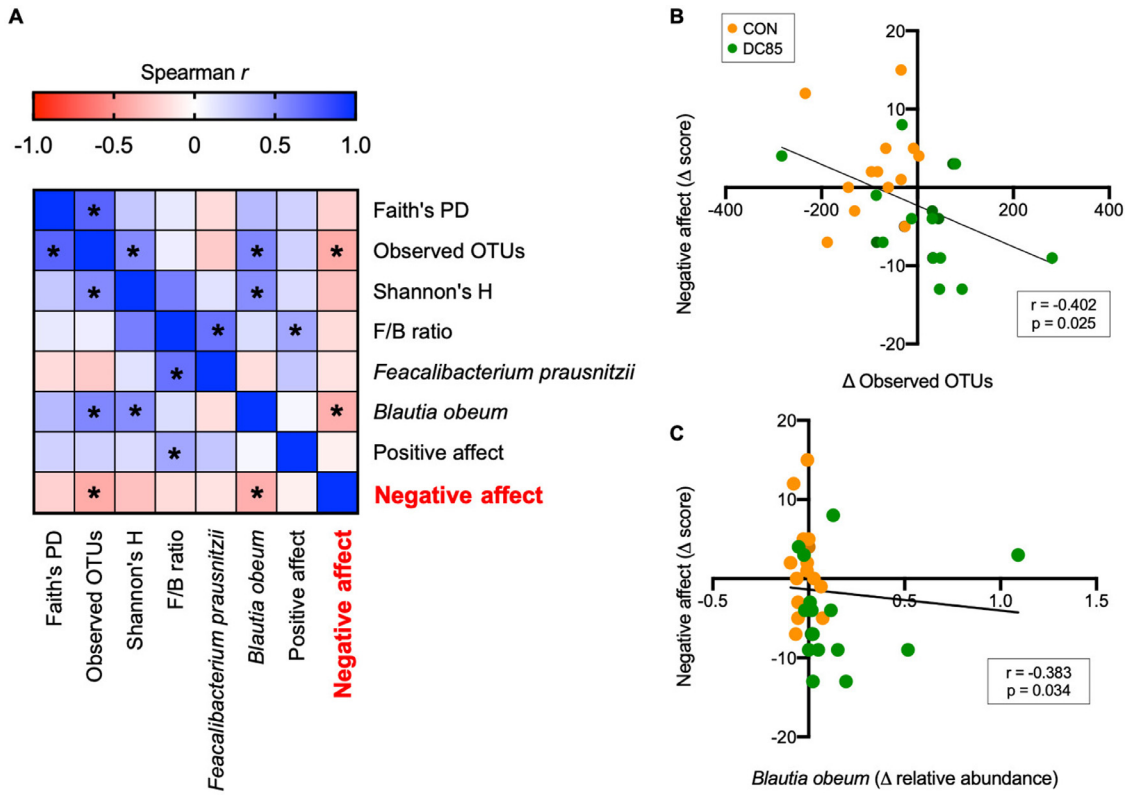

Fig. 3. Correlation of changes in gut microbial diversity and taxa with mood state**.** (A) Heatmap representing the results of Spearman's correlation analysis showing the correlation between gut microbial indices and mood scores in the DC85 group (85% cocoa chocolate) compared with the CON group (control). *$P<.05$ by Spearman's correlation analysis. Correlation of (B) changes in the bacterial diversity index (number of observed OTUs) and (C) relative abundance of specific bacterial taxa (*Blautia obeum*) that were significantly regulated by dark chocolate consumption with changes in negative affect scores. The Spearman's correlation coefficient (r) and level of significance (p) are indicated within each graph. Each point in the graphs represents data from an individual subject (between time points: after - before the intervention). The orange and green circles represent the CON (control) and DC85 (85% cocoa chocolate) groups, respectively.

on brain function differ depending on the dose of cocoa administered [31,32]. One randomized, placebo-controlled trial showed that high-dose cocoa polyphenol treatment (500 mg of polyphenols) significantly enhanced positive mood, while mood remained unaffected under low-dose treatment (250 mg of polyphenols) [32]. In the present study, in agreement with previous findings, the DC85 group, in which participants consumed approximately 400 mg of polyphenols per day, exhibited remarkable effects on mood compared to the DC70 group, which was treated with 250 mg of polyphenols per day. Therefore, we further investigated the impact of dark chocolate consumption on the gut microbiota in the DC85 group only.

A mounting body of evidence indicates that gut microbial diversity indices, including richness and evenness, may serve as markers of disease status [33]. Few studies have shown that reduced bacterial diversity increases the susceptibility to several diseases, including inflammatory bowel disease, major depressive disorder, and anxiety disorder [34,35]. A species-rich microbiome is stable against invasion by exogenous pathogens, resulting in a resilient gut environment [33]. Gut microbial diversity and composition are also associated with emotional well-being. For example, increased gut microbial diversity has been associated with higher positive emotions and a lower level of loneliness [36,37]. In line with previous research, the current findings highlight that increased microbial diversity by dark chocolate consumption positively influences the emotional status of healthy adults.

Individual bacterial taxa also serve as markers of the host's emotional status [33]. In the present study, we observed a lower abundance of *Faecalibacterium prausnitzii* in the DC85 group. Similarly, one study reported a negative correlation between the abundance of *Faecalibacteirum* and mood [67]. Moreover, our results showed that the DC85 group had a greater abundance of *Blautia*, which was significantly associated with positive changes in mood scores. It has consistently been reported that the microbiota of healthy controls are enriched in *Blautia* in comparison to those of patients with psychiatric disorders such as MDD, autism, and schizophrenia [33,34]. Moreover, pharmacological inhibition of inflammasome signaling in stressed animals increases *Blautia* spp. in a manner that is compatible with a rebalancing of the gut microbiota, indicating that *Blautia* could serve as a microbial target in the gut microbiota-brain axis in psychiatric disorders [35]. *Blautia obeum* produces butyrate [36], a recognized modulator of brain function. Butyrate is known to exert antidepressant-like effects in mouse models [37] and reverse depressive behaviors in rats [38]. We, therefore, suggest that the change in microbiota-derived butyrate may contribute to the positive changes in mood scores in the DC85 group.

The available evidence suggested that the mind-altering effects of cocoa may originate from a variety of polyphenolic compounds in dark chocolate [36,37]. A large epidemiologic study suggested that high dietary intake of polyphenols is inversely associated with depressive symptoms [38]. In addition, a systematic review concluded that dietary polyphenol and isoflavonoid intake is

negatively associated with depressive symptoms [5]. Some putative mechanisms have been suggested, which include the antioxidant and cortisol-lowering effects of polyphenols [37]. For example, catechin and epicatechin, the predominant flavonoid compounds in cocoa, can cross the blood-brain barrier and exert neuroprotective effects via antioxidative activity [39]. In addition, a polyphenol-rich chocolate intervention in adults reduced the level of salivary cortisol, which is known to regulate the hypothalamic-pituitary-adrenal (HPA) axis [40]. Since the HPA axis plays a role in stress-related syndromes such as anxiety and depression [41,42], the reduction in cortisol induced by chocolate consumption may contribute to the mood-regulating effects of cocoa. Furthermore, the bioavailability of polyphenols is determined by the activity of commensal bacteria [43,44]. The majority of dietary polyphenols are present as esters or polymers which have to be transformed to bioactive phenolic compounds by intestinal enzymes or gut microorganisms [43,44]. Thus, intestinal bacteria play a primary role in the abovementioned neuroprotective effects of polyphenols [43,44]. Moreover, polyphenol metabolism by gut bacteria modifies the gut microbial community and the functions thereof, which in turn exerts health benefits in the host [44]. For instance, intake of dietary polyphenols increases *Bacteroidetes* levels, reducing the F/B ratio, as shown in the present study [45]. Collectively, given the role of the gut microbiota in polyphenol bioavailability and metabolism as well as brain function, our findings suggest that daily intake of polyphenol-rich chocolate gradually alters gut microbial diversity, resulting in beneficial impacts on the host's mood.

Use of prebiotics (as a food for gut bacteria) or probiotics that target the gut-brain axis can reportedly ameliorate or eliminate the symptoms of certain psychiatric disorders [46–50]; thus, it is becoming evident that maintenance of gut microbial symbiosis is vital for brain health [46,47]. In the present study, along with changes in mood states, we demonstrated that the *Firmicutes*-to-*Bacteroidetes* ratio was slightly reduced in the DC85 group, though not significantly. *Firmicutes* and *Bacteroidetes* are two major phyla in the gut microbiota, and they may have important roles in the compositional and functional stability of the human intestine [51]. Thus, the F/B ratio has been extensively studied as a microbial marker in association with obesity and other diseases. In particular, several studies showed that patients with major depressive disorder (MDD) had a higher abundance of *Firmicutes* than healthy controls, corresponding to a reduced F/B ratio in MDD [51,52]. These results support our findings that a reduction in the F/B ratio may be involved in positive changes in the composition of the gut microbiome and amelioration of negative emotional states in the DC85 group.

This study has certain limitations. For example, we did not blind the study subjects in the control group; however, the outcomes were objective since the research staff was blinded to treatment allocation to minimize bias. The lack of appropriate placebo controls is another limitation of the current study and should be considered in future research. In addition, the results may be subject to response bias since mood status was self-reported by the participants, although the PANAS test used in the present study is widely used and its reliability and validity have been well-demonstrated [53,54]. Finally, the underlying mechanism by which gut bacteria affected mood status is unclear. This encourages future studies that examine gut-brain signaling following a period of dark chocolate consumption.

Despite its limitations, to the best of our knowledge, this is the first study that provides evidence that dark chocolate consumption in everyday life influences physiological and psychological states. These results suggest that dark chocolate has prebiotic effects by restructuring the diversity and composition of the gut microbiome, which may in turn improve mood via the gut-brain axis.

Declarations of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

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CRediT authorship contribution statement

Ji-Hee Shin: Investigation, Data curation, Formal analysis, Software. **Chong-Su Kim:** Data curation, Formal analysis, Software, Visualization, Writing – original draft. **Jiah Cha:** Data curation, Formal analysis, Software. **Sojeong Kim:** Investigation. **Seokoh Lee:** Investigation. **Suyeon Chae:** Investigation. **Woo Young Chun:** Conceptualization, Methodology, Validation, Supervision. **Dong-Mi Shin:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing.

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