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Manuscripts
Chronic consumption of cocoa rich in procyanidins has a marginal impact on gut microbiota and on serum and fecal metabolomes in male endurance athletes

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Abstract

Cocoa is rich in procyanidins and is used in the sports training world as a supplement because of its antioxidant and anti-inflammatory properties, although there is no consensus on its use. We investigated the effect of cocoa intake on intestinal ischemia, serum lipopolysaccharide (LPS) levels, gastrointestinal symptoms and gut microbiota composition in endurance athletes during their training period on an unrestricted diet. We also performed a metabolomics analysis of serum and feces after a bout of physical exercise before and 10 weeks after cocoa consumption to explore potential mediators of the effect of cocoa on athletes' health. Cocoa consumption had no effect on the intestinal ischemia marker Intestinal fatty-acid binding protein, LPS or on gastrointestinal symptoms. Cocoa intake significantly increased the abundance of Blautia and Lachnospira genera and decreased the abundance of the Agathobacter genus, which was accompanied by elevated levels of the polyphenol fecal metabolites 4-hydroxy-5-(phenyl)-valeric acid and O-methyl-epicatechin-O-glucuronide. Our untargeted approach revealed that cocoa had no significant effects on serum and fecal metabolites and that its consumption had little impact on the metabolome after a bout of physical exercise.

Keywords: Cocoa; exercise; microbiota; serum metabolome, fecal metabolome.
Introduction

The performance of strenuous exercise is often accompanied by an increase in oxidative stress, the release of inflammatory cytokines, muscle damage and, occasionally, by adverse gastrointestinal events including diarrhea, nausea, cramping and gastric pain. Gastrointestinal complaints occur in a significant percentage of athletes and, in some cases, can lead to withdrawal from competition. Several studies have reported that the development of splanchnic hypoperfusion during exercise might lead to intestinal ischemia, which can damage intestinal epithelial cells and compromise the intestinal barrier. The use of nutritional and dietary strategies including amino acids, carbohydrates, polyphenols, prebiotics and probiotics, has been proposed by several studies to alleviate exercise-induced gastrointestinal symptoms. For example, cocoa is a rich source of antioxidant and anti-inflammatory compounds with prebiotic effects on intestinal microbiota, and has proven beneficial effects related to exercise practice.

The more relevant group of polyphenolic compounds present in cocoa are the flavonoids, to which many health benefits have been attributed such as improvements in blood pressure and in platelet and vascular functions, and enhancement of antioxidant and anti-inflammatory activity and insulin resistance. In relation to the gut microbiota, cocoa flavonoids have been shown to increase the presence of Lactobacillus and Bifidobacterium genera, which can improve barrier function and reduce symptoms and possible infections in athletes experiencing gastrointestinal barrier disorders. Changes in the gut microbiota induce changes in microbial metabolites, which become integrated into the human blood metabolome and may also be partly responsible for the observed health benefits of cocoa.

The effects of chronic cocoa intake and its synergetic effect with exercise training has yielded controversial results. In part, this can be attributable to the lack of characterization of the product administered, failure to standardize the physical activity performed, differences in the level of physical activity of the participating athletes, and lack of approximation to real-life situations. We designed a randomized placebo-controlled study to assess the potential benefits of cocoa in endurance athletes using a cocoa supplement with a known nutritional and polyphenol content. We selected a study population based on their high level of physical activity, and we administered the supplement during their controlled training period with no dietary restrictions. We assessed the health and performance effects of chronic cocoa consumption using a laboratory-based controlled exercise protocol. Untargeted and targeted metabolomics approaches were combined with 16S rRNA sequencing to explore the effect of chronic cocoa consumption during training on the gastrointestinal microbiota, and on the blood and fecal metabolome and the gut microbiota. This is the first study to our knowledge to examine the effect of chronic cocoa consumption on metabolomic and microbiota parameters in athletes and the results should provide a valuable resource for further research on the effects of cocoa as a supplementary diet complement.
**Materials and Methods**

**Experimental design**

We designed a randomized, parallel-group, placebo-controlled trial. Participants were recruited from several cross-country athletics teams in Madrid. Fifty-four male athletes met the following inclusion criteria: 18–50 years of age, with a high physical condition (oxygen uptake $[\text{VO}_2] \geq 55$ mL/kg/min), body mass index 18–25 kg/m$^2$, and treadmill experience. Exclusion criteria included smoking, being vegetarian or vegan, on chronic medication, gastrointestinal surgery, or any diagnosed disease, antibiotics intake during 3 months prior to the study, nutritional or ergogenic supplements, probiotics and prebiotics. Subjects were randomized to two equal-size treatment groups using the RAND function of Excel (Microsoft Office Excel, 2019). The cocoa group (CO; n = 22) received 5 g of fat-reduced cocoa containing 425 mg of flavonols and the control group (CT; n = 20) received 5 g of maltodextrin (placebo) (Figure 1). The schedule was 10 weeks.

![CONSORT diagram](image)

**Figure 1:** CONSORT diagram showing the flow of participants through each stage of the randomized trial.

Supplements were provided in identical single-dose paper sachets to dissolve in semi-skimmed milk, which does not affect the bioavailability of cocoa flavonoids $^{16}$. Maltodextrin is used as a carbohydrate supplement by athletes; however, a 5-g bolus provides insufficient carbohydrate or
energy to impact daily macronutrient and energy intake. Sachets were dispensed by postal mail and researchers that collected and analyzed the data were blinded. Supplements were consumed daily during breakfast. During the 10-week intervention period, participants were contacted by telephone every week to check that they were taking the corresponding supplement. All measurements were recorded before and after the 10-week endurance training intervention. The Ethics Committee for Clinical Research of the Community of Madrid Spain approved the study (Ref: 07/694487.9/17), and all procedures were in accordance with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all the volunteers. The study was registered in the US National Library of Medicine (http://www.clinicaltrials.gov) with the accession number NCT04444388. A schematic of the study design is shown in Figure 2.

Figure 2: Schematic diagram of the overall study design and analyses.

Dietary habits

Dietary habits of participants were recorded at the beginning and after the 10-week intervention period. Dietary pattern characterization was performed using a validated food frequency questionnaire with 93 food items, which was analyzed using DietSource software 3.0 (Novartis, Barcelona, Spain) to obtain the total energy ingested (in kcals) of fat, carbohydrates, fiber and proteins.

Exercise test

The exercise protocol was carried out at the beginning of the study and after 10-weeks of cocoa/placebo supplementation. Participants started with a standardized warm-up of 10 minutes of continuous treadmill running (H/P/Cosmos Venus, Nussdorf-Traunstein, Germany) at 60% of their maximum heart rate. After the warm-up, they ran with a slope of 1% at a speed of 10 km/h, with increments of 0.3 km/h every 30 s until volitional exhaustion. Participants were verbally encouraged to give their maximal effort, particularly at the end of test. During the test oxygen consumption values were monitored and the following variables were determined: oxygen uptake \((V'O_2)\), pulmonary ventilation, ventilatory equivalents for oxygen and carbon dioxide, and end-tidal partial pressure of oxygen and carbon dioxide. These variables were used to calculate the absolute maximal oxygen consumption \((V'O_2max\text{ABS})\), relative maximum oxygen consumption
(VO$_{\text{max REL}}$), maximal aerobic speed (MAS), first ventilator threshold and second ventilator threshold. After the exercise bout, the participants performed a 1-km run on an outdoor athletics track at their maximum speed.

Training sessions

The participants were committed to follow a 10-week training intervention period with a polarized endurance-training intensity distribution model based on the classical 3-phase model of Skinner and McLellan. The distribution involves significant proportions of low-intensity (Zone 1) and high-intensity (Zone 3) training, and only a small proportion of moderate-intensity (Zone 2) training. The time expended in each training zone was as follows 75–80% in Zone 1, ~5% in Zone 2, and 15–20% in Zone 3. The total training load was approximately 43%-7%-50% for Zone 1; Zone 2 and Zone 3, respectively. Participants trained in 5 to 6 sessions per week.

Gastrointestinal symptoms

Assessment of gastrointestinal symptoms was performed before and after the exercise bout (T1 and T2, respectively, Figure 2) using the questionnaire described by Pfeiffer et al. The questionnaire assesses upper abdominal problems (reflux/acidity, belching, bloating, stomach cramps/pain, nausea, vomiting), lower abdominal problems (intestinal or lower abdominal cramps, flatulence, urge to defecate, pain/punctures on one side, loose stools, diarrhea, intestinal bleeding) and systemic problems (dizziness, headache, muscle cramps, need to urinate). Each question was assessed on a 10-point scale, ranging from 0 or “no problem at all” to 9 or “the worst it has ever been.”

Serum, plasma and stool sample collection

Venous blood samples were collected in vacutainer tubes immediately before and within 15 minutes of finishing the exercise tests (T1, T2, T3 and T4, Figure 2). Serum was obtained after clotting and centrifugation at 760 × g for 10 min at 4°C; aliquots were immediately frozen and stored at -80°C. Plasma samples were obtained in EDTA vacutainers tubes and centrifuged as before. Stool samples were collected just before and just after the exercise session. Participants were provided with the Fe-col stool collection container device (Alpha Laboratories, Hampshire, UK), an insulated bag and ice blocks to preserve the samples until they were delivered to the laboratory. All post exercise, fecal samples were collected within four hours of the exercise bout to avoid diet interference. On arrival at the laboratory, stool samples were maintained at 80°C until they were processed. Stool samples for metabolomics analysis were lyophilized using the TFD5503 Bench Top freezedryer (ilShin Biobase, Ede, The Netherlands) and stored at -80°C.

Intestinal Fatty Acid-Binding Protein and lipopolysaccharide determinations

Plasma Intestinal Fatty Acid Binding Protein (I-FABP) abundance was determined with the I-FABP Human ELISA kit (Hycult Biotech, Uden, The Netherlands) using a 1:2 dilution. Serum lipopolysaccharide (LPS) quantification of endotoxin units was measured using the Limulus
amebocyte lysate (LAL) chromogenic assay (Pierce® LAL Chromogenic Endotoxin Quantitation Kit, ThermoFisher Scientific, Waltham, MA) using a 1:25 serum dilution. All material used in LPS analysis (microtubes, tips, water and plates) was endotoxin-free.

Fecal bacterial DNA extraction, sequencing and bioinformatics

Fecal bacterial DNA was extracted using the E.Z.N.A. Kit (Omega-Biotek, Norcross, GA) and a bead homogenizer (Bullet Blender Storm, Next Advance, Averill Park, NY). A DNA fragment comprising the V3 and V4 hypervariable regions of the 16s rRNA gene was amplified for sequencing analysis. Amplicons were sequenced on the MiSeq Illumina platform (Illumina, San Diego, CA). Sequence results were analyzed using Quantitative Insights into Microbial Ecology (QIIME2) v2019.7 and were processed with DADA2 for quality control. The classify-sklearn method was used for taxonomy assignment with an in-house customized classifier based on the SILVA reference database. To construct the customized reference database, we extracted the sequences according to our primers (forward primer sequence: CCTACGGGNGGCWGCAG, reverse primer sequence: GACTACHVGGGTATCTAATCC) from the SILVA 132 database clustered at 99% identity. We trained the classifier using our tailored reference reads and SILVA 7-levels for reference taxonomy, including the species probability (weights) likely to be observed for human stool (downloaded from https://github.com/BenKaehler/readytowear).

Chemicals and reagents for untargeted metabolomics

All reference compounds were from Sigma Chemical Co. (Saint Quentin Fallavier, France). The standard mixtures used for the external calibration of the mass spectrometry (MS) instrument (Calmix-positive, for the positive ion mode, consisting of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate; and Ultramark 1621, and Calmix-negative, for the negative ion mode, consisting of the same mixture plus sodium dodecyl sulfate and sodium taurocholate) were from ThermoFisher Scientific (Courtaboeuf, France). Acetonitrile was from SDS ( Peypin, France), formic acid from Merck (Briare-le-Canal, France), methanol from VWR Chemicals (Fontenay-sous-Bois, France) and deionized water from Biosolve chemicals (Dieuse, France).

Untargeted metabolomics analysis by liquid chromatography coupled to high-resolution mass spectrometry

Metabolite extraction from stool and serum samples was performed essentially as described before. Briefly, lyophilized stool samples (2 × 10 mg) were first resuspended in 150 µL of pure water, vortexed and then 600 µL of methanol were added. Samples were subjected to bead-assisted mechanical lysis and left on ice for 90 min. In parallel, serum samples (50 µL) were mixed with 200 µL of methanol and incubated on ice for 90 min to allow complete protein precipitation. After centrifugation, supernatants of both fecal and serum samples were removed and evaporated to dryness under a nitrogen stream at 30°C. Dried aliquots were resuspended in either 100 µL of water/acetonitrile (95:5, v/v) with 0.1% formic acid for C18 analysis or 100 µL of a mixture of 10...
mM ammonium carbonate buffer (pH 10.5) and acetonitrile (40:60, v/v) for HILIC analysis (see below). A volume of 95 µL of the supernatant obtained upon centrifugation was transferred to 0.2 mL-vials and mixed with 5 µL of a mixture of external standards (13C-glucose, 15N-aspartate, ethyl malonic acid, amiloride, prednisone, metformin, atropine sulfate, colchicine, imipramine) to check for consistency of analytical results in terms of signal and retention time stability. Quality control samples were obtained by mixing aliquots of each sample and were injected every 5 to 10 samples throughout the analysis for further data normalization/standardization purposes.

Untargeted metabolomics experiments were performed by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) using a combination of two complementary chromatographic methods, consisting of reversed-phase chromatography (C18 chromatographic column) and hydrophilic interaction chromatography (HILIC) for the analysis of hydrophobic and polar metabolites, respectively. LC-HRMS experiments were conducted on an Ultimate 3000 chromatographic system (ThermoFisher Scientific) coupled to an Exactive mass spectrometer from ThermoFisher fitted with an electrospray ionization (ESI) source and operating in the positive and negative ion modes for C18 and HILIC separations, respectively (designated as C18(+) and HILIC(-), respectively). LC-HRMS conditions have been previously described before by us.

Metabolomic data processing and metabolite annotation

Metabolomic data processing and metabolite annotation were performed essentially as described. Data processing and statistical analysis were achieved using the Workflow4Metabolomics (W4M) platform. Annotation of metabolite features was first performed using our spectral database according to accurate measured masses and chromatographic retention times. Verification of metabolite annotation was then conducted by performing additional LC-MS/MS experiments using a Dionex Ultimate chromatographic system combined with a Q-Exactive mass spectrometer (ThermoFisher Scientific) operated under non-resonant collision-induced dissociation conditions. Obtained MS/MS spectra were both manually and automatically matched using MS-DIAL software to the spectra included in our in-house spectral database, as described. To be identified, ions had to match at least 2 orthogonal criteria (accurately measured mass, isotopic pattern, MS/MS spectrum and retention time) to those of an authentic chemical standard analyzed under the same analytical conditions, as proposed by the Metabolomics Standards Initiative.

Sample preparation and polyphenol metabolite targeted metabolomic analysis

Hexane, acetone, formic acid, acetonitrile, methanol solvents and reagents were all from J.T. Baker (JT Baker Chemical Company, Phillispburg, NJ). Pure chemical standards were obtained from Sigma-Aldrich (St. Louis, MI). One-hundred milligrams of freeze-dried stool samples were homogenized in an IKA-Ultraturrax T10 basic homogenizer (IKA, Wilmington, NC, USA) at 24,000 rpm with a mixture of 1 mL of acetone/water/ hydrochloric acid (70:29.9:0.1) plus 0.1 mL of 4% phosphoric acid and 0.05 mL of 1% ascorbic acid on an ice bath and then sonicated for 10 min in
an ultrasonic ice bath. Samples were centrifuged at 14,000 × g 10 min at 4°C and supernatants were recovered and dried in a vacuum concentrator (Labconco™ Centrivap™, ThermoFisher Scientific), and reconstituted in 1 mL of water that was extracted twice through vigorous vortexing with 0.5 mL ethyl acetate. Both supernatants were pooled and dried in the vacuum concentrator, reconstituted in 0.2 mL of methanol/water/12.1 M hydrochloric acid (50:49.9:0.1), and 2 µL was injected for analysis. Analyses were carried out by ultra-high-performance liquid chromatography (Waters Corp., Milford, MA) coupled to tandem photodiode array detection and an ESI triple-quadrupole mass spectrometer (MS/MS) (Xevo TQS, Waters Corp., Wexford, Ireland). The polyphenolic compounds and metabolites were separated on a Acquity UPLC BEH-C18, 100 mm × 2.1 mm, 1.7-µm column (Waters Corp., Milford, MA), operated at 40°C. The elution gradient included two solvents: acidified Milli Q water with 0.2 % acetic acid (Solvent A) and acetonitrile:acetic acid (98.8:0.2) (Solvent B). Gradient was initial – 0.1% B, 1.5 min 0.1% B; 11.17 min gradient to 16.3% B; 11.50 min gradient to 18.4% B; isocratic to 14 min, B at a flow rate of 350 µL/min. Acquisition was performed in the negative multiple reaction monitoring (MRM) mode. ESI operating conditions for the negative ionization mode were as follows: capillary voltage 2 kV, desolvation temperature 400°C, source temperature 150°C, desolvation gas flow 800 L/h. The liquid chromatography and triple quadrupole control and data processing was done using MassLinx v. 4.1 Software (Waters Corp.).

Statistical analysis

Data are presented as the mean ± standard deviation. Data normality was analyzed using the Shapiro–Wilk test before analysis. Generally, data that met the assumption of normality were analyzed using two-tailed, paired-samples t-tests. For the remaining data, the non-parametric Wilcoxon signed-rank test was used. For metabolomics data, multivariate analyses were used to identify molecular features that discriminate metabolite differences in athletes before and after the supplementation, and in athletes before and after the supplementation after and before a bout of vigorous exercise. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed using the W4M and Metaboanalyst 5.0 platform, and were used to identify features with discriminative power and to maximize variation between the two groups (before and after the test). Also, permutation tests (200 cycles) were conducted to assess the robustness of the PLS-DA model when using a small sample size. Univariate data analyses included a Wilcoxon signed-rank test, corrected for multiple testing by the Benjamini-Hochberg procedure, to assess the statistical significance of each compound. The generation of the clustered heatmap was performed using normalizing data, Pearson distance metric, and hierarchical clustering method. To detect changes in microbiota, beta-diversity analyses were assessed by permutational multivariate analysis of variance (PERMANOVA) and alpha-diversity was compared by the Kruskal-Wallis test. Inter-group differences in gut microbiota were compared using the analysis of composition of microbiomes (ANCOM) whereas longitudinal analyses (time × group) were compared using the q2-longitudinal method. For the longitudinal comparison, we first performed a feature volatility analysis to explore the data. This type of
analysis uses a supervised learning regressor to predict a continuous variable (time in this case) as a function of feature composition (bacterial taxa). Based on volatility analysis, some bacterial taxa were selected for subsequent analyses according to their importance and cumulative average change. Selected taxa were analyzed using a linear mixed-effects model to detect significant bacterial taxa. Cumulative sum scaling normalization was used. Diet macronutrients data, fiber, age and VO\textsubscript{2} were used as covariates in the analyses. All p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate.

RESULTS

Participant characteristics and dietary habits.

In total, 42 male athletes completed the intervention study. Participants characteristics including, age, weight, body mass index and endurance fitness (VO\textsubscript{2}max obtained in the first exercise test) are described in Table 1.

Table 1: Participants’ characteristics.

<table>
<thead>
<tr>
<th></th>
<th>CT (n = 20)</th>
<th>CO (n = 22)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>36.45 ± 9.03</td>
<td>35.18 ± 7.13</td>
<td>0.615</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.16 ± 8.70</td>
<td>71.98 ± 7.90</td>
<td>0.481</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176.20 ± 6.30</td>
<td>177.13 ± 5.84</td>
<td>0.620</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>22.56 ± 2.18</td>
<td>22.92 ± 2.09</td>
<td>0.582</td>
</tr>
<tr>
<td>VO\textsubscript{2}max (mL/kg/min)</td>
<td>59.70 ± 5.13</td>
<td>57.72 ± 5.10</td>
<td>0.217</td>
</tr>
</tbody>
</table>

BMI: Body mass index; VO\textsubscript{2}max: maximal oxygen consumption. CT, control group; CO, cocoa group.

The dietary habits of the participants were determined at baseline and after 10 weeks of supplementation. No differences were observed between groups initially or after 10 weeks, and no significant changes were found in either group over time (Table 2).

Table 2: Energy, macronutrients and fiber intake at baseline and after 10 weeks of intervention.

<table>
<thead>
<tr>
<th></th>
<th>CT T1</th>
<th>CT T3</th>
<th>p</th>
<th>CO T1</th>
<th>CO T3</th>
<th>p</th>
<th>p*</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2496.21 ± 1515.72</td>
<td>2062.26 ± 608.86</td>
<td>0.117</td>
<td>2093.38 ± 609.34</td>
<td>2162.39 ± 589.89</td>
<td>0.669</td>
<td>0.241</td>
<td>0.600</td>
</tr>
<tr>
<td>Carbohydrates (g/1000 kcal)</td>
<td>113.94 ± 20.97</td>
<td>111.47 ± 24.71</td>
<td>0.635</td>
<td>157.72 ± 22.13</td>
<td>103.43 ± 28.63</td>
<td>0.290</td>
<td>0.394</td>
<td>0.348</td>
</tr>
<tr>
<td>Proteins (g/1000 kcal)</td>
<td>68.97 ± 9.43</td>
<td>49.49 ± 9.33</td>
<td>0.835</td>
<td>51.39 ± 11.14</td>
<td>49.76 ± 10.55</td>
<td>0.539</td>
<td>0.460</td>
<td>0.931</td>
</tr>
<tr>
<td>Lipids (g/1000 kcal)</td>
<td>38.42 ± 8.38</td>
<td>37.93 ± 9.84</td>
<td>0.635</td>
<td>38.70 ± 6.58</td>
<td>38.60 ± 11.57</td>
<td>0.964</td>
<td>0.915</td>
<td>0.846</td>
</tr>
<tr>
<td>Fiber (g/1000 kcal)</td>
<td>13.70 ± 4.05</td>
<td>14.09 ± 4.25</td>
<td>0.609</td>
<td>12.44 ± 4.73</td>
<td>13.21 ± 5.62</td>
<td>0.278</td>
<td>0.351</td>
<td>0.584</td>
</tr>
</tbody>
</table>

p = intragroup comparison between T1 and T3; p* = intergroup comparison at T1; p** = intergroup comparison at T3.
Effect of cocoa supplementation on the plasma levels of intestinal fatty acid-binding protein and lipopolysaccharide after a bout of exercise.

The intestinal ischemia biomarker I-FABP has been reported to increase in abundance after a bout of strenuous exercise. To determine whether the strenuous exercise session was sufficient to increase intestinal permeability, we measured the plasma I-FABP before and after the exercise session at baseline and after 10 weeks of cocoa supplementation. We found that I-FABP levels were significantly elevated (up to 3-fold) after the exercise session, both at baseline and after 10 weeks of supplementation (Figure 3A), indicating that the exercise bout triggered a change in intestinal permeability. However, cocoa or placebo supplementation had no effect on I-FABP levels (Figure 3A). Increased intestinal permeability can lead to the release of LPS from gram-negative bacteria to the systemic circulation, which might trigger low-grade inflammation. To determine whether increased intestinal permeability was accompanied by an increase in circulatory LPS, we determined its serum levels using a chromogenic assay. Results showed that the increase in intestinal permeability was not accompanied by an increase in blood LPS levels, either at baseline or after 10 weeks of supplementation, and no effect of cocoa on LPS levels was observed after an acute exercise session (T3 vs T4) or after 10 weeks of supplementation (T1 vs T3) (Figure 3B).

Figure 3: A) I-FABP plasma levels before and after a bout of vigorous exercise initially (T1, T2) and after 10 weeks of supplementation (T3, T4). B) LPS serum levels before and after a bout of vigorous exercise initially (T1, T2) and after 10 weeks of supplementation (T3, T4).

Gastrointestinal symptoms

Data on gastrointestinal symptoms were collected through questionnaires before and after 10 weeks of supplementation, and results were analyzed according to Pfeiffer et al. Symptoms were grouped as explained in upper/lower abdominal problems and as systemic problems. No differences were found between the groups before or after 10 weeks of supplementation, indicating that cocoa supplementation exerted no effects on gastrointestinal symptoms.


Table 3: Gastrointestinal symptoms before and after 10 weeks of supplementation.

<table>
<thead>
<tr>
<th></th>
<th>CT T1</th>
<th>CT T3</th>
<th>p-value</th>
<th>CO T1</th>
<th>CO T3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper abdominal problems</td>
<td>0.82 ± 0.72</td>
<td>0.93 ± 0.72</td>
<td>0.322</td>
<td>0.77 ± 0.76</td>
<td>0.87 ± 0.78</td>
<td>0.242</td>
</tr>
<tr>
<td>Lower abdominal problems</td>
<td>1.51 ± 0.95</td>
<td>1.67 ± 0.99</td>
<td>0.319</td>
<td>1.62 ± 1.31</td>
<td>1.47 ± 0.95</td>
<td>0.536</td>
</tr>
<tr>
<td>Systemic problems</td>
<td>0.95 ± 0.57</td>
<td>0.93 ± 0.97</td>
<td>0.914</td>
<td>0.47 ± 0.52</td>
<td>0.52 ± 0.51</td>
<td>0.624</td>
</tr>
</tbody>
</table>

Effect of chronic cocoa supplementation on gut microbiota

Analysis of the gut microbiota after 10 weeks of cocoa supplementation (between T1 and T3) revealed no changes in the structure of the bacterial community. β-diversity analyses (Bray-Curtis distance, Jaccard index and weighted and unweighted Unifrac) revealed no change in the analyzed parameters (PERMANOVA p > 0.05) (Figure S1). Likewise, α-diversity (observed operational taxonomic unit [OTU], Shannon index and Faith’s index) analyses revealed no significant changes in the microbiota after supplementation (Figure S2). Volcano plots of ANCOM analysis did not reveal any difference in OTU abundance. To evaluate longitudinal changes in bacterial taxa, we used an exploratory analysis of bacterial taxa volatility—an approach that uses machine-learning regressors to establish the important bacterial taxa that predict the T3 state. The accuracy obtained for our model was significant (Mean squared error = 1.4475; R2 = 0.6228; p = 0.0001). The bacterial taxa and those with higher cumulative average change between T1 and T3 identified by the volatility plot were used to test whether the relative abundances of these features were impacted by cocoa supplementation using a linear mixed effects (LME) analysis, in which time and treatment were included as forced predictors (fixed effect) and subject identifier as a random effect. Only four bacterial taxa were identified as significantly and differentially abundant between treatments in T3 (Table 4).

Table 4. LME model parameters calculated to measure changes over time in the cocoa group in comparison with the control group.

<table>
<thead>
<tr>
<th>Bacterial taxa</th>
<th>β-coefficient</th>
<th>SE</th>
<th>Z-score</th>
<th>p</th>
<th>Taxa confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_5__Lachnospira;D_6__gut metagenome</td>
<td>0.001</td>
<td>0.000</td>
<td>6.615</td>
<td>0.022</td>
<td>0.887</td>
</tr>
<tr>
<td>D_5__Agathobacter;D_6__uncultured organism</td>
<td>-0.002</td>
<td>0.001</td>
<td>-2.866</td>
<td>0.004</td>
<td>0.992</td>
</tr>
<tr>
<td>D_5__Blautia;D_6__Blautia sp. Marseille-P3087</td>
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<td>0.000</td>
<td>5.694</td>
<td>0.000</td>
<td>0.936</td>
</tr>
</tbody>
</table>

Parameter estimate (β-coefficient), standard error (SE), Z-score, and p-value for each bacterial taxa. A positive β-coefficient or Z-score indicates a higher relative abundance in T3.
Effect of chronic cocoa supplementation on the polyphenol profile in fecal samples (targeted approach).

The daily intake of 425 mg of cocoa polyphenols during 10 weeks without any dietary restrictions did not substantially change the profile of polyphenols and their derived metabolites in feces, with no differences observed between the CT and CO groups (T1 vs T3) for most of the compounds (Supplementary Table S1). Only 4-hydroxy-5-(phenyl)-valeric acid and O-methyl-epicatechin-O-glucuronide were significantly different (Supplementary Table S1).

Effect of chronic cocoa supplementation on the fecal metabolome (untargeted metabolomics approach).

LC-HRMS with a combination of two complementary chromatographic methods, reversed-phase chromatography C18 (+) and HILIC (-), was used to analyze the effect of the 10 weeks-consumption of cocoa on the fecal metabolome. Under these conditions, 10,866 and 6795 metabolite features were detected, of which 191 metabolite features from the C18(+) and 330 from the HILIC(-) analysis matched the accurate masses and retention times of the metabolites included in our chemical database, yielding 411 unique annotated metabolites in total. The score plot of the PCA and PLS-DA did not reveal any distinctions between T1 (CO) and T3 (CO), and T1 (CT) and T3 (CT) groups (data not shown).

Clustered heatmap analysis of the whole set of annotated fecal metabolites (top 50 according to ANOVA p-values) highlighted poorly consistent metabolic profiles across the population of athletes when studying the effect of cocoa consumption: T1 (CO) and T3 (CO); T1 (CT) and T3 (CT) using HILIC (-) and C18 (+) analyses (Figure 4 and Figure S3). Also, no significant metabolites were found when considering p-values after Benjamin-Hochberg correction. These results might reflect highly variable inter-individual effects of cocoa consumption and/or the difficulty in obtaining homogenous and representative fecal samples. Overall, these data indicate that cocoa does not exert any robust changes in the fecal metabolome after chronic consumption (10 weeks).
Figure 4: Hierarchical clustering heatmaps showing the top 50 fecal metabolites (according to ANOVA p-values) obtained from HILIC (-) LC HRMS analysis in T1 (before dietary intervention) and T3 (after dietary intervention) and in A) cocoa (CO) and B) maltodextrin (CT) groups.

**Effect of cocoa on the serum metabolome after 10 weeks of chronic consumption.**

An experimental set-up similar to that described for untargeted fecal metabolomics was used for the analysis of the serum metabolome. A total of 3195 and 1600 metabolites features using C18 (+) and HILIC (-), respectively, were found, of which 98 and 163, respectively, matched the accurate masses and retention times of the metabolites included in our chemical database, yielding 238 unique annotated metabolites in total. Differences between the two groups (CT and CO) after 10 weeks of intervention study was first investigated using PCA (Figure 5). The serum metabolome profiles of athletes that consumed or not cocoa for 10 weeks remained almost unchanged (Figure 5A), indicating that cocoa did not exert any significant effects on the serum metabolome. Also, and as expected, no differences between the serum metabolome of CT groups were observed before and after the supplementation with maltodextrin (Figure 5B).

![Figure 5: Principal component analysis score plots obtained from HILIC (-) fingerprints of annotated metabolites at T1 [before consumption of cocoa (CO) and maltodextrin CT]] and T3 [after chronic consumption of cocoa (CO and maltodextrin (CT)].](image)

When the univariate clustered analysis heat map was generated using the whole set of annotated metabolites using HILIC (-) and C18 LC HRMS in serum samples (Figure 6, and Figure S4) (top 50 ones by ANOVA p-values), no significant and consistent metabolite pattern was observed between the groups analyzed [T1 (CO) and T3 (CO), T1 (CT) and T3 (CT)]. These data indicate that chronic cocoa consumption does not exert important effects on the serum metabolome.
Effect of chronic consumption of cocoa on the serum metabolome after a bout of exercise.

To assess the effects of chronic consumption of cocoa on athletic performance, athletes were subjected to a bout of intense exercise and the serum metabolome was analyzed from samples before and after the bout of intense exercise [T3 and T4 (CO) and T3 and T4 (CT)] by untargeted metabolomics. Differences in the serum metabolomes were assessed using supervised PLS-DA. As shown in Figures 7A,B, samples before a session of acute exercise (T3) could be distinguished from those after a session of acute exercise (T4) in both cocoa and control groups. The cross-validation parameters R2Y and Q2 indicated the variance and the predictive ability of the model. Permutation tests (200 times) were conducted to assess the robustness of the PLS-DA model when using a small sample size (Figure 7 C,D).
Figure 7: Partial least square discriminant analysis (PLS-DA) of the metabolites at T3 [(before exercise and after 10 weeks of supplementation: cocoa (CO) and Maltodextrin (CT) and T4 (after exercise and after 10 weeks’ supplementation)]. The results are presented as principal component score plots, with each point in the plot representing an individual sample. (A,B). PLS-DA score plots obtained from LC-HRMS data in negative mode HILIC. (C,D). Statistical validation of the PLS-DA model (A,B), showing R2Y (pink dots) and the Q2 (light-blue dots) values from the permutated analysis (bottom left) lower than the corresponding original values (top right).

As we reported previously a bout of intense exercise produced changes in the serum metabolome affecting several metabolic pathways. To distinguish the true effect of cocoa from the metabolic changes induced by the intense bout of exercise, we performed three distinct pairwise comparisons: i) T1 vs T2, to analyze the effect of a bout of intense exercise; ii) T3 CO vs T4 CO, to evaluate the effect of the chronic supplementation of cocoa after a bout of intense exercise; and iii) as a control, the serum metabolome of T3 CT / T4 CT groups was analyzed. As indicated in Figure 8, sixty-two metabolites were common in the three comparisons made, 4 metabolites were found exclusively in the T3 CO / T4 CO group, 2 metabolites only in the T3 CT / T4 CT group, 15 metabolites in the T1 / T2 group, while 9 metabolites were common to the T1 / T2 and T3 CT / T4 CO groups and 3 metabolites were shared by T1 / T2 and T3 CO / T4 CO groups (Table S2).

Figure 8: Venn diagram showing the overlap of serum metabolites significantly distinguishing groups of athletes.

The performance of an exercise bout had the greatest effect on the metabolome of the athletes, modifying a total of 89 metabolites (T1 vs T2, Figure 8). The chronic consumption of cocoa produced only four significantly impacted metabolites during the exercise bout, which were phosphoserine, N-acetyl-L-glutamine, indoxyl sulfate and 5-deoxy-5-(methylthio)adenosine (Figure 9, T3 CO and T4 CO; Table S2).
Figure 9: Serum metabolites whose peak intensity was significant different between T3 CO and T4 CO

Discussion

Intense physical exercise often induces a series of gastrointestinal symptoms, which can be mediated by gastrointestinal ischemia, leading to mucosal damage, increased permeability and bacterial translocation. Some bacteria such as lactobacilli and bifidobacteria have the ability to reinforce the intestinal barrier by increasing the abundance of mucins and tight junction proteins. In addition to the anti-inflammatory, vasodilator and antioxidant properties for which cocoa has been used as a supplement in athletes, it has also been reported to have prebiotic properties. The effects of cocoa on the gut microbiota have been addressed in several in vitro and animal studies, which have shown that it enhances the presence of bacteria with potential health benefits including lactobacilli and bifidobacteria, and promotes gut barrier integrity by up-regulating the levels of the tight junction protein Zonula occludens-1 and the glycoprotein mucin. Studies in humans are, however, scarce. For example, 4 weeks cocoa consumption in healthy humans was found to increase the abundance of Lactobacillus and Bifidobacterium genera and was accompanied by a decrease in pathogenic bacteria; in people with obesity, consumption of dark chocolate triggered an increase in Lactobacillus spp. Owing to its prebiotic properties, cocoa supplementation of diets in athletes performing intense exercise might be a good option to alleviate exercise-associated gastrointestinal symptoms. In addition, the metabolism of cocoa polyphenols by gut microbiota yields metabolites with anti-inflammatory and vasodilator effects, which could also help alleviate gastrointestinal symptoms. For these reasons, the impact of cocoa consumption on the microbiota and metabolome of athletes is of great interest.
We found that an acute exercise session performed by athletes increased the abundance of I-FABP; however, cocoa supplementation had no effect on its levels. Likewise, cocoa had no effect on the gastrointestinal symptoms reported by athletes, or on the gut microbiota. Only three bacterial taxa were affected by cocoa supplementation: an increase in the *Lachnospira* and *Blautia* genera and a decrease in the *Agathobacter* genus. All three genera belong to the *Lachnospiraceae* family, which could indicate that cocoa modulates members of this family of short-chain fatty acid producers, possible providing health-promoting effects. In a previous study in diabetic rats, a diet rich in cocoa was found to increase the proportion of the *Blautia* genus, which was also associated with increased expression of Zonula occludens-1. Regarding the *Lachnospira* and *Agathobacter* (previously known as *Eubacterium rectale*), there is no report of the impact of cocoa on these genera; however, it has been reported that the abundance of the *Blautia coccoides-Eubacterium rectale* group increases after ingestion of red wine (also rich in flavan-3-ols). Some members of the *Lachnospiraceae* family are also related to the metabolism of aromatic amino acids and, therefore, with the production of the uremic toxins indoxyl sulfate and p-cresyl sulfate. Indeed, one of the metabolites increased in the serum of athletes consuming cocoa specifically after a bout of exercise was indoxyl sulfate. This metabolite is derived from the breakdown of tryptophan by tryptonase-expressing bacteria, and it has been associated in one study with the presence of the genus *Blautia*. Indoxyl sulfate is also associated with the progression of kidney and cardiovascular disease, although the data on its toxicity in humans, or its effects on healthy people, are not entirely clear. However, it should be noted that the athlete population has a high protein intake, and so the increase of indoxyl-sulphate-producing bacteria could have undesirable health effects. Our results also showed a significant increase in 5′-deoxy-5′-methylthioadenosine (MTA) following cocoa intake. MTA is an endogenous metabolite derived from polyamine metabolism, to which neuroprotective and immunosuppressive effects have been attributed. Nevertheless, it has not been previously related to polyphenol consumption, nor have the other metabolites changed in the cocoa group, N-acetyl-L-glutamine and phosphoserine.

The minimal effect of cocoa supplementation on the microbiota of the athletes found here might be due to several factors. One such factor is the composition of cocoa, which in turn depends on other variables such as variety, cultivation and processing methods. More specifically, the degree of polymerization of the procyanidins present in cocoa determines their bioavailability; those procyanidins with a lower degree of polymerization are more bioavailable, while those with a higher degree are less well absorbed and reach the colon, but the information about the procyanidins degree of polymerization (DP) is not usually provided in the studies carried out with cocoa. In our study, the mean degree of polymerization (mDP) of procyanidins was not high (mDP = 2.45); nevertheless, we detected significant increases in two metabolites in feces: O-methyl-epicatechin-O-glucuronide and 4-hydroxy-5-(phenyl)-valeric acid. O-methyl-epicatechin-O-glucuronide is a phase II metabolite that has been previously described in human plasma and urine, and is likely excreted in feces via the enterohepatic circulation. 4-hydroxy-5-(phenyl)-valeric acid is a microbial metabolite derived from procyanidins, and its presence could
indicate that part of the cocoa flavanols reach the colon where they are metabolized by the microbiota. However, the athletes’ diet was not restricted in terms of any food containing polyphenols with the intention of making the study closer to real life. Furthermore, the diet was rich in fruits and vegetables (data not shown), and this could be another reason why we failed to observe an effect of cocoa supplementation. In a previous study, we observed that the diet of sedentary people is less rich in fruits and vegetables than that of physically active people; accordingly, enhanced polyphenol intake may not be as relevant as in previous studies conducted in obese or healthy people. This is, however, not verifiable, as the diet in the aforementioned studies was not reported. Another factor that may influence the effect of cocoa on the microbiota is the food matrix itself. In our study, cocoa was consumed dissolved in milk, and although some studies indicate that the bioavailability of cocoa is not affected by milk or its proteins, other studies report that at least the urinary excretion of cocoa-derived metabolites is lower.

We found that cocoa consumption for 10 weeks did not change the metabolomic profile of the plasma and feces of athletes. In agreement with our results, a study by Martin et al. examining the effects of 4-week cocoa consumption on the plasma metabolite profile of free-living subjects also found no significant differences. Similarly, we found that cocoa consumption did not produce evident changes in the metabolic profile when athletes performed an exercise bout. This is the first study to address this issue.

Conclusions

Metabolomic and metagenomic studies are useful procedures to discern the metabolic pathways underlying the physiological effects associated with food intake and to examine for links between consumption and health benefits. Although other studies indicated a beneficial effect of cocoa consumption in athletes, here we found that the 10-week consumption of polyphenol-rich cocoa had only marginal effects on gut microbiota and plasma and fecal metabolomes in male athletes. As we move towards personalized nutrition and dietary strategies for specific populations, a deeper characterization of the foods tested and specific dietary framework will be needed.

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Introduction.


