Although host-related factors are important for the formation of gut microbiota, environmental factors cannot be ignored

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Running Head: Factors influencing human gut microbiota

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ABSTRACT

The gut microbiome is essential to human health. However, little is known about the influence of the environment versus host-related factors (e.g. genetic background, sex, age, and body mass) in the formation of human intestinal microflora. Here, we present evidence in support of the importance of host-related factors in the establishment and maintenance of individual gut assemblages. We collected fecal samples (n = 249) from 44 Korean naval trainees and 39 healthy people living in Korea over eight weeks and sequenced the bacterial 16S rRNA genes. The following hypotheses were tested: 1) microbiome function is linked to its diversity, community structure, and genetic host-related factors, and 2) preexisting host-related factors have a more significant effect on gut microbiome formation and composition than environmental factors. For each individual, the difference between the initial gut microbiota and that after eight weeks was negligible even though the 44 naval trainees lived in the same area and received the same diet, the same amount of exercise, and the same amount of physical stress during the study. This suggests that host-related factors, rather than environmental factors, is a key determinant of individual gut microflora. Moreover, eight weeks of physical training and experiencing the same environmental conditions resulted in an increase in the species *Bifidobacterium*, *Faecalibacterium*, and *Roseburia* in most trainees, suggesting a healthier intestinal environment.

IMPORTANCE In order to understand the role of human gut microbiome, it is important to know how individual's gut microbiota are formed. In this study, we tested the host-related factors versus environmental factors to affect gut microbiome and found that the former have a more association. However, we also found that the controlled environment give an effect on the gut microflora as well. This study provides preliminary evidence that differences in the formation and diversity of gut microbiota within a population could be determined by host-related factors rather than environmental factors.

Introduction

The human body harbors complex and diverse microbial communities (about 10–100 trillion microbes), including bacteria, archaea, eukaryotes, and viruses. These communities may be described as the "normal flora" in healthy individuals (1–3). These organisms perform a variety of metabolic functions. For example, organisms residing in human and animal colons, with an estimated density of 10^{11}–10^{12} cells/g (4–6), have been implicated in niche-specific metabolic, defense, and reproductive functions (1). They also influence their hosts during homeostasis and disease development through existing immunological factors, coexisting interacting networks of other microorganisms, and environmental influences (7).

The human gut microbiota is a reservoir of microorganisms and is crucial to health (3) through its involvement in metabolic interactions (e.g., food decomposition and nutrient intake) (8, 9), drug metabolism (10, 11), energy production and storage (12), and protection against pathogens (13). The gut microbiome provides signals that influence the development of the host immune system and stimulate the maturation of immune cells (14, 15). However, the gut microbiota is not only associated with human well-being but also with human disease conditions, including metabolic diseases, growth disorders, mental illness (such as autism), and obesity (16–20). Consequently, gut microbes affect human physiology directly and indirectly (13). Moreover, abrupt changes to the delicate balance of the microbial assemblage can result in unexpected consequences.

Despite the importance of the gut microbiome to human well-being, very little is known about the interactions between the host and the intestinal microorganisms (21). Previous studies of host interactions with intestinal microbes found that gut

81 microorganisms play a fundamental role in nutrient metabolism by converting dietary
82 components that escape digestion and polymers excreted by the host into easily
83 accessible nutrients and other compounds, such as vitamins (22). Moreover, gut
84 microbiota regulate host immune system interactions. For example, Bacteroides fragilis
85 induces a specific IgA response that is dependent on commensal colonization factors
86 regulation of surface capsular polysaccharides and enhances epithelial adherence (23).
87 Considering that the in vivo production of IgA is necessary for single-strain stability,
88 mucosal colonization, and epithelial aggregation, this bacterial influence on the host
89 immune system is vital. Adaptive immunity has evolved to rely on an intimate
90 association with members of the gut microbiome (23).
91 Due to host-bacterial mutualism, humans have not needed to evolve some
92 metabolic pathways, such as the ability to harvest otherwise inaccessible nutrients (24).
93 Therefore, investigating factors that influence the diversity and composition of the
94 human gut microbiota increases our understanding of how these communities are
95 structured, and to predict their response to environmental change. Microbial
96 establishment in the human intestine begins at birth. Subsequently, the intestinal
97 microflora continues to develop through successive microbial communities, until the
98 microbial climax community colonizes the intestine. Moreover, due to co-evolutionary
99 interactions, microbes undergo further functional modifications (24). However, the
100 initial gut microbiome of an infant at birth is similar in composition to the mother's
101 (25). In addition to environmental and genetic factors (26–29), other factors, including
102 lifestyle, diet, stress, and probiotics (30–32) have also been implicated in controlling the
103 establishment of gut microbiota. However, it is still poorly understood whether

104 environmental or host-related factors play a dominant role in shaping the human gut
105 microbial community.

106 Most gut microbiome studies to date have focused on Westerners (mostly
107 Europeans and North Americans); therefore, there is a gap in our knowledge about the
108 gut microbiota of people from other parts of the world (33). In this study, we used next-
109 generation sequencing of bacterial 16S rRNA genes to analyze the intestinal bacterial
110 community in South Korean naval trainees during a time when the trainees were
111 experiencing relatively similar environmental conditions.

112 Our first objective was to determine whether host-related factors are linked to
113 the community structure and diversity of the intestinal microbiota of naval trainees.
114 Given the broad range of interactions that are commonly associated with gut microbiota,
115 we aimed to identify key functional processes. Glendinning and Free suggested that
116 these functions could range from providing additional metabolic functions to
117 modulating the immune system (34). Our second objective was to determine whether
118 preexisting host-related factors have a more significant effect on the formation and
119 composition of the intestinal microbial community than environmental factors. Turpin
120 et al. suggested that almost one-third of fecal bacterial taxa in the intestine are heritable
121 (35). Furthermore, ecological concepts like dispersal, species diversity and distribution,
122 community assembly and contamination, and genetic makeup may influence the
123 formation, development, and maintenance of the gut microbiota. Through this objective,
124 we sought to investigate which set factors, host-related or environmental, exerts a more
125 significant influence on the composition of the gut microbiota.

Results

The aim of our study was to determine whether host-related background or environmental factors play a more significant role in the formation and diversity of the intestinal microbial community. Therefore, we first identified the composition of the intestinal microbial communities of the OCS trainees, who had different host-related and environmental backgrounds, on the day they were admitted (T = week 0). After living together for eight weeks at the naval OCS and being exposed to the same environmental conditions (e.g., same diet, same physical exercise, and same sleeping regimes), their intestinal microbial communities were reanalyzed at T = 4 weeks and T = 8 weeks. As a control sample, intestinal microflora samples from healthy Koreans with different host-related and environmental backgrounds were also analyzed. Theoretically, if the host-related background is more significant in determining the intestinal microbiota, the initial composition and diversity of microbial communities of naval trainees would be maintained, with negligible differences between samples collected at the beginning and end of the experimental period, and samples would remain equally distant from each other (Figure 1a). However, if environmental factors are more significant in shaping the intestinal microbiota, then the composition of microbial communities would shift, the bacterial compositions between samples would become similar, and PCoA plots would show clustering of samples (Figure 1b).

From 132 fecal samples collected from the 44 naval trainees (OCS), we obtained 2218 OTUs at 97% similarity from 1.49 million reads. The average number of reads per sample was 38 443 (ranging from 27 652 to 48 155). Similarly, 117 stool samples were collected from 39 healthy Korean adults as the control group. We obtained 1927 OTUs at 97% similarity from 3.19 million reads. The average number of reads per sample was 27 102 (ranging from 7331 to 45 842).

Composition and diversity of the gut microbial community at week zero

Based on the demographic questionnaire and the FFQ, we compared characteristics of the control and experimental groups at the beginning of the experiment. We found no significant differences among most of the individuals except for age, regular exercise, recent weekly exercise time, and hours of sleep (Table 1). We then performed PCoA ordination based on weighted UniFrac distances to determine the beta diversity of the intestinal microbial communities of the experimental and control groups. The PCoA plot shows that both the experimental and control groups had distinct intestinal microbial communities, and all samples remained distant from each other (Figure 2a and 2b). The adonis function was used to determine whether host-related factors, such as sex, age, weight, height and BMI, and environmental factors, including exercise, sleep and daily nutrient intake, have a major effect on the composition of the intestinal microbial community. Adonis results show that the majority of environmental factors studied did not significantly affect the gut microbial community. Sex ($P = 0.006$, $R2 = 0.131$), height ($P = 0.031$, $R2 = 0.085$), weight ($P = 0.001$, $R2 = 0.174$), BMI ($P = 0.001$, $R2 = 0.079$), and number of bowel movements per week ($P = 0.005$, $R2 = 0.142$) show significant association with the intestinal microbiome in the control group. In the experimental group, sex ($P = 0.029$, $R2 = 0.064$), height ($P = 0.008$, $R2 = 0.081$), and weight ($P = 0.007$, $R2 = 0.089$) show significant association with the intestinal microbiome (Table 2). Alpha diversity was also calculated before admission to the navy center and was significantly higher in the experimental group than in the control group (Figure 2c–d). The Shannon index ($P = 0.010$, Mann Whitney test), which is the most commonly used alpha diversity index, shows an average diversity of 5.38 (3.91–7.14) for the control group, and 5.81 (3.88–6.84) for the experimental group. Phylogenetic diversity ($P = 0.016$, Mann Whitney test), which measures the phylogenetic relationship

between OTUs, has a median value of 27.08 (15.35–43.83) for the control group, and 29.43 (10.10–37.94) for the experimental group.

Composition and diversity of gut microbial community after eight weeks of training

In the experimental group, one host-related factor and majority of environmental factors show significant differences between the first day of admission to the OCS (T = week 0), four weeks (T = 4 weeks), and eight weeks later (T = 8 weeks). The factors that differed were BMI, exercise time, the number of housemates, and daily nutrient intake (carbohydrates, cholesterol, and fiber). However, a host-related factor (BMI) and environmental factors did not statistically differ in the control group (Table 3). The intestinal bacterial communities of the experimental group did not differ among the first day of admission to the OCS (T = week 0), four weeks (T = 4 weeks), and eight weeks later (T = 8 weeks). This finding is confirmed by the PCoA plot showing dissimilarities in bacterial communities and no clustering of samples according to the measured environmental factors (Figure 3a–b). The composition of bacteria in the intestines of trainees and ordinary people remain distinct, suggesting that host-related factors have a greater effect than environmental factors in determining the composition of the intestinal microbial community. Similarly, alpha diversity indices show no significant differences at the end of the experiment in experimental and control groups (Figure 3c–f). The Shannon index shows no significant change between 0, 4, and 8 weeks (control group: $F = 0.585$, $P = 0.559$, repeated-measures one-way ANOVA; experimental group: $P = 0.431$, Friedman test) and neither does the phylogenetic diversity (control group: $F = 0.504$, $P = 0.602$, repeated-measures one-way ANOVA; experimental group: $P = 0.084$, Friedman test).

Changes in the intestinal microbiota for each experimental period were measured using the distance matrix (Figure 4).

This study was designed to determine the distribution of the intestinal microbial community for each subject at each sampling time. The average distance between all samples was used to determine the degree to which the intestinal microflora of subjects differed at each time point (Figure 4a).

For the control group (Figure 4b), there is no significant change (P = 0.204, Kruskal-Wallis test) between T = 0 weeks (mean = 0.47, 0.38–0.71), T = 4 weeks (mean = 0.47, 0.37–0.60), and T = 8 weeks (mean = 0.45, 0.38–0.54).

The experimental group (Figure 4c) also shows no significant change (P = 0.238, Kruskal-Wallis test) between T = 0 weeks (mean = 0.30, 0.24–0.53), T = 4 weeks (mean = 0.31, 0.24–0.44), and T = 8 weeks (mean = 0.32, 0.25–0.53). Despite highly controlled environmental factors, the composition of the intestinal microbial community does not seem to be affected.

The distance matrix was again used to illustrate changes in the intestinal microbial community (Figure 5).

Changes in intestinal microflora over time were tracked for each subject (Figure 5a). The results showed that there is no significant difference (P = 0.154) in the individual distance matrix between T = 0 weeks and T = 4 weeks. The mean distance in the control group is 0.26 (ranging between 0.07 and 0.73), and in the experimental group, the mean distance measures 0.20 (ranging between 0.07 and 0.50), as shown in Figure 5b. Similarly, the individual distance matrices do not differ between the control group (mean = 0.24; 0.07–0.31) and the experimental group (mean = 0.20; 0.08–0.50) between T = 0 weeks and T = 8 weeks (P = 0.516), as shown in Figure 5c. However, between T = 4 weeks and T = 8 weeks, the individual distance matrices are significantly

different (P = 0.033) between the control group (mean = 0.23; 0.09–0.55) and the experimental group (mean = 0.19; 0.07–0.53), as shown in Figure 5d. This suggests that environmental factors do not exert major effects on the composition of the intestinal microbial community but they do have some influence.

Shifts in intestinal microbial communities after eight weeks

The majority of bacterial sequences recovered in our study belonged to the following phyla: Firmicutes (mean = 5413.70, 3104–6925) and Bacteroidetes (mean = 2319.19, 219–3343), which do not differ significantly between T = 0 weeks and T = 8 weeks.

The relative abundance of the top five groups (at the phyla, class, order, and family levels) was compared between T = 0 weeks and T = 8 weeks in the experimental group (Figure S1). The class Actinobacteria (P = 0.022), the order Bifidobacteriales (P = 0.026), and the families Bifidobacteriaceae (P = 0.026) and Ruminococcaceae (P = 0.019) increase significantly at T = 8 weeks. However, the phylum Proteobacteria (P = 0.003), the class Coriobacteriia (P = 0.0001), the order Coriobacteriales (P = 0.0001), and the family Lachnospiraceae (P = 0.042) decrease significantly at T = 8 weeks. At the genus level, *Bifidobacterium* sp. (P = 0.026), *Faecalibacterium* sp. (P = 0.045), and *Roseburia* sp. (P = 0.020) significantly increase at T = 8 weeks. However, *Blautia* sp. (P = 0.015), *Collinsella* sp. (P = 0.0001), and *Ruminococcus* sp. (P = 0.022) decrease significantly at T = 8 weeks (Figure 6). In the control group, intestinal microorganisms do not change significantly, unlike the experimental group (Figure S2).

Discussion

The primary goal of microbiome research is to elucidate the factors that determine microbial structure and composition within and upon a host (36). To this end, the current study demonstrated that variations in the formation and composition of the human intestinal microbiota are primarily determined by host-related factors rather than environmental factors. Previous studies (37, 38) support our findings. Moreover, the results of Kolde et al. suggest that host genetic factors are vital in maintaining gut microbiome interactions (39). Furthermore, it has been proposed that the genetic background of the host may influence the composition and formation of the intestinal microbial community.

One host-related factor (age) and some environmental factors, including regular exercise and exercise time, and sleep time, showed significant differences between the experimental and control groups (Table 1). In the case of the experimental group, we propose that these differences are because the subjects were enlisted as military officer candidates. Alpha diversity was significantly higher in the experimental group than in the control group (Figure 2c–d). This may be due to differences in specific environmental factors in the experimental group. According to Clarke et al. (40), athletes frequently have high intestinal microbial diversity. In addition, we found that similarity in intestinal microbial communities occurs more frequently when specific host-related factors (sex, height, weight, BMI) and an environmental factor (the number of bowel movements per week) are similar (Table 2). Our results mirror the findings of Oki et al. (41), and Haro et al. (42), who report that host-related and environmental factors have a notable effect on the formation of the intestinal microbiome in various cohorts. Compared with the control group, the experimental group was not significantly

different in BMI and the number of bowel movements per week. This is probably because the majority of individuals in both groups had similar values for these factors.

Hypothesis 1: The structure and diversity of the gut microbial community are linked to genetic host-related factors

The results reported in this study (Figure 3) support our hypothesis that host-related factors control the host gut microflora. Our results also showed that host-related factors had a considerable effect on the composition of the intestinal microbial community. This is shown in the PCoA plot, which clusters intestinal bacterial communities by individual subjects under a controlled environment. The results showed that samples remained distinct from each other after trainees shared identical environmental conditions for eight weeks (Figure 3b). Bacterial diversity showed the same pattern as community composition, with no significant changes at the end of the experimental period (Figure 3b). Together, these findings suggest that genetically-driven intrinsic factors exist that may organize the gut microbiota structure and participate in the formation of the microbial community. Although the cause-and-effect relationships behind these links have yet to be elucidated, host genetics does have an impact on host health (43). Although our results showed that the environmental influence on the gut microbiota in our cohort is limited, we suggest that greater interaction between genetic and environmental factors exists, which may contribute to the genetic influence observed in this study. Small et al. investigated the importance of genotype-environment interactions in understanding host genetic mechanisms and complex molecular relationships between hosts and their resident microbes (44). Similar studies using genetic techniques have demonstrated that the gut microbiota is influenced by both environmental and genetic factors (29, 45, 46). Genetic factors have been proposed to control the composition and diversity of gut microbiota under controlled

294 environmental conditions. However, genetics cannot be the only factor that influences
295 the intestinal microbial community because the intestine can only be colonized by
296 microorganisms present in the environment (47).

297 Our findings are also consistent with a study by Org et al. (48). They reported
298 that genetic background, under controlled environmental conditions, plays a
299 considerable role in the composition and diversity of the intestinal microbial
300 community. Host genes that are associated with the gut microbiota regulate diet-
301 sensing, metabolism, and host immunity (49). These background interactions may, in
302 turn, influence the nature and structure of the overall host gut microbiota. Other studies
303 have demonstrated that the host genetic background has a significant effect on the
304 composition of the gut microbial community. Henao-Mejia et al. (50) and Peng et al.
305 (51) showed that mice with diabetes or mutations in inflammatory signaling genes differ
306 in their gut microbial composition relative to wild-type mice. Functionalities associated
307 with the genome encoded by the gut microbiome assemblage expand the host's
308 physiological potential by increasing digestive capabilities, priming the immune system,
309 producing vitamins, degrading xenobiotics, and resisting colonization by pathogens
310 (49). Governing these host gene-gut microbiota interactions are ecological and
311 evolutionary consequences that often emerge from complex interspecies relationships
312 (44). In a related finding, Buhnik-Rosenblau et al. (52) reported that because the gut
313 microbiota is strongly associated with the health status of the host, its composition may
314 be affected by environmental factors, such as diet and maternal inoculation. Moreover,
315 the operational functions behind the host's genetic control over gut microbiota are
316 consistent with the broader effects of evolutionary divergence of the gut microbiota
317 composition (29).

Hypothesis 2: Preexisting host-related factors have a more significant effect than environmental factors on the formation and composition of the intestinal microbial community

Our hypothesis was again supported by our results because the abundance of certain types of microbial taxa was consistently and significantly associated with host-related factors. The relative abundance of the two most abundant microbial taxa (i.e., Bacteroidetes and Firmicutes) showed slight differences at the beginning and end of the experiment, with variation between study groups (Figure S1–S2). This finding is consistent with that reported by Richards et al. (53) when they sought to identify host genes responsible for microbiome regulation. Koliada et al. (54) also found that Bacteroidetes and Firmicutes are abundant in the gut microbiota of healthy obese individuals in Ukraine. However, in the present study, no enrolled subjects were obese at enrollment and did not have a history of obesity. This agrees with the findings of Clarke et al. (55) that Bacteroidetes and Firmicutes are found in both lean and obese individuals in varying proportions. However, the relative abundance of these taxa in the present study was not related to lean or obese status. Correlations between the relative abundance of gut microbes and genetic loci have been found in mice following the same diet (29, 45) and in humans with Crohn's disease (27). It is likely that variations in the gut microbiome are governed by molecular mechanisms, such as changes in gene regulation in the host epithelial cells that directly interface with the gut microbiota (53).

Environmental factors also affect intestinal microbial community formation

Controlling for environmental factors, such as regular sleep, a well-balanced diet, and steady exercise, the genera *Bifidobacterium*, *Faecalibacterium*, and *Roseburia* significantly increased in abundance by the end of the experimental period (Figure 6). *Bifidobacterium* is a significant component of gut microflora and plays a crucial role in

343 human health (56). Furthermore, *Bifidobacterium* regulates intestinal homeostasis,
344 modulates local and systemic immune responses, and protects against inflammatory and
345 infectious diseases (57, 58). The abundance of *Bifidobacterium* was found to
346 significantly increase in rats undergoing moderate exercise (59). *Bifidobacterium* is also
347 associated with a healthier status in adults; Aizawa et al. (60) reported a decrease in
348 *Bifidobacterium* in patients with severe depression relative to people without severe
349 depression. Similarly, *Faecalibacterium*, which promotes a healthy digestive tract by
350 producing butyrate and lowering the oxygen tension of the lumen (61), significantly
351 increased in our experiment. These results mirror the findings of Campbell et al. (62),
352 who reported an increase in *Faecalibacterium prausnitzi* in rats after physical exercise
353 and concluded that *F. prausnitzi* might protect the intestine through oxygen
354 detoxification by a flavin/thiol electron shuttle. *Roseburia* also plays a role in
355 maintaining intestinal health and immune defense systems (e.g., regulatory T cell
356 homeostasis) through the production of butyrate (63). The abundance of *Roseburia*
357 decreases in many intestinal diseases and has been used as an indicator of intestinal
358 health (64). These bacteria ferment insoluble fiber as an energy source (65). The
359 abundance of *Roseburia* decreases as the host intake of carbohydrate decreases (66) or
360 fat increases (67). In our results, carbohydrate intake significantly decreased after
361 training, but the abundance of *Roseburia* showed an opposite pattern. We propose that
362 the increased fiber intake during training produced these results. *Faecalibacterium*
363 *prausnitzii* and *Roseburia* are known as key organisms in the formation of a healthy
364 microbiota (19, 68).

365 Some taxa decrease significantly during training. For example, the abundance of
366 Proteobacteria had significantly decreased by the end of the study. Bacteria belonging to
367 the Proteobacteria phylum cause dysbiosis in the intestinal microflora (69). They are

368 found in many patients with irritable bowel disease and are known to cause
369 inflammation (70). Inflammation increases oxygen levels in the large intestine, which
370 reduces the absolute anaerobic bacteria that constitute most of the intestinal microflora,
371 resulting in dysbiosis (71). Our results indicated that, due to the controlled living
372 conditions imposed during training, the abundance of microorganisms that produce
373 short-chain fatty acids increased. Therefore, the internal anaerobic condition was
374 maintained, resulting in a decrease in Proteobacteria.

375 The abundance of bacteria of the genera *Collinsella* and *Ruminococcus*
376 decreased by the end of the study. *Collinsella* was increased in obese pregnant women
377 with low fiber diet group (72). It is also reported that this genus increases as
378 carbohydrate intake decreases (73). Diet changes during training could explain the
379 decrease in *Collinsella*. *Ruminococcus* showed the same pattern as *Collinsella* because
380 *Ruminococcus gnavus* and *R. torques* decreased significantly during training (Figure
381 S3). This result suggests that the controlled living routine of the OCS training increases
382 the abundance of bacteria that have a beneficial effect on the host and reduces the
383 abundance of bacteria that have a harmful effect. *Ruminococcus* is an enterotype that
384 enters the intestine (74) and has the ability to ferment complex carbohydrates, such as
385 cellulose, pectine, and starch (73, 75), and is a producer of acetate and propionate (76,
386 77). The *Ruminococcus* genus is quite heterogeneous, including both beneficial and
387 harmful species. For example, *Ruminococcus bromii* is known to exert beneficial effects
388 on health (77), whereas other *Ruminococcus* species are proinflammatory (78, 79).
389 Recently *Ruminococcus gnavus* and *R. torques* are reported to be associated with
390 allergic diseases, Crohn's disease in infants, and autism spectrum disorders (80–82).
391 Overall, the increases in *Bifidobacterium*, *Faecalibacterium*, and *Roseburia* observed in
392 our study indicate an improvement in the health of the intestinal environment. The

393 effects of dietary changes on gut metabolism due to the strict naval training regime that
394 the subjects of this study followed may have caused the proliferation of these bacterial
395 taxa. This has previously been noted by Thomas et al. (83), who investigated the effects
396 of inter-individual microbiota differences, focusing on the presence or absence of
397 keystone species involved in butyrate metabolism.

398 Further studies in large population-based cohorts are necessary to gain a greater
399 understanding of the relationship between the host genetic profile, gut microbiome
400 composition, and host health (39, 43). Moreover, it will be important to map the loci
401 that control microbiota composition and prioritize the investigation of candidate genes
402 to improve our understanding of host-microbiota interactions (48).

403 The present work is a preliminary study showing that differences in the
404 formation and diversity of intestinal microbial communities within a population are
405 primarily determined by host-related factors rather than environmental factors. Long-
406 term experiments, more controlled environmental conditions, and more detailed
407 metadata are required to elucidate the factors that control the function of the gut
408 microbiota. However, it is challenging to standardize human environmental conditions
409 and to ignore the influence of unique lifestyle factors on a community. The results
410 reported here support findings from recent studies (29, 36, 44, 48) suggesting that the
411 microbiome depends more on host-related factors and less on related environmental
412 factors for its variation, composition, and control. Finally, according to Kolde et al. (39)
413 and Richards et al. (53), manipulating the microbiome alter the expression of the host
414 genes. Therefore, knowledge of healthy microbiota for each genetic types suggests
415 future therapeutic routes for human wellness.

Materials and Methods

Sampling

The present study was approved by the Institutional Review Board of Kyungpook National University (KNU 2017-84), and Armed Forces Medical Research Ethics Review Committee (AFMC-17-IRB-092), Republic of Korea. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Fecal samples were collected from 44 trainees of the naval officer candidate school (OCS) on the first day of enlistment (week 0) and at four and eight weeks after their admission to the naval center. The trainees lived in the same environment for eight weeks, ate the same food at regular intervals, and participated in similar training and sleeping regimes. Fecal samples were also collected from 39 healthy people living in Korea at the same sampling points as the OCS as controls. All samples were collected by participants using Transwab tubes (Sigma, Dorset, UK) and sent to the laboratory, where they were stored at −80 °C until DNA extraction.

Data Collection

Participants were asked to complete a self-administered questionnaire to collect demographic, lifestyle, and physical activity data at weeks 0, 4, and 8. Dietary consumption was assessed by a food frequency questionnaire (FFQ) used in the 2017 Korea National Health and Nutrition Examination Survey conducted by the Korea Centers for Disease Control and Prevention. The FFQ was completed by the control and the experimental groups at week 0 and in the experimental group at weeks 4 and 8. Reported intakes below 500 kcal/d or >5,000 kcal/d for controls and >6,000 kcal/d for trainees were determined inaccurate and excluded from further analyses. The OCS provided naval trainees' menus which were analyzed for nutrient content using the

computer-aided nutritional analysis program (CAN Pro 5.0, Korea Nutrition Society). The daily nutrient intake for foods consumed by trainees that were not available in CAN Pro 5.0 were calculated by referencing the Korean Food Composition Database, Version 9.1 (2019, Rural Development Administration). Meals were served buffet style, thus the analyzed nutrients were based on the ideal diet intake for trainees. Five day menus immediately prior to each naval trainees' fecal collection date were used to estimate mean daily nutrient intake for weeks 4 and 8 of the naval trainees.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from approximately 500 μL (wet weight) of each sample using QIAamp PowerFecal DNA Isolation kits (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted DNA was assessed for quality by electrophoresis and was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). DNA isolated from each sample was amplified using the universal primers, 515 F (5′-barcode-GTGCCAGCMGCCGCGGTAA-3′) and 907 R (5′-barcode-CCGYCAATTCMTTTRAGTTT-3′), targeting the V4-V5 regions of prokaryotic 16S rRNA genes. The barcode is an eight-base sequence unique to each sample. PCR experiments were performed under the following conditions: 95 ºC for 5 min, 5 cycles of 57 ºC for 30 s and 72 ºC for 30 s, and 25 cycles of 95 ºC for 30 s and 72 ºC for 30 s. PCR was performed in duplicate in 24 μL reaction volumes, consisting of 20 μL Emerald AMP GT PCR 1X Master Mix (Takara Bio, Shiga, Japan), 0.5 μL (10 μM) of each barcoded PCR primer pair, and 3 μL of DNA template (10–50 ng DNA). PCR products were purified using an AMPure XP bead purification kit (Beckman Coulter, Brea, CA, USA) and pooled in equal concentrations. An Agilent 2100 Bioanalyzer

(Agilent Technologies, Santa Clara, CA, USA) was used to confirm the correct concentration needed for sequencing. Each amplified region was sequenced on an Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA) using a MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA), according to the manufacturer's protocols.

Sequence processing

Raw FASTQ files were processed using QIIME version 1.9.1[84] and quality filtered with Trimmomatic (85) using the following criteria: MINLEN:250 CROP:250. After chimera removal, all sequences were clustered into operational taxonomic units (OTUs) at 97% identity and classified against the SILVA database (v132) for 16S rRNA genes using the VSEARCH pipeline. To correct for differences in the number of reads, which can bias downstream analyses estimates, all samples were rarefied at an even sequencing depth of 7,331 reads per sample.

Statistical analysis

Alpha and beta diversities and phylogenetic diversity were calculated in QIIME. PCoA plots were generated using the weighted UniFrac distance to visualize the beta diversity. CALYPSO software was used for the interpretation and comparison of taxonomic information from 16S rDNA datasets (Davenport, 2016). The D'Agostino-Pearson Omnibus test was used to determine the distribution of data in RStudio 1.0.153 (https://www.rstudio.com/).

The Pearson chi-square test or Fisher's exact test was used to compare categorical variables. Statistical analysis was performed by repeated-measures one-way analysis of variance (ANOVA), ordinary one-way ANOVA, Friedman, and Kruskal-Wallis tests for multiple comparisons. Paired or unpaired Student's t-tests with Welch's

487 correction were used to analyze normal data, and a Wilcoxon matched-pairs signed-rank
488 test or Mann-Whitney U-test was used to analyze non-normal data. A p-value of < 0.05
489 was considered significant. Analyses were performed using Prism 8 software (GraphPad
490 Software, San Diego, CA).

491 Data were tested to determine whether diversity indices and relative abundances
492 of taxonomical groups were significantly different between samples collected at
493 different time points. Variations in the composition of microbial communities were
494 analyzed using the adonis function (a nonparametric method that is analogous to
495 ANOVA) with a weighted UniFrac distance and 999 permutations. All analyses were
496 performed using the "vegan" package in RStudio 1.0.153 (https://www.rstudio.com/).

497 ***Data Availability Statement***

498 The datasets generated during and/or analyzed during the current study are available
499 from the NCBI Sequence Read Archive database under accession numbers
500 PRJNA596059 (Experimental group) and PRJNA596112 (Control group).

501 ***Ethics Statement***

502 The present work was approved by the Institutional Review Board of Kyungpook
503 National University, South Korea (KNU 2017-84), and by the Korea Armed Forces
504 Medical Research Ethics Review Committee (AFMC-17-IRB-092).

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511 No potential conflicts of interest were disclosed.

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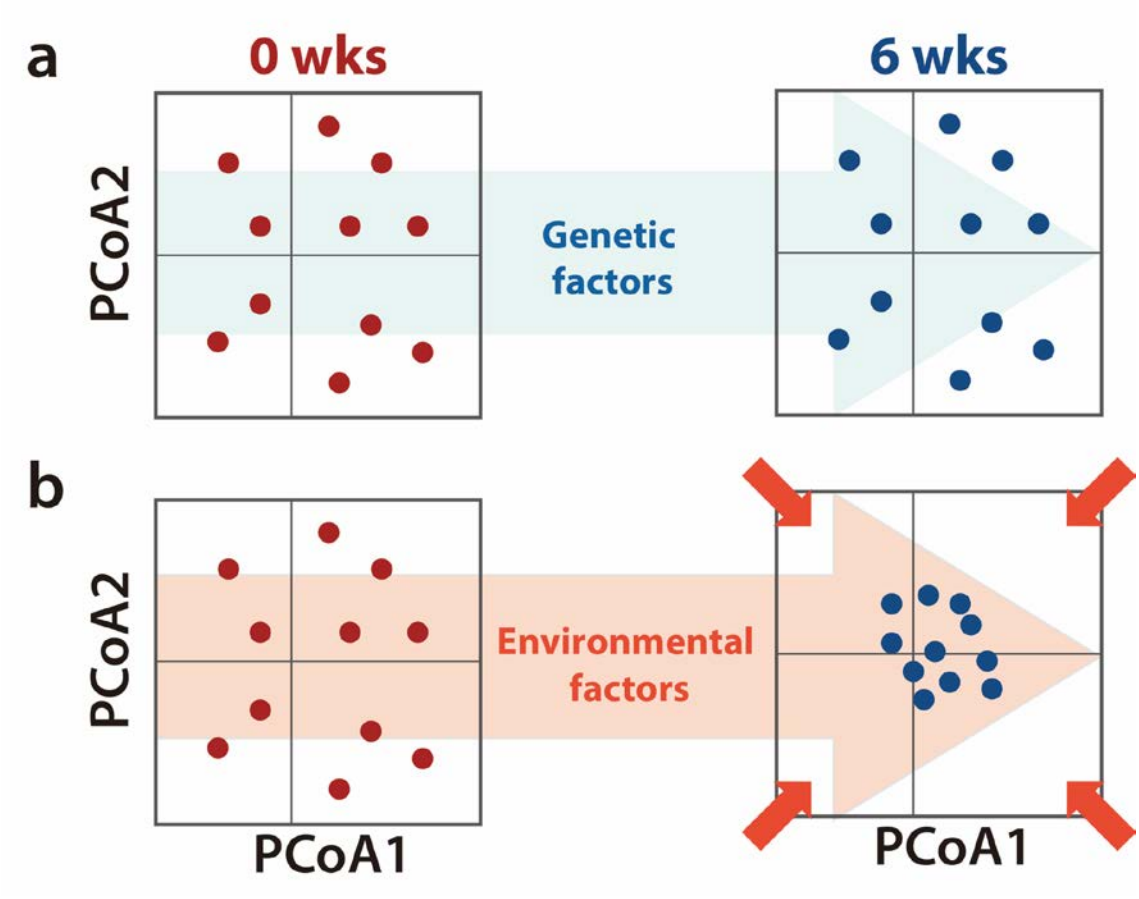
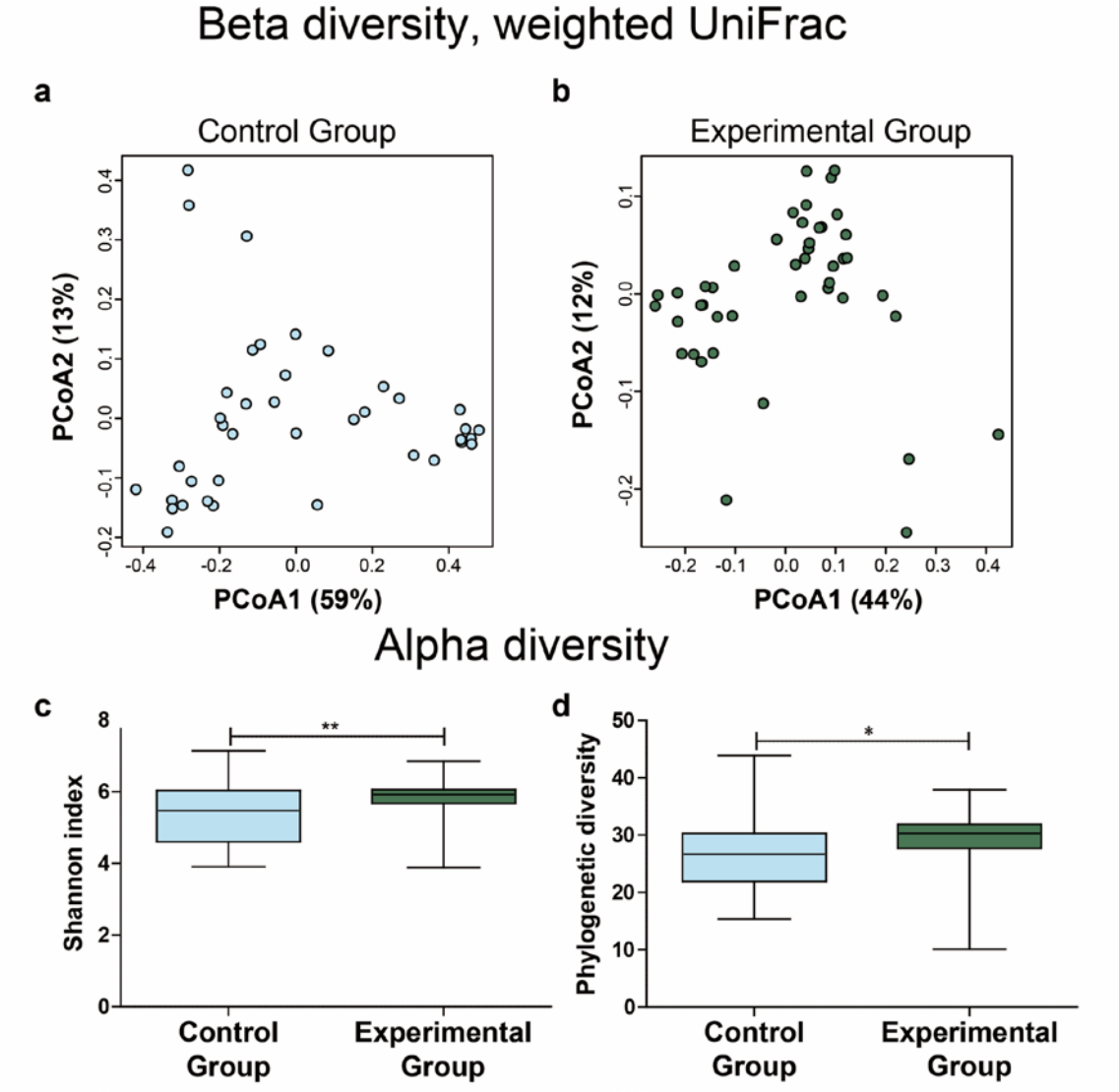


795

796 Figure 1. A schematic representation of how genetic and environmental factors affect
797 the composition and structure of the host intestinal microbial community differently. (a)
798 If genetic factors have a more significant effect, then the intestinal microbial community
799 in each individual is maintained, and samples remain distant from each other. (b) If
800 environmental factors have a more significant effect, then the intestinal microbial
801 community in each individual changes, and samples will cluster together.



802

803 Figure 2. Individuals have distinctive intestinal microbial communities. PCoA plots of
804 weighted UniFrac distances in (a) control and (b) experimental groups at T = 0 weeks.
805 The first and second principal components (PCo1 and PCo2) are plotted. The
806 percentage of variance in the dataset explained by each axis is reported. Variation of
807 bacterial alpha diversity indices in intestinal microbiota between control and
808 experimental groups: (c) Shannon index and (d) phylogenetic diversity. Data are shown
809 as mean ± SEM; *$p < 0.05$, **$p < 0.01$ by the Mann Whitney U test.

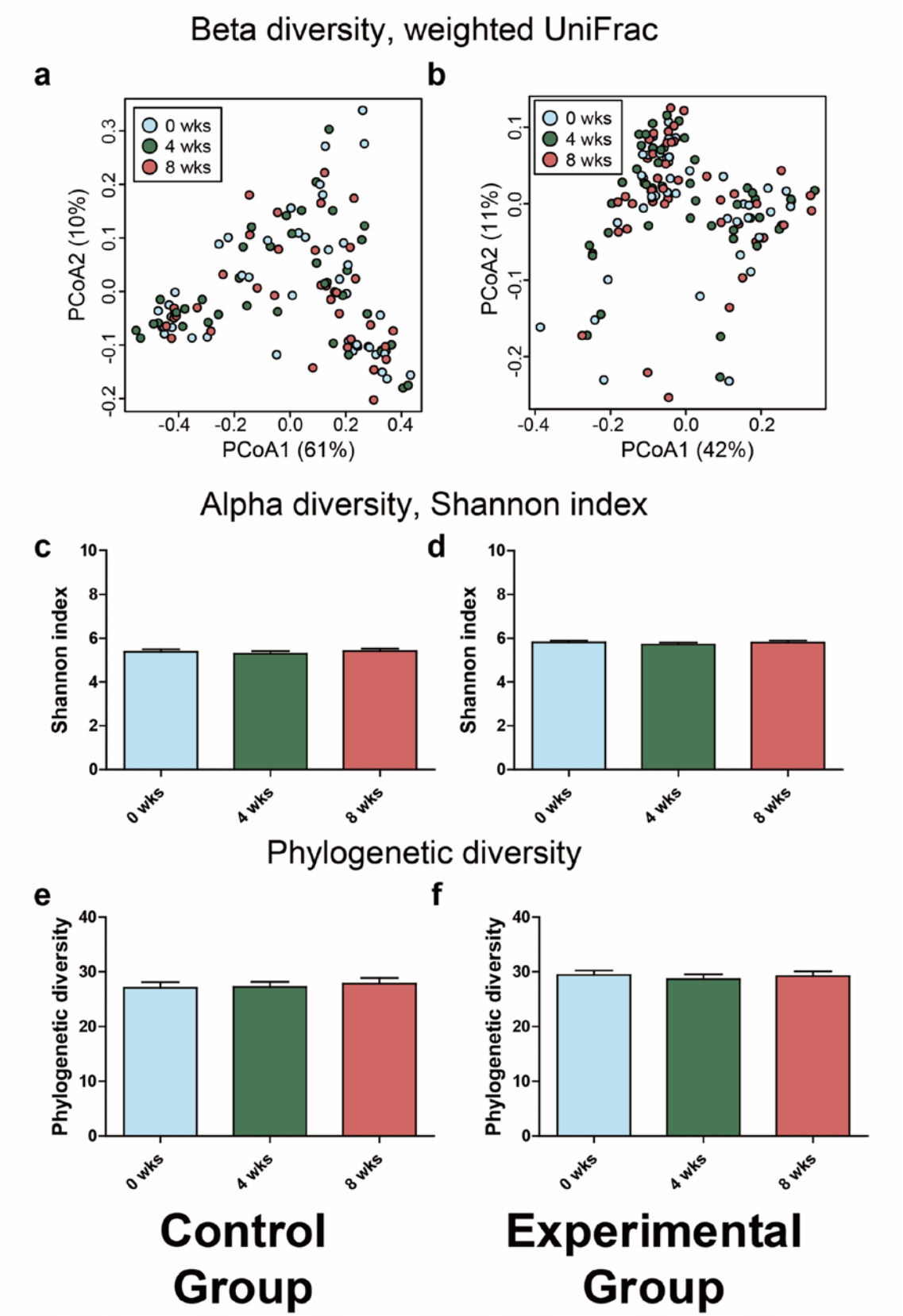
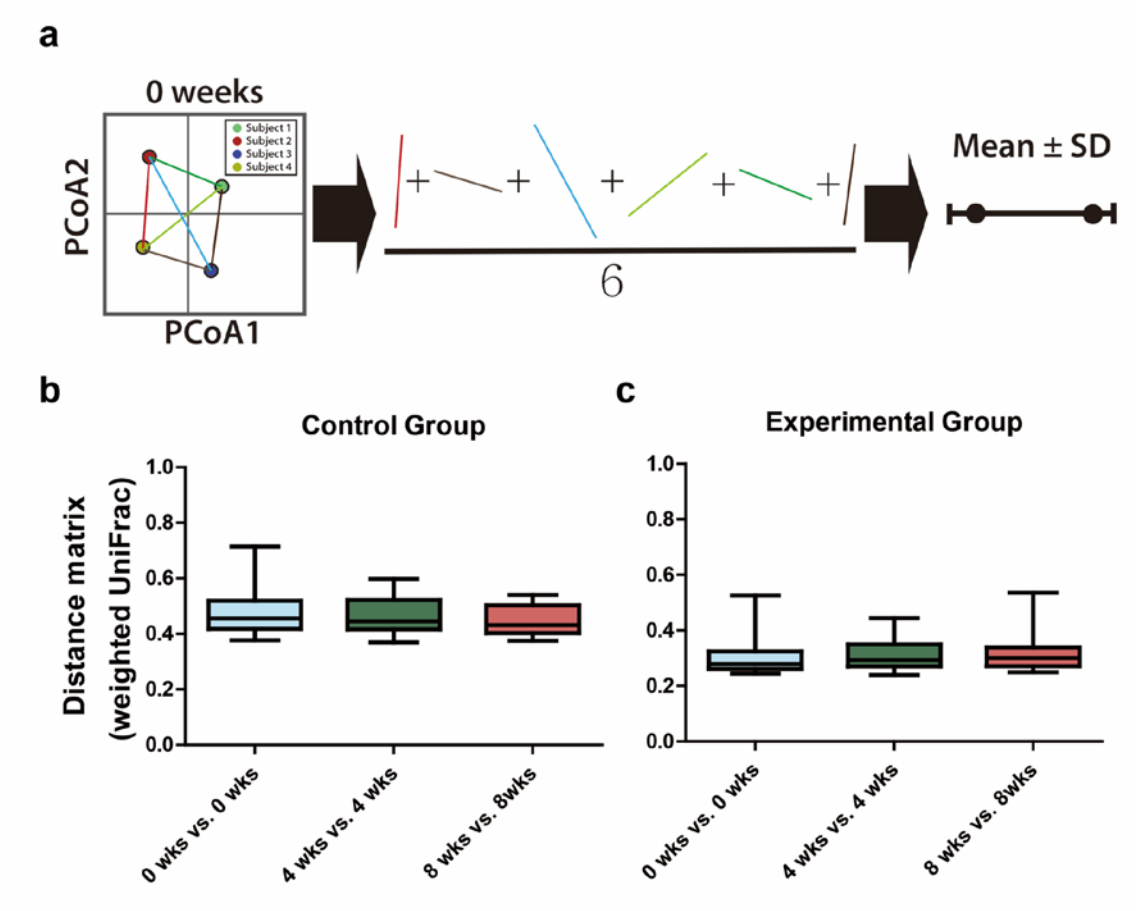


Figure 3. Environmental factors have negligible effects on the gut microbiome. PCoA plots of weighted UniFrac distances in (a) control and (b) experimental groups at T = 0

weeks, T = 4 weeks, and T = 8 weeks. Variation of bacterial alpha diversity indices in intestinal microbiota at T = 0 weeks, T = 4 weeks, and T = 8 weeks: Shannon index of (c) control and (d) experimental groups and phylogenetic diversity index of (e) control and (f) experimental groups. Data are shown as mean ± SEM. The alpha diversity of the control group was analyzed using repeated-measures one-way ANOVA. The alpha diversity of the experimental group was analyzed using the Friedman rank-sum test.



819

820 Figure 4. Even when environmental factors are controlled, individual intestinal
821 microbial communities are maintained. (a) A schematic representation of the average
822 weighted UniFrac distance on the composition of the intestinal microbial community.
823 The average distance between all samples was used to determine how differentiated the
824 intestinal microflora of subjects were at each time point. The average weighted UniFrac
825 distances among (b) control and (c) experimental groups within each time point are
826 shown in the box plot. Data were analyzed using the Kruskal-Wallis test.

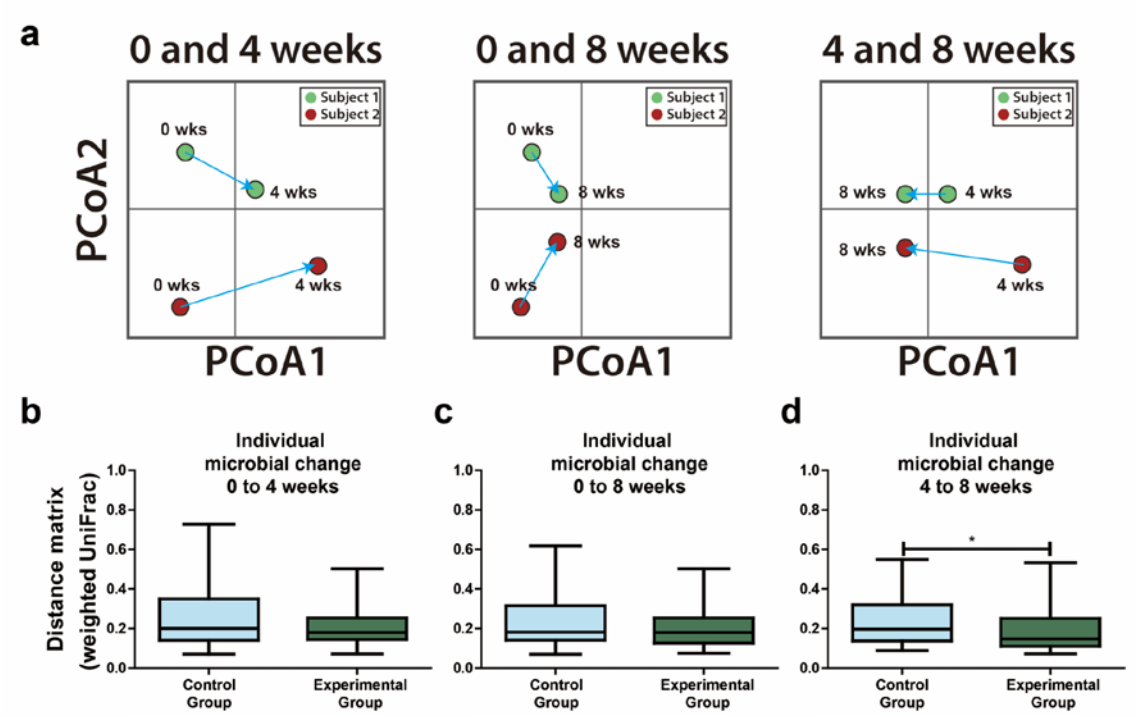


Figure 5. Regular living stabilizes the gut microbiome in a healthy direction. (a) A schematic representation of the variation in individual weighted UniFrac distances according to sampling time, explaining how individual intestinal microflora changes over time. Changes in gut microbiome between (b) T = 0 weeks and T = 4 weeks, (c) T = 0 weeks and T = 8 weeks, and (d) T = 4 weeks and T = 8 weeks of the control and experimental groups. *$p < 0.05$ by the Mann Whitney U test.

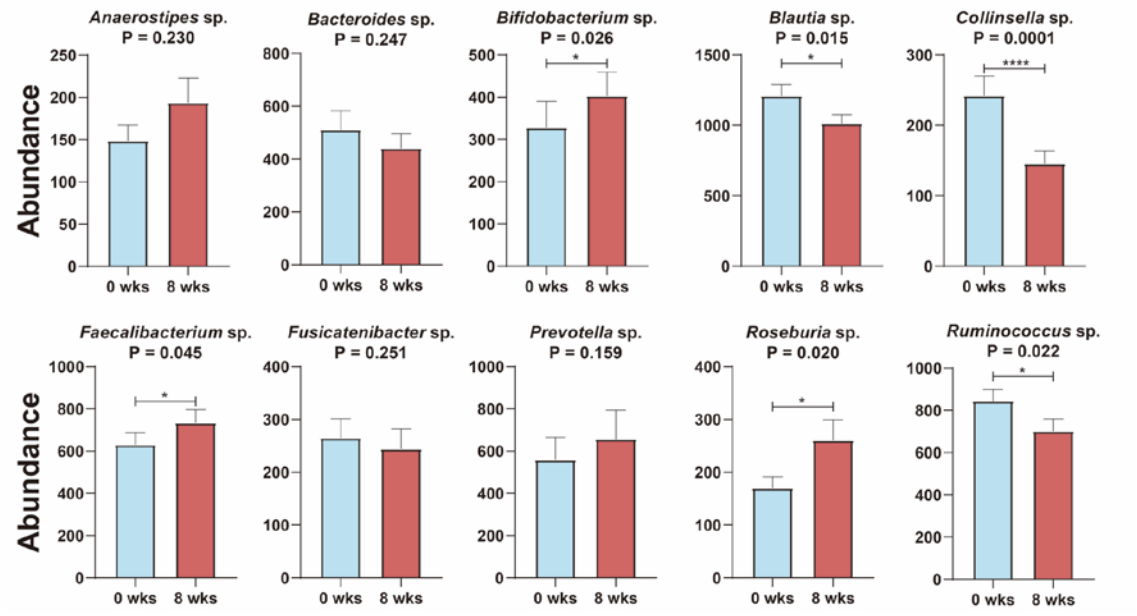
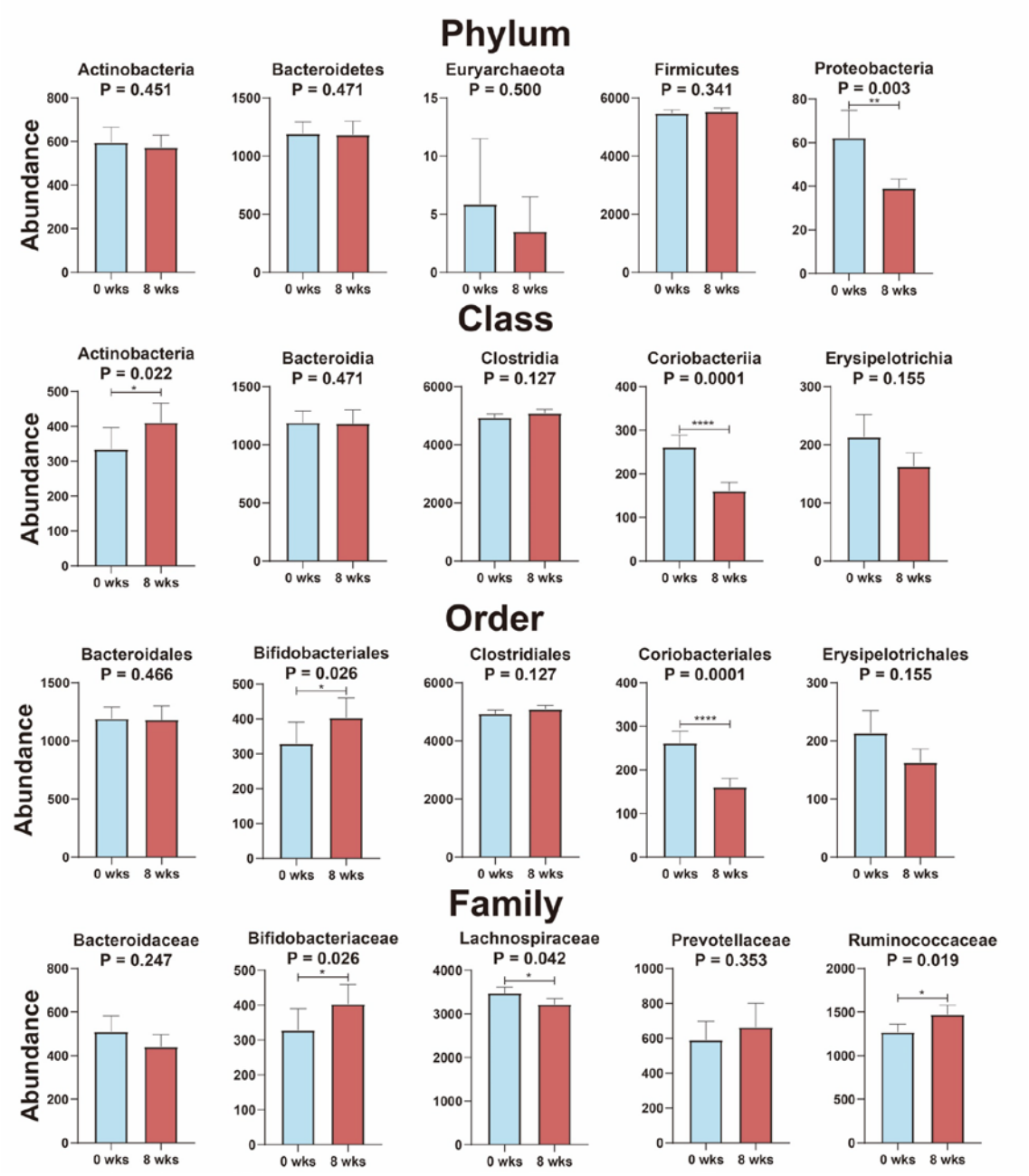


Figure 6. Controlled living conditions of the OCS training cause beneficial changes to the gut microbial community. Abundance of intestinal bacteria by family. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. *Blautia*, *Fusicatenibacter*, and *Ruminococcus* were analyzed using a paired t-test. *Anaerostipes*, *Bacteroides*, *Bifidobacterium*, *Collinsella*, *Faecalibacterium*, *Prevotella*, and *Roseburia* were using the Wilcoxon matched-pairs signed-rank test.



841

842 Figure S1. Comparison of the abundance of the detected phyla, classes, orders and
843 families in the intestine of experimental gorup at T = 0 week and T = 8 weeks. Data are
844 shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
845 Bacteroidetes, Firmicutes, Bacteroidia, Clostridia, Bacteroidales, Clostridiales,
846 Ruminococcaceae were using paired t-test. Actinobacteria (phylum), Euryarchaeota,
847 Proteobacteri, Actinobacteria (class), Coriobacteriia, Erysipelotrichia, Bifidobacteriales,
848 Coriobacteriales, Erysipelotrichales, Bacteroidaceae, Bifidobacteriaceae,

849 Lachnospiraceae and Prevotellaceae were using Wilcoxon matched-pairs signed-rank

850 test.

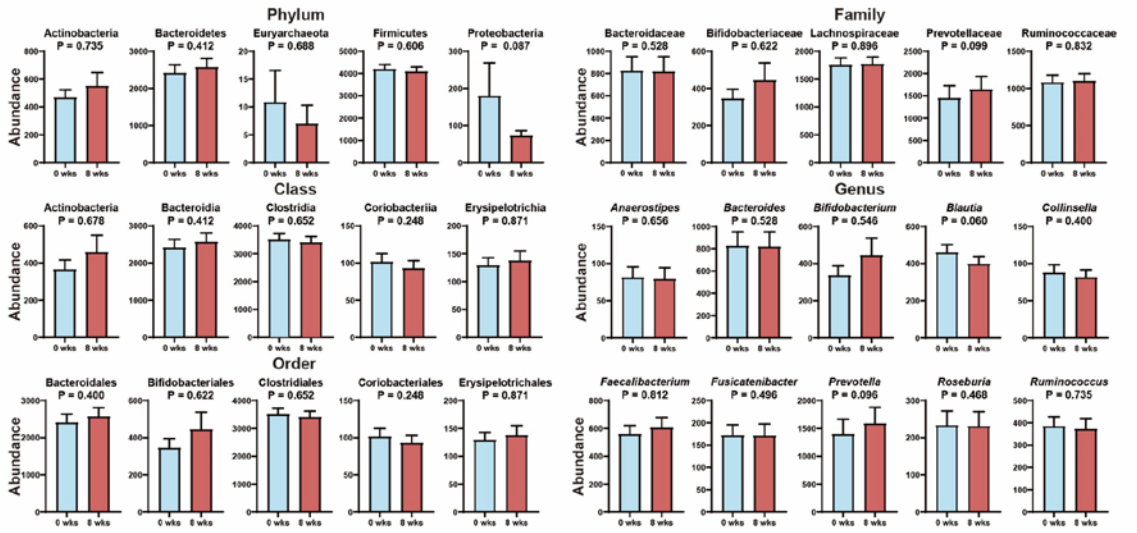


Figure S2. Comparison of the abundance of the detected phyla, classes, orders, families and genera in the intestine of control group at T = 0 week and T = 8 weeks. Data are shown as mean ± SEM. Bacteroidetes, Firmicutes, Bacteroidia, Clostridia, Bacteroidales, Clostridiales, Lachnospiraceae and Ruminococcaceae were using paired t-test. Actinobacteria (phylum), Euryarchaeota, Proteobacteri, Actinobacteria (class), Coriobacteriia, Erysipelotrichia, Bifidobacteriales, Coriobacteriales, Erysipelotrichales, Bacteroidaceae, Bifidobacteriaceae, Prevotellaceae, *Anaerostipes*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Collinsella*, *Faecalibacterium*, *Fusicatenibacter*, *Prevotella*, *Roseburia* and *Ruminococcus*.

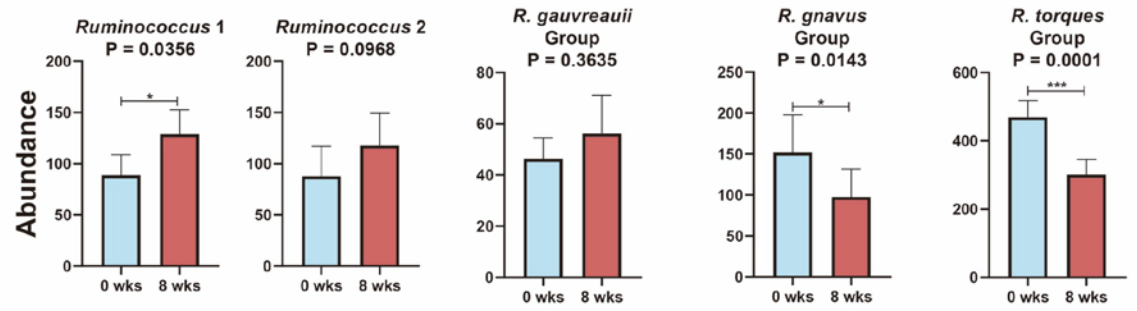


Figure S3. Comparison of gut microbiota within the genus *Prevotella* at T = 0 week and T = 8 weeks. *p < 0.05, **p < 0.01, ***p < 0.001 by Willcoxon matched-pairs signed-rank test.

865 Table 1. Baseline characteristics of participants in the study. Data are shown as
866 mean ± SEM; p-values are obtained by unpaired t-test (Welch's correction) or Mann-
867 Whitney U test (continuous variables) or chi-square test (proportions).

Characteristics	Control Group (n = 39)	Experimental Group (n = 44)	p-value
Host-related factors			
Sex, % (n)			0.056[c]
Male	58.97 (23)	77.27 (34)	
Female	41.02 (16)	22.72 (10)	
Age, (years)	29.92 ± 1.39	24.25 ± 0.30	<0.001[b]
Height, (cm)	169.54 ± 1.33	171.00 ± 1.08	0.400[a]
Weight, (kg)	66.21 ± 2.08	71.30 ± 1.71	0.063[a]
Body mass index (BMI)			0.679[c]
<18.50	5.13 (2)	2.27 (1)	
18.50-24.99	69.23 (27)	65.90 (29)	
25.00-29.99	25.64 (10)	31.82 (14)	
>30.00	0.00 (0)	0.00 (0)	
Environmental factors			
Do you Smoke?, % (n)			0.924[c]
Yes	28.20 (11)	27.27 (12)	
No	71.79 (28)	72.73 (32)	
Do you exercise regularly?, % (n)			0.019[c]
Yes	33.33 (13)	59.09 (26)	
No	66.66 (26)	40.91 (18)	
If you do regular exercise, what kind of exercise do you do?, % (n)			0.904[c]
Weight training	15.38 (2)	26.92 (7)	
Cardio	15.38 (2)	7.69 (2)	
Both	69.23 (9)	65.38 (17)	
How much time did you exercise during the past week?, (min)	96.03 ± 27.00	208.10 ± 37.34	0.008[b]
How many hours a day do you sleep?, (hr)	6.38 ± 0.21	7.69 ± 0.19	<0.001[b]
How is your sleep quality?, % (n)			0.212[c]
Very Bad	7.69 (3)	0.00 (0)	
Bad	10.26 (4)	6.82 (3)	
Normal	30.77 (12)	50.00 (22)	
Good	41.03 (16)	31.82 (14)	
Very Good	10.26 (4)	9.09 (4)	
No Answer	0.00 (0)	2.27 (1)	
How many people do you live with?, (n)	2.69 ± 0.24	3.25 ± 0.21	0.083[a]
Do you have pets?, % (n)			0.222[c]
Yes	12.82 (5)	22.72 (10)	
No	87.17 (34)	75.00 (33)	
No Answer	0.00 (0)	2.27 (1)	
How many times a week do you have a bowel movement?, (n)	5.49 ± 0.45	6.18 ± 0.24	0.052[b]
How do you feel after a bowel movement?, % (n)			0.880[c]
Bad	5.13 (2)	2.27 (1)	
Normal	35.90 (14)	40.91 (18)	
Good	48.72 (19)	45.46 (20)	
Very Good	10.26 (4)	11.36 (5)	
No Answer	0.00 (0)	0.00 (0)	
Daily nutrient intake[¶]			
Energy (kcal)	3377.48 ± 182.33	3924.18 ± 254.79	0.089[a]
Carbohydrate (g)	519.24 ± 29.66	584.84 ± 46.31	0.241[a]
Protein (g)	117.02 ± 8.40	138.24 ± 9.821	0.108[a]
Fat (g)	79.89 ± 6.48	95.52 ± 6.92	0.106[a]
Cholesterol (g)	473.82 ± 41.44	603.84 ± 68.07	0.112[a]
Fiber (mg)	28.11 ± 2.04	28.77 ± 2.09	0.822[a]

868 [¶] Daily nutrient intake were adjusted subjects number; control group (n = 28), experimental group (n =
869 21); FFQ non-responders were excluded, [a] p-values were obtained by the unpaired t-test (Welch's
870 correction), [b] p-values were obtained by the Mann-Whitney U test, [c] p-values were obtained by
871 the chi-squre test

872 Table 2. Adonis function results for host-related and environmental factors (T = 0
873 weeks) based on weighted UniFrac distance. Weighted UniFrac beta diversity analysis
874 was performed using the adonis function to identify significant differences in
875 microbiota composition.

Characteristics	Control Group (n = 39)		Experimental Group (n = 44)	
	p-value	R^2	p-value	R^2
Host-related factors				
Sex (male, female)	0.006	0.131	0.029	0.064
Age (years)	0.770	0.012	0.106	0.042
Height (cm)	0.031	0.085	0.008	0.081
Weight (kg)	0.001	0.174	0.007	0.089
Body mass index (BMI)	0.001	0.161	0.778	0.968
Environmental factors				
Smoker (yes, no)	0.050	0.071	0.821	0.012
Regular exercise (yes, no)	0.085	0.058	0.173	0.033
Recent weekly exercise time (min)	0.500	0.045	0.657	0.019
Sleep time (hr)	0.420	0.023	0.070	0.048
Quality of sleep (very bad, bad, normal, good, very good)	0.159	0.041	0.678	0.016
Number of housemates (n)	0.514	0.019	0.789	0.012
Raise pet (yes, no)	0.211	0.038	0.192	0.033
Number of bowel movements per week (n)	0.005	0.142	0.149	0.041
Feeling after a bowel movement (very bad, bad, normal, good, very good)	0.552	0.017	0.959	0.008
Daily nutrient intake[¶]				
Energy (kcal)	0.101	0.060	0.554	0.019
Carbohydrate (g)	0.178	0.045	0.701	0.015
Protein (g)	0.160	0.050	0.523	0.020
Fat (g)	0.164	0.046	0.488	0.020
Cholesterol (g)	0.121	0.055	0.492	0.020
Fiber (mg)	0.061	0.071	0.738	0.014

876 [¶] Daily nutrient intake were adjusted subjects number; control group (n = 28), experimental group (n =
877 21); FFQ non-responders were excluded

878 Table 3. Characteristics of participants at T = 0 weeks, T = 4 weeks, and T = 8 weeks.

879 Data are shown as mean ± SEM; p-values are obtained by repeated-measures one-way

880 ANOVA.

Characteristics	Control Group (n = 39)				Experimental group (n = 44)			
	0 wks	4 wks	8 wks	p-value	0 wks	4 wks	8 wks	p-value
Body mass index (BMI)	22.83 ± 0.48	22.73 ± 0.46	22.77 ± 0.47	0.996	24.22 ± 0.41	23.96 ± 0.38	23.25 ± 0.31	<0.001
Recent weekly exercise time (min)	88.33 ± 27.35	99.87 ± 25.52	89.23 ± 24.81	0.734	208.10 ± 37.34	3252.00 ± 32.35	1311.00 ± 24.43	<0.001
Sleep time (hr)	6.38 ± 0.21	6.51 ± 0.19	6.44 ± 0.19	0.835	7.69 ± 0.19	7.51 ± 0.10	7.63 ± 0.09	0.552
Number of housemates (n)	2.69 ± 0.24	2.39 ± 0.23	2.38 ± 0.22	0.543	3.25 ± 0.21	4.00 ± 0.00	4.00 ± 0.00	<0.001
Number of bowel movements per week (n)	25.44 ± 0.46	6.08 ± 0.56	6.09 ± 0.61	0.865	6.17 ± 0.24	5.55 ± 0.44	5.36 ± 0.28	0.083
Daily nutrient intake[¶]								
Energy (kcal)	3377.48 ± 182.33	3330.89 ± 182.47	3284.30 ± 189.63	0.209	3924.18 ± 254.79	3474.01 ± 11.03	3369.69 ± 24.78	0.055
Carbohydrate (g)	519.24 ± 29.66	512.84 ± 29.07	506.43 ± 30.12	0.367	584.84 ± 46.31	468.66 ± 1.08	464.84 ± 3.92	0.017
Protein (g)	117.02 ± 8.40	116.24 ± 8.43	115.45 ± 8.54	0.332	138.24 ± 9.82	139.70 ± 0.88	135.12 ± 1.36	0.694
Fat (g)	79.89 ± 6.48	78.08 ± 6.42	76.28 ± 6.53	0.091	95.52 ± 6.92	112.22 ± 0.54	104.29 ± 0.62	0.051
Cholesterol (g)	473.82 ± 41.44	466.13 ± 41.17	458.44 ± 41.25	0.052	603.84 ± 68.07	836.35 ± 11.31	733.68 ± 21.46	0.006
Fiber (mg)	28.11 ± 2.04	27.69 ± 2.02	27.26 ± 2.03	0.095	28.77 ± 2.09	37.99 ± 0.57	39.81 ± 0.62	<0.001

881 [¶] Daily nutrient intake were adjusted subjects number; control group (n = 28), experimental group (n =

882 21); FFQ non-responders were excluded