

**The impact of almonds and almond processing on gastrointestinal physiology, luminal microbiology and gastrointestinal symptoms: a randomized controlled trial and mastication study**

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**Running head:** Almonds and gut health

### **Conflicts of interest**

AC was funded by a PhD studentship funded by Almond Board of California. ED has received an education grant from Alpro, research funding from the British Dietetic Association, Almond Board of California, the International Nut and Dried Fruit Council and Nestec Ltd and has served as a consultant for Puratos. MR has received research funding from Almond Board of California, Danone, International Dried Fruit and Nut Council, is the co-inventor of volatile organic compounds in the diagnosis and dietary management of IBS, and co-founder of Bio&Me a gut health food brand. TG supervises PhD students partially funded by Mondelez and McCain Foods Ltd, and has previously received research funding from The Almond Board of California. SEB has received grant funding from Almond Board of California, Malaysian Palm Oil Board and ZOE Ltd, and receives consultancy and options from ZOE Ltd. KW has received research funding from Almond Board of California, Danone, International Dried Fruit and Nut Council, and is the co-inventor of volatile organic compounds in the diagnosis and dietary management of IBS. EH, CP, JMB, JRM and SMS declare no conflicts of interest.

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Data described in the manuscript, code book, and analytic code will be made available upon request pending application.

**Abbreviations:** Amplicon sequence variant, ASV; analysis of covariance, ANCOVA; analysis of variance, ANOVA; body mass index, BMI; bowel movement per week, BM/wk; Bristol stool form scale, BSFS; colonic transit time, CTT; gastric emptying time, GET; gastrointestinal symptoms rating scale, GSRS; ileo-cecal junction, ICJ; intention-to-treat, ITT; particle size distribution, PSD; per-protocol, PP; principle component analysis, PCA; quality of life, QoL; randomized controlled trial, RCT; short-chain fatty acids, SCFA; small bowel transit time, SBTT; volatile organic compounds, VOC; whole-gut transit time, WGTT; wireless motility capsule, WMC

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## Abstract

**Background** Almonds contain lipid, fiber and polyphenols and possess physicochemical properties that impact nutrient bioaccessibility, which are hypothesized to impact gut physiology and microbiota.

**Objective** Investigate the impact of whole almonds and ground almonds (almond flour) on fecal bifidobacteria (primary outcome), gut microbiota composition and transit time.

**Design** Healthy adults (n = 87) participated in a parallel, 3-arm randomized controlled trial. Participants received whole almonds (56 g/d), ground almonds (56 g/d) or an isocaloric control muffin in place of habitual snacks for 4 weeks. Gut microbiota composition and diversity (16S rRNA gene sequencing), short-chain fatty acids (gas-chromatography), volatile organic compounds (gas-chromatography mass-spectrometry), gut transit time (wireless motility capsule), stool output and gut symptoms (7-day diary) were measured at baseline and endpoint. The impact of almond form on particle size distribution (PSD) and predicted lipid release was measured in a subgroup (n=31).

**Results** Modified intention-to-treat analysis was performed on 79 participants. There were no significant differences in abundance of fecal bifidobacteria following consumption of whole almonds (8.7%, SD 7.7%), ground almonds (7.8%, SD 6.9%) or control (13.0%, SD 10.2%;  $q=0.613$ ). Consumption of almonds (whole and ground pooled) resulted in higher butyrate (24.1  $\mu\text{mol/g}$ , SD 15.0  $\mu\text{mol/g}$ ) in comparison to control (18.2  $\mu\text{mol/g}$ , SD 9.1  $\mu\text{mol/g}$ ;  $p=0.046$ ). There was no effect of almonds on gut microbiota at the phylum level or diversity, gut transit time, stool consistency or gut symptoms. Almond form (whole versus ground) had no effect on study outcomes.

Ground almonds resulted in significantly smaller PSD and higher predicted lipid release (10.4%, SD 1.8%) in comparison to whole almonds (9.3%, SD 2.0%;  $p=0.017$ ).

**Conclusions** Almond consumption has limited impact on gut microbiota composition but increases butyrate concentrations in adults, suggesting positive alterations to microbiota functionality. Almonds can be incorporated into the diet to increase fiber consumption without triggering gut symptoms.

**Keywords:** Almonds, gut microbiota, bifidobacteria, gut transit time, SCFA, Butyrate, mastication, particle size

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## Introduction

Diet is an important modifiable factor exerting a profound effect on the composition of the gut microbiota [1]. The majority of randomized controlled trials (RCTs) exploring diet-microbiota interactions have focused on individual nutrients, with strong evidence for the modulatory impact of fiber [2]. Fiber is derived from plant foods whose diverse components (macronutrients, micronutrients, non-nutrient bioactives) may interact to synergistically impact the gut microbiota, while their physical food matrix may alter nutrient availability for both host and microorganisms [3]. Considering the diverse roles of gut microbiota in human health [4], the identification of whole foods that impact the gut microbiota is important to inform public health messages and clinical practice guidelines.

Early studies suggested a prebiotic effect of almonds on fecal bifidobacteria *in vitro* [5–7], and *in vivo* [8]. Subsequently, a systematic review of randomized controlled trials (RCTs) of nuts reported almond-specific effects including increased bacterial  $\alpha$ -diversity and relative abundance of several bacteria at the genus level [9]. However, there is a lack of consensus between RCTs on the impact of almonds on fecal bifidobacteria,  $\beta$ -diversity and stool output that is likely due to limitations in study design that impact the assessment of gut microbiota, such as the use of crossover design, which is not ideal because the effects of diet on microbiome can persist in the short-term following discontinuation of the intervention [8] and because cross-over trials can compromise blinding or masking of food interventions [10]. Other limitations include washout periods of insufficient duration [11,12] and lack of power [11–14]. In addition, few studies addressed the impact of almond consumption on gut function or clinical outcomes [12,13].

The mechanisms responsible for a prebiotic effect of almonds remains unclear, but potentially include their high fiber and polyphenol content and their low lipid bioaccessibility due to storage as lipid droplets within tough cell walls that are resistant to human digestion [15]. Following mastication of whole almonds, the incomplete fracture of cell walls [16,17] results in almond cells and their intracellular lipid reaching the colon intact where they are available for metabolism by the microbiota. In contrast, it is hypothesized that processing into ground almonds (almond flour) would result in higher lipid bioaccessibility due to decreased particle size prior to consumption, and therefore less lipid delivery to the colon. Maintenance of an intact almond cell matrix may therefore represent a unique method of delivering a rich supply of fermentable nutrients to the gut microbiota.

To address the lack of consensus between studies regarding the prebiotic effect of almonds and the potential impact of processing on the effect, a parallel-design RCT was conducted, powered to investigate the impact of whole or ground almonds on abundance of fecal bifidobacteria (primary outcome), gut microbiota composition and gut transit time. To elucidate components responsible for the previously observed effects, we investigated the role of almond processing by comparing the effects of whole almonds (low bioaccessible lipid) and ground almonds (high bioaccessible lipid) on study outcomes.

## Methods

### Study population

Study participants were healthy adult males and females (aged 18-45), who consumed snacks regularly (self-reported  $\geq 2/d$ ) and did not follow a moderate or high-fiber diet ( $< 22$  g/d; assessed by Block Fruit/Vegetable/Fiber screener [18]). Volunteers were excluded if they fulfilled any of the following criteria: allergy, intolerance, dislike or regular consumption of almonds as snacks, history of gastrointestinal disorders, history of other chronic medical condition, history of abuse of alcohol, drugs or other medications, antibiotic therapy in the past 4 weeks, on-going therapy with medication known to impact gastrointestinal motility, presence of medical devices, pregnant, planning pregnancy or lactating, consumption of prebiotics or probiotic supplements in the past 4 weeks, consumption of prebiotic or probiotic foods in the past 2 weeks, very high physical activity levels (assessed by the international physical activity questionnaire [19]), body mass index  $< 18.5$  or  $> 29.9$  kg/m<sup>2</sup>, or unintentional weight loss in the past 6 months.

Participants were recruited via circular emails to staff and students of King's College London and other London universities; advertisements in local newspapers, social media, clinical trials databases; and recruitment posters and leaflets between June 2018 and September 2019. Written informed consent was obtained prior to enrolment in the study. Trial procedures were approved by the King's College London Research Ethics Committee (RESCM-17/18-5341) and conducted in accordance with the Declaration of Helsinki. The study was registered on [clinicaltrials.gov](https://clinicaltrials.gov) (NCT03581812).



## Study design

This was a free-living, 4-week, 3-arm, parallel-design RCT conducted as a snack-replacement study to minimize impact on habitual background food intake (**Supplementary Figure 1**). The study was powered to detect differences in the primary outcome of fecal bifidobacteria abundance between groups based on a previous observation of an effect of almonds in comparison to control in a non-randomized human trial (10.47% vs 9.75%; [8]) with 90% power. A significance level ( $\alpha$ ) of 0.0166 was used to allow for a post-hoc Bonferroni correction for multiple testing. This calculation resulted in the requirement of 72 participants (24 per group), but with an anticipated attrition of 15-20% we aimed to recruit 87 participants (29 per group). The study was also powered to detect differences in whole-gut transit time (WGTT) based on a previous observation of the effect of fiber in comparison to control on WGTT (22.5 h vs 31.4 h; [20]) with 80% power and an attrition rate of 15-20%. Thus, 48 participants (16 per group) were required to undergo measurement of WGTT.

Volunteers were screened via telephone and in person. Eligible participants attended study visits at the beginning and end of the 4-week intervention period for collection of data, samples and, in a sub-group, measurement of WGTT. All study visits took place at the Metabolic Research Unit, King's College London.

At baseline, participants were randomly allocated to receive either whole almonds (56 g/d), ground almonds (56 g/d) or an energy-matched control snack muffin (2/d) using block randomization with a block size of six. Randomization was conducted by an independent researcher using a randomization website

(www.sealedenvelope.com) with sex (male, female), age (18-30, 31-45 years) and willingness to undergo measurement of WGTT (yes, no) as stratification variables. Allocations were concealed from study researchers in opaque sealed envelopes that were only opened following completion of baseline measurements.

Blinding of participants to the intervention was not feasible for numerous reasons: almonds are easily identifiable; it was impossible to design a placebo identical to almonds, but without any active components; and it was necessary to exclude participants who had an allergy/intolerance or dislike to almonds. However, all efforts were made to mask participants to the true intervention by advertising the trial as a “snack replacement trial” testing the impact of a variety of snack foods on gut health, and avoiding any mention of almonds in advertising materials. Researchers were blinded to intervention allocation for data analysis through recoding of participant data.

Trial design and procedures are illustrated in **Supplementary Figure 1**. Outcomes assessed at baseline and endpoint were: relative abundance of fecal bifidobacteria (primary outcome) and secondary outcomes were fecal microbiota composition (phyla and genera) and diversity ( $\alpha$  and  $\beta$ ), fecal short-chain fatty acids (SCFA), fecal volatile organic compounds (VOC), WGTT, regional gut transit times, gut pH, stool output (frequency and consistency), gut symptoms. Potential confounders were monitored at baseline and endpoint: diet, body composition and body mass index (BMI). Compliance and acceptability were assessed at endpoint visit.

### **Dietary interventions**

Participants were required to consume study snacks in place of habitual snack foods, twice a day for 4-weeks. Two almond arms were included: whole almonds (2 x 28g/d)

and ground almonds (2 x 28g/d). This dose was selected as 28g is the established amount for a single serving of almonds, and has been used in studies previously [14] and this duration was selected as it was considered sufficient to allow for changes to gut microbiota [21], while also allowing for adaptation to the increase in fiber from almonds, and accounting for the impact of hormone fluctuations associated with the menstrual cycle on metabolism and GTT [22,23].

To facilitate consumption, participants were instructed to consume the ground almond intervention mixed with 15 ml of water. Whole and ground natural almonds with skins (Nonpareil) were provided (Almond Board of California, California, US). The control group received an iso-caloric muffin intervention (2 x muffin/d), that was developed at King's College London and used as a control in previous studies [24]. The muffins were designed to provide a macronutrient profile based on the average nutrient intake from snacks (excluding fruit) from the UK National Diet and Nutrition Survey [25]. Muffins were prepared by study researchers at King's College London. Macronutrient profiles for snacks consumed in each arm are included in **Table 1**.

All participants were instructed to consume study snack interventions in place of usual snacks between meals and with a minimum of 100ml water [26]. Participants were asked to avoid consuming nuts, prebiotics, probiotics and additional snacks, and to maintain habitual diet, exercise, and smoking habits.

### **Stool sample collection and processing**

Stool samples were collected by participants according to Standard Operating Procedure for Fecal Sample Self-collection [27], delivered to investigators and processed within 4 hours. Briefly, samples were kept on ice, homogenized for 2 min

each side (Steward Laboratory Blender Stomacher 400), aliquoted for later analyses and stored at -80°C.

### **DNA extraction and sequencing**

DNA was extracted from stool using the DNEasy PowerLyser PowerSoil DNA Isolation Kit (Qiagen, UK). DNA quality and quantity were confirmed using Nanodrop™ (Thermo Fisher Scientific, USA). Sample libraries were prepared by amplifying the V1-V2 region of the 16S rRNA gene following the 16S Metagenomic Sequencing Library Preparation protocol [28] with the following modification; the index PCR reactions were cleaned and normalized using the SequalPrep normalization plate kit (Life Technologies, UK). Sample libraries were quantified using the NEBNext library quant kit for Illumina (New England Biolabs, UK). Sequencing was performed on an Illumina MiSeq platform (Illumina, UK) using the MiSeq reagent kit V3 (Illumina, UK) with paired-end 300bp chemistry. Raw sequencing data was processed following the DADA2 pipeline in R [29].

### ***Taxonomic and diversity analysis***

Taxonomy was assigned using the SILVA database version 132 [30]. Bacterial sequences were rarefied to an even sampling depth of 3195 sequences per sample. To reduce noise in the data caused by the presence of low abundance/rare strains, a filter was applied to remove amplicon sequence variants (ASVs) with abundance lower than 0.1% and presence in <10% of all samples. Centered log ratio transformation was applied prior to statistical analysis.

Analyses of bacterial diversity were conducted on processed sequencing data both before and after the application of the filter, to test the effect of removal of low abundance/rare strains on diversity indices. As both analyses produced similar

results, and to maintain consistency with taxonomic comparisons, results are presented for filtered data only.  $\alpha$ -diversity was measured using Chao1 index, Shannon's index, and Simpson's index [31] and  $\beta$ -diversity was calculated using unweighted and weighted UniFrac and Bray-Curtis dissimilarity [14,32].

### **Short-chain fatty acids**

SCFA were extracted using a buffer (0.1%  $\text{HgCl}_2$  (Sigma, UK), 1%  $\text{H}_3\text{PO}_4$  (Merck, Germany), 4.5% of the internal standard 2,2-dimethylbutyric acid (Sigma, UK)). The extracted SCFA were quantified by gas-liquid chromatography performed on a 7890a Agilent technology GC system with flame ionization detector, and a BP21 25 m fused silica capillary column with a 220  $\mu\text{m}$  internal diameter and a film thickness of 0.25  $\mu\text{m}$  (Trajan Scientific and Medical, Australia). The carrier gas was  $\text{N}_2$ , and the oven was initially set to 80°C and programmed to increase by 10°C/min up to 145°C and 100°C/min up to 200°C to complete the elution. The injected sample volume was 0.2  $\mu\text{l}$ , followed by a 1.2 % formic acid wash solution (Merck, Germany) to minimize carryover from the previous sample. A blend of six pure SCFA at six different concentrations were first run to produce calibration curves for quantitative analysis. All samples were analyzed in duplicate. The concentrations of individual SCFA were expressed as  $\mu\text{mol/g}$  of wet feces and total SCFA concentrations were calculated as the sum of the individual SCFA concentrations.

### **Volatile organic compounds**

VOCs were extracted from fecal samples by solid phase micro-extraction coupled to gas-chromatography mass-spectrometry as described in detail previously [33].

## **Gut transit time and luminal pH**

Whole and regional gut transit times and pH were measured using the SmartPill® wireless motility capsule (WMC; Medtronic, UK). The procedure for administration and analysis of the WMC has been described previously [34]. In brief, the WMC was ingested orally and continuously measured gastrointestinal pH, temperature and pressure via sensors encapsulated in an indigestible polyurethane shell. Data from sensors was transmitted to a receiver worn by the participant. Two researchers independently identified the following landmarks using proprietary software (MotiliGI™, Medtronic): ingestion (abrupt drop in pH, rapid rise in temperature); pyloric-duodenal transition (sharp, sustained rise in pH of >3 pH units); passage through the ileo-cecal junction (ICJ, fall in pH of  $\geq 1$  pH unit, sustained for at least 10 min); pill exit (sharp drop in temperature with pressure and pH signal termination). Regional and whole-gut transit times were derived from time intervals between landmarks: gastric emptying time (GET; from ingestion to pyloric-duodenal transition), small bowel transit time (SBTT; from pyloric-duodenal transit to passage through the ICJ), colonic transit time (CTT; from passage through the ICJ to pill exit) and WGTT (from ingestion to pill exit). Mean values for regional transit times and pH in the small bowel and colon, calculated from both researchers, were used for analysis. Normal ranges for transit times and pH have been published previously [35].

## **Stool output and symptoms**

Participants completed two 7-day stool and symptom diaries, incorporating the Bristol stool form scale (BSFS) for assessment of stool output [36] and the Gastrointestinal

symptoms rating scale (GSRS) for assessment of common gastrointestinal symptoms [37,38]. Summary measures for stool output were: stool frequency (total bowel movement/week; BM/wk); stool consistency (mean BSFS score over 7 days); normal stools (proportion of stool types 3, 4, 5 over 7 days).

The GSRS consists of 16 items rated on a Likert scale in terms of severity (0 absent; 1 mild, 2 moderate, 3 severe) and was measured at the end of each day for 7-days. Incidence was calculated as the number of days of mild, moderate, or severe symptoms and severity was calculated as the mean score over 7 days.

### **Monitoring of confounders, quality of life and compliance**

Participants completed two 7-day estimated food diaries, one at baseline immediately prior to the intervention and one during the final week of the intervention. Diaries were the standard food diaries used in the UK National Diet and Nutrition Survey, including detailed instructions and visual aides to assist completion [39]. Data from food diaries was entered into nutrition analysis software (Nutritics research edition; version 5.6; Nutritics, Ireland) for analysis based upon the McCance & Widdowson composition of foods integrated dataset.

Bodyweight and body composition were measured using bioelectrical impedance (BC-410MA; Tanita Ltd., UK). Height was measured using a wall-mounted stadiometer for calculation of BMI.

Health-related quality of life (QoL) was measured at baseline and endpoint using the SF-36 questionnaire [40].

Participants were contacted weekly by telephone to encourage compliance. At the final visit participants returned all unused snack foods. Adequate compliance was

defined as the consumption of  $\geq 75\%$  of snacks ( $\geq 42$  snacks) as this is a common compliance threshold and would provide  $>5$  g/d fiber. Participants who fulfilled this criterion were included in both intention-to-treat (ITT) and per protocol (PP) analyses. Those who consumed  $<75\%$  of snacks ( $<42$  snacks) were included in the ITT analyses only. At the final visit participants completed an acceptability questionnaire developed for use in dietary intervention studies at King's College London.

### **Mastication study**

Participants had to opt in to take part in the mastication phase of the study, requiring an additional study visit. The objective of this study was to assess the impact of almond form (whole almonds vs ground almonds) on particle size distribution (PSD) and lipid release following mastication as an exploratory outcome.

Mastication sample collection and measurement of PSDs by mechanical sieving were conducted as described previously [41]. Almonds were consumed in the same form (whole almonds or ground almonds mixed in 15 ml water) as in the snack provided in the feeding study. For mechanical sieving, the following sieve aperture sizes were used: 3350, 2000, 1000, 500, 250, 125, 63, 45, 20  $\mu\text{m}$  (Endecotts Ltd., UK) and the proportion of masticated almonds retained on each sieve was calculated (% weight). Lipid bioaccessibility was predicted using a theoretical model developed and validated previously [42–44].

### **Statistical analysis**

For the majority of study outcomes statistical analysis was performed using IBM SPSS Statistics (version 26; IBM, UK). All data were checked for normality and outliers using Q-Q plots and the Shapiro-Wilk statistic. Descriptive statistics were



calculated; mean (SD) or median (IQR) for continuous outcomes and n (%) for categorical variables.

Differences between the three groups at endpoint were assessed using an analysis of covariance (ANCOVA), with baseline values as a covariate or an analysis of variance (ANOVA) for change from baseline values. Where the tests were significant, comparison on two groups were performed using a Bonferroni *post-hoc* correction. For non-normally distributed data, the Kruskal–Wallis test with *post-hoc* Mann-Whitney test was applied. Categorical variables were assessed using chi-squared test. The following planned contrasts were conducted; 1) analysis of almond groups pooled (whole almond and ground almond) vs. control; 2) analysis of whole almonds vs. ground almonds. Groups were compared by independent samples t-test or Mann-Whitney test.

The primary analysis was based on the ITT data set consisting of all participants randomized. A modified-ITT analysis set (laboratory ITT), consisting of participants who provided sufficient stool at baseline and endpoint such that laboratory analyses could be completed, was used for the following outcomes: gut microbiota composition and diversity, SCFA and VOC. A PP data set consisted of participants who completed the trial, maintained adequate compliance (>75%) and provided primary outcome data (stool). Missing data was assumed to be completely missing at random and no imputation was performed.

Analyses of gut microbiota composition were conducted using the MicrobiomeAnalyst software package [45]. Taxonomic comparisons were conducted on relative abundance of taxa at the phylum and genus levels with differences between groups assessed by non-parametric tests. *P*-values were corrected for multiple comparisons

using the Benjamini-Hochberg FDR ( $q$ ). A UniFrac weighted distance matrix was generated using the phangorn package in R [46] that was used to create non-metric multidimensional scaling plots and PERMANOVA  $p$ -values using the Vegan library package in R [47].

Statistical analysis of fecal VOC was conducted in Metaboanalyst (version 4.0) [48].

Missing data were replaced by 1/5 minimum value for each compound. The data were normalized to the sample median, log transformed and then auto-scaled.

Differences in abundance of fecal VOCs between groups at baseline and endpoint were analyzed by one-way Analysis of Variance and corrected for multiple testing using the Benjamini-Hochberg FDR ( $q$ ). Principle component analysis (PCA) plots generated from log-transformed data were used to visually compare VOC profiles between groups.

Differences in PSDs from mechanical sieving were assessed by two-way repeated measures ANOVA with sieve aperture size and almond form (whole or ground) as factors. Where there was a significant interaction, simple main effects were analyzed at each level of particle size (paired  $t$ -test) and  $p$ -values were corrected for multiple comparisons using a Bonferroni *post hoc* correction. Paired  $t$ -test was used to detect differences in predicted lipid bioaccessibility between whole and ground almonds.

For all analyses  $p$ -values and  $q$ -values of  $<0.05$  were considered statistically significant.

## Results

### Recruitment and participant characteristics

Three hundred and thirty-eight volunteers were screened for eligibility; 87 were randomized and included in the ITT analysis of which 81 completed the intervention (CONSORT in **Figure 1**). Participants included in the modified-ITT group for laboratory analysis were; fecal microbiota (n = 80), SCFA (n = 73) and VOC (n = 79). A subgroup of 48 participants undertook measurement of gut transit time, pH and pressure using the WMC, with 47 participants completing the test at baseline, and 41 participants completing at endpoint. A subgroup of 31 participants opted to take part in the mastication analysis.

The demographic characteristics and baseline dietary intakes of the total cohort are presented in **Table 2**. The majority of eligible participants were female (86.2%), with a mean age of 27.5 years (SD 6.2), mean BMI of 22.9 kg/m<sup>2</sup> (SD 2.8) and mean fiber intake of 20.7 g/d (SD 7.7; from baseline food diary). There were no differences between study groups in baseline characteristics, or baseline outcome measurements (with the exception of baseline relative abundance of genus *Oscillibacter*).

### Compliance

Seven (8.0%) participants were non-compliant (consumption of <75% of snacks) to whole almond (n = 3), ground almond (n = 3) or control muffin (n = 1) regime.

Therefore 74 participants were included in the PP analysis set. There were no differences in the number of compliant participants between groups ( $p = 0.464$ ; Chi-square test).

Overall compliance was 86.7% (SD 27.7) corresponding to mean consumption of 49 (SD 15.5) snacks throughout the intervention period. Compliance in the control group (93.5%, SD 21.8) was significantly greater than in the ground almond group (80.7%, SD 27.8;  $p = 0.028$ ).

### **Fecal microbiota composition**

A total of 2,697,014 high quality paired 16S rRNA gene sequences were obtained from all samples, an average of 16,149 per stool sample (range 3,195 – 41,293). Sequences were rarefied to an even sampling depth of 3,195 sequences per sample and resolved into a total of 9,131 ASVs. Following the application of filters to remove low abundance and rare taxa, 417 ASVs remained and were included in analysis. The modified ITT analysis consisted of 79 participants at the end of the intervention, due to removal of one participant (ground almond group) for insufficient sequencing quality.

There were no significant differences in phyla or genera between groups at baseline, except for the genus *Oscillibacter* ( $q = 0.042$ ), which was significantly higher in the control group (0.115%, SD 0.132) in comparison with the whole almond group (0.024%, SD 0.087;  $p = 0.005$ ).

In contrast to the primary hypothesis that almonds would increase abundance of fecal bifidobacteria, there was significantly lower abundance following whole almonds (8.7%, SD 7.7) and ground almonds (7.8%, SD 6.9) versus control muffin (13.0%, SD 10.2;  $p = 0.031$ , Kruskal-Wallis test). However, this did not remain significant following FDR adjustment ( $q = 0.613$ ; **Supplementary Table 1**). An additional four taxa were significantly different across the groups (*Lachnospiraceae\_UCG\_001*, *Phascolarctobacterium*, *Tuzzerella*, *Tyzzereella*; all  $p < 0.05$ ). However, there were no

significant differences for any bacteria at the phylum or genus level following FDR adjustment (all  $q > 0.05$ ; **Supplementary Table 1**). There were no significant differences between groups for any taxa under the PP analysis, or planned contrasts.

There were no other significant differences observed in microbiota analyses, or  $\alpha$ -diversity or  $\beta$ -diversity (**Figure 2**).

### **Gut microbiota metabolites**

In the ITT analysis, there were no significant differences between groups for total or individual SCFA, when analyzed as either absolute or change in concentrations ( $\mu\text{mol/g}$  feces; **Table 3**). However, in the PP analysis set, butyrate was significantly higher following almonds (whole and ground pooled; not included in Table;  $24.1 \mu\text{mol/g}$ , SD 15.0) in comparison to control ( $18.2 \mu\text{mol/g}$ , SD 9.1;  $p = 0.046$ ). For remaining sensitivity analyses, there were no significant differences between groups of any other SCFA.

Following removal of low copy features from raw data, 118 VOCs were identified from all samples. PCA plots at baseline and endpoint (**Figure 3**) revealed no evidence of clustering of groups based on VOC profile. There were no differences between groups for any individual VOCs identified at baseline or endpoint.

Planned contrasts were conducted to compare almonds (whole almond and ground almond groups pooled) vs. control muffins and there was no evidence of clustering of groups from the PCA plot (**Figure 3**). Three VOCs were increased following almond consumption (whole and ground pooled) in comparison to control muffins: 2-methylbutanoic acid ( $p = 0.004$ ,  $q = 0.057$ ), 3-methylbutanoic acid ( $p = 0.006$ ,  $q = 0.057$ ) and nonanal ( $p = 0.007$ ,  $q = 0.057$ ), which even following correction for multiple testing (FDR) approached traditional statistical significance (**Supplementary**

**Table 2).** Comparison of whole almond and ground almond groups revealed no evidence of clustering of groups from the PCA plots (**Figure 3**) and no difference between groups for any VOCs following correction for multiple testing.

### **Gastrointestinal transit time and pH**

Comparison between the three groups at the end of the intervention revealed no significant differences in WGTT ( $p = 0.940$ ; **Table 4**). Similarly, no significant differences between groups were identified for GET, SBTT, or CTT. There were no significant differences for any transit outcome variable in the PP analysis or planned contrasts (Table 4).

There were no significant differences in small bowel pH or colonic pH between control muffins, whole almond and ground almond groups, or planned contrasts in either the ITT or PP analyses (**Table 4**).

### **Stool output and symptoms**

Stool frequency and stool consistency (BSFS score and % normal stools) are presented in **Table 5**. There was a significant difference in change in stool frequency at the end of the intervention period ( $p = 0.017$ ), with greater change in the whole almond group (+1.5, IQR 3.0) in comparison to the control group (-1.0, IQR 3.0;  $p = 0.034$ ) and in comparison to the ground almond group (-0.5, IQR 4.3;  $p = 0.061$ ). No other significant differences were observed. There were no group differences in incidence or severity of common gastrointestinal symptoms (**Supplementary Table 3 and 4**).

## **Nutrient intake**

At the end of the intervention there were significant differences between the groups in intakes of many nutrients (**Supplementary Table 5**), with *post-hoc* testing revealing intakes of absolute energy, protein, total fat, MUFA, total fiber, NSP, potassium, magnesium, phosphorus, zinc and manganese were higher in the whole almond group in comparison to control group, whilst intakes of MUFA, total fiber, NSP, magnesium and manganese were higher in the ground almond group in comparison to control muffins.

## **Body composition**

There were no significant differences in body weight, BMI, or body composition between groups at the end of the intervention (**Supplementary Table 6**) or for any domain assessed by the SF-36 questionnaire (**Supplementary Table 7**).

## **Quality of life and acceptability**

Most participants in both whole almond and ground almond groups reported favorable opinions of the interventions flavor (75.9% and 41.7% respectively) and portion size (63.3% and 38.5% respectively). For whole almonds, many participants had favorable opinions of the snacks' texture (60%) and mouth feel (53.3%) in contrast to ground almonds, with the majority of participants reporting a dislike of the snacks' texture (42.3%) and mouth feel (36%).

### **Mastication, particle size distribution and lipid bioaccessibility**

A total of 31 participants completed the mastication phase. Analysis of PSDs revealed a significant interaction between almond form (whole or ground) and particle size on PSD ( $p < 0.001$ ).

Following mechanical sieving, significantly more smaller particles were retained on sieves for masticated ground almonds in comparison to whole almonds (20  $\mu\text{m}$ ,  $p = 0.009$ ; 45  $\mu\text{m}$ ,  $p = 0.018$ ; 63  $\mu\text{m}$ ,  $p < 0.001$ ; 125  $\mu\text{m}$ ,  $p < 0.001$ ; 500  $\mu\text{m}$ ,  $p < 0.001$ ) and significantly more larger particles retained for whole almonds in comparison to ground (1000  $\mu\text{m}$ ,  $p < 0.001$ ; 2000  $\mu\text{m}$ ,  $p < 0.001$ ; 3350  $\mu\text{m}$ ,  $p < 0.001$ ; **Figure 4**).

Lipid bioaccessibility of masticated whole and ground almonds predicted using a theoretical model [42] revealed a significantly greater lipid bioaccessibility for masticated ground almonds (10.4%, SD 1.8) versus masticated whole almonds (9.3%, SD 2.0;  $p = 0.017$ ).



## Discussion

In this first appropriately powered, parallel-design RCT to investigate the impact of almonds and almond processing on gut microbiota and incorporating clinical and gut function endpoints, we observed that almonds may impact gut microbial metabolism and stool output. However, contrary to our hypothesis, results of this RCT show that consumption of whole or ground almonds for 4-weeks had no impact on fecal bifidobacteria numbers; indeed the abundance was numerically lower in the almond groups. These findings are in agreement with previous RCTs that have reported no effect of almond consumption on bifidobacteria [13,14], but are in contrast to one RCT that found a significant decrease in bifidobacteria following a pooled analysis of four processed almond forms (whole natural, whole roasted, chopped, butter) in comparison to control [11]. As outlined, previous RCTs had significant limitations, which were overcome in the current trial and therefore our results can be considered robust.

There were no significant differences between groups for any taxa at the phylum or genus level at the end of the intervention period. This is despite good compliance to whole almonds (86.5%, approximately 48 g/d) and ground almonds (80.7%, approximately 45 g/d). It is widely acknowledged that gut microbiota composition is subject to large inter-individual variability [49], therefore, while the RCT was powered to detect changes in bifidobacteria it is possible that secondary outcomes such as bacterial abundance at the phylum and genus levels were insufficiently powered to detect significant effects should they occur.

Several members of the family *Lachnospiraceae* were altered by almond consumption in this RCT, but the observed effects did not remain significant after

correction for multiple testing. *Lachnospiraceae* are among the main producers of colonic SCFA [50], and members of this family have been reported to be influenced by almond consumption in a meta-analysis of almond interventions [9]. Interestingly, we also observed significant increases in the SCFA butyrate and several VOCs following almond consumption (whole and ground pooled) in comparison to control muffins in the first RCT to assess the impact of almonds on bacterial metabolites. It was anticipated that almonds would increase production of SCFA, which would result in a more acidic pH, particularly in the right side of the colon. However, contrary to these findings, there was no impact of almonds on colonic pH. Therefore, we must interpret these results with caution, due to uneven group sizes and potential for type 1 error. Nonetheless, they indicate potentially important outcomes for future investigations. In particular, butyrate plays a role in multiple processes relating to human health [51]. For example, 2-methylbutyric acid is produced by bacteria when carbohydrates are limited [52], indicating a transfer from saccharolytic to proteolytic metabolism potentially due to increased availability of almond proteins. Meanwhile nonanal has been identified in roasted almonds following storage, and is considered an indicator of shelf life [53], representing an area for future investigation as an objective biomarker of almond intake.

There was no impact of almond consumption on  $\alpha$ -diversity or  $\beta$ -diversity by any metric, in agreement with previous RCTs that also reported no effect of almonds on  $\beta$ -diversity [11,14]. In contrast, a previous RCT reported an effect of almonds on  $\alpha$ -diversity [14], whereby snacking on almonds for 8 weeks resulted in increases in both Chao1 index and Shannon's index in comparison to control. Similarly, the meta-analysis of almond RCTs reported consumption of almonds resulted in increased Shannon's index that was borderline statistically significant [9]. Despite these

conflicting results, it is worth emphasizing that evidence for an effect of gut microbiota diversity on human health is limited to observational trials and therefore its importance as an outcome in dietary intervention trials remains unclear [54].

Duration of interventions is an important consideration in diet-microbiome studies, a factor that may explain the variability in results between studies [9]. The long-term impact of almonds on gut microbiota remains to be evaluated. It has been suggested that although short-term changes in diet (2 days – 12 weeks) rapidly alter gut microbiota, it is possible that long-term changes in dietary habits (>6 months) have the greatest potential influence on gut microbiota composition and subsequent clinical benefits associated with their modulation [55]. Dose may be important as shown from a previous meta-analysis [9] but was not possible to explore in the current study.

This was the first RCT to investigate the impact of almonds on whole and regional gut transit time and luminal pH. Contrary to our hypothesis that almonds would result in faster WGTT, our findings indicate no effect on this outcome. A plausible explanation for this has been illustrated by a systematic review of 65 intervention trials that reported that the effect of fiber on transit is dependent on baseline WGTT, whereby reductions are most pronounced in those with baseline WGTT of >48 hr [56]. In this study, mean WGTT at baseline was normal (37.4 hr, SD 21.4; [35]) potentially accounting for the lack of overall effect of almonds on WGTT. Our results indicate that almonds have a small impact on increasing stool frequency in healthy people but have no effect on stool consistency (when assessed by both subjective and objective measures) or gut symptoms. This finding confirms that whole almonds and ground almonds, consumed as a snack twice a day for four weeks, are well tolerated by healthy adults with low fiber intake.

Finally, almond processing had no impact on any study outcome. The hypothesis that, following mastication, ground almonds would have a PSD with greater proportions of smaller particles in comparison to whole almonds, and that this would subsequently influence predicted lipid bioaccessibility, was investigated in the mastication study. Our results confirmed that following mastication, ground almonds had more particles of smaller size in comparison to whole almonds. Despite this, and although significant, the difference in lipid bioaccessibility between these almond forms was very small (mean difference 1.1%, SD 2.3). Therefore, these findings support the comparable effect of whole and ground almonds on study outcome measures. It can be concluded that commercial grinding of almonds does not result in clinically meaningful differences in nutrient bioaccessibility

The main limitation of this trial is the sex distribution, which is predominantly female (86.2%) and young (27.5 y, SD 6.2) and therefore results are not representative of male and older populations.

## **Conclusion**

Almond consumption does not exert a prebiotic effect on fecal bifidobacteria abundance or result in major changes in other gastrointestinal microbiota, gastrointestinal transit, pH, pressure, stool output or gut symptoms in healthy adults. Therefore, their incorporation into the diet of low fiber consumers in the public, to increase fiber intake, would likely be well tolerated. Almond consumption may influence the family *Lachnospiraceae*, and aspects of bacterial metabolism, in particular fecal butyrate. These outcomes warrant further investigation in future RCTs, which should focus on confirming these findings in cohorts of older adults with

an even sex distribution. Commercial processing of almonds increases predicted lipid bioaccessibility to a limited degree but did not appreciably influence gut health.

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## Author contributions

KW, SB, ED and MR, designed research; AC and EH, conducted research; TG provided the spreadsheet containing equations of the theoretical model for assessment of almond lipid bioaccessibility; AC, ED, CP, JMB, TG, JRM, and SMS analyzed data or performed statistical analysis; AC, ED, SB and KW wrote paper; KW had primary responsibility for final content; all authors read and approved the final manuscript.

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ORIGINAL UNEDITED MANUSCRIPT

**TABLE 1 Nutritional composition of almonds (whole, ground) and control snack muffins**

	Whole almonds	Ground almonds	Control snack muffin <sup>1</sup>
<b>Serving size</b>	56 g	56 g	2 muffins
<b>Energy (kcal)</b>	324	324	318
<b>Protein (g)</b>	12	12	7
<b>Fat (g)</b>	28	28	12
<b>SFA (g)</b>	2	2	5
<b>MUFA (g)</b>	17	17	5
<b>PUFA (g)</b>	5	5	2
<b>Carbohydrate (g)</b>	12	12	44
<b>Sugars (g)</b>	2	2	24
<b>Starch (g)</b>	9	9	20
<b>Fiber (g)</b>	7	7	< 1

Data from The Almond Board of California; <sup>1</sup>Based on the macronutrient profile of the average UK snack: 9.4 % protein, 35.2 % fat, 55.5% carbohydrate [25]; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

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**TABLE 2 Demographic characteristics of participants**

	<b>All</b>	<b>Control</b>	<b>Whole almond</b>	<b>Ground almond</b>
	<b>(n = 87)</b>	<b>(n = 26)</b>	<b>(n = 33)</b>	<b>(n = 28)</b>
<b>Age (years)</b>	27.5 (6.2)	27.9 (5.0)	27.5 (6.8)	27.0 (6.5)
<b>Female, n (%)</b>	75 (86.2)	25 (96.2)	27 (81.8)	23 (82.1)
<b>Weight (kg)</b>	63.9 (10.1)	65.0 (8.1)	64.4 (11.9)	62.4 (9.6)
<b>BMI (kg/m<sup>2</sup>)</b>	22.9 (2.8)	23.6 (2.7)	22.6 (2.9)	22.7 (2.9)
<b>Fat (%)</b>	27.9 (7.1)	29.9 (6.0)	26.9 (6.7)	27.2 (8.2)
<b>Fat free mass (kg)</b>	45.3 (5.5)	45.3 (4.5)	45.6 (6.2)	45.0 (5.8)
<b>Energy (kj)</b>	8143.1 (1949.8)	8148.8 (2006.8)	8205.1 (2021.7)	8064.7 (1877.4)
<b>Energy (kcal/d)</b>	1941.3 (465.4)	1942.3 (479.2)	1956.7 (482.4)	1922.2 (448.0)
<b>Protein (g/d)</b>	75.4 (20.3)	74.6 (21.1)	72.7 (19.6)	79.3 (20.4)
<b>Fat (g/d)</b>	81.0 (23.8)	79.1 (24.8)	84.9 (26.1)	78.2 (20.0)
<b>Carbohydrate (g/d)</b>	214.9 (64.0)	218.7 (63.8)	214.6 (66.6)	211.7 (63.1)
<b>Fiber intake (g/d)</b>	20.7 (7.7)	20.9 (7.8)	21.9 (8.4)	19.2 (6.7)
Data are mean (SD) unless otherwise stated; BMI, body mass index				

ORIGIN

**TABLE 3 Short-chain fatty acids ( $\mu\text{mol/g}$  wet feces) at baseline and end of intervention, and change from baseline to end of intervention in the modified intention to treat and per protocol analysis**

	Control			Whole almond			Ground almond			<i>p</i> -values	
	Baseline	Endpoint	Change	Baseline	Endpoint	Change	Baseline	Endpoint	Change	ANCOVA <sup>1</sup>	ANOVA <sup>2</sup>
<b>Intention to treat</b>	n = 22	n = 22	n = 22	n = 27	n = 27	n = 27	n = 24	n = 24	n = 24		
Total SCFA	110.1 (40.8)	122.1 (44.9)	12.1 (45.8)	142.2 (62.0)	144.9 (65.2)	2.7 (58.4)	117.3 (52.5)	127.1 (63.2)	9.8 (66.1)	0.915	0.836
Acetate	67.2 (25.9)	72.5 (28.5)	5.3 (30.3)	82.1 (32.9)	82.5 (32.8)	0.5 (33.3)	66.9 (29.2)	71.9 (36.1)	5.0 (40.7)	0.858	0.859
Propionate	17.6 (6.7)	21.1 (9.8)	3.6 (7.9)	23.5 (13.5)	24.8 (12.3)	1.3 (9.8)	22.3 (11.9)	22.5 (12.3)	0.2 (13.5)	0.826	0.562
Butyrate	15.5 (7.8)	17.7 (9.0)	2.2 (8.9)	24.9 (16.0)	25.5 (17.1)	0.6 (14.7)	16.8 (11.0)	20.9 (12.8)	4.2 (12.2)	0.752	0.589
Isobutyrate	2.2 (1.5)	2.3 (1.0)	0.1 (1.2)	2.6 (1.4)	2.7 (1.7)	0.1 (1.7)	2.6 (1.4)	2.5 (1.3)	-0.0 (1.6)	0.861	0.947
Valerate	3.0 (1.8)	3.6 (1.6)	0.6 (1.7)	3.8 (2.5)	3.9 (2.3)	0.0 (2.1)	3.4 (2.0)	3.9 (2.3)	0.6 (1.7)	0.747	0.461
Isovalerate	4.6 (3.4)	4.8 (2.5)	0.2 (2.8)	5.6 (3.2)	5.6 (3.9)	0.3 (3.4)	5.4 (3.2)	5.4 (2.9)	-0.0 (3.5)	0.847	0.945
<b>Per protocol</b>	n = 22	n = 22	n = 22	n = 27	n = 25	n = 25	n = 21	n = 23	n = 21		
Total SCFA	109.5 (50.2)	122.6 (44.7)	11.0 (46.6)	137.9 (62.9)	148.5 (66.1)	7.5 (58.0)	117.5 (50.2)	134.6 (63.6)	10.2 (69.8)	0.746	0.978

Acetate	66.7 (26.5)	72.2 (28.7)	4.6 (30.9)	78.8 (33.3)	84.3 (33.2)	3.6 (32.5)	68.3 (29.3)	77.6 (39.3)	4.5 (42.7)	0.717	0.994
Propionate	17.5 (6.7)	21.1 (9.8)	3.5 (8.1)	22.8 (13.7)	25.3 (12.6)	2.0 (9.8)	21.7 (11.2)	23.7 (12.8)	0.6 (13.6)	0.760	0.683
Butyrate	15.5 (7.7)	18.2 (9.1)	2.0 (9.0)	24.1 (16.3)	26.3 (17.4)	1.5 (14.9)	16.2 (9.2)	21.7 (11.7)	4.5 (13.0)	0.631	0.694
Isobutyrate	2.2 (1.5)	2.4 (1.1)	0.1 (1.2)	2.7 (1.4)	2.8 (1.8)	0.1 (1.7)	2.5 (1.3)	2.5 (1.0)	-0.0 (1.7)	0.717	0.921
Valerate	3.0 (1.8)	3.6 (1.6)	0.6 (1.7)	3.9 (2.4)	4.0 (2.3)	0.1 (2.2)	3.4 (1.7)	4.0 (2.1)	0.7 (1.8)	0.752	0.506
Isovalerate	4.6 (3.4)	5.1 (2.7)	0.2 (2.9)	5.5 (3.3)	5.8 (4.0)	0.3 (4.0)	5.3 (2.8)	5.2 (2.3)	-0.1 (3.7)	0.740	0.926

All values are mean (SD); n, number of participants with available data; <sup>1</sup>ANCOVA is the *p*-value following comparison of endpoint values with baseline values as a covariate; <sup>2</sup>ANOVA is the *p*-value following comparison of change values; Intention to treat population was modified to include only those who provided sufficient sample for analysis. Numbers in each group: modified ITT (n = 73) and PP (n = 74).

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**TABLE 4 Whole and regional gut transit times and gastrointestinal pH at baseline and endpoint in the intention to treat and per protocol populations**

	Control				Whole almond				Ground almond				p-values		
	n	Baseline (hh:mm)	n	Endpoint (hh:mm)	n	Baseline (hh:mm)	n	Endpoint (hh:mm)	n	Baseline (hh:mm)	n	Endpoint (hh:mm)	ANCOVA	Pooled almond vs. control <sup>1</sup>	Whole vs. ground <sup>1</sup>
<b>Intention-to-treat</b>															
WGTT	14	36:28 (20:43)	13	35:06 (18:02)	18	40:46 (25:56)	15	34:31 (12:51)	15	34:17 (16:25)	13	36:12 (23:20)	0.940	0.955	0.487
GET	14	02:46 (00:37)	12	04:17 (04:32)	18	02:38 (00:43)	15	03:18 (01:35)	15	02:52 (00:55)	13	03:32 (04:14)	0.769	0.082	0.442
SBTT	14	05:21 (01:18)	12	05:34 (00:52)	18	05:14 (01:27)	14	05:01 (01:01)	14	04:15 (01:09)	13	04:34 (01:22)	0.325	0.055	0.349
CTT	14	28:20 (19:55)	12	26:19 (19:36)	18	32:46 (25:20)	15	25:52 (12:20)	14	27:56 (16:37)	13	28:05 (21:03)	0.578	0.637	0.906
Small bowel pH	14	7.1 (0.3)	12	7.2 (0.4)	18	7.1 (0.3)	14	7.1 (0.4)	14	7.1 (0.3)	13	7.3 (0.3)	0.578	0.763	0.331
Colonic pH	13	6.7 (0.8)	12	6.5 (0.7)	18	6.5 (0.6)	14	6.5 (0.5)	13	6.6 (0.7)	13	6.5 (0.8)	0.937	0.819	0.845
<b>Per protocol</b>															
WGTT	12	38:58 (21:26)	12	35:18 (18:49)	14	41:39 (26:58)	14	35:22 (12:54)	12	28:57 (11:17)	12	33:14 (21:41)	0.728	0.936	0.241
GET	12	02:42 (00:38)	11	02:59 (00:28)	14	02:43 (00:44)	14	03:22 (01:37)	12	02:46 (00:56)	12	02:22 (00:40)	0.091	0.076	0.181
SBTT	12	05:34 (01:16)	11	05:31 (00:53)	14	05:19 (01:30)	13	05:02 (01:04)	11	04:24 (01:02)	12	04:31 (01:25)	0.548	0.091	0.301

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CTT	12	30:41 (20:40)	11	27:59 (19:38)	14	33:36 (26:31)	14	26:36 (12:27)	11	22:16 (11:13)	12	26:21 (20:59)	0.875	0.974	0.584
Small bowel pH	12	7.1 (0.3)	11	7.1 (0.4)	14	7.2 (0.3)	13	7.1 (0.4)	11	7.1 (0.4)	12	7.3 (0.3)	0.370	0.467	0.238
Colonic pH	11	6.8 (0.8)	11	6.5 (0.7)	14	6.5 (0.6)	13	6.4 (0.6)	10	6.6 (0.7)	12	6.5 (0.8)	0.878	0.746	0.851

All values are mean (SD); n, number of participants with available data; *p*-values are the result of ANCOVA with baseline values as a covariate; <sup>1</sup>*p*-values are the result of an independent samples t-test on endpoint values.

CTT, colonic transit time; GET, gastric emptying time; ITT, intention to treat; PP, per protocol; SBTT, small bowel transit time; WGTT, whole-gut transit time.

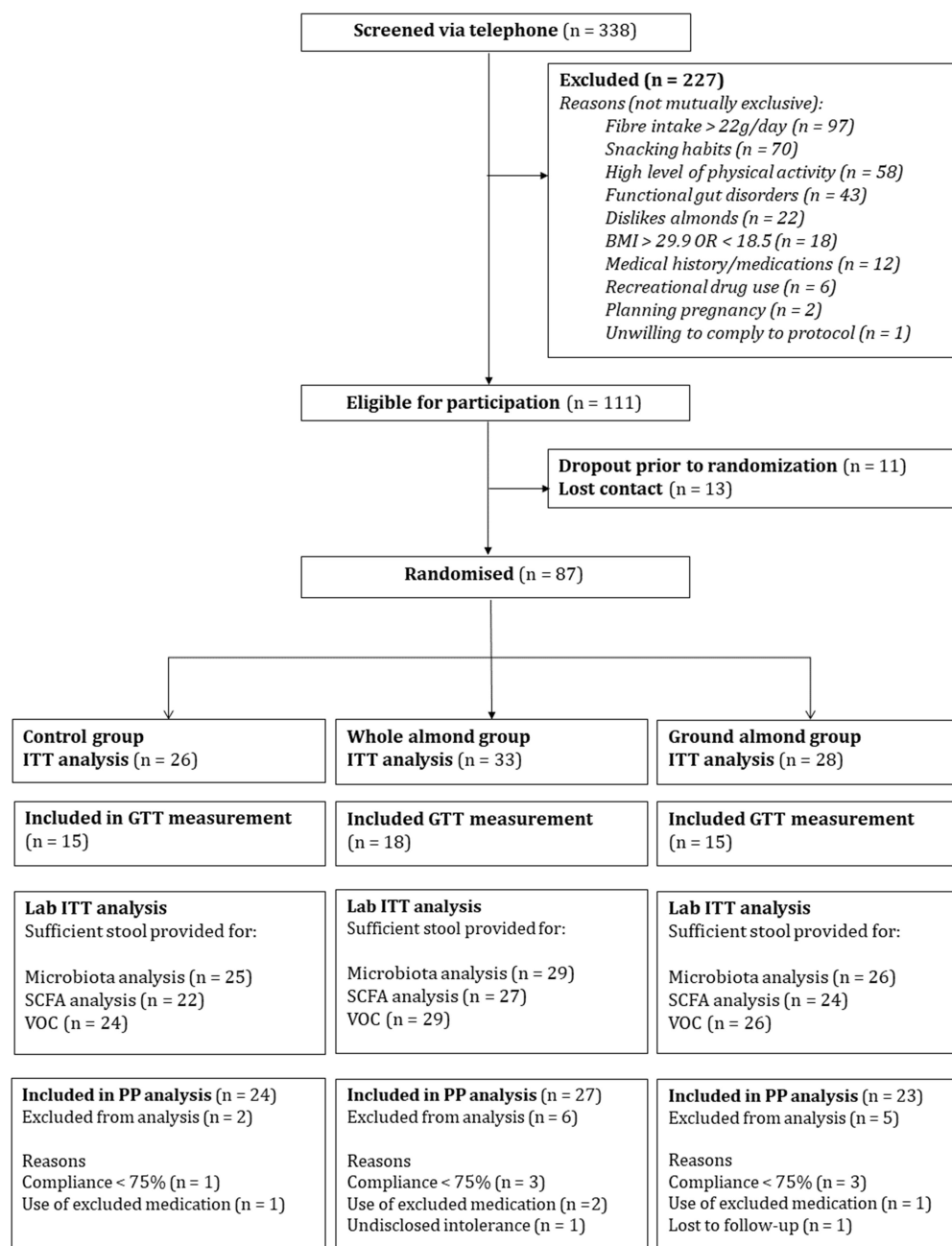
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**TABLE 5 Stool frequency in the intention to treat population**

	Control			Whole almond			Ground almond			p-values	
	Baseline (n=26)	Endpoint (n=25)	Change <sup>1</sup> (n=25)	Baseline (n=33)	Endpoint (n=30)	Change <sup>1</sup> (n=30)	Baseline (n=28)	Endpoint (n=26)	Change <sup>1</sup> (n=26)	ANCOVA <sup>2</sup>	Kruskal- Wallis <sup>3</sup>
Stool frequency (BMs/wk)	8.3 (3.9)	7.7 (3.1)	-1.0 (3.0)	7.9 (3.5)	9.2 (3.7)	1.5 (3.0) <sup>4</sup>	9.4 (3.5)	9.1 (4.6)	-0.5 (4.3)	0.070	<b>0.017</b>
BSFS score	4.0 (0.7)	3.7 (0.8)	-0.3 (0.8)	3.9 (1.0)	3.8 (0.8)	0.0 (1.5)	3.8 (0.6)	3.8 (0.8)	0.0 (0.7)	0.508	0.368
Normal stools (%)	88.2 (34.1)	85.7 (36.1)	0.0 (25.3)	77.8 (47.2)	89.2 (35.0)	1.4 (25.0)	87.1 (32.7)	97.1 (25.9)	0.0 (22.4)	0.617	0.213

Values are mean (SD) unless otherwise stated; <sup>1</sup>Values are median (IQR); n, number of participants with available data; BMs/wk, bowel movements/week; BSFS, Bristol stool form scale; <sup>2</sup>p-values are the result of ANCOVA on endpoint values with baseline values as a covariate; <sup>3</sup>p-values are the result of Kruskal-Wallis H test on change values; <sup>4</sup>Significantly greater change in comparison to control,  $p = 0.034$ ; Numbers in each group: ITT (n = 87), control = 26, whole almond = 33, ground almond = 28

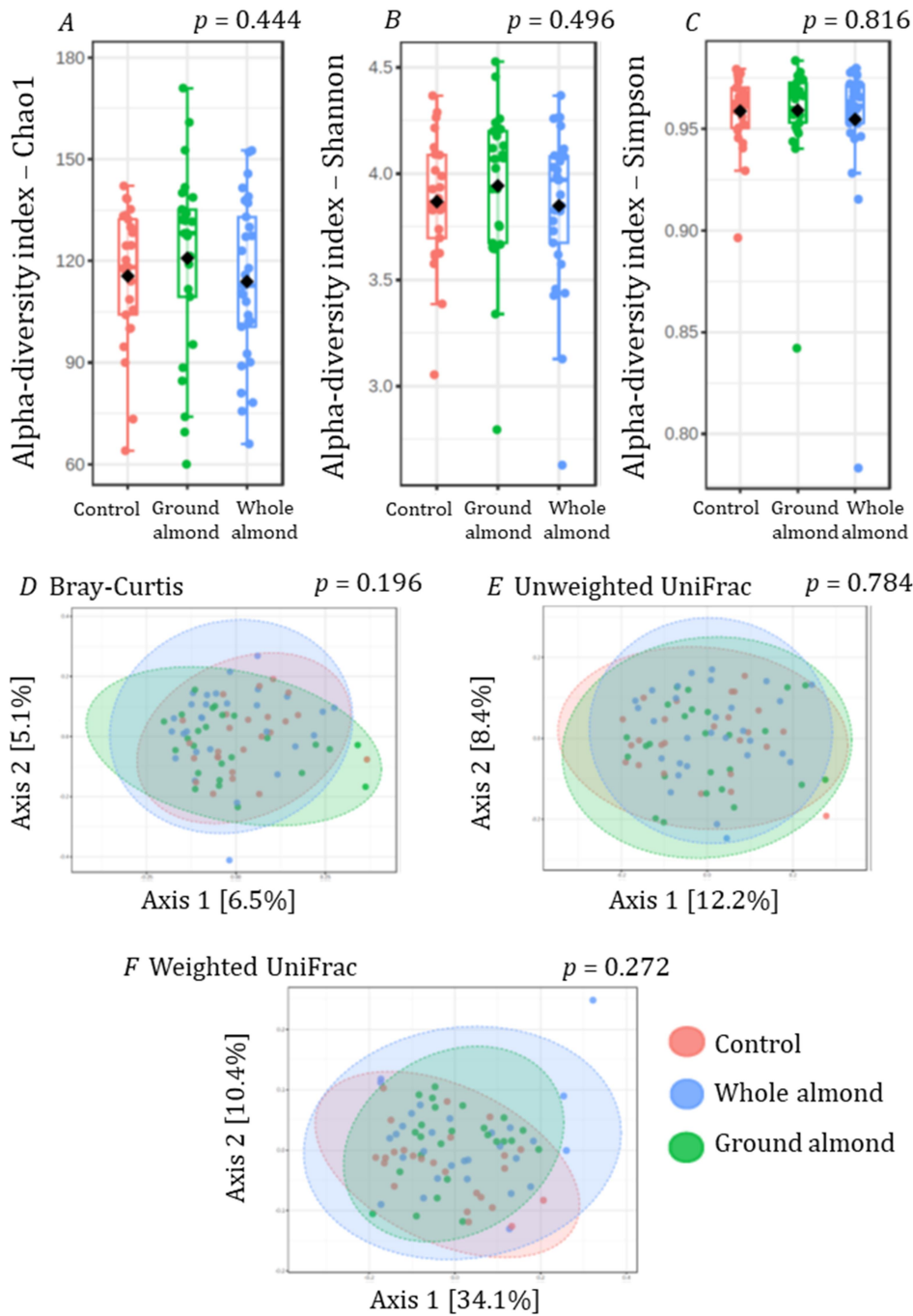
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**Figure 1** Consort diagram

GTT, gut transit time; ITT, intention to treat; PP, per protocol

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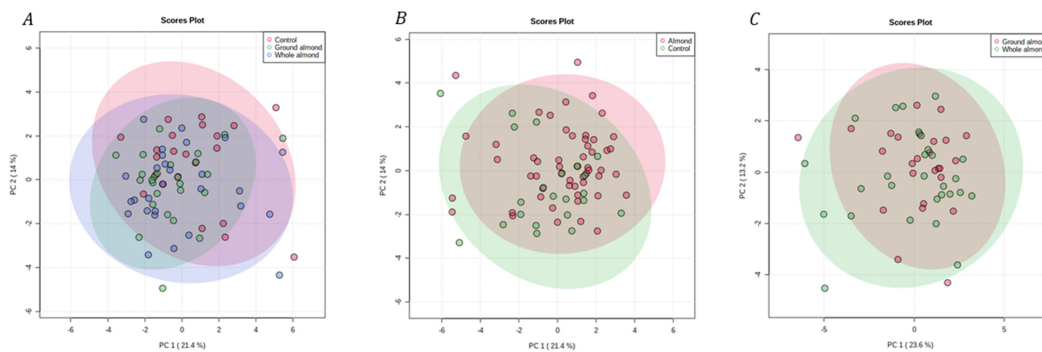
**Figure 2** Gut microbiota diversity indices at the end of the intervention.

All indices calculated on rarefied abundance data following application of filter to remove low abundance/rare taxa;  $\alpha$ -diversity indices (A) Chao1 Index; (B) Shannon index; (C) Simpsons index. Boxplots are median and interquartile range with the

sample mean represented by the black diamond. Individual sample values are represented by colored dots.  $p$ -values are the result Kruskal-Wallis test.  $\beta$ -diversity indices (D) Bray-Curtis Dissimilarity; (E) Unweighted UniFrac distance; (F) Weighted UniFrac distance in the whole almond, ground almond and control groups;  $p$ -values are the result PERMANOVA.

Modified ITT (n = 79), control = 25, ground almond = 25, whole almonds = 29

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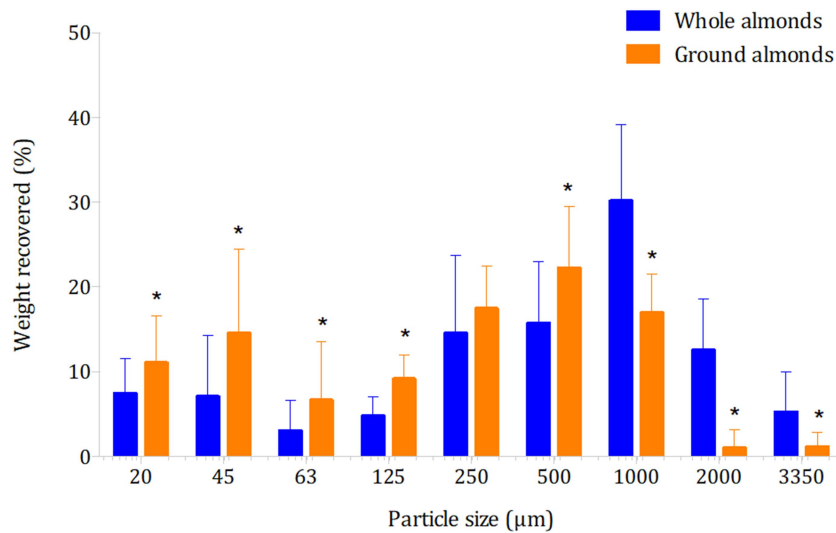


**Figure 3** PCA plot of fecal volatile organic compound profile in groups that consumed A) whole almond vs ground almond vs control muffin; B) almond (whole and ground pooled) vs. control and C) whole almond vs ground almond groups.

Modified ITT (n = 76), control = 22, ground almond = 26, whole almonds = 28

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**Figure 4** Particle size distributions of masticated whole and ground almonds measured by mechanical sieving.

Bars are mean weight recovered; error bars are standard deviation;  $n = 31$  participants provided paired data; significant interaction between almond form (whole or ground) and particle size on PSD from two-way repeated measures ANOVA ( $p < 0.001$ ); (\*) indicates significant difference between whole and ground almonds following simple main effects (paired  $t$ -test) at that level of particle size ( $p < 0.05$ ).